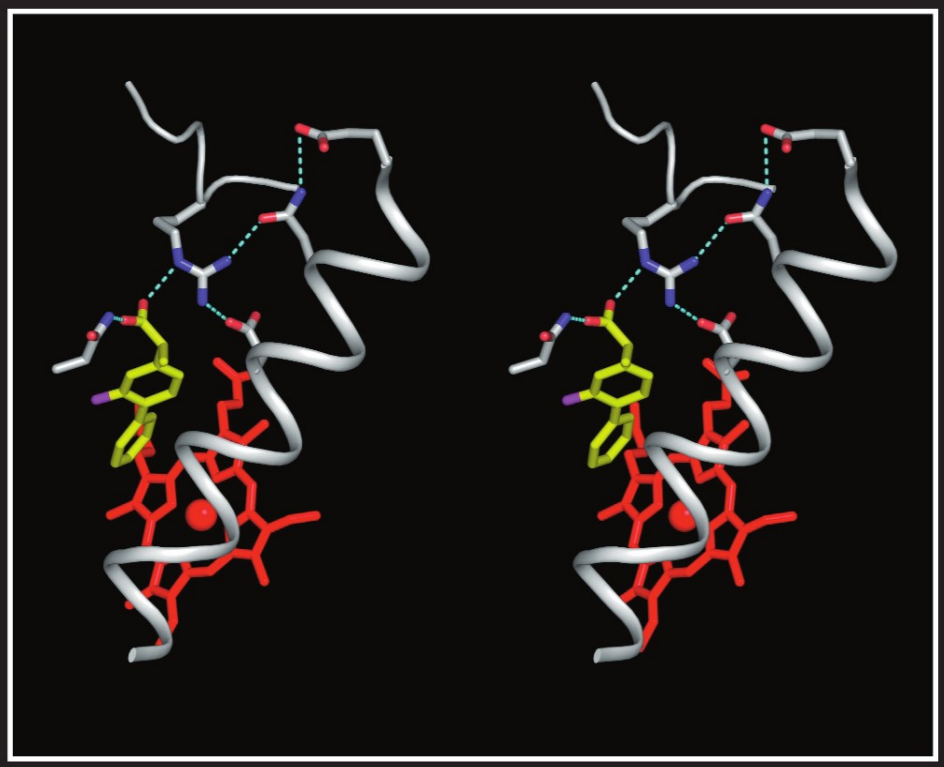
CYTOCHROME P450

Structure, Mechanism, and Biochemistry

THIRD EDITION



Edited by Paul R. Ortiz de Montellano

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Preface

In Dantean terms, three is a magic number, and this is the third edition of this book. Two decades ago the first edition appeared at a time when the first crystal structure of a P450 enzyme had just been determined, the multiplicity of P450 isoforms was just beginning to be realized, and determination of the human genome was not even a dream. The first edition surveyed a field that was young and awkward but full of promise. Ten years later, when the second edition appeared, enormous progress had been made in all aspects of P450 chemistry and biology. The structures of several bacterial P450 enzymes were then available, a systematic nomenclature system had brought order to the chaos engendered by the rapidly growing number of isoforms, and the mechanisms involved in regulation of P450 activity were beginning to yield their secrets. The subsequent 10 years have brought the field to the maturity reflected in this third edition of the book. The dream of obtaining crystal structures of the mammalian P450 enzymes has become a reality, the human genome has defined the number of P450 enzymes in *homo sapiens*, and P450 enzymes have taken center stage in the pharmaceutical industry because of their critical roles in the success or failure of new therapeutic agents. The field continues to be exciting and vigorous, but it is now the excitement of maturity and fulfillment. The increasing sophistication of both the questions that can be asked and the experimental tools available for their investigation has led to a deeper, more satisfying, understanding of the P450 system and, in some cases, to a reexamination of earlier conclusions.

In order to accommodate the new areas of P450 biology that have come into their own in the past decade it has been necessary to eliminate the chapters on the peroxidases, nitric oxide synthases, and related proteins that in earlier editions placed the P450 system in its hemoprotein context. The first section of the book, which collects the work on the structure and mechanism of the P450 enzymes, includes chapters on the model systems used to elucidate P450 chemistry (Chapter 1) and on recent developments in computational chemistry that rationalize the sometimes conflicting mechanistic observations (Chapter 2). These chapters are followed by an up-to-date discussion of bacterial and mammalian crystal structures (Chapter 3), a current review of the electron transfer partners (Chapter 4), a detailed discussion of the mechanism for activation of molecular oxygen (Chapter 5), and a general review of substrate oxidation mechanisms (Chapter 6). This broad introduction to P450 structure and mechanism closes with an updated review of the inhibition of P450 enzymes (Chapter 7). The second section of the book, which focuses on the biology of mammalian P450 enzymes, includes a discussion of our current understanding of the induction of P450 enzymes (Chapter 8), a review of the hormonal influences on P450 expression and activity (Chapter 9), a very extensive summary of the biology of all the human cytochrome P450 enzymes (Chapter 10), and a chapter on the oxidation of arachidonic acid and eicosanoids (Chapter 11). The final section of the book is comprised of a chapter on non-mammalian—primarily plant—P450 enzymes (Chapter 12) and a review of bacterial P450 enzymes and their biotechnological potential (Chapter 13). The book, as before, closes with an appendix containing practical experimental information for individuals doing research in the P450 field.

It is my hope that this third edition will turn out to be as useful as the prior editions. Perhaps, with luck, it may even prove to be sufficiently special to justify the folkloric expectations of a third effort.

I gratefully dedicate this third edition to my brother Bernard, who brought me into chemistry; to my wife Kirby, who for the past 30 years has gracefully sacrificed Saturday companionship to the demands of the laboratory; to Almira Correia, who has made P450 a congenial experience at UCSF for almost as long; and to my daughters, Lara and Maya, who decided to pass on P450 but continue to amaze me.

Paul R. Ortiz de Montellano *San Francisco*

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1

Models and Mechanisms of Cytochrome P450 Action

John T. Groves

1. Introduction

The reactions catalyzed by the cytochrome P450 family of enzymes have challenged and intrigued chemists for more than three decades. Alkane hydroxylation and olefin epoxidation, particularly, have attracted a sustained worldwide effort, the allure deriving both from a desire to understand the details of biological oxygen activation and transfer and, as well as the sense that the development of new, selective catalysts, based on these principles could be of considerable economic value. The focus of this chapter is on the advances in our understanding of the mechanisms of the remarkable oxygenation reactions mediated by oxometalloporphyrins in both enzymatic and in small molecule model systems. Particular emphasis is on the period since the publication of second edition of this monograph in 1995.

The activation and transfer of molecular oxygen into its substrate by an iron-containing enzyme was first demonstrated by Hayaishi in the 1950s[1]. It was shown, in some of the first mechanistically informative oxygen isotopic measurements, that both the inserted oxygen atoms in the conversion of catechol to *cis*-muconic acid derived from O_2 and not water. These findings challenged the then firmly held view that oxygen in biological molecules was derived exclusively from water via hydration processes. The biosynthesis of cholesterol and its precursor, lanosterol, from the hydrocarbon squalene were also shown to derive their oxygen functionality from molecular oxygen[2]. Here, a single oxygen atom derived from molecular oxygen while the other was transformed to water. Later, the prostaglandins were shown to derive from the incorporation of two molecules of oxygen to form, initially, an alkyl hydroperoxide-endoperoxide. Thus, what appeared at first to be an obscure process of bacteria and fungi became recognized as a major theme of aerobic metabolism in higher plants and animals. The subsequent search for "active oxygen species" and efforts to elucidate and understand the molecular mechanisms of oxygen activation and transfer have been richly rewarding. Novel and unusual iron redox chemistry, particularly those of high-valent metal–oxo and metal–peroxo species, has appeared as our understanding of enzymatic oxidation strategies has developed.

2. Oxygen Activation by Heme-Thiolate Proteins

The heme-containing metalloenzymes cytochrome P450[3], chloroperoxidase (CPO)[4, 5], nitric oxide synthase (NOS)[6], and their relatives catalyze a host of crucial biological oxidation reactions. Highly specific P450s are involved in the selective oxygenations of steroid and prostaglandin biosynthesis. Myeloperoxidase, which is a CPO, is an

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integral part of the immune response, and NOS is the source of the highly regulated signal transducer, nitric oxide (NO). Certain fungal CPOs and bacterial P450s have been genetically engineered for large-scale biotransformations[7–10]. The active sites of these three protein families, known in detail from a number of X-ray crystal structures[4, 11–13], are remarkably similar. All three have an iron protoporphyrin IX center coordinated to a cysteine thiolate. All of them are oxidoreductases that activate molecular oxygen (O_2), in the cases of P450 and NOS, or hydrogen peroxide in the case of CPO, at the iron center and incorporate one of the oxygen atoms into a wide variety of biological substrates. The other oxygen atom is transformed into H_2O. All three proteins are proposed to initiate their chemistry through the oxidation of a resting iron(III) state (**1**) to a reactive oxoiron(IV) porphyrin cation radical intermediate (**2**) (Figure 1.1). A depiction of the CPO active site derived from the crystal structure of this protein from *Caldariomyces fumago* is shown in Figure 1.2. The structure, biochemistry, molecular biology, and the chemistry of cytochrome P450 and related model systems have been extensively reviewed [14–22].

Our understanding of the mechanism of action of these heme proteins comes from the direct

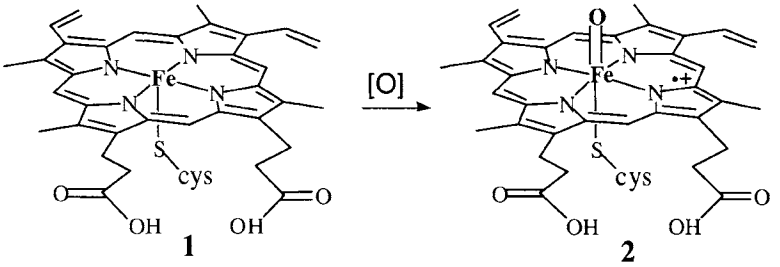


Figure 1.1. Iron(III) protoporphyrin IX with a cysteinate as the axial ligand (**1**), which is typical of cytochrome P450, chloroperoxidase (CPO), and nitric oxide synthase (NOS) enzymes. The active oxygen species of these proteins and related heme enzymes is an oxoiron(IV) porphyrin cation radical (**2**), often called compound I.

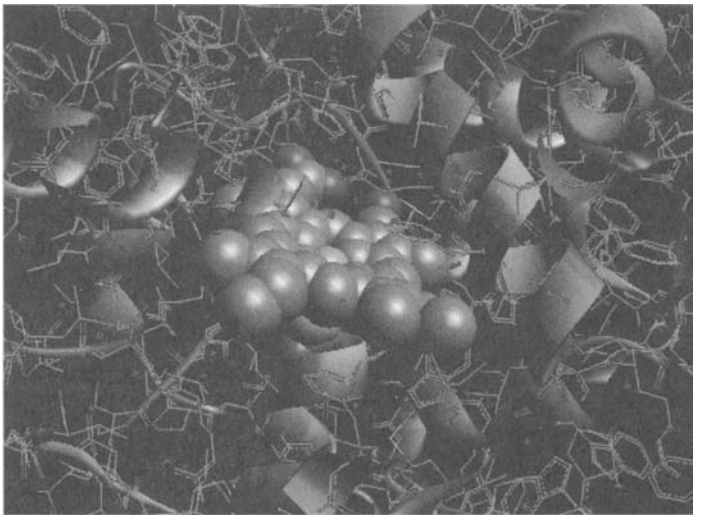
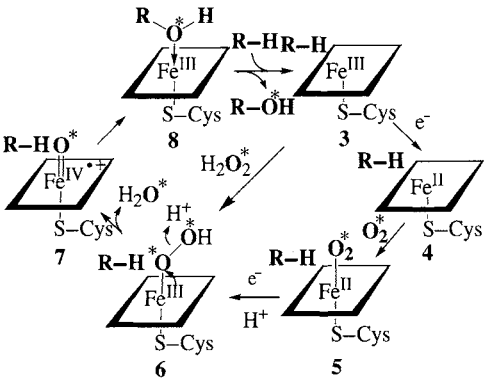


Figure 1.2. Crystal structure of the active site of chloroperoxidase (CPO) (EC 1.11.1.10) from *C. fumago*. Protein framework is shown as ribbons. The heme is buried in a hydrophobic binding pocket containing the iron-coordinating cysteinate ligand. Adapted from the X-ray atomic coordinates of CPO[4].

observation of intermediates in the catalytic cycle through a variety of spectroscopic techniques, the use of diagnostic substrates with mechanistically revealing rearrangements during oxidation, and the parallel development of the chemistry of synthetic metalloporphyrins. The principal features of the consensus mechanism of cytochrome P450[23] are as outlined in Scheme 1.1:

(1) binding of substrate to the enzyme, sometimes accompanied by a spin-state change of the iron, to afford an enzyme–substrate adduct **3**;
(2) reduction of the ferric cytochrome P450 by an associated reductase with an NADPH-derived electron to the ferrous cytochrome P450 **4**;
(3) binding of molecular oxygen to the ferrous heme to produce a ferrous cytochrome P450–dioxygen complex **5**, similar to the situation in oxymyoglobin;
(4) a second one-electron reduction and protonation to arrive at the Fe(III)–hydroperoxy complex **6**;
(5) protonation and heterolytic cleavage of the O–O bond in **6** with concurrent production of a water molecule to form a reactive iron–oxo intermediate **7**;
(6) and, finally, oxygen-atom transfer from this iron–oxo complex **7** to the bound substrate to form the oxygenated product complex **8**. Product dissociation completes the cycle.

There were a number of important realizations in the course of elucidating this mechanism. That hydrogen peroxide, alkyl hydroperoxides, peroxyacids, periodate, and iodosylbenzene were also functional with cytochrome P450 suggested that the chemistry of "oxygen activation" was the two-electron reduction of molecular oxygen to hydrogen peroxide and that, in analogy to the peroxidases, the active oxygen species was a ferryl (or oxene) complex Fe=O, formally iron(V). It was shown that a synthetic oxoiron(IV) porphyrin cation radical species could be formed at low temperature by the oxidation of an iron(III) precursor with peroxyacids (**9** → **10**)[24]. Intermediate **10** did have the requisite reactivity to transfer an oxygen atom to hydrocarbon substrates. It is this oxygen-atom transfer from



Scheme 1.1. Consensus catalytic cycle for oxygen activation and transfer by cytochrome P450.

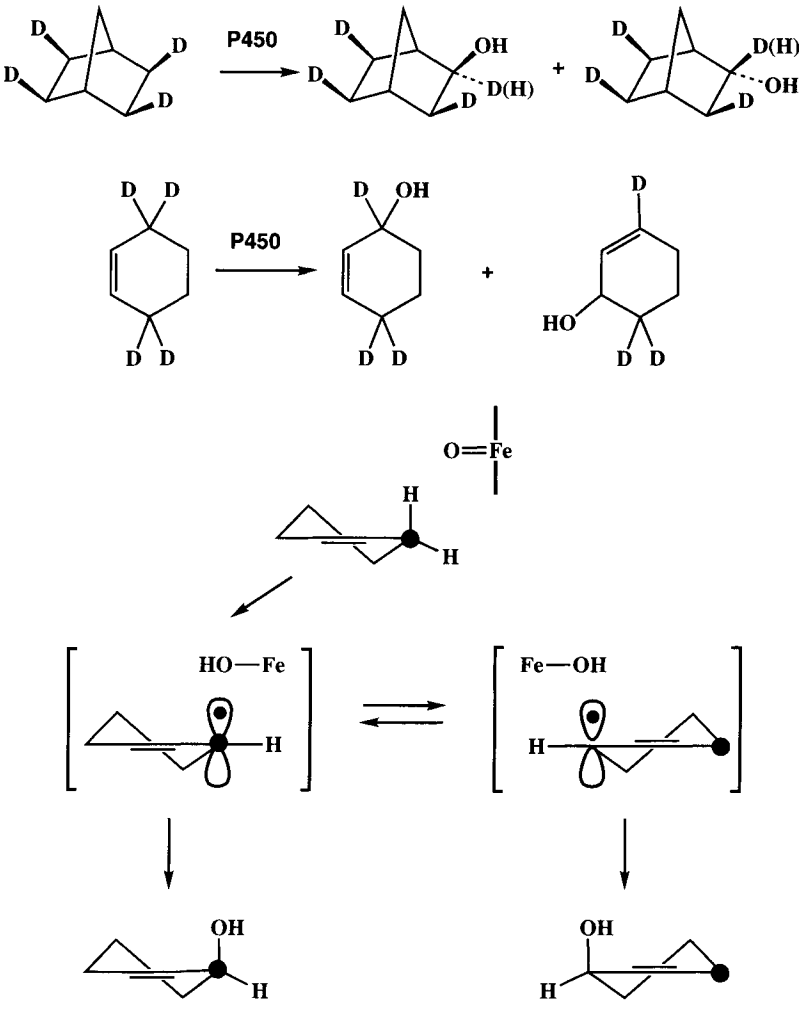
the oxygen donor to form the Fe=O intermediate **7** and the subsequent oxygen transfer to form the substrate complex **8** that has been termed *oxygen rebound*[25]. Such an iron–oxo species (compound I) has been observed for the CPO of *C. fumago*[26] but the active species of cytochrome P450 has remained elusive. Very recently, it has been shown that an intermediate with the spectral properties similar to those of CPO compound I and the model iron porphyrin systems is formed upon the oxidation of Cyp119, a thermostable cytochrome P450, with a peroxyacid, analogous to the model systems[27]. Consistent with the high reactivity expected for P450 compound I, this intermediate decayed with a rate constant of 29 s^{-1} at 4°C. Interestingly, similar experiments with $P450_{cam}$, the camphor-oxidizing enzyme from *Pseudomonas putida*, resulted in an iron(IV)–protein tyrosine radical species, presumably via a one-electron oxidation of Tyr96 which is only 9.4 Å from the iron center[26].

3. Mechanism of Hydroxylation by Cytochrome P450

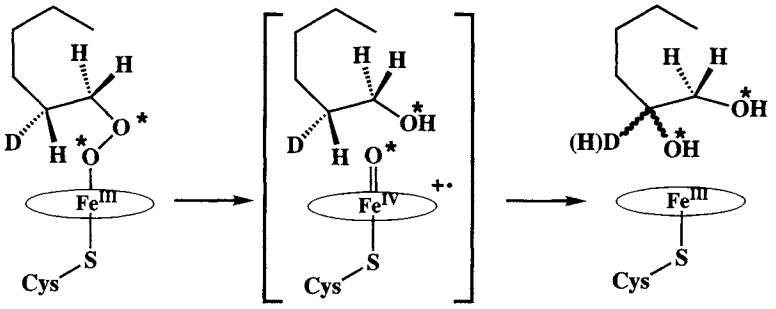
There has been much discussion in the field about the oxygen transfer process **6** → **7** → **8**. The oxygen rebound mechanism in Scheme 1.1 is consistent with the stereochemical, regiochemical, and allylic scrambling results observed in the oxidation of norbornane, camphor, and cyclohexene by cytochrome P450. The hydroxylation of

a saturated methylene (CH_2) in norbornane was accompanied by a significant amount of epimerization at the carbon center[25]. Thus, the hydroxylation of *exo-exo-exo-exo*-tetradeuterionorbornane by P450 2B1 and the hydroxylation of camphor by $P450_{cam}$[28] gave *exo*-alcohol with retention of the *exo*-deuterium label (Scheme 1.2). The hydroxylation of selectively deuterated cyclohexene proceeded with substantial allylic scrambling[29]. The intrinsic isotope effects for the oxygen insertion into a C–H bond are very large, in the range of 10–13.5. These large isotope effects are inconsistent with an insertion process and indicate that the C–H bond is essentially half-broken in a linear [O·H·C] transition state thus providing strong evidence for a nonconcerted mechanism. Significantly, model iron porphyrin systems displayed the same behavior for both the norbornane[30] and cyclohexene[29] substrates. Thus, one concludes that the epimerization and allylic scrambling processes are intrinsic properties of the oxygen transfer event from an oxoiron complex.

Another revealing probe of the nature of P450-mediated hydroxylation is a study of the



Scheme 1.2. Epimerization and allylic scrambling observed for cytochrome P450 catalyzed hydroxylation.

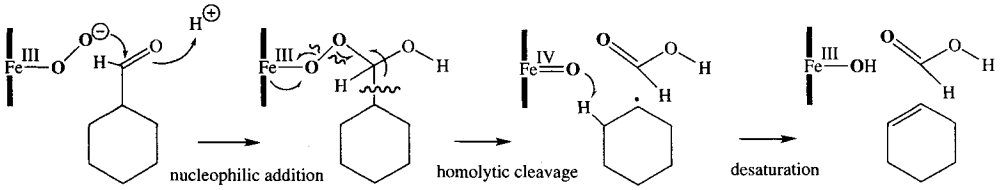


Scheme 1.3. Hydroperoxide isomerase activity of cytochrome P450 is intramolecular.

hydroperoxide isomerase activity of these enzymes. 1-Hydroperoxyhexane has been shown to afford 1,2-dihydroxyhexane upon exposure to P450 2B1[31]. This is an unusual reaction since the oxidizing equivalents of the hydroperoxide have been used in this case to hydroxylate the *neighboring* methylene group. A mixed, double oxygen label experiment established that the rearrangement was *intramolecular* (Scheme 1.3). Thus, the terminal hydroperoxide oxygen was incorporated into the adjacent C–H bond. This reaction pathway is pertinent to the discussion about the nature of reactive P450 intermediates since the same ferryl species (compound I) can be accessed by this "peroxide shunt" pathway. Analysis of the diols derived from chirally labeled 2-deuterio-1-hydroperoxyhexane showed that there was a loss of stereochemistry at the hydroxylated carbon center. Accordingly, the results support a mechanism involving initial peroxide heterolysis, hydrogen abstraction at the adjacent methylene, and radical recombination to afford the product diol. The result is revealing since O–O bond homolysis to form a hexyloxy radical should lead instead to γ-hydrogen abstraction and products derived therefrom.

Kupfer *et al.* have used this P450-hydroperoxide isomerase reaction to explore substrate mobility at the enzyme active site during the hydroxylation event[32]. Isomerase substrates were found to remain in proximity to the P450 oxoferryl intermediate and were rapidly captured by the oxidant with high efficiency. Monooxygenase substrates, by contrast, apparently bind to ferric P450 in multiple orientations and undergo more extensive substrate reorientation prior to oxidative attack. This difference is likely to be due to the requisite prepositioning of the hydroperoxide as a ligand of Fe(III). During turnover via oxygen reduction, however, the positioning of the substrate will be dictated by substrate–active-site interactions. An important conclusion from these studies is that product selectivity can be affected significantly by substrate mobility. Accordingly, *changes in product selectivity*, which have been used to suggest alternative oxidants, need to be interpreted with caution.

The hydroperoxy iron(III) complex **6** has also been suggested to effect substrate oxygenations based on observed changes in product ratios and loss of hydrogen peroxide (uncoupling) upon P450 active-site mutations[33–35]. An important recent advance has been the development of cryospectroscopic studies by Hoffman *et al.* that have allowed the stepwise interrogation of intermediates depicted in Scheme 1.1[36]. Thus, the injection of an electron into complex **5** via γ-radiation, followed by thermal annealing of the sample has produced EPR and ENDOR evidence for the formation of, first, a hydrogen bonded iron–peroxo species and then the iron–hydroperoxo complex **6**. While no ferryl intermediate **7** was observed, the product alcohol was found to be formed with its oxygen atom *coordinated to the iron center and with the substrate-derived proton attached to the product alcohol* as depicted in structure **8** (Scheme 1.1). This arrangement has important mechanistic implications since, if a ferryl species **7** were the immediate precursor of the product complex **8**, then coordination of the product hydroxyl oxygen would be a necessary consequence. By contrast, if



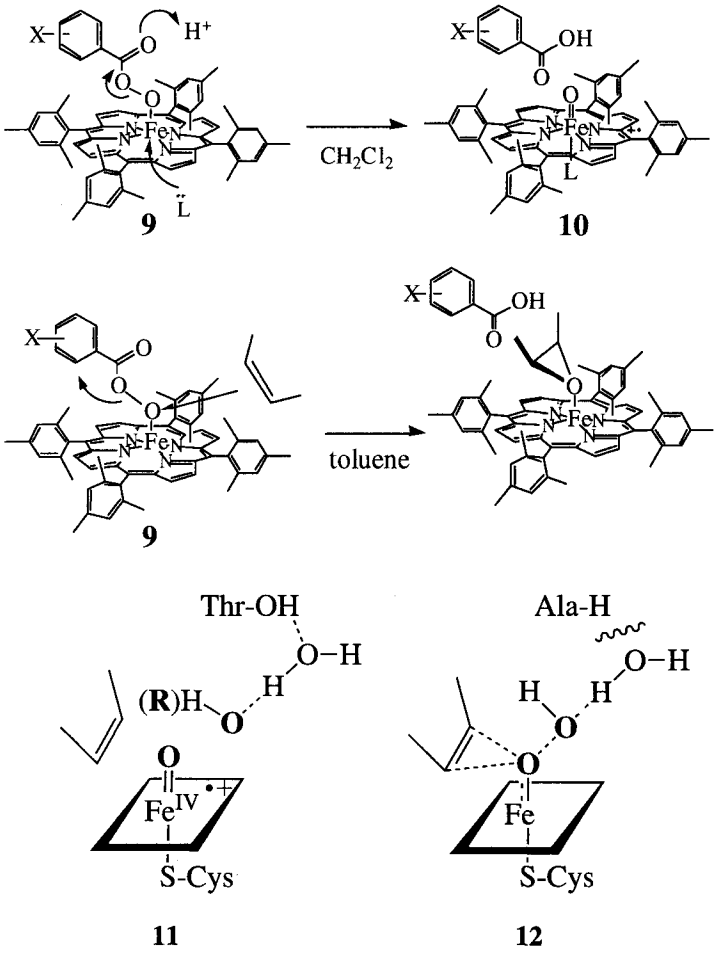
Scheme 1.4. Proposed mechanism for the deformylation typical of P450 aromatase activity.

the hydroperoxo species **6** were the source of the electrophilic oxygen, then *water* would be coordinated to iron rather than the product alcohol. A product complex such as **8** could also be the source of cationic rearrangement products that are sometimes observed during P450 oxygenations.

Significant recent advances in computational approaches to the study of biological catalysis, and the applications of these techniques to the cytochrome P450 mechanism have also been illuminating. Thus, Shaik *et al.*[37] and Yoshizawa *et al.*[38], have presented the results of a density functional theory (DFT) analysis of the reactivity of hydroperoxyiron(III) complexes such as **6**. Both groups conclude that a hydroperoxyiron(III) porphyrin, Fe(III)–OOH, would be an implausible primary oxidant. The protonation and heterolytic O–O bond cleavage of **6** to afford a ferryl species analogous to **7** was found to proceed with almost no energetic barrier, in accord with earlier experimental results for the oxidation of an iron(III) porphyrin **9** to an oxoiron(IV) porphyrin cation radical species **10** with a peroxyacid[39]. Further, the oxygen transfer from **7** to ethylene to form an epoxide proceeded with only a low barrier. It was concluded that the DFT calculations exclude a hydroperoxyiron(III) intermediate such as **6** as a reactive, electrophilic oxidant. Several modes of oxygen transfer from the hydroperoxide intermediate encountered exceedingly high barriers for reaction. The lowest energy of these was an interaction of the substrate ethylene with the *proximal*, iron-bound oxygen of the Fe(III)–OOH ensemble.

Nucleophilic reactions of a hydroperoxyiron(III) intermediate **6**, as have been suggested by Akhtar[40], Robinson[41], and Vaz and Coon[42], for the deformylation reactions characteristic of the P450 aromatase, do seem to be suggested by the significant basicity of the distal, hydroxylic oxygen found in the calculations for the Fe(III)–OOH group. This mode of reactivity is highly analogous to the reactions of enzymes such as cyclohexanone monooxygenase that proceed through a flavin 4a-hydroperoxide[43]. Here, only *electron-deficient* olefins react to afford epoxides even though the flavin hydroperoxide is 2×10^5 times more reactive than a simple alkyl hydroperoxide[44]. The reader is referred to an insightful review by Watanabe for a thorough discussion of the various modes of reactivity of peroxoFe(III) porphyrins (Scheme 1.4)[45].

The hydroxylation of a C–H bond does seem to require the full formation of a reactive ferryl intermediate as in **11**. This applies both for the reductive activation of dioxygen and for the very revealing cases of alkyl hydroperoxide isomerization catalyzed by P450 discussed above[31, 32]. For P450s in which the proton relay system has been disrupted by active-site mutations, one would expect that particularly reactive substrates could interact with the proximal oxygen earlier in this reaction profile as shown in **12**. While similar atomic trajectories and electronic charge redistributions are followed in each case, the former (**11**) is analogous to the S_N1 reaction in organic chemistry, generating a discrete ferryl intermediate, while the latter (**12**) is S_N2-like, requiring assistance from the electron-rich substrate. Indeed, in a recent report by Sligar and Dawson, mutation of the conserved active-site threonine-252 to alanine in $P450_{cam}$ was shown to disable camphor hydroxylation while maintaining some reactivity for more reactive olefinic substrates[46]. Similarly, two reactive intermediates, as suggested by Jones[47] for the reactions of a thioether substrate, and also for model porphyrin systems described by Nam[48], could reasonably derive from a mechanistic spectrum of this type. An important precedent for this behavior is seen in the reactions of peroxyacids with model Fe(III) porphyrins. Thus, Watanabe and Morishima have shown that the iron-coordinated peroxyacid **9** reacted with olefins at the *iron-coordinated oxygen atom* in nonpolar



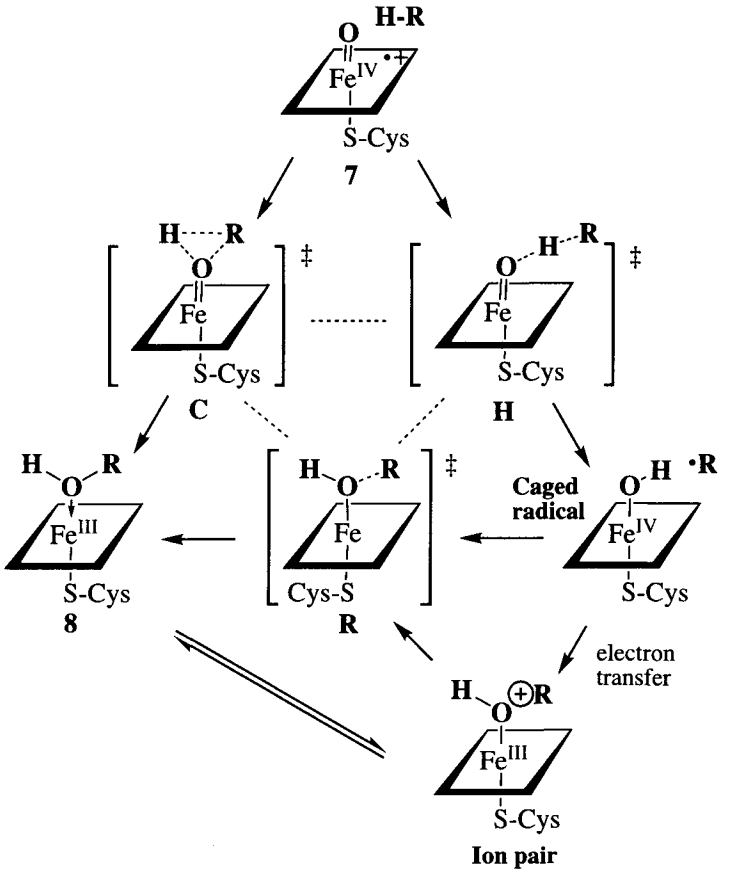
solvents to give epoxides but would not react with saturated hydrocarbons[45, 49]. By contrast, the same oxoiron(IV) porphyrin cation radical, **10**, was formed with a variety of peroxyacids in more polar media. This effect is also seen in model compounds with a thiolate ligand to iron[50]. The protein-derived hydrogen bonds to the axial thiolate ligand to iron in $P450_{cam}$ have been shown to affect the O–O bond cleavage[51].

4. Mechanisms and Molecular Trajectories for Hydroxylation by Cytochrome P450

Among all the varied reactions mediated by cytochrome P450, none has captured the imagination of chemists more than the hydroxylation of saturated carbon centers. Metal–oxo reagents such as chromates and permanganate can perform reactions of this type but are notoriously nonselective and must be used under forcing conditions. The selective hydroxylation of hydrocarbons remains one of the grand challenges for the chemical catalysis community. How can a protein create an iron intermediate reactive enough to hydroxylate even as inert a substrate as cyclohexane and not oxidize the relatively fragile protein superstructure? What is the electronic structure of that intermediate and what are the molecular pathways for oxygen insertion into a C–H bond? Without clear answers to these questions, the chemical catalysis performed by these metalloenzymes will remain an enigma and our

attempts to draw conclusions will be without physical meaning. Without knowledge of the mechanism, we learn nothing of predictive value that could be applied to other systems such as the rational design of enzyme inhibitors or the development of enzymatically inspired catalysts.

Presented in Scheme 1.5 is the range of mechanisms that have been considered as likely candidates for the cytochrome P450-catalyzed hydroxylation of hydrocarbons and those of model iron, manganese, and ruthenium porphyrins. A linear, *homolytic* transition state, as in intermediate **H**, best fits the available data, such as the very large hydrogen isotope effects. Indeed, extensive similarities to the hydrogen abstraction observed by cytochrome P450 and a *t*-butoxy radical have been presented by Dinnocenzo and Jones in support of this view[52]. A nonconcerted pathway for C–H bond cleavage is strongly supported by the observations of a variety of molecular rearrangements that are known to accompany P450-mediated hydroxylation as discussed above. Initially it was clear that the kinds of rearrangements observed were consistent with the formation of a caged substrate radical at the heme active site. The intermediate radical could be trapped in a subsequent step. Both P450 enzymes and model systems showed a nonstereospecificity for the hydrogen removal step from norbornane or camphor substrates. Such a process was counterindicative of a cationic pathway to explain the observed rearrangements. The results rule out *freely diffusing* radicals, but a short-lived substrate radical would explain the observed results.



Scheme 1.5. Pathways for oxygen-atom transfer from the active ferryl species 7 of heme-thiolate enzymes such as cytochrome P450 to form the product alcohol coordinated to the ferric, resting form of the protein (**8**).

It was shown by Ortiz de Montellano *et al.* that bicyclo[2.1.0]pentane was oxidized by rat liver microsomes to a 7 : 1 mixture of *endo*-2-hydroxybicyclo[2.1.0]pentane and 3-cyclopenten-1-ol, consistent with a radical ring-opening reaction[53]. Applications of the "radical-clock" method by Ingold[54] and by Newcomb[55] began to measure the lifetime of the suspected radical cage intermediate. The rate constant for the rearrangement of bicyclo[2.1.0]pent-2-yl radical to 3-cyclopenten-1-yl radical was determined to be $2.4 \times 10^9\ s^{-1}$ at room temperature by using laser flash photolysis techniques[56]. Thus, a rate constant of $k_{OH} = 1.7 \times 10^{10}\ M^{-1}\ s^{-1}$ was estimated for the rebound process. Radical clocks with very fast rearrangement times were shown to produce *less* rearrangement than slower clocks in the P450-mediated hydroxylations, however. The results led Newcomb to question whether a radical pathway existed since the apparent lifetimes revealed by these probes were in the range of 100 fs, too short to represent a bona fide intermediate[57]. Several suggestions have been considered to resolve this dilemma and the question is still an area of active experiment and debate. As shown in Scheme 1.5, the transition state for hydrogen abstraction will position the active oxygen only a few tenths of an Angstrom *farther* from the hydroxylated carbon atom than the transition state for the ultimate C–O bond formation. Thus, the extent of radical rearrangement might be expected to depend critically on the *tightness* of the radical cage and the ensemble of steric and electronic forces experienced by the incipient radical within the cage. Even the molecular makeup of the active site will depend on how the substrate fills the site, leaving room for movement of amino acid side chains in the vicinity of the substrate or allowing additional water molecules into the active-site area. The extent of rearrangement detected by a particular probe may simply reflect a facile molecular trajectory from the hydrogen abstraction transition state to the hydroxylation transition state in this variable environment. For substrates with a very strong C–H bond and a small steric size, both effects would push the reaction coordinate toward a tighter radical cage.

Indeed, it has been shown that the effective *lifetime* of a radical intermediate can even be affected by the stereochemistry of the hydrogen abstraction event[23]. The chiral, binaphthyl porphyrin shown in Figure 1.3 has been found to hydroxylate ethylbenzene with a 70% *ee*. Stereoselective deuteration of the substrate revealed that the pro-*R* hydrogen of ethyl benzene was hydroxylated with nearly complete retention of configuration at carbon while the pro-*S* hydrogen underwent significant racemization (Figure 1.3). Interestingly, the partition ratio, retention/inversion, was nearly the same for the two enantiomers of ethylbenzene-d_1, suggesting similar mobility of the radical intermediate at the active site.

Evidence for a similar type of host–guest complementarity effect has been presented recently by Wüst for the hydroxylation of limonene by the limonene-6-hydroxylase, P450 CYP71D18[58]. The regiochemistry and facial stereochemistry of the limonene hydroxylation was found to be determined by the absolute configuration of the substrate. Thus, (−)-(4*S*)-limonene gave (−)-*trans*-carveol as the only product, whereas (+)-(4*R*)-limonene afforded mostly (+)-*cis*-carveol in a mixture of products. Specifically deuterated limonene enantiomers revealed that (4*R*)-limonene has sufficient freedom of motion within the active site of CYP71D18 to allow formation of either the *trans*-3- or *cis*-6-hydroxylated product. However, the kinetic isotope effects resulting from deuterium abstraction were significantly smaller than expected for an allylic hydroxylation. Significantly, the oxygenation of (4*R*)-limonene gave *trans*-carveol with considerable allylic rearrangement and stereochemical scrambling, while the formation of (+)-*cis*-carveol proceeded with high stereospecificity for C6 hydrogen abstraction and little rearrangement. These results are analogous to the ethylbenzene hydroxylation by the chiral iron porphyrin described above, in that epimerization and allylic rearrangement apparently depend upon the fit and mobility of the substrate at the active site. Another informative probe of substrate mobility at the active site using kinetic isotope effect has been presented by Jones and Trager[59].

For a reaction that involves a paramagnetic iron–oxo intermediate and proceeds to produce paramagnetic radical intermediates, it is likely that spin-orbit coupling effects and the spin states of reacting intermediates may offer another significant consideration[60]. Schwarz first suggested that the unusually slow reaction of FeO^+ with hydrogen in the gas phase was due to spin-conservation effects that were imposed on these

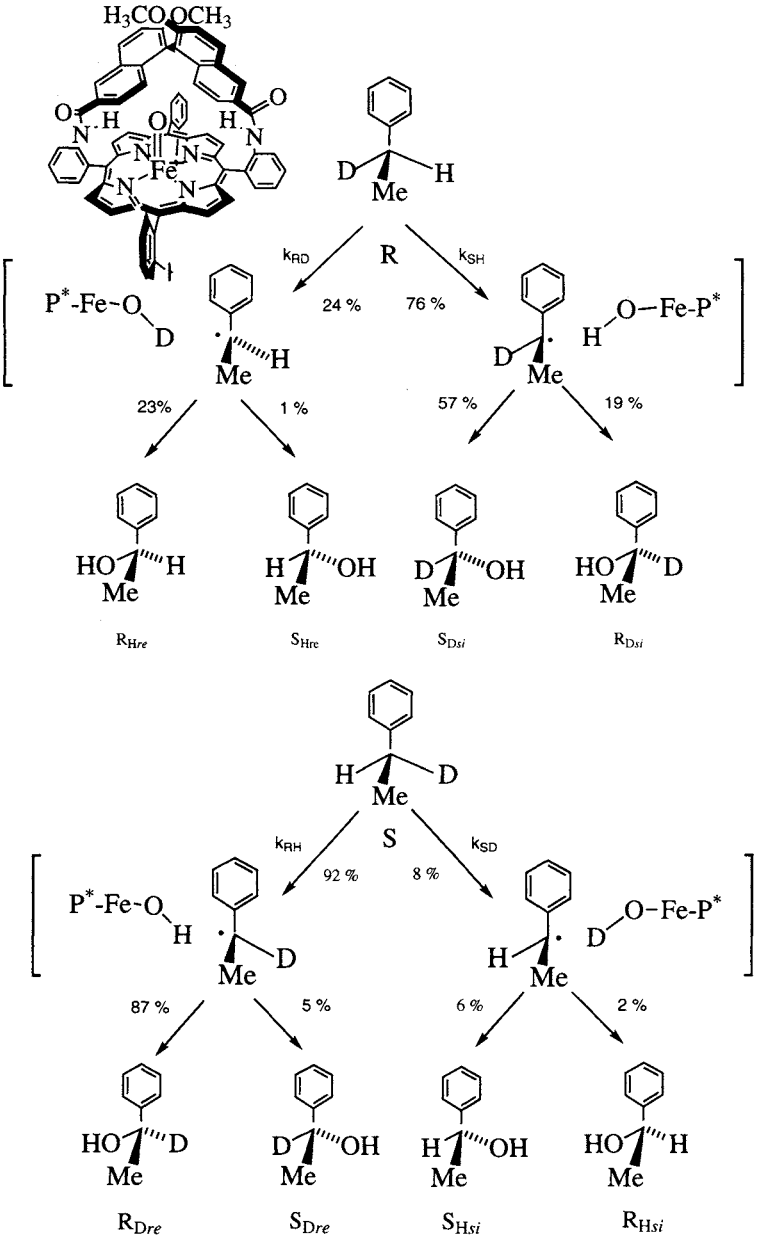


Figure 1.3. Catalyic asymmetric hydroxylation by a chiral, binaphthyl porphyrin. The stereochemical outcome of a hydroxylation depends upon the steric fit of the substrate.

intermolecular encounters[61]. Detailed DFT calculations on this simplest iron–oxo electrophile showed that there was a spin-state crossover during the H–H bond cleavage step to form a species H–Fe–OH^+, and another spin crossover leading to the product $Fe(OH_2)^+$. Thus, the lowest energy pathway for the reaction involved crossing from an initial high-spin, sextet state for the oxidant FeO^+ to a low-spin, quartet state near the transition state for H–H bond cleavage. While such effects are common for first-row elements as, for example, with singlet and triplet carbenes, "spin forbiddenness" has usually been discounted for reactions involving transition metals. However, the successful application of DFT calculations to explain the unusual behavior of FeO^+ suggests that these effects may be significant in the area of oxidative catalysis.

Shaik has applied these considerations to examine interactions of a prototype substrate, methane, with a ferryl intermediate similar to **7** to probe this chemistry of P450[62]. The results are very revealing. The ferryl intermediate was shown to have two nearly isoenergetic electron configurations, doublet and quartet, depending upon whether the unpaired electron in the porphyrin cation radical is ferromagnetically or antiferromagnetically coupled to the triplet ferryl center. Indeed, both situations are known in enzymatic compounds I and model systems. The calculations indicate that the transition state for C–H bond cleavage does look like the extended arrangement **H** in Scheme 1.5. Here, however, the molecular trajectories for the high-spin and low-spin reaction coordinates diverge. For the high-spin pathway, there was a discernable intermediate caged radical state with the carbon center interacting weakly with the iron–hydroxide. A significant energy barrier was found for collapse of this high-spin intermediate to the product via formation of a carbon–oxygen bond. By contrast, the low-spin trajectory could proceed to products without encountering this barrier. This two-state hypothesis could provide a way out of the mechanistic dilemma presented by the radical clock results since the apparent timing of the clocks would depend upon the relative importance of the high- and low-spin pathways that would likely vary from substrate to substrate.

Evidence for short-lived substrate radicals has been presented recently for the oxidation of the mechanistically diagnostic probe molecule norcarane by cytochrome P450[63]. Among the products found with P450 BM3, was 1.3% of the radical rearrangement product hydroxymethylcyclohexene while the cation rearrangement product 3-cycloheptenol was not observed with that isozyme (Figure 1.4). An alternate interpretation of similar data, involving unusual behavior of the probe molecule at the active site, has also been presented[64]. In all known cases of reactions involving a radical intermediate, this norcarane probe produces a product derived from the 3-cyclohexenylmethyl radical, as the major rearrangement product. The rate constant for the radical rearrangement of the 2-norcaranyl radical has been found to be $2 \times 10^8\ s^{-1}$. By contrast, for reactions proceeding through discrete carbocations, rearrangement leads instead to 3-cycloheptenol as the *major rearrangement product.*

The extent of observed rearrangement with a panel of P450 enzymes leads to a radical lifetime in the picosecond to nanosecond regime, certainly long enough to be considered an intermediate (Figure 1.5). A consistent timing was found for several similar probes that were all small, aliphatic hydrocarbons. Smaller amounts of cation-derived products were also observed and were attributed

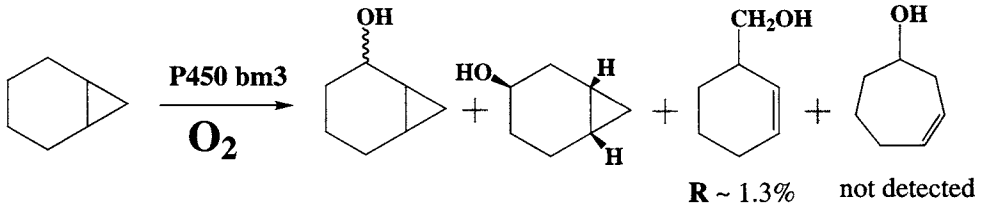


Figure 1.4. Norcarane as a molecular probe of radical intermediates during C–H hydroxylation by cytochrome P450 BM3.

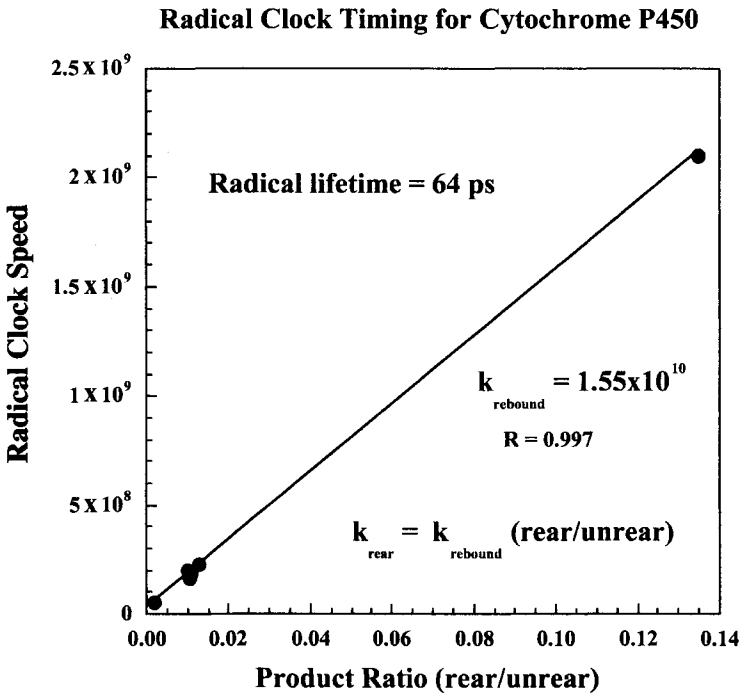


Figure 1.5. Plot of radical rearrangement rate constant vs observed product ratios for P450-mediated hydroxylation of bicyclo[2.1.0]pentane, norcarane, and spiro[2,5]octane.

to a competing electron-transfer oxidation of the incipient radical, a well-precedented process. By contrast, the hydroxylation of norcarane with a ruthenium porphyrin catalyst that proceeds through a reactive oxoruthenium(V) porphyrin intermediate, afforded *no detectable rearrangement.*

DFT calculations on the ruthenium-mediated hydroxylation show that the low-spin reaction trajectory is preferred throughout, in accord with general expectations for the behavior of second-row transition metals[65, 66]. The ruthenium analog was found to be more electrophilic than its iron complex, having lower hydrogen abstraction barriers. Thus, the data for the iron and ruthenium porphyrin systems is in accord with the predictions of theory that a radical rebound process is viable for iron which has an accessible high-spin state but not for ruthenium which is always low-spin.

The hydroxylation of camphor by an oxoferryl porphyrin has also been described by Kamachi and Yoshizawa[67]. While two spin states of the reactive intermediate were also found in this work, it was the high-spin quartet state of the oxoferryl that was lower in energy. Also significant in these calculations, were the findings that there was an interaction between the incipient substrate radical upon hydrogen abstraction and the Fe–OH center at the P450 active site and that there was a 3.3 kcal mol^{-1} activation energy for the highly exothermic radical rebound to form the product alcohol (Figure 1.6(a)). Such an interaction would be expected to *retard* radical rearrangement rates, providing another possible avenue for the mis-timing of the clocks. The reaction profile for the oxygen rebound pathway of cytochrome P450 computed by Shaik is presented in Figure 1.6(b) for comparison. Computations on the details of C–H bond cleavage in camphor by a P450 model described by Friesner have revealed an unusually low energy barrier for this process[68]. The primary contribution to stabilization of the transition state

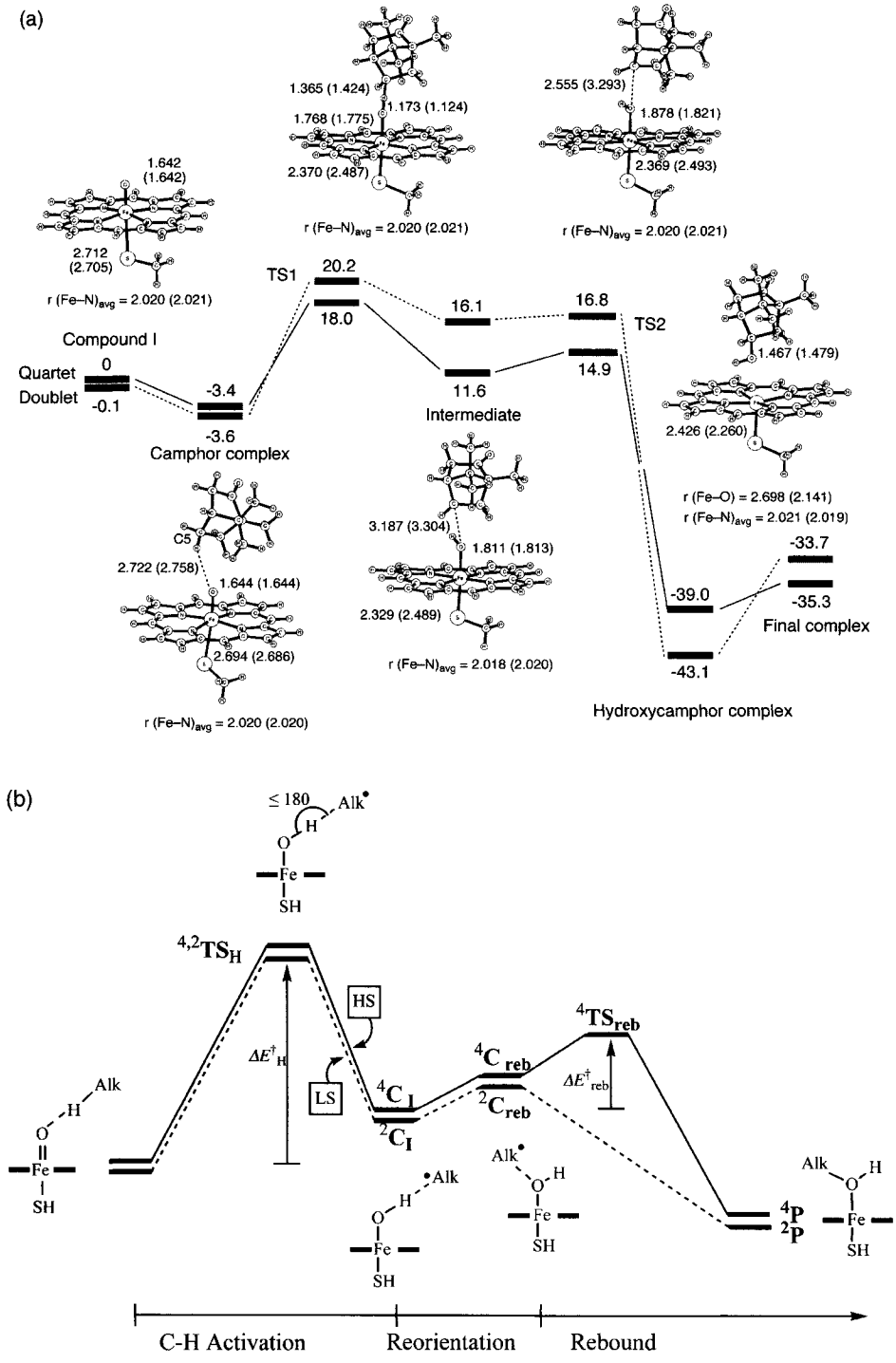


Figure 1.6. (a) Energy level diagram and reaction coordinate computed by Yoshizawa *et al.* for the hydroxylation of camphor by a ferryl–porphyrin cation radical (7). Adapted from ref. [67]. (b) Reaction profile for oxygen rebound computed by Shaik *et al.* Adapted from refs [60], [62] and unpublished material from S. Shaik.

was attributed to the interaction of positively charged residues in the active-site cavity with carboxylate groups on the heme periphery. Additional experiments on oxoferryl species of known electronic configuration would seem to be necessary to address these questions.

Other, more exotic factors such as nonstochastic behavior[69] and tunneling effects[70], could also be involved in causing the mistiming of events during C–H bond hydroxylation. Indeed, a carbene ring-expansion reaction was very recently found to have a large quantum-tunneling effect that significantly affected the observed rate[71]. High-level calculations indicated that a thermal, over-the-barrier process, and quantum tunneling of *carbon* were still competitive even at room temperature. Applied to C–H hydroxylation by a reactive oxidant, this situation could give the appearance of multiple oxidants and non-Arrhenius behavior. Further, computations have suggested that the speed of radical clocks can be made to run fast via interactions with even simple metal ion centers such as Li^+ (ref. [72]). Thus, for a stepwise reaction via the caged radical intermediate in Scheme 1.5, a spectrum of apparent lifetimes, perhaps dependent on such effects as weak dipolar interactions and even vibrational state, might be observed for rebound through transition state **R** to intermediate **8**. Consideration of the energy landscape for C–H hydroxylation (Figure 1.7) suggests that the C–H bond cleavage and concomitant FeO–H bond formation will occur *on a high-energy plateau*, since the scissile C–H bond should be similar in energy to the forming FeO–H bond. Accordingly, the intrinsic exothermicity of the hydroxylation reaction will be expressed in the C–OH bond-forming step. In such a scenario, it becomes more clear as to how small changes in bond energies and weak interactions of the reaction ensemble along the reaction coordinate could have a significant effect on the outcome, for example, positional or stereochemical scrambling, by shifting the position of the transition states along the reaction coordinates.

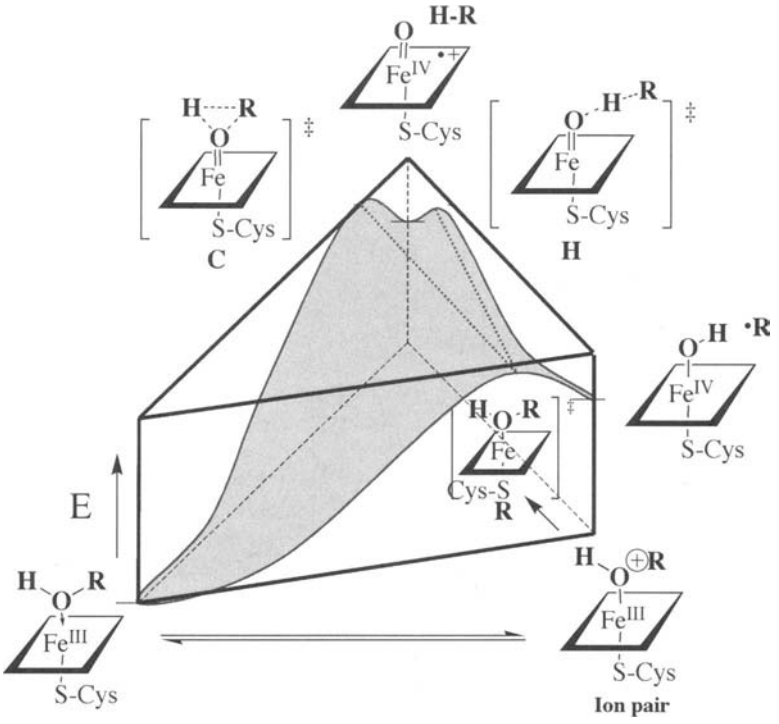


Figure 1.7. Energy landscape for aliphatic hydroxylation by cytochrome P450.

The nonheme diiron hydroxylases, such as methane monooxygenase (MMO)[73] and AlkB, the ω-hydroxylase from *P. putida*, have also yielded to similar structural, spectroscopic, and mechanistic probes. Interestingly, there are striking similarities between the consensus mechanism for the heme and nonheme iron proteins (Figure 1.8). For MMO, the resting enzyme has both iron centers in the ferric state. Reduction and binding of oxygen again produces a peroxo intermediate which is oxidized to a reactive species, compound Q, that has been characterized as a bis-μ-oxoiron(IV) intermediate. Both AlkB[74] and MMO[64, 75] have been interrogated recently with the diagnostic probe norcarane and both have shown the radical rearrangement product, hydroxymethylcyclohexene. With MMO, it was possible to show that it was the reactive intermediate Q that was interacting with the substrate probe. For the histidine-rich hydroxylase, AlkB, the results were particularly striking since 15% of the product was indicative of the radical rearrangement pathway. Similar

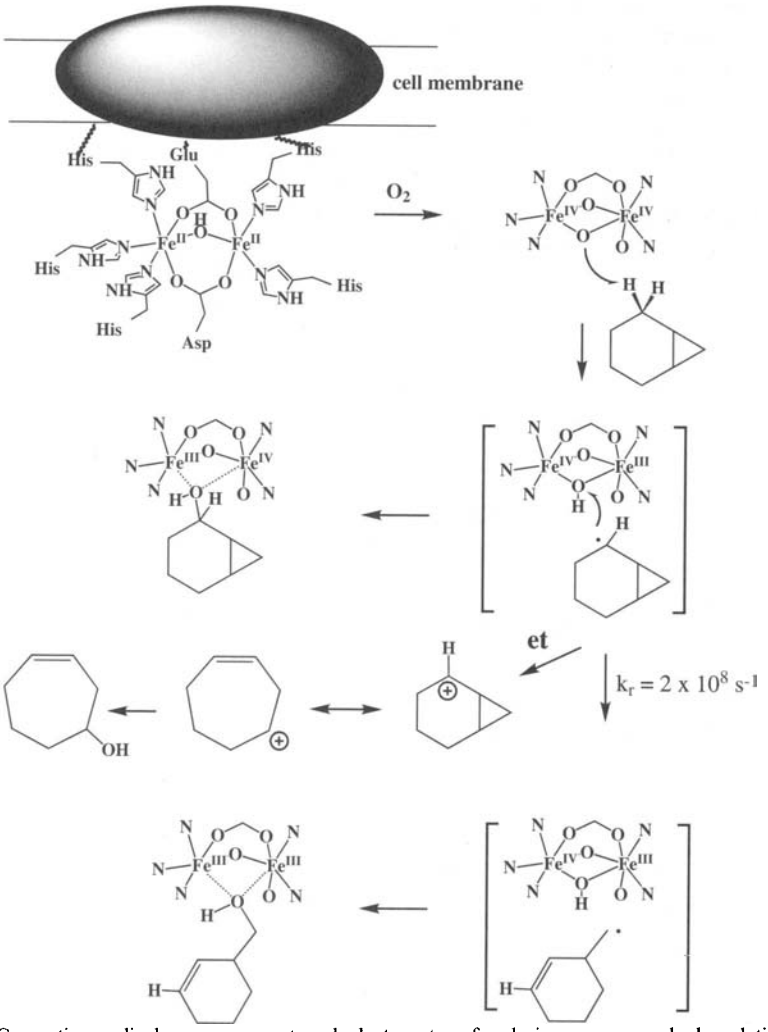


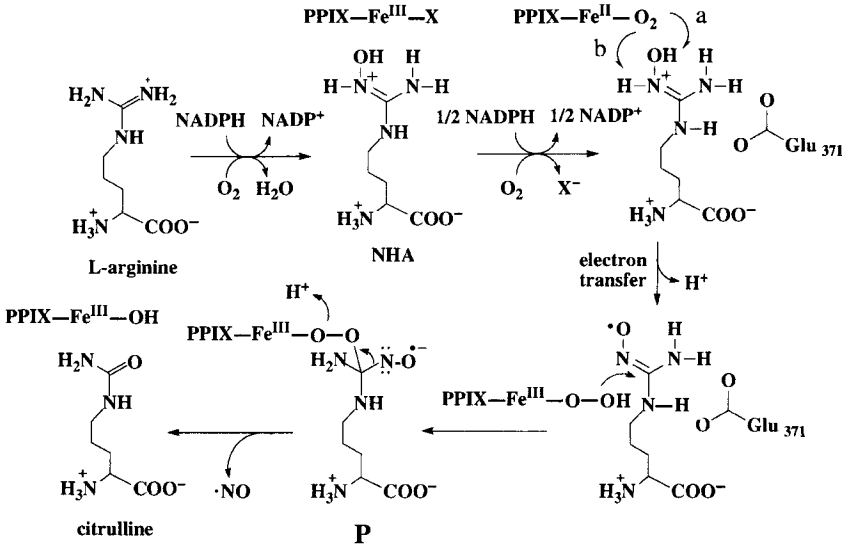
Figure 1.8. Competing radical rearrangement and electron transfer during norcarane hydroxylation by the histidine-rich hydroxylase XylM.

results have been obtained recently for the related histidine-rich, diiron hydroxylase XylM[76]. A significant aspect of this work was that it was performed on whole cells and clones into which the AlkB and XylM genes had been introduced. Thus, mechanistically informative biochemistry can be obtained from this type of biological screen.

5. On the Mechanism of Nitric Oxide Synthase

Nitric oxide (NO) is produced by the heme-containing metalloenzyme NOS (EC 1.14.13.39). Several NOS isoforms are homodimers with each monomer containing binding sites for NADPH, FMN, FAD, calmodulin, tetrahydrobiopterin (H4B), and a heme group[6]. Similar proteins are found in animals, plants, and bacteria indicating that this is a widely distributed and highly conserved process in nature. The H4B cofactor is especially important, serving structural, allosteric, and redox functions[77–80]. The X-ray crystal structures of substrate-bound NOS show that both the substrate and H4B are bound at the heme site with a substantial network of hydrogen bonds[11–13]. NOS catalyzes the two-step, five-electron oxidation of L-arginine via *N*-hydroxyarginine (NHA) to citrulline and NO (Scheme 1.6). The initial *N*-hydroxylation of L-Arg to NHA by O_2 is similar to the *C*-hydroxylations of P450 described above. The second step of the NOS reaction is unusual because it is a three-electron, aerobic oxidation of NHA to NO and citrulline[81, 82].

Our current understanding of these processes is constrained by the fact that the consensus mechanism (Scheme 1.6) contains several unknown intermediates and unprecedented processes in the second step. There have been a number of significant recent advances in the mechanistic enzymology of NOS and the structures of the enzyme–substrate complexes. However, while these results have provided confirmation of the basic tenets for the *N*-hydroxylation of arginine in the first part of the consensus mechanism, the results raise important questions regarding the oxidation of NHA and the release of NO. Thus, Poulos has shown that the X-ray structure of NOS with NO bound to the heme iron center as a structural surrogate for O_2, places the NO oxygen within hydrogen-bonding distance to the ω-N–H[13]. This juxtaposition provides support for the notion that the arginine proton assists the heterolysis of the FeO–O bond during oxygen activation to afford the ferryl intermediate in a P450-like process. However, the same structure would have



Scheme 1.6.

difficulty accommodating both the hydroxyl group of NHA where it would have to be, and the O_2 of the next cycle. Significantly, very recent EPR/ENDOR results by Hoffman *et al.*[83] have indicated that the incipient hydroxyl of NHA is formed *bound to the heme iron* in a manner similar to C–H hydroxylation by P450. This unsuspected arrangement is consistent with an oxygen rebound scenario but inconsistent with the X-ray structure of NHA bound to the active site of NOS obtained by Tainer *et al.*[11], which shows the *N*-hydroxy group to be displayed away from the heme iron. Thus, it appears that NHA, as biosynthesized from arginine, may be formed in *a non-equilibrium configuration* with respect to the NOS heme active site. In this light, the observation by Silverman[84] that oxime ethers of the type NHA–OR are active substrates for NOS and that NO is produced from these species is very informative. The NHA–NOS crystal structure suggests that NHA–OR derivatives can be accommodated at the active site in the configuration shown in Figure 1.9[85]. Thus, the O–H of NHA may not be mechanistically significant because the mechanism of the *N*-hydroxylation of L-Arg to afford NHA is still available to the oxime ethers.

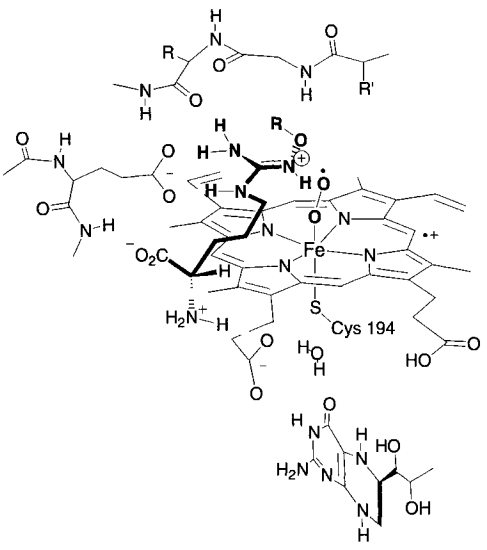


Figure 1.9. Structure suggested for the active site of RO-NHA-bound murine iNOS.

6. Synthetic Oxometalloporphyrins as Models for Cytochrome P450

Studies using synthetic metalloporphyrins (Figure 1.10) as models for cytochrome P450 have afforded important insights into the nature of the enzymatic processes[86, 87]. Indeed, each of the intermediates shown in Scheme 1.1 has been independently identified by model studies using synthetic analogs, especially *meso*-tetraaryl porphyrins[86, 88].

The first report of a simple iron porphyrin system that effected stereospecific olefin epoxidation and alkane hydroxylation was reported in 1979 (Scheme 1.7). This system introduced the use of iodosylbenzene as an oxygen-transfer agent to mimic the chemistry of cytochrome P450[89].

It was later discovered that the reactive intermediates in the iron porphyrin model systems were high-valent oxoiron porphyrin complexes. A green oxoiron(IV) porphyrin cation radical species (**13**) has been well characterized by various spectroscopic techniques, including visible spectroscopy, NMR, EPR, Mössbauer, and EXAFS (Figure 1.11)[90–98]. It has recently been shown by Nam and Que that the oxygen-atom transfer from certain iodosylarenes is reversible with some iron porphyrins. For the case of 1,2-difluoro-4-iodobenzene both an oxoferryl species and an iodosyl–ferric species were observed to be in equilibrium[99].

A family of oxoiron(IV) porphyrin cation radical species (**13**) with different axial ligands has been reported, each of which displayed a characteristic ^{1}H–NMR for the pyrrole protons. Addition of methanol replaced each of these ligands with a solvent molecule ($\delta = -22.8$)[100]. A report of the imidazole and *p*-nitrophenolate complexes of **13** has also appeared, affording closer model complexes for the compounds I of peroxidase and catalase, respectively[101]. The oxoiron(IV) porphyrin cation radical complexes **13** were shown to be highly reactive as oxygen-atom transfer agents toward olefins and hydrocarbons, that is, reacting with olefins to afford epoxides and with alkanes to give alcohols[102–104]. Notably, **13**-4-Me-Im was also reactive toward norbornene, giving a 67% yield of the corresponding epoxide[100]. Significant variations have been observed in the reactivity and

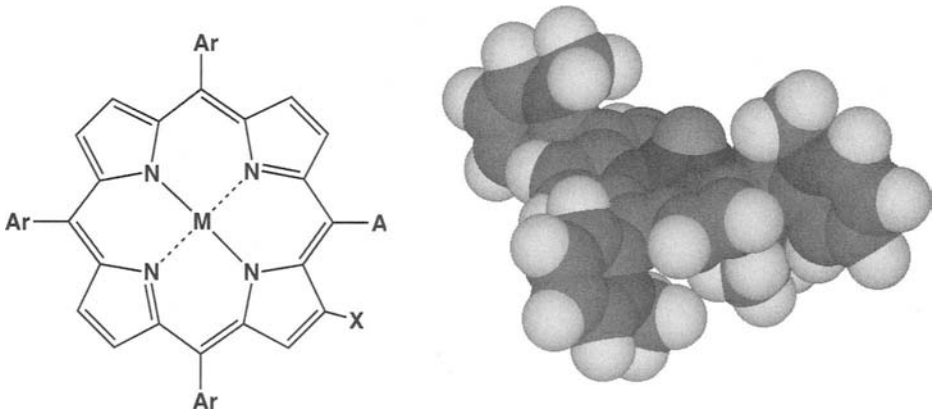
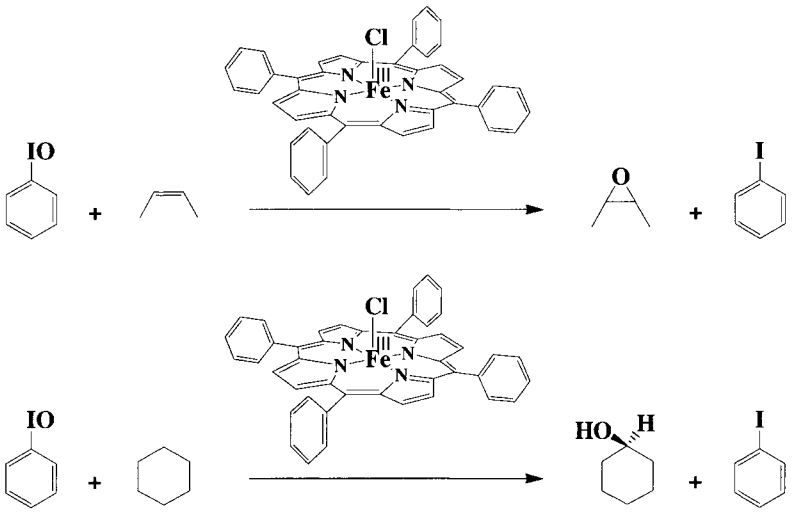
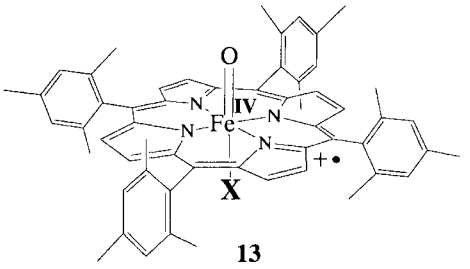


Figure 1.10. Typical synthetic tetraaryl porphyrins.



Scheme 1.7. Olefin epoxidation and alkane hydroxylation catalyzed by an iron porphyrin, $Fe^{III}(TPP)Cl$.



13

X = Cl δ = -9.01
O-Bz δ = -15.6
OMe δ = -16.2
4-Me-Im δ = -13.7

Figure 1.11. The family of oxoiron(IV) porphyrin cation radical species, $Fe(IV)(O)(TMP)^{+\bullet}(X)$ and the characteristic proton NMR resonances of the pyrrole protons.

selectivity of the complex **13** as a function of the axial ligand[105–107]. By contrast, the reactivity and stereospecificity of the corresponding oxoiron(IV) porphyrin complexes were low[86, 108, 109].

Nam has described studies using observed changes in product ratios and ^{18}O-labeling to suggest that *both* oxoferryl complexes such as **13** and the Fe(III)–O–X precursors are reactive oxidants[48, 110]. The nature of the anionic ligand was shown to affect both product selectivities and the efficiency of ^{18}O exchange. It is difficult, however, to discern the cause of the changes observed, since the two-oxidant scenario proposed by Nam and the anionic ligand effect on the reactivity of the oxoferryl complex itself described by Gross, both would seem to explain the results. The two most-well-characterized intermediates, $Fe(IV)(O)(por)^{+\bullet}(X)$, ("compound I") and (por)Fe(IV)=O ("compound II") are known to react with olefins to afford epoxides with different stereoselectivities. The former is known to produce a high *cis/trans* ratio of epoxide from *cis* olefins, while the latter gives mostly *trans* epoxide via a stepwise process. The effect of axial ligands would then be on the *lifetime* of $[(por)Fe(IV)=O]^{+\bullet}$ (high *cis/trans* epoxide ratio), which easily decays to (por)Fe(IV)=O (low *cis/trans* epoxide ratio). Thus, while an iron(III)(por)–peroxyacid complex has been demonstrated to be reactive toward organic substrates such as olefins, as discussed above, there is no unambiguous evidence as yet from the model studies that a hydroperoxoiron(III) porphyrin species, HOO–Fe(III)(por), is a reactive, electrophilic oxidant.

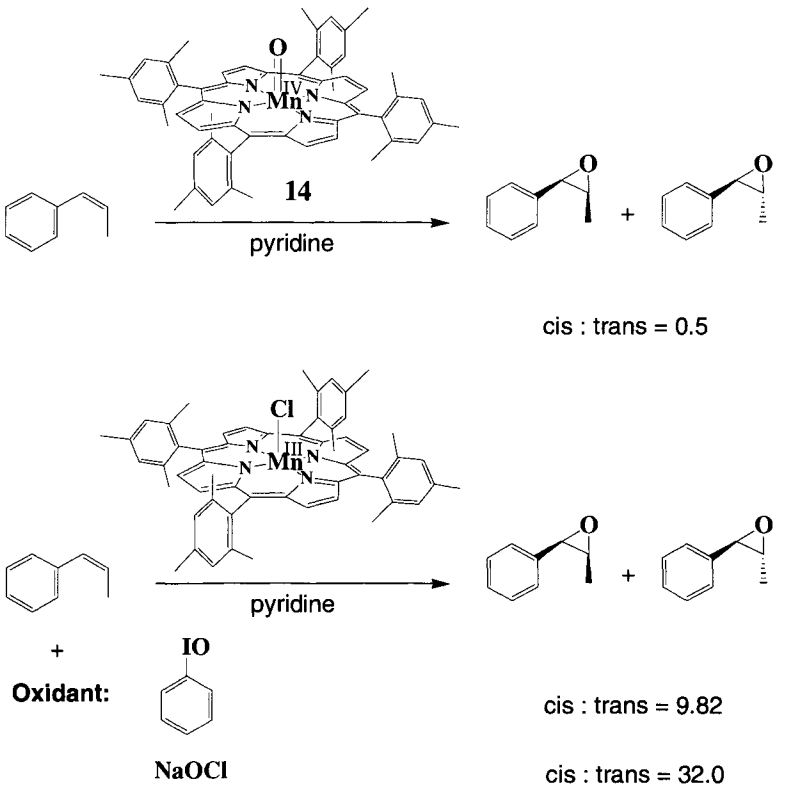
7. Manganese Porphyrins in Catalytic Oxidations

Manganese porphyrins have been shown to have unusually high reactivity toward olefin epoxidation and alkane hydroxylation[111–117]. However, the physical characteristics of the putative oxomanganese(V) porphyrin species remained particularly elusive[90, 118] because of its high reactivity and transient nature. Stable oxomanganese(V) complexes are few, the only examples involving the use of tetraanionic ligands to stabilize the high-valent manganese center[119–122].

Structurally related to the porphyrins, manganese salen catalysts have shown wide applicability for the epoxidation of unfunctionalized olefins. First described by Kochi[123], this system has been particularly effective for the asymmetric epoxidation of prochiral olefins with readily available complexes such as **11**[124]. Evidence for an oxomanganese(V) salen complex[125] and an oxoiron(IV) salen complex $[O{=}Fe(IV)(salen)]^{\bullet+}$(ref. [126]) have been presented. The area has been thoroughly reviewed[127, 128]. The reader is also referred to the growing literature on high-valent metallocorroles[129–132].

The intermediacy of reactive oxomanganese(V) porphyrin complexes has long been implicated in olefin epoxidation and alkane hydroxylation because of the distinct reactivity patterns and $H_2{}^{18}O$-exchange behavior[133–139], as compared to that of the relatively stable oxomanganese(IV) porphyrin **14** intermediates which have been isolated and well characterized[118, 140]. The oxomanganese(IV) porphyrin complex transferred oxygen to olefins with little stereoselectivity, while a transient oxomanganese(V) complex underwent oxygen transfer to olefins with predominant retention of configuration (Scheme 1.8)[134, 141]. Further, the oxomanganese(IV) species exchanged the oxo ligand with water slowly while the positively charged oxomanganese(V) complex readily exchanged the oxo ligand with added ^{18}O water[134].

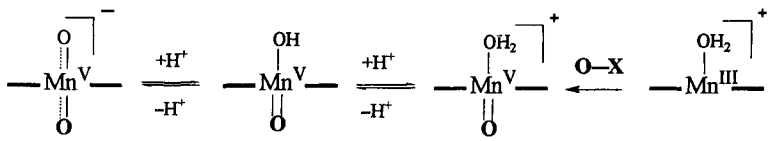
Oxometalloporphyrin studies in aqueous media have allowed the study of reactive intermediates and metal–oxo–aqua interchange. The small peptide–porphyrin fragment, microperoxidase 8, has afforded evidence of reactive metal–oxo intermediates upon reaction with oxidants such as hydrogen peroxide[142, 143]. Important insights into this oxo–hydroxo tautomerism were first reported by Meunier[18, 137–139, 144]. It was shown in these studies that metal–oxo species are able to transfer an oxygen atom originating from either the oxygen source or from water. Because the intermolecular exchange of metal–oxo with water is slow, an intramolecular exchange of labeled oxygen atoms occurred, which is reminiscent of a carboxylic acid. This mechanism involves a rapid, prototropic equilibrium, probably via a *trans*-dioxoMn(V) intermediate[145], that interconverts an oxo group on one face of the metalloporphyrin with an aqua or hydroxo group on the other face. This type of rearrangement was revealed by performing a catalytic oxygenation catalyzed by a manganese porphyrin in



Scheme 1.8. Reactivity and stereoselectivity of oxomanganese(IV) and oxomanganese(V) (generated by Mn(III) and oxidants *in situ*) in olefin epoxidation.

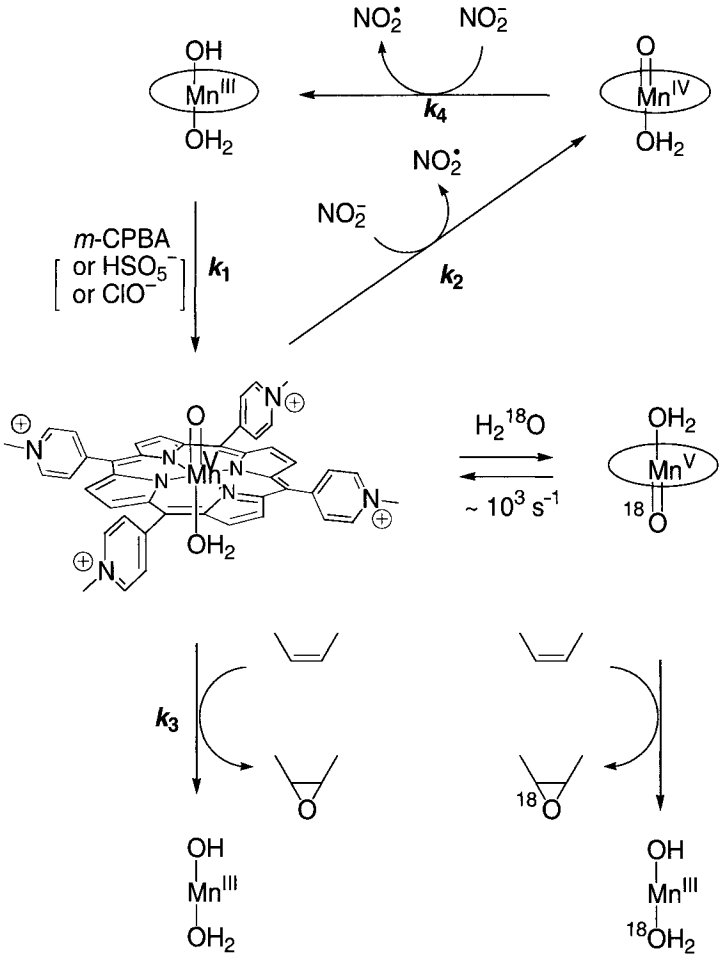
^{18}O-labeled water but with a ^{16}O oxidant such a Oxone or a peroxyacid. The oxo–hydroxy tautomerism would allow as much as 50% water-derived ^{18}O oxygen transfer to a substrate, while the other 50% of the oxygen would derive from the peroxide.

$[Mn^{III}(TMPyP)]$ with a variety of oxidants, *m*-CPBA, HSO_5^-, and ClO^-, has been shown to produce the same, short-lived intermediate. An oxoMn(V) porphyrin structure was assigned to this intermediate. The rate of formation of oxoMn(V) from $Mn^{III}(TMPyP)$ followed second-order kinetics,



The first direct detection of an oxomanganese(V) porphyrin intermediate under ambient catalytic conditions was achieved by using rapid-mixing stopped-flow techniques[46, 146]. A direct assessment of its reactivity in both one-electron and oxygen-transfer processes was made possible by these observations. The reaction of tetra-*N*-methyl-4-pyridylporphyrinatomanganese(III) first-order in Mn(III) porphyrin, and first-order in oxidant. The rate constants have the following order: *m*-CPBA ($2.7 \times 10^7\ M^{-1}\ s^{-1}$) > HSO_5^- ($6.9 \times 10^5\ M^{-1}\ s^{-1}$) ~$ClO^-$ ($6.3 \times 10^5\ M^{-1}\ s^{-1}$). Once formed, the intermediate oxoMn(V) species was rapidly converted to oxoMn(IV) by one-electron reduction with a first-order rate constant of $5.7\ s^{-1}$. The oxoMn(IV) species was relatively

stable under the reaction conditions and was shown by EPR spectroscopy to have a high-spin quartet ground state. The one-electron reduction of oxoMn(V) to oxoMn(IV) was greatly accelerated by nitrite ion ($k = 1.5 \times 10^7\ M^{-1}\ s^{-1}$). However, the reaction between nitrite and oxoMn(IV) is much slower. The oxoMn(V) intermediate was shown to be highly reactive toward olefins, affording epoxide products. In the presence of carbamazepine, a water-soluble olefin, this oxygen transfer was extraordinarily rapid with a second-order rate constant of $6.5 \times 10^5\ M^{-1}\ s^{-1}$). By contrast, oxoMn(IV) was not capable of effecting the same reaction under these conditions. With *m*-CPBA as the oxidant in the presence of $H_2{}^{18}O$, the product epoxide was shown to contain 35% ^{18}O, consistent with an O-exchange-labile oxoMn(V) intermediate. Nitrite ion inhibited the epoxidation reaction competitively by one-electron reduction of the oxoMn(V) intermediate to the unreactive oxoMn(IV). In this way, it was possible to show that the oxo–aqua exchange rate was about $10^3\ s^{-1}$ for the *coordinated water*. Significantly, bulk water exchange was slower than oxo-transfer to the olefin under these conditions. This simple observation explains a body of often confusing data and claims in the literature in this field.

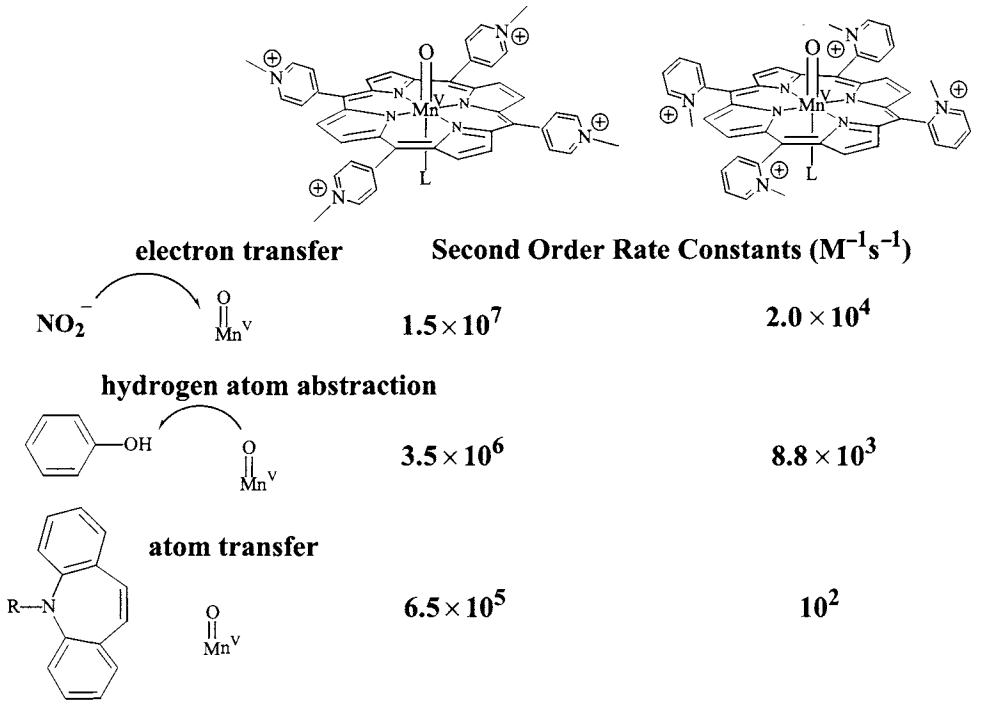


Scheme 1.9. (a) Rate constants: $k_1 = 2.7 \times 10^7\ M^{-1}\ s^{-1}$; $k_2 = 1.5 \times 10^7\ M^{-1}\ s^{-1}$; $k_3 = 6.5 \times 10^5\ M^{-1}\ s^{-1}$; $k_4 = 1.4 \times 10^2\ M^{-1}\ s^{-1}$; ^{18}O exchange $\sim 10^3\ s^{-1}$. (b) ^{18}O incorporation: 35% ^{18}O labeling from the solvent (95% $H_2{}^{18}O$) into the epoxide.

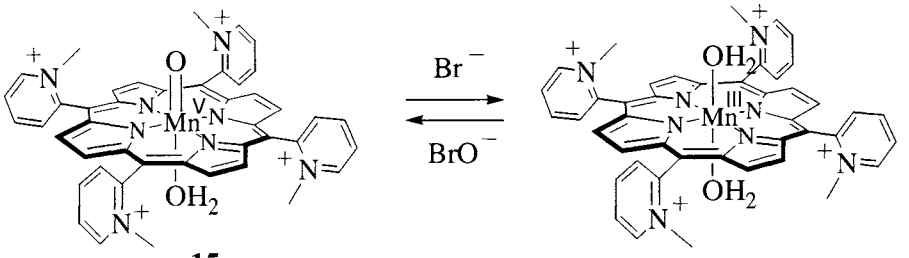
Peroxyacids will not exchange the peroxo oxygen with water at all and neither does the product epoxide. Thus, in those cases for which ^{18}O-incorporation is observed into the product epoxides or alcohols, an oxo–metal species in strongly implicated as the oxygen donor. However, failure to see ^{18}O-exchange is not a definitive result since the exchange rate may simply be too slow to compete with oxygen-atom transfer. These transformations and the ^{18}O-exchange process are summarized in Scheme 1.9. It has been shown that hydrogen peroxide is also an effective oxidant of water-soluble manganese porphyrins, affording a reactive oxoMn(V) intermediate as well[147]. With water-soluble iron porphyrins, hydrogen peroxide was able to epoxidize olefins[148]. Significantly, the efficiency of epoxidation dropped drastically above pH 5, suggesting that an acid-catalyzed heterolytic O–O bond cleavage is part of the oxygen-transfer process.

Surprisingly, the 2-*N*-methyl pyridyl isomer oxoMn(V)(TM-2-PyP) was found to be unusually stable allowing its characterization by ^1H–NMR[46]. Reaction rate constants for oxoMn(V)(TM-2-PyP) toward electron transfer, hydrogen atom abstraction, and epoxidation were all *several orders of magnitude slower* than the corresponding 4-*N*-methylpyridyl species (Scheme 1.10). This unusual effect, which did not appear in the corresponding reactions of the Mn(IV) and Mn(III) states, was ascribed to the low spin, d^2 electronic configuration of oxoMn(V). Thus, a spin-state crossover is required during reduction with the promotion of an electron from d_{xy} to $d_{xz,yz}$.

Oxomanganese(V)-5,10,15,20-tetrakis(*N*-methyl-2-pyridyl)-porphyrin (**15**) was found to transfer its oxo ligand efficiently to the bromide ion. Furthermore, this oxo transfer is *rapid and reversible*[145]. The forward reaction mimics the halide oxidation reaction catalyzed by haloperoxidases, while the reverse reaction is the catalyst activation step in substrate oxygenation by manganese porphyrins. This well-behaved equilibrium has allowed the assignment of a free energy change for this reaction and is the first clear determination of the thermodynamics of an oxo transfer reaction for a metal catalyst of this type. As can be seen in the Nernst plot in Figure 1.12, the



Scheme 1.10. Reaction rates for OxoMn(V)TM-4-PyP and OxoMnT(Y)M-2-PyP.



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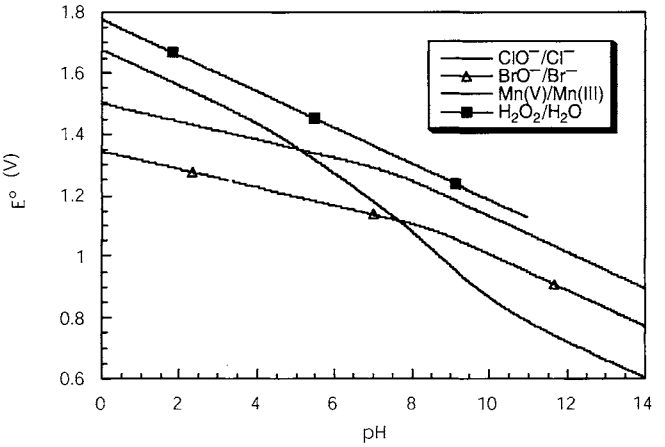


Figure 1.12. Reversible oxygen transfer from hypobromite provided an energy calibration for oxomanganese(V) porphyrins.

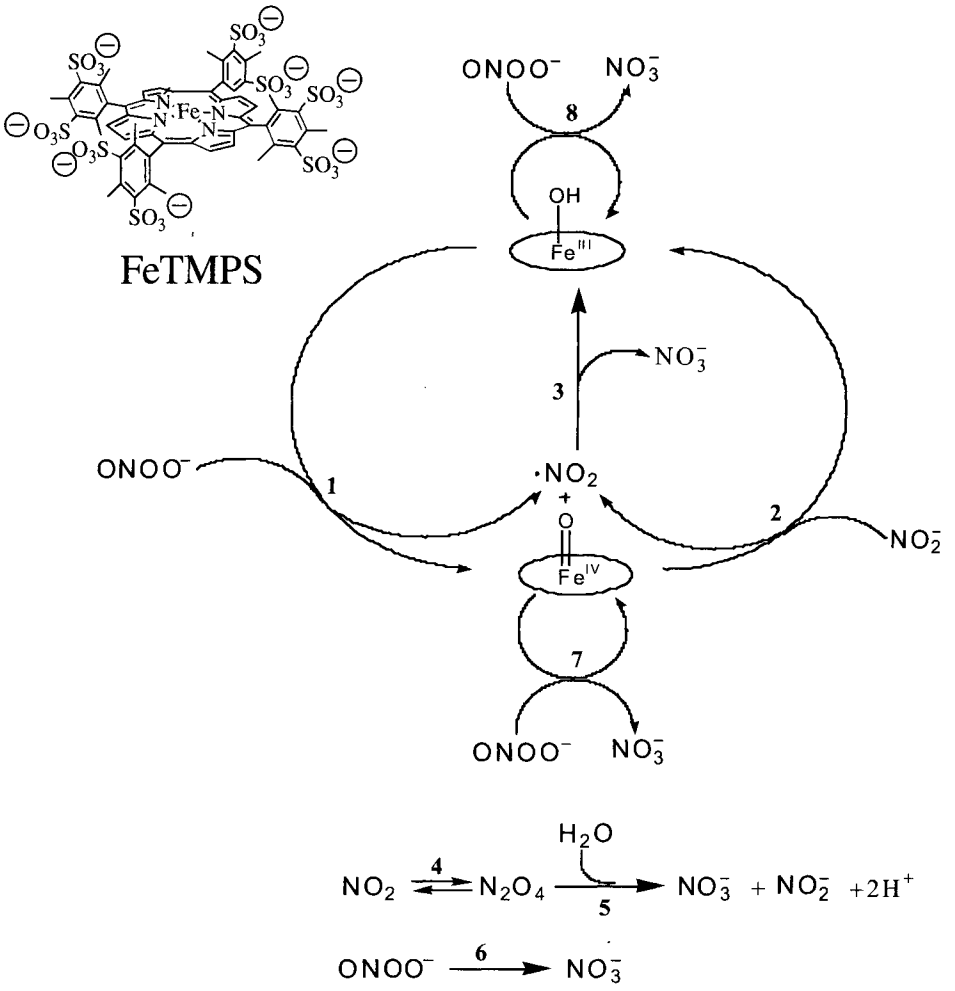
oxoMn(V) species is able to oxygenate bromide ion below pH 8 and chloride ion below pH 5, the increase in oxidizing power being due to the protonation of the oxoMn(V) intermediate. As can be seen, this species is tantalizingly close to the oxidation potential of water/hydrogen peroxide at low pH.

8. Metalloporphyrins as Detectors and Decomposition Catalysts of Peroxynitrite

The rapid reaction rates of peroxides with metalloporphyrins and the detection of observable oxometalloporphyrin species, especially in the reactions of manganese porphyrins with various oxidants (*m*-CPBA, NaOCl, $KHSO_5$)[146], have also inspired the use of these porphyrins as detectors for a transient biological oxidant, peroxynitrite ($ONOO^-$). $ONOO^-$, a strong one- and two-electron oxidant, has been implicated in a number of pathological conditions[149]. Structurally, $ONOO^-$ is the conjugate base of peroxynitrous acid and, like other similar peroxides, it rapidly oxidizes manganese porphyrins[136, 150, 151]. Thus, its reactions with manganese porphyrins to generate oxomanganese intermediates allowed the sensitive detection of this transient molecule under cell-like conditions[136, 150]. Using manganese porphyrins as $ONOO^-$ detectors, it has been shown that $ONOO^-$ can quickly diffuse across biological membranes and react with its biological targets[150, 152].

The fast oxidation of manganese(III) porphyrins to oxomanganese(IV) species by $ONOO^-$ satisfied the prerequisite for the porphyrins to be efficient $ONOO^-$ decomposition catalysts[150]. Indeed, manganese porphyrins, redox-coupled with biological antioxidants (such as ascorbate,

glutathione, Trolox®), rapidly reduce $ONOO^-$ and thus prevent the oxidation and nitration of biological substrates by this toxic oxidant[151, 153]. Amphiphilic analogs of the water-soluble metalloporphyrins have been developed that are suitable for liposomal delivery[154]. Further, these amphiphilic metalloporphyrins in sterically stabilized liposomes are highly active in decomposing $ONOO^-$ (ref. [154]), and thus are potential therapeutic agents for the treatment of $ONOO^-$-related diseases. Iron(III) porphyrins are also rapidly oxidized by $ONOO^-$, and Stern *et al.* have reported that water-soluble iron porphyrins have the ability to decompose $ONOO^-$ by isomerizing it to nitrate and these compounds, moreover, have shown significant biological responses in animals[155]. The mechanism of action of the iron porphyrins is significantly different than the manganese case. Significantly, *both* Fe(III) and oxoFe(IV) states are catalytically active toward $ONOO^-$ (Scheme 1.11)[156, 157]. Compounds of this sort have been shown to be highly potent in animal models of inflammation related pathologies such as diabetes and colitis[158].



Scheme 1.11. Mechanism of peroxynitrite decomposition by FeTMPS.

9. Synthetic Metalloporphyrins as Stereoselective Catalysts

Understanding the mechanism of cytochrome P450 through the study of the synthetic model systems offers an opportunity to develop practical regioselective and stereoselective catalysts. Such catalytic systems have been extensively surveyed in numerous reviews[14, 20, 86, 159–162]. Space does not permit a discussion of all the elegant catalysts developed, but a few systems are shown here as examples.

By analogy to the natural enzymes which utilize a protein scaffold to effect substrate recognition and stereoselectivity, special steric features have been introduced into the synthetic porphyrin catalysts to achieve regio- and enantioselective oxidation. The success of such systems relies on the steric interactions between the substrates and porphyrin catalysts, which position the substrates specifically toward the reactive metal–oxo center. Breslow *et al.*[163, 164] have recently reported a remarkably selective, catalytic steroid hydroxylation using an artificial cytochrome P450 enzyme. The synthetic strategy to induce selectivity in the model system was the attachment of four cyclodextrin appendages to a synthetic manganese porphyrin used in place of the heme center of the natural enzyme. These donut shaped heptamylose sugars have a hydrophobic central cavity, which is known to bind aromatic molecules. The substrate steroid was modified with such an aromatic group at either end of the molecule. The host–guest complex obtained from these designed partners, Figure 1.13, displays a limited region of the substrate steroid in the vicinity of the catalytic manganese center. What is most significant about this artificial enzyme is the fact that hydroxylation occurred *only* at carbon 6 to give the 6α-hydroxysteroid, even though there are many sites in this molecule with similar intrinsic reactivity. This high selectivity was found to depend critically on the precise arrangement of the aromatic groups of the substrate. Moreover, the manganese porphyrin–cyclodextrin construct was able to release the product sterol and hydroxylate at least four steroid molecules. Catalytic turnover with such high positional selectivity is highly unusual for this kind of model system[167].

Collman *et al.* reported a series of "picket basket" porphyrins (Figure 1.14) which show extremely high shape selectivity (>1,000 for *cis*-2-octene vs *cis*-cyclooctene) and relatively high enantioselectivity in olefin epoxidation (*ee* around 70–85%)[159, 166]. Chiral, binaphthyl straps between adjacent *o*-aminophenyl groups have afforded very good enantiomeric selectivities for styrene epoxidations[167].

The first use of a chiral porphyrin to carry out asymmetric epoxidation was reported in 1983, giving 50% *ee* with *p*-chlorostyrene[168]. The *ee* was improved to ~70% for epoxidation of *cis*-β-methylstyrene with the use of a very robust chiral vaulted binaphthyl porphyrin **16** (Figure 1.15)[169, 170]. More significantly, this catalyst afforded the first case of catalytic asymmetric hydroxylation by a model system, giving a ~70% *ee* for hydroxylation of ethylbenzene and related hydrocarbons[169, 170].

Further developments of this system have led to studies of the binaphthyl-peptide-strapped porphyrin **17**[171]. For styrene epoxidation this chiral catalyst afforded an *ee* greater than 90% in the initial stages of the reaction, with the (*R*)-styrene oxide predominating. NMR T_1 relaxation studies with the copper(II) derivative of the same ligand

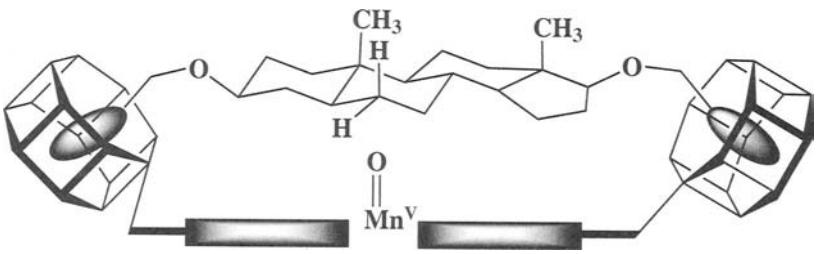


Figure 1.13. Steroid-manganese porphyrin host–guest complex.

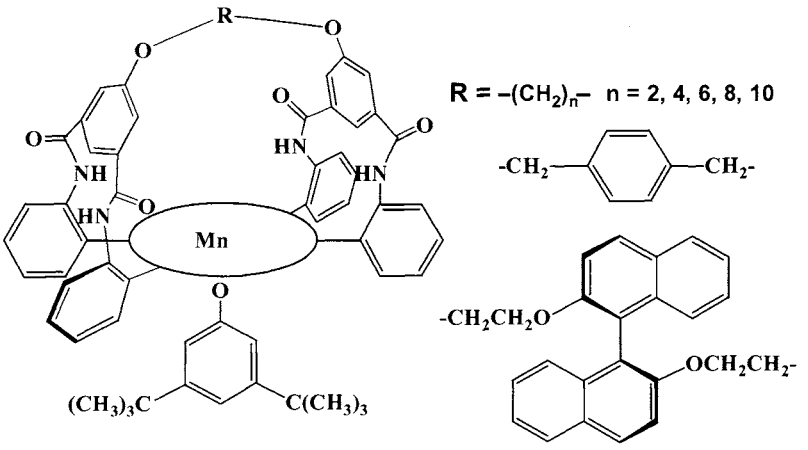


Figure 1.14. Chiral "picnic basket" porphyrins.

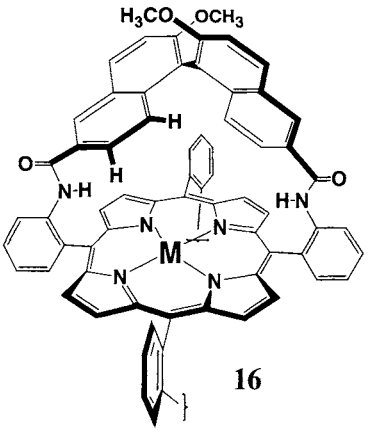


Figure 1.15. A chiral vaulted binaphthyl metalloporphyrin.

showed that it was the protons of the major enantiomer of the product epoxide that were more efficiently relaxed in an inversion-recovery experiment with **Cu-17**. Comparative T_1 data are shown in Figure 1.16. Accordingly, the major product must fit better into the chiral cavity of this catalyst. Thus, one can conclude that the enantioselectivity observed can be attributed to a simple lock-and-key process in which the transition state for epoxidation must look rather like the coordinated epoxide.

An interesting functional active-site model system for prostaglandin H synthase has recently been described by Naruta[172, 173]. In this "twin coronet" structural motif, four pendant hydroxynaphthyl groups provide a sterically encumbered, hydrophobic substrate-binding cavity (Figure 1.17). Upon treatment with *m*-chloroperoxybenzoic acid an oxoiron(IV)–naphthyloxy radical species was formed and detected by EPR. This radical intermediate reacted with a 1,4-diene and oxygen in a single-turnover reaction to afford 5-*trans*-7-*cis*-undeca-5,7-diene-4-ol in 156% yield. The formation of the aryloxy radical was shown to be essential to this conversion in close analogy to the enzymatic process.

10. Ruthenium Porphyrins in Oxidative Catalysis

The controlled oxygenation of alkanes, alkenes, and aromatic hydrocarbons is one of the most important technologies for the conversion of crude oil and natural gas to valuable commodity chemicals[174]. Biomimetic studies of metalloporphyrins have led to important advances in practical catalysis, especially with ruthenium porphyrins. Reaction of *m*-CPBA, periodate, or iodosylbenzene with Ru(II)(TMP)(CO) produced Ru(VI)(TMP)$(O)_2$[175]. Remarkably, Ru(VI)(TMP)$(O)_2$ was found to catalyze the aerobic epoxidation of olefins under mild conditions[176]. Thus, for a number of olefins including cyclooctene, norbornene, *cis*-, and *trans*-β-methyl styrene 16–45 equivalents of epoxide were

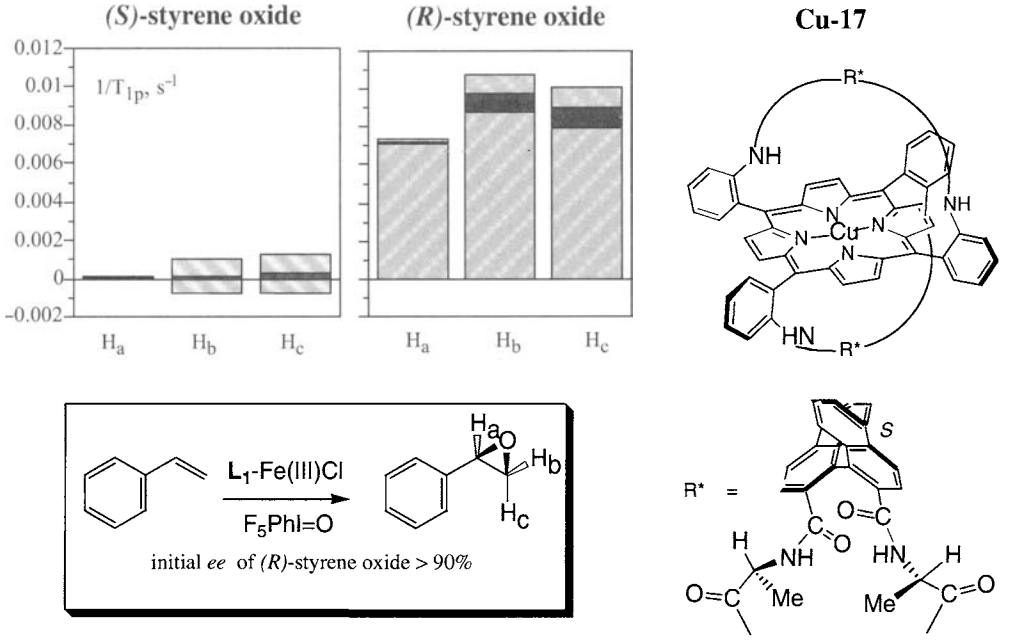


Figure 1.16. NMR T_1 relaxation data compared to stereoselectivity.

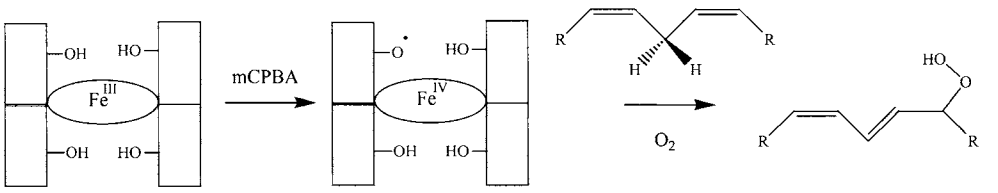


Figure 1.17. Twin coronet model prostaglandin H synthase.

produced per equivalent of the ruthenium porphyrin complex over a 24-hr period at room temperature under 1 atm of dioxygen.

Dioxygen activation by Ru(II) porphyrins is likely to involve the formation of a μ-peroxo-ruthenium(III) dimer followed by homolysis of the O–O bond to give two equivalent of oxoruthenium(IV) species. Nakamoto *et al.*[177, 178] detected such a μ-peroxoruthenium(III) intermediate by resonance Raman spectroscopy (ν_s(O=Ru) at 552 cm^{-1}) in the toluene solution of Ru(II)(TPP) saturated with O_2 at −80°C. Upon raising the temperature of the solution a ν_s(O=Ru=O) band corresponding to Ru(VI)(TMP)$(O)_2$ appeared at 811 cm^{-1}.

X-ray data obtained for a closely related Ru(VI)(TDCPP)$(O)_2$, definitively established a structural precedent for the *trans*-dioxoRu(VI) porphyrin complexes[179]. The structure revealed a nearly planar arrangement of the porphyrin macrocycle and two relatively long Ru=O bonds of 1.729 Å (Figure 1.18). Marchon and coworkers have published an X-ray structure of *trans*-dihydroxoruthenium(IV) complex of H_2TDCPP[180], also obtained from the reaction of Ru(II)(TDCPP)(CO) with *m*-CPBA. The IR spectrum of the *trans*-dihydroxoruthenium(IV) complex was reported to be different from that of the *trans*-dioxoruthenium(VI) complex. Thus, Ru(IV)(TDCPP)$(OH)_2$ showed a Ru–O stretch at 760 cm^{-1} in the IR spectrum

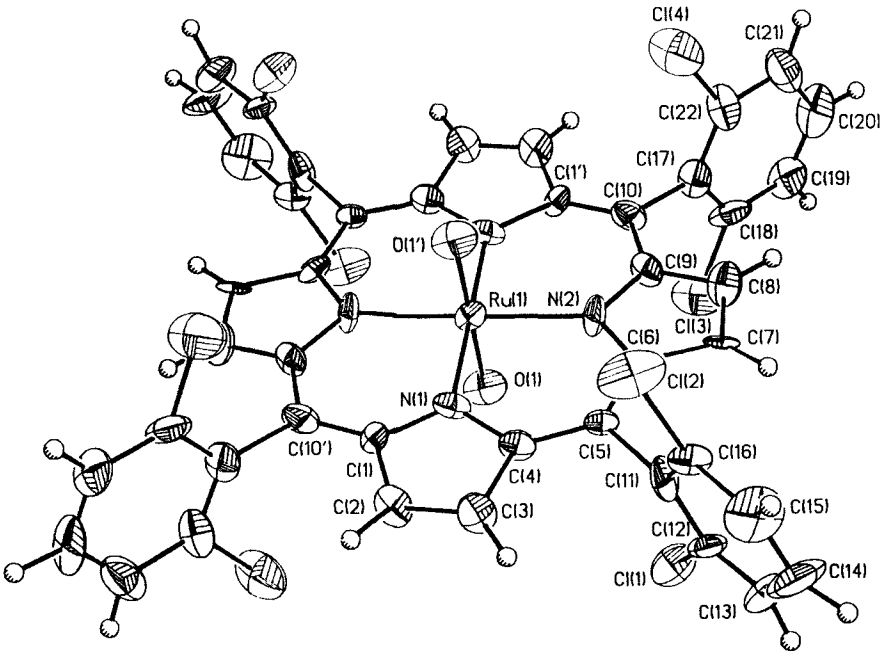


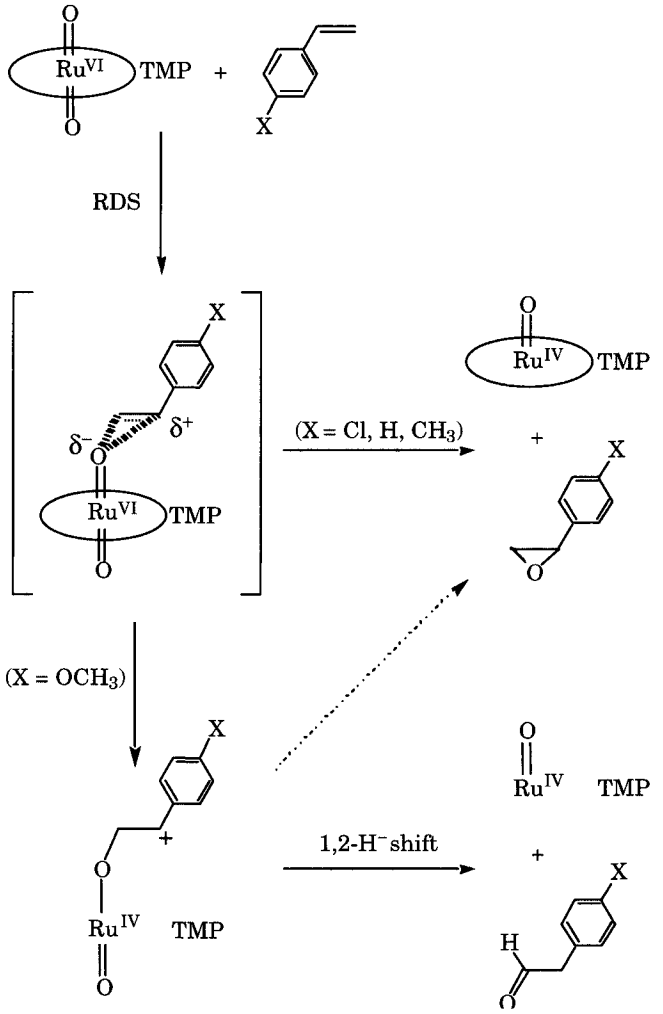
Figure 1.18. Molecular structure of $Ru(VI)(TDCPP)(O)_2$.

while the Ru=O band of $Ru(VI)(TDCPP)(O)_2$ appeared at 828 cm^{-1}. The length of the Ru–O bonds in the X-ray structure of $Ru^{IV}(TDCPP)(OH)_2$ was 1.790 Å which is somewhat longer but not significantly different from the length of the Ru=O bonds in $Ru(VI)(TDCPP)(O)_2$[179].

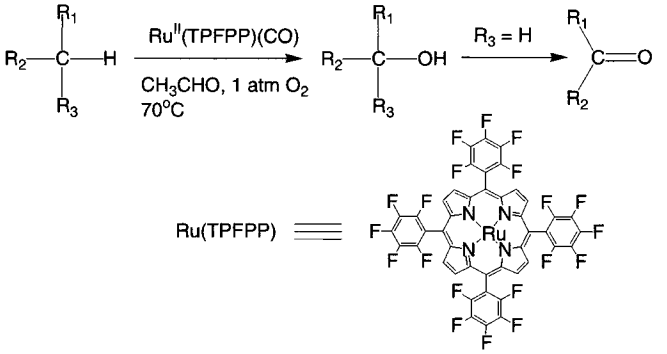
Nonlinear, upward U-shaped Hammett plots were obtained for the stoichiometric oxidation of substituted styrenes with $Ru(VI)(OEP)(O)_2$ and $Ru(VI)(TPP)(O)_2$. The shape of the Hammett correlations indicated a change in the structure of the transition state of epoxidation with these complexes when switching from electron-donating to electron-withdrawing substituents[181]. However, the Hammett treatment of data for a series of competitive aerobic oxidations of *para*-substituted styrenes catalyzed by $Ru(VI)(TMP)(O)_2$ gave a good linear correlation with a ρ^+ value of -0.93 against σ^+ set of parameters[179]. Only *para*-nitrostyrene deviated from the linear trend. This ρ^+ value was typical of the results obtained for iron and manganese-porphyrin-mediated epoxidations with iodosyl arenes and hypochlorite, suggesting a similar, relatively small charge separation in the transition states of these reactions[30, 166, 182, 183]. Significantly, in epoxidations with the $Ru(VI)(TPP)(O)_2/O_2$ system, the electron-rich styrenes gave more *para*-substituted phenylacetaldehyde byproducts than the electron-poor styrenes. For example, 19.5 equiv of the aldehyde and 3.8 equiv of the epoxide was produced in the oxygenation of methoxystyrene while the epoxidation of chlorostyrene produced less than 1 equiv of the aldehyde and 37 equiv of the chlorostyrene oxide based on catalyst. In order to explain these data, a mechanism has been proposed (Scheme 1.12) in which the rate-limiting-step of the initial partial charge transfer is separated from the product-forming step. Phenylacetaldehydes are believed to be formed by a 1,2-hydride shift of the cationic intermediate. This intermediate would be more favored by electron-donating *para*-substituents in agreement with the distribution of products.

Murahashi and coworkers have studied the aerobic oxidation of alkanes catalyzed by polyfluorinated metalloporphyrins including $Ru^{II}(TPFPP)(CO)$ in the presence of acetaldehyde (Scheme 1.13)[184, 185].

The catalytic oxygenation of cyclohexane and adamantane at 70°C under 1 atm dioxygen gave *c.* 200 turnovers in 24 hr. The ruthenium porphyrin



Scheme 1.12. Proposed mechanism of oxygen-atom transfer from Ru(VI)(TMP)$(O)_2$ to substituted styrenes.



Scheme 1.13. Aerobic oxidation catalyzed by Ru(II)(TPFPP)CO, see references 184 and 185.

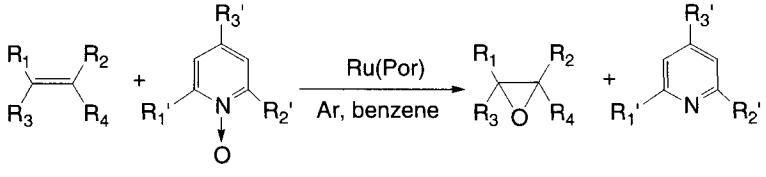
demonstrated high stability toward oxidative degradation with turnover numbers as high as 14,000, achieved in the oxygenation of cyclohexane when a low catalyst concentration was used. The normalized 3°/2° C–H bond preference in oxidation of adamantane with this system was about 20. Competitive experiments with cyclohexane and cyclohexane-d_{12} resulted in an isotope effect of 13. The mechanism of catalysis was not clear. It is known that the reaction of aldehydes with dioxygen in the presence of metal complexes produces peroxyacids[186]. The authors suggested an oxometal species which is formed by reaction of peroxyacid with ruthenium porphyrin as the active intermediate although no good evidence in favor of such species was presented. The possibility of a radical autoxidation mechanism cannot be excluded.

Labinger and coworkers[187] have investigated the catalysis of aerobic olefin oxidation by a highly electron-deficient perhalogenated ruthenium porphyrin complex $Ru^{II}(TPFPPPCl_8)(CO)$. The oxidation of cyclohexene under 1 atm dioxygen at room temperature proceeded mostly in the allylic positions affording 58% 2-cyclohexen-1-ol and 27% 2-cyclohexen-1-one along with only 15% of cyclohexene oxide. Three hundred turnovers of oxidation were achieved in 24 hr and 90% of the catalyst was recovered at the end of the reaction. Oxygenation of styrene gave benzaldehyde (3 turnovers in 24 hr) as the only product. By contrast, the oxidation of cyclooctene produced cyclooctene oxide as the major product (42 turnovers in 24 hr). With iodosylbenzene as oxidant $Ru^{II}(TPFPPPCl_8)(CO)$ demonstrated significantly higher selectivity for epoxidation with 81% styrene oxide and 42% cyclohexene oxide produced, based on the total amounts of oxidation products. The *trans*-dioxoruthenium(VI) complex $Ru^{VI}(TPFPPPCl_8)(O)_2$ prepared by oxidation of $Ru^{II}(TPFPPPCl_8)(CO)$ with *m*-CPBA was found to be a less efficient catalyst for the aerobic oxygenations and therefore an unlikely intermediate in the catalytic cycle. The authors noted that the distribution of products in a cyclohexene oxygenation with $Ru^{II}(TPFPPPCl_8)(CO)$ was identical to the ratio of allylic oxidation vs epoxidation products obtained in the aerobic oxidation with $Fe^{III}(TPFPPBr_8)Cl$[188]. For the iron porphyrin catalyst these reactions have been established to follow a radical autoxidation mechanism[188, 189].

Nitrous oxide is produced as a byproduct in multimillion lb/year quantities in nylon manufacture worldwide. Currently, there is a great interest toward the utilization of N_2O due to the environmentally hazardous nature of this gas with respect to the greenhouse effect and ozone layer depletion. In addition to their ability to utilize dioxygen for catalytic hydrocarbon oxidations, ruthenium porphyrins have been shown to activate nitrous oxide[190] which is an extremely inert molecule[191] and a poor ligand.[192a] Groves and Roman have found that N_2O reacted with $Ru^{II}(TMP)(THF)_2$ in toluene to produce $Ru^{VI}(TMP)(O)_2$[190]. The *trans*-dioxoRu(VI) complex can in turn epoxidize a suitable substrate such as *trans*-β-methyl styrene. This system was subsequently shown to be catalytic under appropriate conditions[192b].

Hirobe and coworkers were the first to use an unusual class of oxidants, 2,6-disubstituted pyridine *N*-oxides, in conjunction with ruthenium porphyrins for oxidation of olefins (Scheme 1.14), alcohols, and sulfides[193–197].

The epoxidation of styrene with 2,6-dichloropyridine *N*-oxide was efficiently catalyzed by of $Ru(VI)(TMP)(O)_2$ or Ru(II)(TMP)(CO) to afford styrene oxide with high selectivity and nearly quantitative yields based on substrate used[196]. Moderate yields of the epoxide (26%) were obtained with Ru(II)(TPP)(CO). Turnover numbers up to 16,500 were achieved in epoxidation of norbornene in the presence of catalytic amounts of $Ru(VI)(TMP)(O)_2$. The catalytic reactions were



Scheme 1.14. RuPor = Ru(II)(TMP)(CO), Ru(II)(TPP)(CO) or $Ru(VI)(TMP)(O)_2$.

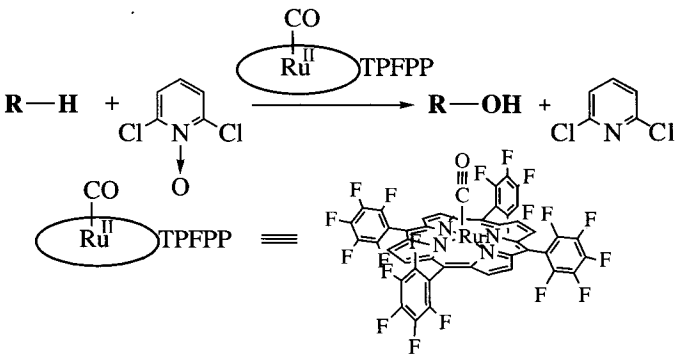
characterized by high chemoselectivity and stereospecificity. For example, when a 1:1 mixture of *cis*- and *trans*-stilbene was reacted with 2,6-lutidine *N*-oxide in the presence of Ru(VI)(TMP)$(O)_2$, *cis*-stilbene oxide was produced in 87% yield, while only 1% of *trans*-stilbene was epoxidized[198]. Further, the *cis* disubstituted double bond of *trans,cis,trans*-1,5,9-cyclododecatriene was predominantly oxidized under these conditions. *Cis*-stilbene was stereospecifically epoxidized with 2,4,6-trimethylpyridine. Only ruthenium porphyrins displayed catalytic activity with respect to the oxygen transfer from pyridine *N*-oxides. Other metalloporphyrins, such as Mn, Fe, Co, Mo, and Rh porphyrin complexes, were found to be inactive with these oxidants[193, 196].

While alkanes and aliphatic alcohols were inert with respect to the RuPor/*N*-oxide system, the catalytic activity of ruthenium porphyrins was dramatically enhanced in the presence of protic acids such as HBr[196, 198, 199]. Hydroxylation of adamantane with 2,6-dichloropyridine *N*-oxide in the presence of Ru(II)(TMP)(CO) and HBr afforded over 100,000 turnovers of the tertiary C–H oxidation products, 1-adamantanol and 1,3-adamantanediol, with a turnover frequency of 5.6 turnovers s^{-1}. Methylcyclohexane was selectively hydroxylated to give 1-methylcyclohexanol in 94% yield. *Cis*-9-decalol was the major product in oxidation of *cis*-decalin with no stereoisomer *trans*-9-decalol detected. Oxygenation of a relatively inactive alkane such as cyclooctanol afforded *c.* 300 turnovers of cyclooctanone (77% yield based on substrate)[196].

Nagano *et al.* have studied the oxidation of steroids with 2,6-dichloropyridine *N*-oxide catalyzed by Ru(II)(TPP)(CO), Ru(II)(TMP)(CO), and Ru(II)(TDCPP)(CO) in the presence of HBr[200]. Tertiary C–H positions of steroidal substrates were hydroxylated with complete retention of configuration of the asymmetric centers. The regioselectivity of oxidation significantly depended on the steric bulk of the *ortho* substituents of the aromatic rings at the *meso* position of the porphyrin catalysts and on the electronic properties of the porphyrins.

A very promising system for hydrocarbon oxygenation with 2,6-dichloropyridine *N*-oxide in the presence of electron-deficient, polyhalogenated ruthenium porphyrins has been reported[201, 202]. The oxidations with this system were characterized by high rates and selectivity even under mild conditions and, most significantly, could be carried out in a nonacidic reaction media. Carbonyl (5,10,15,20-tetrapentafluorophenylporphyrinato) ruthenium(II)—Ru(II)(TPFPP)(CO) showed unusually high activity with 2,6-dichloropyridine *N*-oxide as the oxygen donor (Scheme 1.15).

Adamantane and *cis*-decalin were hydroxylated with high selectivity, complete stereo-retention, extraordinarily high rates (up to 800 turnovers min^{-1}), and high efficiency with up to 15,000 turnovers. Similar conversions were obtained when Ru(VI)(TPFPP)$(O)_2$ and Ru(VI)(TPFPBr_8P)$(O)_2$ were used as catalysts. Oxygenation of less reactive substrates such as benzene and cyclohexane proceeded with lower but still significant turnover numbers (100–3,000). Tertiary vs secondary selectivity in adamantane oxidation was above 210. No rearrangement products were detected in *cis*-decalin hydroxylation.



Scheme 1.15. Fast catalytic hydroxylation with a fluorophenylruthenium porphyrin.

The kinetics of product evolution in a typical reaction of adamantane hydroxylation showed an initial induction period followed by a fast, apparently zero-order phase with the maximum rate and highest efficiencies (Figure 1.19). Deviation from linear behavior took place only after 90% of the oxygen donor and 80% of the substrate had been consumed. When Ru(VI)(TPFPP)$(O)_2$, prepared by reaction of Ru(II)(TPFPP)(CO) with 3-chloroperoxybenzoic acid was used as the catalyst, no induction time was detected and zero-order kinetics were observed as well. The well-defined and characteristic UV–vis spectra of metalloporphyrins provide an invaluable tool for the mechanistic studies. Thus, monitoring the state of the metalloporphyrin catalysts during the course of both model reactions by UV–vis spectroscopy revealed that the initial form of the catalyst remained the predominant one throughout the oxidation, that is, in the Ru(II)(TPFPP)(CO)-catalyzed reaction *c.* 80% of the porphyrin catalyst existed as Ru(II)(TPFPP)(CO) and in Ru(VI)(TPFPP)$(O)_2$-catalyzed reaction more than 90% of the catalyst was still in the form of Ru(VI)(TPFPP)$(O)_2$ despite the high turnover numbers reached (~400 turnovers). The fact that Ru(II)(TPFPP)(CO) demonstrates similar and even higher maximum turnover rate of 4.9 turnovers min^{-1} in adamantane hydroxylation vs 4.0 turnovers min^{-1} for Ru(VI)(TPFPP)$(O)_2$ under the same conditions indicates that an active catalyst species *other than* Ru(VI)(TPFPP)$(O)_2$ is involved in the fast catalytic hydroxylation.

Therefore, a typical *trans*-dioxoRu(VI)–oxoRu(IV) catalytic cycle can be ruled out as the primary reaction pathway in case of rapid catalytic oxygenation. The apparent zero-order kinetics observed are consistent with a steady-state catalytic regime accessible from different initial states of ruthenium metalloporphyrin. Indeed, common oxidants, other than aromatic *N*-oxides, such as iodosylbenzene, magnesium monoperoxyphthalate, Oxone®, and tetrabutylammonium periodate produced the *trans*-dioxoRu(VI) species from Ru(II)(TPFPP)(CO) under reaction conditions but were ineffective for the rapid catalysis.

A two-electron oxidation of Ru(II)(TPFPP)(CO) would produce oxoRu(IV) porphyrin and eventually dioxoRu(VI). What is the alternative pathway for Ru^{II}(TPFPP)(CO) activation? It is known that ruthenium(II) π-cation radicals are formed from the corresponding carbonyl compounds by chemical or electrochemical one-electron oxidation[203]. Such species have been

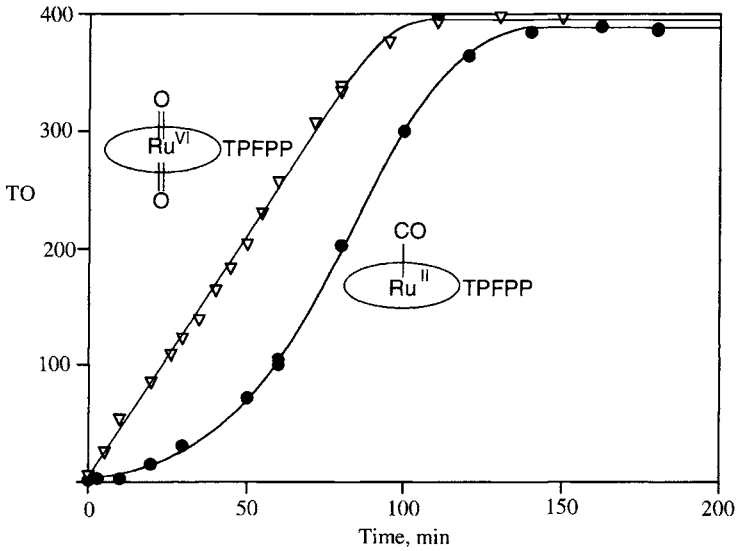


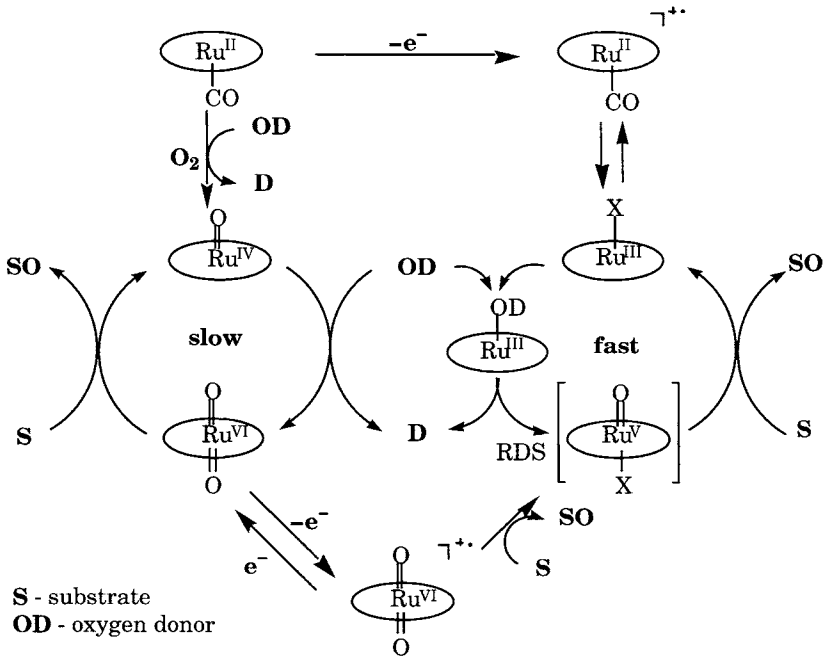
Figure 1.19. Adamantane hydroxylation catalyzed by Ru(II)(TPFPP)(CO) and Ru(VI)(TPFPP)$(O)_2$, [adamantane] = [$pyCl_2NO$] = 0.02 M, [catalyst] = 50 μM, CH_2Cl_2, 40°C. TO (Turnovers) = moles of product/moles of catalyst.

shown to undergo intramolecular electron transfer upon axial ligation and removal of CO to give ruthenium(III) porphyrins[204]. An emerald green solution of Ru(II)(TPFPP)(CO)$^{+\bullet}$ radical cation with a strong EPR signal (g = 2.00) was quantitatively obtained when Ru(II)(TPFPP)(CO) was oxidized with ferric perchlorate in methylene chloride. An EPR signal typical of a ruthenium(III) species ($g_{\|}$ = 2.55, $g_{\perp}$ = 2.05) was detected after the addition of 2,6-lutidine *N*-oxide to the solution of the radical cation.

Metastable Ru(III) and oxoRu(V) species have been proposed as key intermediates in the catalytic cycle of "rapid oxygenation" which can be viewed as a part of the general scheme of diverse oxidative chemistry of ruthenium porphyrins (Scheme 1.16). The aerobic oxygenation pathway, involving the even oxidation states, is shown in the left half of Scheme 1.16. The new fast catalytic process on the right half of the figure depicts the chemical interconnectivity between the fast and slow catalytic regimes.

Enantioselective epoxidation of unfunctionalized olefins is an important area of asymmetric synthesis[185, 205, 206]. Metalloporphyrins represent an attractive foundation for the design of the new chiral epoxidation catalysts due to their high intrinsic stability with respect to oxidative degradation. Thus, extremely large turnover numbers in the oxygenation of hydrocarbons, approaching 10^5–10^6, have been achieved with achiral metalloporphyrin catalysts[199, 207].

Gross *et al.* reported the first use of a chiral ruthenium porphyrin Ru^{II}(L_1)(CO) as a catalyst for styrene epoxidation[208]. Chiral ruthenium porphyrin systems have also been reported by Che *et al.*[209, 210]. The utilization of another chiral ruthenium porphyrin, Ru^{II}(L_2)(CO) as a catalyst for enantioselective epoxidation of olefins with 2,6-dichloropyridine *N*-oxide has been described by Berkessel and Frauenkron[211]. The highest enantiomeric excesses of the oxiranes were obtained in the epoxidation of tetrahydronaphthalene and styrene, 77% and 70% *ee*, respectively, with high yields (up to 88%). Terminal aliphatic olefins and *trans*-disubstituted olefins, represented by 1-octene and *trans*-stilbene, were sluggish substrates and gave low *ee*'s. The epoxidation of tetrahydronaphthalene with iodosylbenzene catalyzed by Ru(II)(L_2)(CO) produced only 52% *ee*



Scheme 1.16. Mechanisms of ruthenium porphyrin oxidation catalysis.

of the epoxide. Interestingly, the results were similar to those published by Gross *et al.*[107] in that the enantioselectivity of epoxidation with Ru(II)(L_2)(CO) was different when iodosylbenzene or pyridine *N*-oxides were used as primary oxidants but did not depend on the nature of the pyridine *N*-oxide. Under the comparable reaction conditions ~72% *ee* of tetrahydronaphthalene oxide was reported for the catalytic oxidation of tetrahydronaphthalene with 2,6-dichloropyridine *N*-oxide, 2,6-dibromopyridine *N*-oxide, and *N*-methylmorpholine *N*-oxide.

11. Conclusion

Cytochrome P450 has been called the Rosetta Stone of iron proteins. Perhaps nowhere else in the biological sciences has the rich interplay between structural, spectroscopic, mechanistic, computational, and chemical modeling techniques led to such a detailed level of understanding of such an important system. The central paradigm of biological oxygen activation is now recognized to involve the formation a ferryl, or oxoiron intermediate. Oxoiron(IV) porphyrin cation radicals have been observed in peroxidase, cytochrome oxidase, CPO, cytochrome P450, and in a variety of model systems. Model system studies, especially those of iron, manganese, and ruthenium porphyrins and related ligands, have led to important advances in catalysis and in catalytic asymmetric oxygenation. Advances in computational studies of such complex, open-shell systems have begun to provide a rigorous physical underpinning for the body of complex and sometimes confusing experimental results. In this chapter, I have tried to weave together all of these aspects to provide for the reader a unified picture of the current understanding in the field of cytochrome P450 research. More detailed presentations are to be found in the chapters that follow.

Acknowledgments

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2

Computational Approaches to Cytochrome P450 Function

Sason Shaik and Samuël P. De Visser

This chapter describes computational strategies for investigating the species in the catalytic cycle of the enzyme cytochrome P450, and the mechanisms of its main processes: alkane hydroxylation, alkene epoxidation, arene hydroxylation, and sulfoxidation. The methods reviewed are molecular mechanical (MM)-based approaches (used e.g., to study substrate docking), quantum mechanical (QM) and QM/MM calculations (used to study electronic structure and mechanism).

1. Introduction

The action of cytochrome P450 (P450) enzymes has been for years a very active arena of research that led to important insights, and generated lively debates over the nature of the various species and their reactivity patterns[1]. The active species of the enzyme is based on an iron ligated to a protoporphyrin IX macrocycle and two additional axial ligands (Figure 2.1): one, called proximal, is a thiolate from a cysteinate side chain of the protein and the other, called distal, is a variable ligand that changes during the cycle. When the distal ligand becomes an oxo group, the species is called Compound I (Cpd I), which is the reactive species of the enzyme and one of the most potent oxidants known in nature.

One of the pioneering theoretical studies on Cpd I was carried out by Loew *et al.*[2], using an empirical QM method to calculate electronic structure and derive the Mössbauer parameters for the species. However, it was only after the development of density functional theoretic (DFT) methods that QM theory came of age and offered a tool that combines reasonable accuracy with speed. More recently, even better tools became available when DFT calculations were combined with MM approaches, leading to hybrid QM/MM methods that enable the study of active species in their native protein environment. All these developments have had a considerable impact on the field and an ever-growing surge of theoretical activity. It was therefore deemed timely to review the results and insights provided by these methods. The chapter starts with a very brief summary of the theoretical methods, and follows with a description of the various species in the catalytic cycle and the main mechanisms by which the reactive species of the enzyme transfers oxygen into organic compounds. As a matter of policy, the main emphasis of the chapter is on QM-based methods; while other methods are mentioned, they receive less coverage.

2. Methods

The recent monograph of Cramer[3] provides an excellent exposition of the theoretical methods,

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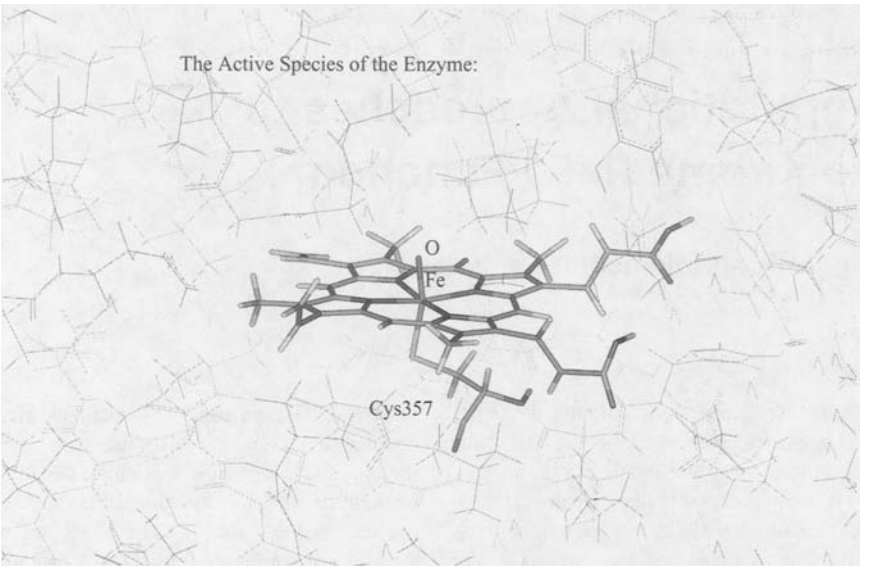

Figure 2.1. The active site of Cytochrome P450, showing Cpd I.

and the interested reader may consult this book for further details than the meager summary given here. There are three generic theoretical categories: *ab initio* methods, semiempirical methods, and MM methods. Both *ab initio* and semiempirical methods derive from QM theory; they provide electronic structure information and can be applied to the study of complete reaction pathways. By contrast, MM is a classical method: it provides only three dimensional structures and energies, and is limited to the study of related structures, for example, conformers. In *ab initio* methods, once one selects a basis set (a set of functions) that describes the atomic orbitals of the atoms in the molecule, the functional (in DFT) and everything else is calculated starting from scratch; DFT is such an approach. Semiempirical methods calculate part of the terms while others are imported from "appropriate" experimental data. MM too, uses a mix of empirical and theoretically calibrated data (e.g., force constants, atomic size parameters, strain energies, van der Waals parameters, etc.) to calculate the energy as a function of geometry, and the parameters are usually system specific, for example, to proteins, hydrocarbons, etc. As a crude generalization, there is an inverse relationship between the speed of the method and its reliability/accuracy, such that the *ab initio* methods, which are the most time consuming, are generally the most accurate, while MM is the fastest and also the least accurate.

The *ab initio* methods are split into two parts: the wave mechanical approaches and the DFT methods. In the wave mechanical approach, one starts with the Schrödinger equation and minimizes the energy by optimizing the orbitals in a wave function that represents the system at a certain level of accuracy; the latter is defined by the degree of coverage of electronic correlation. The resulting wave function defines a specific electronic state, characterized by certain orbital occupation, total spin and energy, from which all the properties of the molecule can be derived. The lowest *ab initio* level, called Hartree-Fock, describes the wave function by a single determinant built from doubly occupied orbitals, and as such does not include electron correlation. This method fails completely for transition metal containing molecules, which require multideterminantal methods that incorporate static and dynamic electron correlation, such as the complete active space self consistent field (CASSCF) method and its further augmentation by second-order perturbation theory, the CASPT2 method that also incorporates dynamic electron correlation, or the CCSD(T) method that corresponds to

coupled cluster with single and double excitations and perturbative triple excitations. At the time of writing this chapter, CASSCF, CASPT2, and CCSD(T) methods are still too time consuming for P450 species, if one wishes to optimize the geometries of the species or to trace their potential energy surfaces for a given reaction[4].

DFT methods also use a wave function, but this wave function serves merely to obtain the electron density of the molecule, and it is from the density that the energy and all molecular properties are subsequently derived. Even though the auxiliary wave function in DFT has a single determinant form, the energy expression extracted from it incorporates static as well as dynamic electron correlation. Consequently, the DFT procedure is faster than the *ab initio* procedures; its time consumption scales like Hartree-Fock theory, but its accuracy is much better, and is sometimes competitive even with CASPT2[4]. As such, DFT can treat systems of up to *c.*100 or more atoms and obtain results with decent accuracy for an entire potential energy surface of an enzymatic reaction.

Pure functionals with gradient correction of the density, for example, BP86, BLYP, BPW91, etc., are considered to be suitable for enzymatic species. However, much better are hybrid density functional methods, such as B3LYP or B3PW91, which can reproduce experimental enthalpies of formation within 3 kcal mol^{-1}, while the highly elaborate wave mechanical methods reproduce the same set of data within 1.6 kcal mol^{-1}. Reaction barriers are more difficult to reproduce with experimental accuracy, and here too B3LYP is considered to be better than the other DFT procedures. As such, B3LYP has become the standard method for carrying out biological and P450 related research[4]. To instill some uniformity in this chapter, many of the calculations were repeated here by us, using B3LYP with a double zeta basis set, composed of LACVP for Fe and 6-31G for all other atoms, hence LACVP(Fe)/6-31G(H,C,N,O,S). This basis set gives qualitatively reliable results for the assemblage of P450 species.

Semiempirical QM methods are also based on wave mechanics. However, unlike the *ab initio* methods, here many of the terms are not calculated but taken from some empirical data, which are further recalibrated to fit a set of properties. In principle, semiempirical methods provide the same information as *ab initio* methods with the exception of accuracy. The current method that can be employed for transition metal compounds is SAM1 that is based on the older AM1 method. While the method seems to be very good for zinc compounds, it is still inaccurate for many of the transition metals. Another semiempirical method used for spectroscopic properties is INDO/S/CI that also includes configuration interaction (CI). These semiempirical methods are very fast and can be used for very large systems, but their accuracy remains questionable.

MM expresses the total energy of a molecular system as a sum of bond energy terms, electrostatic terms, and van der Waals interactions. The bond energy terms include bond stretching angular deformation, and torsional deviation that are evaluated based on a force-field derived from parameters calibrated for these bond types. The electrostatic interactions involve classical electrostatics; for proteins, usually, the partial charges are kept fixed during the calculations. The van der Waals interactions involve a Lennard–Jones potential with $1/r^6$ and $1/r^{12}$ terms for the interaction energy. Since MM calculations are cheap, they can be used to run molecular dynamics (MD) calculations to study multiple conformations, as well as to map and sample an entire potential energy surface and to determine free energies. The dynamics is calculated according to Newton's laws of classical mechanics. However, due to the dimensionality of the problem, there are many algorithms for running dynamics and for sampling the configuration space. These MM calculations require computer resources and can be applied to large systems, such as enzymes and proteins. Thus, MM and MD studies are useful for the studies of large systems, in which one, for instance, explores the entrance and exit channels of substrates/products into an enzyme[5, 6] and the preferred location of substrate binding[7, 8]. By contrast, the electronic structures of all species of P450 involve unpaired electrons, different spin states, and unusual mixed-valent states, which can be studied only with QM methods.

The QM/MM method combines the advantages of QM and MM. In QM/MM calculations, the system is divided into two subsystems that are treated at different levels. The small subsystem involves the active species that is computed with a QM method such as a semiempirical or DFT.

The larger subsystem is the protein, which does not actively take part in the studied reaction, but influences the active species by providing an electrostatic field and a hydrogen bonding machinery, as well as a sterically constrained matrix with van der Waals interactions. The protein subsystem is considered to be "classical" and is treated with MM. The interactions between the QM and MM subsystems are split into electrostatic and van der Waals types. The electrostatic interactions are incorporated into the QM calculations by embedding the MM charges into the QM Hamiltonian; in this manner, the QM subsystem undergoes polarization in response to the electric field and hydrogen bonding machinery of the protein environment. The van der Waals interactions are treated classically. QM/MM calculations that involve DFT for the QM subsystem are still very demanding and elaborate, and, at the time of writing this chapter, are limited to a few species of P450. Moreover, the method is still limited to QM/MM geometry optimization, but cannot yet be used for proper sampling of configuration space, which is required in order to derive free energy quantities.

3. The Catalytic Cycle of P450

The enzyme cytochrome P450 operates by means of a catalytic cycle[9] that is schematically depicted in Figure 2.2, where the cysteinate proximal ligand is abbreviated as L, and the porphyrin macrocycle is symbolized by the two bold lines flanking the iron. The cycle follows a beautiful chemical logic that highlights the impact of chemistry on the field. The resting state of the enzyme is the ferric (Fe^{III}) complex (**1**) that possesses a water molecule as a distal ligand. With six coordination, the d-block orbitals of the complex are split into three-below-two molecular orbitals (MOs). Consequently, the five d-electrons of the complex occupy the lower MOs leading to a low-spin (LS) species. The entrance of the substrate (RH) into the protein pocket displaces the water molecule and generates the five-coordinated ferric species (**2**). The result is that the iron pops out of the plane of the porphyrin, and thereby weakens the interaction of the d-orbitals with the ligands, resulting in a narrow d-block. Consequently, the five d-electrons occupy the d-block in a high-spin (HS) fashion, and the complex itself becomes a good electron acceptor. This triggers a one-electron transfer from the reductase domain that reduces **2** to the HS ferrous (Fe^{II}) complex **3**. Ferrous porphyrin is a good dioxygen binder, and this leads to the binding of molecular oxygen to produce the LS ferrous-dioxygen complex, **4**. The latter species is again a good electron acceptor and this causes another electron transfer from the reductase to give rise to the twice-reduced ferric-dioxo species (**5**). The ferric-dioxo complex is now a good Lewis base, and therefore undergoes protonation to yield the ferric peroxide complex **6** that is also referred to as Cpd 0. Since Cpd 0 is still a good Lewis base, it undergoes a second protonation and releases a water molecule leading to the reactive species **7**, which is a high-valent iron-oxo complex also known as Cpd I. Cpd I, in turn, transfers the distal oxygen atom to the substrate, which is released and is replaced by a water molecule to regenerate the resting state of the enzyme (**1**). Alternative species, such as the $Fe–OH_2–O^-$ intermediate[10] and Cpd II that results from the one-electron reduction of Cpd I (ref. [11]), have been suggested to participate in the cycle. However, as of now there is no experimental evidence for the existence of these intermediates in the P450 cycle itself, albeit Cpd II is well known for related heme enzymes[12].

There are two additional key features in the cycle. First is the good electron donor ability of the proximal thiolate ligand, which is thought to have a significant impact on the entire cycle, and especially on the generation of Cpd I. This property of the thiolate is also called the "push effect"[13]. A second important feature that controls the efficacy of the cycle is the hydrogen-bonding interaction in the proximal side with the thiolate ligand in the cysteine loop. Thus, as shown by Poulos[14] and subsequently by Schlichting *et al.*[15], in $P450_{cam}$ the sulfur ligand is hydrogen bonded to three amidic hydrogens due to the side chains, Gln_{360}, Gly_{359}, and Leu_{358}, while a fourth hydrogen bond is donated by Gln_{360} to the carbonyl group of the cysteine. These hydrogen-bonding interactions are implicated in the stability of the enzyme and are thought to have an effect on the generation of Cpd I from Cpd 0. Other interactions in the distal side, for example, with Thr_{252} and Asp_{251}, are implicated in the proton relay mechanism that leads from **5** to **7**.

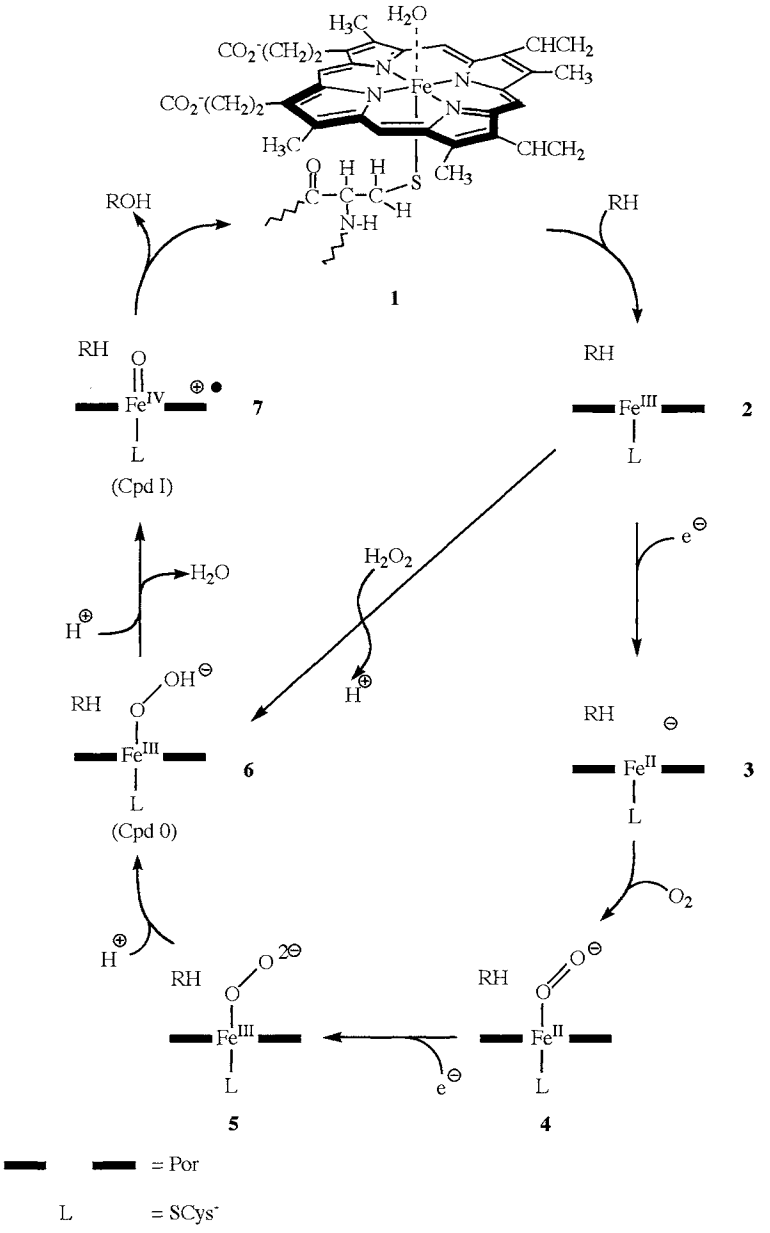

Figure 2.2. The catalytic cycle of Cytochrome P450.

The next two sections describe the results of theoretical calculations on the intermediates **1**–**7** in the catalytic cycle. Most of the literature in the field up to the year 2000 can be found in a review by Loew and Harris[16] and Loew[17]. Extensive work of Giler and Clark [18] modeled the complete catalytic cycle of P450 with methyl mercaptide (SCH_3^-) as an axial ligand using the semiempirical SAM1 method, which found good overlap between their results and experimental assignments. The five-coordinated Fe^{III} complex **2** was found to exist in a quartet ground state, whereas

the true one is a sextet. The semiempirically calculated Cpd I species is not well described, having too short an FeO bond and hence a different electronic structure compared with *ab initio* methods and experimental characterization of this species as triradicaloid. As such, we do not describe details of the semiempirical results and defer the discussion until the methods become suitable for treating iron compounds.

Each of the intermediates **1**–**7** has several closely lying spin states due to the dense orbital manifold of the iron-porphyrin system. As a typical example of the important orbitals that figure in the catalytic cycle, we depict in Figure 2.3 d-block iron and the highest lying porphyrin orbital for Cpd I (**7**). The five iron d-orbitals on the left exhibit the well-known three-below-two splitting, much like the t_{2g} and e_g sets in the purely octahedral symmetry. The three lower orbitals include the nonbonding $\delta(d_{x^2-y^2})$ orbital, which is followed by a pair of almost degenerate π^*_{FeO} (π^*_{xz} and π^*_{yz}) orbitals made from the d_{xz} and d_{yz} orbitals of iron in antibonding relationship with the ligand p_x and p_y orbitals. The upper two are σ^* orbitals that have strong antibonding relationship with the ligand orbitals; σ^*_{xy} is Fe–N antibonding, in the plane of the porphyrin ring, while $\sigma^*_{z^2}$ is antibonding along the O–Fe–S axis. Without a ligand bound to the distal position, as in **2** and **3**, or with a ligand that has only one p_π lone pair as in **6**, one of the π^* orbitals becomes a nonbonding d_{xz} orbital, while the other remains a π^*_{yz} MO and possesses antibonding interaction across the Fe–S linkage. These alternative energy orderings of the d-block orbitals, for complexes with different ligand types, are shown in the insets in Figure 2.3.

A high-lying porphyrin orbital that acquires single occupancy in some of the species is a_{2u} that is depicted in the center of Figure 2.3. In the presence of the thiolate, the a_{2u} orbital mixes strongly with the σ_S-hybrid orbital on the sulfur. The porphyrin-proximal ligand mixture of the a_{2u} orbital depends on the nature of the axial ligand. In contrast to thiolate that mixes strongly, an imidazole axial ligand mixes weakly with the porphyrin a_{2u} orbital. Finally, an orbital that may play a role is the p_π-type sulfur lone pair depicted in the far right of the diagram and is labeled π_S.

Generally, QM modeling cannot describe the complete enzyme, and one needs to truncate models that mimic faithfully the active species. Most of these models truncate the side chains on the porphyrin and use porphine, while the proximal ligand is truncated either to thiolate (SH^-), methyl mercaptide (SCH_3^-), or to cysteinate anion ($CysS^-$). The experience is that SH^- gives results closer to reality than the methyl mercaptide (SCH_3^-) ligand as revealed by QM/MM calculations[19]. This choice is due to the hydrogen-bonding machinery that stabilizes the thiolate and decreases its donor ability.

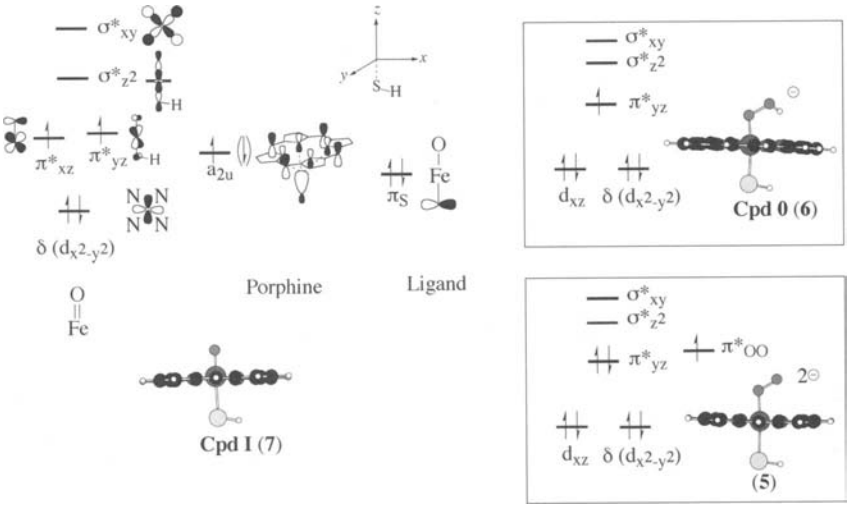

Figure 2.3. Orbital occupation of some critical species in the catalytic cycle.

To appreciate the role of thiolate in P450, it is instructive to also look at related heme-containing enzymes like HRP or catalase, where iron is coordinated to imidazole from a histidine side chain or to phenoxyl from a tyrosine side chain.

Apart from O_2 and water as ligands, calculations have been reported on other small molecules bound to iron-porphyrin complexes, among which are carbon monoxide[20] and NO[21, 22]. Calculations of metal-free porphyrin systems were reviewed by Ghosh[23] and Ghosh and Taylor[4], while DFT calculations on oxo-iron porphyrin without axial ligand[24] and studies of oxo-manganese porphyrins with different axial ligands[25] were also reported. Another complex that was studied is the hydrogen peroxide ferric complex, $Fe^{III}(H_2O_2)$, which in the P450 cycle is thought to lead to decoupling[26], while in HRP it is thought to be an intermediate in the formation of Cpd I[27, 28].

3.1. The Resting State (1)

One of the most thoroughly studied intermediates in the catalytic cycle of P450 is the resting state (**1**). Experimentally, the ferric-water complex was characterized by electron spin echo envelope modulation (ESEEM) spectroscopy and found to have a doublet ground state[29]. Based on a previous study[30] of the resting state of cytochrome *c* peroxidase (CCP), Harris and Loew[31, 32] studied the state for P450 by an early phase of the QM/MM method. They employed initial MM minimization of the X-ray structure and subsequently used INDO/ROHF/CI semiempirical methods with, and without, inclusion of the electric field of the protein in the QM procedure. The electric field-free calculations predicted that the ground state should be the sextet state, while the doublet state was found to lie significantly higher (3.84 kcal mol^{-1}). With the electrostatic field included, the doublet state descended below the sextet state by -1.59 kcal mol^{-1}. The authors concluded that the LS ground state is due to the electric field of the protein and is not an intrinsic feature of the water complex. Some support for this conclusion was provided by synthesis of a model ferric-water complex with a thiophenoxide ligand[33], which exhibits a ground state with a sextet spin, whereas the doublet state becomes the ground state only when water is replaced by a ligand with a stronger field such as imidazole.

This conclusion was contested by Green[34] who calculated the resting state of cytochrome P450 using DFT and the B3LYP functional. Green's model system had a water ligated iron-porphine and a methyl mercaptide proximal ligand. The sextet–doublet energy difference was calculated with a series of basis sets; the result was found to converge at the 6-311+G* basis set which predicted a doublet ground state. It was reasoned that the sextet state that involves the electronic configuration $\delta^1 \pi^{*1}_{xz} \pi^{*1}_{yz} \sigma^{*1}_{z^2} \sigma^{*1}_{xy}$ (Figure 2.3) is destabilized with basis set improvement, due to the increased Fe–S antibonding character of the $\sigma^*_{z^2}$ orbital. Thus, Green concluded that the doublet state is an intrinsic property of the resting state of P450.

Green's study[34], however, did not involve geometry optimization. An early DFT study by Filatov *et al.*[35], used the pure functional BP86, and partial geometry optimization. The ground state was found to be a doublet state that existed in two conformations; in the "upright" conformation, the water molecule points upward away from the porphyrin ring, while in the "tilted" conformation, the water molecule forms hydrogen bonds to the nitrogen atoms of the porphyrin ring. The water–porphyrin hydrogen bonds were found to stabilize the "tilted" complex by about 6.6 kcal mol^{-1}. Our present B3LYP/LACVP(Fe),6-31G(H,C,N,O,S) calculations, shown in Figure 2.4[36], support this conclusion, but shows that after geometry optimization, the energy differences are small, *c.*1.1 kcal mol^{-1}. An additional frequency analysis of both species showed that while the "tilted" conformation is a true minimum, the "upright" structure is a saddle point since it has one imaginary frequency, driving it back to the "tilted" form. Clearly therefore, DFT calculations indicate that the resting state has an intrinsic preference for a "tilted" conformation due to the propensity of the water ligand to undergo hydrogen bonding with a suitable acceptor. Within the protein pocket, this interaction can be supplied by the protein side chains that will stabilize the "upright" conformation, which might become the ground state or, at least be, in equilibrium with the "tilted" conformer. The ESEEM results fit better the "upright" conformation. A QM/MM study using BP86 functional for the QM subsystem was

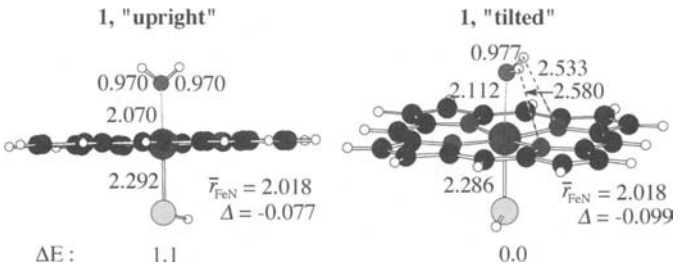

Figure 2.4. Orientations of the water ligand in the resting state (**1**). Here and elsewhere the parameter Δ specifies the deviation (in Å) of Fe from the porphyrin plane, while $r_{Fe\text{-}N}$ is an average distance of the four bonds.

performed by Scherlis *et al.*[37] and Scherlis *et al.*[38] using X-ray data without geometry optimization. The studies confirm the previous DFT findings that the doublet state is indeed the ground state. However, the study started with the "upright" conformer, so that the conformational question is still open. Future QM/MM calculations with full geometry optimization will be required to resolve this issue.

The related resting state of peroxidase enzymes, involving iron-porphine with water and imidazole as axial ligands, was studied using CASSCF(5,5) calculations[39], in which all the electronic configurations that can be generated by distributing the five d-electrons into the five d-orbitals, were included in the active space. The lowest lying state was found to be the sextet spin state with $\delta^1\ \pi^{*1}_{xz}\ \pi^{*1}_{yz}\ \sigma^{*1}_{z^2}\ \sigma^{*1}_{xy}$ configuration. The quartet and doublet states were found to be 2.21 eV (51.0 kcal mol^{-1}) and 3.41 eV (78.6 kcal mol^{-1}) higher in energy than the sextet ground state. The doublet state is characterized by short Fe–O and Fe–$N_{imidazole}$ distances of 2.09 and 2.04 Å, respectively. By contrast, the Fe–O distances in the sextet and quartet states are 2.32 and 2.34 Å, respectively. This is mainly the result of occupation of the $\sigma^*_{z^2}$ orbital which is antibonding across the O–Fe–$N_{imidazole}$ axis. These results suggested that the actual spin state would depend on the protein environment that can control the spin state by imposing bond length restriction.

Comparison of the resting states of P450 vs those of peroxidase highlights the difference between the thiolate and imidazole ligand. Thiolate mixes more strongly with the iron d-orbitals and hence the resulting $\sigma^*_{z^2}$ orbital is raised in energy, making the sextet state less favorable than the doublet. By contrast, imidazole mixes less strongly with the d-orbitals and the corresponding $\sigma^*_{z^2}$ orbital is sufficiently low to stabilize the sextet ground state by virtue of its higher exchange stabilization.

3.2. The Pentacoordinate Ferric-Porphyrin (2) and Ferrous-Porphyrin (3) Complexes

Ogliaro *et al.*[40] calculated the pentacoordinated ferric- and ferrous-porphyrin and quantified the "push effect" of the thiolate ligand, using B3LYP hybrid functional. Figure 2.5 provides optimized structures and energy separation of some of the lowest lying states. At the UB3LYP/LACV3P+* level of theory, the ground state of **2** was found to be the sextet state with $d_{x^2-y^2}{}^1\ d_{xz}{}^1\ \pi^{*1}_{yz}\ \sigma^{*1}_{z^2}\ \sigma^{*1}_{xy}$ configuration, whereas the quartet and doublet states were higher by 4.21 and 4.23 kcal mol^{-1}, respectively. Experimentally, the HS and LS forms of **2** are in equilibrium[41, 42] which suggests that their energy separation is somewhat smaller than the value predicted by the calculations, and is perhaps modulated by the electric field or steric constraints in the protein pocket. Other complications in the protein pocket are the presence of the substrate and the entropic driving force due to the expulsion of a few water molecules upon the formation of **2**. These features will have to await an appropriate QM/MM study.

Reduction of ^{6}**2** fills the $d_{x^2-y^2}$ orbital and generates a quintet ground state, ^{5}**3** for the reduced pentacoordinated ferrous complex. Another quintet state with a doubly filled d_{xz} orbital lies only 2.14 kcal mol^{-1} higher, while the triplet $d_{x^2-y^2}{}^2$

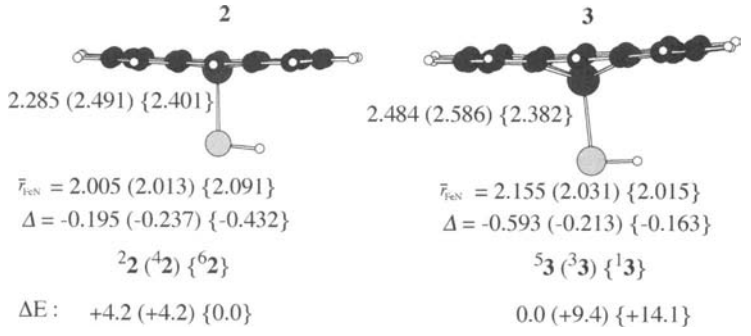

Figure 2.5. Optimized (UB3LYP/LACVP) geometries of **2** and **3**.

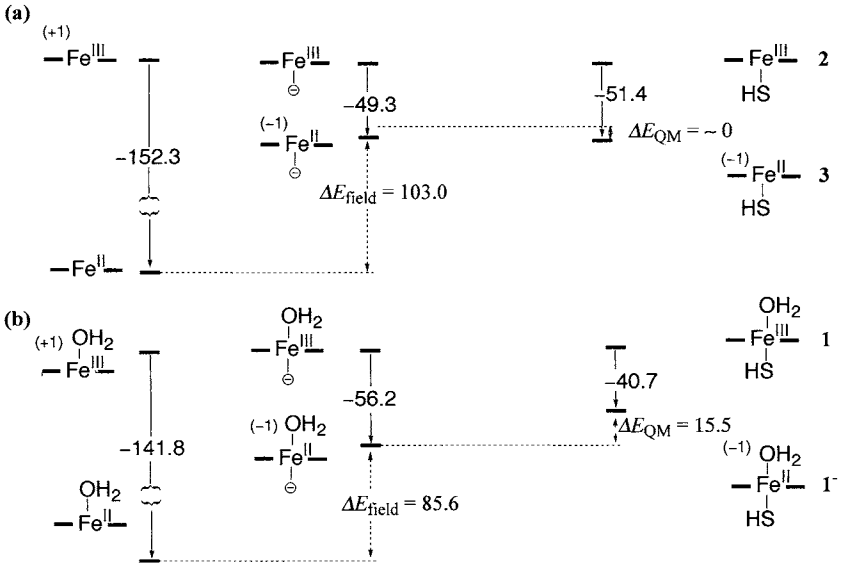

Figure 2.6. Calculated (UB3LYP/LACV3P^{+*}) push effect of the thiolate ligand (a) on the reduction energy **2** → **3**, and (b) on the reduction of **1** → **1**$^-$.

$d_{xz}^2\ \pi^{*1}_{yz}\ \sigma^{*1}_{z^2}$ state lies 9.4 kcal mol^{-1} higher. The singlet state, $d_{x^2-y^2}^2\ d_{xz}^2\ \pi^{*2}_{yz}$, lies considerably above the quintet ground state by 14.1 kcal mol^{-1}.

The process ^{6}**2** → ^{5}**3** was found to be exothermic by 51.4 kcal mol^{-1} (Figure 2.6(a)), while in the absence of the thiolate ligand, the reduction energy would have been exothermic by 152.3 kcal mol^{-1}. Thus, the thiolate makes the pentacoordinated complex a much poorer electron acceptor by the "push effect" that is a measure of its electron donation capability toward the iron-porphyrin. It was reasoned[40] that since the reduction involves filling of the $d_{x^2-y^2}$ orbital, which does not mix with the thiolate orbital, this large "push effect" originates from the electrostatic interaction between the negatively charged thiolate and the iron-porphyrin moiety. To ascertain the origins of the "push effect," the thiolate was replaced by a point negative charge placed at precisely the same position as the thiolate[40]. As shown in Figure 2.6(a), the resulting reduction energy for the pseudo complex (−49.3 kcal mol^{-1}) was almost identical to the pentacoordinated complex. It was therefore concluded that during the reduction ^{6}**2** → ^{5}**3**, the "push effect" is indeed a pure electrostatic field

effect and not of quantum chemical origin. The effect of the protein electric field was estimated by embedding the bare species in a medium with a dielectric constant of $\varepsilon = 5.7$. This was found to reduce the "push effect" from 103 kcal mol^{-1} for the bare system to 33 kcal mol^{-1} for the embedded system[40]. Although significantly reduced, still the "push effect" is substantial; it makes the reduction of ^{6}**2** selective such that only the reductase can perform it and thereby trigger the initiation of the cycle.

3.3. The Gating of the Catalytic Cycle

To understand the reason why it is that only the pentacoordinated complex, **2**, and not the water complex, **1**, is reduced during the catalytic cycle, Ogliaro *et al.*[40] studied the reduction of the ferric-water complex ^{2}**1**, with and without a thiolate ligand. The results are shown in Figure 2.6(b), and project once more that without thiolate, the ferric complex is a much better electron acceptor, by *c.*101.1 kcal mol^{-1}. It was further determined by the same technique as above, that most of this energy effect (ΔE_{field}), *c.*85.6 kcal mol^{-1}, comes from the electrostatic field effect, and a small part, *c.*15.5 kcal mol^{-1} (ΔE_{QM}) is contributed by QM mixing with the p_π orbital of the thiolate ligand, which raises the electron accepting orbital π^*_{yz} (ref. [40]).

Comparison of the reduction energies in Figures 2.6(a) vs (b) reveals that the reduction of the pentacoordinated complex is more exothermic than that of the ferric-water complex by 10.7 kcal mol^{-1}. This difference safeguards the resting state against reduction by the reductase, such that a single water molecule can gate the catalytic cycle. Note however that the ligand that actually controls this gating is the thiolate; it makes all the species poorer electron acceptors, leading thereby to a selective reduction of the pentacoordinated species by the reductase. Without the thiolate ligand, all the complexes are such good electron acceptors that most reducing agents would have reduced all the species with no selectivity whatsoever. Thus, the property of a gated cycle by a single ligand (water) is achieved due to the "push effect" of the thiolate[40].

3.4. The Ferrous-Dioxygen (4) and Ferric-Dioxygen (5) Complexes

Early CASSCF calculations of a ferric-dioxygen species (**4**) with ammonia as an axial ligand were carried out by Yamamoto and Kashiwagi[43], using a minimal basis set and no geometry optimization. The lowest singlet state was found to possess a major weight of 64% of the Pauling configuration that involves coupling of Fe^{II} with the neutral O_2 moiety in its singlet situation. The state also has some $Fe^{III}O_2^-$ character due to the mixing of higher configurations.

The first DFT calculations on **4** and **5** were reported by Harris and Loew [26] and Harris *et al.*[44], using the BPW91 and BLYP pure functionals with a basis set of double-ζ plus valence polarization quality (DZVP); the two functionals gave virtually the same results, and in good agreement with experimental data. The most stable form of ferrous-dioxygen (**4**) is an end-on complex in accord with the experimental predictions[45] while the symmetrically bridged isomer was found to be much higher in energy, by *c.*28 kcal mol^{-1}. Reduction of **4** to **5** resulted in elongation of the Fe–O and Fe–S bonds, while leaving the O–O bond length intact, albeit the O–O bond order decreased from 1.20 to 0.87 (ref. [26]). In agreement with its silent ESR behavior, **4** was found to have a singlet ground state, but the triplet state to lie only 1.1 kcal mol^{-1} higher. By comparison, **5** was reported to have a doublet ground state with spin densities distributed over both oxygen atoms, in agreement with ESR data. Electronic spectra of both the ferrous- and ferric-dioxygen species, calculated with the semiempirical INDO/S/CI method[44], exhibit, in agreement with experiment, a split Soret band. The corresponding Mössbauer parameters of **4** and **5** were calculated too, and those for **4** show a good fit to experimental data.

Figure 2.7 compares the structures of ^{1}**4** and ^{2}**5** as derived by us, using the B3LYP functional with the LACVP(Fe)/6-31G(H,C,N,O,S) basis set[36], *vis-à-vis* the BPW91/DZVP results of Harris *et al.*[44] in brackets. The structures show a general fit, with the exception of **4** that appears more open in B3LYP compared with BPW91. Transformation of the DFT orbitals to natural orbitals for **4** revealed that its electronic structure is the open-shell singlet $\delta^2 d_{xz}^2 \pi^{*1}_{yz} \pi^{*1}_{OO}$ configuration.

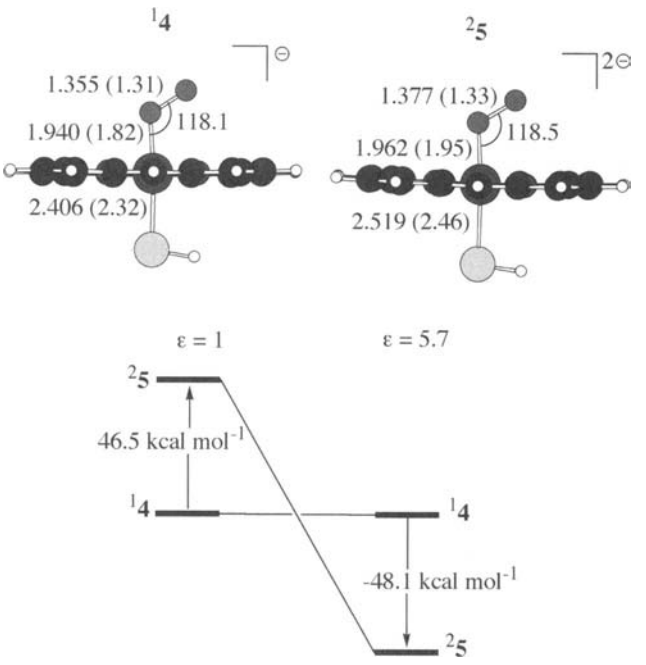

Figure 2.7. Top: optimized geometries of ^{1}**4** and ^{2}**5**. Values out of parentheses are taken from De Visser and Shaik[36], while in parentheses from Harris *et al.*[44]. Bottom: the reduction energies ^{1}**4** → ^{2}**5** (ref. [36]).

The π^*_{yz} orbital has a strong mixing with the corresponding π and π^* orbitals of the dioxygen moiety, and as such, an appropriate description of ^{1}**4** is a resonating mixture of the ferrous and ferric forms, $Fe^{II}O_2$ and $Fe^{III}O_2^-$. This conjugation requires that the dioxygen moiety, in the $Fe^{II}O_2$ form, will be in its singlet state, so that the empty π^*(O–O) orbital can mix with the doubly occupied d_{yz} (Fe) orbital; as such there is a very low lying triplet state, ^{3}**4**, only 1.1 kcal mol^{-1} higher than ^{1}**4** (ref. [44]). The electronic structure of ^{1}**4** suggests its origin from the excited singlet state of **3** and $^1\Delta_g$ state of O_2. This is further supported by the fact that **4** has low-lying HS states, for example, quintet and heptuplet states which originate from the coupling of ^{5}**3** with the triplet and singlet states of O_2. In view of the fact that both moieties of **4** have HS ground states, an interesting feature of the O_2 binding must be the spin crossover process that is yet to be properly elucidated by theory.

A natural orbital transformation analysis reveals that the odd electron in ^{2}**5** populates predominantly the π^*(O–O)-type orbital of the dioxygen ligand (Figure 2.3), as found by Harris *et al.*[44]. In reasonable agreement with Harris and Loew[26], the reduction ^{1}**4** → ^{2}**5** was found to be endothermic, by 46.5 kcal mol^{-1}. However, in the presence of an electric field modeled by a dielectric constant of ε = 5.7, the reduction energy becomes exothermic by 48.1 kcal mol^{-1}.

The effect of the protein environment on ^{1}**4** and/or ^{2}**5** was studied initially by MD simulations of $P450_{cam}$ and $P450_{eryF}$ (refs [46], [47]) using MM/MD calculations. The study showed that the ferrous-dioxygen species is stabilized by a hydrogen bond from Thr_{252} (ref. [46]). In the case of the species ^{2}**5** of $P450_{eryF}$, Harris and Loew[47] found that the distal oxygen of the twice-reduced species ^{2}**5** is linked through a hydrogen-bonding network involving neighboring amino acids, such as Ala_{241} and Ser_{246}, and several water molecules. This stable hydrogen-bonding network was implicated as the root of the double protonation that eventually converts **5** to Cpd I.

3.5. The Protonation Mechanism of Ferric-Dioxygen (5) to Cpd 0 (6)

BPW91 DFT calculations[26] showed that ^{2}**5** is an extremely strong base with a proton affinity of 422 kcal mol^{-1}, and as such it may undergo protonation by a water molecule. This study was pursued by Guallar *et al.*[48] who carried out a QM(DFT)/MM investigation of the protonation mechanism, leading from **5** to Cpd 0 (**6**) in $P450_{eryF}$. This was followed by a full quantum dynamics simulation of the proton transfer through a one-dimensional profile. The study of the QM subsystem used B3LYP with a mixed basis set, and focused on three different protonation mechanisms, by: (a) a single water molecule (W519), (b) an array of two water molecules (W519 and W564), and (c) an array of W519, W564, and an ethanol that mimics the Ser_{246} amino acid. The latter model is depicted in Figure 2.8(a), and seems to be highly conserved in many P450 isozymes that exhibit sequestered array of water molecules hydrogen bonded to a polar amino acid residue near the protein surface.

The computed energy profile[48] changed gradually from an endothermic one (+20 kcal mol^{-1}) (a) to an exothermic one (−10.7 kcal mol^{-1}) for (c) with a concomitant decrease of the barrier to 1.8 kcal mol^{-1}. The dramatic effect caused by the Grotthuss-like mechanism is very likely due to the diminishing repulsion between the doubly negative ^{2}**5** species and the incipient anion of the protonating species. Indeed, the study showed incisively that hydronium ions are not needed to initiate the protonation of the twice-reduced species, ^{2}**5**, and ruled out any putative protonation of the ferrous-dioxygen complex, ^{1}**4**, by the W519-W564-Ser_{246} array. This result is consistent with the large kinetic solvent isotope effect[50] that was observed for the reduction of ^{1}**4** to ^{2}**5**, which indicated that the reduction of ^{1}**4** and protonation of ^{2}**5** to Cpd 0, ^{2}**6**, are nearly commensurate events. The MD study[48] further showed that the initial protonation by W519 is completed within 500 fs and is the rate-determining step that triggers a sequential protonation from W564.

In a subsequent paper, Harris[51] extended the DFT study (B3LYP/ LACVP**(Fe)-6-31G*(H,C, N,O,S)) of the protonation process and analyzed its features by calculating proton affinities and transition states (TS) for protonations by various candidate acids. His studies showed, *inter alia*, that the hydronium ion can indiscriminately protonate both ^{1}**4** and ^{2}**5**, and is therefore ruled out as the source of protons. By contrast, serine, threonine, or their clusters with two water molecules (e.g., W519, W564 in $P450_{eryF}$) can protonate ^{2}**5**, but are only capable of donating a hydrogen bond to ^{1}**4**; the protonation of the HS species ^{4}**5** encounters a high barrier despite its identical proton affinity to ^{2}**5**; this was ascribed to the reduced negative charge on its distal oxygen. Using an extended array of two water molecules and alanine, the

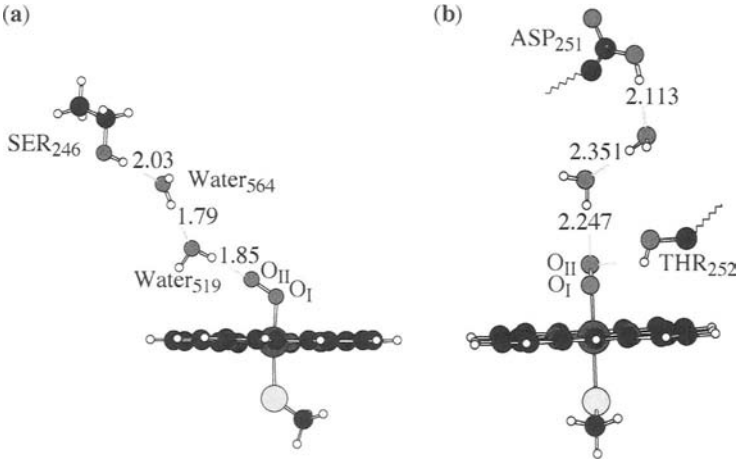

Figure 2.8. Protonation models of ^{2}**5** → ^{2}**6** taken from (a) Guallar *et al.*[48], (b) Kamachi and Yoshizawa[49].

barrier for proton transfer to ^{2}**5** was found to decrease to 1.3 kcal mol^{-1} and the product of the hydrogen-bonded array showed characteristics of low-energy hydrogen bonds that may contribute to the process facility. In this manner, the hydrogen-bonding network in the pocket creates a gentle as well as spin-state selective protonation process.

An alternative and more potent protonation mechanism was very recently studied by Kamachi and Yoshizawa[49], who calculated a model fashioned after Vidakovic *et al.*[52], with two water molecules sequestered between the acidic end, CO_2H, of Asp_{251} and the side hydroxyl group of Thr_{252}. This study involves both doublet and quartet states of **5**, and the model is shown in Figure 2.8(b) alongside the model of Guallar *et al.*[48]. The protonation is initiated by proton transfer from the CO_2H group of Asp_{251} to the adjacent water that further transfers a proton to the water molecule adjacent to the distal oxygen of ^{4,2}**5**, which completes the transfer and generates ^{4,2}**6** spontaneously. The Kamachi–Yoshizawa process was found to be much more exothermic, −61.5 (−50.1) kcal mol^{-1}, than the model of Guallar *et al.*[48], as would be expected from the stronger acid that relays the initial proton. Although barriers were also reported, one cannot avoid the conclusion that there are multiple protonation pathways that must be taken into account via QM/MM with proper sampling.

3.6. Cpd 0: The Ferric Peroxide Complex (6)

Harris and Loew[26] used BPW91 calculations and subsequently Ogliaro *et al.*[53] performed B3LYP studies of ferric peroxide, **6**, and its proximally protonated isomer, **6**-iso, shown in Figure 2.9. In both studies, **6** was found to be the stable isomer by *c.*18.4 (23.3) kcal mol^{-1} and to possess a doublet ground state, labeled $^{2}\Pi_{yz}$ (Fe^{III}), a symbol that denotes its singly occupied π^{*}_{yz} orbital[53]. An interesting feature of the ferric peroxide is the internal hydrogen bond between the hydroxo proton and the nitrogen of the porphyrin ring[53]. Application of an electric field simulated by a dielectric constant of $\varepsilon = 5.7$ further shortened the Fe–S bond and the OH---N hydrogen bond[53]. Whether this hydrogen bond will or will not survive in the protein pocket is an interesting question. But already it is clear that the porphyrin is an internal base that may participate in deprotonation/ protonation events.

3.7. Protonation of Cpd 0 and Formation of Cpd I (7)

DFT calculations show that ferric peroxide, Cpd 0 (**6**), is a fairly strong base with a high proton affinity; PA = 334 kcal mol^{-1} at BPW91 and with CH_3S^{-} proximal ligand[26] or 330.1 kcal mol^{-1} at B3LYP and with HS^{-} as the proximal ligand[40, 53]. Both studies found that the protonated species yields Cpd I (**7**) spontaneously without a barrier. In any event, the study of Davydov *et al.*[9] shows that mutation that replaces Thr_{252} does not prevent the formation of Cpd 0, but prevents, or at least slows down, its subsequent protonation to yield Cpd I. This suggests that the protonation mechanisms that lead to **5** and **6** are different. The protonation mechanism is thought to occur from a hydronium ion sequestered in $P450_{cam}$ by Asp_{251} and Thr_{252}

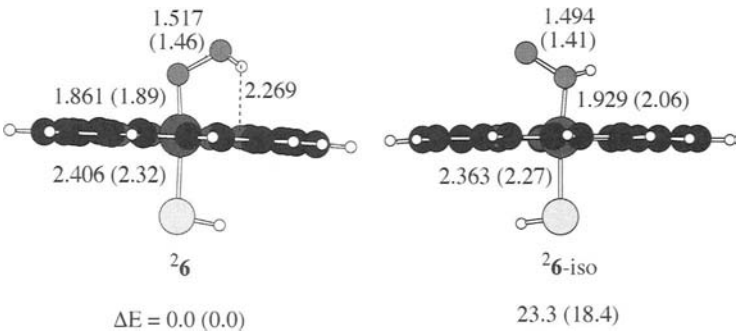

Figure 2.9. Optimized geometries of Cpd 0 (^{2}**6**) and its isomer ^{2}**6**-iso. Values out of parentheses are from Ogliaro *et al.*[53], values in parentheses are from Harris and Loew[26].

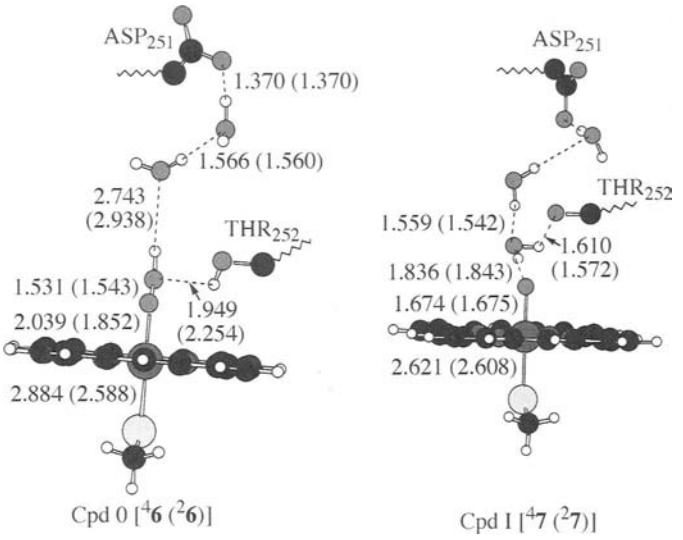

Figure 2.10. Hydrogen bonding set-up for protonation of Cpd 0 to Cpd I, from Kamachi and Yoshizawa[49].

(ref. [52]). A recent study of Kamachi and Yoshizawa[49] used a similar model to the one proposed by Vidakovic *et al.*[52] with two water molecules sequestered between the carboxylate end, CO_2^-, of Asp_{251} and the hydroxyl side chain of Thr_{252}, as shown in Figure 2.10. The protonation starts with a proton transfer from Thr_{252} to the distal oxygen of **6**, followed by a departure of a water molecule that is trapped through hydrogen bonding to the Thr_{252} anion and the array of the two waters that are linked by hydrogen bonding to the Asp_{251} anion. This mechanism predicts that the protonation of **6** and formation of **7** is exothermic by 13.1 and 5.5 kcal mol^{-1} for the quartet and doublet states, respectively. The entire protonation process from ^{2,4}**5** to ^{2,4}**7** by this Asp_{251}-Thr_{252} machinery was calculated to be exothermic by 74.6 (55.6) kcal mol^{-1} (ref. [49]). However, the barrier was not calculated for this process. Alternatively, the protonation mechanism may be nascent from a hydrogen-bonding array similar to the one discussed by Guallar *et al.*[48] and Harris[51]. This, to the best of our knowledge, has not been addressed by Harris[51].

In principle, protonation of Cpd 0, **6**, can occur on either the proximal or the distal oxygen of the complex. Distal protonation results in the departure of a water molecule and generation of Cpd I. Alternatively, proximal protonation gives a hydrogen peroxide complex. The latter complex is able to release H_2O_2 and return to the resting state by coordination with a water molecule, thereby leading to decoupling. Energetically, the formation of the hydrogen peroxide complex from **6** is only 6 kcal mol^{-1} less exothermic than that of Cpd I from **6** (ref. [26]), such that the decoupling reaction is a serious competitor with the productive process that leads to Cpd I. Not much is yet known about this competition. Clearly, a future theoretical study is required to explore alternative protonation pathways that can reveal the intricate behavior of the wild-type and mutant enzymes.

3.8. The "Push Effect" on the O–O Cleavage Process

The thiolate ligand was implicated as a crucial factor in the O–O bond cleavage process through its "push effect" that leads to Cpd I (ref. [54]). Ogliaro *et al.*[40] have addressed this issue by comparing the proton affinity of **6** to a reference complex without a thiolate ligand. The thiolate ligand was found to increase the proton affinity of ferric peroxide by 81 kcal mol^{-1}. At the same time, the protonated reference complex devoid of thiolate loses water spontaneously as well. Thus, the "push" effect of the thiolate does not concern

the mechanism of the O–O cleavage as such, if there could exist a strong enough acid to protonate the ferric peroxide devoid of a thiolate ligand. The "push" effect is expressed on the thermodynamics of the protonation, and by raising the proton affinity by 81 kcal mol^{-1}, the thiolate enables the protonation of **6** by moderate acids such as those that exist in the protein pocket. The roots of this "push" effect were analyzed and were found to consist of a combination of a field effect and an orbital effect[40]. The field effect originates in the electrostatic interaction of the negatively charged thiolate with the ferric peroxide moiety, while the orbital effect results from the mixing of the σ-hybrid of the thiolate with the a_{2u} orbital of the porphyrin (see Figure 2.3), which raises the energy of this orbital.

Another aspect of the "push" effect that was addressed by Ogliaro *et al.*[40] is the putative reduction of **6** by electron transfer. It was found that the reduction of **6** to $\mathbf{6}^-$ is highly endothermic by 43.3 kcal mol^{-1}, whereas in the absence of thiolate such a reduction would be exothermic by 35.1 kcal mol^{-1}. This very large change in the reduction energy is mostly due to the field effect of the negatively charged thiolate. This can be compared to the high proton affinity of **6**, which is very high due to the same "push" effect. Thus, the thiolate ligand endows ferric peroxide with selectivity to undergo protonation rather than accepting an additional electron.

3.9. Cpd I (7)

Initial DFT studies of Cpd I were done for the bare molecule, that is, in vacuum or gas phase conditions, and led to controversial results regarding the electronic structure of the ground state. These results showed sensitivity to the thiolate used to model the cysteinate ligand, as well as to the functional used to calculate the species. Nevertheless, all the calculations agreed that the species is a triradicaloid with three singly occupied orbitals. Two of these are the π^*_{xz} and π^*_{yz} orbitals, depicted above in Figure 2.3, which appear in all calculations including the CASSCF study of Cpd II[55]. However, the various studies differ significantly in the description of the third orbital, depending on the manner in which the study models the thiolate proximal ligand[16, 26, 35, 56–61]. The studies, especially those using mercaptide[60] or cysteinate anion devoid of its internal hydrogen bonding[61] predicted that the third singly occupied orbital is the p_π lone pair orbital on sulfur leading to $^{4,2}\Pi_S$ ($\pi^{*1}_{xz}\ \pi^{*1}_{yz}\ \pi^1_S$) states (Figure 2.3) with spin density almost exclusively on the sulfur while the porphyrin is closed shell. Other studies[57, 58] that used HS^- or cysteinate with its internal hydrogen-bonding interactions, found that the third singly occupied orbital is a_{2u} strongly mixed with the sulfur σ-hybrid (see Figure 2.3); this occupancy leads to $^{4,2}A_{2u}$ states, with spin density distributed over the porphyrin and sulfur. It was found[57, 62], that with HS^- or cysteinate as ligands, the $^{4,2}\Pi_S$ electronic states are more than 5 kcal mol^{-1} higher in energy than the $^{4,2}A_{2u}$ state, whereas with mercaptide all the four states were condensed to within 1 kcal mol^{-1}. This difference is not only academic but also has clear physical manifestations. Had Cpd I been a $^2\Pi_S$ ground state, it would have been red, as the Cpd II species with closed shell porphyrin, while if the ground state had been $^{4,2}A_{2u}$ type, the compound would have been green.

These considerations and other findings, which showed that the nature of the state is highly dependent on the Fe–S bond length[62], prompted a DFT study of the effect of the NH---S hydrogen bonding and the protein electric field on the nature of Cpd I, using simple modeling of these effects[58, 63]. Figure 2.11 depicts a typical result, by comparing the key bond lengths and spin densities (ρ) for the bare molecule, the molecule in an electric field characterized by a dielectric constant, ε = 5.7, and when the bare molecule is coordinated to two ammonia molecules by NH---S hydrogen bonds. As can be seen, under all conditions, the FeO moiety has two spins, while the third spin is distributed over the sulfur and porphyrin ligands in proportions that are highly dependent on the conditions. In the bare molecule, the third electron resides more on the sulfur than on the porphyrin, for example, ρ(Por) = 44% in the $^4A_{2u}$ state. With just two NH---S hydrogen bonds, the unpaired electron shifts mostly to the porphyrin, and so is the situation in a polarizing electric field (mimicked by a dielectric constant, ε = 5.7). Another interesting feature of Cpd I is that whereas most bond lengths do not change significantly with the application of hydrogen bonding and polarity, the Fe–S linkage gets shorter by almost 0.1 Å and its bond

dissociation energy increases significantly. These results led to the conclusion that Cpd I is a *chameleon species* that can change its character and electronic state in response to the environment to which it must accommodate[58, 63].

Recent QM(DFT)/MM calculations of Cpd I of $P450_{cam}$ (ref. [19]) used B3LYP, a variety of basis sets, three different thiolate ligand models, and four different snapshots selected from the MD trajectory after equilibration had been established (200 ps simulation). These calculations retrieved the important NH---S hydrogen bonds, donated to the sulfur by Leu_{358}, Gly_{359}, and Gln_{360}, and assigned Cpd I in a definitive manner as the doublet $^2A_{2u}$ state with a very closely lying $^4A_{2u}$ state; the same QM/MM description applies to the Cpd I irrespective of whether the proximal ligand was HS^-, CH_3S^- or a more extensive chunk of the cysteine loop. Thus, in accord with experimental results on the analogous Cpd I species of the enzyme chloroperoxidase[64], Cpd I of P450cam is a doublet state and it corresponds to the "green species."

The QM/MM calculations also confirmed the chameleonic nature of Cpd I, as can be gleaned from Figure 2.11. Thus, in the gas phase situation, the Fe–S bond was long and the third spin was located mostly on the sulfur, whereas in the protein environment, the Fe–S underwent shortening and the spin transferred to the porphyrin; in both respects, the gas phase situation with HS^- was closer to the QM/MM results[19] than

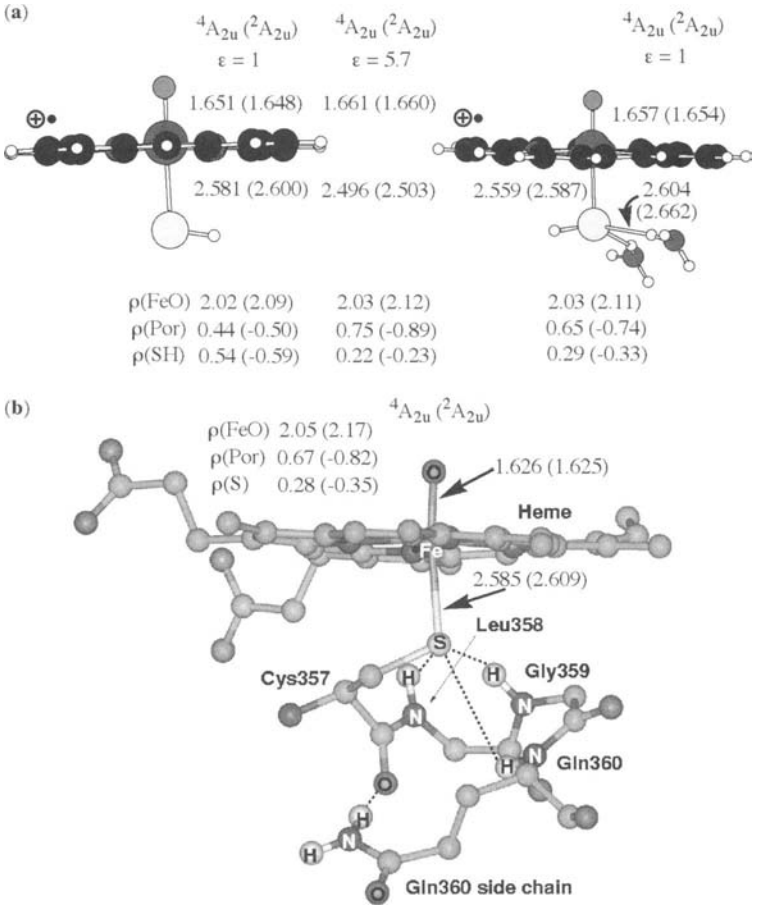

Figure 2.11. Hydrogen bonding (NH---S) and polarity effects (ε is the dielectric constant) on geometrical parameters and group spin densities (ρ) of Cpd I. (a) Model calculations[63]. (b) QM/MM calculations[19].

the more extensive proximal ligand model, for example, mercaptide or cysteinate anion. A very interesting feature of the QM/MM study[19] is the variation in sulfur/porphyrin spin densities and Fe–S bond length, imparted by the NH---O=C hydrogen bond, donated to the carbonyl group of the cysteine ligand by the side-chain Gln_{360}. As this hydrogen bond was allowed to intensify and change gradually toward its X-ray position, so did the porphyrin spin density increase, the sulfur's spin density decrease, and the Fe–S bond length gets shorter. Thus, fine-tuning the hydrogen-bonding interaction around the thiolate ligand fine-tunes the electronic structure of Cpd I and its Fe–S bond length; Cpd I is indeed a chameleon species that will be different for different P450 isozymes.

A simple valence bond (VB) model was used to account for this chameleon nature of Cpd I, as shown in Figure 2.12[19, 57, 63]. The electronic structure of Cpd I can be constructed from two resonance structures, |**a**> and |**b**>; |**a**> describes a thiolyl radical and a closed-shell iron-oxo porphyrin, while |**b**> is an ion pair composed of a thiolate anion and an iron-oxo porphyrin cation radical. In the gas phase and at infinite Fe–S distance, |**a**> is much lower than |**b**>. However, at the equilibrium distance in the gas phase, |**b**> gets stabilized by electrostatic interactions and closely approaches |**a**>, but is still above |**a**>. The mixing of the resonance structures will lead to a state that is |**a**>-like with a preponderant S• character. This character in the gas phase for the molecule will depend strongly on the donor capability of the thiolate ligand; it will be larger for a model like CH_3S^- and smaller for a model like HS^- that is a relatively poorer donor. In a polarizing electric field and in the presence of NH---S hydrogen bonds, the ion-pair structure |**b**> gets stabilized and descends below |**a**>; the mixed state is now |**b**>-like and has a $S:^-Por^{\bullet+}$ character. It is apparent that this model predicts that as the hydrogen-bonding situation and strength of the polarizing field increase so will the $Por^{\bullet+}$ character. It is also apparent from Figure 2.12(b) that by changing from S•-like to $S:^-$-like, the sulfur changes its Fe–S bonding from a weak one-electron bond to a strong two-electron bond. Thus, the VB model shows that Cpd I is a mixed-valent state and as

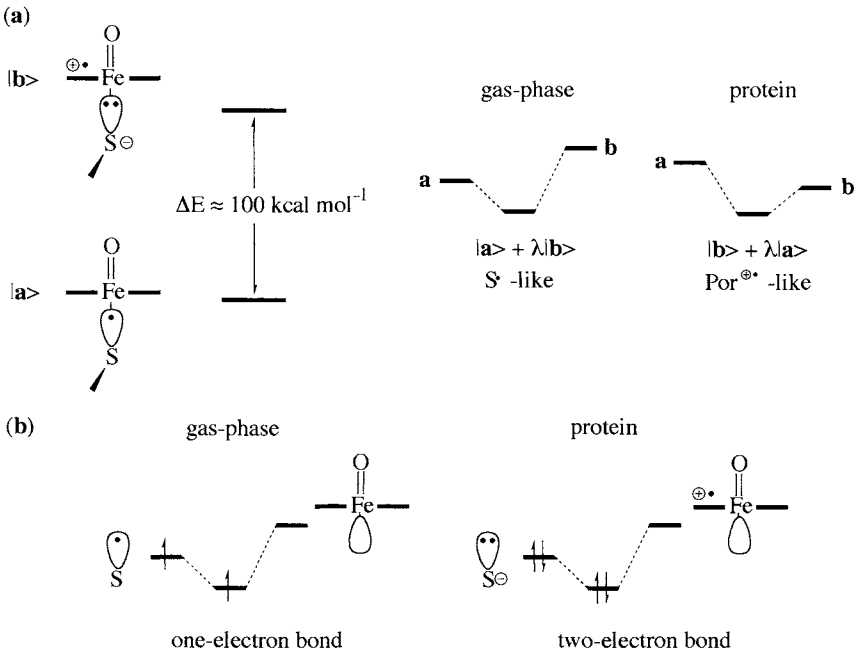

Figure 2.12. Modeling the influence of hydrogen bonding and polarity effects on the electronic structure of Cpd I: (a) Valence bond mixing of the contributing structures. (b) The Fe–S bond orbital, its occupancy and the type of the Fe–S bond.

such will change its electronic structure and Fe–S bond length depending on the hydrogen-bonding machinery and the electric field of the protein pocket that accommodates it; it will behave as *a chemical chameleon*.

Many other Cpd I species for different enzymes and model systems were studied, and it is worthwhile to mention some of these even if they are not P450 species. Ohta *et al.*[61] performed DFT-B3LYP calculations with a methoxide axial ligand and found a LS ground state ($^2A_{2u}$) that contained 64–69% unpaired spin density on the axial ligand and 22–33% on the porphyrin ring. It is most likely that being a good electron donor, methoxide will endow its Cpd I with a *chameleonic behavior*, which has not yet been studied. DFT calculations with the iron substituted by manganese[65] or by ruthenium[66] showed that these systems have different ground states than Cpd I of P450, and therefore will show differences in reactivity as shown subsequently by Sharma *et al.*[67]. These differences do not arise from changes in the nature of the orbitals, shown above in Figure 2.3, but rather from the relative energy of the orbitals being modulated by the transition metal. In particular, in the ruthenium substituted Cpd I species with HS^- as a proximal ligand, the ground state involves Ru^V with a single unpaired electron in the π^*_{xz} orbital labeled $^2\Pi_{xz}(Ru^V)$[66]. This state was found to be 4.58 kcal mol^{-1} lower lying than the $^2A_{2u}$ state. In contrast, in the case of Cpd I with iron, the $^2\Pi_{xz}(Fe^V)$ state was found to be 22 kcal mol^{-1} higher in energy than the $^2A_{2u}$ ground state. However, in the case of Cpd I (Ru) too, the $^{4,2}A_{2u}$ states exhibit a chameleonic behavior, and become the ground states when medium polarity effect is taken into account. Thus, the Cpd I(Ru) species offers a wonderful opportunity to tune the nature and identity of the ground state and possibly also the reactivity patterns, by changing the proximal ligand, by substituting the porphyrin, and by changing the polarity of the medium[66, 67].

Replacing the cysteinate axial ligand with either imidazole[68] or phenolate[69] models the related enzymes HRP and catalase, respectively. In contrast to cysteinate, an imidazole ligand hardly interacts with the porphyrin a_{2u} orbital. As a result, the spin densities of the singly occupied a_{2u} orbital in Cpd I(HRP) are primarily located on the porphyrin ring, while in Cpd I(P450), the spin density is spread over the porphyrin and cysteinate groups. Kuramochi *et al.*[12] found an energy gap of 0.15 eV (3.46 kcal mol^{-1}) between the ground state $^4A_{2u}$ and the excited $^4A_{1u}$ states of a HRP model Cpd I with imidazole as the axial ligand. Subsequently, Deeth[70] studied the same species and showed that the most stable isomer involves saddling of the porphyrin that stabilizes the molecule by 2.5 kcal mol^{-1}. No saddling was observed for thiolate ligands with porphine or octamethyl porphyrin[58]. However, with *meso*-tetramethylporphyrin, a significant saddling was observed[71] even when the proximal ligand was thiolate. The saddling of the *meso*-tetra-substituted-porphyrinated Cpd I species was interpreted as the means to relieve the steric repulsion between the *meso* substituent and the hydrogen substituents on the α and β positions[71].

The influence of the neighboring amino acids on the stability of Cpd I for HRP was studied by Wirstam *et al.*[72] using DFT calculations. Their model used oxo-iron porphyrin and an imidazole ligand replacing His_{175}, a formate anion replacing Asp_{235} and an indole group instead of Trp_{191}; all these three amino acids are located on the proximal side of the porphyrin. In their optimized, geometry, the indole moiety (of Trp_{191}) is protonated and the formate group (of Asp_{235}) is negatively charged forming hydrogen bonds with both the imidazole and the indole groups. It was found that in the HS state (S = 3/2), two spins are located on the FeO unit, while the third one is shared between the porphyrin and indole groups 0.48 and 0.47, respectively. Once again, it is apparent that the porphyrin cation radical is eager to share its hole with other good donors. Perhaps the chameleon behavior is general for Cpd I species, even when the proximal ligand itself cannot participate in electron donation to the "hole," other, better donor moieties will take its role.

Green[69] calculated the low-lying electronic states of Cpd I for a catalase model, with phenolate as the proximal ligand. He found that the ground state had the LS $\pi^{*1}_{xz}\ \pi^{*1}_{yz}\ \pi_L{}^1$ configuration, with π_L being a lone-pair orbital on the phenoxy ligand. Two spins were located on the FeO moiety and the third almost exclusively on the phenoxy ligand. A hydrogen bond donating to the oxygen of the phenolate ligand, or the placement of cationic species that mimic the presence of a charge-relay system in the protein, caused

a significant change and shifted half of the spin density from the phenoxy ligand to the porphyrin[69]. These results are similar to the ones obtained for Cpd I of P450[19, 58, 63] and indicate that the *chameleon* concept may well be a general paradigm for heme enzymes.

3.10. What Makes the Catalytic Cycle Tick? A Summary

Two factors, mentioned above, emerge from the calculations to strongly influence the catalytic cycle: one is the "push" effect of the thiolate and the other is the hydrogen-bonding machinery that is involved in protonation mechanisms of **5** and **6** as well as in stabilization of Cpd I. The "push" effect is associated with the strong electron donor property of the thiolate ligand. The calculations of Ogliaro *et al.*[40] showed that the "push" effect is responsible for gating the cycle by a single molecule of water; for the O–O cleavage; for the preference of the twice-reduced species **5** and the ferric peroxide Cpd 0 species, **6**, to undergo protonation rather than reduction; and for the propensity of Cpd I to participate in hydrogen abstraction or bond-making processes over electron transfer process. Without the thiolate ligand or with one that is a much lesser electron donor than thiolate, the resting state, as well as **5** and **6**, would have been prone to reduction, the O–O bond cleavage process would have been highly endothermic, and Cpd I would have been an extremely powerful electron acceptor. Thus, the thiolate creates selectivity toward reduction and thereby contributes to a stable cycle with a tightly gated reducibility and basicity of the various species.

The calculations of Guallar *et al.*[48] and of Harris[51] demonstrate that the hydrogen-bonding machinery provides the means for a gentle protonation that can protonate the twice-reduced species, **5**, without touching its precursor ferrous-dioxygen complex, **4**. This gentle machinery awaits, therefore, patiently the second electron transfer and, hence, ultimately enables the generation of Cpd I. The study of Kamachi and Yoshizawa[49] offers an alternative protonation mechanism that is more potent and exothermic than the one advocated by Harris[51], and which applies to both the doublet and the quartet states of **5**. This mechanism is based on a proposal of Vidakovic *et al.*[52] and involves the acidic CO_2H proton of Asp_{251} that is transferred via an array of two waters. Protonation of ferric peroxide species, **6**, appears, however, to proceed by a gentler machinery[49] with exothermicity of *c.*13.1 and 5.5 kcal mol^{-1} (Figure 2.10), respectively, for the quartet and the doublet states. These multiple protonation pathways suggest very strongly that both the doublet and the quartet states of Cpd I may be formed separately, and those mutations may affect the production of the two states in a different manner.

4. MM and MM/MD Studies of P450 Reactivity Aspects

This section reviews MM/MD theoretical work, which addresses the entrance of the substrate to the pocket, its binding, and the exit of the substrate. Much theoretical work that rely on quantitative structure activity relations, QSAR, is not reviewed, but can be found in the authoritative treatment of Lewis[73].

4.1. Studies of Substrate Entrance, Binding, and Product Exit

P450 enzymes usually have an active-site pocket that is equipped with a substrate binding and O=O cleavage machineries[74, 75]. Figure 2.13 shows the QM/MM calculated camphor within the pocket of $P450_{cam}$, in the presence of Cpd I[76]. In accord with experiment, the calculations reveal that two amino acids participate in the substrate binding; Tyr_{96} holds camphor by an OH---O=C hydrogen bond and Val_{295} interacts with the bridge methyl groups of camphor and thereby sequesters the substrate. Other P450 isozymes have their own specific machineries and still others have larger and less selective pockets. Substrate access to the pocket, binding, and product exit have been probed by a variety of experimental techniques, and have been theoretically studied by means of docking and MM/MD simulation techniques of various types.

Docking calculations followed by MM/MD and Monte Carlo simulations on the catalytic metabolism of the insecticide carbofuran by

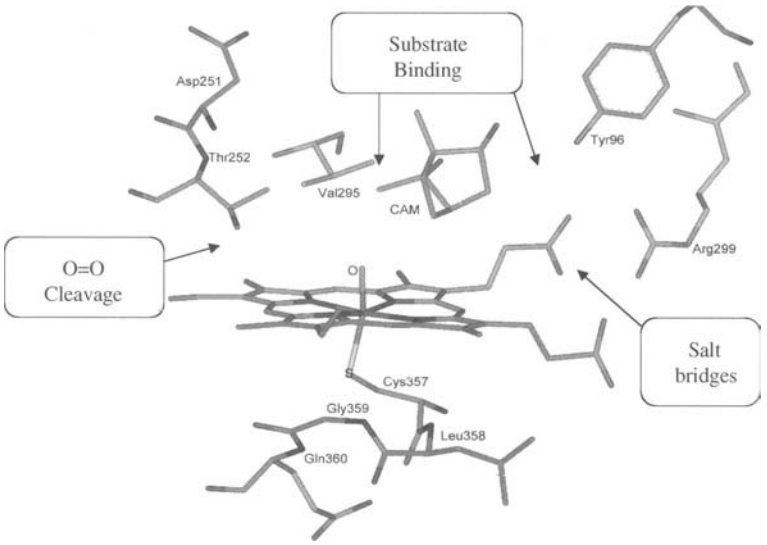

Figure 2.13. The active site of $P450_{cam}$ showing functions of various residues.

$P450_{cam}$ were performed by Keserü *et al.*[77] and Keserü *et al.*[78]; the tight binding of carbofuran by hydrogen bonding to Tyr_{96} and its steric confinement by Val_{247} and Val_{295} were found to fit the preferred regioselectivity and stereospecificity of hydroxylation at the C3 atom of carbofuran. Cavalli and Recanatini[79] used docking simulations to study the selectivity of P450 inhibitors that carry imidazole groups, and found that the inhibitors bind to the heme via the lone pair of nitrogen in the imidazole group. This theoretical approach, and similar ones not reviewed here, tacitly assumes that the preferred position of the substrate *vis-à-vis* the active species can foretell the regio- and stereospecificity of the monooxygenation process, as well as efficacy patterns of inhibitors.

Other approaches, which combined experimental and theoretical studies, revealed some generalities on binding modes and motions within the pocket. NMR results[80] of perdeuterated adamantane in $P450_{cam}$ indicate that the substrate is conformationally mobile on the timescale of the enzymatic turnover. This mobility was further revealed by docking studies[81] of various substrates and inhibitors of $P450_{cam}$. It was found that the general strategy of rigid docking, which seeks substrates that fill the active site completely, fails to reproduce their set of experimental data about the enzyme function. A successful strategy was found to require, *inter alia*, active site plasticity and considerable movement of the substrate, which thereby enable substrate positioning and catalytic function. Other studies revealed that some of the substrate motions are more constrained than others and depend on the topography of the pocket and the substrate. Thus, the experimental results of Atkins and Sligar[82] show that during camphor hydroxylation by $P450_{cam}$ the hydrogen is initially abstracted from both *exo* and *endo* C–H bonds, but the process only produces the *exo* alcohol product. This result indicates that after the hydrogen abstraction, the alcohol formation step is sufficiently fast to reveal the restriction of the camphor by its binding in the pocket. Similarly, MD simulations and experimental studies[83] of the kinetic isotope effect (KIE), for hydroxylation of xylenes and 4,4′-dimethylbiphenyl, having equivalent, but isotopically distinct, methyl and deuterated methyl groups indicated that the mode that switches the position of the two methyl groups is restricted in proportion to the distance between the groups. This restriction masks the intramolecular KIE value for 4,4′-dimethylbiphenyl for which the distance is 11.05 Å, and the KIE drops from *c.*7–10

to *c*.2.7. In summary, the above studies show that the outcome of a given experiment depends on the interplay of the rate constants for substrate binding, de-binding, monooxygenation, and tumbling. Being "free" or "restricted" has a relative meaning depending on the timescale of the various processes.

Still other approaches use MM/MD simulation to study the dynamics of various processes associated with the water content of the protein pocket and the entrance and exit of substrates therefrom. The hydration of the protein cavity was studied by Helms and Wade[84] using MD simulations of the substrate-free $P450_{cam}$ followed by thermodynamic integration. It was found that although the cavity could hold, in principle, 10 water molecules, the thermodynamically most favorable water content in the pocket was 6 molecules; most of them occupied the site of the sixth axial ligand (distal ligand) and one was coordinated to Tyr_{96}. The water molecules in the pocket were spread over a larger volume than in bulk water, and were therefore more mobile than bulk water molecules. MD simulations of the substrate entrance and exit channels of $P450_{cam}$, $P450_{BM-3}$, and $P450_{eryF}$ were studied by Lüdemann *et al.*[5, 6] and Winn *et al.*[85]. In all cases, the major access channels were found to coincide with the ones predicted from crystallographic data based on thermal fluctuation factors (B factors) near the F/G loop and adjacent helices. All these mechanisms involve backbone motions and rotations that are specifically tailored to the physico-chemical properties of the substrate. In $P450_{cam}$, the channel is typified by small backbone displacements (1.8–2.4 Å) and aromatic side-chain rotations of Phe_{87}, Phe_{193}, and Tyr_{29}. In $P450_{BM-3}$, the positively charged Arg_{47} located in the entrance of the channel makes a salt-link that guides the negatively charged substrate via its carboxylate group, while in $P450_{eryF}$, the Arg_{185} residue rotates and, by making intraprotein hydrogen bonds, gates the channel opening. Will such entrance/exit studies eventually account for the specificity of the P450 isozymes, as hoped by the MM modeling community? It is a question that merits a proof of principle. Should this turn out to be the right approach to the problem, then all the chemical details of P450 activation would be immaterial to its action. As shown below, this is certainly not the case.

4.2. MM and MM/MD Studies of Regioselectivity

An MD simulation technique was applied by Audergon *et al.*[83] to analyze the regio- and stereoselectivities of hydroxylation of the (1*R*)- and (1*S*)-norcamphor by $P450_{cam}$. X-ray structures of the substrate–enzyme complex for the two enantiomers of camphor showed that (1*R*) is oriented with its C5 atom pointing toward the heme iron, whereas (1*S*) exhibits significant disorder. Despite the different substrate-binding conformations of the two enantiomers, both are known to give exclusively *exo*-C5-hydroxylation. To resolve this apparent inconsistency between the two sets of experimental results, Das *et al.*[86] performed MD simulation on the substrate binding of these two camphor enantiomers to $P450_{cam}$. The results[86] for both enantiomers revealed the strong orienting effect of the hydrogen bond to Tyr_{96}. However, while the (1*R*) enantiomer gave one stable structure, the (1*S*) enantiomer had greater mobility in the active-site pocket. In addition, the (1*R*) enantiomer was found to orient with its C5 atom pointing toward the heme iron, whereas this was not the case for the (1*S*) enantiomer. These differences accounted for the X-ray structural findings. To address the apparent inconsistency between the experimental X-ray and reactivity data, the simulation was repeated with a water molecule as the sixth ligand. The presence of the water molecule was found to reorient the (1*S*) enantiomer with a C5 contact to the heme. This result was interpreted by the authors as a resolution of the inconsistency, based on the contention that regioselectivity ultimately depends on the orientation of the substrate *vis-à-vis* the ferryl oxygen of Cpd I. The same technique was employed for the monooxygenation of styrene by $P450_{cam}$ (ref. [87]) where a good fit was obtained between product distribution and the docked conformation.

MD simulation studies by Harris and Loew[88] were used to rationalize the regiospecificity of hydroxylation of camphor and a variety of other substrates. A series of trajectory calculations were performed for the enzyme–substrate interactions, and these results were coupled with relative stability of the organic radical intermediates to predict the product distributions. In a recent paper, Park and Harris[89] employed an integrated

modeling approach that involves comparative modeling (sequence and SCR alignment), a *de novo* loop construction and MD equilibration, to reconstruct a model of $P450_{2E1}$ of sufficient accuracy to ascertain the geometric determinants of diverse substrate metabolism via configurational sampling techniques. Energy-based docking was shown to be an adequate predictor of binding modes correlated with experimentally deduced metabolites. An MD-configurational sampling based on the low-energy docked configurations was found to be a more accurate predictor of geometric factors. In this manner, it was possible to screen many substrates and locate the lowest energy enzyme–substrate complexes. Assessment of the relative hydroxylation efficiency of three prototypical substrates at their various functional groups was carried out by combination of MD sampling, of the docked configurations, and an energy criterion of the relative DFT-calculated energies of the radical intermediates produced in the reaction by hydrogen abstraction (see mechanisms below). The relative energies of the radicals were found to be good predictors of the relative barriers of the C–H abstraction step. In several instances, these workers found that while equivalent geometric exposure of metabolical sites occurred, *an accurate prediction of the metabolite pattern could be made only by means of electronic and energetic factors deduced from DFT.*

In summary, investigations of P450 mechanistic problems by reliance on the modes of substrate entrance and binding as the determinants of all the subsequent chemistry, while being a tempting and an economical approach to the problem, are, in our view, not well founded. Importantly, such approaches miss the crucial factors concerning the electronic structure determinants of the processes, as discussed in the rest of the review. The recent results of Park and Harris[89] support this conclusion and highlight the crucial nature of the fundamental mechanistic investigations by means of QM calculation, as reviewed in the remainder of this chapter.

5. QM Studies of P450 Reactivity Patterns

Cpd I is considered to be the primary reactive species of P450 enzymes. However, Cpd 0 (**6**) has been implicated as a second oxidant that functions alongside Cpd I, or in its absence, for example, in mutant enzymes where the O–O cleavage machinery has been impaired[90, 91]. Recent results on the mutant enzyme of $P450_{cam}$ (ref. [92]) show that the mutant $P450_{cam}$ (T252A), where the threonine that is responsible for the efficient protonation machinery is mutated to an alanine, does not hydroxylate camphor, but does epoxidize camphene, albeit much less efficient than in the wild-type enzyme. It was postulated that, in the absence of Cpd I, Cpd 0 was the likely oxidant but that it is a much less efficient oxidizing species than Cpd I. The next few sections outline the results of QM and QM/MM calculations on some of the major reactions of P450: alkane hydroxylation, alkene epoxidation, benzene hydroxylation, and sulfoxidation. These reactions were studied using Cpd I as the electrophilic oxidant. Two reactions, ethene epoxidation and sulfoxidation, were studied with both Cpd I and Cpd 0.

5.1. Reactivity of Cpd I: General Considerations of the Origins of Two-State Reactivity (TSR) of Cpd I

As seen already, Cpd I is a triradicaloid with singly occupied π^*_{xz}, π^*_{yz} and a_{2u} orbitals and, hence, has a virtually degenerate pair of ground states ($^{4,2}A_{2u}$). As such, it is expected that at least these electronic states will participate in the reactions, and will lead thereby to two-state reactivity (TSR)[93–95]. In TSR, each state may produce its specific set of products with different rate-constants, regio- and stereoselectivities and lead thereby to apparently controversial information when viewed through the perspective of single-state reactivity (SSR). It is our contention that TSR resolves much of the controversy that has typified the P450 field of reaction mechanism in recent years[95], and opens new horizons for reactivity studies.

5.2. A Primer to P450 Reactivity: Counting of Electrons

As a prelude to the reactivity discussion, it is worthwhile to appreciate an important generalization, namely that the synchronous oxene insertion

by Cpd I is a forbidden reaction[96, 97], and that we expect, therefore, to deal with essentially nonsynchronous processes even if some of the mechanisms may turn out to be effectively concerted. To assist us in keeping track of the electron count during this formal "two-electron oxidation" process, we present in Figure 2.14, an oxidation state-orbital occupancy diagram that follows the electronic reorganization and accounts for the formal oxidation states of Cpd I and the substrate at various phases of the process. The substrate is chosen to be one that can undergo hydroxylation or epoxidation and is symbolized by its two main active orbitals, the σ_{CH} orbital that figures in hydroxylation and π_{CC} that is important for epoxidation. Thus, initially Cpd I involves Fe^{IV} and porphyrin radical cation ($Por^{+\bullet}$), that is, the effective oxidation state is Fe^{V}. As Cpd I and the substrate make one bond (O–H or O–C), a single electron shifts from the appropriate orbital of the substrate (σ_{CH} or π_{CC}) to either the a_{2u} orbital of the porphyrin (**1**), or to the π^*_{xz} orbital of the FeO moiety (**2**), leaving a singly occupied orbital labeled as ϕ_C on the substrate. Now, the effective oxidation state of the heme species is reduced to Fe^{IV} (either $Fe^{III}Por^{+\bullet}$ or $Fe^{IV}Por$) accounting for

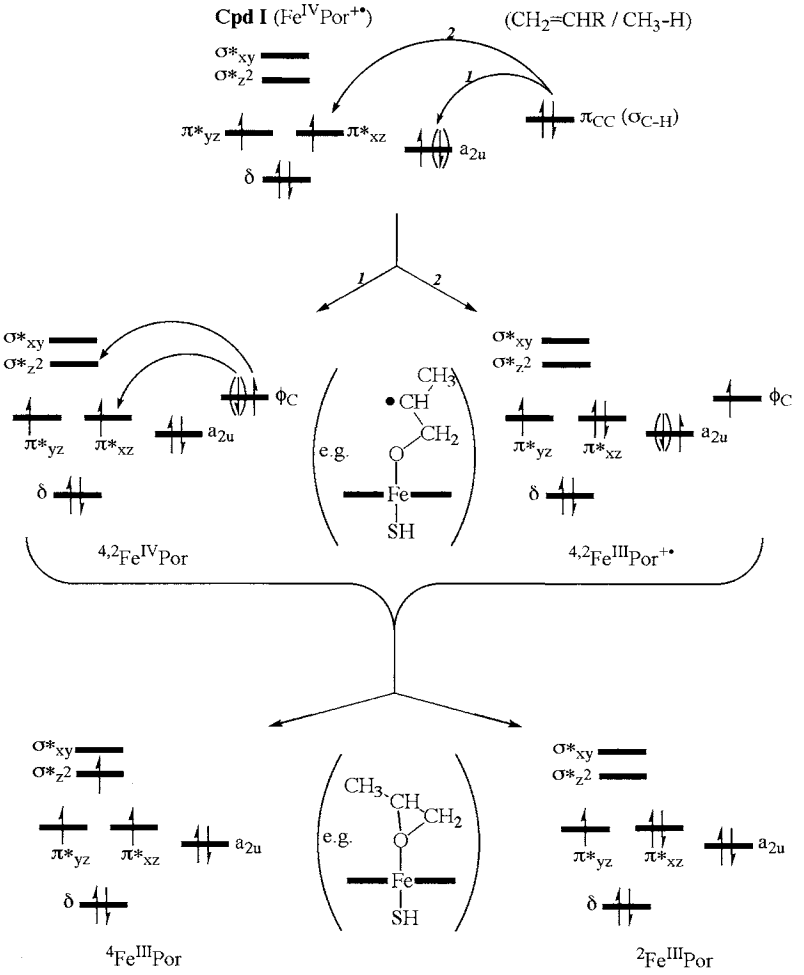

Figure 2.14. Oxidation-states and orbital occupancy diagrams in various stages of alkene epoxidation and alkane hydroxylation. Occupations within parentheses show the alternative spin arrangement, of the odd electron, in the corresponding low-spin state.

the first oxidation equivalent. In the second phase, the substrate forms a second bond with the oxo group of the heme, and a second electron is shifted from the singly occupied substrate orbital ϕ_C to the heme to populate either the $\sigma^*_{z^2}$ orbital, which results in a quartet state of the product complex, or to the π^*_{xz} orbital of $Fe^{IV}Por$ or the a_{2u} orbital of $Fe^{III}Por^{+\bullet}$; the latter two options give the doublet state of the product complex. The last step accounts for the second oxidation equivalent, and the effective oxidation state of the heme is further reduced to Fe^{III}.

5.3. Alkane Hydroxylation

The mechanism of alkane hydroxylation is called the "rebound" mechanism. It transpires via an initial hydrogen abstraction, followed by rebound of the alkyl radical onto the oxygen of the iron-hydroxo species, Figure 2.15(a)[98]. This mechanism accounts for two key observations: (a) a small but detectable amount of stereochemical scrambling, and (b) a large KIE due to replacement of the hydrogen by deuterium in the C–H bond undergoing hydroxylation. The first measurement of radical lifetime by Ortiz de Montellano and Stearns[99] indicated that the radical derived from bicyclo[2.1.0]pentane had a finite, albeit short, lifetime. Everything looked fine for the rebound mechanism until Newcomb *et al.*[91, 100] used their ultrafast radical clocks to determine radical lifetimes. Figure 2.15(b) depicts a typical probe substrate used by Newcomb and its rearrangement pattern; the corresponding lifetime is determined from the inverse of the rate constant (k_R) of radical rearrangement and the ratio [***R/U***] of rearranged to unrearranged alcohol products. Using this method and determining only those [***R/U***] quantities that do not involve carbocation rearrangement, the resulting lifetimes quantified by Newcomb were in the order of 80–200 fs (ref. [100]). Since these lifetimes are too short to correspond to a real intermediate, Newcomb concluded that radicals are not present during the reaction and questioned the validity of the rebound mechanism.

This problem was taken on by the Jerusalem group who used DFT (B3LYP) computations to model the mechanism of methane hydroxylation[62, 101–103], allylic hydroxylation of propene[104, 105], and recently also of camphor hydroxylation by QM and QM/MM calculations[76]. In all these cases, we could not locate a TS for a concerted oxene insertion because the process possesses barriers that

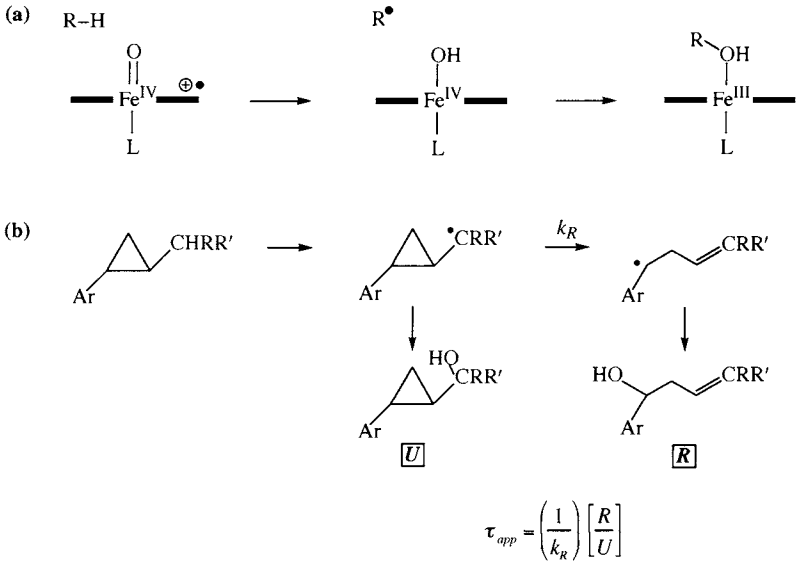

Figure 2.15. (a) The rebound mechanism. (b) Derivation of the apparent lifetime (τ_{app}) of a putative radical intermediate from the ratio of rearranged (***R***) to unrearranged (***U***) alcohol products produced from P450 hydroxylation of a substrate probe.

are too high and transition structures that are not real TSs; for example, they are second-order saddle points. The lowest energy mechanism was found to involve a hydrogen-abstraction like TS (TS_H), as exemplified in Figure 2.16 for camphor hydroxylation[76, 106]. However, the calculations reveal, as conjectured above, that the mechanism involves TSR nascent from the degenerate ground state of Cpd I that was modeled by the simplest system (with a porphine macrocycle and HS^- as a proximal ligand). Subsequent studies of ethane and camphor hydroxylation by the Yoshizawa group[49, 107–111] and of methane hydroxylation by Hata *et al.*[112], used porphine macrocycle and CH_3S^- as a proximal ligand, and arrived at basically the same conclusion, that the mechanism is typified by TSR.

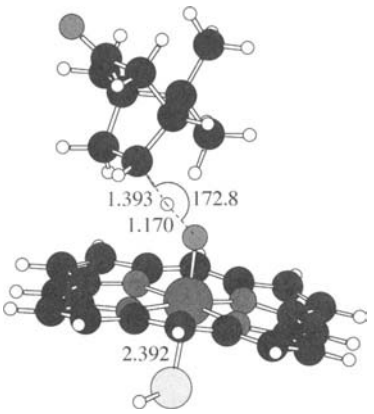

Figure 2.16. Camphor hydroxylation high-spin TS.

A typical reaction mechanism is shown in Figure 2.17, where one can see the doubling of the profile due to the HS and LS states. The reaction pathway involves three phases: (a) a C–H abstraction phase that leads to an alkyl radical coordinated to the iron-hydroxo complex by a weak OH---C hydrogen bond, labeled as $^{4,2}C_I$. (b) an alkyl (or OH) rotation phase whereby the alkyl group achieves a favorable orientation for rebound, and (c) a rebound phase that leads to C–O bond making and the ferric-alcohol complexes, $^{4,2}P$. The two profiles remain close in energy throughout the first two phases and then bifurcate. Whereas the HS state exhibits a significant barrier and a genuine TS for rebound, in the LS state, once the right orientation of the alkyl group is achieved, the LS rebound proceeds in a virtually barrier-free fashion to the alcohol. As such, alkane hydroxylation proceeds by TSR, in which the HS mechanism is truly stepwise with a finite lifetime for the radical intermediate, whereas the LS mechanism is effectively concerted with an ultrashort lifetime for the radical intermediate. A recent study of camphor hydroxylation[49] identifies a rebound TS for the LS process; this TS has a barrier of 0.7 kcal mol^{-1} but is merely the rotational barrier of the camphor to the rebound position.

By referring to Figures 2.15(b) and 2.17, it is possible to rationalize the clock data of Newcomb in a simple manner. The apparent lifetimes are determined from the rate constant of free radical rearrangement and the ratio [***R/U***] of rearranged to unrearranged alcohol product, assuming a

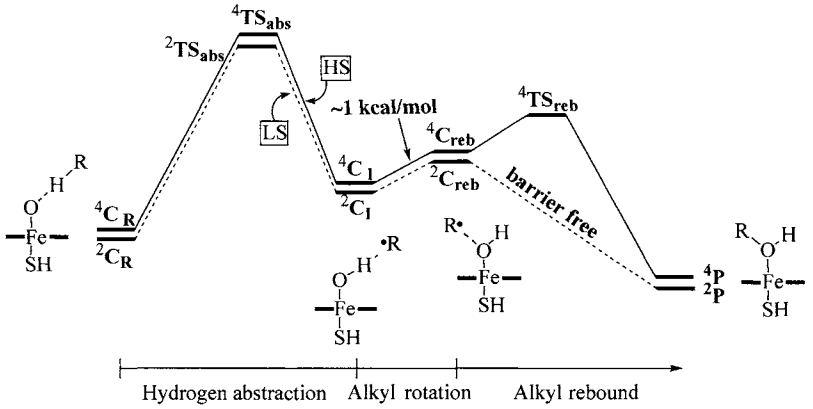

Figure 2.17. A two-state reactivity potential energy surface for alkane (R–H) hydroxylation by Cpd I.

single-state reactivity (SSR). However, in TSR, the rearranged product, ***R***, is formed only on the HS surface, while the unrearranged product, ***U***, is formed on the LS and possibly also on the HS surface. Therefore, the ratio, [***R/U***], is associated with the relative yields of the HS *vis-à-vis* the LS reactions and not with the radical lifetime as such[62, 95]. A simple TSR scheme leads to the following expression for the ratio of the real to the apparent lifetimes:

$$\tau_{REAL}(\mathrm{TSR})/\tau_{APP} = \frac{\{[\boldsymbol{U/R}]\,(1+F)\}}{\{[\boldsymbol{U/R}]-F\}} > 1, \qquad F = [\boldsymbol{LS/HS}], \quad (1)$$

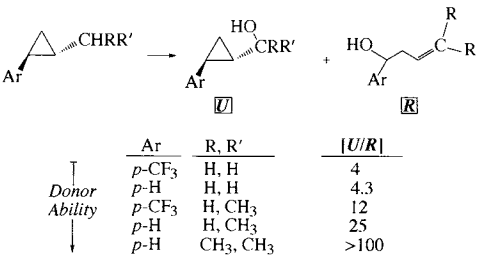

Figure 2.18. Experimentally determined ratios of unrearranged to rearranged product of various probes. The probes are arranged from top to bottom in order of increasing donor ability.

where the quantity F is the relative yield of the LS to HS reactions. It is clear that the real lifetime of the radicals on the HS surface is longer than the apparent lifetime in proportion to the value of F, the LS *vis-à-vis* HS yield. Since in the calculations the LS bond activation barrier is lower than the corresponding HS barrier, the F is larger than unity[62]; in the case of allylic hydroxylation, it reaches values of the order of 10 when the effect of the protein electric field and hydrogen bonding are taken into account[104, 105]. As such, the apparent radical lifetimes will be unrealistically short compared with the real lifetimes. Furthermore, analysis of the rebound process itself[62, 95, 102] showed that the quantity [***U/R***] should increase significantly as the alkane and its derived radical become better electron donors. Therefore, in such a series, the [***U/R***] quantity gradually increases, such that at some critical donor ability of the radical when the HS rebound barrier altogether vanishes, the [***U/R***] quantity converges to infinity, and the apparent lifetime becomes strictly meaningless. Such a trend has been observed in the series of probe substrates (*trans*-alkylarylcyclopropanes) used by Newcomb *et al.*[100] where the substrate, which is the best donor and which leads to the best donor radical, exhibits virtually no rearrangement. This Newcomb series is shown in Figure 2.18, which arranges the substrates in the order of increasing donor ability and displays the corresponding [***U/R***] quantity that exceeds 100 for the best donor situation.

Calculations of Yoshizawa *et al.*[108–111] using CH_3S^- as a proximal ligand retrieved the TSR scenario, and recently also the rebound barrier behavior of the LS and HS states[49]. However, these calculations reverse the ordering of the bond activation $^{2,4}TS_H$ species from "LS-below-HS" as in Figure 2.17, to "HS-below-LS." In addition, the LS pathway involves Fe^{III}, whereas by contrast, the HS pathway is Fe^{IV} type. We think that this inversion of the HS–LS ordering originates in the powerful electron donor property of the CH_3S^- ligand, such that its gas phase calculations do not represent as well as the HS^- ligand the actual situation of the cysteinate within the protein pocket[19]. Thus, much the same as in the case of Cpd I where the use of CH_3S^- as a proximal ligand in a gas phase calculation leads to a wrong assignment of the ground state (as a $^2\Pi_S$ state), this ligand also misrepresents the LS–HS energy difference of the bond activation TS_H species. In fact, QM/MM calculations of camphor hydroxylation by $P450_{cam}$ (ref. [76]) lead to a TS-situation of LS below HS, in agreement with the model studies of Ogliaro *et al.*[62, 103] and De Visser *et al.*[104, 105] using HS^- as a proximal ligand.

Figure 2.19 displays bond activation $^{2,4}TS_H$ structures for methane, ethane, propene, and camphor hydroxylation. It is apparent that irrespective of the alkane and proximal ligand model, all the TSs exhibit an almost linear O---H---C triad of atoms. These species closely resemble the genuine hydrogen abstraction TSs by alkoxyl radicals, one of which is also displayed in the figure. The computed KIE (T = 300 K) values of the P450 TSs range between 5.1 and 10.5 for the various substrates and models[49, 103, 105, 110] and are in good agreement with experimental values[95, 103].

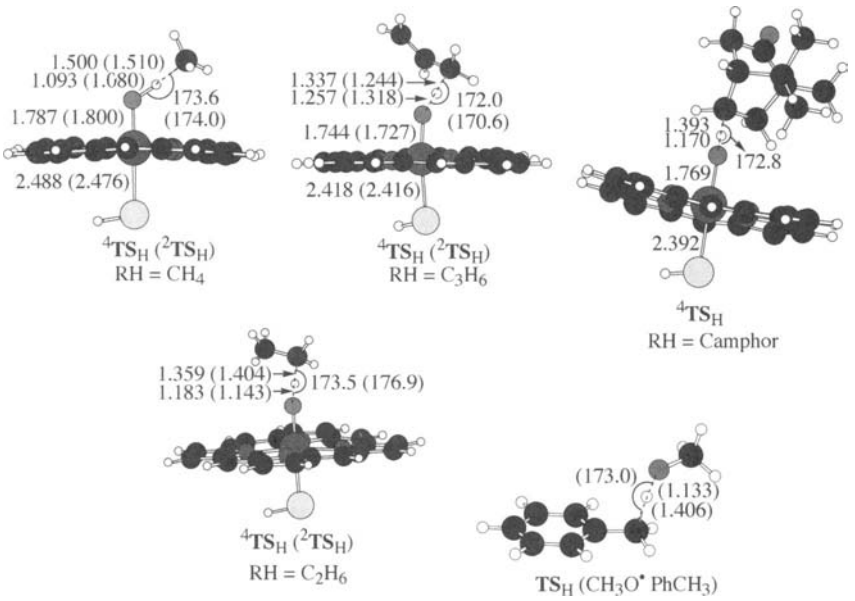

Figure 2.19. Hydrogen abstraction transition states (TSs) for some model reactions. The structures for methane and allylic hydroxylation are taken from Ogliaro *et al.*[62, 103] and De Visser *et al.*[105], the structure for H-abstraction from toluene from Ogliaro *et al.*[103], while for ethane hydroxylation from Yoshizawa *et al.*[111]. Key geometric parameters outside parentheses correspond to the high-spin TS, while those within to the corresponding low-spin structure.

The hydrogen abstraction barriers in alkane hydroxylation shown in Table 2.1 exhibit a high sensitivity to the donor property of the alkane and the C–H bond energy. They range from 26.7, 26.5 (HS, LS) kcal mol^{-1} for methane down to 13.5 kcal mol^{-1} for allylic hydroxylation. This trend was analyzed and shown to conform to hydrogen abstraction and oxidative character of the bond activation step, as outlined above in the oxidation state-orbital population diagram in Figure 2.14[62, 105]. Another feature in Table 2.1 is the reduction of the hydrogen abstraction barrier when zero point energy (ZPE) is included. This arises due to the loss of the ZPE for the C–H bond that is cleaved in the bond activation $^{2,4}TS_H$ species. Another feature in Table 2.1 is the very significant entropic contribution to the free-energy barrier. Much of this arises due to the loss of translational and rotational degrees of freedom and structural stiffening in the $^{2,4}TS_H$ species. Substrate binding within the enzyme is, to a large extent, entropically driven, because it causes expulsion of the water molecules from the binding pocket. Since the hydroxylation begins with the bound substrate,

Table 2.1. Hydrogen Abstraction Barriers

Substrate	$\Delta E^‡$	$\Delta(E+ZPE)^‡$	$\Delta H^‡$	$\Delta G^‡$
CH_4[a]				
HS	26.69	22.76	22.55	31.74
LS	26.54	22.31	21.68	32.35
C_2H_6[b]				
HS	19.4			
LS	16.2			
CH_2=CH–CH_3[c]				
HS	13.53	10.63	10.92	21.21
LS	13.52	10.83	11.28	21.35
Camphor[d]				
HS	18.8			
LS	17.3			
Camphor[e]				
HS	18.0			
LS	20.2			

Notes:
[a]Ogliaro *et al.*[62].
[b]Yoshizawa *et al.*[111].
[c]De Visser *et al.*[105].
[d]Cohen *et al.*[76].
[e]Kamachi and Yoshizawa[49].

at least a good part of this entropic effect, due to restriction in the $^{2,4}TS_{H}$ species, will not contribute to the free-energy barrier. *Thus, the protein machinery that utilizes mobile water molecules absorbs much of the entropic cost of establishing a TS.* Therefore, the gas phase quantities that are more informative of the situation in the protein are not $\Delta G^{\ddagger}$ but $\Delta(E+ZPE)^{\ddagger}$ and $\Delta H^{\ddagger}$. However, a thorough discussion of this feature is impossible at the time of the writing of this manuscript and will have to await QM/MM calculations with real sampling and thermodynamic integration.

5.4. The Rebound Process: More Features than Meet the Eye

The iron-hydroxo intermediate that is formed during the bond activation step exists in two close-lying electromers[101], which differ in the oxidation state of the metal and porphyrin ligand. By reference to the orbital diagram in Figure 2.14, these electromers differ in the orbital occupancy of the d-block and porphyrin a_{2u} orbitals; the Por+•Fe^{III}OH electromer has close lying singlet and triplet $\pi^{*2}_{xz}\ \pi^{*1}_{yz}\ a_{2u}^{1}$ configurations, while the PorFe^{IV}OH state has a triplet $\pi^{*1}_{xz}\ \pi^{*1}_{yz}\ a_{2u}^{2}$ configuration. These two electromeric situations are close in energy, and small changes such as substituents on the porphyrin ring or replacement of the axial ligand can reverse their ordering[101]. Coupling with the alkyl radical leads to five states of the corresponding HS and LS $Por^{+\bullet}Fe^{III}OH/R^{\bullet}$ and $PorFe^{IV}OH/R^{\bullet}$ species. Indeed, gas phase calculations give as ground states either electromer, as for example found recently by Kamachi and Yoshizawa[49] for camphor hydroxylation, where the LS electromer was of the $Por^{+\bullet}Fe^{III}OH/R^{\bullet}$ variety, while the HS electromer was of the $PorFe^{IV}OH/R^{\bullet}$ variety. All the five states can in turn participate in rebound, and as was repeatedly found[62, 105] that the HS intermediates rebound with a significant barrier ($2 < \Delta E^{\ddagger}_{reb} \leq 6$ kcal mol^{-1}), while the LS intermediates rebound without a barrier past the orientation phase (Figure 2.17) that occurs by combined rotation of the alkyl and OH groups.

The origin of the rebound barrier on the HS surface was analyzed[62, 102, 113] and shown to result from the need to shift an electron from the alkyl radical to the heme and populate the high-lying $\sigma^{*}_{z^2}$ orbital; this orbital is antibonding in the Fe–S and Fe–O linkages (see Figure 2.14). In line with the population of the $\sigma^{*}_{z^2}$ orbital, both Fe–S and Fe–O bonds are seen from Figure 2.20 to undergo lengthening in the $^{4}TS_{reb}$ species (relative to the values in the iron-hydroxo radical clusters, shown below the TSs). This bond lengthening is the origin of the HS rebound barrier. By contrast, in the LS process, the electron, shifted from the alkyl radical, fills only low-lying orbitals, either the π^{*}_{xz} orbital of iron or the porphyrin a_{2u} orbital depending on electromeric identity (Fe^{III} or Fe^{IV}). The rate of the rebound process depends much on the electron donor

$^{4}TS_{reb}$; R = CH_3
r_{FeO} = 1.816
r_{FeS} = 2.480

$^{4}TS_{reb}$; R = C_3H_5
r_{FeO} = 1.804
r_{FeS} = 2.356

Figure 2.20. Key geometric parameters for high-spin rebound transition states in methane hydroxylation[62] and allylic hydroxylation[105]. The quantities below the structures correspond to the geometric parameters of the corresponding iron-hydroxo/alkyl radical clusters ($^{4}C_{1}$ in Figure 2.17).

capability of the radical and its C–O bond strength (see above), but also on the acceptor properties of the iron-hydroxo species, its electromeric state, and height of the $\sigma^*_{z^2}$ orbital; these properties depend on the polarity and acidity of the protein pocket and its steric constraints on the Fe–S bonding. The role of the $\sigma^*_{z^2}$ orbital was recently highlighted in the study of methane hydroxylation by the ruthenium analog of Cpd I (ref. [67]), where a very high $\sigma^*_{z^2}$ orbital led to a very high rebound barrier of *c.*11.9 kcal mol^{-1}. An additional factor revealed by the calculations[49, 105, 113] is that the rebound process may occur either by OH rotation around the Fe–O bond, as in the case of the methane monooxygenase enzyme[114], or by rotation of the alkyl group around the same bond, or still by some combination of the two modes. In allylic hydroxylation, the prominent mode was found to be the rotation of the allyl group about the Fe–OH bond[105], while in camphor hydroxylation, both OH and alkyl rotations take part in the rebound[49]. It is very clear that the topography of the protein pocket and the mode of substrate binding will play major roles in the rebound process, by selectively constraining/preferring some of the rebound modes over others. The results of Atkins and Sligar[82] that during camphor hydroxylation both *exo* and *endo* C–H bonds are activated, but the only product is an *exo*-alcohol, is indicative of the manifestations of such selective constraints. All in all, the rebound process is more intricate than meets the eye, and certainly more than QM or even QM/MM calculation can resolve at present. Here we have to look forward for a combination of QM/MM with MD calculations.

5.5. Alkene Epoxidation

The DFT (B3LYP/LACVP) computed mechanistic scheme for ethene epoxidation by the simplest model Cpd I species is summarized in Figure 2.21[97, 115, 116]. The first step involves bond activation and leads to the iron-alkoxy radical intermediate that appears in both Fe^{III} and Fe^{IV} electromers of the HS and LS varieties. In a subsequent phase, these intermediates undergo ring-closure to form the epoxide complex. The alternative synchronous concerted oxygen insertion was also tested[97] but ruled out as a viable mechanism. Precisely the same features were obtained for propene epoxidation[105], with the exception that the bond activation barriers are *c.*4 kcal mol^{-1} lower than those, shown in Figure 2.21, for ethene epoxidation.

The lowest energy TSs for bond activation for ethene and propene activation are shown in Figure 2.22. Since the bond activation involves an electron shift from the alkene to the heme (consult Figure 2.14), propene, which is a better electron donor than ethene, has lower barriers, 10.0 and 10.6 kcal mol^{-1} *vis-à-vis* 13.9 and 14.9 kcal mol^{-1} (HS, LS). In accord, the TSs of propene are seen to be earlier than those of ethene, with less C=C activation, etc. An interesting feature of the two

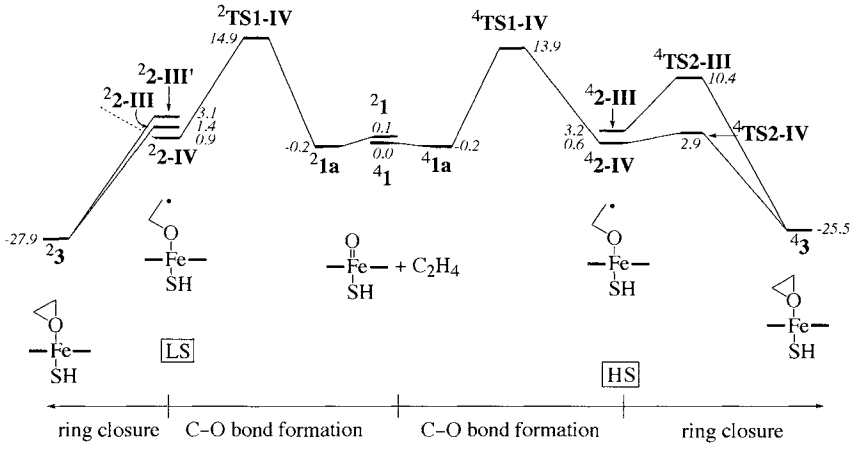

Figure 2.21. Two-state reactivity potential energy surface for ethene epoxidation[115].

TSs is their propensity for an upright orientation of the alkene moiety *vis-à-vis* the plane of the porphyrin. This gas phase conformation avoids the steric repulsion with the porphyrin. However, in the protein pocket, the upright conformation may encounter repulsion from the side-chain amino acids, and a compromise may be achieved in the parallel conformation, which is generated, in Figure 2.22, from the gas phase TS by a simple rotation that achieves a dihedral FeOCC angle of 90°. This structure is related to the one proposed by Groves *et al.*[117] to account for the preferred reactivity of *cis* compared with *trans* isomers, due to enhanced steric repulsion of the substituents in the latter isomer with the porphyrin ring (see the corresponding distances in Figure 2.22).

Past the bond activation phase, in Figure 2.21, the radicals undergo ring closure. As in the hydroxylation, the LS radical complexes undergo ring-closure in a virtually barrierless fashion, whereas on the HS surface, the radicals encounter significant barriers. These barriers for the HS Fe^{IV} electromer are smaller than those for rebound in alkane hydroxylation. However, the ring-closure barriers are large for the HS Fe^{III} electromers. This implies that the radical intermediate complexes, and especially those for the Fe^{III} electromer, will have a significant lifetime only on the HS surface, where they may give rise to rearranged products or lead to side reactions. For instance, rotations around the C–C or the C–O bonds were found to cost less than 1.5 kcal mol^{-1}. Thus, C–C rotation on the HS surface will result in the production

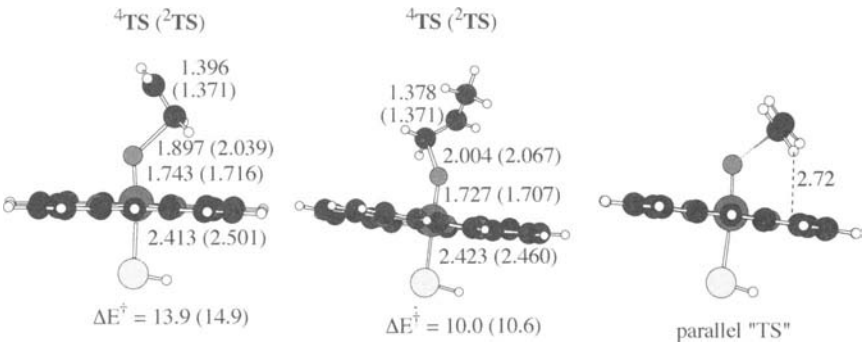

Figure 2.22. Computed structures of the C–O bond activation transition states, and barriers, for ethene[115] and propene epoxidation[105]. The putative "parallel" TS for ethene epoxidation is generated by rotating the computed one around the O–C bond.

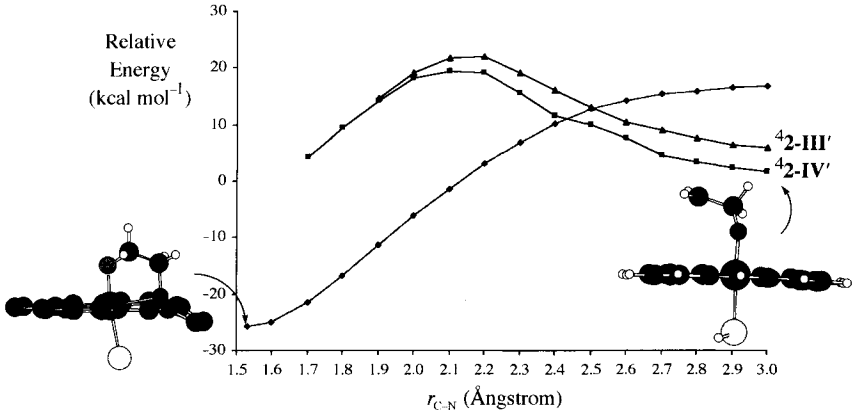

Figure 2.23. Geometry scan for the formation of the suicidal complex by crossover from the high-spin Fe^{III} intermediate (^{4}**2-III′**) to the state generated by promoting an electron from the methylene radical group to the σ^*_{xy} orbital of iron (see Figure 2.3).

of both *cis*-epoxide and *trans*-epoxides from *cis*- or *trans*-alkenes, whereas the LS surface will essentially retain the original isomeric identity of the alkene.

Similarly, rotation around the C–O is facile and will bring the radical center to a position for heme alkylation, where the alkylation barriers are small enough to compete with rebound. The DFT calculations[116] show that the HS radical complex of the Fe^{III} electromer can cross over to a state that is initially higher lying, but which is adiabatically connected with the suicidal complex in Figure 2.23. This crossing point is $\leq$10 kcal mol^{-1} above the Fe^{III} radical intermediate, so that this process may be able to compete with the ring closure of the Fe^{III} electromer to epoxide (with a barrier of 7.2 kcal mol^{-1}). The state, leading to the suicidal complex, is obtained by shifting an electron from the CH_2 moiety of the radical complex, which thereby becomes a carbocationic center, to the σ^*_{xy} orbital of the heme, which thereby becomes anionic with loose Fe–N bonds. As such, the state of the suicidal complex is an internal ion pair that undergoes cation (C^+) anion (N^-) combination, which is likely to be facilitated by the polar environment of the protein pocket. We have preliminary results for the mechanism of formation of the aldehyde side product, which is formed in a related mechanism to the suicidal complex[118]. The rebound barriers as well as those for the side product formation are subject to polarity and NH --- S hydrogen bonding effects[105].

5.6. Hydroxylation of Arenes

One of the long-standing controversies concerns the hydroxylation mechanism of arenes, which apart from phenol can produce also ketone and arene oxide as side products. A universal feature of arene-hydroxylation is the so-called NIH-shift, which accounts for the fact that the original hydrogen atom in the activated C–H bond is retained in the reaction products[1, 54]. A recent DFT investigation[119] addressed the mechanism of benzene hydroxylation. A rebound mechanism, analogous to alkane hydroxylation, and an electron transfer mechanism were ruled out due to their high-energy costs, while the lowest energy mechanisms were found to involve π-attack as summarized in Figure 2.24. In accord with deductions from experimental data[120, 121], the computations show that the bond activation proceeds via two mechanisms; one leads to a radical σ-complex ($^2\sigma$-C$^\bullet$) and the other to a cationic σ-complex

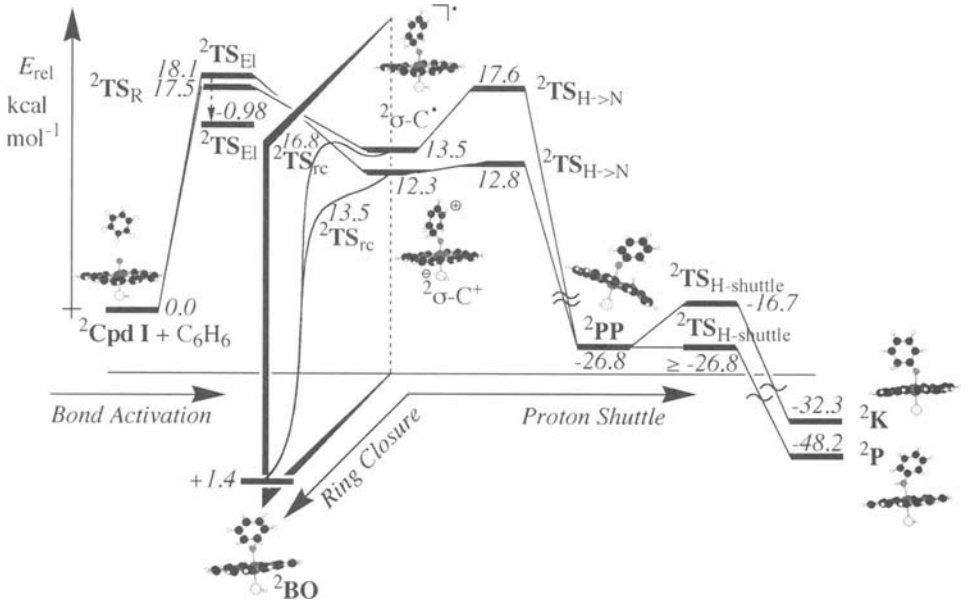

Figure 2.24. Potential energy surface for the competing oxidation mechanisms of benzene by Cpd I (ref. [119]).

($^2\sigma$-C+). Both the radical and electrophilic mechanisms involve the LS states, while the HS states give rise to TSs that are too high to compete with the LS states. Polarity and NH---S hydrogen bonding prefer the electrophilic pathway compared with the radical one. Thus, the calculations predict that the major pathway for arene hydroxylation will be the electrophilic pathway.

The so-formed intermediates, in Figure 2.24, subsequently bifurcate either to the ferric benzene oxide (^{2}**BO**) complex by ring closure, or to the N-protonated porphyrin intermediate (^{2}**PP**) by proton transfer from the *ipso* carbon to one of the nitrogens of the porphyrin. In turn, the latter intermediate reshuttles the proton either to the oxo group to give phenol (^{2}**P**) or to the *ortho* carbon to give the ketone (^{2}**K**). Since both $\mathbf{TS}_{H}$-shuttle species lie well below $\mathbf{TS}_{H->N}$, the excess energy will give rise to both phenol and ketone. This proton-shuttle mechanism accounts for the NIH-shift, since the original hydrogen in the activated C–H bond ends up in the products. It follows therefore that, in addition to phenol production by nonenzymatic protonation of benzene oxide under physiological condition, there should exist an enzymatic pathway that leads directly to phenol and ketone production without the intermediacy of benzene oxide.

5.7. Sulfoxidation of Alkyl Sulfides

Another common reaction of P450 is the sulfoxidation of sulfides[1]. Figure 2.25 summarizes the results of a recent DFT study of the sulfoxidation reaction of dimethyl sulfide with a Cpd I model[122]. The reaction is seen to proceed in a concerted manner via LS and HS pathways. However, in contrast to the hydroxylation of benzene[119], which occurs by a dominant LS potential energy surface, sulfoxidation exhibits a dominant HS pathway, which becomes even more so by inclusion of the effect of medium polarity (using a dielectric constant, $\varepsilon = 5.7$). Unlike alkane hydroxylation and alkene epoxidation where the TSs are related in their electronic structures and differ in having ferro-, namely antiferromagnetic coupling of the three unpaired electrons, in sulfoxidation, the LS and HS TSs are very different in their geometries and electronic structures because the two oxidation equivalents (Figure 2.14) must be condensed into a single step. Thus, sulfoxidation behaves as HS and LS displacement reactions where the sulfur attacks the oxo moiety and displaces the heme, which in turn rebinds to the sulfoxide via a long Fe–O bond. Different heme-oxo orbitals figure in the

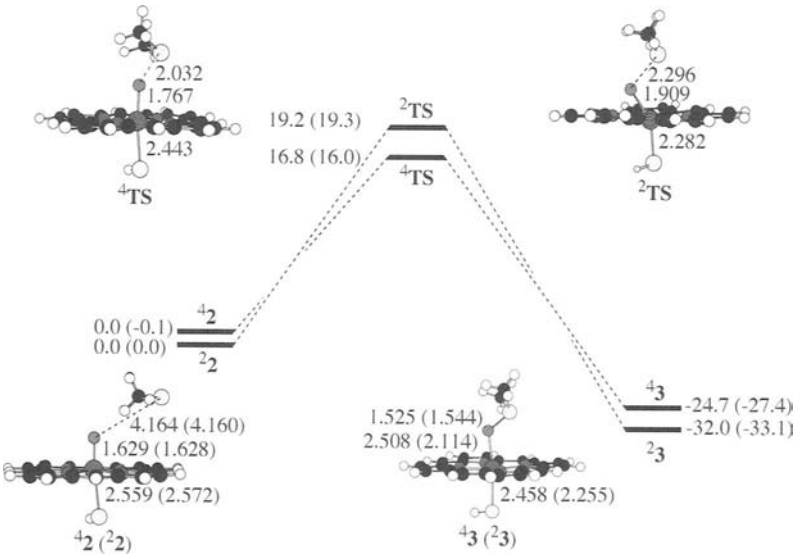

Figure 2.25. Potential energy surface for the sulfoxidation of dimethyl sulfide by Cpd I (ref. [122]). Barriers in parentheses incorporate the effect of a dielectric constant, $\varepsilon = 5.7$.

interaction with the lone pair of the attacking sulfur; in the LS process, these are the π^*(FeO) and a_{2u} orbitals that get filled up during the displacement, whereas in the HS process, the $\sigma^*_{z^2}$ and a_{2u} orbitals accept each one electron through the interaction with the sulfur. It seems reasonable to expect that the sulfoxidation results apply to other heteroatom oxidation processes. Our calculations, done for a single substrate, do not rule out an electron transfer mechanism for substrates that are more powerful donors. However, as a rule, the cysteine ligand makes Cpd I of P450 a relatively poor electron acceptor to participate in pure electron transfer processes with diffusive products[40].

5.8. Can Ferric Peroxide (6) be a Second Oxidant?

This question was addressed by the Jerusalem group using the marker reaction of Cpd 0: alkene epoxidation[53]; ethene served as the alkene. Four different mechanisms were computed, corresponding to concerted and stepwise epoxidations by both distal and proximal oxygen groups of Cpd 0. The barriers for the four mechanisms relative to those obtained by Cpd I that are displayed in Figure 2.26, show that Cpd 0 is by far inferior to Cpd I.

Similar results were obtained for sulfoxidation[122], where Cpd 0 led to barriers in excess of 40 kcal mol^{-1}, more than 20 kcal mol^{-1} higher than the barriers calculated for sulfoxidation by Cpd I. Hydrogen bonding (to an H_2O molecule) or simultaneous protonation (using a cluster of H_3O^+ and H_2O) and oxygen insertion processes were attempted too, and led to barriers which are at least 10 kcal mol^{-1} higher than those by Cpd I[122]. These results clearly show that by itself, Cpd 0 cannot possibly compete with Cpd I because the negative charge makes it a good base and a good nucleophile, but not an electrophile[53]. Its activation by a proton source improves the situation[122], but even then it appears that the Cpd 0 is a much poorer oxidant than Cpd I.

5.9. Competitive Hydroxylation and Epoxidation in Propene

The competition between C–H hydroxylation and C=C epoxidation for a given substrate was addressed[104, 105] using propene as a model. The reaction profiles for in-vacuum conditions are shown in Figure 2.27, which exhibit the already known features of TSR with effectively concerted LS pathways and stepwise HS mechanisms. First, the barriers are seen to be extremely small and they get even smaller when NH---S hydrogen bonding is included (e.g., the barrier for the LS hydroxylation becomes *c*.8.96 kcal mol^{-1} only). With these small barriers, we may anticipate that the substrate binding will control the regioselectivity, so that whichever moiety is bound to Cpd I will be the one to react, and will do so too fast to allow the observation of the competing reaction. Indeed, propene undergoes exclusive epoxidation

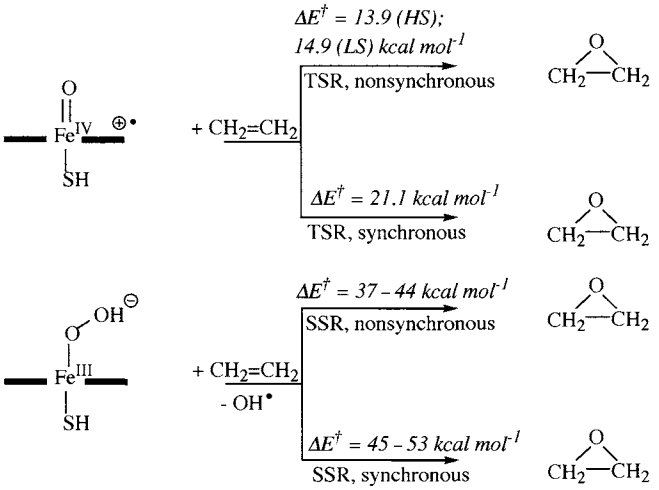

Figure 2.26. Epoxidation barriers of ethene by Cpd I and Cpd 0 (refs [53, 115]).

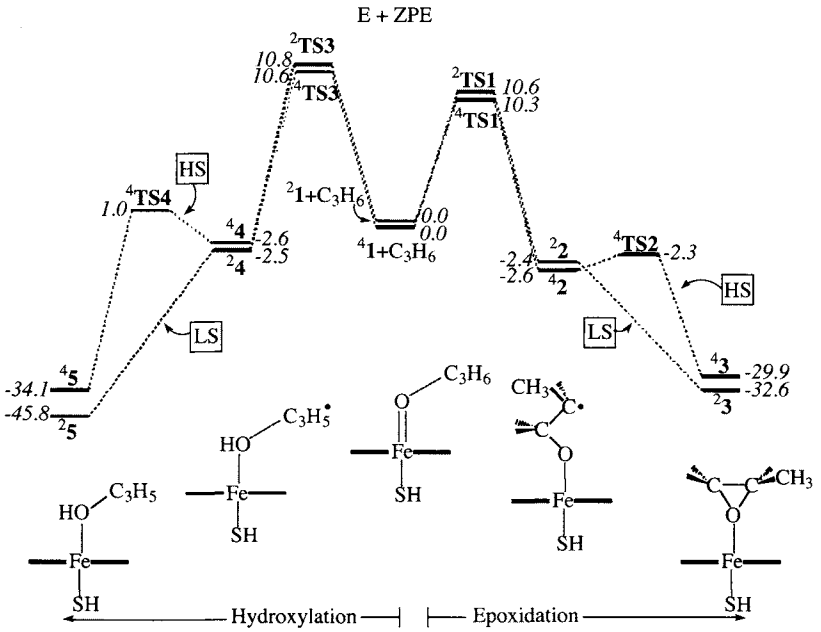

Figure 2.27. Potential energy landscape for the hydroxylation and epoxidation pathways of propene in the gas phase[105]. Relative energies include zero point energies (ZPE).

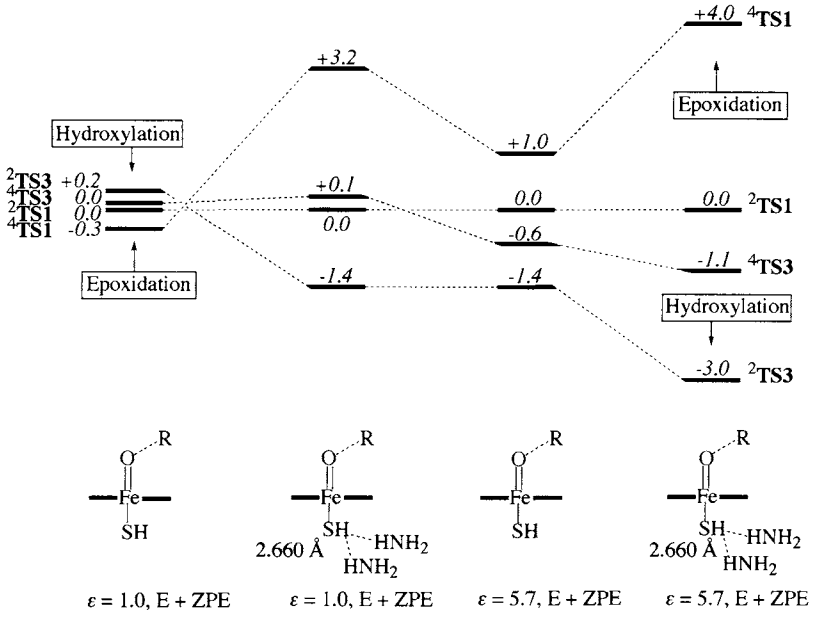

Figure 2.28. Relative energy of the transition states (TSs) for hydroxylation and epoxidation in the gas phase and under the influence of NH---S hydrogen bonding and polarity; the latter is mimicked by a dielectric constant $\varepsilon = 5.7$ (adapted from ref. [104, 105] with permission).

with $P450_{LM2}$ (ref. [123]), but this is not the case for other simple alkenes, for example, cyclohexene[124]. As such, our results with propene should be regarded as a model study of factors affecting regioselectivity and stereoselectivity rather than a specific study of a given substrate. In this respect, the calculations show that the bond activation phase is the rate-limiting step for both processes.

Figure 2.28 displays the four-bond activation TSs under different conditions. In the gas phase, the four species are condensed within 0.6 kcal mol^{-1}, with a slight preference for the epoxidation species. This in turn means that the gas phase reaction will exhibit (a) a low regioselectivity of C=C over C–H and (b) low stereospecificity due to HS/LS scrambling. The addition of just two NH --- S hydrogen bonds is sufficient to render the LS hydroxylation TS the lowest one by a wide margin. Adding the effect of a polar environment (mimicked by a dielectric constant of $\varepsilon = 5.7$) creates a clear preference for hydroxylation over epoxidation, by 3.0 kcal mol^{-1}. In addition, for each process, now the LS pathway has a lower barrier than the HS pathway. These effects correspond to a regioselectivity reversal by almost three orders of magnitude in favor of hydroxylation. In addition, since these effects favor the LS pathways, they also induce improvement in the stereoselectivity of both hydroxylation and epoxidation by almost three orders of magnitude. It follows therefore that the factors that mimic the polarity of the protein pocket and its hydrogen-bonding machinery have a major impact on the selectivity patterns of Cpd I. In fact, since Cpd I is a chameleon species, it also acts as a chameleon oxidant that tunes its reactivity patterns in response to the polarity and hydrogen-bonding machinery of the protein pocket. Thus, along with substrate binding, the hydrogen-bonding machinery and polarity of the protein serve as means by which the enzyme tunes its selectivity. One wonders whether this factor would not play a role in the great versatility of the superfamily of P450 enzymes.

5.10. An Overview of Reactivity Features of Cpd I

Cpd I *is a chameleon and two-state oxidant* and as such exhibits TSR that can be tuned by the polarity of the pocket and its hydrogen-bonding machinery. The gas phase (in vacuum) calculations show that, while alkane hydroxylation and alkene epoxidation feature TSR, by contrast, benzene hydroxylation exhibits a dominant LS reactivity and in sulfoxidation it is the HS state that dominates reactivity. As a rule of thumb, we may say that whenever the substrate deformation is small or identical for the two states, they will have similar electronic structures and be energetically close, as for the reactant species Cpd I. This appears to be the case in epoxidation and hydroxylation, which exhibit clear TSR. The dominance of the LS state in benzene hydroxylation was shown[119] to originate in the large deformation energy of benzene, due to the partial loss of the resonance energy, which is *c.*10 kcal mol^{-1} more severe for the HS TSs. In heteroatom oxidation, however, the electronic structures during the HS and LS processes are not the same since the two oxidation equivalents must be condensed into a single step, and for sulfoxidation, the HS state appears to be the lower one of the two. We may therefore anticipate that arene hydroxylation and heteroatom oxidation will feature a SSR with a substrate-dependent spin-state selection.

A feature encountered during benzene hydroxylation is the appearance of both radical and cationic mechanisms. This feature is associated with the stability of the cationic species and will appear in alkane hydroxylation and alkene epoxidation whenever the corresponding radical center has a sufficiently low ionization energy to transfer an electron to the heme. In such an event, the reactivity will be dominated by the LS state. The result of Newcomb and Toy[91], which indicates the presence of carbocations may well belong to this category. Such results are in progress.

The polarity of the pocket and hydrogen-bonding machinery were found to increase the dominance of the LS reactivity in arene hydroxylation and the HS reactivity in sulfoxidation. Similarly, these factors will have a strong impact on the appearance of cationic intermediates during hydroxylation and epoxidation. More intriguing is the result that these properties of the protein pocket have a major impact on the regioselectivity of C–H hydroxylation versus C=C epoxidation as well as on the stereospecificity of both processes. Time will show whether these are generalities or isolated findings.

Theory also shows that the porphyrin ring is not a spectator ligand but plays quite a few roles during the enzymatic reaction. It acts as an electron sink by accepting the excess electrons generated during the oxidation, and as a proton sponge by reshuttling protons needed for substrate rearrangement. These are in addition to its adverse role in enabling heme alkylation. In our calculations, we find that heme alkylation is more facile with ligands that are not good electron donors as the thiolate is. Thus, the thiolate ligand protects the porphyrin against heme alkylation, and at the same time, renders its nitrogen more basic and, hence, improves its catalytic performance as a proton shuttle.

6. Prospective

As shown in this chapter, theoretical treatments of P450 problems have come of age, and they enable the study of many features related to the three-dimensional structures, the electronic structures, substrate binding, reactivity, and dynamics of the various physical processes in the cycle. This ability will only increase with time, as the various techniques will be brought to bear simultaneously on a given problem. Techniques similar to combinatorial synthesis will have to be adopted in order to screen fast many alternative possibilities.

At present, while calculations have elucidated some important features of the cycle, still others remain obscure; especially features which concern the protein machinery and the dynamics of the various reactions, the role of the water molecules that are present in the pocket and leave it upon entrance of the substrate, and the dialogue between the oxygenase and the reductase domains, as well as the thermodynamic aspects of the entire cycle which must be determined by accounting for the changes in the reductase.

Other outstanding issues that remain unattended at present are the following:

(1) The role of spin crossover in the various events in the cycle, including the TSR during oxygenation, which will have to be elucidated.

(2) All calculations of C–H hydroxylation, with the exception of allylic hydroxylation, show a significant barrier of 18–24 kcal mol^{-1}. Such barriers seem to be overestimated. If this is a fault of the theoretical calculations, this is likely to be discovered as soon as calculations become faster and allow the use of more sophisticated methods (e.g., CCSD(T), CASPT2). If however, these are reasonable estimates of the barrier, we will be facing a conceptual dilemma to explain the potency of the enzyme despite the large barriers. While a few such possible scenarios come to mind immediately, we prefer to leave these as open questions that advanced theory will have to deal with in the future.

Acknowledgment

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3

Structures of Cytochrome P450 Enzymes

Thomas L. Poulos and Eric F. Johnson

1. Introduction

Much of what we know about the molecular level structure–function relationships in P450s is based on studies with the camphor monooxygenase system from *Pseudomonas putida*. Given that P450cam was the first P450 to be purified in sufficient quantities for structure–function relationships, it is not too surprising that P450cam was the first P450 crystal structure to be solved. The high-resolution structure of P450cam was published in 1987[1], and remained the paradigm for P450 structure–function studies until the P450BM3 structure was solved in 1993[2]. Since then the rate at which new P450 structures are being solved has increased dramatically and at present there are structures for 20 unique P450s on deposit in the Protein Data Bank with others waiting in the wings. Some of the new advances that have been made since the last edition of this book are the structures of other redox components of P450 monooxygenase systems, of the first electron transfer complex, of new substrate complexes, of the first P450s from thermophilic organisms, and of the first membrane-bound P450. In the present chapter, we summarize some of these more important recent findings with full recognition that the P450 structural field is moving much more quickly than in the time frame of previous editions of this book. As a result, the current chapter must be considered a snapshot in time on where we now stand in P450 crystallography.

2. Overall Architecture

There are now a sufficient number of structures to safely state that the overall P450 fold is quite conservative. Perhaps more surprising is that the P450 fold is unique and despite the many new structures that have been solved since P450cam, no non-P450 structure has yet been found to share the P450 fold. Thus, the P450 fold appears to be uniquely adapted for the heme-thiolate chemistry required for oxygen activation, the binding of redox partners, and the stereochemical requirements of substrate recognition.

The structures of several P450s are shown in Figure 3.1, while Figure 3.2 highlights some of the key secondary structural elements. Although the overall fold is maintained, the precise positioning of various structural elements differs substantially. In general, the closer to the heme, the more conserved the structure, especially helices I and L, which directly contact the heme. As expected, those regions controlling substrate specificity differ the most, especially the B′ helix. For example, in P450eryF, the B′ helix is oriented about 90° from the orientation observed in P450cam. The

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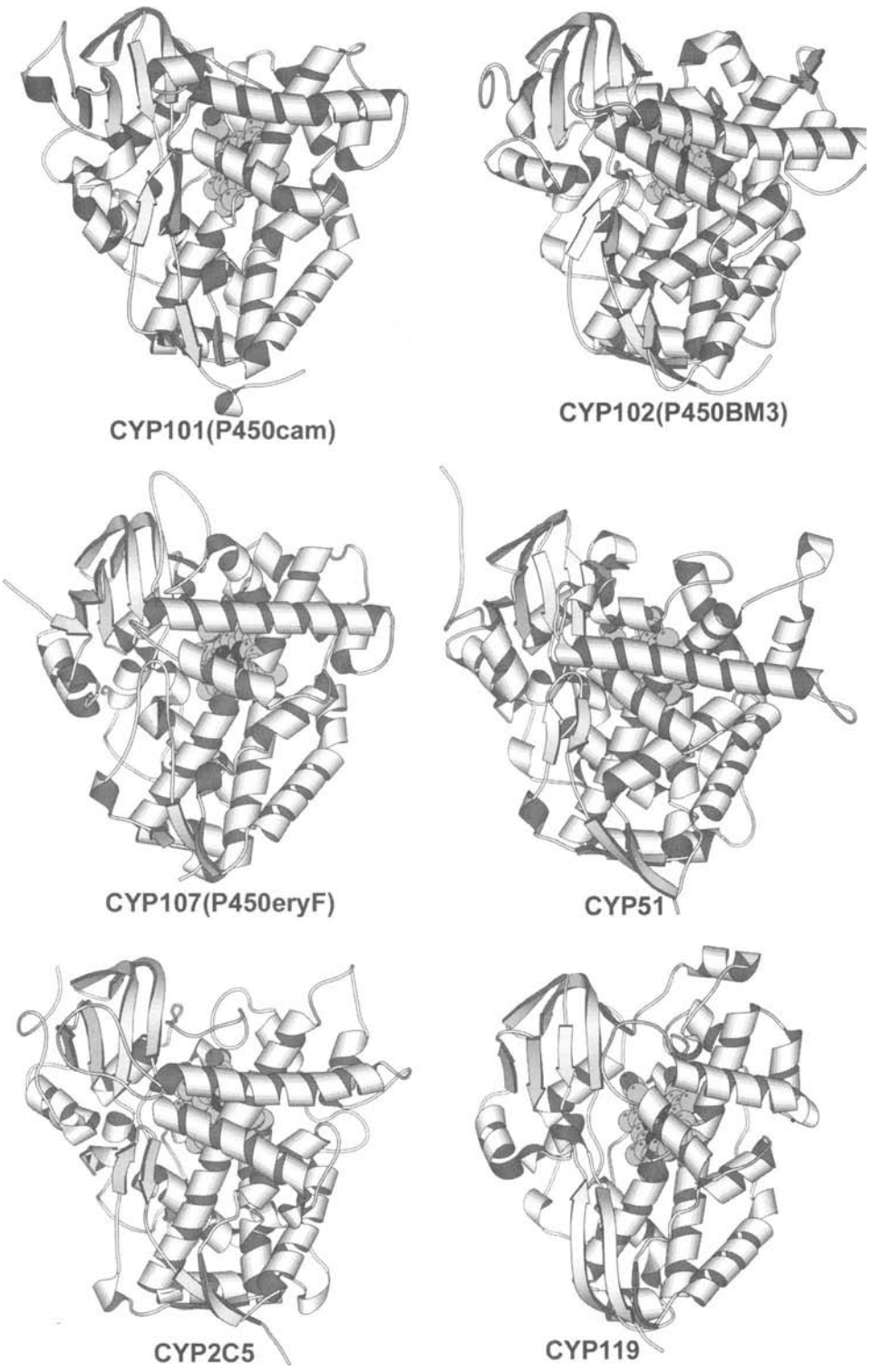

Figure 3.1. A representative example of known P450 structures illustrating the common three-dimensional fold.

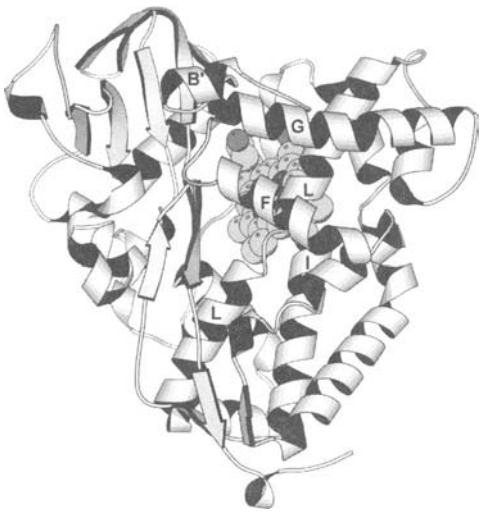

Figure 3.2. The structure of P450cam with key helical segments labeled.

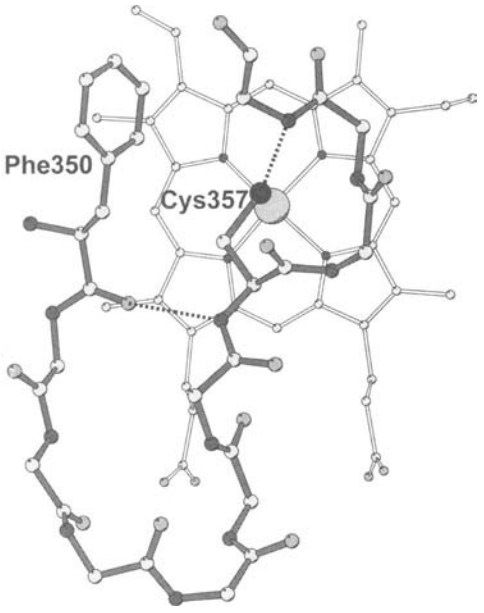

Figure 3.3. The Cys ligand "loop" in P450cam. The dashed lines indicate key hydrogen bonding interactions that aid in stabilizing the Cys ligand.

effect is a substantial change in local environment that is required for substrate selectivity.

Not too surprisingly, the most conserved elements of the P450 structure center on the heme-thiolate oxygen activation chemistry. The most noteworthy is the β-bulge segment housing the Cys ligand (Figure 3.3), just prior to the L helix. This rigid architecture is required to both protect the Cys ligand and hold it in place in order to accept H-bonds from peptide NH groups. This arrangement is not only found in all P450s but in two closely related proteins, nitric oxide synthase (NOS) and chloroperoxidase (CPO). Both NOS and CPO are heme-thiolate enzymes and like P450s catalyze monooxygenation reactions. Exactly as in P450, the Cys ligand in CPO accepts H-bonds from peptide NH groups[3]. NOS is similar except that one of the H-bonds is provided by the indole ring N atom of a conserved Trp residue[4–6]. Such an H-bonding arrangement is not unique to heme thiolate proteins but is a characteristic feature of proteins containing Cys–Fe ligation and was first observed in the ferredoxins[7]. These H-bonds aid in regulating the heme iron redox potential[8, 9]. Without such H-bonds, the redox potential would be too low for reduction by redox partners. Thus, it appears that the protein must provide a suitable electrostatic environment around the Cys ligand in order to maintain the redox potential in a physiologically accessible range. The same is true for a close cousin to P450, the peroxidases. Here His serves as the axial ligand, but in this case, it is necessary to decrease rather than increase the redox potential[10]. As a result, the His ligand H-bonds with a buried Asp residue which imparts greater imidazolate character to the His thus lowering the heme iron redox potential[11–15].

The other highly conserved region involved in O_2 activation is the portion of helix I near the heme Fe (Figure 3.4). Thr252 is involved in a local helical distortion in P450cam such that the Thr side chain OH donates an H-bond to a peptide carbonyl oxygen that would normally be involved in an α-helical H-bond. This Thr is not strictly conserved. For example, P450eryF contains Ala instead of Thr (Figure 3.4). Even so, P450eryF also exhibits a similar distortion in the I helix. In addition, a water molecule in P450eryF takes the place of the Thr side chain OH thus maintaining a very similar H-bonding pattern. This arrangement is thought to be quite important in the proper delivery of protons to the iron-linked oxygen required for cleavage of the O–O bond thus generating the active Fe–O hydroxylating species. The growing consensus is that ordered solvent at the active site serves as the direct proton donor to the iron-linked dioxygen[16, 17]. The most revealing crystal structures that support this view are the P450cam–oxy complex[18] and resting state ferric P450eryF[19]. In the P450cam–oxy

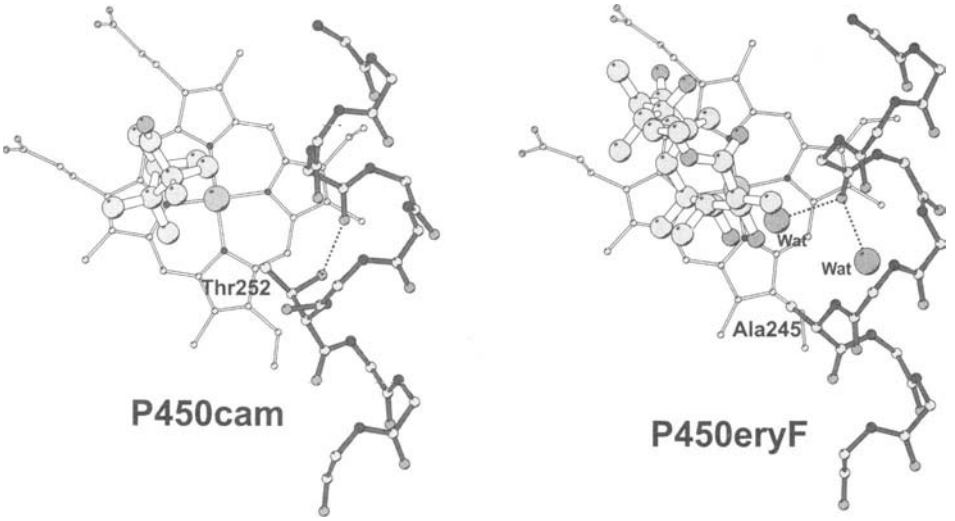

Figure 3.4. A comparison of the I helix region in P450cam and P450eryF. The large gray spheres indicate water molecules that complete the hydrogen bonding network in P450eryF.

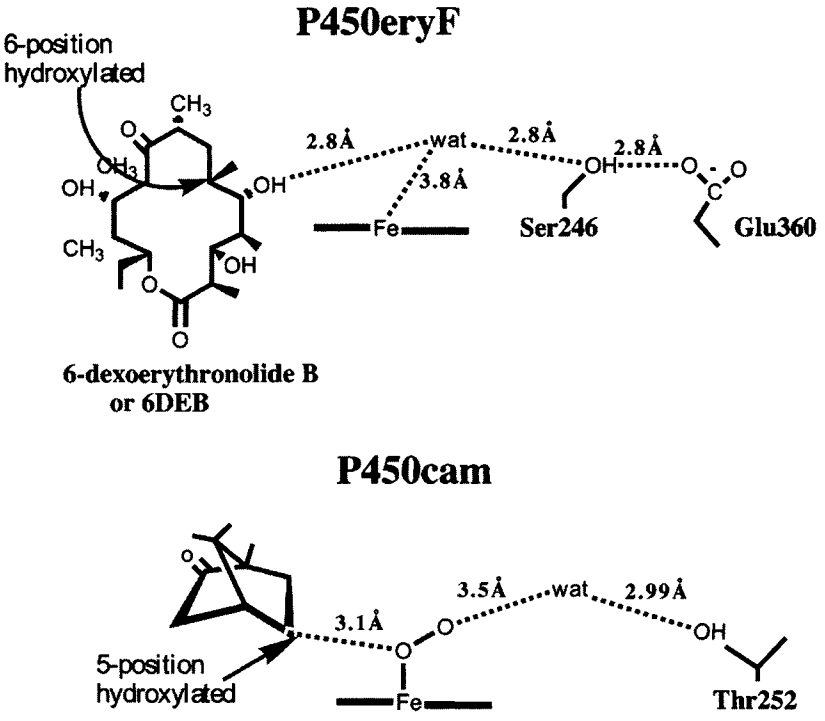

Figure 3.5. A comparison of the solvent-mediated hydrogen bonding network in P450eryF and the oxy-complex of P450cam[18]. In both cases, a critically positioned water molecule very likely serves as the proton donor to bound dioxygen.

complex, two new waters are found in the active site, one of which is shown in Figure 3.5. This new water is close to dioxygen and may participate in relaying protons to dioxygen. P450eryF also has a water similarly positioned (Figure 3.5). P450eryF, however, uses a substrate-assisted mechanism since a substrate OH anchors the key water in place via H-bonding. While the details of the proton shuttle machinery may differ from one P450 to the next, the surrounding protein groups and, in at least one case, the substrate, generally position solvent in the active site for proton delivery to dioxygen resulting in cleavage of the O–O bond.

3. P450s from Thermophiles

Given that P450s are found in such a wide variety of organisms, it is not surprising that P450s also are found in thermophiles. The first to be discovered was CYP119 from the acidothermophilic archaeon *Sulfolobus solfataricus*[20]. Subsequent cloning and expression showed that CYP119 melts near 90 °C compared to P450cam which melts near 50°C[21]. The crystal structure of CYP119 was first solved by Yano *et al.*[22] followed by an independent structure determination by Park *et al.*[23]. The most notable difference between CYP119 and other P450s is that CYP119 is much smaller, consisting of only 368 residues. The difference in size is primarily due to a shorter N-terminal segment and shorter surface loops. A unique clustering of aromatic residues running down one side of the molecule (Figure 3.6) is thought to be the structural basis for thermal stability. Mutating one of these residues, Phe24, to Ser lowers the melting temperature about 10°C[23]. A more extensive mutagenesis analysis of the

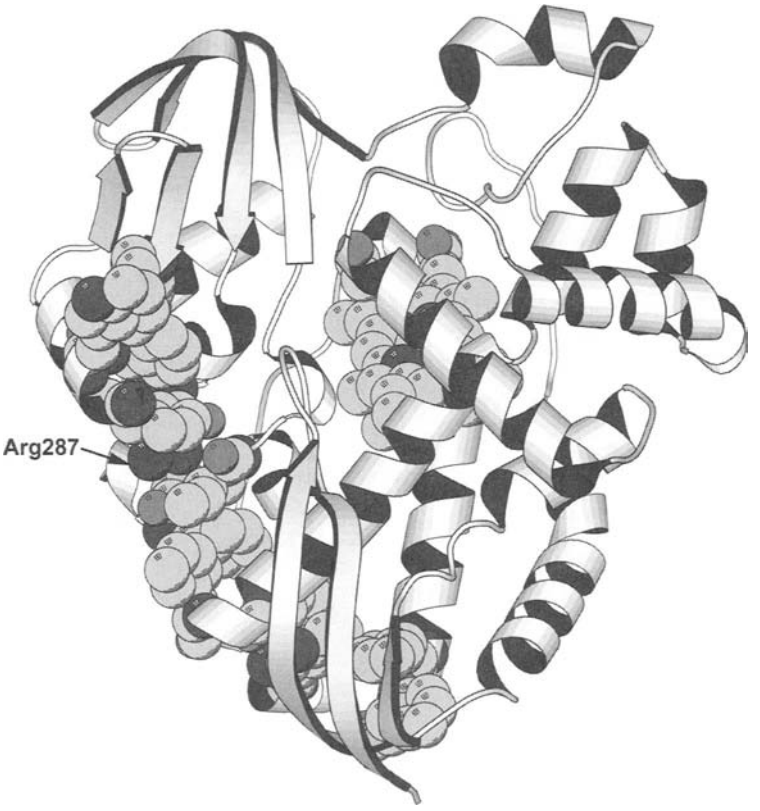

Figure 3.6. The structure of CYP119 showing the aromatic cluster that is thought to be responsible for thermal stability. The cluster also contains an Arg residue (Arg287) which is sandwiched between two tyrosine residues and hence, contributes to the π-stacking interactions of the aromatic cluster.

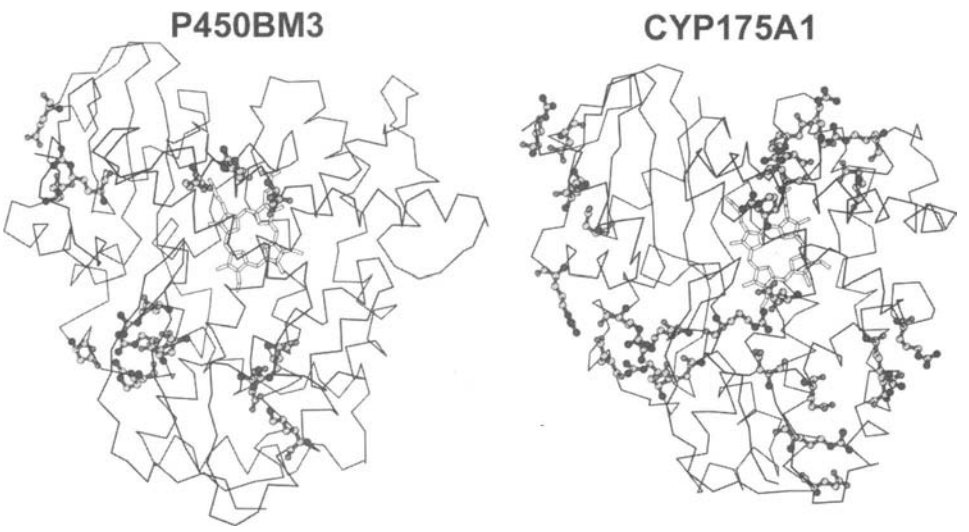

Figure 3.7. A comparison between P450BM3 and the thermal stable P450, CYP175A1, showing the salt bridge networks in both proteins.

aromatic cluster shows that the melting temperature can be lowered 10–15°C[24] further implicating aromatic clustering as a key factor in thermal stability.

The second thermophilic P450 to be discovered was CYP175A1 from *Thermus thermophilus*[25] which melts at 88°C. Like CYP119, CYP175A1 is also shorter than most other P450s and contains 378 residues. CYP175A1 most closely resembles P450BM3 but with shorter surface loops. CYP175A1 lacks the aromatic clustering observed in CYP119 and hence, the structural basis for thermal stability lies elsewhere. Yano *et al.*[25] carried out a detailed comparison of salt bridge networks in various P450s and found that CYP175A1 contains a larger fraction of salt bridges that contain three or more residues. As shown in Figure 3.7, CYP175A1 has 26 residues involved in 8 salt bridge networks while the closest homologue to CYP175A1, P450BM3, has 14 residues participating in 4 salt bridge networks.

4. Membrane P450s

In contrast to prokaryotic P450s, eukaryotic P450s are generally membrane-bound proteins. Most eukaryotic P450s are incorporated into the endoplasmic reticulum. However, several mammalian P450s that participate in the synthesis of sterols, steroids, and bile acids are located on the matrix side of the mitochondrial inner membrane. A longer N-terminal polypeptide chain of roughly 30–50 amino acids precedes the catalytic domain in eukaryotic P450s and mediates membrane targeting. In the case of mitochondrial P450s, the targeting sequences are cleaved during import of the protein into mitochondria[26]. In contrast, the leader sequence of microsomal P450s is retained and is co-translationally inserted into the endoplasmic reticulum[27]. The insertion process stops at the end of a hydrophobic stretch of roughly 20 amino acid residues, and the catalytic domain of microsomal P450s resides on the cytoplasmic side of the endoplasmic reticulum. A short region that generally contains positively charged residues links the catalytic domain to a conserved proline rich motif at the N-terminus of the structurally conserved P450 fold. The N-terminal transmembrane domain extends across the membrane and is unlikely to be closely associated with the catalytic domain (Figure 3.8). This N-terminal domain is not required for function as illustrated by the expression and successful reconstitution of several P450 monooxygenases in which this region was deleted[28–32].

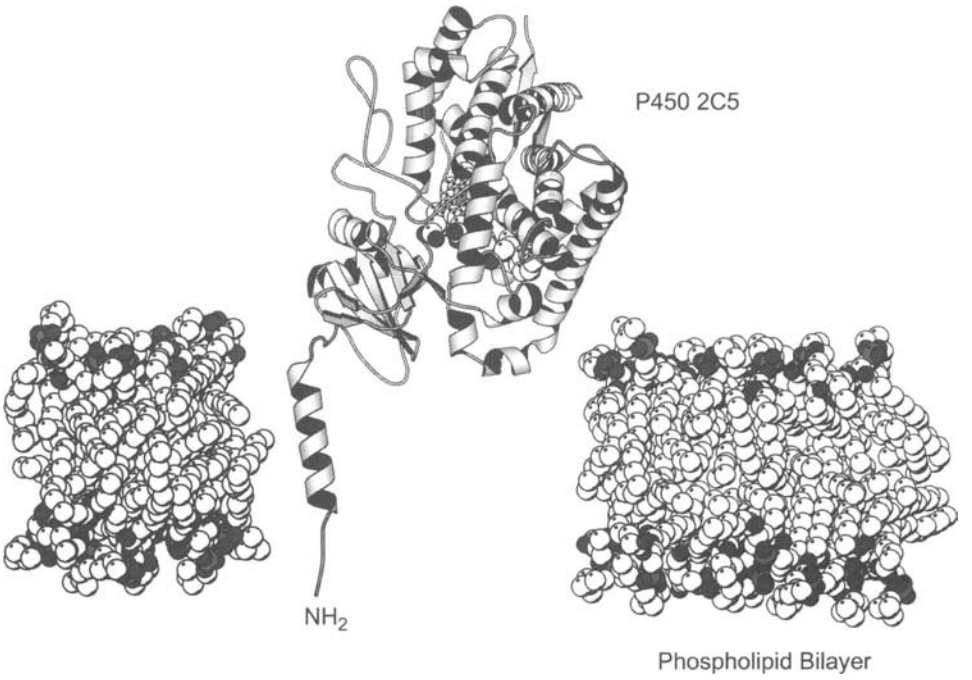

Figure 3.8. Hypothetical model for the membrane binding of microsomal P450s. The cartoon depicts the experimentally determined fold of the modified CYP2C5 catalytic domain, PDB: 1N6B, attached to a hypothetical model for the N-terminal transmembrane helix. The latter is flanked by a modeled array of phospholipid molecules depicted as CPK atoms. The heme and bound substrate, DMZ, of CYP2C5 are also rendered as CPK atoms.

Another major difference relative to prokaryotic P450s is the insertion of a longer polypeptide chain between helices F and G in eukaryotic P450s. This region exhibits two short helices, F′ and G′, in the structures of CYP2C5 (Figure 3.9) and CYP2B4, a single, longer F′ helix in P450 2C8, and a more relaxed coiled conformation in CYP2C9. This portion of the structure is in close juxtaposition to the region between the proline-rich motif at the N-terminus of the catalytic domain and helix A, and together they form a hydrophobic surface near the N-terminus of the protein[33]. Epitope mapping studies of the related enzyme CYP2B4[34] suggest that this portion of the structure is in close proximity to and possibly buried in the membrane as illustrated in Figure 3.8. Similar interactions may underlie the binding of mitochondrial P450s to the matrix surface of the inner membrane. As mitochondrial P450s lack the N-terminal transmembrane helices found in microsomal P450s, the catalytic domain must interact more extensively than their microsomal counterparts with the hydrophobic core of the lipid bilayer because detergents are required to release mitochondrial P450s from the membrane. In contrast, the catalytic domain of microsomal CYP2C5 is released by high salt buffers when it is expressed as a truncated construct that does not contain the N-terminal transmembrane helix[28, 29].

The model shown in Figure 3.8 is consistent with additional observations. Antibody epitope mapping studies indicate that extensive portions of the surfaces of drug metabolizing P450s are accessible to the antibodies, reviewed in ref. [35]. As shown in Figure 3.10, most of the epitopes reside along the edges of the protein with the exception of the tip of the protein near the N-terminus of the catalytic domain. The tilt of the heme relative to the membrane has been estimated for CYP17 and CYP21 based on the rate of decay of the absorption anisotropy following photodissociation of carbon monoxide complexes of each protein.

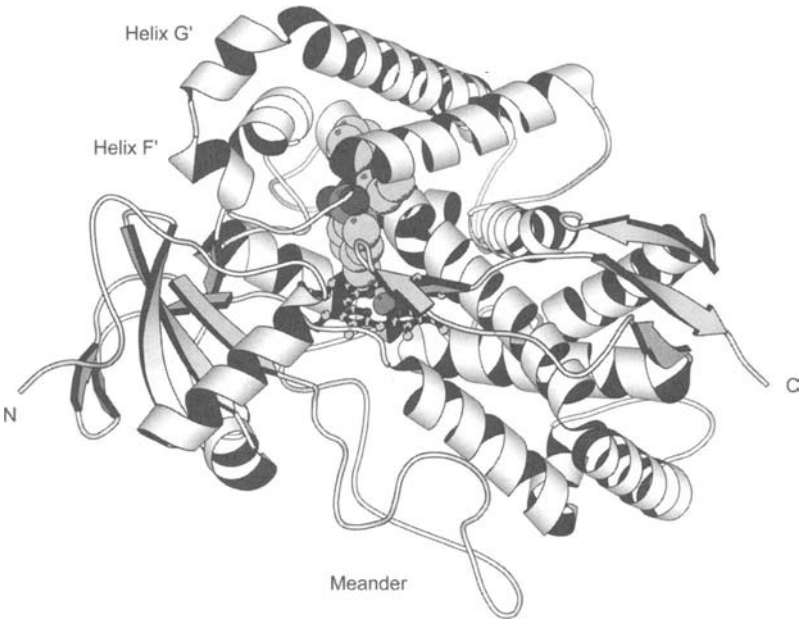

Figure 3.9. A side view showing the overall fold of modified CYP2C5. The largest insertions of additional polypeptide chain relative to prokaryotic P450s occur for the N-terminal region depicted in Figure 3.8, the meander region, and between helices F and G. The latter exhibits two short helical regions labeled F′ and G′.

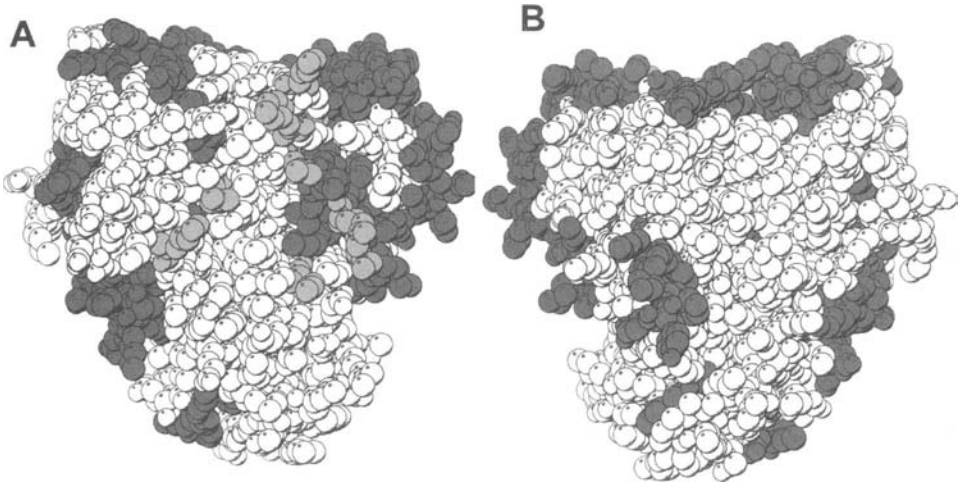

Figure 3.10. CPK rendering of the (A) proximal and (B) distal surfaces of modified CYP2C5. Antibody epitopes recognized when the protein is bound to microsomal membranes are dark gray, as reviewed in ref. [40]. Several conserved amino acid side chains that have been implicated in P450 reductase interactions with CYP2B4[40] are medium gray. The orientation of the protein is similar to that depicted in Figure 3.8 with the N-terminus of the catalytic domain positioned toward the bottom of the figure.

The results of these studies suggest that the angle for the orientation of the heme relative to the membrane surface is either 50° or 70° (See ref. [36]). The latter value is consistent with the orientation seen in Figure 3.8 and leads to significant solvent exposure for the surfaces of the catalytic domain. Atomic force microscopy experiments estimate that the height of P450 2B4 above a model phospholipid membrane is roughly 35–45 nm[37]. This would require a portion of the protein to be buried in the membrane, and this is likely to be the hydrophobic region near the N-terminus of the catalytic domain. Studies of the association of P450 2B4 with Langmuir–Blodget, phospholipid monolayers indicate that the protein displaces an area that is larger than a single transmembrane helix[38]. This result would be consistent with the penetration of the hydrophobic tip of the protein into the lipid layer. A lower angle of the heme plane would juxtapose a larger surface of the helix F–G region with the membrane surface, which is hydrated and largely formed by the zwitterionic phospholipid head groups. A steeper angle would bury the hydrophobic tip deeper into the membrane. The propensity of the helix F to helix G region to form helical structures rich in aromatic residues would enable this region to penetrate more deeply into the membrane because the hydrogen bonding in the helices would diminish the energetic cost of burying the peptide bonds in a hydrophobic environment[39].

Membrane interactions with the catalytic domain of microsomal P450s could promote the transfer of hydrophobic substrates from the membrane to the P450 and could also orient the protein to facilitate interactions with P450 reductase as the two proteins diffuse along the surface of the endoplasmic reticulum. Mutagenesis experiments[40] suggest that a group of relatively conserved, positively charged amino acids on the proximal surface of CYP2B4 interact with the reductase (Figure 3.10). Significant insertions of additional amino acid residues relative to most prokaryotic P450 structures are also present on the proximal surface of the protein, and these are also in the structure of P450BM3. The additional residues form the J′ helix extend a portion of the polypeptide chain termed the meander region that precedes the beta-turn containing the cysteine that provides the axial ligation for the heme. The corresponding region of CYP2C5 is illustrated in Figure 3.9. The functional importance of this insertion is unclear, but it resides in close proximity to the probable site of P450 reductase binding as discussed in the next section.

5. Electron Transfer Complexes

An important advance in understanding P450 monooxygenase electron transfer systems was the solution of the P450 reductase structure[41]. With the addition of the first mammalian P450 structure, CYP2C5, the way is now open for understanding how these two microsomal proteins interact and transfer electrons. Nevertheless, the *Bacillus megaterium* fatty acid monooxygenase system, P450BM3, has so far served as the best model system for understanding reductase–P450 interactions. Although P450BM3 is a bacterial enzyme, P450BM3 is more closely related in sequence, structure, activity, and redox partner to microsomal P450s than to other bacterial P450s. The unique feature of P450BM3 is that the diflavin P450 reductase is linked to the C-terminal end of the heme domain thus giving a catalytically self-sufficient enzyme.

A significant advance was made when Sevrioukova *et al.*[42] succeeded in crystallizing a P450BM3 construct that contains just the heme and FMN domains. The structure (Figure 3.11) shows that the FMN domain docks on the proximal surface of the P450, which was expected based on complementary electrostatic surfaces and mutagenesis studies. Nevertheless, the heme–FMN construct used to solve this structure was missing the FAD domain and the linker connecting the heme and FMN domains had been proteolyzed during crystallization, thus raising the possibility that the structure is an artifact of crystallization. In addition, the structure of the P450BM3 complex is not compatible with the P450 reductase structure. As shown schematically in Figure 3.12, the FMN and FAD in P450 reductase form a direct nonbonded contact. In order for the FMN domain to dock onto the proximal surface of P450, there must be a large structural change which enables the FAD and FMN domains to move apart (Figure 3.12). Indeed, there are good indications that the linker connecting the FAD and FMN domains is quite flexible. One of the more important studies related to the question of domain flexibility is the structure of

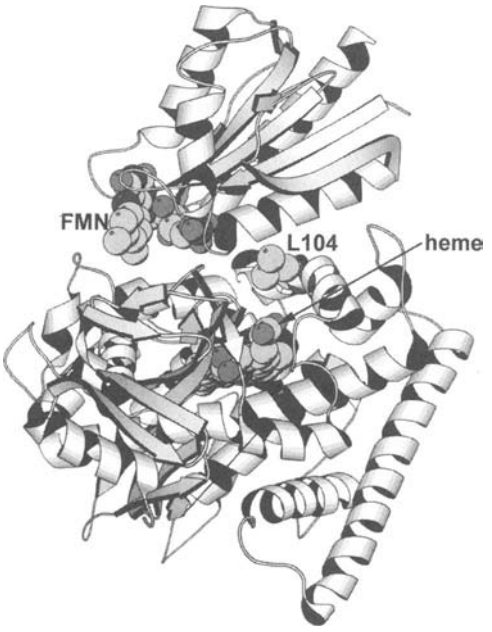

Figure 3.11. Structure of complex formed between the heme and FMN domains in P450BM3. The FMN domain docks into the concave depression on the heme domain proximal surface.

the homologous reductase domain in sulfite reductase. This enzyme resembles P450BM3 in having a heme domain attached to a FMN/FAD reductase domain. The FAD domain of sulfite reductase is very similar to the FAD domain of P450 reductase with an rms deviation of between 1.3 and 1.5 Å[43]. However, the FMN domain is not visible in electron density maps in four different crystal forms indicating that the FMN–FAD linker domain is quite flexible allowing the FMN domain to adopt multiple orientations relative to the FAD domain. As it must, the large void in the crystal lattice can accommodate the FMN module, but packing considerations show that the possible interactions between the FMN and FAD modules are quite different than in P450 reductase[43]. This raises the possibility that the P450 reductase structure may have captured the FMN domain in one possible orientation while the P450BM3 electron transfer complex captured the orientation of the FMN domain required for proper docking to P450.

The P450BM3 electron transfer complex structure also is consistent with mutagenesis and chemical modification studies. Sevrioukova *et al.*[44] changed key residues on the heme domain that lie at the interface between the heme and FMN domains. Replacing Leu104 of P450BM3 with a Cys (Figure 3.11) at the interface should not alter binding or electron transfer because replacing Leu with a smaller side chain should not cause any steric problems in forming the proper complex. However, covalent modification of the mutant Cys104 side chain with a large fluorophore should interfere with electron transfer. For these studies laser flash photolysis was used wherein a laser flash photoreduced a potent reductant, deazariboflavin, which in turn reduces the FMN in the complex. The reduced FMN semiquinone then reduces the P450 heme. As predicted, mutation of Leu104 to Cys had no effect, while chemical modification of Cys104 dramatically decreased the FMN-to-heme electron transfer rate, thus implicating Leu104 as an important residue in forming the proper electron transfer complex.

A second prediction from the P450BM3 electron transfer complex structure that can be tested is the electron transfer path. The heme-FMN domain interface is shown in Figure 3.13. A particularly noteworthy feature at the interface is the participation of water molecules. Waters form H-bonding bridges between the two domains while there are only two direct H-bonds between side chains. The closest point of contact between the two domains places the FMN about 4 Å from the peptide backbone of Gln387. The peptide chain from Gln387 to the heme ligand, Cys400, could constitute an electron transfer path. To test this hypothesis, Gln387 was converted to Cys and modified with (4-bromomethyl-4′-methylbipyridine)[bis(bipyridine)]ruthenium(II)[45]. The covalently attached Ru(II) is photoreduced, and the rate of reduction of the heme Fe(III) to Fe(II) by the photogenerated Ru(I) was followed. The same experiment was carried out with Ru(II) attached to Cys60. Both Cys60 and Cys387 are about the same distance from the heme but electron transfer from Cys60 to Ru(II) must make "through-space" jumps, while there is a continuous covalent connection between Cys386–Ru(II) and the heme ligand, Cys400. In the case of Cys387–Ru(II), the heme iron was reduced at a rate of $4.6 \times 10^5\ s^{-1}$, while Cys60–Ru(II) did not reduce the heme iron. These results indicate that if the crystal structure

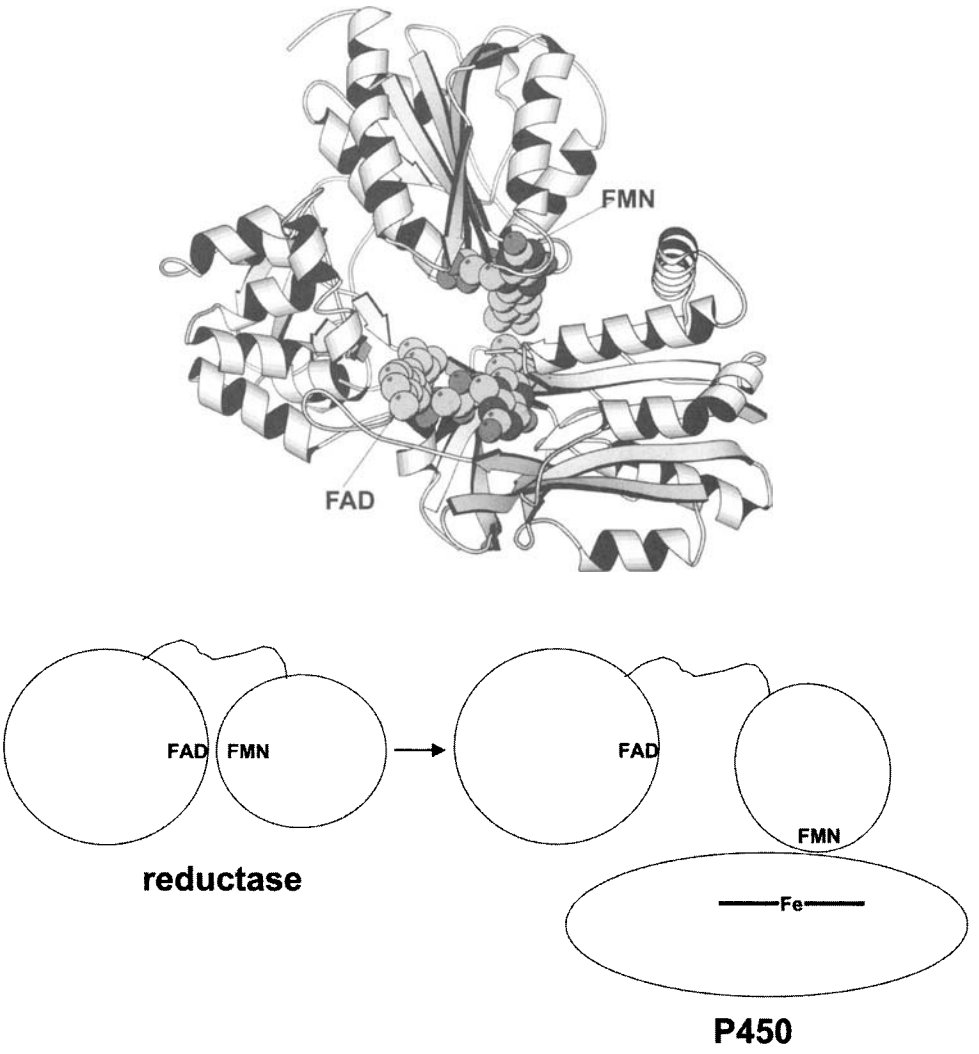

Figure 3.12. The crystal structure of P450 reductase[41]. Note that the FMN and FAD are in direct contact. The schematic diagram illustrates that it may be necessary for the FMN and FAD domains to separate to enable the FMN domain to dock onto the P450 prior to the FMN-to-heme electron transfer reaction.

of P450BM3 electron transfer complex is functionally relevant, then the electron transfer reaction can readily proceed along the direct point of contact between the FMN and heme domain.

Another recent advance in P450 electron transfer is the solution of both the putidaredoxin (Pdx)[46] and putidaredoxin reductase (Pdr)[47] crystal structures. Thus, the structure of all components of the P450cam monooxygenase system now are known. The Pdx and Pdr structures are shown in Figure 3.14 together with the crystal structure of the adrenodoxin–adrenodoxin reductase complex[48]. The Pdx and adrenodoxin (Adx)[49] structures clearly are very similar. However, Pdr and adrenodoxin reductase (Adr)[50] exhibit large differences. The FAD site is less exposed in Pdr owing to an additional β-pair situated near the cofactor. In addition, the C-terminal region of Pdr (Figure 3.14) is positioned very differently than in Adr. Note that this segment in Pdr blocks part of

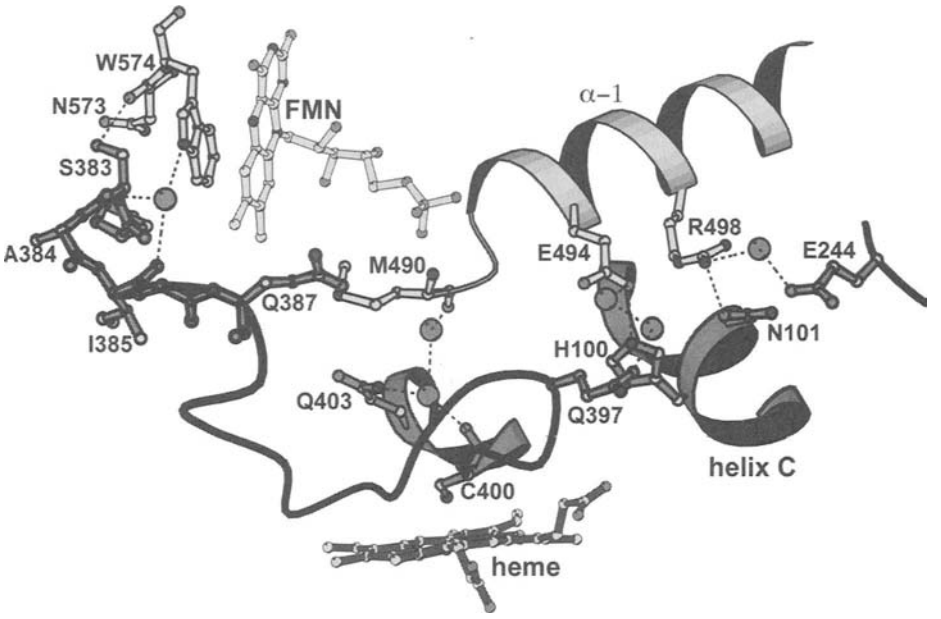

Figure 3.13. A close-up view of the interface formed between the heme and FMN domains in the P450BM3 electron transfer complex. The heme domain is more darkly shaded. There are only two direct H-bonds between the two domains: His100(heme)-Glu494(FMN) and Asn101(heme)-Arg498(FMN). The remainder of the electrostatic interactions are formed by bridging water molecules. Note that the FMN directly contacts the heme domain near Gln387. The section of polypeptide leading from Gln387 to the Cys400 ligand and heme could provide a selective electron transfer conduit.

the binding site for Adx in the Adx–Adr complex. Therefore, if Pdx and Pdr form a complex similar to the Adr–Adx complex, then the C-terminal domain in Pdr must move. This seems unlikely since the C-terminal region that would be required to move is composed of β-sheets that tie this region to the main body of the protein. Therefore, Pdx forms a complex with Pdr that must be quite different than the Adx–Adr complex. Also shown in Figure 3.14 is the glutathione reductase structure[51], which more closely resembles Pdr than does Adr. Note that both Pdr and glutathione reductase have the common domain similarly positioned which limits exposure of the FAD. This similarity is especially interesting since Pdr exhibits an NAD(H)-dependent dithiol/disulfide oxidoreductase activity similar to glutathione reductase activities[52]. Whether or not this unexpected Pdr activity is physiologically relevant remains to be seen.

Although the structures of the various electron transfer complexes in the P450cam system are not known, a good deal has been learned about the primary forces involved in forming the complexes. Isothermal titration calorimetry shows that the Pdr–Pdx complex is entropically driven suggesting that nonpolar interactions dominate[53]. In contrast, electrostatic forces dominate the Pdx–P450cam complex. These equilibrium thermodynamic studies agree with kinetic results obtained using laser flash photolysis methods to measure the P450–Pdx and Pdx–Pdr electron transfer rates[54].

Despite the lack of structural data on the complexes, Pochapsky *et al.* have developed a hypothetical model of the P450cam–Pdx complex using the crystal structure of P450cam and NMR structure of Pdx[55]. The Pochapsky model was developed well before the crystal structure of the P450BM3 complex was solved and was based on data derived from NMR, mutagenesis, and computational studies. Quite interestingly, both the P450cam–Pdx and P450BM3 complexes utilize the same proximal surface of P450 for the docking

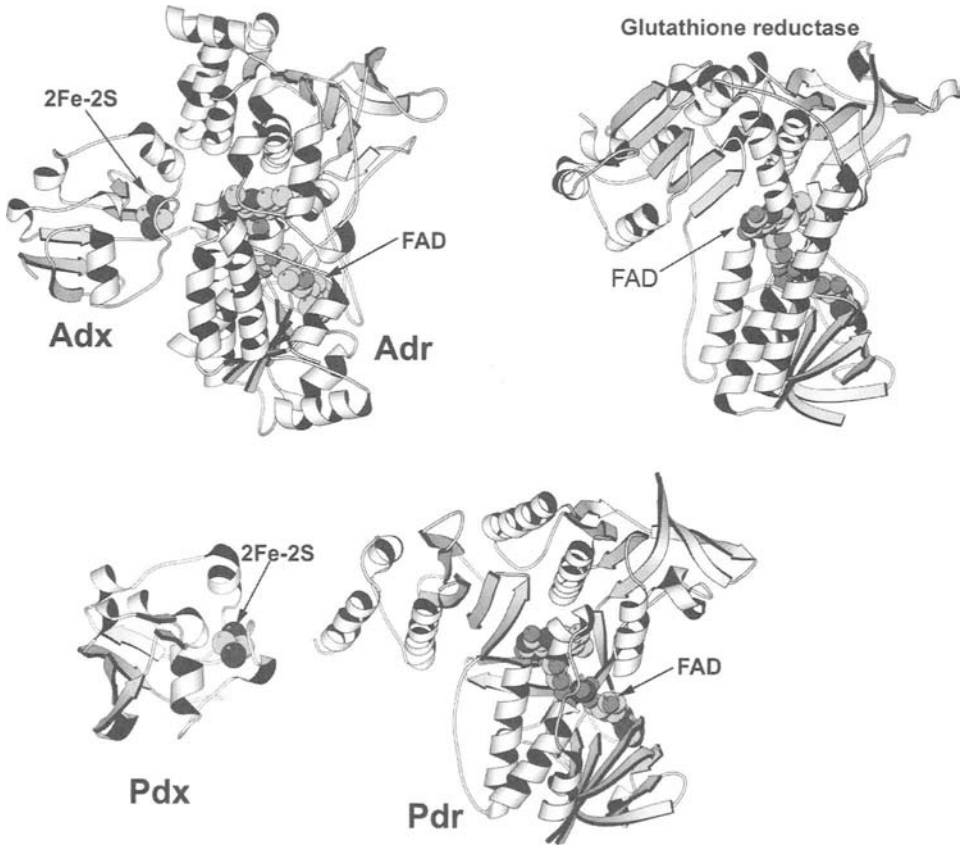

Figure 3.14. Structures of the adrenodoxin (Adx)–andrenodoxin reductase (Adr) complex, putidaredoxin (Pdx), and putidaredoxin reductase (Pdr). For comparisons, the structure of glutathione reductase also is shown.

of redox partners (Figures 3.11 and 3.15). This makes sense since the proximal region is the closest approach of heme to the surface and thus affords the closest approach of redox active cofactors.

6. Substrate Complexes

At present, the structure of five prokaryotic P450–substrate complexes are known (Figures 3.16 and 3.17). P450epoK, the newest member of this group[56], catalyzes the 12,13-epoxidation of epothilones (Figure 3.18) in *Streptomyces coelicolor*[57]. Like Taxol, epothilones block microtubule depolymerization thus arresting the cell cycle in the G2-M phase. As a result, epothilones show promise as anticancer agents. P450BSβ from *Bacillus subtilis* is a novel enzyme that has the P450 fold but catalyzes the peroxide-dependent hydroxylation of fatty acids[58]. As shown in Figure 3.17, an OH group is attached to the polar carboxyl end of the substrate which means the carboxyl head group must be positioned deep in the active site. P450BSβ has adapted to accommodate the carboxyl group by having an Arg residue on the I helix that electrostatically stabilizes the carboxyl head group (Figure 3.17). In the proposed peroxide activation mechanism, the fatty acid carboxyl group serves an acid–base catalytic function[58] similar to the distal histidine in peroxidases. P450BSβ provides a fascinating new example on the adaptability of P450s to the requirements of different metabolic pathways.

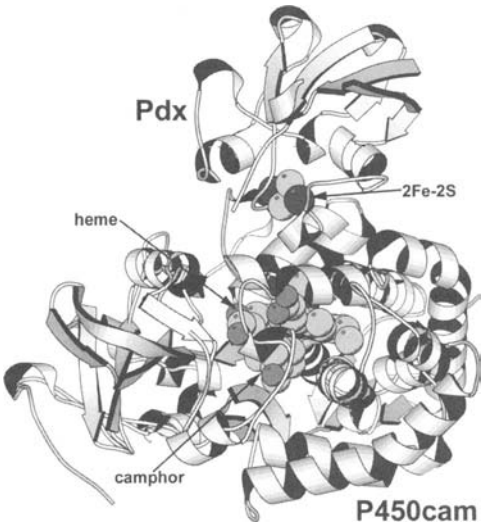

Figure 3.15. The P450cam–Pdx complex based on NMR and modeling studies[77]. Pdx docks onto the proximal surface of P450cam which is similar to the complex formed in P450BM3 (Figure 3.11).

The size and shape of the various substrates shown in Figure 3.16 are sufficiently diverse that the structural basis for what controls substrate specificity can, at least in part, be understood. As expected, all substrates are situated such that the atom to be hydroxylated is within 4–5 Å of the heme iron. Thus, regio- and stereo-selective hydroxylation by the hypothetical Fe(IV)-O species is achieved by specific protein–substrate interactions that hold the substrate in the correct position. The exception is P450BM3. The structure of the P450BM3 heme domain with palmitoleic acid[59] and N-pamitoylglycine[60] show that the fatty acid substrate is ≈7–8 Å from the iron which is too far for hydroxylation. However, NMR results indicate that the substrate moves to be within 3 Å of the iron upon reduction from Fe(III) to Fe(II)[61]. Precisely how reduction is linked to such a large repositioning of the substrate remains unknown.

P450cam and P450epoK represents the two extremes of substrate size and shape. Hence, a comparison between these two structures provides some insights on which regions of the structure change most in response to the requirements of substrate specificity. The two regions that differ the most between P450epoK and P450cam are the F, G, B' helices, and the F/G loop (Figure 3.19). The B′ helix is rotated 90° in P450epoK compared to P450cam. This reorientation opens the substrate-binding pocket thus making room for the thiazole ring of the substrate. The F and G helices do not superimpose well and the F/G loop adopts a substantially different conformation.

7. Conformational Adaptations to Substrates and Inhibitors

An unexpected insight into P450 dynamics came from the *S. solfataricus* CYP119 structure[22], the first known thermal stable P450[20]. The structure was solved in two crystal forms: one with imidazole and one with phenylimidazole coordinated to the heme iron. Compared to the phenylimidazole complex, in the imidazole complex, the C-terminal end of the F helix unwinds which increases the length of the F/G loop thus allowing the loop to dip down into the active and interact with imidazole ligand (Figure 3.20). In effect, the protein shapes itself around the ligand.

Conformational adaptations also contribute to the capacity of mammalian drug and xenobiotic metabolizing enzymes to recognize structurally diverse substrates. Rabbit microsomal CYP2C5, a drug and steroid metabolizing enzyme, has been co-crystallized with substrates of different sizes, flexibility, and polarity. Figure 3.21 depicts the binding of the anti-inflammatory drug diclofenac to CYP2C5 that catalyzes the 4′-hydroxylation of the dichlorophenyl ring of diclofenac. Diclofenac is positioned in CYP2C5 so that the flat surface of the dichlorophenyl ring faces the heme Fe[62]. The 4′-hydroxylation of diclofenac is likely to proceed through the formation of an epoxide intermediate formed by the addition of the iron oxo intermediate to the pi-electron system. The epoxide intermediate would rearrange to form the 4′-hydroxy product. The 3′ and 4′ carbons are 4.4 and 4.7 Å from the heme Fe, respectively[63].

Multiple sites of oxidation are frequently seen for substrates of drug metabolizing enzymes. This is likely to reflect either the capacity of the active site to bind the substrate in more than one orientation/location or motion of substrate within the active site cavity. The former case is evident for the structure of CYP2C5 complex with DMZ,

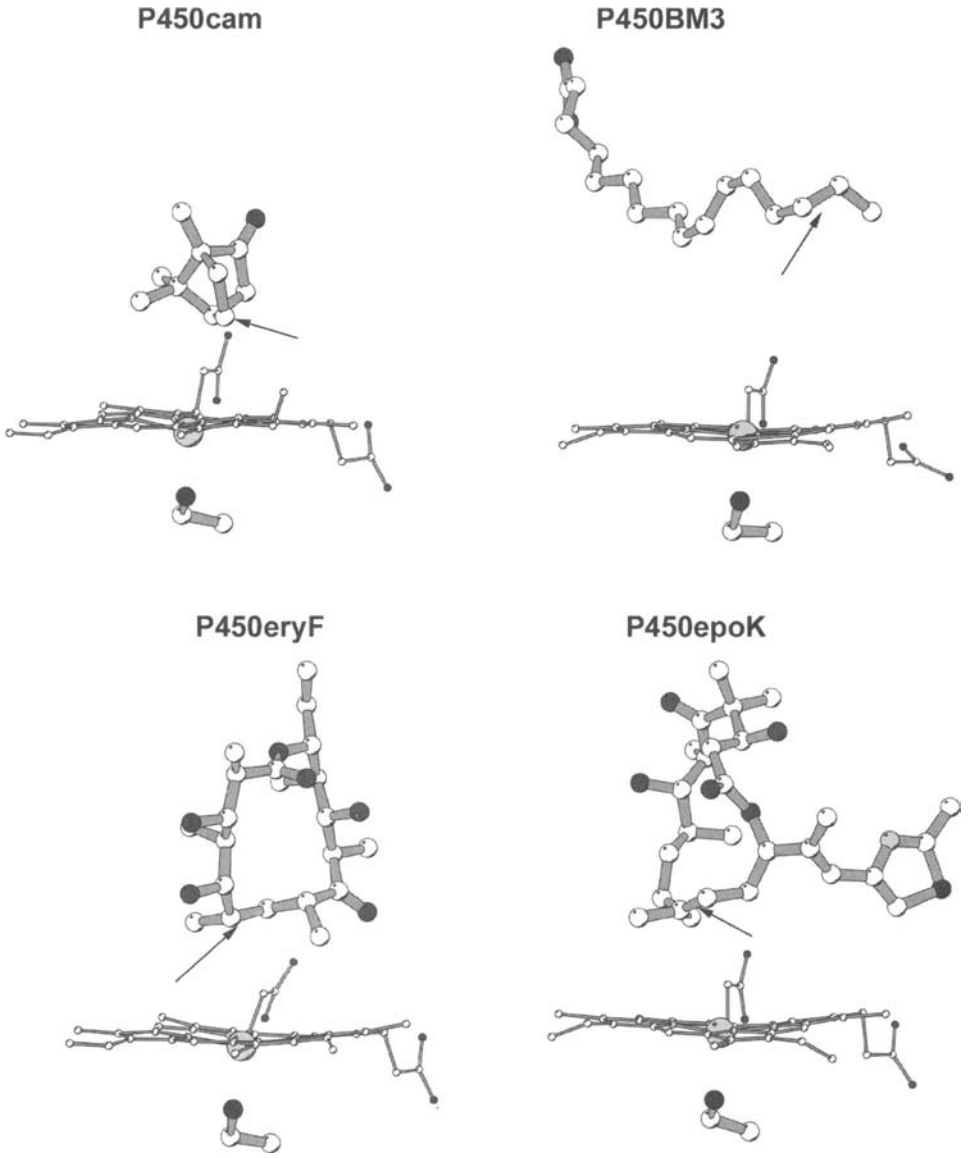

Figure 3.16. Active site structures of various P450–substrate complexes. The arrow indicates the carbon atom that is hydroxylated.

4-methyl-*N*-methyl-*N*-(2-phenyl-2*H*-pyrazol-3-yl)benzenesulfonamide (see ref. [63], Wester *et al.* 2003b). DMZ is a substrate for CYP2C5 as well as for each of the four human CYP2C enzymes[64]. Electron density maps and *in silico* docking studies indicate that the substrate binds in two alternate conformations, Figure 3.22. For DMZ, the primary site of hydroxylation (>98%) is the benzyllic methyl group. One conformation of DMZ places the benzylic methyl group 4.4 Å from the heme iron, and the reaction is likely to occur efficiently by a hydrogen abstraction mechanism. The second conformation places the other end of the molecule closest to the heme. However, DMZ is

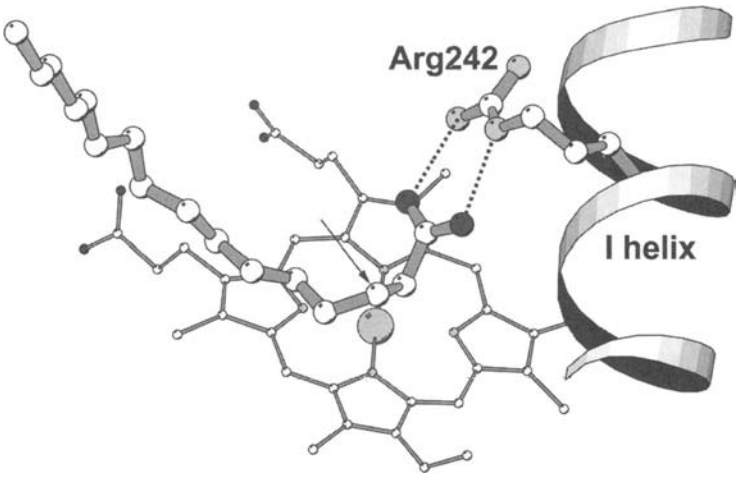

Figure 3.17. The substrate complex in P450BSβ[58]. This P450 utilizes peroxide to oxidize the atom indicated by the arrow. The I helix utilizes an arginine to help stabilize the carboxyl group of the substrate.

not located optimally for hydroxylation in this conformation as it is >5.9 Å from the heme iron, and the phenolic product accounts for <1% of the total products. The two conformations of DMZ overlap but occupy distinct portions of the active site cavity, which is larger than the substrate.

CYP2C5 exhibits adaptive changes when substrates bind. The largest, adaptive changes involve alterations in the conformations of the helix B–C region, in the locations of helices F and G, and the conformation of the region between the F and G helices. A comparison of the diclofenac and DMZ complexes illustrates differences that occur when bound substrates that are different in size, shape, and polarity (Figure 3.23). The change in the position of the F helix reflects differences in the space occupied by the two substrates near the heme Fe. Changes in the conformation of the B′ helix reflect adaptations that maximize interactions with each substrate and, in the case of diclofenac, that accommodate residual hydration in the distal portion of the active site that is not occupied by the substrate (Figure 3.23). These active site water molecules are ordered in a pattern that permits extensive intermolecular hydrogen bonding between water molecules, the protein and the substrate. This hydrogen bonding network is anchored by the polar carboxyl group of diclofenac and polar side chains in the distal portion of the active site. These interactions contribute to the affinity of the enzyme for diclofenac and the regioselective hydroxylation of the dichlorophenyl group. Human CYP2C9 exhibits similar regioselectivity for the oxidation of diclofenac and displays a high catalytic efficiency for the reaction. In contrast, the other human CYP2C enzymes generally exhibit a much lower catalytic efficiency and a more relaxed regioselectivity in that they hydroxylate both rings of the substrate.

8. Conformational Dynamics for Substrate Access

Substrate binding may involve rather large conformational changes. Once the P450cam structure became available, an immediate puzzle was how camphor gains access to the active site since the substrate is buried and there is no obvious opening. The substrate-free and -bound structures showed no differences although substrate-free P450cam exhibited much higher thermal motion in the B′, F, and G helices suggesting that the these regions must move to allow substrate to enter the active site[65]. That this region is quite flexible was demonstrated by binding of a ferrocene label to Cys85[66]. The positioning of the ferrocene into the active site results in unfolding of the B′ helix (Figure 3.24) and exposure of the active site. Although these changes may not mimic what happens when

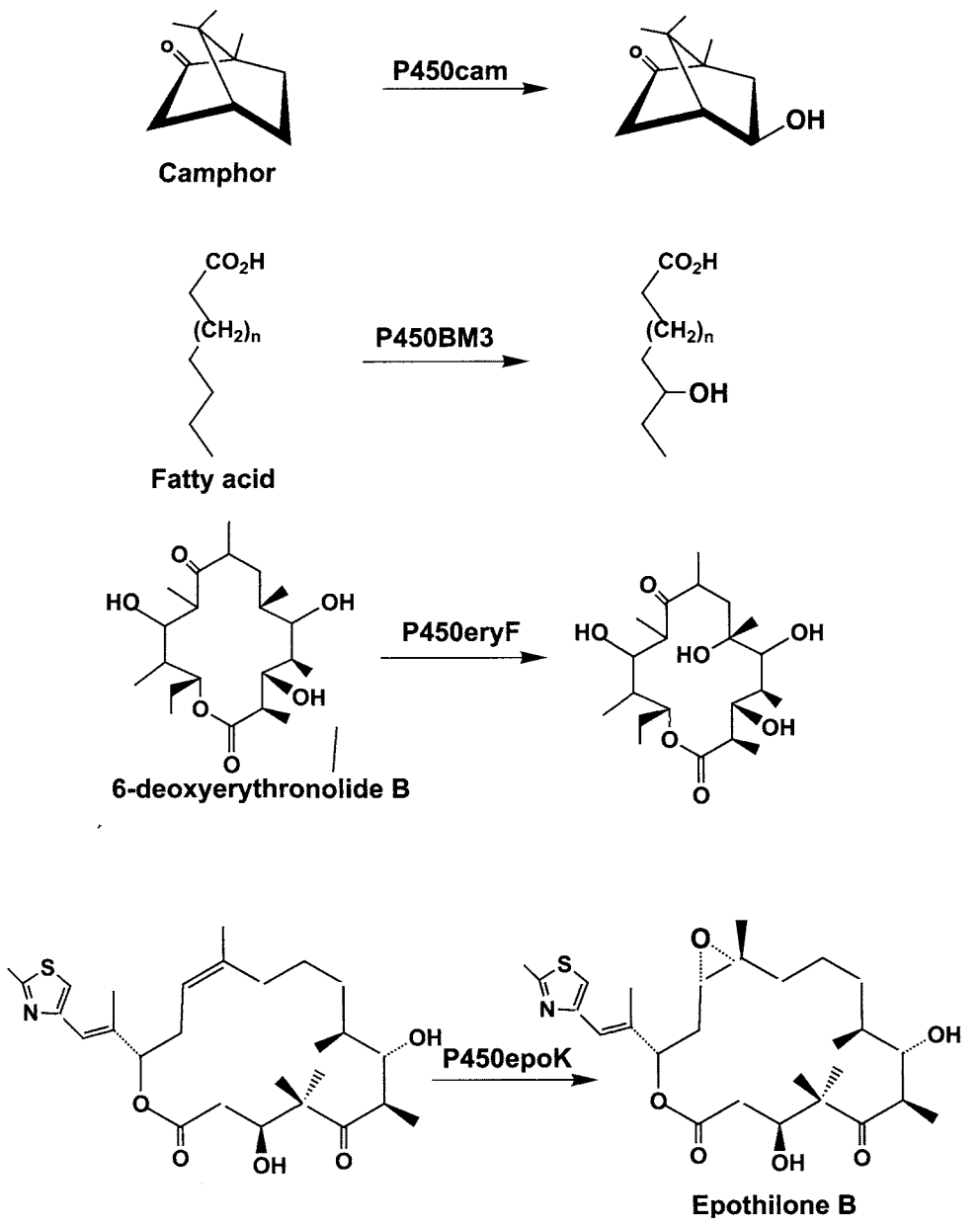

Figure 3.18. Reactions catalyzed by various P450s.

substrate binds and product leaves, this result does support the view that the B′ helix region can open to allow substrate to enter. Additional support for this view is provided by the structures of P450cam complexed with tether compounds. The tether compounds are designed to position a fluorescent reporter group at the enzyme surface by attaching it to a substrate analog that binds in the active site using a linker of the approximate length and size of the substrate access channel[67, 68]. As shown in Figure 3.25, the tether molecule occupies a continuous channel from the active site to the surface of

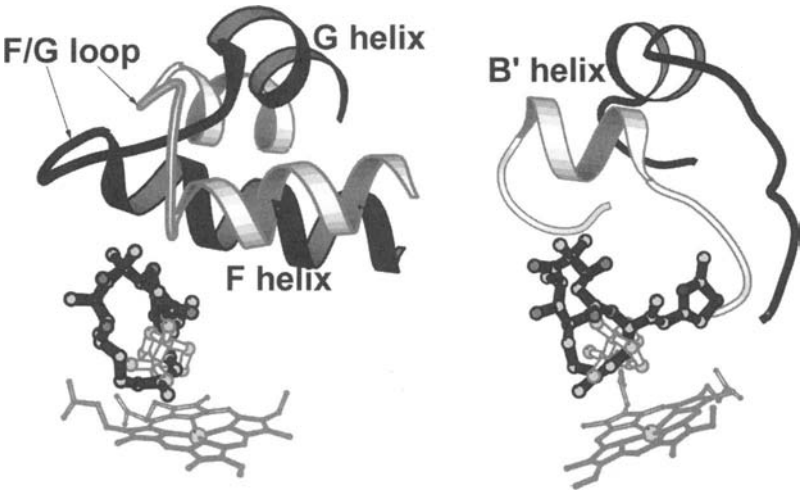

Figure 3.19. Differences between elements of structure that shape the substrate-binding pocket in P450cam (light shading) and P450epoK (dark shading). Note the differences in the position of the F/G loop and B′ helix.

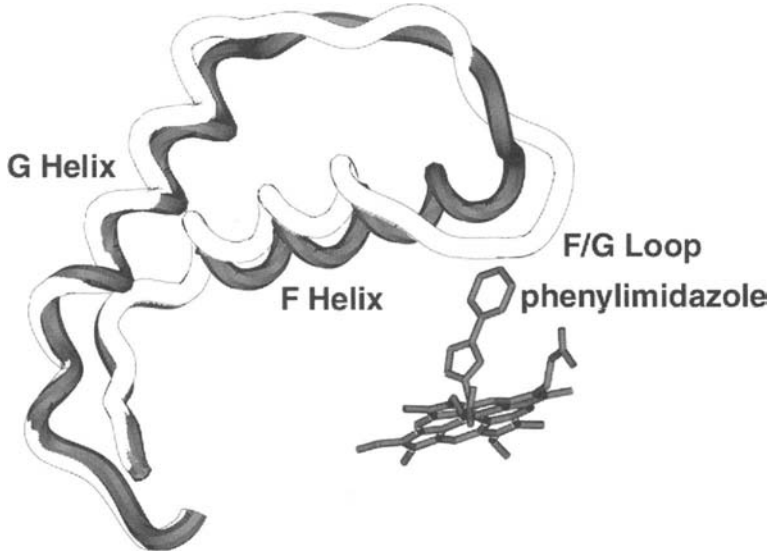

Figure 3.20. Ligand-induced conformational changes in CYP119. Compared to the phenylimidazole complex (dark shading), the C-terminal end of the F-helix in the imidazole complex unfolds which lengthens the F/G loop thus allowing the F/G loop to dip into the active site and interact with the iron-linked imidazole. Since phenylimidazole is larger than imidazole, the F/G loop cannot remain positioned in the active site complex. Therefore, the F/G helical region and loop "shapes" itself around the ligand bound in the active site.

P450cam that is created by displacement of helix B′ and the F–G loop.

The early work with P450cam, however, presented some experimental limitations. The initial diffraction quality crystals of P450cam had DTT bound in the active site. To obtain the substrate-free and -bound structures, DTT had to be back-soaked out or camphor soaked in. The relatively tight crystal lattice of P450cam very likely prevents the substrate-free structure from adopting

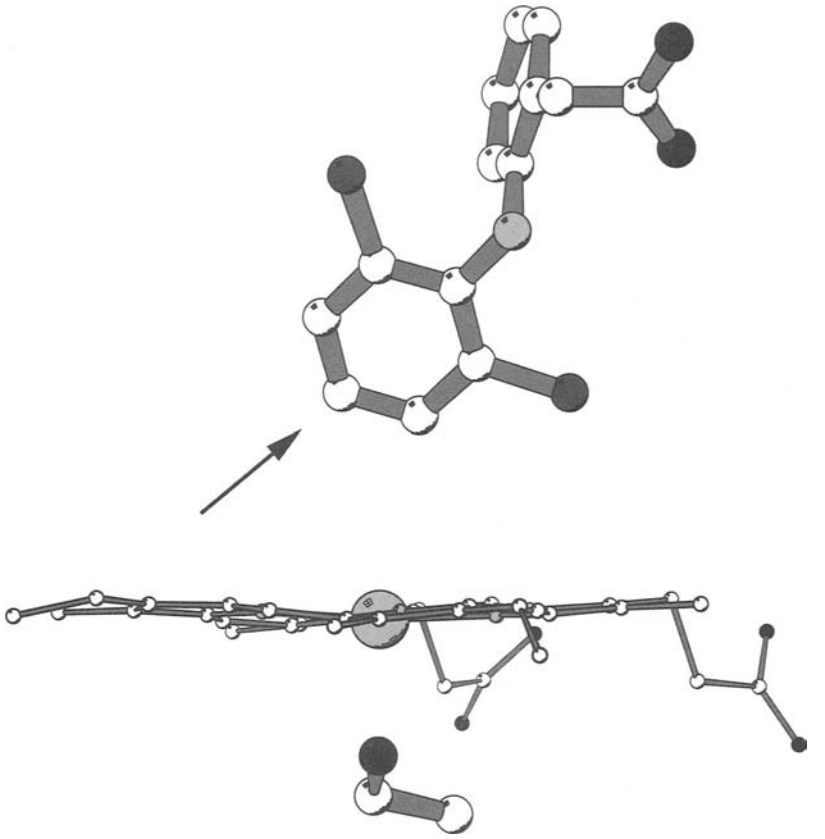

Figure 3.21. Diclofenac bound to CYP2C5, PDB: 1NR6. Hydroxylation of diclofenac at the 4′ carbon is likely to proceed through an intermediary epoxidation of the 3′–4′ carbon bond of the substrate. The 3′ and 4′ carbons are positioned at 4.4 and 4.7 Å, respectively, from the heme Fe. Polar interactions of the substrate carboxylate lead to a high degree of regioselectivity for oxidation of diclofenac by CYP2C5.

the open structure, although it remains unclear whether or not an open conformation is stable or is only a transient conformer.

The first clear indication that conformational changes are important in substrate binding was the structure of palmitoleic acid bound to P450BM3[59] which was followed by a higher resolution structure[60]. A solvent accessible surface diagram (Figure 3.26) illustrates how the substrate access channel is open in the substrate-free structure and closed in the substrate-bound structure. Quite interestingly, the experimentally observed conformational change was correctly predicted based on computational methods[69, 70] before the substrate-bound crystal structure was solved. The main motion involves the F and G helices sliding over the surface of the I helix. This motion closes off the entry channel indicating that substrates enter near the F/G loop region which is similar to P450cam.

Structures of several P450s exhibit open structures in the absence of substrates. P450nor is a novel, soluble eukaryotic P450 that reduces nitric oxide. The enzyme is directly reduced by NADH which binds directly to an open cleft between the F–G loop and the N-terminal β-sheet system of the enzyme[71]. Two recently determined structures for prokaryotic P450s that are thought to be involved in the oxidation of relatively large antibiotic compounds exhibit more open structures. One is OxyB[72], a P450 that is thought to be involved in the synthesis of vancomycin by *Amycolatopsis*

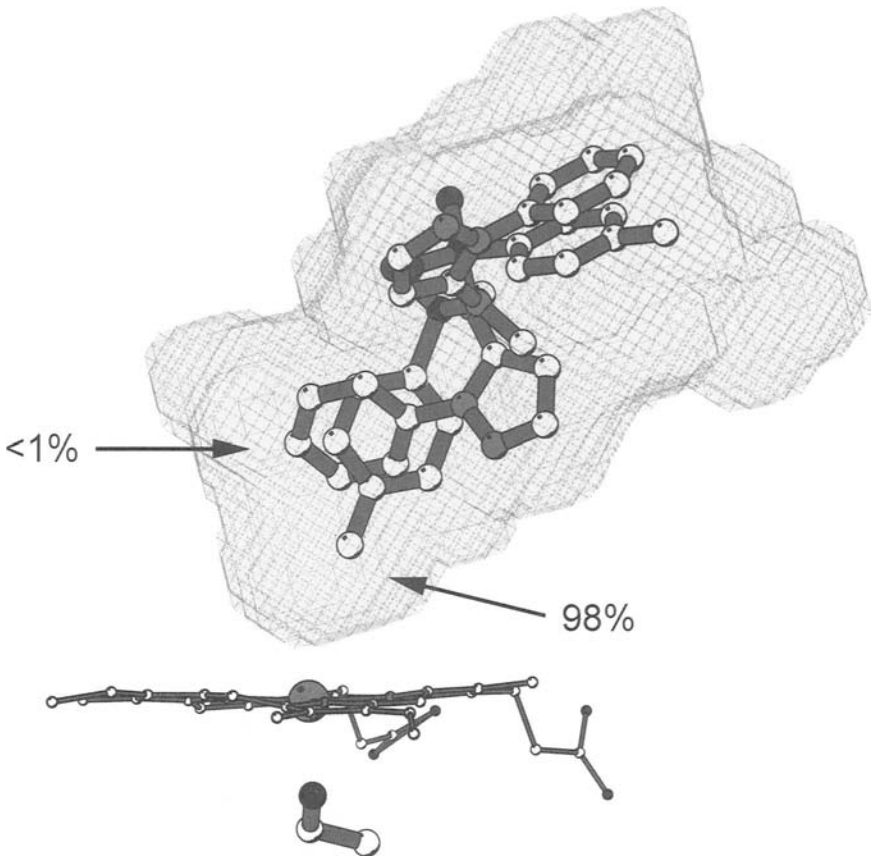

Figure 3.22. Binding of DMZ to CYP2C5, PDB: 1N6B. DMZ binds to the enzyme in two distinct locations depicted by the DMZ molecules with light and dark gray bonds. One orientation (light gray bonds) places the benzyllic methyl group of DMZ 4.4 Å from the heme iron, and oxidation of the benzyllic methyl group accounts for >98% of the observed products. The alternate orientation (dark gray bonds) places the phenyl ring at >6 Å from the heme Fe. Oxidation of the phenyl ring accounts for 1% of the products. A solvent accessible surface for the substrate-binding cavity is depicted by the light gray mesh.

orientalis. Helices F and G are rotated out of the active site in this structure. It is thought that the natural substrate of the enzyme is a relatively large, glycosylated heptapeptide. A similar positioning of helices F and G is also seen in the structure of CYP154C1 from *S. coelicolor A3(2)* (Figure 3.27) that oxidizes macrolide substrates[73]. In contrast to the structure seen for P450BM3, the region between helices B and C separates from the G helix to form a cleft that opens along helix I.

P450epoK presents a different picture. Here the substrate-free and -bound structures were separately crystallized[56], yet there is very little difference in structure. Substrate binding causes a slight tightening of the active site but there is no indication of the sorts of large motions observed with P450BM3. Caution must be exercised here because the energetics of adopting the open conformation must be balanced with the energetics of crystallization. With P450BM3, the open conformation was trapped due in part to crystal contacts. It may be that P450epoK simply prefers to crystallize in the "closed" form with or without substrate bound. The assumption here, of course, is that P450s must undergo open/close motions to allow substrate access even if we only observe the closed form in crystal structures.

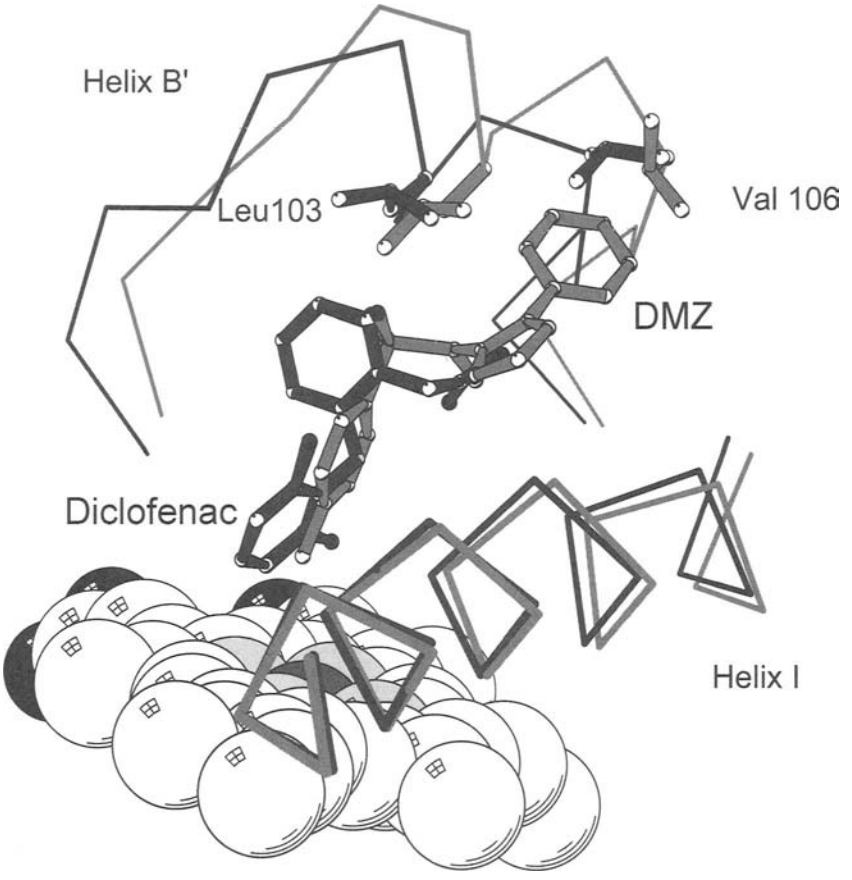

Figure 3.23. Conformational adaptations for substrate binding to CYP2C5. Among other changes, the position of helix B′ adapts to the presence of diclofenac and DMZ that differ in size and polarity. The DMZ complex is rendered in light gray, and the diclofenac complex is depicted in dark gray. Cα traces are shown for the helix B to C region and a portion of the helix I with the heme rendered as CPK atoms. The different positions of two active site side chains, Leu103 and Val106, are rendered as ball and stick figures, as are the two substrates.

While it would appear that the F/G loop region may provide the point of substrate entry and conformational dynamics for many P450s, the CYP51 structure[74] indicates that other access channels may be used. While CYP51 exhibits the normal P450 fold, there is an unprecedented break in the I helix (Figure 3.28). This break creates a new opening that runs roughly parallel to the heme as opposed to the F/G loop entry point which is perpendicular to the heme. Podust *et al.*[74] suggest that if the F/G loop region were to open, then the new opening must close. This offers the possibility that a concerted opening of one channel and closure of the other might provide a means for substrate to enter via one route but product depart via the other.

Eukaryotic P450s generally exhibit longer polypeptide chains between helices F and G than are seen in prokaryotic P450s, and this region interacts more extensively with the N-terminal β-sheet region, potentially limiting the opening of the protein in this direction. In addition, this region is likely to interact with the membrane. In contrast, the B′ helix region is not closely associated with the rest of the protein, suggesting that the flexibility of the helix B to helix C region is likely to shift

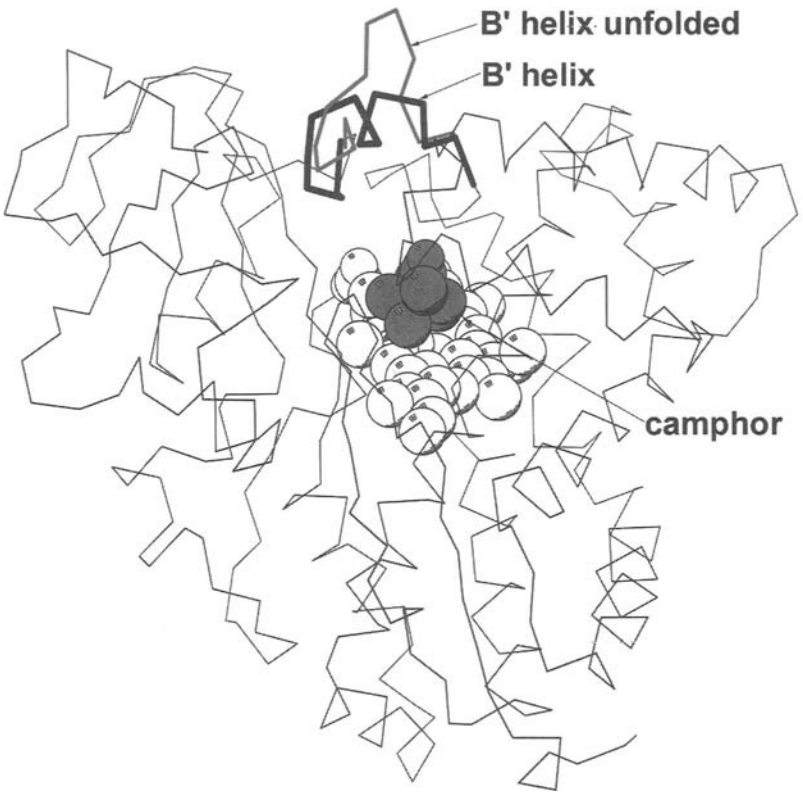

Figure 3.24. A comparison of the structures of B′ helix region in P450cam (dark shading) and the ferrocene attached to Cys85[66]. To accommodate the bulky ferrocene, the B′ helix must unfold (light shading) and move further away from the main body of the protein.

the opening to the active site to a direction along the helix I between the helix B′ to helix C loop and helix G. This is supported by the observation that progesterone can be soaked into CYP2C5 in crystals where the N-terminal β-sheet region and helices F and G are highly constrained by crystal packing. The solvent channel is closed by a single H-bonding interaction between K241 of helix G to the backbone carbonyl of V106 in helix B′ as well as a relatively weak van der Waals contact of V106 with H230 of helix G. Crystallization of CYP2B4 in an open conformation[75] supports this view; an open cleft is observed in this structure between the helix B′ to C loop, helix I and helix F to helix G regions.

A theoretical analysis of substrate-binding routes has helped to clarify the picture[76]. The computational approach predicts that the main substrate channel near the F/G loop is the same as that derived from the crystal structure but also predicts other possible routes of entry in P450cam, P450BM3, and P450eryF. In addition, novel routes of ligand exit were found. These pathways may be favored in other P450s that exhibit different packing interactions between the flexible components of the distal surface of the enzyme. These studies have also provided insights into the energetically accessible motions available to the various P450s.

Overall, the current picture is that while the P450 fold is conservative, it is quite flexible and can undergo rather large changes in response to the requirements of substrate specificity. The F/G helical region and B′ helix are subject to the greatest structural variation as well as flexibility. This is understandable considering the importance the

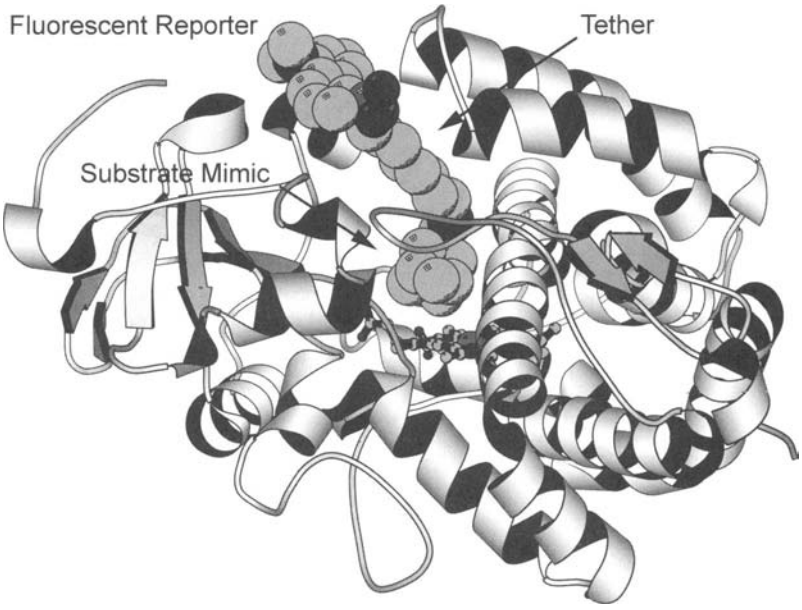

Figure 3.25. The structure of P450cam complexed with a tether compound adamantane-1-carboxylic acid-5-dimethylaminonaphthalene-1-sulfonylamino-octyl-amide rendered as CPK atoms, PDB: 1LWL. The heme is rendered as a ball and stick figure. The tether compound occupies an open channel between helices F and G, helix B′ and the β-sheet domain with the fluorescein moiety residing on the surface and the adamantane moiety positioned in the substrate-binding site.

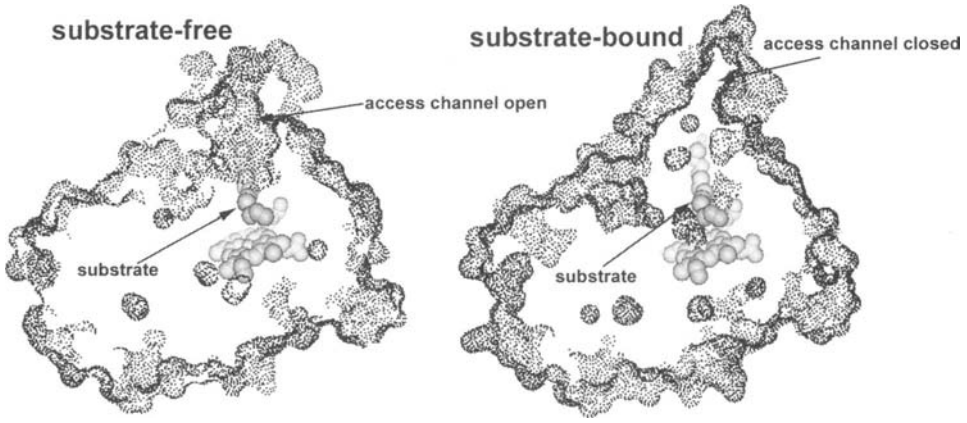

Figure 3.26. Solvent accessible surface diagrams of P450BM3 in the substrate-free and -bound forms.

F/G region and the B′ helix play in substrate entry and binding. Toward the goal of understanding selectivity, homology modeling has become an increasingly popular tool in P450 research. There are simply too many interesting P450s to expect the various crystal structures to be solved in a timely fashion. However, the structures we have in hand show quite clearly that homology modeling has a major challenge because the most difficult regions to predict are precisely those regions that are functionally most important. In many cases, the low degree of amino acid identity renders threading of these sequences onto experimentally determined structures ambiguous. This problem

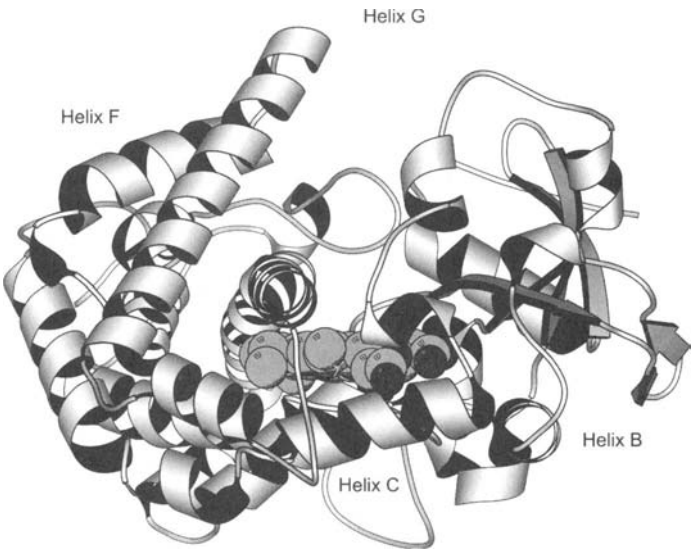

Figure 3.27. The structure of CYP154C1, PDB: 1GWI. A view along the N-terminal end of helix I is shown depicting the rotation of helices F and G up and away from the substrate-binding cavity. An open cleft runs through the molecule above the heme. The helix B to C segment resides on the opposite of the cleft in association with the β-sheet 1. The heme is rendered as CPK atoms.

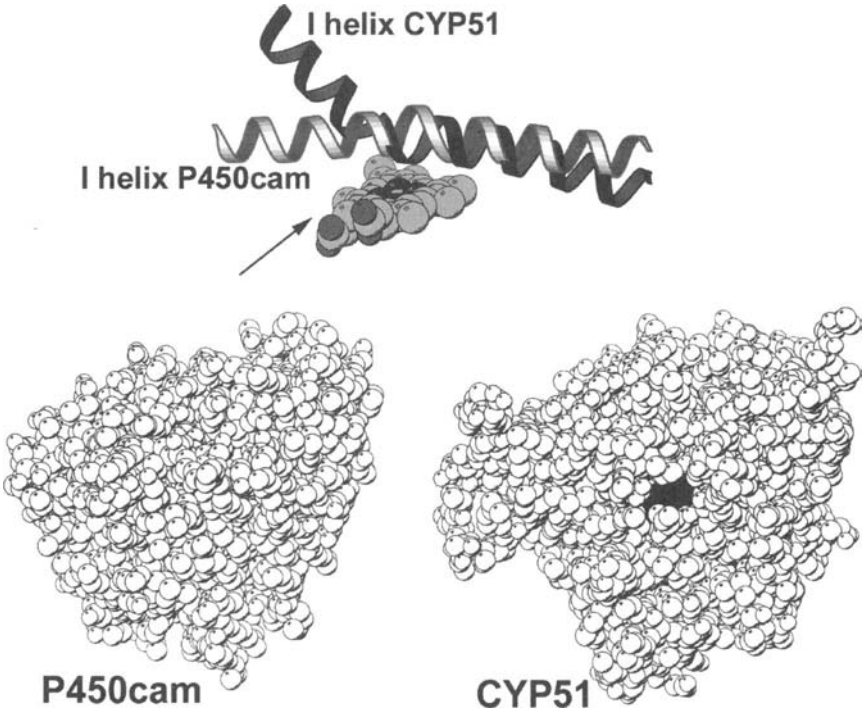

Figure 3.28. The top diagram shows the I helix in CYP51 (dark shading) and P450cam (light shading). The CPK diagrams are viewed along the plane of the heme and illustrate how the break in the I helix leaves the heme pocket open in CYP51.

has been largely overcome for the relatively large number of drug metabolizing enzymes in family 2 because alignments with known structures such as those of CYP2C and CYP2B families are relatively straightforward. However, the experimentally determined structures of the family 2 enzymes exhibit significant differences in the conformations of the flexible portions of the catalytic sites that underlie their functional diversity. Thus, a critical need to determine structures of specific drug metabolizing enzymes remains in order to accurately model substrate enzyme interactions.

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4

Electron Transfer Partners of Cytochrome P450

Mark J.I. Paine, Nigel S. Scrutton, Andrew W. Munro, Aldo Gutierrez, Gordon C.K. Roberts, and C. Roland Wolf

1. Introduction

Cytochromes P450 contain a heme center where the activation of molecular oxygen occurs, resulting in the insertion of a single atom of oxygen into an organic substrate with the concomitant reduction of the other atom to water. The monooxygenation reaction requires a coupled and stepwise supply of electrons, which are derived from NAD(P)H and supplied via a redox partner. P450s are generally divided into two major classes (Class I and Class II) according to the different types of electron transfer systems they use. P450s in the Class I family include bacterial and mitochondrial P450s, which use a two-component shuttle system consisting of an iron–sulfur protein (ferredoxin) and ferredoxin reductase (Figure 4.1). The Class II enzymes are the microsomal P450s, which receive electrons from a single membrane-bound enzyme, NADPH cytochrome P450 reductase (CPR), which contains FAD and FMN cofactors (Figure 4.1). Cytochrome b_5 may also couple with some members of the Class II P450s family, notably CYP3A4, to enhance the rate of catalysis[1].

Although P450 redox partners are usually expressed independently, "self-sufficient" P450 monooxygenase systems have also evolved through the fusion of P450 and CPR genes. These fusion molecules are found in bacteria and fungi, the best-known example being P450 BM3, a fatty acid ω-2 hydroxylase from *Bacillus megaterium*, which comprises a soluble P450 with a fused carboxyl-terminal CPR module (recently reviewed by Munro[2]). BM3 has the highest catalytic activity known for a P450 monooxygenase[2] and was for many years the only naturally occurring fused system known until the identification of a eukaryotic membrane-bound equivalent fatty acid hydroxylase, CYP505A1, from the phytopathogenic fungus *Fusarium oxysporum*[3]. A number of novel P450 systems are starting to emerge from the large numbers of genome sequencing projects now underway[4].

In this chapter, we review the most recent advances being made in understanding the function of P450 redox partners and the electron transfer process. Special attention is paid to CPR, which occupies a particularly important position because of its central involvement in human drug metabolism.

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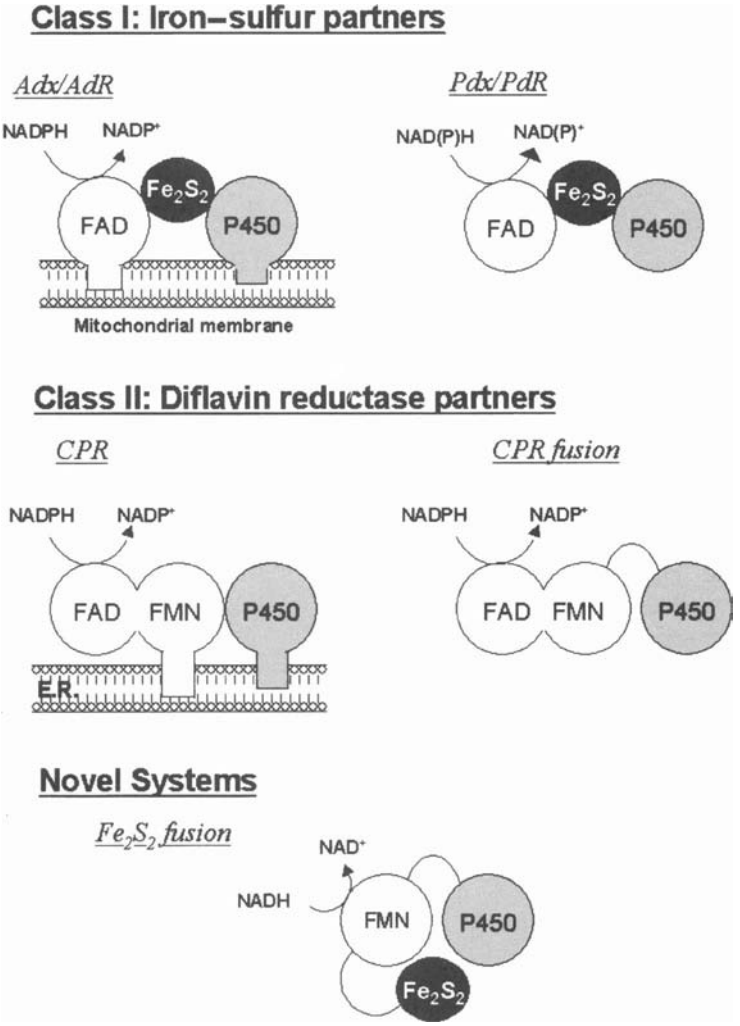

Figure 4.1. Electron transfer partners of cytochrome P450. In Class I systems, electrons are shuttled from NAD(P)H through an FAD-containing ferredoxin reductase and an iron–sulfur containing ferredoxin to P450; in prokaryotes these are typified by putidoredoxin reductase (PdR) and putidoredoxin (Pdx), and in eukaryotes by mitochondrial membrane associated adrenodoxin reductase (AdR) and adrenodoxin (Adx). Class II systems are driven by electrons delivered from NADPH through diflavin (FMN- and FAD-containing) reductases. In eukaryotes these are bound to the endoplasmic reticulum, while fused systems such as P450 BM3 exist in bacteria and fungi. Novel systems now include P450RhF, which contains an FMN-containing reductase fused with a ferredoxin-like center and a P450.

2. NADPH-Cytochrome P450 Reductase and the Diflavin Reductase Family

2.1. Background

In view of the key role that cytochrome *c* has recently been found to play in regulating apoptosis and cellular homeostasis[5, 6], it is interesting that CPR was first isolated from yeast as an FMN containing NADPH-dependent cytochrome *c* reductase[7]. A mammalian equivalent was later isolated from pig liver and reported to contain an FAD cofactor[8]. By 1962, the enzyme was shown to be localized at the endoplasmic reticulum[9], and the flavin cofactors to be involved in the reduction

of cytochrome c[10]. The true physiological redox partner was eventually discovered as P450 through the reconstitution of laurate ω-hydroxylase activity from a detergent solubilized preparation of cytochrome P450, NADPH cytochrome c reductase[11, 12], and a heat stable component, which was later identified as the phospholipid phosphatidylcholine[13].

The initial difficulties in identifying a physiological role for CPR were due to the purification methods used, which incorporated trypsin or lipase treatment[8, 9]. These resulted in the cleavage of the amino-terminal membrane anchoring domain, which constitutes the first 60 or so amino acid residues and is responsible for interactions with the phospholipid bilayer and P450[14]. Thus, while proteolytically cleaved CPR is fully functional and capable of reducing cytochrome c and a range of artificial electron accepting compounds, it is unable to reconstitute with P450s. The intact form of the reductase was eventually purified from liver microsomes using detergent solubilization procedures and found to have a molecular weight of 76–80 kDa and to support P450-dependent reactions[12, 15].

In the early 1970s, the enzyme was shown to contain one molecule each of FMN and FAD[15, 16]. A useful feature of flavins is that their absorption spectra are altered by changes in their reduction state. Thus, the reduction state can be examined by measuring changes in the visible absorbance range (Figure 4.2). This unique property stimulated research into the redox properties[17, 18], of the enzyme and the complex processes of hydride/electron transfer from NADPH, across the flavins and on to P450, discussed later in this chapter.

The cDNA and corresponding primary amino acid sequences of several CPRs including rat[19], rabbit[20], and human[21] were obtained by the mid-1980s, and the development of *Escherichia coli* expression systems paved the way for detailed molecular characterization of the polypeptide through site-directed mutagenesis. The three-dimensional structure of rat CPR was determined by X-ray crystallography in 1997 by Kim and coworkers[22], providing the structural prototype for dual flavin oxidoreductases.

2.2. The Diflavin Reductase Family

CPR is the prototype for a small family of diflavin reductases which are believed to have

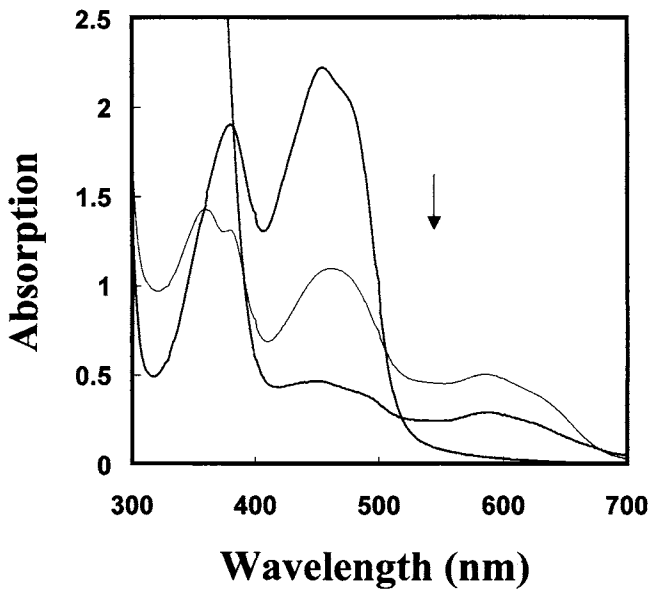

Figure 4.2. Absorbance spectra of oxidized and reduced human CPR. The absorption maxima for the oxidized enzyme are located at 380 nm and 454 nm. The direction of the arrow shows the absorption changes that occur upon reducton of the flavins, in this case using a 2-fold and 20-fold excess of NADPH.

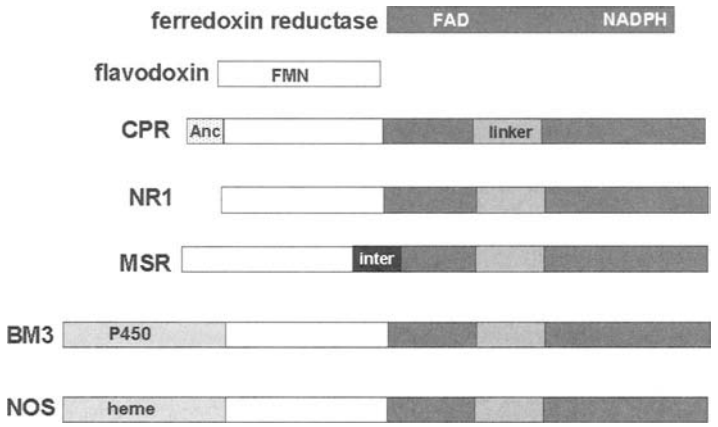

Figure 4.3. Schematic outline of the diflavin reductase family. Members contain an N-terminal FMN-binding flavodoxin-like domain and a C-terminal FAD/NADPH-binding ferredoxin reductase-like domain, which contains an additional linker region. Shown are: CPR, which has an amino-terminal membrane anchor region (Anc); NR1 (Novel reductase 1); MSR (methionine synthase reductase), which contains an additional interdomain sequence; P450 BM3, which is fused to a P450 domain; and NOS (nitric oxide synthases), which has linked to a heme-containing oxygenase domain that is structurally distinct from the P450s.

evolved as a result of a gene fusion event between ancestral FMN- and FAD-containing flavoproteins[23]. Indeed, CPR is one of only four mammalian enzymes that contain both FMN- and FAD-binding domains, the other three being methionine synthase reductase[24], NR1[25], and the nitric oxide synthases[26] (Figure 4.3). Bacterial members of the family include the *B. megaterium* cytochrome P450 BM3[27] and the reductase subunit of sulfite reductase[28]. While these are all structurally related, there exist some key differences. For instance, methionine synthase reductase and NR1 both contain the same domain organization as P450 reductase but lack the membrane anchoring sequence, and are thus located in the cytosol. Methionine synthase also has a much larger interdomain linker than CPR, although the functional significance of this is unclear. In nitric oxide synthase, the reductase region is fused with a heme domain, and has acquired an extra calmodulin-binding domain to regulate electron transfer.

Experimental evidence for the gene fusion concept comes from work of Smith *et al.*[29], who dissected the FMN and FAD/NADH-binding domains of human CPR and expressed them as discrete functional units in *E. coli*[29]. The individual domains not only folded correctly but also could be reconstituted to form an active complex capable of reducing cytochrome *c* and donating electrons to the P450 monooxygenase complex, albeit with greatly reduced efficiency[29]. A number of other diflavin reductases have been dissected into functional units including: rat CPR[30], BM3[31,32], NR1[33], and methionine synthase reductase[34]. The ability to separate CPR and other diflavin reductases into their component parts has greatly facilitated structural and functional studies of these enzymes.

2.3. CPR Genes

Apart from plants, which contain multiple CPR genes[35–37], most organisms contain a single CPR gene. At the time of writing, a relatively modest total of 20 CPR genes have been identified, which will doubtlessly increase as a result of the many genome sequencing projects currently underway. New sequences can be monitored using the website www.icgeb.trieste.it, which provides sequence updates on P450s and related enzymes. The rat gene was cloned in 1990[38] and comprises 16 exons, with the first exon being non-coding. Amongst the coding exons there is a general correspondence with the structural domains, with exons 2 and 3 encoding the membrane anchor region, exons 4–7 the FMN-binding domain, and exons 8–16 the FAD/NADPH-binding region.

A similar intronic arrangement is found for the mouse gene located on chromosome 6[39, 40]. The human gene is located on chromosome 7[21].

Although the regulation of expression of individual P450s in response to exogenous and endogenous factors has been studied extensively, the factors controlling the expression of the CPR gene are less well understood. Reductase expression may be induced by a number of cytochrome P450 inducers such as phenobarbital[41], but the regulation of reductase gene expression is independent of that of cytochrome P450[42]. The upstream regulatory region of the rat CPR gene has a number of interesting features[43]. Unlike many drug-metabolizing enzymes, the promoter does not contain either a TATA or CCAAT box, it is GC rich, contains multiple Sp1 consensus sequences, and utilizes two major transcription start sites.

The CPR gene is regulated by thyroid hormone, which stimulates CPR expression via the thyroid response element (TRE) in the upstream promoter[44, 45]. Most recently, peroxisome proliferators, which regulate a battery of rodent P450 genes, have also been shown to regulate CPR levels[46], possibly through a putative DNA binding site for PPARα that exists in the mouse CPR promoter between −568 and −556 bp[46]. Interestingly, following exposure to peroxisome proliferators CPR gene transcript levels appear to increase, while levels of CPR protein are decreased[46]. Thus, opposing transcriptional and translational or post-translational mechanisms appear to play a role in the regulation of CPR by peroxisome proliferators[46].

2.4. Probing the Physiological Role of CPR

The reductase gene has been knocked out in mice[39, 40]; this leads to embryonic lethality, thus demonstrating that CPR is essential for normal development in higher organisms, presumably because it plays a key role in the biosynthesis of signaling factors such as retinoic acid, sterols, prostaglandins, and steroids that are dependent on P450 catalysis. The precise role of CPR in embryogenesis is still unclear and the effects of gene deletion on the developing embryos are complex. Embryos lacking CPR do not survive beyond 8–9 days and display a number of defects including neural tube, cardiac, eye, and limb abnormalities, and generalized defects in cell adhesion[40].

Kasper's group has examined the effects of CPR gene disruption by removing the natural translation initiation site and deleting the membrane-binding domain of CPR[40]. However, some homozygous null embryos ($CPR^{-/-}$) were found to produce a truncated 66 kDa cytoplasmic form of CPR, presumably through initiation at an alternative start site. That these still produced the spectrum of embryonic defects leading to mid-gestational lethality provides strong evidence that the microsomal location of CPR is essential for the physiologically important functions of the P450s.

The problem of the lethality of a CPR knockout has been resolved by applying the regulatable Cre/loxP system[47] to generate mice in which CPR can be deleted post-natally in the liver[39]. Such "conditional knockout" mice, in which all the hepatic P450s are essentially inactivated, provide the means of evaluating P450 function in normal homeostasis, drug pharmacology, and chemical toxicity *in vivo*. Hepatic CPR-null mice exhibit many intriguing phenotypes[39]. Due to an inability to produce bile acids, they no longer break down cholesterol, and whereas hepatic lipid levels were significantly increased, circulating levels of cholesterol and triglycerides are severely reduced. There are also profound changes in the *in vivo* metabolism of pentobarbital and acetaminophen, demonstrating the predominant role of the hepatic P450s in the pharmacology and toxicology of these compounds, and illustrating the power of transgenic models in understanding P450 function.

One of the remarkable aspects of the hepatic CPR deletion in mice was the fact that they live and reproduce normally. This means that in adult mice at least, the hepatic P450 system is not essential for life, and indicates most strongly a fundamental role in providing protection against toxic environmental agents. The data from these transgenic models[39, 40] also demonstrate that potential alternative electron transfer pathways for P450s, such as the cytochrome b_5/b_5 reductase systems that are discussed later, play only a minor role, if any, *in vivo*.

The earliest CPR gene deletions were of course carried out with yeast, and the data offer some interesting contrasts with mammalian deletions[48, 49]. The deletion of the CPR gene in

Saccharomyces cerevisae is not lethal but instead produces a viable mutant with an increased sensitivity (>200-fold) to ketoconazole, an azole inhibitor of the fungal P450 CYP51, a lanosterol 14α-demethylase involved in the essential ergosterol biosynthesis pathway[48]. Although this hypersensitivity is suggestive of inefficient sterol biosynthesis, it has been observed that the *cpr*$^-$ strains still accumulate significant amounts of ergosterol, around 25% of those in the wild-type parent[49]. This indicates that, unlike mice, an alternative electron donor is effective in delivering two electrons for P450 activity[49]. In contrast to Shen's observations with *cpr*-deleted mice[40], a cytosolic truncated version of native CPR with the N-terminal membrane anchor removed effectively reversed the phenotypes associated with *cpr* deletion in yeast[49]. Thus, there appear to be fundamental differences in the mechanisms of P450 interactions between yeast and mammalian CPRs.

While the major role of CPR is associated with cytochrome P450 and the phase I metabolism of xenobiotic compounds, the enzyme also has the ability to reduce other electron acceptors such as cytochrome c[9], cytochrome b_5 (ref. [50]), and heme oxygenase[51]. In addition, it has recently been implicated, along with cytochrome b_5, in the bioreductive activation of methionine synthase[52], thus pointing to a possible role in the regulation of methionine synthesis. CPR has also been shown to be involved in the regulation of oxidative response genes such as hypoxia-inducible factor 1 (ref. [53]).

P450 reductase plays a role in the bioactivation of therapeutic prodrugs through its ability to reduce a range of one-electron acceptors such as the quinone drugs doxorubicin[54] and mitomycin c[55] and aromatic *N*-oxides such as the novel benzotriazine, tirapazamine[56]. Evidence is also emerging that other members of the CPR family including nitric oxide synthases[57] and NR1[25] may play a similarly important role in the metabolism of chemotherapeutic drugs.

Overall, the biological relationships of CPR with proteins other than P450 are not especially well established. However, attention is starting to shift toward understanding the role of CPR in other redox pathways. In particular, data is accumulating on CPR interactions with cytochrome b_5 (ref. [58]) and heme oxygenase[59]. Consistent with the findings for cytochrome P450 enzymes, molecular investigations on the binding of CPR to human heme oxygenase-1 (hHO-1) establish that ionic interactions contribute to the binding of these proteins[59]. Like P450, positively charged hHO-1 surface residues contribute to the binding of this molecule to CPR, most likely via the negatively charged residues in the FMN domain of CPR.

2.5. Structure of CPR

The crystal structure of rat CPR lacking the N-terminal membrane-binding sequence[22] shows that the catalytic region comprises three distinct domains: an FMN-binding domain structurally similar to flavodoxins; an NADPH/FAD-binding domain similar to ferredoxin–NADP$^+$ reductase; and a "linker" domain, present as an insert in the sequence of the NADPH/FAD-binding domain (Figure 4.4). The "linker" sequence is the major structural feature that distinguishes these molecules from the single-domain ferredoxin reductases, and is likely to play a structural role in positioning the FMN- and FAD-binding domains correctly for direct electron transfer. This is supported by mutagenesis studies in which comparison of wild-type and mutant structures shows significant differences in the relative position of the two flavin domains of rat CPR[60]. Furthermore, there is increasing evidence from structural studies with other dual flavin reductases that the interplay between NADPH, flavin cofactors, and heme is directed by conformational changes. This is exemplified by nNOS, where structural relaxation appears to be an important part of the calmodulin-dependent activation mechanism[61].

2.5.1. The FMN-Binding Domain

The FMN-binding domain interacts with cytochrome P450 to transfer electrons during catalysis. In addition to rat CPR, detailed structural studies of the isolated human FMN domain have been carried out using NMR[62], and X-ray crystallography[63]. The isoalloxazine ring of FMN is sandwiched between two aromatic side chains, Tyr140 and Tyr178[22, 62, 63]. Tyr178 lies parallel to the *si*-side of the FMN ring, while the second aromatic, Tyr140, located on the *re*-side, lies at an angle of ~40° to the isoalloxazine ring (Figure 4.5). A similar arrangement is found in the FMN-binding domain of P450 BM3 and in

most flavodoxins, apart from *Clostridium* flavodoxin where the *re*-side aromatic side chain residue is replaced by methionine[64]. Removal of the aromatic side chains of Tyr140 and/or Tyr178 from rat liver CPR by site-directed mutagenesis results in decreased affinity for FMN[65].

In addition to these two key residues, a third aromatic side chain, Phe181, lies close to the FMN isoalloxazine ring on the *si*-side at the pyrimidine end, although not in contact with it (Figure 4.5). This residue is highly conserved in P450 reductase and related enzymes, indicating a possible role in FMN binding. Mutations that remove the aromatic side chain result in a 50-fold reduction in affinity for FMN, and NMR spectra show significant changes in amide chemical shifts of residues in

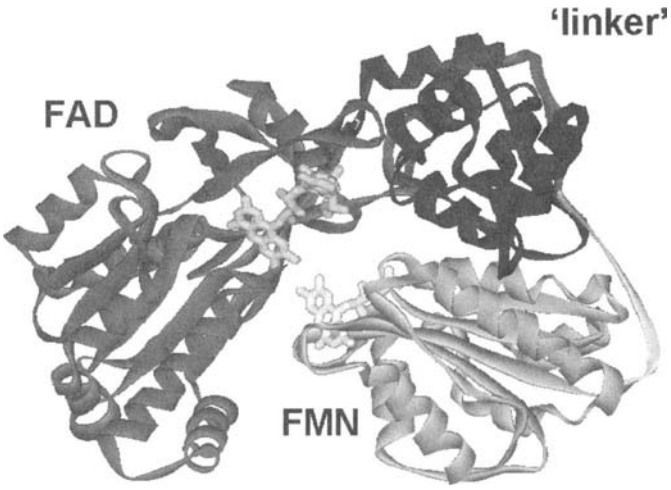

Figure 4.4. Schematic representation of rat CPR domains (protein data base [pdb] code 1AMO). In the crystal, the two flavins, FMN and FAD, are 4 Å apart.

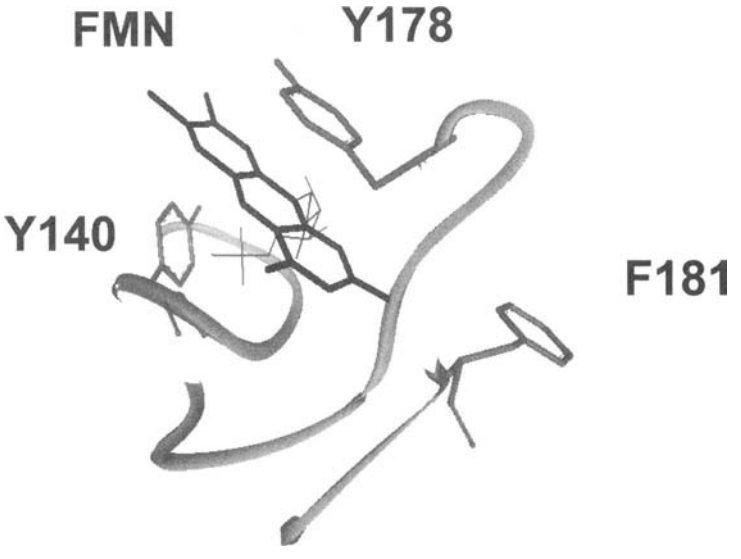

Figure 4.5. Close up of the FMN isoalloxazine ring showing the aromatic residues, Tyr140 (Y140), Tyr178 (Y178), and Phe181 (F181) associated with FMN binding in human CPR (pdb code 1B1C).

the β4–α6 loop. This loop includes Phe181, the key FMN-binding residue Tyr178, and three other residues that form hydrogen bonds to the isoalloxazine ring, as well as residues in the adjacent β5–α7 loop[66]. These are consistent with local changes in structure on substitution of Phe181, and with the idea that the packing of this conserved residue plays a significant role maintaining the orientation of residues which interact with the isoalloxazine ring and hence in the formation of the FMN-binding site.

2.5.2. FAD/NADPH-Binding Domain

Like FNR, FAD is bound in an extended conformation in rat CPR[22]. Key residues that serve to regulate flavin binding and reactivity are Arg454, Tyr456, Cys472, Gly488, Thr491, and Trp677[22]. Site-directed mutagenesis of these residues has been carried out to examine their role in FAD binding and catalysis[67]. Trp677 is stacked against the *re*-face of FAD, while Tyr456 lies at its *si*-face. Deleting Trp677 reduces catalytic activity 50-fold but has little effect on FAD content. However, mutations to Tyr456, which also hydrogen bonds to the ribityl 4′-hydroxyl, decreases affinity for FAD by around 8,000-fold. Side chains from Thr491 and Arg454 stabilize the pyrophosphate group of FAD. Substitution of Thr491 has a relatively modest effect on FAD binding (~100-fold decrease) compared with mutation of Arg454, which decreases affinity 25,000-fold.

NMR studies show that the pro-R-hydrogen attached to the C-4 atom of NADPH is transferred directly to FAD as a hydride ion[68]. However, an understanding of NADPH binding and the factors involved in hydride transfer have been hampered by the spatial disorder in this region of the molecule in the crystal structure. While the electron density of the adenosine moiety of the coenzyme is well defined in the structure, that from the nicotinamide ring is not, and is consistent with at least two different positions for this ring, neither of which is in contact with the isoalloxazine ring of FAD[22]. Similar observations have been made in enzymes of the FNR family which are structurally similar to the FAD/NADPH-binding domain of CPR[69–71]. An aromatic amino acid residue is located at the carboxyl-terminus in all members of the FNR family. In rat and human CPR, this position is occupied by Trp677 and Trp676, respectively. The planar side chain of the tryptophan stacks against the isoalloxazine ring of FAD, and structural rearrangements are thought to be essential to enable access of the nicotinamide ring to the isoalloxazine ring of FAD for hydride transfer (Figure 4.6). The proposed mechanism for binding involves the tryptophan residue flipping away from FAD to be replaced by the nicotinamide region of NADPH[22].

There has been extensive site-directed mutagenesis of amino acid residues in the vicinity of the NADPH-binding region to examine their role in cofactor binding and hydride transfer (Figure 4.7). Removal of the aromatic side chain from Trp676 shows that it plays an essential role in the discrimination between NADPH and NADH as cofactors for CPR[72, 73], notwithstanding the fact that it does not interact with the 2′-phosphate which is the sole difference between the two cofactors. Investigation of the steady-state kinetics shows that substitution of Trp676 with Ala decreases the K_M for NADH from 48 to 0.3 mM, and increases the k_{cat} to ~4,000 min^{-1}, while the K_M^{NADPH} drops to 0.2 μM and k_{cat} to 10 min^{-1}. The Trp676Ala mutant therefore binds both coenzyme molecules more tightly, the change in cofactor specificity (expressed as a k_{cat}/K_M ratio) being predominantly a reflection of changes in k_{cat}. A similar change in cofactor specificity also occurs with pea FNR with the analogous mutation Tyr308Ser[74, 75]. Both in pea FNR[75] and in CPR[60] substitution of the C-terminal aromatic residue allows the nicotinamide ring of the cofactor to bind close to the isoalloxazine ring, the thermodynamically unfavorable flipping no longer being required. Hence, removal of this physical rate-limiting step produces enzymes that have lower apparent K_M values for nicotinamide cofactors. Further kinetic evidence for a critical role for this Trp residue in regulating NADPH binding and hydride transfer is detailed in Section 2.6.1.

Several conserved amino acids including serine 596, arginine 597, and lysine 602 have been proposed to be involved in binding the adenosine-ribose moiety of NADPH (Figure 4.7), and specific charge–charge interactions between these and the 2′-phosphate of NADPH are thought to be responsible for discrimination against NADH[22, 76–78]. Working on the hypothesis that mutations to these residues might reduce the affinity of the enzyme for NADP(H) and thus prevent inhibition by $NADP^+$, Elmore and Porter[78]

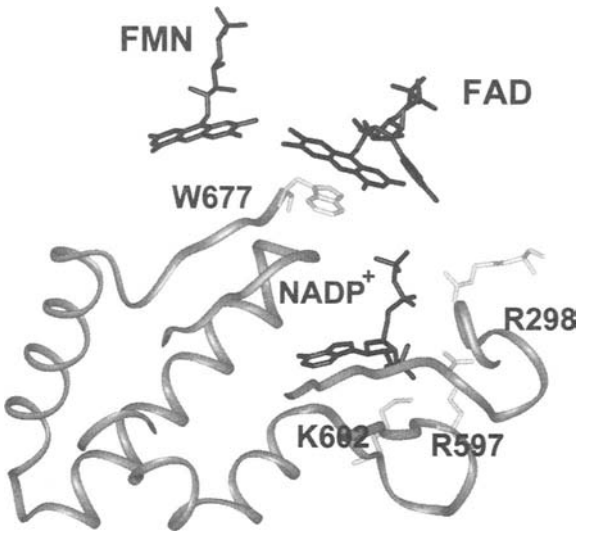

Figure 4.6. Arrangement of FMN, FAD, and $NADP^+$ in the rat CPR crystal structure (pdb code 1AMO). The Trp677 aromatic ring has to move away from the FAD isoalloxazine ring to allow access of nicotinamide ring to FAD for hydride transfer.

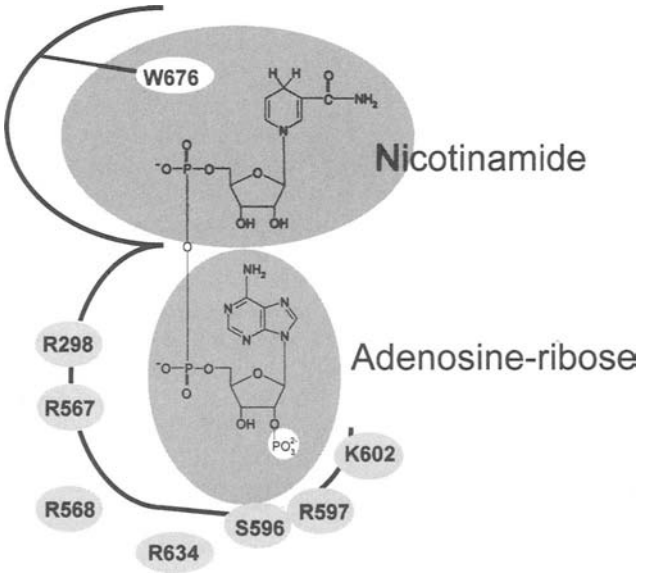

Figure 4.7. Schematic diagram of residues associated with NADPH binding in human CPR (taken from Dohr[72]).

have coupled mutations to residues in the 2′-phosphate-binding site with the W677 substitution. The mutant combinations R597M/W677A and R597M/K602W/W677 A showed a significant 4-fold reduction in inhibition by $NADP^+$ relative to W677A, while their catalytic efficiency with NADH was increased at least 500-fold.

Three residues Ser457, Asp675, and Cys630, lie in close proximity to the FAD isoalloxazine ring in CPR, and have been closely examined

in relation to hydride transfer. Non-conservative substitutions of Ser457, Asp675, and Cys630 produce large decreases in cytochrome *c* reductase activity[60, 76, 79, 80]. These parallel changes in the rate of flavin reduction are associated with large NADPD isotope effects[81], thus pointing to impaired hydride transfer from NADPH. Based on spectral analysis of flavin reduction of mutants at residues Ser457, Asp675, and Cys630 and analysis of the kinetics and pH dependence of cytochrome *c* reduction, it was proposed that Cys630 acts a proton donor/acceptor to FAD, and Ser457 and Asp675 interact to stabilize both the transition state and the FAD semiquinone[80]. This is supported by crystallographic analysis of CPR mutants, which shows that both Ser457 and Asp675 are directly involved in interaction with the nicotinamide group of NADP(H) and likely to orient the C-4 atom of NADP(H) into an optimal position for hydride transfer[60]. The catalytic triad of Ser457, Asp675, and Cys630 is highly conserved in all diflavin oxidoreductases apart from NR1, which has a nonconservative Ala549 corresponding to Cys630[25] and a greatly decreased (~100-fold) rate of reduction relative to CPR.

2.6. The Electron Transfer Mechanism

CPR catalyzes the transfer of electrons along the pathway NADPH → FAD → FMN → P450, and there are two striking features of its involvement in the monooxygenase system. First, the flavin redox potentials are such that electron transfer from NADPH to FAD is moderately unfavorable (the physiological direction of electron transfer in the structurally related ferredoxin reductase is in the direction of NADPH formation). Second, NADPH is an obligate two-electron donor, but these two electrons must be delivered to P450s individually at the appropriate points in the catalytic cycle. CPR orchestrates the electron supply from NADPH to the P450 cytochromes by stabilizing the one-electron reduced form of the flavin cofactors FAD and FMN. This stabilization gives rise to the blue semiquinone species ($FADH^{\bullet}$/$FMNH^{\bullet}$), which is observed in both kinetic and equilibrium studies of CPR[16, 82–84]. CPR and the aforementioned related structural isoforms of nitric oxide synthase[85], methionine synthase reductase[24], and protein NR1[25] can accept a maximum of four electrons. The different redox states of these enzymes have distinct spectral characteristics. However, the fact that the oxidised, semiquinone and reduced states of both FAD and FMN have virtually indistinguishable spectra means that kinetic and equilibrium studies of the diflavin reductases is complicated. This is alleviated, however, by using the genetic approaches discussed previously to dissect the enzymes into their component domains[29, 33, 34, 86]. This strategy has been used successfully with human CPR, human methionine synthase reductase, and human oxidoreductase NR1. Molecular dissection of these diflavin enzymes removes ambiguity in spectral assignment during redox titration, potentiometry, and kinetic analysis[33, 34, 87–90]. With CPR, the domains have also formed a basis for more detailed study of the kinetic and thermodynamic properties of the full-length enzymes[73, 87, 88]. The use of FMN-depleted full-length enzyme has also facilitated assignment of reduction potentials and individual kinetic phases in other mammalian CPRs[82].

Each isolated flavin-binding domain of human CPR (and of related family members) is soluble, binds flavin, and is redox active[29, 87]. The midpoint reduction potentials of the flavin couples are similar in the isolated domains and full-length CPR[88], indicating that the isolated domains are good mimics of domain properties in full-length CPR. The blue semiquinone on the FMN is both kinetically and thermodynamically stabilized, and the reduction potential of the oxidized-semiquinone redox couple is much more positive than those of the other redox couples in CPR (Figure 4.8). This accounts for the "blue/green" appearance of purified CPR prior to chemical treatment with exogenous oxidants. The midpoint reduction potentials measured for human CPR[88] are in broad agreement with earlier studies performed with rabbit CPR[17]. The reductive half-reaction of the isolated FAD domain has been studied by stopped-flow methods[87]. Hydride transfer is reversible, and reduction of $NADP^+$ by $FADH_2$ is more rapid (8 s^{-1}) than FAD reduction by NADPH (3 s^{-1}), consistent with the relative values of the midpoint reduction potentials for the 2-electron couples for $FAD/FADH_2$ and $NADPH/NADP^+$. The more rapid reduction of $NADP^+$ probably reflects the physiological role of the ancestral FNR-like protein, which evolved to catalyze $NADP^+$ reduction in photosynthetic electron transfer. The isolated FAD domain transfers electrons to the isolated FMN domain, but the

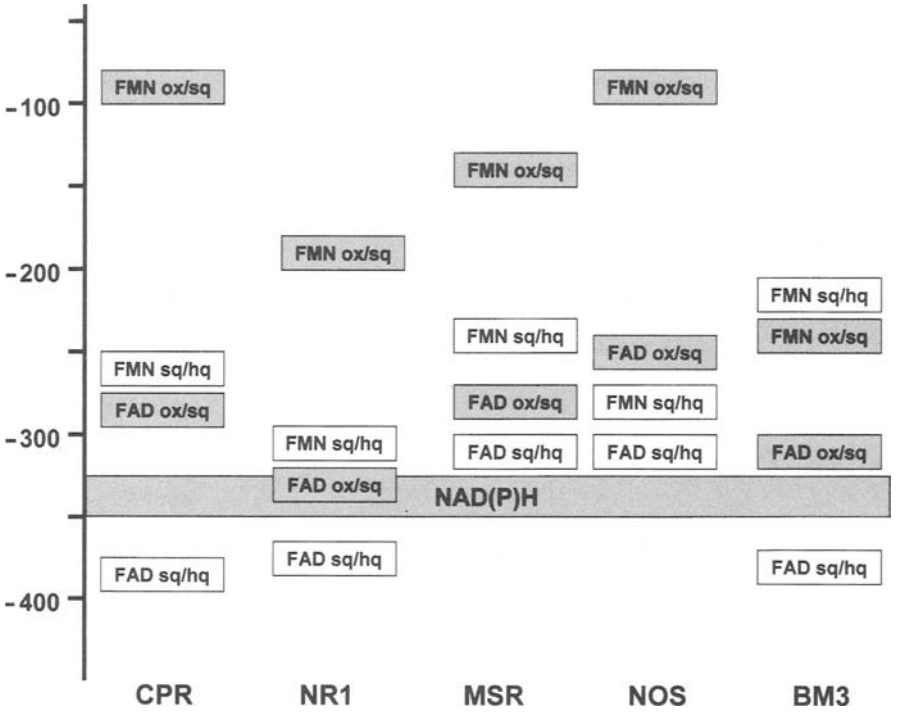

Figure 4.8. Midpoint reduction potentials of the various flavin couples in CPR and related diflavin enzymes. Values are taken from Munro[88] (CPR), Finn[33] (NR1), Wolthers[34] (MSR), Noble[91] (NOS), and Daff[89] (BM3).

observed rate is slow and shows a second-order dependence (second-order rate constant 9.5×10^4 $M^{-1}\,s^{-1}$) on domain concentration, reflecting a bimolecular reaction. This indicates the importance of covalently tethering the two flavin-binding domains of CPR to enable rapid interdomain electron transfer.

Reduction of the isolated FAD domain by NADPH occurs in a series of discrete kinetic steps including at least two different charge-transfer complexes involving NADPH and FAD (Figure 4.9). Partial flavin reduction occurs in the initial phase ($\sim 200\ s^{-1}$) and this displays a kinetic isotope effect with *A*-side NADPD. FAD is then further reduced in the slower phase ($\sim 20\ s^{-1}$). $NADP^+$ release is thought to gate complete reduction of the flavin in the slower phase, consistent with a reversible scheme for flavin reduction in the isolated FAD domain and full-length CPR (Figure 4.9). Similarly, it is proposed that the rate of flavin reduction is regulated by $NADP^+$ release in rat neuronal nitric oxide synthase[61, 92] and the adrenodoxin reductase homolog FprA from *Mycobacterium tuberculosis*[93]. A number of the early kinetic phases are obscured in studies with full-length CPR[87], thus emphasizing the importance of conducting detailed studies of electron transfer mechanism in single domains of CPR.

The relatively high midpoint reduction potential of the FMN oxidized-semiquinone couple (−66 mV) provides the driving force for interdomain electron transfer in full-length CPR[88]. This enables electron flow from NADPH to the CPR FMN. Electron transfer then occurs from the FMN to the cytochrome P450 enzymes (or other redox acceptors) and is a key aspect of the electron transfer mechanism in virtually all diflavin reductase enzymes[33, 34, 94]. Kinetic studies show that the transient accumulation of the blue disemiquinoid species of CPR occurs at a rate identical to hydride transfer from NADPH to FAD[87]. This indicates that interdomain electron transfer is relatively fast. The blue disemiquinoid species subsequently decays following a second hydride transfer from NADPH (Figure 4.9), as the flavins are reduced to their hydroquinone states.

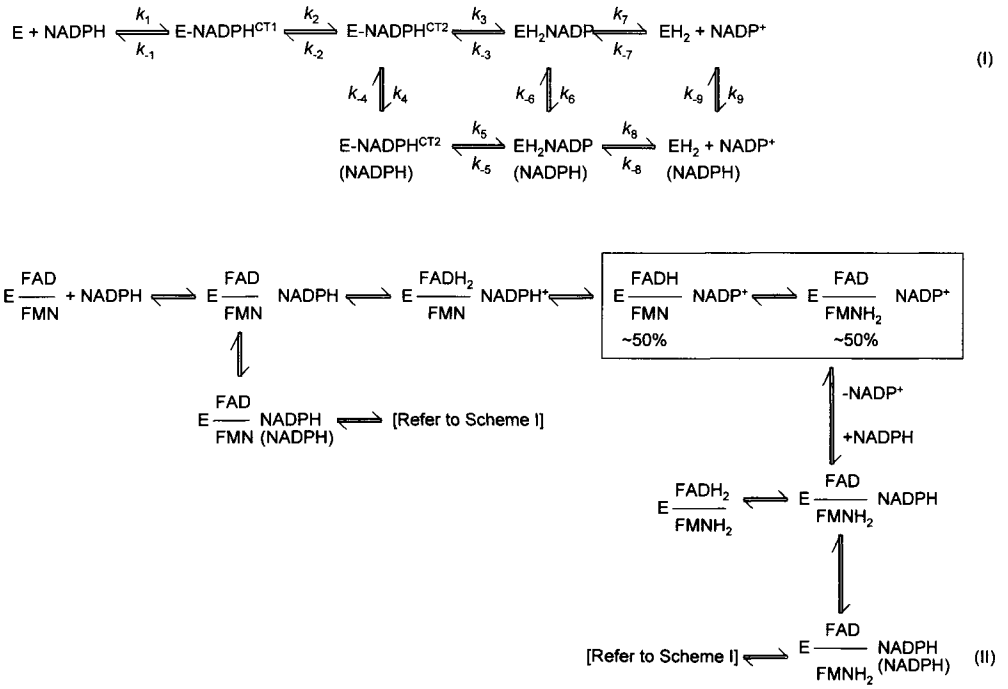

Figure 4.9. Kinetic schemes for the isolated FAD domain (upper scheme) and full-length CPR (lower scheme) derived from stopped-flow studies (taken from Gutierrez[87]). The reversible nature of the reaction is indicated, as is the presence of the second nicotinamide coenzyme-binding site (indicated in parenthesis for the FAD domain). For clarity, only enzyme intermediates bound with a single coenzyme are shown in the lower scheme for CPR, but reference to the upper scheme indicates that a second molecule of NADPH has the capacity to bind to the enzyme.

2.6.1. Trp676 and FAD Reduction

As discussed above, the C-terminal aromatic residue (Trp676 in human CPR) is positioned over the *re*-face of the FAD isoalloxazine ring in such a way that it would sterically prevent hydride transfer from NADPH to FAD in the absence of side chain movement (Figure 4.6). In human CPR, rapid changes in tryptophan fluorescence emission accompany hydride transfer in the isolated FAD-binding domain and in CPR[87]. The complex fluorescence transients observed in CPR as two hydride equivalents that are transferred to the enzyme are largely absent in a W676H mutant CPR, suggesting that Trp676 is the origin of the fluorescence signal in wild-type enzyme[73] and that the observed transients may reflect the postulated movements of this side chain. Substitution of Trp676 by alanine substantially compromises the rate of hydride transfer in CPR, revealing a key role for the side chain of Trp676 in enzyme reduction. In Trp676His CPR the rate of FAD reduction is modestly affected, but importantly the enzyme is reduced only to the two-electron level in rapid-mixing experiments. Reduction beyond the two-electron level is prevented owing to the slow release of $NADP^+$, indicating a role for Trp676 in the release of oxidized coenzyme from the FAD-binding domain. A double-mixing stopped-flow method in which Trp676His CPR is reduced initially with stoichiometric NADPH, and following a suitable delay (100 ms) mixed with excess NADPH, effects reduction to the four-electron level[73]. In the delay period, $NADP^+$ can escape, albeit at a relatively slow rate, allowing binding of the second NADPH to the catalytic site.

2.6.2. Binding of Two Coenzyme Molecules

Rapid-mixing studies of the kinetics of hydride transfer in both the isolated FAD-binding domain and in CPR have led to a kinetic model (Figure 4.9) that invokes the existence of two kinetically distinct binding sites for NADPH[87]. Only one site is catalytic; binding to the second site attenuates FAD reduction, probably by interfering with $NADP^+$ release from the catalytic site. In wild-type CPR, $NADP^+$ release from the catalytic site is sufficiently rapid that binding to the second noncatalytic site does not prevent reduction to the four-electron level in direct-mixing stopped-flow experiments with excess NADPH. Following the identification of two kinetically distinct coenzyme binding sites in CPR and the unusual mechanism for electron transfer to FAD, similar dual binding-site models have been proposed for nitric oxide synthase[92] and the adrenodoxin reductase homolog FprA isolated from *M. tuberculosis*[93].

The presence of two coenzyme-binding sites is unexpected since they cannot be inferred solely from the crystal structure of CPR[22]. Kinetic studies with wild type and W676H CPR at different concentrations of NADPH have, however, provided further support for the existence of two sites[73, 87]. The rate of flavin reduction in the isolated FAD domain and CPR increases as NADPH is decreased from molar excess to stoichiometric concentrations. At stoichiometric concentration, the second noncatalytic site is predominantly vacant and the partial inhibition on the rate of flavin reduction from the catalytic site is therefore relieved (Figure 4.9). Occupation of the noncatalytic site occurs at NADPH concentrations in excess of the enzyme concentration, and impairs $NADP^+$ release from the catalytic site. This in turn partially inhibits flavin reduction, the rate of which is gated by $NADP^+$ release. Preincubation of the enzyme with a stoichiometric amount of adenosine 2′,5′-diphosphate does not lead to inhibition of the flavin reduction rate[95]. We infer that the binding of adenosine 2′,5′-diphosphate prevents NADPH from binding to the noncatalytic site. This observation also suggests that it is the nicotinamide-ribose-phosphate portion of NADPH bound at the second site that hinders $NADP^+$ release from the catalytic site. Clearly, these new data indicate that the coenzyme plays a role in the control of CPR catalytic activity as well as acting as a substrate. Models that assume a single coenzyme-binding site for steady-state turnover to explain competitive inhibition by coenzyme fragments might be inappropriate. The effect of the second coenzyme-binding site on steady-state turn-over and inhibition might lead to revised, and by necessity more complex, steady-state kinetic models.

2.6.3. Internal Electron Transfer

Although a thermodynamic and kinetic framework for electron transfer in CPR has emerged from rapid-mixing kinetic and potentiometry studies[87, 88], alternative approaches have been required to access the rate of internal electron transfer between the flavins. Tollin and coworkers have used flash photolysis studies employing photoexcited 5-deazariboflavin to investigate the kinetics of inter-flavin electron transfer in rabbit CPR[96]. CPR is reduced rapidly by photoexcited 5-deazariboflavin ($6.8 \times 10^7\ M^{-1}\ s^{-1}$) and this phase is followed by a slower ($70\ s^{-1}$) partial loss of blue semiquinone signal that reports on inter-flavin electron transfer from FAD_{sq} to FMN_{ox}. Pre-reduction of rabbit CPR prior to flash photolysis yields a rate constant for internal electron transfer of $15\ s^{-1}$. This slower rate for pre-reduced enzyme (FAD_{ox}/FMN_{sq}) is consistent with the smaller driving force for electron transfer from FAD_{sq} to FMN_{sq} following electron donation by 5-deazariboflavin. Studies with human CPR have used temperature-jump relaxation kinetic methods to follow inter-flavin electron transfer[95, 97]. As with flash photolysis, relaxation kinetic methods bypass preceding steps that might limit the rate of inter-flavin electron transfer in rapid-mixing approaches (e.g., the preceding FAD reduction step). Application of relaxation kinetic methods to CPR has allowed direct measurement of the rate of inter-flavin electron transfer in enzyme reduced at the two- and three-electron level, and also opened up studies of conformational gating and ligand effects on this reaction[97]. A key requirement of the temperature-jump approach is to poise the equilibrium of a reaction, such that perturbation of the equilibrium by rapid heating, effects an absorption change that can be assigned to an

individual reaction step (i.e., internal electron transfer from FAD to FMN in the case of CPR). This is especially difficult with CPR where slow thermodynamic relaxation would lead to an equilibrium mixture of several different redox forms of the enzyme. The experimental conditions were selected carefully to minimize any uncertainty arising from the identity of the enzyme species in the final equilibrium distribution. Potentiometry studies indicate that in two-electron-reduced enzyme the electrons are distributed as an equilibrium between the two enzyme species FAD_{sq}/FMN_{sq} and FAD_{ox}/FMN_{hq}, and that ~50% of each enzyme species is populated[88]. A rapid temperature increase shifts this equilibrium toward the FAD_{ox}/$FMNH_2$ form, and the kinetics of this process report on the rate of inter-flavin electron transfer[97]. Inter-flavin electron transfer in human CPR is relatively slow. For CPR reduced at the two-electron level with NADPH, two kinetic phases are seen in temperature-jump experiments. Fluorescence and absorbance studies with dithionite-reduced CPR indicate that the fast phase ($1/\tau = 2{,}200 \pm 300\ s^{-1}$) is not associated with electron transfer, but is attributed to local conformational change in the vicinity of the FAD when CPR is reduced with NADPH. The slow phase ($1/\tau = 55 \pm 2\ s^{-1}$) reports an internal electron transfer, FAD_{sq}–$FMN_{sq} \rightarrow FAD_{ox}$–$FMN_{hq}$. An intrinsic electron transfer rate of $\sim 10^{10}\ s^{-1}$ is predicted based on the separation distance of the two flavins in rat CPR[22, 98]. The modest transfer rates observed in the temperature-jump studies indicate that electron transfer is gated. Possible origins of the gating reaction have been considered. For example, the reaction involves the deprotonation of the FAD blue semiquinone, but solvent isotope effects do not accompany the reaction, ruling out a rate-limiting deprotonation reaction. Electron transfer is compromised in the presence of glycerol (75% w/v glycerol), suggesting conformational gating[97] and a conformational search for domain orientations that maximize electronic coupling between the flavins is consistent with the atomic structure of CPR (and related diflavin enzymes), which are consistent with a high degree of interdomain mobility[22, 60, 71]. The equilibrium distribution of enzyme species is poised differently by reduction of CPR at the three-electron level[97]. Relaxation experiments with three-electron reduced CPR give access to the kinetics ($1/\tau = 20 \pm 0.2\ s^{-1}$) of electron transfer in the nonphysiological direction ($FAD_{sq}/FMN_{hq} \rightarrow FAD_{hq}/FMN_{sq}$).

In the W676H mutant CPR, this rate is substantially increased ($1/\tau = 263 \pm 3\ s^{-1}$), but the rate of electron transfer in the physiological direction ($FAD_{sq}FMN_{sq} \rightarrow FAD_{ox}FMN_{hq}$) is decreased by only a factor of ~2 ($1/\tau = 27 \pm 1\ s^{-1}$). This therefore points to another important role for Trp676 in controlling electron transfer in CPR in favoring the transfer of electrons in the physiological direction (NADPH → FAD → FMN → heme).

Although the initial equilibrium is the same, inter-flavin electron transfer rates for CPR reduced by NADH ($1/\tau = 18 \pm 0.7\ s^{-1}$) are ~3-fold less than those for CPR reduced by NADPH ($1/\tau = 55 \pm 0.5\ s^{-1}$). This cannot be attributed to thermodynamic differences since the reduction potentials of the reducing coenzymes are essentially identical. This suggests a role for coenzyme binding in modulating inter-flavin electron transfer[95, 97]. This is supported by the observation that the rate of inter-flavin electron transfer in dithionite-reduced CPR ($1/\tau = 11 \pm 0.5\ s^{-1}$) is clearly less than that for CPR reduced with NADPH ($1/\tau = 55 \pm 0.5\ s^{-1}$)[95]. Furthermore, addition of 2′,5′-ADP to dithionite-reduced enzyme effects a ~3-fold increase in the rate of inter-flavin electron transfer ($1/\tau = 35 \pm 0.2\ s^{-1}$), and this is accompanied by an increase in the amplitude of the absorption signal change[95]. Thus, the binding of the adenosine moiety of NADPH, and in particular the 2′-phosphate group, is a major factor in enhancing the rate of inter-flavin electron transfer. Potentiometry measurements have shown that the binding of 2′,5′-ADP does not perturb the midpoint reduction potentials of the four flavin couples. We propose that ligand binding in the coenzyme-binding pocket effects a conformational change involving domain movement, the rate of which controls inter-flavin electron transfer.

2.6.4. Interaction with and Electron Transfer to P450

The microsomal P450 monooxygenase complex is localized to the endoplasmic reticulum membrane. Like P450, CPR contains an N-terminal hydrophobic region that spans the lipid membrane and anchors it to the surface. In yeast,

the soluble form of the enzyme can support P450 activity[49], but soluble mammalian CPRs that have had the hydrophobic anchoring peptide removed are incapable of coupling with cytochrome P450. The anchoring of these enzymes to the membrane surface thus appears to be the principal factor required for correct spatial orientation of the redox centers for effective electron transfer.

Studying the architecture and topology of membrane proteins on the phospholipid bilayer is experimentally extremely challenging. However, new developments are being applied to analyze the topography of the monooxygenase complex on a phospholipid bilayer. Sligar and coworkers have used 10-nm scale phospholipid bilayer disk structures to orient CPR and cytochrome P450 2B4 on a surface for visualization by AFM[99, 100]. The height of CYP2B4 protruding above the surface is estimated at 3.5 nm, which is consistent with a hydrophobic tip of the molecule being partially inserted into the membrane. The exact spatial relationship with CPR is not yet clear, but it is thought that CPR lies in an orientation such that both the FMN and FAD/NADPH domains lie close to the membrane surface, which would allow close communication between the FMN and the P450 heme[22, 100].

Transient monooxygenase complexes are formed on the membrane surface as a result of collisions between P450s and CPR as each diffuses laterally within the membrane of the endoplasmic reticulum. The role of the membrane in mediating these interactions is poorly understood. However, early work has shown that the phospholipid component of the membrane can affect intermolecular interactions of the monooxygenase complex[101, 102] and influence substrate binding[13, 103] and may therefore be important in maintaining efficient electron transfer from CPR to P450.

The structure and sequence of rat and human CPR[22, 63] show some interesting features that might influence the orientation of the protein at the membrane surface. One is a cluster of basic residues $R^{45}KKK$, which lies at the membrane anchor: FMN domain junction. Their functional significance is unclear, but it can be speculated that the basic Arg and Lys side groups might interact with anionic phospholipid head groups, possibly to restrict the movement of CPR on the membrane surface. The FMN-binding domain has clusters of positive and negative charges at opposite ends, leading to a strong electric dipole moment (677 Debye)[63], which may influence the orientation of the reductase at the membrane surface. The patch of positively charged residues exposed at the surface of the human FMN-binding domain (K72, K74, K75, R78, R97, K100, H103, and R108) in particular could form an additional membrane-binding site.

Protein–protein interactions are essential to enable electron transfer from the reduced FMN of the flavoprotein to the substrate-bound ferric form of the P450. Since P450 is present in a 10–25-fold molar excess over CPR in the liver microsome[104, 105], rapid association and dissociation of P450 : CPR complexes is important for the system to work effectively. A number of lines of evidence point to electrostatic interactions being the driving force behind the binding of P450 with CPR. For instance, it has been demonstrated that specific positively charged lysine and arginine residues on the rat P450, CYP1A1, are involved in forming an electron transfer complex with CPR[106]. It has also been reported that CYP2B1 and CYP2B4 interact with CPR through complementary charge interactions[107, 108]. In the case of CYP2B4, site-directed mutagenesis has identified a series of lysine and arginine residues on the proximal surface near the heme ligand that interact with CPR[108]. Neutralization of carboxylate groups on CPR by chemical modification inhibits both cytochrome *c* reductase activity and P450-dependent monooxygenation[42, 109]. Chemical cross-linking studies indicated that a cluster of acidic amino acids on CPR were involved in the interaction with rat CYP1A1[110].

The FMN domain may be assumed to provide a major portion of the docking surface for P450s, although as discussed above some reorientation of this domain from its position in the crystal structure may be required. Electrostatic potential measurement of the surface of the human CPR–FMN-binding domain shows three distinct clusters of acidic residues that could form ion-pair interactions with the electron transfer partners[63, 111]. Cluster 1 contains Asp207, Asp208, Asp209; cluster 2, Glu213, Glu214, Asp215; and cluster 3, Glu142, Asp144, Asp147. The first two clusters correspond to the region of CPR that was cross-linked to a lysine residue in cytochrome *c*[112], and have been investigated by site-directed mutagenesis in rat[111] and human[63] CPR. The results of these experiments

show that while cluster 1 mutations do not affect cytochrome *c* binding, removing the charge from Asp208 alters the binding of cytochrome P450. In contrast, cluster 2 mutations affected the binding of cytochrome *c*, but not P450. Mutations of residues in cluster 3 produce a modest decrease in P450 activity with no effect on cytochrome *c* reductase activity[63]. Thus, discrimination is apparent between residues involved with the interaction of CPR with cytochrome *c* and with P450.

The finding that mutation of residues in clusters 1 and 3 on either side of the FMN-binding site affect P450 activity, suggests that cytochromes P450 bind at the tip of the FMN domain in such a way as to cover the FMN cofactor[63]. Examination of the crystal structure of rat CPR indicates that structural rearrangements during catalysis of the kind discussed above would be required to bring the redox centers of CPR and P450 into an appropriate configuration for efficient electron transfer[63] (Figure 4.10). A similar structural rearrangement has also been suggested for the FMN and FAD domains of BM3[113].

While it is generally considered that charge-pair interactions are important in docking and electron transfer reactions, this is not always reflected in the ionic-strength dependence of P450 reduction *in vitro* with reconstituted monooxygenase systems. For instance, it has been shown that in the presence of high concentrations of salt, which should neutralize electrostatic interactions, the reduction rate of P450 by CPR may increase[114–116]. It is possible that the observed effects of ionic strength reflect the combined result of perturbations of CPR–P450 interactions and of domain–domain interactions within CPR.

Most recently Davydov *et al.*[117] have compared the role of electrostatic interactions in the association of rabbit CPR with CYP2B4 and the heme domain of P450 BM3, which is normally fused with a reductase domain[117]. They found an increase in K_d (10–90 nM) for the CPR–CYP2B4 complex with increased ionic strength (50–500 μM), consistent with the involvement of charge pairing. Interestingly a reverse relationship was observed with BM3-heme and BM3–CPR domains, which showed low affinity for each other. It is postulated that this may reflect the fact that P450 BM3 is a tethered complex, where there is no strong requirement for charge pairing.

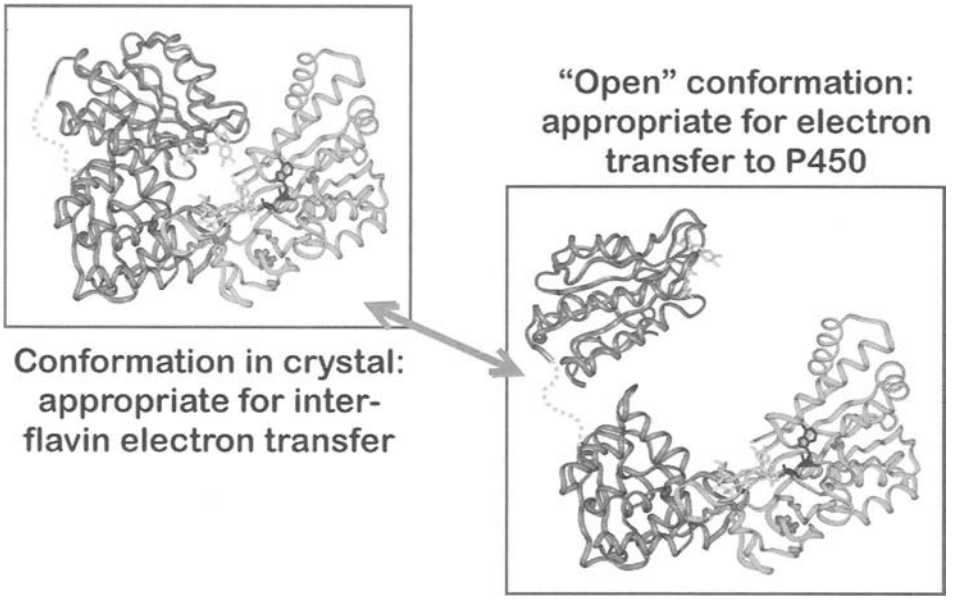

Figure 4.10. Schematic model for the domain movement required for opening CPR for efficient electron transfer to P450.

2.7. Cytochrome P450 BM3

Since its discovery in the 1980s, flavocytochrome P450 BM3 (CYP102A1) has undergone intensive biochemical study and has established itself as a model P450 system to rival P450cam. The key aspect of the structure of P450 BM3 that has led to this rise to prominence is the fact that it was the first prokaryotic P450 enzyme recognized to interact with a eukaryotic-like P450 reductase redox partner (i.e., a FAD- and FMN-containing NADPH-cytochrome P450 reductase) rather than with an iron–sulfur ferredoxin and FAD-containing ferredoxin reductase, long considered to represent the "typical" bacterial P450 reductase system[118]. Thus P450 BM3, a soluble P450, offered great promise as a tool for understanding redox partner interactions and electron transfer in an experimentally more tractable system than was the case for the membrane-bound eukaryotic P450s and P450 reductase[2]. An even more attractive feature of P450 BM3 was that it was a natural fusion protein—with the reductase linked to the P450 in a single 119 kDa polypeptide. Structural, spectroscopic, and enzymological studies of P450 BM3 in the two decades since its discovery have been extremely informative as regards P450 structure and mechanism, establishing P450 BM3 as a vitally important "model" system for the P450 superfamily[2].

P450 BM3 was discovered as a result of studies by Armand Fulco's group on fatty acid hydroxylase systems from the soil bacterium *B. megaterium*[119]. As implied by the name, BM3 was the third enzyme with fatty acid hydroxylase activity identified in the bacterium. P450 BM3 became a major focus of Fulco's studies following the finding that the gene was strongly induced by addition of phenobarbital to the growth medium[27], suggesting that the gene was under transcriptional control akin to the types of regulation seen previously for mammalian drug-metabolizing P450 isoforms[120]. Subsequent studies have shown the importance of a repressor protein (BM3R1) in the control of CYP102A1 expression, with barbiturate drugs and a range of other hydrophobic molecules being able to displace the repressor to promote production of P450 BM3[121, 122]. Fatty acids are likely to perform the same role *in vivo*, and have also been shown to facilitate expression of a homolog of P450 BM3 in *Bacillus subtilis*. The overproduction of P450 BM3 facilitated by addition of phenobarbital to *B. megaterium* growth medium simplified purification of the enzyme, and cloning of the gene allowed its expression and purification from *E. coli*[123]. It quickly became apparent from analysis of both gene and protein that P450 BM3 was an unusual fusion protein comprised of a fatty acid-binding P450 (homologous to the eukaryotic CYP4 fatty acid hydroxylase family) fused to a CPR[124]. Analysis of the catalytic properties of the purified enzyme showed it to have a very high catalytic center activity relative to its eukaryotic fatty acid hydroxylase relatives—with turnover numbers of several thousand per minute with various long-chain fatty acids[86, 125]. This was rationalized as being due to the efficiency of the electron transfer system covalently associated with the P450, and opened the door to mechanistic and kinetic studies on the enzyme[90, 125, 126]. In the BM3 system, NADPH reduces the FAD flavin transiently to the hydroquinone form. Electron transfer to the heme is mediated by the FMN cofactor, which shuttles electrons one at a time between the FAD and heme[2].

Genetic dissection provided further proof for the multidomain nature of the enzyme-producing first sub-genes encoding stable P450 (first ~472 amino acids of P450 BM3) and CPR domain[86, 127], and then constructs encoding the individual FAD/NADPH-binding and FMN-binding domains of the CPR[128]. Domain dissection also enabled the determination of the atomic structures of the heme (P450) domain of the enzyme and also the structure of a nonstoichiometric complex of the heme and FMN domains of the enzyme, arising from proteolysis of a construct containing the heme domain and the FMN-binding domain[113].

The atomic structure of the heme domain has been solved in substrate-bound and substrate-free forms, showing that a large structural change in the protein accompanies the binding of substrate (palmitoleic acid). Binding of fatty acid substrates to the heme domain causes a shift in the heme iron spin-state equilibrium toward the high-spin form, which is accompanied by a large increase in reduction potential for the ferric/ferrous transition of the heme iron[86, 89, 127]. Binding of fatty acid thus facilitates electron transfer to the heme iron and triggers catalysis. In the absence of bound substrate there is negligible electron transfer

between the FMN and heme cofactors. When P450 BM3 binds its most efficient substrates, that is those fatty acids, such as arachidonic acid, that induce the greatest degrees of spin-state transition and promote the fastest turnover[129, 130], FMN-to-heme electron transfer is rapid (*c.* 250 s^{-1} with lauric acid) and catalysis occurs[90]. The extent of the increase in reduction potential accompanying saturation with fatty acid is approximately 130 mV, a change which elevates the heme reduction potential above that of the ultimate reductant in the system (NADPH: $E^0 = -320$ mV cf. -427 for BM3 heme in absence of substrate and -289 in presence of arachidonate) and within range of that of the FMN cofactor ($E^0 = -203$ mV for its oxidized/hydroquinone couple)[89, 131].

2.7.1. Electron Transfer Properties of BM3 Reductase

Interestingly, the BM3 reductase is the only member of the diflavin reductase class of enzymes which does not stabilize a neutral, blue semiquinone on its FMN. Formation of a blue semiquinone during reductive titration is a feature typical of flavodoxins, and retained in other members of the diflavin reductase family (e.g., nitric oxide synthase, methionine synthase reductase, and other eukaryotic CPRs). The fact that the semiquinone is destabilized with respect to the hydroquinone in BM3's CPR and FMN domains has been rationalized on the basis of the structure of the FMN domain. The presence of basic residues (particularly Lys572 and Lys580) may stabilize the anionic hydroquinone form and destabilize the semiquinone[113]. The redox state of the flavins is of great importance to the catalytic properties of BM3, since early studies on the enzyme showed that there was strong inhibition of fatty acid hydroxylase activity following preincubation of the enzyme with NADPH[27]. It appears that an unfavorable three-electron (and possibly four-electron) reduced form of the enzyme accumulates within a few minutes of incubation of the enzyme with NADPH, and that electron transfer in this species is diminished and results in a catalytic rate of fatty acid hydroxylation less than 20% of the maximal rate. Analysis of the redox state of the flavins during turnover suggests that both may exist predominantly in a semiquinone state as catalysis progresses (a neutral semiquinone in the case of the FAD and a red, anionic semiquinone in the case of the FMN), suggesting that the CPR domain of the enzyme cycles favorably between 0–2–1–0 electron-reduced states during catalysis[132]. The situation appears similar for the housefly CPR, but contrasts with that reported for many eukaryotic CPRs, where the cycle appears to be 1–3–2–1[133]. However, the precise reason for the apparent inactivation of BM3 in an "over-reduced" form remains obscure. Since electron transfer to exogenous electron acceptors via the flavins remains largely unaffected[123], it appears to be the case that the catalytic step perturbed is that of first or second electron transfer to the heme iron. This may indicate that the reduction state and/or coenzyme-binding affects the conformational status of the reductase domain, in particular the movement of the FMN-binding domain relative to the FAD-binding domain discussed above.

Stopped-flow absorption spectroscopy has provided information on some of the internal electron transfer processes in P450 BM3[90]. In particular, the rate of electron transfer between NADPH and the FAD (in itself a composite of rates for NADPH association, orientation and the actual hydride transfer event) is very fast (500 s^{-1} at 5°C) relative to those measured for mammalian CPRs, nitric oxide synthase, methionine synthase reductase, and novel reductase 1 (NR1)[33, 34, 95]. The rate of internal (inter-flavin) electron transfer remains to be determined, but should be greater than 250 s^{-1} (the turnover rate with arachidonate). The rapid electron transfer from NADPH to flavin is diminished considerably for the NADH-dependent reaction, indicating the importance of the 2′ phosphate group of NADPH in both recognition and binding of the pyridine nucleotide, and in the actual electron transfer event. Comparison of the thermodynamic properties of the BM3 reductase with those of the other members of the diflavin reductase family (Figure 4.8) indicates that increased driving force does not underlie the enhanced rate of NADPH-to-FAD electron transfer observed for this isoform[89]. For instance, in human CPR there is a more favorable thermodynamic balance for electron transfer between NADPH ($E^{0\prime} = -320$ mV) and the FAD cofactor ($E^0 = -328$ mV for the two-electron reduction to the hydroquinone form) and an apparent rate of

electron transfer of ~3 s^{-1} at 25°C with excess NADPH[87, 88]. For BM3, the midpoint reduction potential for the FAD cofactor is virtually identical to that for human CPR (−332 mV), and yet the rate of electron transfer is apparently ~200–300-fold that of human CPR under similar conditions. Obviously, structural differences in BM3 reductase underlie its capacity to drive electron transfer so efficiently with respect to other diflavin reductases. However, these differences await clarification through structural elucidation of the BM3 reductase and/or its FAD/NADPH domain. In view of the proximity of the redox couples of NADPH and the FAD flavin in BM3 (and other diflavin reductases), reversibility of the electron transfer process (i.e., hydride transfer from FAD hydroquinone to $NAD(P)^+$) might be expected. This has been shown to occur for both mammalian CPR and for BM3 reductase[87, 88, 134].

Many aspects of the electron transfer reactions in P450 BM3 are now reasonably well understood, and the first mutagenesis experiments on the enzyme ruled out the involvement of a tryptophan residue adjacent to the heme in the flavin-to-heme electron transport process[134]. However, even after two decades of study of this enzyme, several fundamental issues remain unresolved. Key among these are the reasons why the electron transfer reactions in the reductase domain of the enzyme (and between FMN and heme) are so efficient by comparison with the eukaryotic P450 and CPR systems. Thus, further detailed rapid kinetic and structural studies are critical to gain a complete understanding of this efficient electron transfer system.

2.8. Artificial CPR–P450 Fusion Constructs

Many attempts have been made to increase the catalytic efficiency of mammalian P450s to the level of BM3 through artificial fusions with CPR. The first such artificial fusion between rat P450 CYP1A1 and rat CPR was constructed by Murakami *et al.*[136]. Since then, several fusions with rat CPR have been constructed including bovine CYP17α (ref. [137]), human CYP3A4 (ref. [138]), human CYP1A1 (ref. [139]), CYP4A1 (ref. [140]), and rat CYP2C11 (ref. [141]). In addition, yeast CPR has been fused to rat CYP1A1 (ref. [142]), and human CYP3A4 (ref. [143]). Most recently, a fusion comprising entirely human genes was constructed between CYP2D6 and human CPR[144].

As yet none of these artificial systems approach P450 BM3 in terms of its high catalytic activity. Indeed, most artificial mammalian fusions achieve no greater turnover levels than their physiological counterparts in which the reductase and P450 form noncovalent complexes. This may indicate that the exceptional catalytic rate exhibited by P450 BM3 arises from the details of its mechanism rather than simply from the fact that the reductase and P450 are covalently linked. Nevertheless, such catalytically self-sufficient P450–CPR fusion enzymes are extremely useful for probing the biochemical mechanisms involved in catalysis and electron transfer between P450 and CPR. They also have important practical applications in the development of efficient P450-based biocatalysts and high throughput screening systems.

3. Electron Transfer to P450s from Cytochrome b_5

Cytochrome b_5 was originally discovered in silkworm larvae[145] and has since been most extensively studied in mammals[1, 146]. Like cytochromes P450 and CPR, cytochrome b_5 is an integral membrane protein located on the cytosolic side of the endoplasmic reticulum where it is principally involved in lipid biosynthesis. It functions along with cytochrome b_5 reductase as the electron donor to microsomal desaturases that synthesize unsaturated fatty acids, plasmalogens, and sterols[147]. Indications of the involvement of cytochrome b_5 in P450 reactions date from early studies that showed that NADH stimulates NADPH-supported metabolism of drugs in microsomes[148, 149]. Since then, evidence has accumulated that cytochrome b_5 has a significant role in a number of P450 systems[150], most significantly CYP3A4 (ref. [151]), which is involved in the majority of human drug oxidations.

Cytochrome b_5 is a small polypeptide (~17 kDa) containing 134 residues, which are divided into an amino-terminal hydrophilic heme domain and a carboxyl-terminal hydrophobic

membrane-binding region[152, 153]. While the membrane-bound form of b_5 participates in elongation and desaturation of fatty acids[50, 154, 155], cholesterol biosynthesis[156], and drug metabolism, soluble forms of b_5 and b_5 reductase are also found in erythrocytes, where they catalyze methemoglobin reduction[157]. Mutations to either the b_5 gene or the b_5 reductase gene can cause congenital methemoglobinemia[158]. Soluble b_5 has also been purified as a cytosolic component in the NAD(P)H-reductive activation pathway for porcine methionine synthase[52].

Like CPR, it is generally accepted that charge-pair interactions drive the formation of P450:b_5 complexes[159–162]. This is supported by site-directed mutagenesis of rabbit CYP2B4, which has identified several positively charged residues on the P450 surface (R122, R126, R133, K139, and K433) as being involved in binding b_5 (ref. [58]). However, the precise mechanism by which b_5 supports P450 catalysis is not yet clear. As the redox potentials of cytochrome b_5 and ferric P450 are approximately +25 mV and −300 mV[163] respectively, a role in the first electron transfer step of the P450 catalytic cycle is unlikely, suggesting that b_5 may only be involved in the donation of the second electron[147, 148]. Since electrons may be supplied to cytochrome b_5 by either NADH cytochrome b_5 reductase or CPR[50], the pathway of electron movement is difficult to establish. Furthermore, studies that show that apocytochrome b_5 can stimulate the catalytic activity of CYP3A4[164, 165], 2C9, 4A7, and 17[146] question a formal role in electron transfer. Thus, over the years several different mechanisms have been suggested by which b_5 supports P450 catalysis. These include: electron transfer from NADPH through CPR to cytochrome b_5 and thence to P450[166, 167]; electron transfer from NADH through b_5 reductase to cytochrome b_5 and thence to P450[168]; electron shuttling between P450 and b_5 resulting in a tighter coupling between electron flow and product formation[169]; reduction in the uncoupled generation of superoxide or hydrogen peroxide[170]; and an allosteric effect not dependent on electron transfer[164, 165].

Much of the early data on cytochrome b_5 interactions with P450 has come from *in vitro* analysis using reconstituted systems. However, the expression in *E. coli*[151], insect cells[171], or yeast[172] of a complete mammalian P450 monooxygenase complex comprising cytochrome b_5, CPR, and P450 has provided a means of studying the actions of the individual components in a more physiological context. Using yeast systems, Perret and Pompon[169] have shown that the redox properties of b_5 were strictly required to enhance CYP3A4 activity, with results of rapid kinetic analysis of b_5 reduction following NADPH addition suggesting that b_5 was reduced by the 3A4 ferrous–dioxygen complex and reoxidized by subsequent P450 oxygenated intermediates[169]. In *E. coli*, cytochrome b_5 significantly enhances the testosterone and nifedipine activity of CYP3A4 and results in the stabilization of the P450 during substrate turnover[151]. This is consistent with the proposal that b_5 interaction reduces uncoupling and superoxide anion release[169, 173].

With the increasing sophistication in the genetic tools now available it is becoming possible to investigate the complex interrelationships of the monooxygenase system in the correct context of the whole organism. For instance, the absence of any detectable P450 activity in mice where CPR has been knocked out of the liver provides compelling evidence that the alternative cytochrome b_5/b_5 reductase electron transfer pathway does not play any significant role *in vivo*[39]. We now await the construction of b_5 knockout mice to provide further clues as to the precise role of b_5 in the physiological function of P450s in higher vertebrate organisms.

4. Iron–Sulfur Electron Donors: Adrenodoxin, Putidaredoxin, and their Reductases

4.1. General

The Fe_2S_2-type ferredoxins can be arranged into three distantly related classes based on amino acid sequence homologies: bacterial-, plant-, and vertebrate-type[174]. Extensive information on the function and mechanisms of this system has been gained through work on the bacterial P450cam system in *Pseudomonas putida*, which catalyzes the conversion of *d*-camphor to 5-exo-hydroxycamphor[175]. In *P. putida*, the iron–sulfur protein is putidaredoxin (Pdx), a 106 amino acid residue ferredoxin. For catalysis, two reducing equivalents are sequentially transferred from NADH

to P450cam via an FAD-containing enzyme, putidaredoxin reductase (Pdr) and Pd. The putidaredoxin : P450cam binary complex forms with a K_d that is dependent on the oxidation states of the proteins and on whether substrate is bound to the P450[176]. In the first electron transfer, reduced Pd interacts with ferric *d*-camphor-bound P450cam and reduces it to the ferrous form. In the second electron transfer, reduced Pd forms a complex with ferrous dioxygen and *d*-camphor-bound P450cam to transfer the second electron and reductively cleave bound dioxygen, producing hydroxycamphor and water[177]. While most structural and biophysical studies of P450/ferredoxin interactions have focused on the Fe_2S_2 putidaredoxin from *P. putida* (and its cognate P450cam), other types of bacterial ferredoxins are recognized to interact productively with P450s. For instance, the Fe_3S_4 ferredoxin SoyB is a redox partner for the *Streptomyces griseus* P450 Soy (SoyC)[178] and the Fe_4S_4 ferredoxin from *B. subtilis* (fer) has been shown to undergo redox reactions with P450 BioI from the same bacterium[179].

In animals, ferredoxins are primarily involved in electron transfer interactions involving mitochondrial P450s. This fact is in agreement with the theory of the prokaryotic origin of this organelle. Much work has been done with adrenodoxin (Adx) and the transfer of electrons from NADPH-dependent adrenodoxin reductase (AdR) to CYP11A1, which is involved in cholesterol side chain cleavage, and to CYP11B, involved in the formation of cortisol and aldosterone[174]. Bovine adrenodoxin mRNA is translated as a 186 amino acid precursor molecule, which undergoes proteolytic processing upon import into the mitochondria to remove the N-terminal 58 residues[180]. The mature Adx comprises 128 amino acid residues and has a molecular weight of 14.4 kDa.

In plants, [2Fe–2S] ferredoxins are involved in conjunction with ferredoxin-reductase in photosynthesis reactions and the transfer of electrons from photosystem I to $NADP^+$[181]. However, evidence for involvement of ferredoxins in electron transfer to plant P450s awaits a more detailed characterization of the multiple P450 systems present in the biotechnologically important plant species.

Ferredoxins receive their electrons from ferredoxin reductase, which represents the first component of the electron transfer system. These enzymes contain FAD and belong to a large group of electron transferases that includes bacterial-type putidaredoxin reductase (PdR)[182], animal-type adrenodoxin reductase (AdR)[183–185], and plant-type ferredoxin-$NADP^+$ reductases[70]. An AdR-type reductase system (FprA) has recently been characterized in the pathogen *M. tuberculosis*[93] and the structure reveals extensive similarity with the mammalian enzyme[186]. In humans, AdR is most abundant in the steroid-producing cells of the adrenal cortex, the ovary, and the testis[187].

4.2. Interactions with P450

The structures and models of structures of several bacterial, animal, and plant ferredoxins are available (e.g., putidaredoxin[188] and the Fe_4S_4 ferredoxin from *Bacillus thermoproteolyticus*[189]). In particular, details of the electron transfer mechanisms have developed through kinetic and mutational analysis of the Pdx–P450cam system[176]. These indicate that Pdx interacts with proximal surface of P450cam through electrostatic interactions involving the acidic Pdx residues Asp38 and Asp34 and the basic P450cam residue Arg 112[190, 191]. Additionally, Arg112 appears to form the electron transfer route in the P450cam–Pdx complex with Cys39 and Asp38 of Pdx[191, 192]. A role for the C-terminal amino acid of Pdx (W106) in electron transfer to P450cam was suggested on the basis of diminished enzymatic activity in systems with Pdx W106 mutants, but later studies of various W106 mutants showed that this residue primarily affects the K_d of Pdx for the P450[193]. Studies of the interactions between cytochrome b_5 and P450cam show that b_5 and Pdx compete for the same binding site on the P450, suggesting a common mode of charge–charge pairing that is the major force driving interactions at the same surface on the P450[194].

However, isothermal calorimetry experiments show that the energetics of the P450cam/Pdx association are most similar to those of binding reactions of antibody to antigen complexes[195], suggesting that van der Waals and hydrogen-bonding interactions may predominate in the formation of P450cam–Pdx complexes. This study also suggested that binding between Pdr and Pdx might be dominated by hydrophobic interactions. Notwithstanding these data, a variety of mutagenesis studies along with the fact that increased ionic

strength markedly increases the K_m of Pdx for the P450, highlight the important influence of electrostatics on the docking process[196, 197].

Catalytic interactions between Pdx and P450cam are considered to follow a ping-pong mechanism[198]. The flavin (FAD) of Pdr is reduced directly to its hydroquinone by interaction with the obligatory two-electron donor NADH, but negligible formation of semiquinone is observed during the successive single-electron transfer reactions between Pdr and Pdx in steady-state turnover of P450cam. This indicates destabilization of the flavin semiquinone with respect to the hydroquinone and oxidized forms[198]. Rapid (laser flash photolysis) kinetic studies indicate transient formation of a blue semiquinone on the Pdr FAD, which disproportionates rapidly[199].

Upon complex formation, Pdx induces conformational changes in P450cam[191], which convert the high-spin state of ferric P450cam to low-spin state and stretch the heme-axial ligand[191]. NMR studies of the Pdx–P450cam complex also show that Pdx binding perturbs several regions involving the substrate access channel in P450cam[200] and induces a tilt in the heme plane, leading to the movement of *d*-camphor and the axial Cys to the heme-iron by 0.1–0.5 Å and facilitating heterolysis of the O–O bond[201]. It is thus proposed that in addition to its redox function, Pdx invokes structural changes that facilitate the oxygen activation reaction[201]. Only Pdx is capable of reducing the oxy form of P450cam, in agreement with this hypothesis[196].

The rate-limiting steps in the entire catalytic cycle of P450cam are the electron transfers between Pdx and the P450. The precise rate-limiting step appears to change from the first electron transfer to the second electron transfer as the concentration of Pdx in the system is reduced toward levels considered typical of the situation *in vivo*[202].

With respect to the mammalian adrenodoxin/adrenodoxin reductase system, crystal structures of truncated and full-length oxidized bovine Adx[203, 204] have been determined, along with NMR structures of both oxidized and reduced full-length forms of Adx[205]. Adrenodoxin, contains a large hydrophobic core region that houses a single Fe_2S_2 cluster, and an interaction domain that contains acidic residues responsible for the recognition of the redox partners, CYP11A1 and AdR[206, 207]. (Figure 4.11). The single Fe_2S_2 cluster is ligated by four cysteinyl thiolate ligands, Cys46, Cys52, Cys55, Cys92, replacement of any

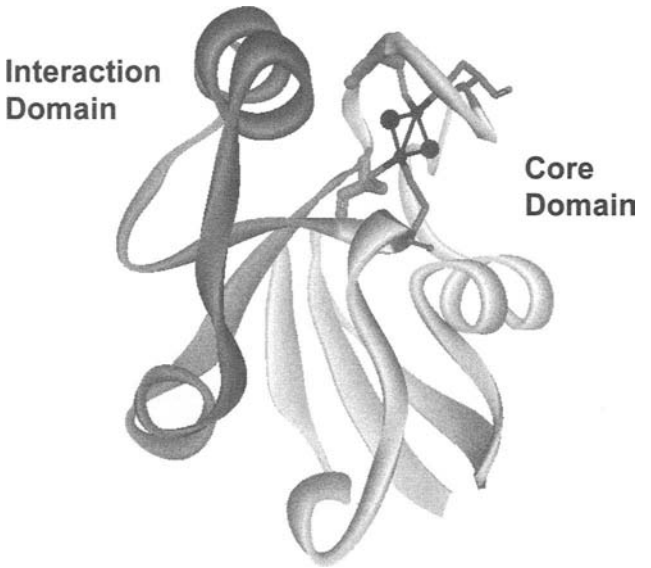

Figure 4.11. Ribbon representation of bovine Adx (pdb code1AYF). The Fe_2S_2 cluster is shown in black ligated to cysteines 46, 52, 55, and 92. The Fe and S atoms are represented as small and large spheres respectively.

of which, produces a nonfunctional apoprotein[208]. Sites involved in redox partner binding are also present in the core domain centered around an acidic patch at position Asp59[207].

Two different models have been described to explain the electron delivery system mediated by Adx: (a) an organized cluster model comprising AdR : Adx : P450 ternary/quaternary complexes[209] and (b) a shuttle system where Adx sequentially transports one electron at a time from AdR to P450[210]. Of these, the shuttle hypothesis is most favorable since the formation of a ternary complex formed by AdR, Adx, and P450 is unlikely from a structural perspective due to overlapping interaction regions[203, 211]. The sequential model is also that favored for the well-characterized P450cam system. Following the resolution of the bovine AdR crystal structure it has become possible to fully investigate the structural mechanisms driving the protein complex formation[185]. The distinguishing feature of AdR is a large groove between α9 and β12 at the surface (Figure 4.12), which can accommodate the C-terminal chain end of adrenodoxin[185]. Both Adx and AdR contain highly asymmetric surface charge distributions, which indicates that interactions between these molecules and P450 are mediated by electrostatic interactions[174]. The structural data are largely confirmatory of the earlier data from mutagenesis and modification studies, showing the importance of charge pairing in the binary complex between AdR and Ad[212]. Most recently, the structure of the AdR/Ad complex was solved at 2.3 Å resolution[212]. Interactions sites were confirmed as involving predominantly acidic residues of Adx (D76, 79, 72, and 39) with basic side chains of Adr (R211, 240, 244, and K27).

In the P450cam system, our understanding of the nature of the interactions between the Pdr and Pdx components is not fully developed. However, Cys73 of Pdx has been reported as important in the docking interaction between Pdx and Pdr[190].

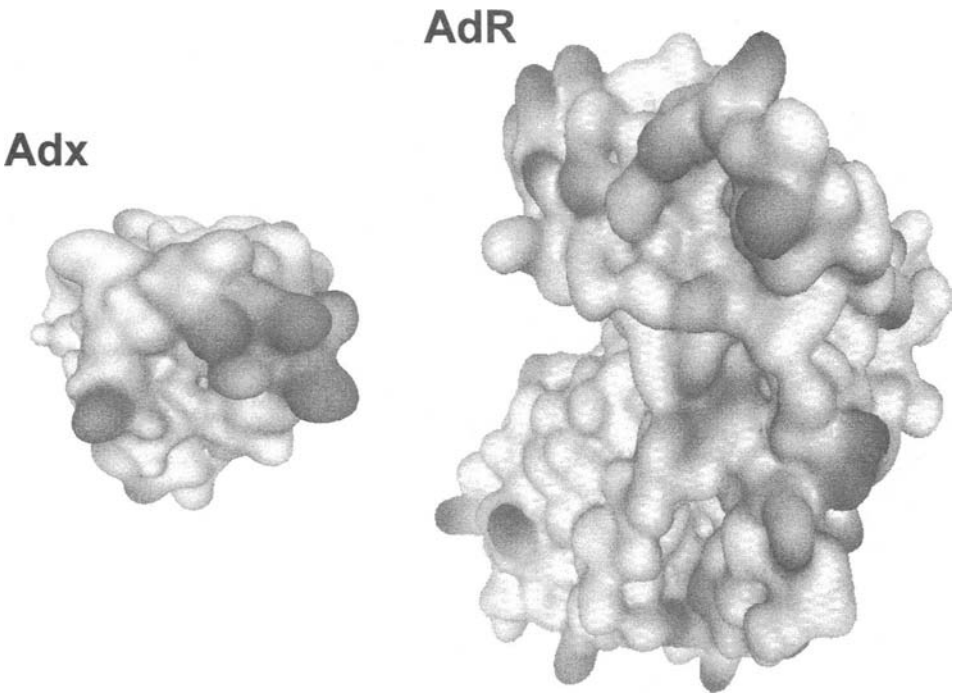

Figure 4.12. Surface representations of Adx and AdR (pdb codes 1AYF and 1CJC respectively). The molecules are oriented according to their proposed docking positions[184], which involves charge-pair interactions between acidic residues at the C-terminal end of Adx and basic residues around the docking groove of AdR.

Mutations to the adjacent Pdx surface residue Glu72 also affect electron transfer reactions in the P450cam redox system[213]. The generation of catalytically efficient putidaredoxin reductase–putidaredoxin–P450cam triple fusion protein demonstrates that a much more simple single component camphor hydroxylase system can be produced, with obvious biotechnological implications. The addition of short peptide linkers between the respective polypeptides was compatible with strong expression of fusion proteins in an *E. coli* system and efficient electron transfer reactions between the redox centers[214]. Although much structure/function work remains to be done on the Pdr component of the P450cam system, a novel finding regarding its activity was made recently with the demonstration that Pdr has NAD(H)-dependent dithiol/disulfide oxidoreductase activity[182]. However, much structural and kinetic work remains to be done to provide a clear description of the nature of interactions between the Pdr and Pdx components of the P450cam system, and how these relate to those in the analogous Adr/Adx mitochondrial system.

5. Novel Redox Systems

The identification of the P450 BM3 system led to the realization that alternative types of P450 redox systems (beyond those typified by mammalian hepatic class and P450cam) exist in nature. In recent years, a variety of alternative modes of P450 reduction have been reported—including P450s that require no electron transfer at all (i.e., catalyze molecular rearrangements without oxygen chemistry) such as the dehydratase allene oxide synthase[215] and a P450 that appears to interact directly with hydrogen peroxide to facilitate fatty acid oxidation in *B. subtilis*[216]. Various types of ferredoxins have been shown to be competent redox partners for selected P450 enzymes[217] and flavodoxins may also be physiologically relevant mediators of electron transfer to P450s in bacterial (and possibly plant) systems[218, 219]. More recent studies suggest more exotic types of P450 fusion enzymes may exist—including fusions of P450s to ferredoxin and phthalate dioxygenase reductase-like domains[220, 221]. It is highly likely that further insights into biodiversity of P450 redox systems will be gained as ongoing genome sequencing projects reveal ever more diverse means by which this intriguing class of enzymes can operate and obtain reducing power.

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5

Activation of Molecular Oxygen by Cytochrome P450

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Life depends on the kinetic barriers to oxygen reactions
R.J.P. Williams, in the preface to the *Oxygen Chemistry* by Donald T. Sawyer[1]

1. Introduction to Oxygen Activation

The cytochromes P450 have occupied a central focal point in biochemistry, pharmacology, toxicology, and biophysics for more than three decades. This rich history is faithfully conveyed in accompanying articles appearing in this volume as well as those of the first and second editions of *Cytochrome P450* edited by Ortiz de Montellano[2,3]. In addition, there have been innumerable review articles and book chapters devoted to the subject[4,5]. Interest in the cytochromes P450 perhaps began due to the importance of these catalysts in human physiology and pharmacology. The interest of chemists and biophysicists peaked due to the many unique spectral features of these heme enzymes. One of the most intriguing aspects of P450 systems is the range of difficult chemistry efficiently catalyzed, the importance of oxygen as a cosubstrate, and the thoughts of overall common mechanisms with the heme monooxygenases and other systems that effect reductive metabolism of atmospheric dioxygen[6,7]. Thus, from the very beginning, the phrase "active oxygen" was used to label a holy grail of cytochrome P450 research: the documentation of the intermediate states of oxygen and heme iron that give rise to the diversity of metabolic chemistries observed.

The chemistry of "oxygen activation" catalyzed by a variety of metal centers has a long and rich history, and as many as eight variations of structurally different metal–oxygen complexes were discussed as early as 1965[8]. This publication of *Cytochrome P450, Third Edition* is particularly timely. During the past 3–5 years, a great deal of progress has been realized in the direct observation of P450 intermediate states and the evolution of a detailed understanding of the structural

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features of the protein, which control the population and stepwise movement through the reduction and protonation states of heme and oxygen. The veil surrounding "oxygen activation" has been lifted through the systematic application of a variety of biochemical and biophysical tools, such as mutagenesis, high-resolution cryocrystallography, multiparameter spectroscopic studies of intermediate states isolated using cryogenic or fast kinetic techniques, and many rigorous quantum chemical and molecular dynamics computational studies. Equally important has been the explosion of genomic and proteomic breakthroughs. No longer are we dealing with a single microbial P450 and a few membrane bound mammalian counterparts. Rather, with over 3,000 P450 genes known, we can find examples from unique environmental niches where the enzymatic reaction cycle yields newly observable features. Thus today, the current view of oxygen activation mechanisms catalyzed by metal centers in heme enzymes ensures a much better opportunity to develop recurring catalytic paradigms than was possible at the time of the *Cytochrome P450, Second Edition.*

A broader view of heme-oxygen catalysis with the understanding of how proximal ligand, protein structure, and subtle control of electron/proton stoichiometry contribute to very different reactivities is now within reach[9]. Common mechanistic hypotheses for oxygen activation in metalloenzymes are currently emerging. Recent progress in mechanistic studies on other heme enzymes, which use different forms of so-called "active oxygen intermediates," such as peroxidases[10, 11], heme oxygenases (HOs)[12, 13], catalases[14], nitric oxide synthases (NOS)[15], and peroxygenases[16, 17], have provided an impressive degree of unification. A fundamental question that has always plagued the interpretation of experimental data has been the functional differentiation of these enzymes, which efficiently catalyze a range of diverse chemistries yet utilize similar highly reactive heme-oxygen complexes. The comparison of similar reactive intermediates in different enzymes has helped to distinguish between the essential features of each of the enzymes. This in turn has provided important clues into the role of protein structure in the heme-oxygen catalytic events. The recent progress in actually observing, through kinetic isolation and cryogenic techniques, these fundamental intermediate states in the cytochrome P450s brings us to a much deeper understanding of P450 monooxygenase catalysis that was available at the time of the previous edition of this volume.

In discussing the reaction cycle of the cytochrome P450s, it is useful to incorporate similar states which appear to be more or less stable in other heme-oxygen systems. The sequential steps of "oxygen activation" are those that follow the formation of the ferrous reduced iron-porphyrin state that is generated by electron transfer from the physiological redox donor. These steps are formally:

1. Dioxygen binding to the ferrous heme center to form an oxygenated complex which can be written as either Fe^{2+}–OO or Fe^{3+}–OO^{-}.
2. One-electron reduction of this complex to form the Fe^{3+}–OO^{2-}, ferric-peroxo complex.
3. Protonation of the distal oxygen of this peroxo anion to generate the Fe^{3+}–OOH^{-}, ferric-hydroperoxo complex.
4. A second protonation of the Fe^{3+}–OOH^{-} complex at the distal oxygen atom to form a transient oxo-water adduct which immediately undergoes heterolytic scission of the O–O bond. This process generates a single oxygen atom containing iron-porphyrin species, which is formally two redox equivalents above the ferric heme starting state. This intermediate has been variously termed the "iron-oxo," "ferryl-oxo porphyrin π-cation radical," or "Compound I," the latter after the pioneering discoveries made by Theorell, Keilin, George, and Chance while studying the peroxidases and catalases[18].

The intermediate states so defined have counterpoints in nonheme oxygen activation and have been also studied and reviewed in numerous articles over past decades. We note that a search of the term "oxygen activation" in *SciFinder Chemical Abstracts Database* returns almost 29,000 references published after 1995! Thus, we will not attempt to link nonheme iron chemistry, nor oxygen activation in multicenter metalloenzymes which was recently reviewed by Siegbahn[19], with that of the P450 systems in this chapter. Rather, we focus on those features which directly address the important aspects of the oxygen activation cascade in P450. The structure and properties of relevant porphyrin complexes, including models of cytochrome P450 and other oxygen-activation enzymes, are extensively reviewed in the recent edition of the *Porphyrin Handbook*[20–25].

2. General Features of Dioxygen Activation in Heme Enzymes

As discussed in the first section of this chapter, the P450 cytochromes, NOS, HOs, and cytochrome oxidases are all heme enzymes which are able to "activate" molecular oxygen. Related heme enzymes, such as the peroxidases and catalases, work with partially reduced dioxygen species. It has always been intriguing that each of these enzymes uses the same cofactor, heme or Fe-protoporphyrin IX, but performs a distinct physiological function.

In heme enzymes that are able to activate molecular dioxygen, the porphyrin and proximal ligand provide the heme iron with five coordination sites. Thus, the side-on coordination of peroxide, which is common for nonheme metalloenzymes and oxygen activation mechanisms, is not usually observed in heme enzymes and is also rare in model metalloporphyrins[23, 26–29].

A common role of the porphyrin ring in oxygen activation is the donation of an electron during the overall process of O–O bond cleavage[30]. The relatively easy oxidation of Fe-protoporphyrin IX, which serves as an additional buffer supply of electrons to the dioxygen ligand, is a common feature in the mechanisms of related heme enzyme families, such as peroxidases, heme catalases, cytochromes P450, NOS, and peroxygenases, which catalyze O–O bond cleavage of a peroxide ligated to the heme iron[19, 31]. Donation of one electron from the porphyrin to the peroxide ligand initiates the effective heterolytic scission of the O–O bond and results in formation of a cation radical on the porphyrin ring, a definitive feature of a "Compound I" state[31].

Oxygen activation in all of these systems begins with the binding of dioxygen as an axial ligand to the Fe^{2+} heme iron, or with binding of H_2O_2 to the Fe^{3+} heme. Thereby hydroperoxoferric heme complex is formed through different pathways as a common intermediate state, shown in Figure 5.1. Thus, a current focus of research is this common "peroxo," both in the pathways for its formation as well as in the control of

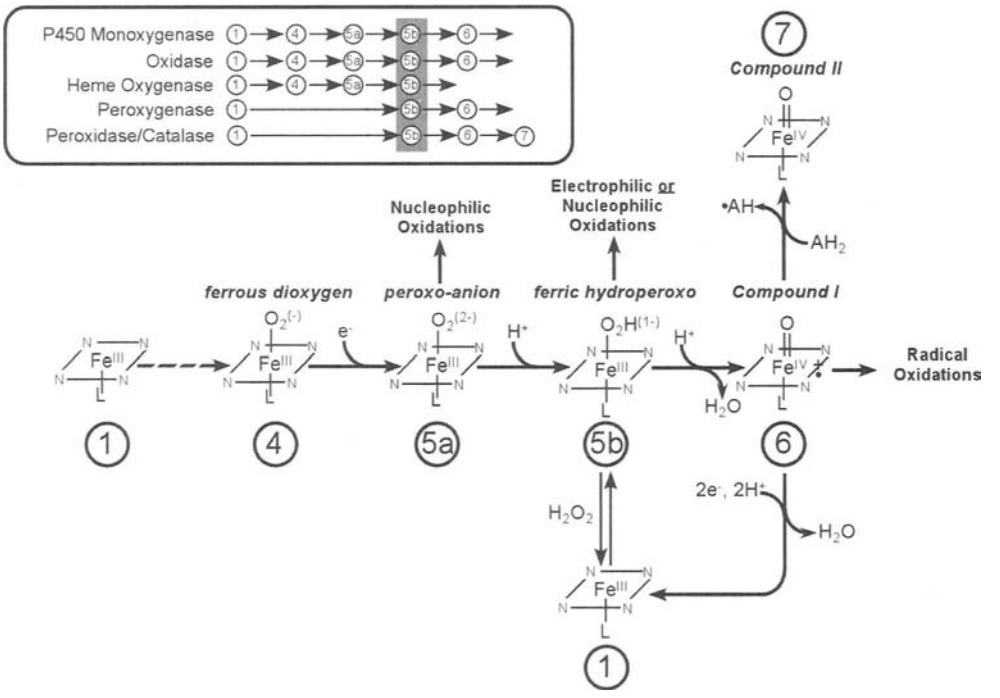

Figure 5.1. Common reactive intermediates in bioinorganic catalysis. The catalytic pathways of various heme enzymes are shown in the inset.

proton delivery events, which dictate the inherent reactivity of the resulting "active oxygen" intermediates. In the classic P450 reactivities of a "Compound I like" intermediate, it is also this critical acid–base chemistry which initiates dioxygen bond scission to form the higher valent iron-oxo state.

In addition, there are other redox active pathways operant in the cytochromes P450 (Figure 5.1). First is an "oxidase" pathway using atmospheric dioxygen and two additional redox equivalents in addition to the two required for heterolytic O–O bond cleavage. Additionally there is a "peroxidase/peroxygenase" pathway wherein hydrogen peroxide serves as an oxygen donor. A detailed analysis of these pathways with respect to oxygen activation in the cytochromes P450 was provided in earlier editions of this volume[2, 3]. Obviously, these two mechanisms differ by the overall redox state of oxygen vs peroxide and the need in two additional reduction steps (one-electron reductions by the physiological redox partner) in the oxidase pathway. Perhaps most important, however, is the difference in the protonation states that necessitate specific delivery of two protons to a bound peroxo intermediate when beginning with dioxygen as opposed to a 1–2 proton shift to form the transient Fe–O–OH_2 precursor when starting with hydrogen peroxide and ferric heme.

2.1. The Oxidase/Oxygenase Pathway in Cytochrome P450

The pivotal role of different protonation pathways to bound oxygen derivatives in bioinorganic chemistry has been shown in various mechanistic studies[19, 32, 33]. The same concept has also been considered for the case of catalysis involving H_2O_2, in which the correlation between the ability to efficiently deliver the proton to the metal-bound hydroperoxide anion, and the efficiency of catalytic decomposition of hydrogen peroxide has been noted[34]. In heme-enzyme catalysis, however, the ability to supply the necessary one or two protons for the generation of the proper active intermediate from the iron-bound peroxide/hydroperoxide anion state is an essential and crucial aspect of the detailed structural "fine-tuning" provided by the enzyme active center. Thus, the relative spatial orientation of catalytic residues and active-site water molecules may facilitate or suppress various branches of the microscopic pathways that contribute to the possible chemical reactions involving these activated oxygen compounds. An encompassing scheme that graphically illustrates the commonality of these various processes is depicted in Figure 5.1.

Many laboratories have focused on the role of protein structure in defining the pathways and control points outlined in Figure 5.1. Recent results, which are discussed in this chapter, have greatly improved the understanding of these events. For example, a hydrogen-bond network has been implicated as the critical component of the enzymatic mechanism which defines both catalytic activity and the effective branching points between productive and nonproductive pathways for the camphor hydroxylation catalyzed by cytochrome P450 CYP101[35–42]. Modification of this network, either through mutagenesis of the CYP101 enzyme, or by the use of substrate analogs, points to the subtle active-site features of the protein, which can in some cases have dire consequences on the productive monooxygenase pathway with resultant abortive processes that short circuit the classic P450 mechanistic "wheel" (Figure 5.2). These additional pathways, since they bleed redox equivalents and dioxygen from the pure monooxygenase stoichiometry, have been termed "uncoupling" events. While the mechanisms of uncoupling may have either a thermodynamic and/or kinetic origin, it is most certainly governed by the role of a protein-provided "proton relay" system. These pathways of proton delivery can involve not only backbone and side-chain moieties from the protein structure, but also localized water molecules, which are appropriately stabilized in the active center of the enzyme. The realization of how Nature uses specialized water alludes to analogous discussion of proton delivery in diverse enzymes such as cytochrome oxidase[43, 44], and introduces these concepts into the vernacular of P450 specialists. Again, genomics and proteomics have helped identify critical structural features, for instance the famous "acid–alcohol pair," Asp251–Thr252 in the cytochrome P450 CYP101. Only most recently, however, has structural information shed critical light on how these residues work in concert to deliver protons at the right place and time.

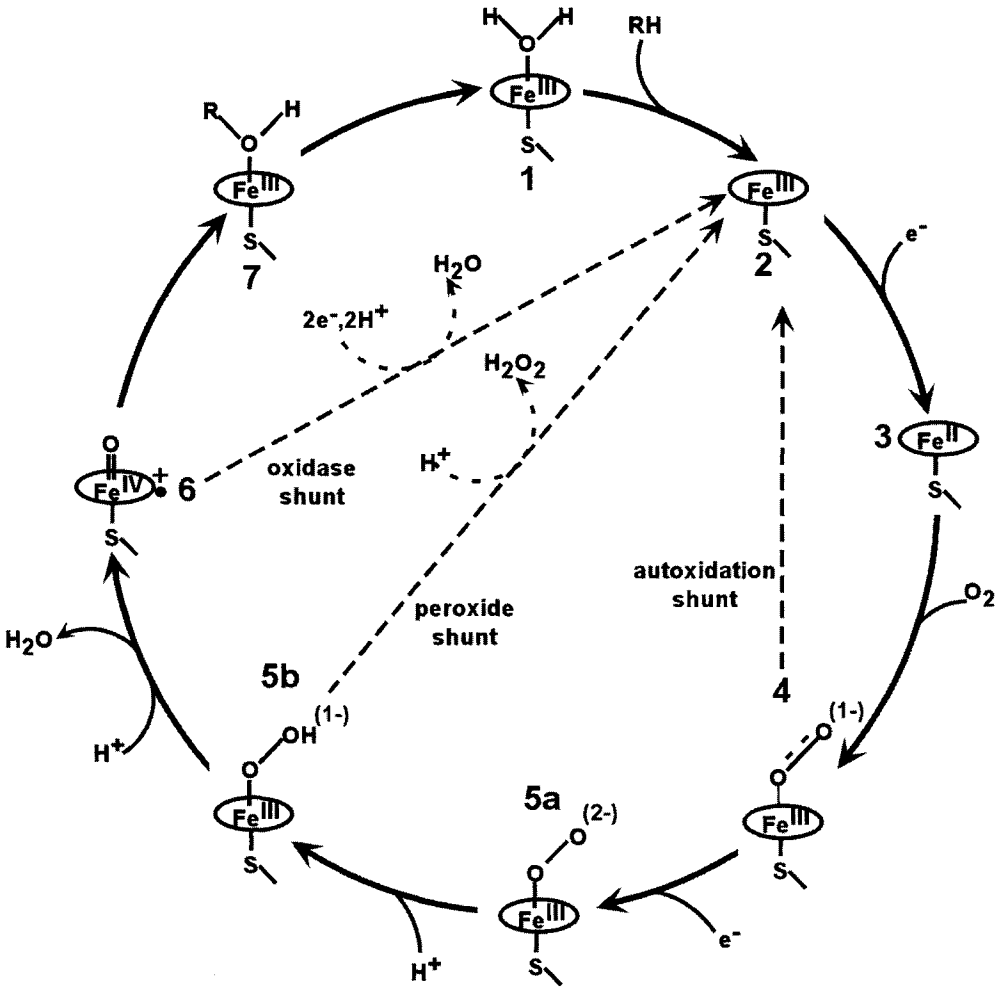

Figure 5.2. The P450 catalytic cycle.

An oxidase mechanism of oxygen activation supported by NADH has also been suggested as an alternative activity in the peroxidases, but was later disproved[45]. Both the cytochromes P450 and peroxidases are thought to share a high-valent "ferryl-oxo" porphyrin cation radical intermediate[18, 46]. In P450 catalysis, atmospheric dioxygen binds to the reduced heme iron of the enzyme, and this ferrous-oxygen, or ferric-superoxide complex accepts one more electron from its protein redox partner to form the peroxo-ferric intermediate. This in turn is quickly protonated to generate the hydroperoxo-ferric intermediate[47, 48]. The formation of this intermediate from the ferrous-oxy complex, and its subsequent transformations were recently investigated in detail using the method of cryogenic radiolytic reduction[41, 49, 50]. The same iron-hydroperoxo intermediate is assumed to be a common state in the formation of "Compound I" in peroxidases and a variety of other systems such as the HOs[12, 13, 51–53]. Contrary to P450 and HO, in peroxidases the natural formation of this active intermediate involves the hydrogen peroxide starting material. In contrast to

dioxygen, H_2O_2 brings the two reducing equivalents and two protons necessary for the "Compound I" generation to the ferric heme in one step. The key step in the enzymatic cycle of the peroxidases, therefore, involves the abstraction of a proton from the proximal (closest to the iron center) oxygen atom as the H_2O_2 molecule is ligated and the delivery of a proton which is necessary to initiate O–O bond scission[54, 55]. The formation of the "oxo" complex and water is essentially a barrierless process once protonation occurs[56]. A feature of the peroxidase mechanism is the fact that it proceeds with no other external source of electrons and protons, with an imidazole side chain from a histidine residue in the distal pocket thought to serve as the species responsible for this proton shift. The distal histidine thus serves, first, as a proton acceptor from the proximal oxygen, and then, as a donor of the same proton to the distal oxygen at the second step of the reaction.

Defining the parameters involved in the binding and proton-mediated transformation of the peroxide-coordinated ferriheme complex is critical in defining the properties of these states in both the peroxidase and related oxygenase mechanisms. The pK_a of the Fe-coordinated HOO(H) was estimated as 3.6–4.0 for horseradish peroxidase (HRP) and cytochrome c peroxidase (CcP)[57, 58]. Thus, the iron-coordinated peroxide is in an anionic form at neutral pH. The polarity of the peroxidase distal pocket vs the dioxygen carrier, myoglobin, or even the rather hydrophobic nature of the P450 cytochromes, stabilizes the resulting charge separation upon deprotonation.

The resulting hydroperoxo-ferriheme complex is nonetheless unstable, as the peroxide ligand weakly ligates the heme iron[59], and the dissociation rate constant for the peroxide anion as the sixth ligand in Fe-microperoxidase-8 (Fe-MP8) and Mn-MP8 is in the neighborhood of 10–20 s^{-1} (ref. [60]). Similar estimates can be made from other kinetic studies[45, 61] which measured the K_m and k_{cat} values for the formation of "Compound I" in reaction of HRP with hydrogen peroxide. The reported millimolar K_m values and the observed k_{cat} on the order of $10^7\ M^{-1}\ s^{-1}$ suggest that the dissociation rate of HOO(H) may be as high as $10^3\ s^{-1}$.

In contrast, the cytochromes P450 face the challenge of "activating" dioxygen, with the delivery of two protons in addition to the two redox equivalents which are consumed per one O_2 molecule. While the reduction of the heme iron is provided by the protein redox partner, it is an essential part of the P450 enzyme to catalyze the transfer of two protons to the distal oxygen atom of the bound peroxide anion through a sophisticated proton relay mechanism. As noted in the introduction, this pathway was shown to include protein-bound water molecules which are stabilized at the enzyme active site through specific hydrogen-bond interactions[39, 42, 62]. Through mutagenesis of the residues involved in this network, it was shown that the overall enzyme kinetics and the observed efficiency of coupling redox equivalents into product was very sensitive to the hydrogen-bonding properties of these sites[63]. Similarly strong effects, including drastic changes in the formed product ratios, were found in other enzymes of the P450 superfamily[64–67]. Kinetics of the productive and nonproductive turnover by human cytochromes P450 with various substrates were extensively studied by Guengerich and coauthors[68–70]. Their results directly show the existence of multiple steps, each potentially rate limiting depending on the specific reaction being catalyzed.

A linked event is the resultant chemical processing that occurs after proton delivery. Following second proton transfer, the O–O bond order is significantly diminished, so that heterolysis is ensured, with release of water and generation of the higher valent "Compound I" state. Without efficient delivery of this second proton, one has to consider the possibility of the radical process of O–O bond homolysis. Homolytic and heterolytic scission of O–O bond in alkylperoxides and in hydrogen peroxide catalyzed by various heme enzymes have been extensively studied[71–75]. In the cytochromes P450, the ratio of homolytic/heterolytic cleavage of the O–O bond has been addressed through numerous investigations[72, 73, 75–78]. The vast majority of these studies utilized the exogenous oxidant driven pathways, with a variety of substituted peroxides and peroxo acids. Significantly, the results were found to depend on the ability of the leaving group to stabilize a radical; the more stable this species, the greater the percentage of homolytic O–O bond scission[79]. The product distribution with a cumene hydroperoxide driven reaction was used as a measure for

the relative ability of particular isozyme to form a "Compound I" intermediate[73, 78, 80]. Attempts were also made to correlate the "push" electron-donating effect[81] from the proximal thiolate with the spectroscopic markers of the porphyrin ring and with the formation of "Compound I" (i.e., the heterolysis of O–O bond) and product[80]. The formation of hydroxyl radicals via homolytic cleavage of the O–O bond in the reconstituted P450 CYP2B4 system resulted in covalent modification of the protein and degradation of the heme[82].

The general acid catalysis of heterolytic O–O bond scission via protonation from solvent water was suggested by many authors[72]. In the absence of additional catalysis of heterolytic scission through the second protonation of the distal oxygen atom, heterolysis/homolysis of O–O bond is governed by the general thermodynamic stability of the reactant and product, as predicted by Lee and Bruice[79] for porphyrin models, and recently analyzed by Que and Solomon[32, 33, 83–88]. If the iron-peroxide complex is predominantly in a high-spin (HS) state, as it is very often in nonheme metal enzymes with weak or moderate ligand field, homolysis of the O–O bond is favorable, as the HS state of the iron in both the reactant and product forms is conserved, with the process involving release of a hydroxyl radical.

For heterolytic O–O scission following a second protonation, in which the departing oxygen atom leaves as water, electron density is withdrawn from the porphyrin moiety, and a porphyrin pi-cation radical is formed. The heterolysis/homolysis ratio and overall product distributions are thus coupled in the native enzyme systems. Various parameters such as bulk and local pH, ligation state of the metal, structure, and redox properties of porphyrin and peroxide species certainly play important roles in controlling the spin state, O–O bond order, and proton delivery events.

Perhaps the most important is the enzyme's ability to control the delivery of protons to the developing negative charge of the iron-dioxygen complex as electrons are introduced into the center. The protonation/deprotonation events at the bound superoxo, peroxo, and hydroperoxo anions were theoretically analyzed and claimed to account for the definitive reaction sequence in the formation of the active oxo-ferryl porphyrin pication hydroxylating intermediate in cytochrome P450 and peroxidases[89]. Despite their common use of intermediate states, the oxygenases and peroxidases have distinct functions, beginning their active cycle with different heme ligand complexes[89–98]. The variability of different pathways of oxygen activation provides one with a tool to alter the performance of the enzyme through different mutations, or even to directly design new types of activity into well-known systems[99, 100]. However, only in the last decade have the proton delivery mechanisms in P450 catalysis been directly revealed[35, 39–42, 62, 92–94, 101–104]. These important details of P450 catalysis will be discussed in detail in the remainder of this chapter.

3. Enzymatic Cycle of Cytochrome P450

It is humbling to note that an outline of the common catalytic cycle for the cytochromes P450 was proposed as early as 1968[4, 105]. The critical features of an atmospheric dioxygen binding event interspersed between two single-electron transfer events remain as a generally accepted outline of the P450 catalytic "wheel"[63, 106] (Figure 5.2). The sequential two-electron reduction of cytochrome P450 and the existence of multiple intermediates of substrate binding and reduction was documented in bacterial P450 CYP101 (refs [107]–[109]) and in microsomal systems[110, 111]. Substrate binding to a resting state of the low-spin (LS) ferric enzyme **[1]** perturbs the water, coordinated as the sixth ligand of the heme iron if there is a close fit to the active-site pocket, and alters the spin state to favor the HS substrate-bound complex **[2]**. In P450 CYP101, the HS Fe^{3+} form of the protein has a more positive redox potential, is a more efficient electron acceptor from its redox partner, and accompanies a correspondingly tighter association of the substrate to the reduced form of the protein **[3]**[112]. In other systems, it was observed that this spin shift is absent or incomplete for some combinations of P450 isozyme and substrate[113]. Oxygen binding leads to an oxy-P450 complex **[4]**, which is the last quasi-stable and observable intermediate in the reaction cycle. Subsequent sequential steps are reduction of the FeO_2 complex, formation of the

peroxo-ferric intermediate **[5a]**, its protonation yielding a hydroperoxo-ferric intermediate **[5b]**, a second protonation at the distal oxygen atom and subsequent heterolysis of the O–O bond to form the iron-oxo "Compound I" **[6]** and water, and finally the oxygenation of the substrate to form a product complex **[7]** and its subsequent release. These steps have, over the years, been addressed by many methods, but direct observation of key intermediates was difficult due to the high inherent reactivity of these states and their lack of accumulation in kinetic studies. For instance, **[5a]**, **[5b]**, and **[6]** had not been unambiguously observed until recently, and alternative bidentate structures of the "reduced oxyP450" had been proposed[26, 29].

A beautiful feature of the overall reaction pathway for the cytochrome P450s is the rich chemistry afforded by the various steps in the reductive metabolism of the heme-oxygen center. These include nucleophilic, electrophilic, and hydrogen-abstracting species. In addition, there are at least three branchpoints where multiple side reactions are possible and realized *in vivo* (Figure 5.2). Abortive reactions include: (a) autoxidation of the oxy-ferrous enzyme **[4]** with concomitant production of a superoxide anion and return of the enzyme to its resting state **[2]**, (b) a "peroxide shunt," where the coordinated peroxide **[5b]** dissociates from the iron and forms hydrogen peroxide, thus completing the unproductive (in terms of substrate turnover) two-electron reduction of oxygen, and (c) an "oxidase uncoupling pathway," whereby the "ferryl-oxo" intermediate **[6]** is reduced to water by two additional electrons in lieu of hydrogen abstraction and subsequent substrate oxygenation. This results in an effective four-electron reduction of the dioxygen molecule with a net formation of two molecules of water, thus anointing the P450s with a "cytochrome oxidase" activity. The oxidase activity in P450, in comparison to those enzymes involved exclusively in bioenergetics, proceeds at a drastically reduced rate and without a productive proton-pumping cycle. Thus the physiological processing of dioxygen and substrates via reductive metabolism is a rich and varied set of events. The control of the specific reactivities is determined by proton delivery in the enzyme.

3.1. The Ferrous-Dioxygen Complex

Oxygen binding to reduced P450 yields the species **[4]**, Fe^{2+}–OO (ferrous-dioxygen) or Fe^{3+}–OO^- (ferric-superoxide) complex. The chemical structure of oxy-P450 is similar to analogous complexes in oxygen-carrier-heme proteins (hemoglobin and myoglobin) and other heme enzymes (HRP, HO, etc.). This ferrous Fe^{2+}–OO complex is EPR silent, but shows Mossbauer quadruple splitting of the heme iron center consistent with a ferric state[114], similar to that observed for "Compound III" in HRP[115], and for oxy-Hb[116]. The low frequency of the O–O stretch band, observed at 1140 cm^{-1} in the resonance Raman spectra of P450 CYP101[117], is also typical for a superoxide complex. These features have often prompted discussion about the correct electronic assignments of these complexes and more sophisticated models, such as a three-centered four-electron ozone-like bond that was envisioned almost 30 years ago[118].

The properties of "oxy-P450" were first characterized in P450 CYP101 and other isozymes using optical absorption[119–124], resonance Raman[117, 125, 126], and Mossbauer[114] spectroscopy. The electronic structure of P450 and chloroperoxidase (CPO) complexes with O_2, CO, and CN^-, together with their magnetic circular dichroism (MCD) spectra were also analyzed[81, 127]. Theoretical explanation of the specific split Soret band for the complexes of the ferrous P450 and CPO with diatomic ligands was also provided in the mid-1970s[128, 129] for the case of carbon monoxide, and later experimentally extended to other electron-rich ligands with a strong back-donation (dioxygen, cyanide, thiolate)[81, 127].

The absorption spectra and autoxidation properties of oxygen complexes for several cytochromes P450[50, 121, 122, 130, 131], and related enzymes, such as NOS[132] and CPO[133], have also been reported. While all of these states are not particularly stable, and are quickly autoxidized, the autoxidation rates for P450 and other thiolate-ligated enzymes (CPO and NOS) are significantly higher than for the oxygen carriers Mb and Hb. This can be aided by a general acid catalysis in the heme monooxygenases, through facile protonation of the coordinated oxygen[134]. As will be

discussed in later sections, active proton delivery to the bound oxygen or peroxide ligand is a salient feature of the P450 mechanism of oxygen activation, and similar mechanisms could be responsible for faster autoxidation of the ferrous–oxygen intermediate in these enzymes.

The recent use of cryocrystallographic techniques has enabled the direct visualization of intermediate states in the P450 cycle, and thus the modulation of active-site moieties within the catalytic timeframe. With regards to dioxygen ligation, however, the X-ray structure of Fe^{2+}–OO complex of P450 CYP101 determined recently[42] demonstrates that the structure of the heme ferrous-oxy complex of P450 appears very similar to analogous complexes of other heme enzymes[135, 136]. Oxygen is found to be coordinated in the bent "end-on" mode (with a Fe–O–O angle of 136°). This structure provides a starting point for the formation of the end-on bound peroxo-ferric complex, which is the first of three unstable and highly reactive intermediates in the P450 catalytic cycle.

3.2. Reduction of Oxy-Ferrous P450 and Formation of Peroxo-Ferric Complexes: Properties, Stability, and Spectroscopy

The stability of iron-peroxo complexes is marginal in heme systems in the presence of a strong proximal ligand (His, Cys, or Tyr), and the aqueous solution at a near-neutral pH. The numerous attempts to isolate such complexes obtained in reactions of hydrogen peroxide with P450 at ambient conditions have failed because of the inherent low stability and fast conversion to "ferryl-oxo" species with O–O bond scission[137]. There have been successful isolations of the ferriheme-peroxide complex in myoglobin, however[138].

Chemical models of the Fe^{3+}–OOH^- and Fe^{3+}–OOR^- complexes have been prepared by reacting metalloporphyrins with peroxides at low temperatures (either 200–230 K in solutions or freeze-quenched at 120 K and below) and studied by EPR, NMR, and optical spectroscopic methods[139–142]. Similar results were obtained with heme proteins[143], including cytochrome P450[144]. Particularly noteworthy is the signature LS EPR spectra with narrow g span (g = 2.25–2.31, 2.16–2.21, 1.93–1.96) and the red shift of Soret band (as compared to the spectrum of oxy-ferrous precursor) of the Fe^{3+}–OOH^- complexes in heme systems. The direct reactions of superoxide anion with free Fe^{2+}-porphyrins in the absence of strong proximal ligand usually afford the HS Fe^{3+}–OO^{2-} complex with a side-on bound peroxide and iron displaced out of the porphyrin plane toward the bound ligand[26, 29, 142]. In order to realize such a structure in a heme protein, it would be necessary to break the proximal ligand bond to the iron and force a π-bonded configuration, acting against the steric hindrance of the heme prosthetic group. It turns out that the presence of the strong proximal ligand, which favors the LS state in hexacoordinated Fe^{3+}–OOH^- complexes, is an important restriction on the chemistry of oxygen activation in the heme enzymes. Structure and properties of model Fe^{3+}–OOH^- complexes in heme enzymes have been extensively studied theoretically[56, 89, 91, 93, 96, 97, 145, 146]. These investigations, together with analogous studies on heme[147] and nonheme enzymes[19] have played a definitive role in establishing the currently accepted view of the importance of the second protonation step of the distal oxygen atom of the peroxide ligand for the efficient heterolytic cleavage of the O–O bond and "Compound I" formation.

Early attempts by Estabrook and Peterson to create and stabilize the reduced oxy-ferrous, or "peroxo" complex in cytochrome P450 were first made more than 30 years ago[4, 111]. Subsequent efforts included steady-state and stopped-flow studies of reconstituted systems[148–150], replacing dioxygen by superoxide[151, 152] or peroxides as oxygen donors, or the use of alternative chemical, photochemical[153, 154], and pulse radiolytic[155, 156] methods for fast and efficient reduction of the preformed oxy-ferrous P450. However, only in recent years have reproducible, stable, and high-yield preparations of Fe^{3+}–OOH^- complexes been obtained with cytochrome P450 and other heme systems. This was achieved using the methods of radiolytic reduction of oxy-ferrous precursors in frozen solutions at 77 K[40, 50, 157–159]. Irradiation with high-energy photons from a ^{60}Co gamma-source or using ^{32}P enriched phosphate as

an internal source of high-energy electrons[49, 159] generates radiolytic electrons, which reduce a preformed oxy-ferrous complex which is stabilized against autoxidation by low temperature. Similar approaches have long been used by physicists and chemists in matrix isolation chemistry of highly reactive intermediates[160–162]. In the solid state, at the temperatures below the glass transition where translational and rotational diffusion is minimized, the peroxo complex is stable due to the impediment of trap migration and proton transfer events.

Historically, in biological systems, cryogenic radiolysis was first used as a tool for studies of the nonequilibrium intermediates in heme proteins in the early 1970s[163]. For example, radiolysis of several ferric proteins with different ligands in frozen aqueous-organic solutions at 77 K was shown to produce the corresponding ferrous species, which then could be annealed at elevated temperatures, and the conformational and chemical relaxation processes monitored by EPR and optical spectroscopy methods[164–169]. The first reports on cryoradiolysis of oxy-ferrous complexes in heme proteins and formation of the Fe^{3+}–$OO(H)^{2-(-)}$ "peroxo" complex in hemoglobin, myoglobin, and HRP[158, 170–178] established the characteristic EPR spectrum of Fe^{3+}–$OO(H)^{2-(-)}$ complexes with a signature narrow span of g values (2.3–2.25, 2.2–2.14, and 1.94–1.97). Optical absorption[173, 174, 176], Mossbauer[116], and EPR analysis have been used to characterize the peroxo intermediates in heme enzymes including cytochrome P450 CYP101[158, 176].

Recently, this approach was further expanded to understand the detailed electronic structure and stability of peroxo-ferric intermediates in heme[40, 41, 49, 50, 53, 157, 159, 179–181] and nonheme systems[27, 182–188]. As a result, the direct spectral identification of the intermediates **[5a]** and **[5b]** in several heme proteins has been clearly achieved. Importantly, the EPR spectra were found to be sensitive to the protonation state of the peroxide ligand, but less responsive to the nature of the *trans*-proximal ligand (His or Cys), Table 5.1. UV spectroscopy shows a weak response to the protonation state of the peroxide with the Soret and Q bands shifting by only a few nanometers with iron-peroxo protonation, but high sensitivity to the identity of the proximal ligand. For example, the Soret band of the **[5b]** intermediate appears at 440 nm in the thiolate-ligated cytochrome P450, but at 420 nm in HRP and HO, Figure 5.3.

The radiolytic reduction of **[4]** at 77 K yielded, in most cases, an already protonated hydroperoxo-ferric complex **[5b]**. In several proteins, however, such as oxy-Mb, oxy-HRP, and the D251N variant of cytochrome P450 CYP101, it was possible to observe the unprotonated species **[5a]**. Interestingly, the irradiation of oxy-P450 at 4 K in liquid helium yielded the unprotonated form **[5a]**[41], suggesting that an activation process of proton transfer occurs between liquid helium and ligand nitrogen temperatures. Most exciting is the direct observation of the protein-catalyzed proton transfer event, **[5a]** to form **[5b]**, as the temperature is raised. A second protonation and catalytic conversion of the substrate to a product complex then ensues[41]. Alternatively, a separate uncoupling channel can be opened with peroxide release and direct transition to the resting state of the enzyme, demonstrating the subtle control of proton delivery provided by the protein matrix. The lack of "Compound I" formation on the oxidase pathway in HRP[181] is explained by the inability of this enzyme to deliver this second proton to bound dioxygen, despite the facile formation of the "Compound I" from hydrogen peroxide.

Thus, during the last decade, the method of cryoradiolytic reduction has emerged as a new tool to investigate critical intermediates of redox systems. X-ray-induced radiation chemistry is also increasingly recognized both as a potential source of misinterpretations due to measurement-induced changes of the sample, and as a new, important tool in X-ray crystallography, where the irradiation of protein crystals may be used to deliberately alter the redox state of metals, flavins, disulfides, and other cofactors[42, 189–191]. The chemical details of the radiolytic process in frozen solutions and protein crystals may, however, be quite different. In the former, there is usually a high concentration of glycerol or ethylene glycol present as a cosolvent to improve the optical glass quality of the sample at low temperature. These cosolvents are necessary as effective quenchers of hydroxyl radicals generated by the radiolysis of water. On the other hand, protein crystals can sometimes contain a much lower concentration of organic cosolvents, thus potentially altering the processes operating during low temperature radiolysis and thermal annealing. For example, it was shown[182]

Table 5.1. Electron Paramagnetic Resonance (EPR) Parameters of Mononuclear Peroxo-Ferric Complexes from Select Synthetic Models and Enzymes

Model	Ligands	O_2/e^- donor	g-value	Assignment	References
Synthetic models					
Bztpen(OOH)$^{2+}$	N_4	H_2O_2	2.22, 2.18, 1.97	FeOOH (η_1)	[277]
Bztpen $(O_2)^{2+}$	N_4	H_2O_2, base	7.6, 5.8, 4.5	FeOO (η_2)	[277]
Fe(SMe2N4tren)	N_4S	H_2O_2	2.14, 1.97	FeOOH (η_1)	[278]
Fe(rtpen)	N_5	H_2O_2, acid	2.20, 2.16, 1.96	FeOOH (η_1)	[279]
Fe(rtpen)	N_5	H_2O_2, base	7.6, 5.74	FeOO (η_2)	[279]
Fe(rtpen)	N_5	Methanol	2.32, 2.14, 1.93	FeOCH3 (η_1)	[279]
trispicMeen	N_5	H_2O_2	2.19. 2.12, 1.95	FeOOH (η_1)	[280], [281]
trispicMeen	N_5	H_2O_2, acid	7.4, 5.7, 4.5	FeOO (η_2)	[280], [281]
TPEN	N_5	H_2O_2	2.22, 2.15, 1.97	FeOOH (η_1)	[281]
BLM	N_5	H_2O_2	2.26, 2.17, 1.94	FeOOH (η_1)	[281]
trispicen	N_5	H_2O_2	2.19, 2.14, 1.97	FeOOH (η_1)	[281]
FePMA	N_4	H_2O_2	2.27, 2.18, 1.93	FeOOH (η_1)	[282]
TMC	N_4	KO_2	8.5, 4.23	FeOO (η_2)	[283]
OEC	N_4	KO_2	8.8, 4.24	FeOO (η_2)	[283]
MemMemxyl	N_4	H_2O_2 or KO_2	2.23, 2.16, 1.93 2.25, 2.10, 1.97	FeOOH (η_1)	[284]
FeTPP	N_4O	BuOOH	2.31, 2.16, 1.96	FeOOR (η_1)	[285]
FeEDTA	N_2O_3	H_2O_2	4.2, 4.15, 4.14	FeOO (η_2)	[286]
FeOEP	N_4	Me_4NO_2	9.5, 4.2	FeOO (η_2)	[287]
Enzymatic intermediates					
Horseradish peroxidase	N_4, Histidine	O_2, γ	2.32, 2.18, 1.90 2.27, 2.18, 1.90	FeOOH (η_1)	[181] FeOO (η_1)
Nitric oxide synthase	N_4 Cysteine	O_2, γ	2.26, 2.16, 1.95	FeOO (η_1)	[179]
P450	N_4 Cysteine	O_2, γ	2.25, 2.16, 1.96 2.30, 2.17, 1.96	FeOO (η_1)	[40], [41], [158] FeOOH (η_1)
P450	N_4 Cysteine	BuOOH	2.29, 2.24, 1.96	FeOOR	[144]
Superoxide reductase	His_4 Cysteine	H_2O_2	4.30, 4.15	FeOO (η_2)	[288]
Heme oxygenase	N_4 Histidine	O_2, γ	2.25, 2.17, 1.91 2.37, 2.18, 1.92	FeOO (η_1) FeOOH (η_1)	[54]
Hemoglobin	N_4 Histidine	O_2, γ	2.25, 2.15, 1.97 2.31, 2.18, 1.94	FeOO FeOOH (η_1)	[171], [177]
Myoglobin	N_4 Histidine	O_2, γ		FeOO (η_1) FeOOH (η_1)	[177], [178]
Myoglobin (H64N,V)	N_4 Histidine	H_2O_2	2.29, 2.16, 1.91	FeOOH (η_1)	[138]

that the yield of cryoradiolytically reduced ribonucleotide reductase increases 100-fold when the glycerol concentration is raised from 0% to 50%. An insufficient concentration of the quencher of $OH^{\cdot}$ radicals in the protein crystal will result in oxidative, instead of reductive, modification of redox centers, as occurs during radiolysis at room temperature[192]. The presence of the broad absorption band in the visible and near infrared, characteristic for the trapped electrons in irradiated frozen samples, is an indication of the successful entrapment of hydroxyl radicals which would

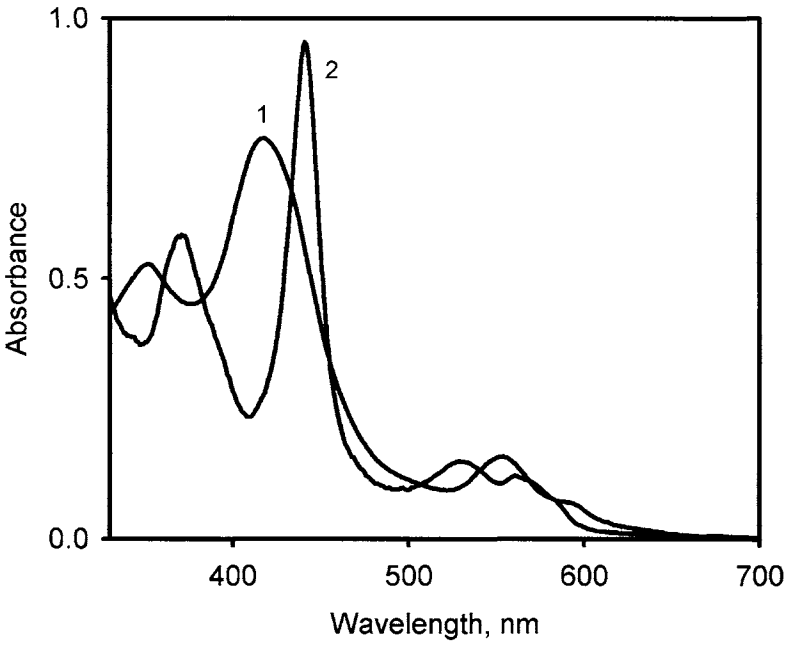

Figure 5.3. Absorption spectra of ferrous-oxy (1) and hydroperoxo-ferric (2) CYP101 at 80 K in 70% glycerol/phosphate buffer.

otherwise recombine with radiolytically produced electrons and thus change the overall radiolysis products observed[159].

EPR spectroscopy has been a powerful tool to investigate radiolytic events in nonheme complexes[178, 193]. Very recently, the signature EPR spectrum for the Fe^{3+}–OOH^- intermediate in activated bleomycin was calculated[85, 194], and the analysis of electronic structure of this complex provides a detailed insight into the chemical stability of Fe^{3+}–OOH^- moiety. Neese also noted that the side-on coordinated iron-peroxide is not activated for O–O bond cleavage[195].

3.3. The Second Branchpoint of P450 Catalysis: Uncoupling with Hydrogen Peroxide Production or Dioxygen Bond Scission

The formation of the hydroperoxo-ferric heme intermediate is a common step in the different pathways, in oxygenases and peroxidases. In heme enzymes, this intermediate can undergo several different transformations, including dissociation from the metal center as a hydroperoxide anion. The dissociation rate for the hydroperoxo-ferric intermediate was estimated for microperoxidase as 10–20 s^{-1} (ref. [60]), and the half-life of these complexes at ambient conditions is likely to be much less than a second. The dissociation of the peroxide ligand, termed the "peroxide shunt," is shown in Figure 5.2 as a conversion of intermediate **[5b]** to **[2]**. It is actually a reversible process since a "peroxygenase" reaction wherein **[2]** is converted to **[5b]** with the addition of exogenous peroxide is also possible. Homolytic cleavage of the O–O bond generating a hydroxyl radical and forming "Compound II," a ferryl-oxo porphyrin complex, or heterolytic scission of the O–O bond to produce water and "Compound I" are also competing processes. The rate constants for the latter two are difficult to measure.

In peroxidases, both protons are essential for heterolytic scission of the O–O bond and subsequent formation of "Compound I." Hence, in order for a similar mechanism in P450 to occur, the enzyme must deliver two protons to provide

the same reaction stoichiometry. If the second proton is not available, the hydroperoxo-ferric complex can still undergo O–O bond scission, but must compete with dissociation of the complex to form hydrogen peroxide. The weakness of peroxide ligand affinity provides a straightforward explanation for the possibility of uncoupling at this stage. The low binding affinity for the peroxide to the heme presumes the shift of the equilibrium **[5b]** $\leftarrow\rightarrow$ **[2]** (Figure 5.2) to the right at low peroxide concentrations. In the case of protonation of the distal oxygen, the O–O bond is cleaved with little or no apparent barrier to the reaction[145]. The resultant "Compound I" **[6]** then reacts with the substrate or undergoes an "oxidase shunt" process to be discussed in a subsequent section.

Unfortunately, the only method of observing the intermediate **[6]** to date in P450 enzymes has been through the reaction of the ferric enzyme with exogenous oxidants such as with *m*-CPBA[196–198]. Direct evidence for the quasi-stable existence of a "Compound I" state, through measurement of both formation and breakdown kinetics, in the cytochromes P450 has been provided only recently[196]. This work utilized hyperthermostable CYP119 and low concentrations of peroxyacid to prevent secondary reactions. The attempts to detect "Compound I" in annealing experiments with cryogenically generated peroxo- and hydroperoxo-ferric complexes in P450, wherein atmospheric dioxygen was sequentially reduced, have not been successful. Possible reasons include the very high rate of substrate oxygenation by this active intermediate, and the subtleties of competing proton delivery pathways. Recently, Friesner *et al.* (personal communication) estimated the barrier height of the reaction **[6]** $\rightarrow$ **[7]** in a P450 model to be only a few kcal mol^{-1}. Thus, the catalytic step of hydrogen abstraction and oxygen rebound[199] may be too rapid to allow intermediate buildup. It is worth noting that **[6]** does not accumulate even during the annealing of cryogenically irradiated oxy-samples at low temperatures 180–200 K. However, the use of solvent exchange and ENDOR spectroscopy[41] of the product complexes was able to provide important indirect evidence for the operation of a "Compound I" like species. The attempts to detect these intermediates in experiments with substrate-free P450 have also proved frustrating.

4. Structural Input into the Mechanisms of P450-Catalyzed Dioxygen Activation

While the purely chemical characterization of P450 reaction intermediates has largely focused on redox-linked changes in heme-iron systems, a complete understanding of intermediate evolution and reactivity must involve the role of the protein scaffold in dioxygen scission. In the case of P450 monooxygenation chemistry, this goal has been formulated into ascertaining the role of the distal pocket in the control of concerted proton transfer events to a reduced oxy-ferrous intermediate, resulting ultimately in heterolytic cleavage to produce the high-valent oxo-ferryl, or "Compound I" intermediate. While numerous processes in the P450 catalytic scheme have been linked to both protons and the ionization state of particular residues, we focus specifically on those proton transfer events that result in the transformation of the reduced ferrous-dioxygen complex, to yield the hydroperoxo intermediate. It is critical not only for any structural model to explain a fully functional "wild-type" P450 reaction chemistry, but also to give a structural basis for the compilation of kinetic and spectroscopic parameters that result from alteration of the active site by mutagenesis or by utilization of a variety of alternate substrate structures. This then translates into knowledge of the inherent reactivity of peroxo-ferric complexes on the pathway to "Compound I" formation, the control of branchpoints and their specific chemistries providing oxidant in nucleophilic, electrophilic, and radical oxidations. The entire P450 reactivity landscape cannot be unified into a strict and inflexible structural model, as some aspects of proton delivery and dioxygen activation are "finely tuned" according to the structural requirements of a particular P450 isozyme active site and are moderated by particular substrate recognition events. Nonetheless, many common structural motifs of the enzyme distal pocket have been evidenced which link the P450 superfamily both mechanistically and structurally.

In order to resolve structural components potentially involved in proton donation to a reduced ferrous-dioxygen or peroxo-anion species,

at least three potential mechanisms of proton delivery should be addressed. The first involves ionization in which the relay of protons involves a change in the charge state of the participating amino acid side chain or solvent moieties. The assignment of participating side chains involved in proton transfer can be inferred by their local pK_a values. In a second type of process, the two participating moieties undergo simultaneous protonation and deprotonation in a Grotthuss mechanism[200], and all moieties participating in hydrogen-bonding interactions can be potential proton donors in the enzyme active site. Finally, the involvement of the peptide bond itself in proton-transfer reactions has been implicated in the proton-transfer mechanism of CcO[201] and model peptides[202].

The identification of potential proton-transfer conduits in P450 catalysis was aided by the first P450 crystallographic structure of CYP101 from *Pseudomonas putida*[203, 204]. While this structure played an indispensable role in visualizing the structural features that years of P450 spectroscopic studies had suggested, such as confirmation of the ligands of the HS substrate bound ferriheme species[114, 205], the positive identification of proton donors in the distal pocket was elusive. In fact, the P450 CYP101 distal pocket was shown to be mostly hydrophobic in nature, in sharp contrast to the polar active sites of other heme enzymes, such as CcP[206] and catalase[207]. Of particular interest was the absence of charged residues in the immediate vicinity of the heme-oxygen binding site, such as the well-characterized acid–base pairs (His–Arg and His–Asp) in the peroxidase and catalase structures, which were thought to mediate dioxygen scission reactions. Readily apparent from these early structures of CYP101 however, was the interruption of normal hydrogen-bonding pattern in the distal I-helix, and hydrogen bonding of the Thr252 side chain with the carbonyl oxygen of Gly248. The resultant "kink" in the I-helix has proved to be a common structural element of most P450 isozymes, and was postulated to create a pocket in which dioxygen could easily bind[203].

4.1. A "Conserved" Alcohol Side Chain in the Active Site of P450

Upon examination of aligned sequences of P450 isozymes, it has become clear that an acid–alcohol side-chain pair is observed in a large majority of P450 genes. These are typically threonine, or in some cases serine, and either aspartate or glutamate[208, 209]. In P450 CYP101 these residues are Asp251 and Thr252. Given their placement in proximity to a heme-dioxygen binding site, a plethora of mutagenesis experiments provided mechanistic suggestions as to their role in stabilizing distal pocket hydrogen-bonding networks and contributing to proton delivery to a peroxo-anion complex. For example, upon mutation of the conserved threonine in CYP101 to a hydrophobic residue, one observes normal kinetic parameters of pyridine nucleotide oxidation and dioxygen consumption, yet the enzyme is fully uncoupled, resulting almost entirely in hydrogen peroxide production rather than the oxidation product hydroxycamphor[210, 211]. This phenotype is certainly not exclusive to the case of CYP101, as similar mutations across the space of P450 phylogeny have shown similar effects[212, 213]. The roles assigned for the "conserved" threonine have included a direct proton source for the peroxo-anion species, or a secondary effect such as stabilizing critical hydrogen bonding and water placement which in turn serves in proton delivery and dioxygen bond scission. Several mutagenesis experiments were designed to discriminate between these two possibilities. Ishimura and colleagues, for instance, demonstrated that the uncoupling reaction resulting in peroxide formation could be avoided if the Thr residue was altered to a side chain which could participate in a hydrogen-bonding interaction[102, 214]. This is particularly well evidenced in the cases of Asn252 and Ser252 mutations in P450 CYP101, where both substitutions retain more than half of the enzyme's activity with regards to pyridine nucleotide oxidation and hydroxylation turnover. Furthermore, through the utilization of unnatural amino acid mutagenesis[215], this avenue could be explored without the stringency of naturally occurring side chains. In an important experiment, the hydroxyl side chain on the conserved alcohol side chain (Thr252 in CYP101) was replaced with a methoxy group[216, 217], although the modified protein was not structurally characterized. Interestingly, the mutant enzyme still retained relatively normal kinetic parameters and nearly full coupling of reducing equivalents into product. Some caution is needed, however, since a common activity of the cytochromes P450 is oxidative

demethylation[218, 219] and a single turnover could result in restoration of a functional hydroxyl at the enzyme active site. Nonetheless, this result has often been interpreted to imply that the Thr252 side chain itself is not directly involved in donating protons to a reduced oxygen intermediate but rather provides its functionality by providing hydrogen-bonding interactions that stabilize active-site waters that are the immediate source of catalytic protons[102].

High-resolution X-ray crystallographic studies of the Thr252Ala mutant have also proved essential in dissecting its role in catalysis and peroxide evolution[35]. Despite mutating this residue to one unable to form a hydrogen bond to the carbonyl oxygen of Gly248, the I-helix "kink" is still observed in the mutant enzyme. The observed new solvent molecules in this mutant structure[35], in particular Wat720, have been inferred as a source of peroxide uncoupling in the mutant. This could arise either by decreased stability of the peroxo ligand in protic solvents or due to the delivery of protons to the proximal oxygen, both of which would result in the release of hydrogen peroxide[93]. The inclusion of extra waters as a potential source of uncoupling has likewise been structurally inferred with a series of camphor analogs bound to CYP101 (ref. [36]) and in the substrate-free enzyme[220]. The use of alternate substrates for the enzyme displayed a varying degree of hydroxylation regiospecificity and uncoupling, resulting either in the production of peroxide or water through an "oxidase" channel[221–223]. The former can often be explained through an increased substrate mobility owing to perturbed hydrogen-bonding interactions with Tyr96[224, 225], but a consistent water-mediated mechanism was difficult to understand for the latter. Nonetheless, the localization and superposition of active-site waters has emerged as being of critical importance in evaluating the proton-aided dioxygen scission and uncoupling chemistries. The crystallographic structure of Thr252Ala also demonstrated that, both the mutated residue and Asp251 residue adopt different conformations[35]. For example, in the Ala252 mutant, the C_β atom moves 1.4 Å away from the dioxygen-binding pocket. In addition, the Asp251 backbone carbonyl has flipped (120°) toward the distal helix. The participation of solvent in mediating P450 catalysis, and the conformational adaptation of the acid–alcohol pair in the distal pocket, have since been shown to be important in understanding peroxo-ferric stabilization and reactivity.

The implication of "extra" water as a potential source of uncoupling has also been included as a parameter in the redesign of the substrate-binding pocket to oxidize hydrocarbon substrates of varying sizes. In the reaction of CYP101 with ethylbenzene, for example, the effective coupling to monooxygenase activity is highly sensitive to the sterics of the substrate-binding pocket[37, 226]. Although a redesign in the distal pocket to accommodate different substrates certainly diminished unwarranted uncoupling pathways, both at the peroxide and oxidase branchpoints, a strict mapping of potential solvation pathways leading to peroxide uncoupling was difficult. Active-site mutagenesis also served as a starting point for the redesign of the active sites in other P450 isozymes to oxidize a number of substrates of varying polarity and size[227–229]. While the precise structural details behind the uncoupling pathways in these "redesigned" enzymes is not yet realized, an important independence of substrate-binding parameters and efficient coupling to monooxygenation is clear.

Even though the presence of an active-site alcohol functionality is highly conserved throughout the landscape of P450 isozymes, one important exception has further established the importance of an active-site hydrogen-bonding network in dioxygen catalysis. CYP107 (P450eryF), which catalyzes the hydroxylation of 6-deoxyerythronolide B in the erythromycin biosynthetic pathway, has an alanine (Ala245) instead of threonine at the conserved position[230, 231]. Despite the absence of the I-helix threonine, a kink or cleft is still retained in the structure and the side chains of Glu360 and Ser246 and a series of ordered water molecules contribute a distal pocket hydrogen-bonding network[232, 233]. Interestingly, the 5-hydroxyl group of the macrolide substrate provides a key H-bonding interaction to a water molecule (Wat564). This water is in a nearly identical position as the threonine hydroxyl moiety in P450 CYP101, thus suggesting a similar proton delivery network via "substrate-assisted catalysis"[234]. Alteration of the substrate C5 and C9 side chains, which normally donates a hydrogen bond to crystallographically observable active-site water (Wat563), results in dramatic effects on catalytic efficiency. Through reintroduction of the active-site hydroxyl in the

Ala245Ser and Ala245Thr mutant enzymes, it appears as though the loss of catalytic activity[235] in the mutant forms correlates with the loss or repositioning of Wat519 (ref. [234]). Nonetheless, these mutants are still able to metabolize the substrate testosterone at different sites[235], implying that a very intricate hydrogen-bonding interplay exists between substrate and enzyme.

Molecular dynamics simulations performed by Harris and Loew[93] provide additional evidence regarding the role of active-site hydrogen bonding in CYP107. By subjecting the peroxo-anion species in the substrate-bound active site of the enzyme to molecular dynamics simulation, two-proton-donating partners, the C5 hydroxyl of the bound substrate, and a bound water Wat519 are seen to form-up hydrogen bonds on the distal oxygen atom of the ligand within 25 ps from initiation of the simulation[93]. Furthermore, connectivity of this hydrogen-bonding network to other exchangeable groups, namely to Wat564, Ser246, and Glu360 suggests a specific proton relay pathway in the enzyme.

In order to properly assess the molecular basis of peroxide forming chemistries in the Thr252X mutants, one must first adequately assess the operant chemical mechanism or mechanisms that give rise to the observed uncoupling. As described earlier, the use of cryogenic irradiation has proven to be an invaluable tool in dissecting the particular intermediate state involved in both P450 monooxygenation and peroxide evolving chemistries. Using low temperature radiolytic reduction of the Thr252Ala mutant ferrous-oxy complex, the enzyme directly forms the protonated peroxo anion[40, 41] as is readily identified by the characteristic shift in g-tensor upon protonation of the distal oxygen[170, 177]. This result is congruent in terms of the unmodified rates of pyridine nucleotide oxidation seen in room temperature studies. First, despite mutation, the Thr252Ala mutant of CYP101, like the wild-type enzyme, is able to protonate the distal oxygen atom of the peroxo-anion species. This in turn, in the course of temperature annealing, decays to the ferriheme resting state with no intervening intermediates. In order to further assign the hydroperoxo-ferric species as the branchpoint of peroxide forming and monooxygenation studies, analogous experiments were performed with the double mutant, Thr252Ala/Asp251Asn, in which the stoichiometric ratio of the two pathways is equal at room temperature[103]. As primary proton transfer is also visible at low temperature in this mutant construct, prior to decay to the resting state, one is able to definitely assess that the protonated peroxo intermediate serves as the branching intermediate of these two pathways.

4.2. The "Conserved" Acid Functionality

As in the case of the conserved hydroxyl moiety in the P450 active site, a conserved acid residue has similarly been implicated as the critical proton transfer vehicle in the P450-catalyzed oxygen activation. Upon mutagenesis of Asp251 in P450 CYP101 to asparagine, a phenotype quite distinct from that of the alcohol mutagenesis is observed[62, 101]. Instead of the normally high, but decoupled, rate of pyridine nucleotide consumption seen in the Thr252Ala mutant, the enzymatic turnover activity of the Asp251Asn mutant is decreased by more than one order of magnitude[39, 62]. With close examination of the separate catalytic parameters of the P450 reaction wheel (Figure 5.2), this decreased activity was specifically localized to second electron transfer and linked protonation events following formation of the ferrous-oxy complex[62, 103]. Unlike the conserved hydroxyl moiety in the immediate vicinity of the heme iron, a number of mutations at this site have revealed that the observed phenotypical variance is poorly explained by a discrete physicochemical property of the amino acid side chain that is inserted. For example, both the Ala251 and Gly251 substitutions behave similarly to that of the isosteric charge reversal Asp251Asn[103]. While these phenotypical consequences of Asp251 mutation are similar throughout many P450 sequences, such as CYP107, the kinetic efficiency of the mutant enzymes often depends on the identity of the substrate being metabolized[150, 236–238].

Some indication of active-site acidic functionality in P450 dioxygen scission can be revealed by the comparative evaluation of kinetic parameters in protonated and deuterated waters. The use of kinetic solvent isotope effects (KSIEs) has been valuable in the determination of potential proton-linked dioxygen activating enzymes such as P450[38, 39], methane monooxygenase (MMO)[239],

and the cytochrome oxidases[240]. It is important to prove that the observed isotopic effects are specifically linked to proton transfer events in the dioxygen cleavage reaction. In the case of cytochrome P450 CYP101, detailed kinetic studies demonstrated that only the second electron transfer rate, and the linked concomitant catalytic steps were sensitive to solvent isotope content[38]. This in turn has allowed the comparable gauging of isotopic sensitivity of the dioxygen cleavage reaction simply through the rate ratio measurements of product formation. Through the comparative analysis of KSIEs and proton inventories of the wild-type and the Asp251Asn mutant CYP101 enzymes, a startling difference in these parameters is observed[39]. In the wild-type enzyme, by fitting the proton inventory to a range of fractionation factors, it was shown that at least two protons are in flight during the observed transition state process. This is consistent with the previously discussed schemes involving distal pocket waters and the hydrogen-bonding Thr252. Furthermore, a 4-fold increase of the KSIE in the Asp251Asn mutant, as well as linear dependences of NADH oxidation rate on bulk proton concentration, intimates that the proton delivery conduit in this mutant is drastically altered, perhaps mediated by a chain of water molecules[39].

In structural terms, crystallographic analysis of the Asp251Asn mutant has provided additional evidence for its involvement in proton delivery[39]. In the wild-type ferric structure of CYP101, the Asp251 side chain itself points away from the distal pocket, and instead interacts in a bifurcated salt-bridge involving Arg186 and Lys178. Although other structural features of the distal pocket remain intact, mutagenesis to Asn251 results in the simultaneous rotation of the Asn251 and Lys178 side chains to the protein surface, resulting in loss of their intermolecular contacts. This in turn is compensated by a new interaction of the Asn251 side chain with Asp182. These multiple motions speak to a highly plastic active site with a resulting open access of the heme iron from the protein surface. While it is difficult to translate the observed KSIE and pH dependence data of the Asn251 single mutant to a rigorous structural model, in part due to difficulty in visualizing active-site mobile waters, it nonetheless remains consistent with a proton delivery mechanism modulated by additional solvent molecules.

As in the case of the Ala252 mutation, the annealing profile of cryogenically reduced oxyferrous Asn251 mutant in P450 CYP101 corroborates the kinetic data just discussed. Unlike the wild-type and Ala252 mutant enzymes, which form the hydroperoxo-ferric complex upon irradiation at 77 K, EPR g-tensors and ^{1}H ENDOR couplings reveal that the primary reduced complex of Asn251 is the unprotonated peroxo-anion species[40, 41]. Upon annealing to the glass transition temperature (190 K), protonation of the peroxo anion occurs, confirming that the phenotypic effects seen in this mutant are localized at primary proton delivery. Moreover, the temperature-dependent annealing profile likewise implies an active-site conformational change in the mutant protein is required to enable efficient proton transfer. This reorientation of the bifurcated salt-bridge during CYP101 catalysis has additionally been implicated by the dependence of turnover rate on hydrostatic[241] and osmotic[242] pressures, as well as detailed photoacoustic calorimetry measurements[243, 244] of both wild-type and mutant cytochromes. These results implicate a distinct and localized re-solvation of active-site residues during substrate turnover. However, localization of these dependences to protonation of the peroxo anion is complicated as it is mixed with analogous reorientations and conformational changes occurring during other catalytic steps such as substrate binding. Nevertheless, the implication of a rotameric flexibility of the Asp251 side chain, stabilized by discriminating electrostatic interactions, presents an important theme in the development of a dynamic model of P450 dioxygen scission.

4.3. Crystallographic Studies of P450 Reaction Intermediates

As with spectroscopic characterization of the peroxo-ferric intermediates in CYP101, one of the most revealing studies of P450 dioxygen activation has been the recent structural resolution of P450 intermediates using cryocrystallography[42]. This work not only provided a molecular picture of P450 intermediate states, but also a road-map for the participation of the distal pocket in the effective delivery of protons to a ferrous-dioxygen species.

Analogous to the spectroscopic work outlined in previous sections, these studies rely on cryogenic radiolytic reduction in the form of exposure to long-wavelength X-rays in order to isolate normally reactive heme-oxygen intermediates. However, in contrast to the spectroscopic analysis of low-temperature reduced samples, the X-ray crystallographic analysis of the X-ray reduced sample is complicated by the fact that the X-rays not only serve as a pulse but also as probe. Therefore, sample radiolysis cannot be totally controlled or eliminated. Diffusion of dioxygen into dithionite-reduced crystals of CYP101 at low temperature, and using short wavelength X-rays to minimize radiolysis, revealed the heretofore structurally uncharacterized P450 ferrous-dioxygen complex. These structural studies nicely complement the low-temperature spectroscopic characterizations.

Structural resolution of oxy-ferrous P450 reveals important differences from the ferrous precursor. The accompaniment of dioxygen ligated in an end-on (η_1 coordination geometry) to the heme-iron correlates to a number of structural nuances. First, and perhaps most obvious, is the presence of two new water molecules, WAT901 and WAT902, in the active site of the enzyme. One of these, WAT901, appears both within hydrogen-bonding distance of the dioxygen ligand and the Thr252 hydroxyl side chain (Figure 5.4). This water molecule is unique to the ferrous-dioxygen structure, but may in fact represent the stabilized water implicated in proton delivery in the threonine mutagenesis and solvent isotope-effect studies outlined above. In addition, the backbone amide of Thr252 occupies a "flipped" orientation allowing not only additional stabilization of this bound water molecule through new hydrogen-bonding interactions with the peptide backbone, but also a new inter-action of the Asp251 backbone carbonyl with the amide and amino groups of Asn255.

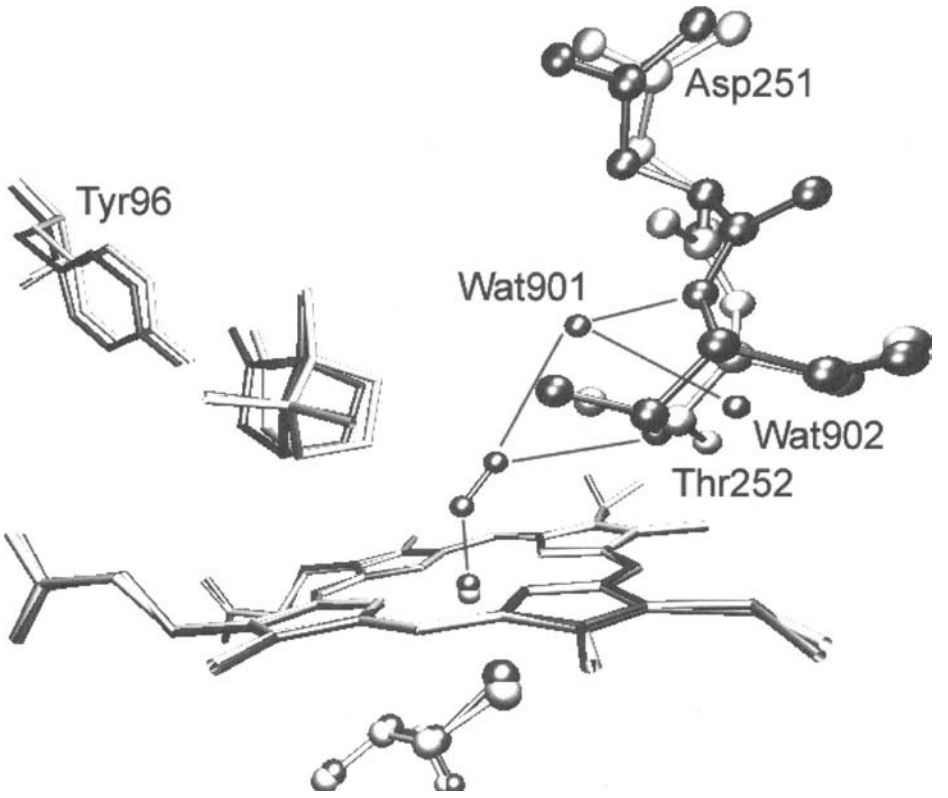

Figure 5.4. Overlaid crystal structures of the ferrous (light) and oxy-ferrous (grey) wild-type CYP101.

The observation of this new hydrogen bond network in the crystals of the ferrous-dioxygen complex provides a satisfying structural explanation of the observed changes in coupling and turnover upon mutation of Asp251Asn and Thr252Ala. In the case of the latter, the threonine hydroxyl, in addition to the backbone amide, donates key stabilization interactions with both bound dioxygen and WAT901. One might expect that the removal of these interactions in the Thr252Ala mutant would have effects at multiple branchpoints of the P450 catalytic cycle. Elimination of this H-bonding interaction would result in a relative destabilization of the oxy-ferrous complex, resulting in an increased rate of autoxidation in the Thr252Ala mutant[211]. In addition, the newly observed water molecule in the ferric Thr252Ala structure, WAT720, is localized about 1 Å further from the dioxygen moiety than in the wild-type oxy-ferrous counterpart. While efficient primary-proton transfer is observed in this mutant in radiolytic studies, the crucial proton transfer step, resulting in water formation and heterolytic cleavage to form "Compound I," is perturbed by the modification of these interactions. Interestingly, the flip of the Asp251 carbonyl is also seen in the Thr252Ala ferric structure, although the rotation is enhanced even more (120° vs 90°).

The structure of the CYP101 wild-type ferrous-dioxygen complex also provides insight into the diminished efficacy of primary proton delivery in the Asp251Asn mutant, as observed in both room temperature kinetics and EPR annealing profiles. The "flip" of the Asp251 backbone is synergistically coupled with the rotation of the Thr252 backbone. In the ferric structure of Asp251Asn, the mutated Asn251 side chain forms new interactions with Asp182 and Asn255, with the latter effectively blocking the interaction with the 251 carbonyl in the "flipped" conformation typical for the oxy complex. Examination of the oxy-ferrous structure of Asp251Asn corroborates this finding, as neither of the catalytically critical solvent molecules is observed (Schlichting *et al.*, unpublished).

The differential solvation of the active site in the Asn251 and Ala252 mutants is also observed in resonance Raman spectra of the ferric cyanide adducts[245]. Specifically, while the wild-type enzyme shows exclusive sensitivity to isotopic exchange in the bent conformer, those of the mutants show spectral changes upon D_2O exchange in both linear and bent conformations. As the bent conformer better represents the ferrous-oxy complex as observed in structural studies, one would expect that it is exclusively subject to isotopic exchange in the wild-type enzyme. Meanwhile, as mutation would cause drastic changes in hydrogen-bond patterning in the distal pocket, it is reasonable to expect isotopically induced vibrational changes of both conformers in the mutants. Crystallography of the cyanide complex of wild-type CYP101, in which the bent conformer is observed, illustrates the interaction of the cyanide ligand with both the threonine hydroxyl and WAT901 (ref. [246]).

By irradiating oxy-ferrous P450 crystals with longer X-ray wavelengths, catalytic processing in the active site can be initiated and structurally resolved. Radiolytic reduction of the oxy-ferrous crystal of CYP101 results in distinct changes localized in the distal pocket. These are perhaps best illustrated in overall electron density difference maps[42]. Most importantly, following introduction of an electron to the preformed oxy-complex is the appearance of a negative difference density localized at the distal oxygen atom of the iron-bound dioxygen. This suggests that oxygen–oxygen bond cleavage has occurred leaving behind a single oxygen atom ligated to the heme iron. The resulting Fe–O bond distance appears shortened to about 1.67 Å, consistent with an iron-oxo "Fe=O" type structure in analogy with that observed in CcP (1.66 Å)[247], HRP (1.70 Å)[191], and catalase (1.76 Å) (Protein Databank entry 1MQF). In these cases, the iron is seen to move to a position slightly above the heme plane. Frustratingly, despite numerous attempts, the resulting "ferryl-oxo" structure does not have a fully occupied oxygen atom and could not be precisely refined without constraints. Nonetheless, the resulting picture is extremely exciting as it shows that the twice-reduced iron-oxygen complex is catalytically competent. Indeed, warming the crystal to a point above the glass transition temperature reveals a structure with clear positive electron density corresponding to a 5-*exo* alcohol hydroxycamphor product. In the product complex, the protein displays the resting state conformation with respect to water and protein structure. No "extra" water molecules appear in the active site and the Thr251–Asp252 backbone conformation

has moved back to that found in the ferric and ferrous camphor-bound complexes.

Further differences between studies performed on frozen solutions and crystals can be attributable to crystal contacts that may have a crucial impact on the dynamics of the system, thereby changing relative rates of subsequent reaction steps. For example, the lack of accumulation of **[6]** in cryoradiolytically reduced frozen solutions[41, 49] but apparently not in crystals[42] may be related to a crystal contact involving the potassium binding site and the carbonyl oxygen atom of Tyr96 in the monoclinic crystal form of CYP101 (Figure 5.5). The hydroxyl group of Tyr96 forms a hydrogen bond with the keto group of camphor, thereby controlling its position, mobility, and thus the distance between the C5 atom of camphor and the reactive oxygen species.

Given the dynamic nature of both intermediates and the isomerization of amino acids of side chains occurring during catalysis, it is not surprising that a number of computational studies have provided subtly different structural mechanisms for the acid–alcohol pair in P450 catalysis. Molecular dynamics simulations of the ferrous-dioxygen complex, prior to its structural resolution described above, suggested a possible role for Thr252 (ref. [92]). During the course of the simulation, the Thr252 hydroxyl, instead of interacting with the Gly248 backbone as modeled initially, preferentially participates in a hydrogen-bonding interaction with the distal oxygen atom. While this interaction persists throughout the simulation, no significant movement of the Asp251 residue was observed. A more direct role for the conserved acid residue in protonation events has very recently been made by Hummer and colleagues[248]. Through the examination of allowable side chain and water fluctuations, utilizing the ferrous-dioxygen state as a precursor, an interesting model for the active-site modulation of proton networks emerges. The authors note that the movement of the Asp251 side chain toward heme-bound dioxygen is almost unrestricted and that the correlated movement of this residue and WAT901 provides effective proton connectivity between these two moieties. The addition of one new water molecule links this local network to the surface of the protein and bulk solvent. While such a dramatic rotation of the Asp251 side chain was

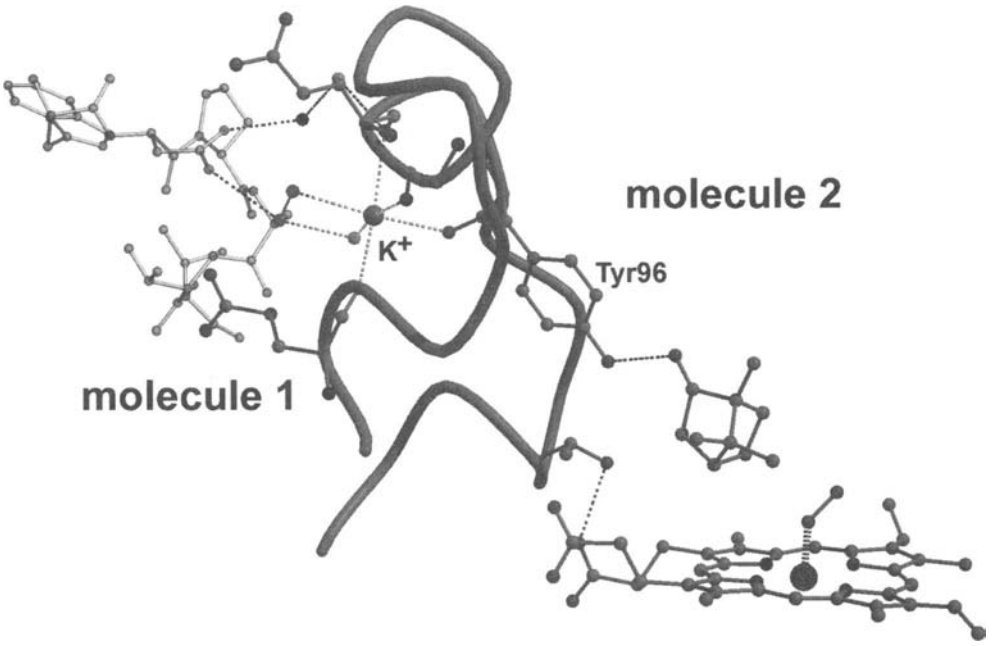

Figure 5.5. A crystal contact at the potassium binding site may influence the dynamics of active-site moieties, particularly Tyr96, and thus indirectly, camphor.

not observed in static crystal structures of resting or dioxygen bound form, such localized conformational changes can obviously significantly modulate proton delivery[101].

4.4. Mechanism-Based Specificity of Proton Transfer

Conformational changes in the distal pocket reorientation can result in drastic effects on the concerted delivery of protons to heme-bound dioxygen. Coupled with these movements is the mechanism-based specificity of proton transfer as determined with the identity of the substrate to the specific P450. The site directed transformation of various heme enzymes to accommodate novel reactivities, both in substrate recognition and through mechanistic inferences of proton delivery, has led to a successful redesign of protein peroxo-iron chemistries. These have been documented in histidine- and thiolate-ligated peroxidases[249–251]. As a feat in reengineering, the directed alteration of reactivity is perhaps best evidenced in the redesign of the dioxygen carrier myoglobin, normally defunct with regards to heteroatom oxygen transfer reactions, to evolve successful monooxygenation chemistries utilizing both hydrogen peroxide[249, 252–254] and dioxygen[255] as the source of oxidant. Within the family of P450 enzymes, two examples of altered reactivity, that of nitric oxide reductase and peroxygenase activities, illuminate mechanisms of active-site complementarity to a specific ligand.

The role of proton transfer in P450-catalyzed nitric oxide reduction has been implicated in a number of computational[256] and experimental studies[257], even though a complete mechanistic assignment of particular nitrogen ligated states remains elusive. Structural analysis of the P450nor active site from *Fusarium oxysporum* by Shiro and colleagues reveal that, unlike CYP101, the active site is comprised of a number of hydrophilic residues[258–260]. Although the "conserved" threonine is localized in the distal pocket (Thr243), the orientation of its side-chain hydroxyl is subtly different, with the alcohol side chain pointing toward the dioxygen-binding site. Whether this reflects a more natural rotamer configuration not seen in the other P450s structurally examined, or if significantly different mechanisms are present is unclear. Similar differences in the relative positioning or flexibility of the threonine have likewise been inferred in the examination of lysine mutants in various P450s[261, 262]. The connectivity of the threonine hydroxyl in P450nor with the I-helix main chain is retained, however, through a solvent molecule, WAT46, to the peptide carbonyl of the *i-4* residue Ala239. Meanwhile, the threonine is involved in another water channel leading to the distal face, and finally bulk solvent. Such a change in polarity of the distal pocket between P450nor and CYP101 is transmitted even into the observed coordination geometry of bound NO and isocyanide complexes[263, 264].

Mutagenesis of the Thr243 residue in P450nor, like the Thr252, results in a drastic reduction of activity, in this case the NADH dependent reduction of NO to N_2O (ref. [265]). Nonetheless, structural analysis of several mutants at this position show very minor perturbations of a plausible proton delivery network[266]. Thus, while the appearance of new water molecules in mutant forms is certainly correlated with a change in a possible proton delivery network, it is difficult to eliminate the possibility that the residue could be functioning by facilitating electron transfer from NADH, as the enzyme directly catalyzes electron transfer from pyridine dinucleotide to the active site. This potential alteration in the role of a conserved threonine in proton delivery is likewise supported by the absence of a conserved acid residue, Ala at position 242, in P450nor, and may reflect an alteration of the distal pocket network to accommodate its unique mechanism. Based on its closer proximity to the NO binding site and similar abolishment of activity upon mutation, another hydroxyl, Ser286, may instead fulfill the role of the conserved P450 hydroxyl[260, 267]. Unlike the Thr243 mutants, structural analysis of various Ser286 mutants shows drastic changes in hydrogen-bonding networks through the systematic destabilization of water networks[260, 267]. The effects of these water networks involving Ser286 appear critical, as even the minor Ser286Thr mutation results in a rotamer configuration, which destabilizes the water and results in a loss of activity. Thus, the active-site proton delivery networks of these heme-oxygen enzymes could be very plastic with the precise dynamical picture of hydrogen ion delivery not yet revealed.

While P450s are typically poor in the utilization of hydrogen peroxide as a dioxygen/electron/proton surrogate in monooxygenation chemistry, a number of P450s have been shown to utilize the peroxygenase pathway in the hydroxylation of fatty acids[268–271]. Evaluation of these enzymes is important in order to assess the isozyme dependence of various P450s in the utilization of the peroxide shunt pathway in catalytic processes. In order to accommodate this fundamental difference in active-site chemistry with these P450s, as in this case both protons and electrons are delivered to the ferriheme moiety, the enzyme is devoid of the conserved acid–alcohol pair. For example, in P450 CYP152A1 these positions in the active site are occupied by Arg242 and Pro243 respectively. However, mutagenesis has succinctly demonstrated their critical role in both fatty acid binding and in the hydrogen peroxide mediated hydroxylation reaction[272]. Likewise, both the presence and positioning of a substrate carboxylate is similarly crucial presumably in an electrostatic interaction with this conserved arginine in peroxygenase[273, 274]. Recent crystallographic structure determination of this P450 by Shiro and coworkers have suggested a very elegant mechanism of substrate mediated peroxygenase catalysis[275]. The fatty acid carboxylate is found in a similar orientation as the Glu183 residue in chloroperoxidase[276] and thereby seems to serve as the catalyst for O–O bond scission of hydrogen peroxide[251]. While the conserved histidine found in chloroperoxidase is absent in the peroxygenase distal pocket, the role of modulating the basicity of the substrate carboxylate may be served by Arg242 (ref. [275]).

4.5. Summary

The rich mechanistic enzymology of the cytochrome P450s has occupied chemists, biochemists, pharmacologists, and toxicologists for over three decades. Are we near to a detailed molecular understanding? We have attempted to convey in this chapter of *Cytochrome P450* the recent discoveries that fill many of the lacunas in our understanding of P450-catalyzed substrate oxidations. We now have a precise three-dimensional structure of the ferrous-oxygenated state, and ample spectroscopic characterization of the ferric-peroxo anion and ferric-hydroperoxo intermediates. In the exogenous oxidant driven pathway, an archaeal P450 allowed facile observation of the formation and breakdown of the "Compound I" ferryl-oxo state. Yet much remains. Stabilization and characterization of the "Compound I" state in the dioxygen reaction has not yet been achieved. With the ability to separate, through time and temperature, the population of multiple "active oxygen" intermediates in P450 catalysis, it remains to precisely define the reactivity profiles of each state and thereby realize a mapping of observed *in vivo* metabolic profiles to specific states in the reaction cycle. An overriding revelation has been the subtle way in which Nature controls the reactivity of atmospheric dioxygen, electrons, and transition metal through delicate hydrogen-bonding interactions. Thus, in a Periclesian control of mechanism, the cytochromes P450 utilize a variety of proton pathways to finely tune this versatile catalyst for critical physiological processes.

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Substrate Oxidation by Cytochrome P450 Enzymes

Paul R. Ortiz de Montellano and James J. De Voss

1. Introduction

The cytochromes P450 are catalytic hemoproteins in which the heme iron atom is coordinated to a proximal cysteine thiolate. This thiolate ligand is responsible for the characteristic Soret absorption maximum of the Fe^{II}–CO complex at ~450 nm and is critical for P450 catalysis[1]. Early site-specific mutagenesis studies with CYP1A2 and $P450_{cam}$ suggested that replacement of the cysteine thiolate by a histidine ligand gave inactive protein[2, 3]. Detailed studies of the $P450_{cam}$ Cys357His mutant have recently confirmed that this mutant enzyme has an almost undetectable catalytic activity[4, 5]. The low camphor-oxidizing activity is paralleled by a low rate of reduction of the iron, an elevated autooxidation rate, and an observable peroxidase activity[4]. The thiolate ligand is thus clearly critical for $P450_{cam}$ function, although the relative contributions of the electronic vs structural perturbations of the mutation to the low catalytic activity remain unclear. These results agree with the results of experiments with thiolate ligated metalloporphyrin model systems[6, 7] and of computational analyses of the role of the thiolate (see Chapter 2)[8, 9].

The heme iron ligand on the distal side is a water molecule in all the available crystal structures of substrate-free P450 enzymes, including $P450_{cam}$ (CYP101)[10], $P450_{BM-3}$ (CYP102)[11, 12], $P450_{terp}$ (CYP108)[13], $P450_{eryF}$ (CYP107A1)[14], $P450_{nor}$ (CYP55A1)[15], *Sulfolobus solfataricus* CYP119[16], *Streptomyces coelicolor* CYP154C1[17], *Mycobacterium tuberculosis* CYP52[18], *Sorangium cellulosum* P450epoK[19], and the mammalian CYP2C5 (see Chapter 3)[20]. Although the thiolate ligand is always present, the distal water ligand appears to be absent in some mammalian enzymes, either because the water does not bind in those structures or because it is displaced by an endogenous ligand[21].

The cytochrome P450 catalytic cycle is initiated by the binding of a substrate, usually with concomitant displacement of the distal water ligand. The ferric heme is then reduced to the ferrous state using electrons provided by suitable electron donor proteins (see Chapter 4). In cytochrome $P450_{cam}$ and many other P450 enzymes, substrate binding is widely believed to be a prerequisite for the transfer of the first electron to the iron, but in some enzymes electron transfer can occur without the prior binding of a substrate[21]. Reduction of the iron is followed by binding of oxygen to give the ferrous dioxy complex. Transfer of a second electron to this complex produces the ferric peroxy anion ($PorFe^{III}$–OO^-, where Por = porphyrin) or, after protonation, the ferric hydroperoxo complex (Por^{III}–OOH) (Figure 6.1). Heterolytic cleavage of

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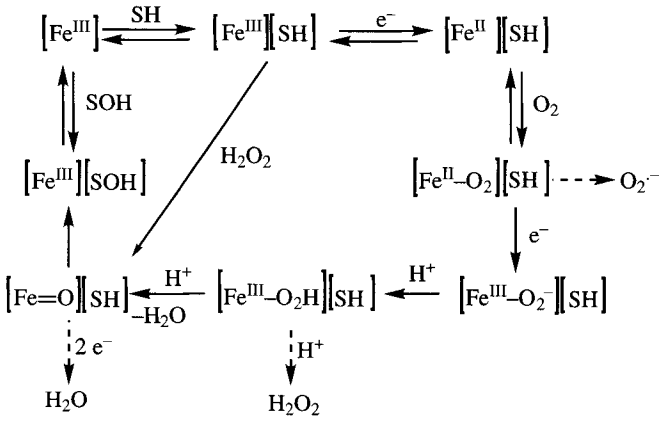


Figure 6.1. The general catalytic cycle of cytochrome P450 enzymes. The [Fe^{III}] stands for the resting ferric state of P450, and SH for a substrate molecule. The shunt pathway utilizing H_2O_2 is shown as are three sites for the uncoupling of the enzyme to give, respectively, $O_2^{\bullet -}$, H_2O_2, or H_2O.

the dioxygen bond in this peroxo intermediate extrudes a molecule of water and forms the putative ferryl oxidizing species (Figure 6.1). Hydrogen bonding of the distal ferric hydroperoxo oxygen, directly or via a water molecule, to a highly conserved threonine facilitates this heterolytic cleavage (see Chapter 5)[22–24]. The ferryl species is thought to be responsible for most P450-catalyzed oxidations, although the ferric peroxo anion and the ferric hydroperoxo complex have been invoked as oxidizing species (see below). It is usually, but not always, possible to circumvent the requirement for activation of molecular oxygen in a so-called "shunt" pathway by employing H_2O_2 or some other peroxide as a co-substrate (Figure 6.1). However, the oxidizing species thus obtained is apparently not identical to that obtained by normal oxygen activation. Thus, peroxides cannot replace molecular oxygen activation in some reactions, they often give product distributions that differ significantly from those obtained by molecular oxygen activation[25–30], and they cause a more rapid degradation of the prosthetic heme group[31].

The P450 oxidation stoichiometry requires one molecule of oxygen and two electrons from NAD(P)H to add one oxygen atom to a substrate. If the ratio of reduced pyridine nucleotide (or oxygen) consumed to product formed is greater than one, the enzyme is said to be uncoupled. Uncoupling occurs when (a) the ferrous dioxy complex reverts to the ferric state by dissociation of superoxide, (b) a molecule of H_2O_2 dissociates from the ferric hydroperoxide complex, or (c) two electrons are used to reduce the ferryl species to a molecule of water before it can be used in a reaction with the substrate (Figure 6.1). The parameters that govern uncoupling at each of the three stages are unclear, but factors that contribute to uncoupling appear to be the degree of uncontrolled water access to the active site, the extent to which the substrate can reside at unproductive distances from the ferryl species, and the presence or absence of sufficiently reactive sites on the substrate molecule[22, 32–34]. The catalytic efficiency of a P450 enzyme can be seriously impaired by uncoupling, as evidenced by the contrast between nearly quantitative coupling in the oxidation of camphor by $P450_{cam}$ and a process that is more than 95% uncoupled when the same enzyme oxidizes styrene[35]. A higher degree of intrinsic uncoupling is often observed in mammalian P450 enzymes, some of which can be suppressed by interaction of the P450 enzyme with reduced cytochrome b_5[36, 37].

2. Activation of Molecular Oxygen

Cryogenic X-ray crystallographic, EPR, ENDOR, and spectroscopic studies have convincingly identified several intermediates in the P450

catalytic cycle (see Chapter 5)[38–40]. These include the ferric, ferrous, ferrous dioxo, and ferric hydroperoxo complexes of $P450_{cam}$. Crystallographic evidence has also been reported for the ferryl species[38], but this intermediate has not been detected by other sensitive cryogenic approaches and its attribution to the ferryl species remains open to question. In low-temperature EPR, ENDOR, and spectroscopic studies, the ferric hydroperoxide intermediate disappears as the hydroxylated camphor product appears without the observation of any intermediate species[39, 40]. All the intermediates in oxygen activation by P450 have thus been observed except for the critical ferryl species, which remains elusive and undefined.

As already mentioned, the activation of molecular oxygen can often be circumvented if peroxides are used as activated oxygen donors. Efforts to identify the reactive oxygen species in these peroxide-supported reactions have been pursued for many years[41–47]. The species that has been spectroscopically detected in these reactions has the spectroscopic signature of a ferryl intermediate[47], but evidence is lacking that this intermediate is the same as that produced by the activation of molecular oxygen. To the contrary, the reactions with peroxides have been shown to produce EPR signals tentatively attributed to tyrosine radicals[41, 45, 46], but no such radicals have been observed under normal turnover conditions. Furthermore, as noted earlier, the peroxide-mediated reactions do not always faithfully reproduce the normal reactions.

Two additional intermediates, the ferric peroxy anion and ferric hydroperoxo complex, have been proposed to substitute for the ferryl as the actual oxidizing species in at least some P450 reactions. The role of the ferric peroxy anion in some reactions is supported by good evidence and is discussed in the section on carbon–carbon bond cleavage reactions (see Section 8), but the proposed role of the ferric hydroperoxide in electrophilic double bond and heteroatom oxidations is discussed here.

The current interest in the ferric hydroperoxo complex as a P450-oxidizing species derives largely from the work by Vaz *et al.*, who observed that mutation of the conserved threonine (Thr303) in CYP2E1 to an alanine decreased the allylic hydroxylation of cyclohexene, *cis*-2-butene, and *trans*-2-butene, but increased the epoxidation of the same three substrates plus styrene[48]. To rationalize this observation, the authors argued that hydroxylation is mediated exclusively by the ferryl whereas epoxidation can be mediated by both the ferryl and ferric hydroperoxide intermediates. Thus, impairing formation of the ferryl species by removing the catalytic threonine would decrease hydroxylation but have little effect upon epoxidation. However, in contrast to the results with the CYP2E1 T303A mutant, the corresponding T302A mutant of CYP2B4 exhibited both decreased hydroxylation and epoxidation rates. This discrepancy does not necessarily contradict the hypothesis, as it could reflect differential changes in the active sites of the two proteins in addition to elimination of the hydrogen bond that facilitates ferryl formation. In a more recent study in which Thr252, the catalytic threonine of $P450_{cam}$, was mutated to an alanine, it was found that camphor hydroxylation was suppressed, but the epoxidation of an olefinic camphor analogue could still be observed[49]. However, the epoxidation reaction occurred at a much slower rate (<20%) despite the expectation that the steady-state level of the ferric hydroperoxide should be elevated. This finding is consistent with the prediction by computational studies that the ferric hydroperoxo complex should be a very poor olefin-oxidizing agent[50]. These results argue that in the wild-type proteins, the ferric hydroperoxide makes no more than a small contribution to epoxidation, and none to hydroxylation.

In a second study, the *N*-oxidation of amines by CYP2B4 and its T302A mutant supported by either NADPH-cytochrome P450 reductase or H_2O_2 was investigated[51]. In contrast to what would be expected if the ferric hydroperoxide were a primary catalytic species, the rates of *N*-demethylation and *N*-oxidation of *N*,*N*-dimethylaniline were both decreased in the mutant. However, as these activities were also decreased when H_2O_2 or phenyliodosobenzene was used in a shunt reaction, little can be said from these results relative to the role of the ferryl vs ferric hydroperoxide species in these reactions. The oxidation of *para*-substituted phenols via an *ipso*-substitution mechanism using the CYP2E1 T303A and CYP2B4 T302A mutants has also given contradictory results (Figure 6.2)[52]. The T303A CYP2E1 mutation increased the rates of *ipso*-substitution with 10 *para* substituents ranging from a chloride to a *tert*-butyl group but did not increase or decrease the rate of reaction of *para*-fluorophenol, by far the most active of the investigated substrates

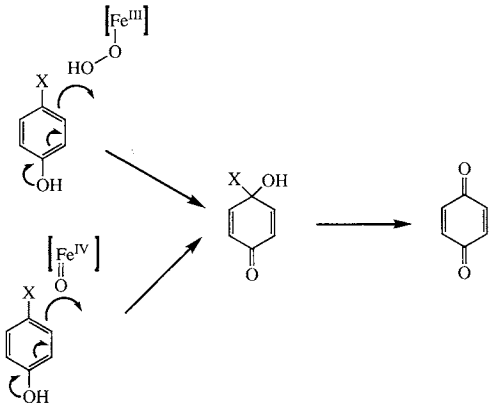


Figure 6.2. Hypothetical *ipso*-substitution mechanism involving the ferric hydroperoxo complex and ferryl species as the potential oxidizing species[52].

for this reaction. Furthermore, although the increase in the reaction rate correlated well with the substituent electronegativity for wild-type CYP2E1, the reaction with the mutant gave a biphasic correlation that included a region in which the reactivity was shown to *decrease* with increasing electron withdrawal[52]. CYP2B4 exhibited only a low activity in this reaction and this activity was not greatly changed when the conserved threonine was mutated to an alanine. It has finally also been proposed that the ferric hydroperoxo complex may play a role in the hydroxylation of saturated hydrocarbons[53–55]. As discussed in Section 3, these studies indicate that a second (or altered) oxidant contributes to the oxidation in threonine mutant enzymes, and suggest that a minor fraction of the reaction products are formed via nonobligate cationic intermediates, but do not specifically implicate the ferric hydroperoxo species in hydrocarbon hydroxylation reactions. It may be relevant to the observation of two oxidizing species that hydrogen bonding to the thiolate ligand, which is very sensitive to structure, has been found in calculations to govern the distribution of unpaired electron density between the porphyrin and protein[56].

In sum, all the reaction intermediates with the exception of the ferryl species have been clearly detected and identified in the catalytic cycle of at least one P450 enzyme. The two instances in which it has been proposed that the ferryl species was detected have shortcomings, one because the finding is not reproduced with other detection techniques, and two because the ferryl produced with peroxides may not be identical to the reactive species formed by oxygen activation. Although it has not been reliably detected as a normal intermediate, the ferryl species is nevertheless almost certainly responsible for the majority of the chemistry supported by P450 enzymes. The circumstantial and contradictory evidence so far available does not provide strong support for significant involvement of the ferric hydroperoxide species in normal P450-catalyzed reactions. Although mutation of the conserved threonine appears in some instances to cause the apparent intervention of a second differentiable oxidizing species, the evidence does not actually indicate the nature of this second species. Computational comparison of the ferric hydroperoxo and ferryl reactivities suggests that the ferric hydroperoxo complex is a poor oxidizing agent unlikely to contribute significantly to P450 catalysis (see Chapter 2)[57, 58].

3. Hydrocarbon Hydroxylation

Proposals on the mechanism of hydrocarbon hydroxylation have become increasingly complex and sophisticated over the past decade. The most widely accepted mechanism involving hydrogen atom abstraction by the ferryl oxygen followed by rebound recombination of the resulting carbon radical with the iron-bound oxygen, first clearly stated in 1978[59], has more recently been challenged, primarily on the basis of work with radical clock probes. As already discussed, the high-valent oxidizing species responsible for most, if not all, cytochrome P450 substrate oxidations is likely to be the iron(IV)oxo porphyrin radical cation ($Por^{+\bullet}Fe^{IV}{=}O$). Recent calculations support this formulation[60, 61], although they suggest that the radical density may reside to a greater or lesser extent on the thiolate iron ligand or other protein residues (see Chapter 2). In the conventional oxygen rebound mechanism, the $Por^{+\bullet}Fe^{IV}{=}O$ species abstracts a hydrogen atom from a carbon of the substrate, producing a $PorFe^{IV}$–OH species and a carbon radical. The Fe^{IV}–OH species, which can also be viewed as a complex of Fe^{III} with a hydroxyl radical, then undergoes a recombination step in which the hydroxyl radical equivalent and carbon radical combine to produce the hydroxylated product. The discrete radical intermediate

proposed in this mechanism readily explains the repeated experimental observation of high intrinsic isotope effects (often >10)[59, 62, 63], partial scrambling of substrate stereochemistry[59, 64, 65], and incidence of allylic rearrangements in P450-catalyzed hydroxylations[64, 65]. Scrambling of stereochemistry has been seen in many situations and includes the early observation that $P450_{cam}$, removes either a 5-*exo* or 5-*endo* hydrogen from camphor but transfers the oxygen exclusively to the 5-*exo* position to yield the 5-*exo*-hydroxy product[65]. Allylic rearrangements, indicative of a delocalized intermediate, have been observed with 3,4,5,6-tetrachlorocyclohexene and other cyclohexenes[66, 68], linoleic acid[67], and a variety of other compounds (Figure 6.3). In the same vein, the recently observed cleavage of a carbon–carbon bond in the P450-catalyzed oxidation of marmesin is most plausibly rationalized by a mechanism involving a carbon radical intermediate (Figure 6.4)[69].

Since 1987[70], major efforts have been made to use radical clocks to estimate the rate of the oxygen rebound step and the lifetime of the radical intermediate in the hydroxylation reaction. In radical clocks, a strained—usually cyclopropyl—ring is directly bonded to the carbon that is the proposed site of the radical intermediate. The ring strain inherent in a cyclopropyl carbinyl (or related) radical leads to a rapid and essentially irreversible rearrangement to the corresponding homoallylic radical. In the case of P450 hydroxylation, the hydroxyl group can be delivered either to the cyclopropyl carbinyl or rearranged homoallylic radical, and the ratio of the two resulting products is determined by the relative magnitudes of the rate constants for radical quenching (k_t, Figure 6.5) and rearrangement (k_r, Figure 6.5). As the intrinsic rearrangement rate can be independently measured in nonbiological experiments, one can calculate both the lifetime of the radical and the rate of radical recombination from the ratio of unrearranged to rearranged products. The first experiments with substrates containing simple, unsubstituted cyclopropyl rings ($k_r = 1.3 \times 10^8\ s^{-1}$) only gave unrearranged products[71–73]. However, bicyclo[2.1.0] pentane, which gives a radical that rearranges much faster ($k_r = 2.4 \times 10^9\ s^{-1}$) due to the additional strain in the system[74], was converted by cytochrome P450 into a mixture of rearranged and unrearranged products from which a rebound rate of $1.4 \times 10^{10}\ s^{-1}$ could be calculated[70, 74].

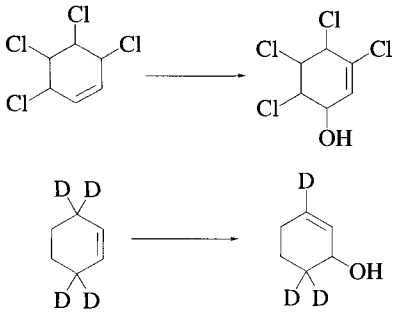


Figure 6.3. Allylic rearrangements observed in the hydroxylation by cytochrome P450 of two substituted cyclohexenes.

Radical clocks of increasing sophistication were subsequently employed to define better the radical intermediate in hydrocarbon hydroxylation[74–79]. Addition of substituents to the cyclopropyl ring increases the rate of the ring-opening reaction. For example, the rearrangement rate for a 2-aryl substituted cyclopropyl carbinyl radical in solution is approximately 1,000-times faster than that of the parent compound without the 2-aryl group[76]. Experiments with these faster radical clocks should have increased the proportion of rearranged products and thus led to a more accurate value for the lifetime of the radical. Contrary to expectation, the measured rate of the radical recombination step appeared to increase in parallel with the rate of the probe rearrangement, resulting in a lower rather than higher proportion of the rearranged

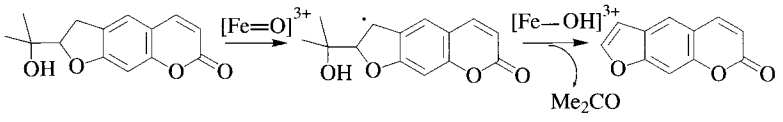


Figure 6.4. Mechanism proposed for the unusual P450-catalyzed carbon–carbon bond fragmentation observed during the biosynthesis of psoralen[69].

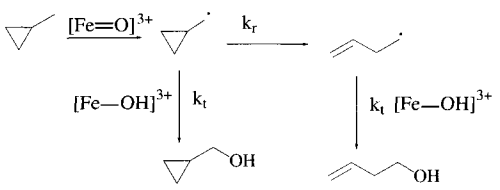


Figure 6.5. The principle of radical clock probes of the cytochrome P450 mechanism based on the methylcyclopropyl radical rearrangement.

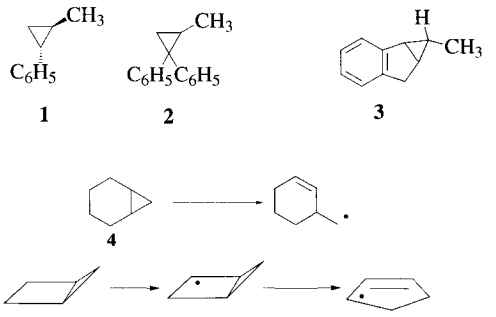


Figure 6.6. Examples of radical clock probes of the cytochrome P450 mechanism, including the radical rearrangements typical of norcarane and bicyclo[2.1.0]pentane.

product[76, 78]. When the very small amount of rearranged product was used to calculate the radical rebound rate and radical lifetime, values were obtained that challenged the existence of a discrete radical intermediate. Thus, the 2-phenyl and 2,2-diphenyl substituted probes **1** and **2** (Figure 6.6) used by Atkinson and Ingold yielded a radical rebound rate of $2\text{–}7 \times 10^{12}\ s^{-1}$ [76], values that approach the limiting rate constant of approximately $6 \times 10^{12}\ s^{-1}$ at 37°C imposed by transition state theory. At these rates, the existence of a discrete radical intermediate is reduced to a question of semantics. The observation of similar high rates with other radical clocks, the demonstration that even large substrates undergo significant motion within P450 active sites, and the observation of intramolecular isotope effects with some of the probes, suggests that the rearrangement is not being suppressed by interaction of the probe with the protein structure[77]. Indeed, analysis of the products formed from the structurally rigid probe **3** gave the unbelievably high rebound rate of $1.4 \times 10^{13}\ s^{-1}$ [78]. These results argued that a radical intermediate was not mandatory in hydrocarbon hydroxylation. However, recent experimental work has again complicated the radical clock results[80, 81]. The oxidation of norcarane (**4**) by four P450 enzymes gave a radical rebound rate of $\sim 10^{10}\ s^{-1}$, a rate very close to that of $1.4 \times 10^{10}\ s^{-1}$ from the original experiments with bicyclo[2.1.0] pentane (Figure 6.6)[70, 74]. Similar experiments with spiro[2,4]octane, a related structure that rearranges much more slowly than norcarane, did not, as expected, detectably yield rearranged products[80].

The discrepancies among the radical clocks, which give credible radical lifetimes in the case of simple cyclopropylmethyl and bicyclic probes, but impossibly short lifetimes with the phenylcyclopropylcarbinyl probes, suggests that the hydroxylation reaction may be more complex than predicted by a simple radical rebound mechanism. Norcarane and bicyclo[2.1.0]pentane differ from most of the radical clocks examined to date in that the radical is located on a secondary rather than primary carbon. This led to the proposal that the extent of rearrangement might depend on both the initial hydrogen abstraction transition state and the subsequent radical recombination transition state, and that the shift from one to the other might be easier for methyl probes because of the tighter transition state due to the higher C–H bond strength and smaller size of the methyl group[80]. However, the finding that four (2-phenyl)cyclopropylalkyl probes, in which the radical also resides on a secondary (or tertiary) carbon, give very high recombination rates is at odds with this explanation[54]. An attractive solution for this dilemma is provided by the two-state reactivity theory, which postulates a competition between two parallel reaction pathways (see Chapter 2). One of these pathways is equivalent to a concerted oxygen insertion and the other akin to a conventional radical recombination mechanism, *and the dominant pathway in any given situation is substrate and environment dependent.* A substrate-dependent reaction mechanism readily rationalizes the differences in the results obtained with the different radical clock probes. Reevaluation of the radical clock results and additional experimental and theoretical work are required to satisfactorily reconcile the contradictory results provided by the probes.

The conclusion from some of the radical clock data that P450 hydroxylation might not proceed

via a radical intermediate led Newcomb to propose that oxygen might be inserted into the C–H via a concerted, nonradical mechanism[75, 79]. According to this proposal, the hydroxylation traverses a bifurcated transition state that allows some of the probes to leak into a radical rearrangement manifold, thus explaining the observation of rearranged products. A shortcoming of this rationale is that it must explain a large diversity of reaction outcomes, including radical clock rearrangements, allylic transpositions, and stereochemical scrambling, by postulating a range of electronically and structurally different bifurcated transition states. Furthermore, even more complex structural rearrangements have been reported that are most consistent with the intervention of a radical intermediate. For example, dieldrin **5** is converted into the intramolecularly bridged ketone **6** and Dolphin has demonstrated that this product is directly produced when **5** is oxidized by a highly halogenated iron porphyrin (Figure 6.7)[82]. The alcohol **7** is also produced metabolically *in vivo* and *in vitro*, but no chemical or biochemical conditions have yet been found that will convert it to the ketone. The most reasonable explanation for the formation of the ketone product involves hydrogen abstraction to give a carbon radical, cyclization of this radical driven by the formation of a chlorine stabilized radical, recombination with the iron-bound hydroxy radical to give the alcohol, and elimination of HCl to give the ketone (Figure 6.7).

A mechanism that reconciles the differences in the radical clock results has been postulated by Shaik on the basis of theoretical calculations. This mechanistic hypothesis, which elegantly combines an essentially concerted insertion and a nonconcerted radical reaction in a single pathway[61, 83–86], is at once complex, intriguing, and satisfying. As discussed in detail in Chapter 2, the oxidizing species is an Fe^{IV}=O porphyrin radical cation that is present as two approximately equienergetic electromers, one in a doublet spin state and the other in a quartet spin state[60, 61]. Intuitively, this small difference can be viewed as arising from the combination of two electrons with unpaired spins in the d orbitals of the iron and a third unpaired electron in the a_{2u} orbital of the porphyrin (Figure 6.8). The quartet and singlet species abstract a hydrogen atom from the hydrocarbon through nearly identical transition states, which readily explains the measured isotope effects and the similarities in the reactivities of the P450 enzymes and the *t*-BuO• radical[87, 88]. The transition states lead to complexes in which the alkyl radical is weakly coordinated to the iron-bound hydroxyl. These complexes, which can be in a doublet or quartet spin state and are again of nearly the same energy, derive from the corresponding doublet and quartet states of the ferryl species. The species in the doublet spin state can collapse to the hydroxylated product in a virtually barrierless (i.e., essentially concerted), nonsynchronous reaction with no true intermediate. The porphyrin cation acts to accept an electron and

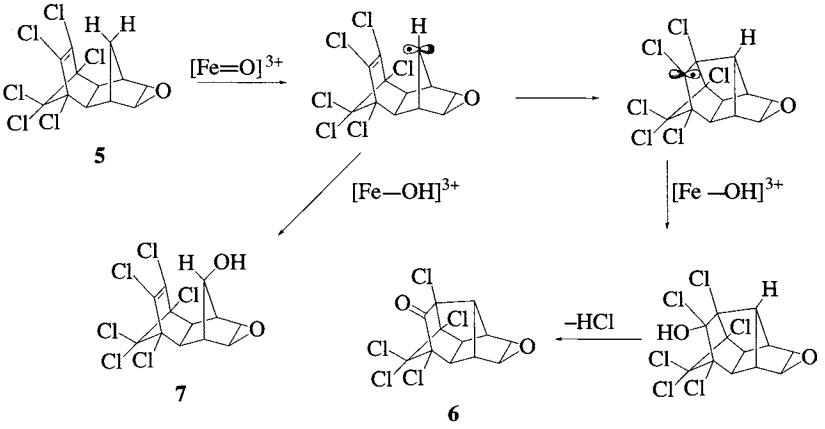


Figure 6.7. Unusual reactions observed in the oxidation of dieldrin.

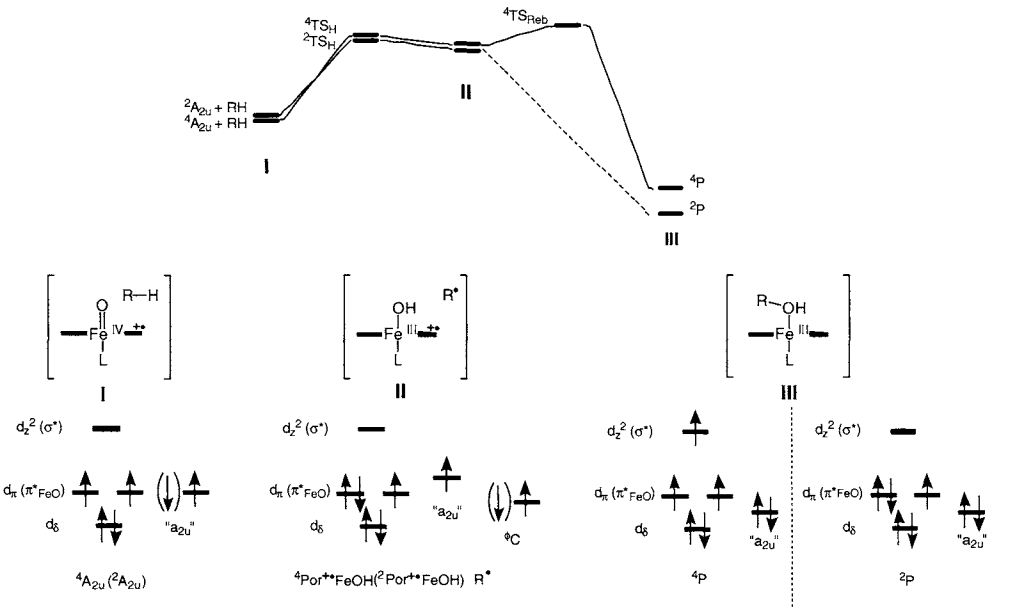


Figure 6.8. The ferryl radical cation–substrate complex (**I**) has two unpaired electrons in the iron d_π orbitals and one in the a_{2u} porphyrin orbital. This may give rise to either a quartet ($^4A_{2u}$) state if all spins are unpaired or, if the spin of the electron in the a_{2u} orbital is inverted, the complex is in a doublet ($^2A_{2u}$) state. (Another possible pair of doublet/quartet configurations has a filled a_{2u} orbital and a single electron in $p\pi$[S] orbital.) In the first step, a hydrogen atom is abstracted from the substrate and an electron is transferred to either the iron, reducing it to the Fe^{III} state (**II**), or to the porphyrin, quenching the radical cation, the former being suggested by calculations. Each of these "intermediates" (**II**) can be in a quartet or doublet state, depending on the electron pairing between the carbon radical R• and the electrons in the iron porphyrin system. Low-spin **II** collapses to a low-spin product (**III**) in a rebound reaction that is virtually barrierless while high-spin **II** traverses a significant barrier to arrive at high-spin **III**. Thus, only high-spin **II** is believed to behave as a true intermediate (radical). The energy diagram corresponding to the transformation is shown above. This scheme is based on the work of Shaik and coworkers.

the thiolate ligand increases its interaction with the iron atom and both of these processes facilitate the effectively concerted nature of the transformation. In contrast, the quartet species must traverse a significant energy barrier to form the product, thus allowing the formation of true intermediate radicals and the consequent reactions (rearrangements) that this implies. The calculated barrier to product formation in this pathway appears to arise from the fact that in the recombination reaction, to maintain the quartet spin state, one electron must reside in a relatively high energy (σ^*) d_z^2 iron orbital and this leads to a loss of bonding across the S–Fe–O axis. In general terms, the above mechanism is a specific example of the two-state reactivity paradigm recently proposed as an important feature in understanding organometallic reactivity (see Chapter 2)[85].

Our discussion of the P450 mechanism to this point has been based on the assumption that the reactive intermediate is a ferryl species. However, the possibility has been raised that the ferric hydroperoxide rather than the ferryl species might be the active agent in carbon hydroxylation[53, 55, 89]. A direct role for the Fe^{III}–OOH intermediate in substrate oxidation was postulated to explain the formation of ring opened products in the oxidation of *trans*-1-methyl-2-(4-trifluoromethyl)phenylcyclopropane (**8**) by CYP2B1 (Figure 6.9)[53]. Radical and cationic pathways could both explain the formation of these ring-opened products, but the authors favored the cationic pathway and proposed that the cationic products arose from an oxidation mediated directly by the Fe^{III}–OOH intermediate. Subsequent studies focused on oxidation of the

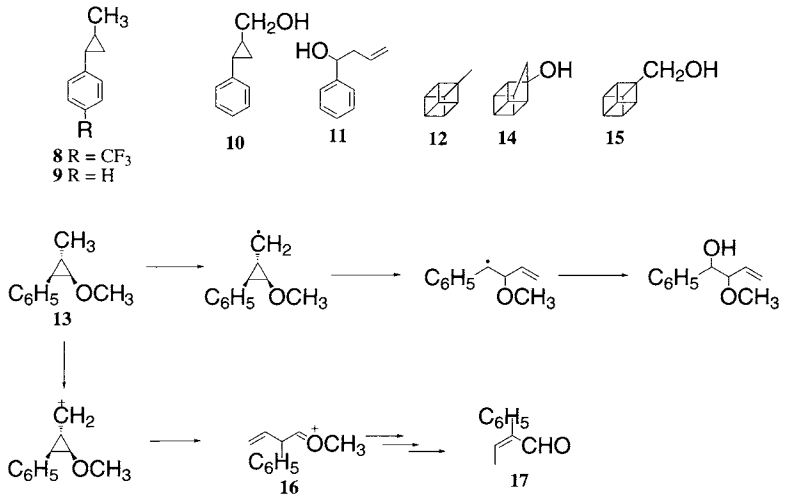


Figure 6.9. Probes of the P450 mechanism, showing the two rearrangement pathways for one of the probes intended to distinguish between radical and cation intermediates.

radical clock probes **8** and **9** by the CYP2B4 T302A mutant, in which the threonine mutant thought to facilitate the formation of the ferryl species by hydrogen bonding to the Fe^{III}–OOH complex was replaced by an alanine. CYP2B4 and its T302A mutant oxidized **9** to products obtained by reaction with the methyl group (including unrearranged **10** and rearranged **11**) and the phenyl ring. Although the same ratio of ring opened to ring closed products was obtained with the wild-type and mutant enzymes, the ratio of phenyl to methyl oxidation products was higher with the T302A mutant. This shift in product profile was interpreted as evidence for the involvement in the mutant enzyme of a second oxiding species that preferentially oxidized the phenyl group. Substitution of the phenyl group in **8** with an electron-withdrawing CF_3 group, which disfavors electrophilic attack on the aromatic ring, resulted in a change in the ratio of ring opened to ring closed products. This change was also interpreted as resulting from an alteration in the nature of the oxiding species, specifically, as evidence for involvement of the Fe^{III}–OOH intermediate in methyl group hydroxylation. However, different explanations are possible for the nature of the two oxidizing species. For example, differences may exist in the proportions of the low spin (LS) and high spin (HS) ferryl species produced by the wild-type and mutant enzymes in the Shaik two-state reactivity model (see Chapter 2), or the ferryl species may be perturbed by differential hydrogen bonding or other environmental factors, giving rise to distinguishable ferryl reactivities. It is important to note, of course, that evidence for a second oxidizing species in the absence of the threonine that is required for normal oxygen activation does not necessarily mean that the second oxidizing species is relevant to catalysis in the presence of the threonine.

In fact, cryogenic ENDOR studies with $P450_{cam}$ provide direct evidence that the ferric hydroperoxide is not involved in camphor hydroxylation[39]. In this work, the ferric hydroperoxide complex of $P450_{cam}$ was prepared at 77 K by radiolytic reduction of the camphor-bound ferrous dioxygen complex. EPR and ENDOR experiments then demonstrated that the hydroperoxide complex was smoothly and quantitatively converted on warming to ~200 K to a complex of the enzyme with the normal 5-*exo*-hydroxycamphor product. The hydroxyl of the 5-*exo*-hydroxycamphor was coordinated to the iron atom in the product formed immediately upon warming, as expected for insertion of a ferryl oxygen into a C–H bond. If the ferric hydroperoxide had been the oxidizing species, the initially formed hydroxycamphor product would incorporate the distal oxygen of the peroxide and the proximal oxygen would have remained bound

to the iron. Coordination of the product hydroxyl to the iron would therefore require displacement of the water ligand, an unlikely exchange reaction at 200 K. Furthermore, ENDOR studies demonstrated that the C-5 hydrogen abstracted from the camphor was bound to the hydroxyl oxygen of the product, in accord with a ferryl insertion mechanism but not with oxidation by the ferric hydroperoxide.

The formation of products from **12** and **13** suggestive of a cationic intermediate is relevant to arguments for participation of the Fe^{III}–OOH species in C–H oxidation, as insertion of the terminal hydroperoxide "HO^+" into a C–H bond would give a protonated alcohol that readily explains the observation of (carbo)cationic rearrangements. Minor amounts of such rearrangement products are obtained in the oxidation of methylcubane **12** and the methylcyclopropane **13** (Figure 6.9)[89]. Formation of small amounts of homocubyl alcohol **14** along with alcohol **15** upon oxidation of the methyl group of **12** provides evidence for cationic intermediates as the cation but not radical derived from the methyl group of **12** rearranges to **14**. However, one shortcoming of **12** as a mechanistic probe, as pointed out by the authors, is that the cationic rearrangement product is readily identified but the products of the radical pathway are too unstable to detect. This is not a shortcoming in the case of **13**. Ring opening of the cyclopropylcarbinyl radical derived from this probe to the benzylic radical gives rise to structurally related products, while the corresponding cation is cleaved in the opposite direction to give first oxonium ion **16** and then aldehyde **17** (Figure 6.9). These radical and carbocationic ring-opening reactions occur with very high regioselectivity: in the case of the cation reaction, the indicated ring opening is favored by >1000 : 1 relative to the direction of ring opening observed with the radical. The traces of aldehyde **17** observed in the oxidation of **13** by a P450 enzyme thus provide credible evidence for at least a minor pathway involving a cationic intermediate. A corollary, however, is that cations cannot be mandatory intermediates in normal hydrocarbon hydroxylation, otherwise, much higher amounts of the rearranged products **12** and **13** would be expected. The cationic intermediate thus diverges from the normal hydroxylation at some branch point in the reaction trajectory.

Two mechanisms have been considered for the formation of cationic intermediates. In the first of these, electron transfer from the radical intermediate in the conventional oxygen rebound mechanism occurs more rapidly than oxygen transfer and produces a cationic intermediate. Direct electron transfer from a substrate to the P450-oxidizing species is proposed to occur in the oxidation of electron-rich nitrogen atoms (see Section 4), and a precedent exists for such electron transfer even in the case of hydrocarbon hydroxylation. Thus, the product formed in the P450-catalyzed oxidation of quadricyclane **18**, a hydrocarbon with a low oxidation potential, is most consistent with electron transfer to give a radical cation that is subsequently trapped by the iron-bound oxygen (Figure 6.10)[90]. An alternative explanation for the formation of cation rearrangement products is that the oxidation is mediated by the P450 Fe^{III}–OOH complex. As already noted, insertion of the hydroperoxide oxygen into a C–H bond would produce the protonated alcohol that could either undergo deprotonation to give the alcohol or ionization to give the carbocation. In this mechanism, the carbocation is not an intermediate in the hydroxylation reaction but rather a result of decomposition of the initial product. The observation that the proportion of cationic products formed in the oxidation of **12** and **13** increases when the catalytic threonine is mutated to an alanine can be taken as evidence for this mechanism[89], but can also be rationalized by environmental perturbation of a ferryl oxidizing species. In any case, the significance of results with the mutants to catalysis by the native enzyme is questionable. A further important observation is that the extent of cationic rearrangement does not parallel the stability of the cation, as shown by studies of a series of 1-aryl-2-alkyl-cyclopropane probes with either a phenyl or *para*-trifluoromethylphenyl aryl group and an ethyl, propyl, or isopropyl alkyl group[55]. Although ring opened

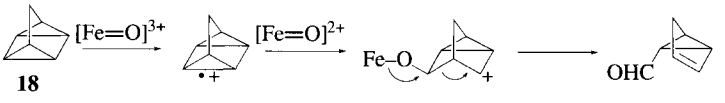


Figure 6.10. The cytochrome P450-catalyzed oxidation of quadricyclane involving an initial electron abstraction step.

products were observed with these probes, substitution with cation-stabilizing groups yielded *less* rather than more rearrangement products, contrary to expectation if a protonated alcohol were the initial oxidation product[55]. This result is not consistent with initial formation of a protonated alcohol product, and therefore does not support the involvement of an Fe^{III}–OOH species in hydrocarbon oxidation. In contrast, the two-state radical recombination model (see Chapter 2) predicts that better electron donors such as a methine hydrogen will favor oxidation through the LS manifold which proceeds without the formation of a true radical intermediate, precluding further oxidation of the carbon to a cation.

In sum, the evidence in hand is most consistent with Shaik's two-state reactivity model for hydrocarbon hydroxylation. Both the LS and HS electromers of the ferryl species first abstract a hydrogen atom. The LS species then follows a barrierless, effectively concerted, pathway to give the unrearranged product, while the presence of a significant energy barrier in the pathway for the HS species leads to the formation of a true radical intermediate. Radical formation readily explains reaction characteristics such as high kinetic deuterium isotope effects, stereochemical scrambling, and structural rearrangement, while the existence of two parallel pathways allows the reactivity pattern to vary both with the substrate and the enzyme. Carbocationic species are not obligate hydroxylation intermediates, as a much higher extent of cationic rearrangements would be expected if they were. Small amounts of cationic products, however, apparently can be formed by a pathway that diverges from that responsible for normal hydroxylation, possibly by a mechanism that involves electron transfer from a radical intermediate to the active oxidizing species.

4. Heteroatom Oxidation and Dealkylation

Heteroatom oxidation can be viewed as part of the hydrocarbon hydroxylation continuum if the reaction outcome is the introduction of a hydroxyl group onto the carbon attached to the heteroatom, an outcome that is usually followed by elimination of the heteroatom with concomitant formation of a carbonyl group. *O*-dealkylation, *N*-dealkylation, *S*-dealkylation, and oxidative dehalogenation are all examples of such processes. However, although in appearance the outcome may be initially similar, that is, carbon hydroxylation, the mechanisms of hydroxylation of a simple C–H bond and a C–H bond adjacent to a heteroatom need not be the same. Whereas carbon hydroxylation involves hydrogen abstraction from the carbon to give a transient carbon radical, hydroxylation adjacent to a heteroatom may proceed via initial electron abstraction from the heteroatom to give the radical cation, deprotonation of the adjacent carbon, and recombination of the resulting carbon radical with the iron-bound hydroxyl radical (Figure 6.11). Such a mechanism is particularly feasible in the case of atoms such as nitrogen that are electron rich and easily oxidized. Of course, if the oxygen rebound reaction is faster than deprotonation of the adjacent carbon, the oxidation will simply result in oxidation of the heteroatom, as in conversion of a trialkylamine to a trialkylamine *N*-oxide or a dialkyl sulfide to a sulfoxide. The reaction outcome, and the mechanism of the reaction (i.e., electron abstraction vs C–H oxygen insertion) would thus be expected to depend on the ease of oxidation of the heteroatom and the relative energies of the various reaction pathways.

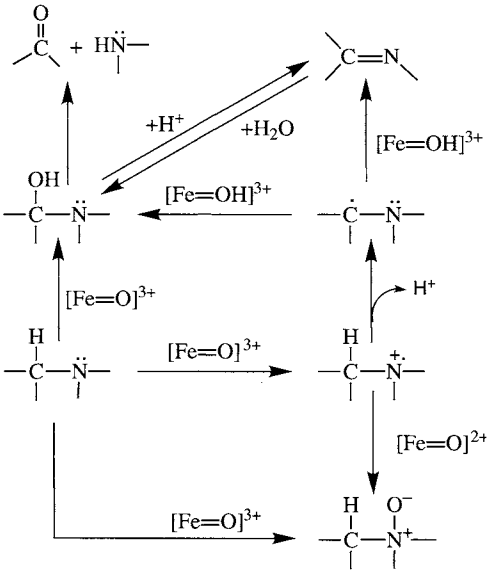


Figure 6.11. The reaction manifold for the oxidation of an amine or related nitrogen compound by cytochrome P450.

In view of the high electronegativity of oxygen and, therefore, the high energy required to remove one of its electrons, it is not surprising that the oxidation of a dialkyl ether occurs by direct reaction of the oxidizing species with the C–H bond, although the distribution of electron density in the hydroxylation transition state is likely to be perturbed by the vicinal oxygen atom. There is no evidence for electron abstraction from the oxygen to give an oxygen radical cation, and also none for transfer of the ferryl oxygen to an ether or alkoxy oxygen to give a 1,1-disubstituted zwitterionic peroxo species. Thus, the *O*-dealkylation of ethers with at least one C–H bond next to the oxygen is most appropriately treated as an extension of carbon hydroxylation. In the absence of a vicinal C–H bond, ether functions are resistant to P450-catalyzed oxidation.

The oxidation of nitrogen compounds gives more diverse products than that of oxygen compounds, and the attendant mechanisms are more varied and controversial. As a result of the lower electronegativity of nitrogen relative to oxygen, oxidation of a nitrogen center can result in hydroxylation of the adjacent carbon (and thus *N*-dealkylation), oxidation of the nitrogen electron pair to an *N*-oxide, or insertion of an oxygen into an N–H bond. The key mechanistic question in the P450-catalyzed oxidation of a nitrogen function is whether it proceeds via initial electron abstraction to give the nitrogen radical cation, followed by either collapse to give the *N*-oxide or deprotonation of the vicinal carbon to give a carbon radical that combines with the iron-bound oxygen to give the alcohol. Oxidation of an N–H bond to a hydroxylamine by a mechanism analogous to that for carbon hydroxylation is also possible. To the extent that the nitrogen radical cation mechanism is operative, *N*-oxide formation and *N*-dealkylation represent the divergent partitioning of a common intermediate (Figure 6.11). The alternative is that nitrogen oxidation and carbon hydroxylation are independent reactions, one involving reaction of the ferryl oxygen with the nitrogen electron pair, and the other a more or less classical hydroxylation of the vicinal carbon.

The ability of P450 enzymes to oxidize nitrogen atoms to radical cations via an initial electron abstraction is supported by a number of experimental results. The finding that the 4-alkyl group of 3,5-(*bis*)carbethoxy-2,6-dimethyl-4-alkyl-1,4-dihydropyridines is transferred to a nitrogen of the prosthetic heme group almost certainly requires initial oxidation of the dihydropyridine nitrogen to a radical cation (see Chapter 7)[91]. This heme alkylation reaction occurs upon oxidation of the dihydropyridine within the P450 active site. However, in incubations with liver microsomes, the dihydropyridine can also be oxidized by trace metals in the solution. This adventitious oxidation releases the 4-alkyl group as a spin-trappable free radical that obscures whatever radical release, if any, occurs in the enzyme-catalyzed reaction[92, 93]. No nitrogen radicals have been observed by EPR in P450 systems that are free of medium-dependent peroxidative reactions except perhaps for the reported observation of nitrogen radicals in the CYP2B1-catalyzed oxidation of *para*-substituted dimethylanilines supported by iodosobenzene rather than NADPH-cytochrome P450 reductase[93]. It is possible that radical formation is detected in this system due to a faster rate of substrate oxidation with phenyliodosobenzene than P450 reductase, but the possibility also exists that the radicals stem from an abnormal process supported by the artificial oxidizing agent.

Differences in the deuterium isotope effects for the oxidation of carbons adjacent to nitrogen vs oxygen suggest that the two reactions are mediated by different mechanisms. The intramolecular isotope effect for *O*-deethylation of deuterium substituted 7-ethoxycoumarin is ~13 and for *O*-demethylation of 4-nitroanisole with a trideuterio methyl group is 10[94, 95]. In contrast, an isotope effect of 2–3 is obtained for the *N*-dealkylation of *N*-methyl-*N*-trideuteriomethylaniline[62, 94]. The intrinsic isotope effects for *O*-dealkylation thus approach those for normal carbon hydroxylation reactions, but the isotope effects for *N*-dealkylation are much lower.

The intramolecular isotope effects observed for *N*-demethylation of *para*-substituted *N*-methyl-*N*-trideuteriomethylanilines increased from $k_H/k_D = 2.0$ to 3.3 in traversing the range from the most electron withdrawing (NO_2) to the most electron-donating (CH_3O) substituent[96]. Similar values were obtained in an earlier study[92]. The *N*-dealkylation rates are also increased by electron-donating substituents[96], in accord with the finding that the rates of oxidation of 12 *para*-substituted *N,N*-dimethylanilines can be fit to the equation $\log V_{max} = 0.41\pi - 1.02\sigma - 0.023MR$

+ 1.72 ($r = 0.953$)[97], where π is the partition coefficient, σ, the Hammett electronic factor, and MR, the molecular refractivity. A strong enhancement of the reaction by electron-donating substituents is indicated by the negative sign and magnitude of the cofactor of the electronic parameter. These results are consistent with a mechanism involving a nitrogen radical cation.

The oxidizing species of cytochrome P450 is thought to have some resemblance to the well-characterized ferryl species of horseradish peroxide (HRP). It is therefore relevant that the rates of reduction of the HRP Compound I by *para*-substituted *N,N*-dimethylanilines and *N,N*-di(trideuteriomethyl)anilines correlate with the oxidation potentials of the anilines[98, 99], and that no kinetic isotope effects are observed in these reactions[99]. Earlier studies measuring the rates of product formation rather than the reduction of the ferryl species led to the conclusion that dimethylaniline *N*-demethylation by HRP is subject to large isotope effects[94, 100]. However, in the case of HRP, product formation involves a disproportionation reaction subsequent to radical cation formation that is subject to a large isotope effect. The more recent results of Goto *et al.*[99] are most consistent with a single electron transfer (SET) mechanism for the *N*-dealkylation mediated by HRP. A similar dependence of the *N*-dealkylation reaction on the substrate oxidation potential was observed in the reactions mediated by $TMP^{+}\bullet Fe^{IV}{=}O$ (TMP = 5,10,15,20-tetramesitylporphyrin dianion), but with this P450 model system, kinetic isotope effects of 3.9 (p-CH_3O) to 6.2 (p-NO_2) and intramolecular isotope effect of 1.3–5.9 for the corresponding *N*-methyl-*N*-trideuteriomethylanilines were observed[99]. The authors argue that isotope effects are observed in this instance due to a competition between back electron transfer from the partially reduced $TMPFe^{IV}{=}O$ species to the nitrogen radical cation and oxygen transfer to the nitrogen radical cation. The implication of a SET mechanism in both the HRP and P450 model reactions agrees with earlier findings on the rates of oxidation of dimethylaniline by HRP, CYP2B1, and two metalloporphyrin systems. Unfortunately, similar spectroscopic rate studies cannot be carried out with P450 itself because the analogous "Compound I" form of cytochrome P450 is not sufficiently stable.

Support for a nitrogen radical cation mechanism in P450-catalyzed *N*-dealkylation reactions is provided by the fact that *N*-demethylation is usually favored over *N*-deethylation. For example, *N*-demethylation is favored over *N*-deethylation by a factor of 16:1 in the CYP2B1-catalyzed oxidation of *N*-methyl-*N*-ethylaniline[94]. Electronic factors would favor deethylation if a direct hydroxylation of the carbon adjacent to the nitrogen were involved because the incipient radical would be better stabilized by hyperconjugation. In contrast, demethylation should be favored if the reaction involves deprotonation of an initially formed nitrogen radical cation because a methyl is more acidic than an ethyl methylene. However, these arguments are not unambiguous because the electronic differences in the two reactions may be masked by the differences in the steric effects of a methyl and an ethyl group. Thus, steric effects possibly account for the observation that *N*-demethylation of *N*-methyl-*N*-alkyl-4-chlorobenzamides is favored over *N*-deethylation by a factor of 2.2, as this reaction (see below) is thought to involve direct oxygen insertion into the C–H bond[101]. Despite this caveat, the observation of intramolecular isotope effects ≤ 2.0 in the *N*-dealkylation of *N,N*-dimethylaniline by CYP2B1, chloroperoxidase, and metalloporphyrin models, but of isotope effects >8 in the corresponding reactions catalyzed by hemoglobin, HRP, and prostaglandin H synthase, provides independent evidence that the iron-bound oxygen removes a proton from the carbon adjacent to the nitrogen radical cation in the first but not the second set of proteins (Figure 6.12)[102]. The pK_a values of the protons next to the trimethylamine and dimethylaniline

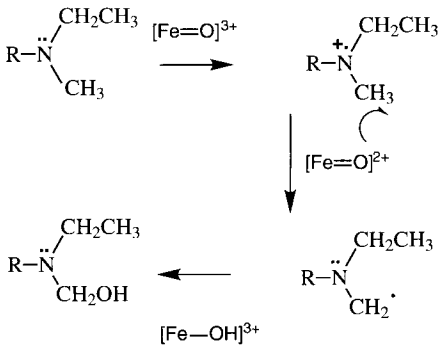


Figure 6.12. The nitrogen radical cation pathway proposed for a P450-catalyzed *N*-dealkylation reaction.

nitrogen radical cations have been estimated to be, respectively, ~15 and 9[102, 103].

It has been proposed that the isotope effects for the P450-catalyzed oxidations of hydrocarbons and alkylamines are similar to those observed in the reactions of the same substrates with the *tert*-butoxyl radical[87, 104]. The finding that the measured kinetic isotope effects for the hydrogen abstraction from benzylic methyl groups fall on the same line as those for the *N*-demethylation of 4-substituted dimethylanilines has, therefore, been advanced as evidence that *N*-dealkylations occur via a hydrogen abstraction (HAT) rather than SET mechanism, in contrast to the evidence for a SET mechanism provided by the already discussed isotope effect and rate data. Recent studies of the rates and isotope effects in the reactions of deuterated 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridines with the *tert*-butoxyl radical suggest, however, that the *tert*-butoxyl radical may not fully mimic the enzymatic oxidizing species, as the *tert*-butoxyl radical did not discriminate between C–H bonds that differed in bond strength by as much as 10 kcal /mol^{-1} [105]. A correlation of reaction rates with bond dissociation energies for a range of alkylamine C–H bonds indicated that entropy factors make a significant contribution to the rate constant. The poor correlation between the absolute rates and C–H bond strengths is caused by differences in the entropy required to align the C–H bond to be broken with the electron pair on the adjacent nitrogen. A correlation between isotope effects and bond strength may nevertheless be observed if the entropy factor is similar for a series of substrates and thus cancels out, but caution must be exercised in interpreting such correlations.

Cyclopropylamines can, in principle, be used to probe the mechanism of nitrogen oxidation because a cyclopropyl substituent on a nitrogen radical cation can undergo ring-opening reactions analogous to those of a cyclopropyl attached to a carbon radical. The inactivation of P450 enzymes by cyclopropyl amines was postulated in early studies to involve formation of the nitrogen radical cation, ring cleavage to give an iminium carbon radical species, and alkylation of the heme group[106, 107]. The observation of a correlation between the one-electron oxidation potentials and the rates of P450 inactivation by a series of nitrogen-, oxygen-, and halide-substituted cyclopropanes offers circumstantial support for an electron abstraction mechanism[108]. Hanzlik has recently examined the oxidation of cyclopropylamine probes by HRP, an enzyme that demethylates *N*,*N*-dimethyl- and *N*-methyl,*N*-isopropylaniline in the presence of H_2O_2 and O_2[109]. *N*-cyclopropyl,*N*-methylaniline (**19**) was oxidized to both *N*-methylaniline and a cyclized product that derives from a radical-based ring opening of the cyclopropyl group (Figure 6.13). However, cyclopropanone was obtained as the product in the P450-catalyzed oxidation of *N*-methyl, *N*-(1-alkylcyclopropyl)aniline[110]. No trace of radical cation products such as those obtained when the same substrate was oxidized by HRP were detected. The results suggest that direct oxygen insertion into the cyclopropyl group occurs faster than oxidation of

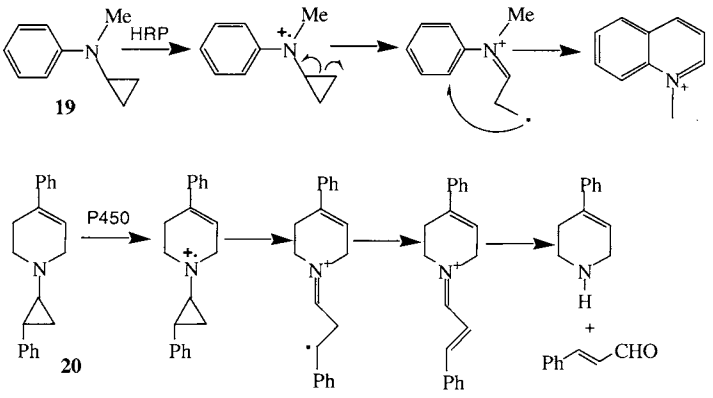


Figure 6.13. Probes designed to investigate whether a nitrogen radical cation is involved in P450-catalyzed *N*-dealkylation reactions.

the nitrogen, and thus is more consistent with a HAT than a SET mechanism.

4-Phenyl-*trans*-1-(2-phenylcyclopropyl)-1,2,3,6-tetrahydropyridine (**20**) is oxidized by rat liver microsomes to cinnamaldehyde and *N*-dealkylated tetrahydropyridine in addition to conventional metabolites (Figure 6.13). The first two metabolites have been postulated to be formed via the nitrogen radical cation, cyclopropyl ring opening, electron abstraction, proton elimination to form the double bond, and hydrolysis of the iminium link to release the aldehyde[111].

The oxidation potential for an amide nitrogen is higher than that of an amine due to the electron-withdrawing effect of the carbonyl group. The P450-catalyzed *N*-dealkylation of amides with a deuterated and undeuterated *N*-methyl substituent, $RCON(CH_3)(CD_3)$, are subject to intramolecular isotope effects of 4–7[112, 113]. The corresponding isotope effect for the *N*-dealkylation catalyzed by a model porphyrin was 5.6[114], a much higher value than that observed for the electrochemical reaction that proceeds via a nitrogen radical cation intermediate[115]. These results suggest that amide *N*-dealkylation occurs by direct carbon hydroxylation as a result of the higher oxidation potential of the amide nitrogen.

P450-catalyzed oxygen transfer to amines to give the *N*-oxide or *N*-hydroxyl product is generally considered to involve nitrogen radical cation formation followed by recombination with the ferryl oxygen (Figure 6.11)[116, 117]. As these reactions are less amenable to direct investigation with mechanistic probes, the postulate of a radical cation mechanism rests largely on the evidence for such intermediates in *N*-dealkylation reactions. However, the reported absence of a systematic relationship between the electronic properties of substituents and the rates of oxidation of anilines and dimethylanilines to hydroxylamines and *N*-oxides, respectively, provides no support for such a mechanism[117, 118]. One as yet unproven explanation for the absence of a correlation is that the stability of the *N*-oxide–iron complex makes dissociation of the *N*-oxide partially rate limiting[117]. Hlavica has also proposed that *N*-oxide formation is mediated by the P450-ferric hydroperoxide rather than ferryl species based, in part, on the observation that the oxidation of *N*,*N*-dimethylaniline to the corresponding *N*-oxide mediated by CYP2B4 is both inhibited by superoxide dismutase and supported by H_2O_2[119]. However, these criteria do not differentiate between the ferric hydroperoxide and ferryl species, as one is the precursor of the other. In the absence of more direct evidence, it is not possible to determine whether *N*-oxide and hydroxylamine formation proceeds by a mechanism other than reaction with the ferryl species to give a transient nitrogen radical cation intermediate.

The conversion of thioethers to sulfoxides or *S*-dealkylated products, as noted for the oxidation of amines, could involve the formation of a transient sulfur radical cation or direct oxygen transfer to either the sulfur or the adjacent carbon. If any function can be oxidized by direct reaction with the P450 ferric hydroperoxide species, it would appear to be a thioether sulfur. The ratio of *S*-dealkylation to sulfoxidation products was reported in early work to correlate well with the acidity of the protons adjacent to the sulfur[120]. Furthermore, electron-donating groups modestly accelerate the rate of formation of sulfoxides from substituted thioanisoles (Hammett $\rho^+ = -0.16$), and of the sulfoxides to the corresponding sulfones (Hammett $\rho^+ = -0.2$)[121, 122]. In an intramolecular competition experiment, it has been found that the thioether of thianthrene-5-oxide is oxidized in preference to the symmetry-related thioether sulfoxide function, confirming the expected higher reactivity of the sulfide than sulfoxide[123]. Unfortunately, although these results indicate that sulfoxidation occurs most readily at electron-rich sulfur atoms, the magnitudes of the effects are such that they cannot be used to unambiguously differentiate between radical cation and oxygen transfer mechanisms for sulfur oxidation.

Bacciochi *et al.* have shown that a radical cation localized on the trimethoxy-substituted phenyl ring is generated when a thioether, with a 2-(3,4,5-trimethoxyphenyl)ethyl on one side and a phenyl group on the other, is chemically oxidized. They have then shown that liver microsomes exclusively oxidize a thioether with a 3,4,5-trimethoxyphenyl group on one of the sulfur-bearing carbons to a sulfoxide rather than to the products expected from formation of the trimethoxyphenyl radical cation[124]. In view of the finding that chemical oxidation yields the trimethoxyphenyl radical cation, they conclude that the sulfoxide is formed by direct oxygen transfer from the P450 to the sulfur because oxygen rebound to the

sulfur should be slow if the unpaired electron is not localized on the sulfur. In contrast, HRP gives both sulfoxidation and radical cation cleavage products, but only the sulfoxide is formed with chloroperoxidase[125]. However, these studies all assume that the ferryl oxidation is equivalent to a chemical oxidation in favoring the trimethoxyphenyl ring over the sulfur. It is possible that the P450-oxidizing species oxidizes the sulfur to the radical cation and recombines with it faster than the electron can be transferred to the trimethoxyphenyl ring. A similar caveat tempers the conclusions that can be drawn from the finding that phenyl cyclopropyl sulfide is oxidized by *Mortierella isabellina* to the sulfoxide without opening of the cyclopropyl ring[126].

In sum, the course of heteroatom oxidation appears to be sensitive to the oxidation potential of the heteroatom, the acidity of hydrogens on the adjacent carbon, and steric factors. The bulk of the evidence suggests that oxidation of the nitrogen in amines generally involves electron abstraction followed primarily by *N*-dealkylation if a labile proton is present, or nitrogen oxidation if it is not. As the nitrogen oxidation potential increases, there is a shift toward direct insertion into the C–H bond, as is thought to occur in the *N*-dealkylation of amides. *O*-dealkylation reactions are mediated by direct insertion of the oxygen into the vicinal C–H bond, as electron abstraction from the oxygen is too difficult due to the high oxygen oxidation potential. Transfer of the P450 ferryl oxygen to an oxygen atom to give a peroxide is not known, presumably for the same reason. The mechanisms of sulfur oxidation remain more uncertain, but the limited evidence suggests that sulfur dealkylation may occur via direct insertion into the vicinal C–H bond, as found for *O*-dealkylation, in a reaction that diverges from that responsible for sulfoxidation.

5. Olefin and Acetylene Oxidation

No critical experimental advances have been made in the past decade toward a fuller understanding of the mechanisms of P450-catalyzed epoxidation reactions, although new insights into the process are emerging from computational studies. The finding that P450-catalyzed olefin epoxidations invariably proceed with retention of the olefin stereochemistry, as illustrated by results on the epoxidation of *cis*-stilbene[127], oleic acid[128], and *trans*-[1-^{2}H]-1-octene[129] supports the view that the reaction occurs by a concerted mechanism. However, retention of stereochemistry does not preclude a nonconcerted mechanism, a point clearly made by the fact that the stereochemistry is retained in most carbon hydroxylation reactions even though they are mediated by a stepwise radical mechanism. Early isotope effect studies also provided evidence for a nonconcerted, or at least asynchronous, reaction mechanism[130]. Thus, substitution of a deuterium on the internal but not terminal carbon of the exocyclic double bond of *p*-methyl- and *p*-phenylstyrene led to the observation of an inverse secondary isotope effect k_H/k_D = 0.93 in the epoxidation reaction. Similar isotope effects would be expected at both carbons if the two carbon–oxygen bonds were formed simultaneously. However, differential secondary isotope effects can be observed if one carbon–oxygen bond is formed to a significantly greater extent than the other in an asynchronous epoxidation transition state. This is clearly shown by the finding that olefin epoxidation by *meta*-chloroperbenzoic acid, a well-established concerted reaction, also gives differential secondary isotope effects, in this instance, the isotope effect being seen on the terminal but not internal carbon[131].

Acetylenes, like olefins, have oxidizable π-bonds, although it is harder to oxidize an acetylenic than an olefinic π-bond because the triple bond is shorter and stronger. Nevertheless, cytochrome P450 readily oxidizes terminal acetylenic bonds to give ketene products in which the terminal hydrogen of the acetylene has migrated quantitatively to the internal acetylenic carbon (Figure 6.14)[132, 133]. The oxirene that would result from "epoxidation" of the triple bond has not been detected and may not form, as oxirenes are highly unstable moieties. If formed, they would be expected to rearrange to the observed products. The finding of a substantial kinetic isotope effect on formation of the arylacetic acid metabolites when the terminal hydrogen of the arylacetylene is replaced by a deuterium indicates that the hydrogen migration occurs in the rate-determining step of the catalytic process[133, 134]. This finding, and the observation that the same products are formed with similar isotope effects in the oxidation of aryl acetylenes by *m*-chloroperbenzoic acid[132, 134],

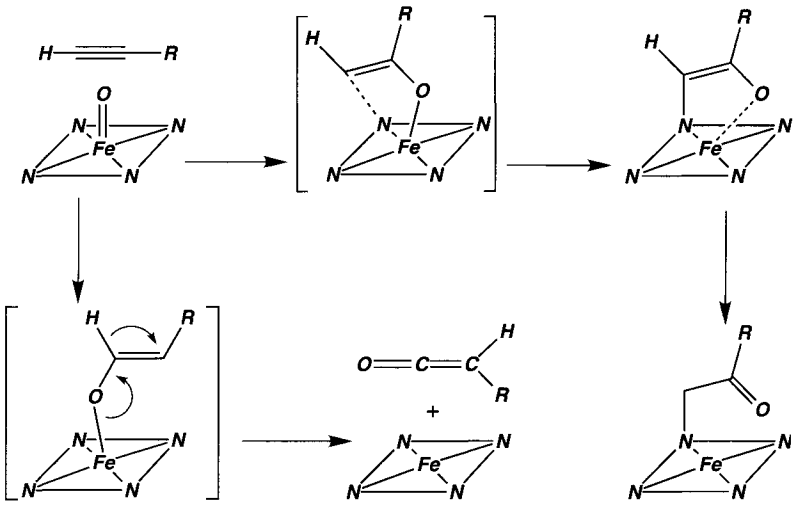


Figure 6.14. Schematic mechanism for the oxidation of a terminal acetylene by cytochrome P450, showing that addition at the terminal end of the triple bond leads to a ketene product, whereas addition to the internal carbon results in alkylation of a nitrogen of the heme group.

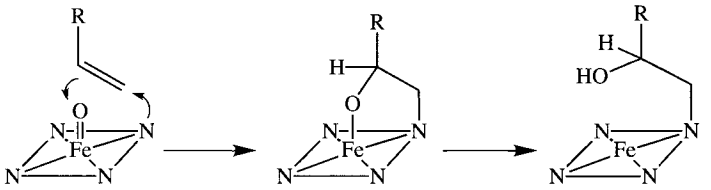


Figure 6.15. Alkylation of a nitrogen of the heme during the P450-catalyzed oxidation of a terminal double bond.

suggest that the hydrogen migrates to the vicinal carbon as the oxygen is transferred to the terminal carbon in a concerted reaction process. The demonstration that 2-biphenylpropionic acid is a minor product in the CYP1A1- and CYP1A2-catalyzed oxidation of 4-(1-propynyl)biphenyl indicates that it is also possible to oxidize a triple bond with the migration of an alkyl group rather than a hydrogen[135].

Two types of evidence, again based on relatively early experiments, provide serious support for the availability of a nonconcerted olefin epoxidation pathway. Thus, the observation that terminal olefins, including ethylene gas, are not only oxidized to the corresponding epoxides but simultaneously alkylate one of the prosthetic heme nitrogen atoms is incompatible with a concerted epoxidation mechanism (Figure 6.15) (see Chapter 7)[136]. Although the structure of the adducts is that which would result from addition of the porphyrin nitrogen to the terminal carbon of the epoxide, control experiments have clearly established that epoxides do not alkylate the heme group[137]. Furthermore, the stereochemistry of the *N*-alkylated products is opposite to that expected from backside attack of the nitrogen on the epoxide[138]. The heme alkylation must, therefore, occur prior to formation of the epoxide metabolite. Given the requirement for catalytic turnover of the enzyme[137] and the fact that the resulting *N*-alkyl group has incorporated an atom from molecular oxygen[138, 139], it is clear that the heme is alkylated by a reactive intermediate generated during the olefin epoxidation reaction (Figure 6.15). The reactive species that alkylates the heme must be a precursor of the epoxide product, or the result of a parallel but divergent olefin oxidation pathway. The partitioning of the common intermediate, or of the flow of oxidation equivalents into two parallel, but

distinct, pathways is defined by the partition ratio between epoxidation and heme alkylation. This partition ratio ranges from values as low as 1–2 (i.e., nearly every oxidation leads to heme alkylation) to values as high as several hundred (i.e., heme alkylation competes poorly with epoxide formation).

Prosthetic heme alkylation is also observed during the oxidation of terminal acetylenes by cytochrome P450 (Figure 6.14). As found in inactivation by olefins, the terminal carbon of the acetylene is bound to a nitrogen of the P450 heme group and an atom derived from molecular oxygen to the internal carbon[129, 140]. The oxygen is present as a carbonyl group due to tautomerization of the enol that would be formed by simple addition of a hydroxyl group to the internal acetylenic carbon. In the case of acetylene oxidation, a clear distinction is possible between the reaction pathway that produces the ketene metabolites and that which yields the *N*-2-ketoalkyl adducts because metabolite formation involves delivery of the oxygen to the terminal carbon, but *N*-alkylation delivery to the internal carbon. In the case of acetylenes, enzyme inactivation can also occur by a different mechanism subsequent to metabolite formation because the initial ketene product can acylate nucleophilic protein residues before it is hydrolyzed to a stable carboxylic acid (see Chapter 7)[141, 142]. The partition ratios for metabolite formation vs heme alkylation are usually smaller for acetylene than for olefin oxidation.

The second type of evidence that strongly argues for the availability of a nonconcerted epoxidation pathway is provided by the finding that carbonyl products are directly formed during the oxidation of a few olefins. As a case in point, the oxidation of trichloroethylene yields both trichloroethylene oxide and trichloroacetaldehyde. As control experiments indicated that trichloroethylene oxide did not rearrange to trichloroacetaldehyde under the incubation conditions, the aldehyde apparently arose by an oxidation pathway distinct from that which generated the epoxide (Figure 6.16)[143, 144]. Similar results were obtained for the oxidation of 1,1-dichloroethylene to the epoxide vs monochloro- and dichloroacetic acids—again the epoxide did not appear in control experiments to rearrange to the acids under physiological conditions[145]. Direct formation of a carbonyl product during the oxidation of an olefin has been observed in a few other situations, notably in the formation of 1-phenyl-1-butanone and 1-phenyl-2-butanone as minor products of the oxidation of *trans*-1-phenylbutene[145], and of 2-phenylacetaldehyde in the oxidation of styrene[146]. Under physiological conditions, these carbonyl products do not appear to be formed by rearrangement of the epoxides. The carbonyl products are consistent with the formation of a carbocation intermediate, possibly through leakage from the normal epoxidation pathway into an alternative pathway within the overall reaction manifold. This could occur, for example, if a competition exists between closing the second epoxide bond and electron transfer from a radical-like carbon intermediate to the iron to give the cation. However, the search for products indicative of radical intermediates in olefin epoxidation reactions has so far been fruitless. Thus, no cyclopropane ring-opened products were observed in the oxidation of *trans*-1-phenyl-2-vinylcyclopropane[147].

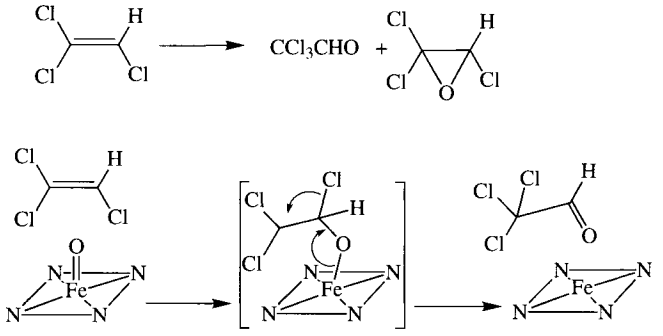


Figure 6.16. The oxidation of some halogenated olefins has been shown to yield the corresponding epoxides and rearrangement products that appear to arise via a reaction path that does not include the epoxide as an intermediate.

The seemingly contradictory evidence for concurrent concerted and nonconcerted epoxidation pathways can be satisfyingly rationalized by the two-state reactivity paradigm advocated by Shaik and colleagues (see Chapter 2). These investigators have shown by density functional theoretical calculations that the P450 ferryl porphyrin radical cation can exist in doublet and quartet spin states that are quite close in energy. The calculations suggest that ethylene epoxidation by both the doublet and quartet oxidizing species involves addition of the oxygen to one carbon of the olefin, leaving the other carbon of the olefin with an unpaired electron (Figure 6.17)[148]. This radical complex can again exist in both doublet and quartet states, although the system is more complex in that each of the two states has two equilibrating electromers that differ in whether the electron contributed by the olefin is used to neutralize the porphyrin radical cation or to reduce the iron to the Fe^{III} state (see Figure 6.8 and Chapter 2). The critical difference between the two states is that there is no barrier to closure of the doublet state to the epoxide, so that epoxidation occurs via an almost concerted trajectory with retention of the olefin stereochemistry even though a true, concerted mechanism is not predicted by the calculations[149]. In contrast, a barrier of 2.3 kcal mol^{-1} is found for closure to the epoxide of the quartet intermediate with the configuration $PorFe^{IV}$–O–$CH_2CH_2^{\bullet}$, and a barrier of 7.2 kcal mol^{-1} for the quartet intermediate with the $Por^{+\bullet}Fe^{III}$–O–$CH_2CH_2^{\bullet}$ structure[148]. These energy barriers are sufficiently high to allow alternative reactions to compete with epoxide ring closure and provide a ready explanation for the experimental observation that the heme undergoes nitrogen alkylation in the epoxidation of some olefins[150]. The partitioning between epoxidation and heme alkylation is largely determined in this model by the proportion of the doublet and quartet transition-state complexes. The critical feature of the two-state epoxidation mechanism is the presence of a doublet and a quartet transition state of sufficiently close energies that the oxidation reaction can proceed via either of the two transition states. This computational model rationalizes the experimental data available on olefin oxidation in a satisfying and comprehensive manner, although the model is difficult to test experimentally.

The discussion of epoxidation has been framed in terms of a ferryl catalytic species. It has been proposed that the ferric hydroperoxo intermediate may contribute to olefin epoxidation[48], but as discussed in Section 2, the support for this postulate is contradictory. Although it appears that the ferric hydroperoxo intermediate can oxidize double bonds at a low rate, the data strongly suggests that the ferric hydroperoxide

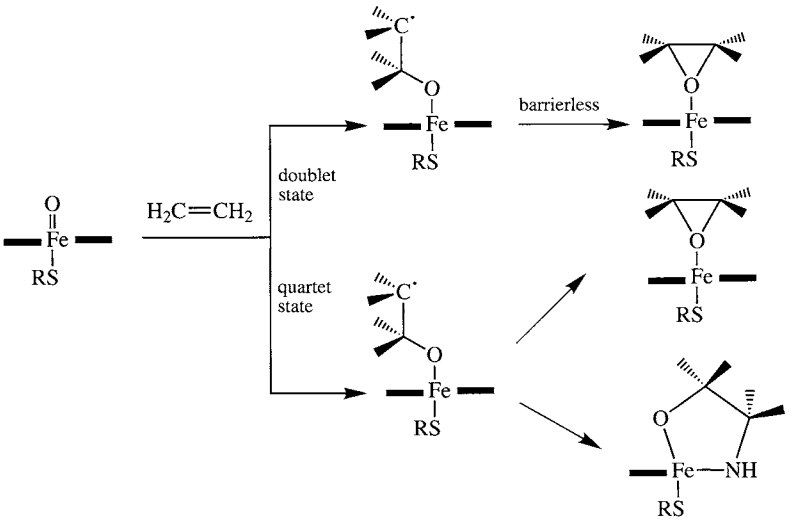


Figure 6.17. Explanation of the dual pathways of olefin oxidation resulting in epoxide formation and heme alkylation in terms of the two-state hypothesis of P450 catalysis extensively described in Chapter 2.

makes little contribution to oxidations catalyzed by the wild-type proteins.

Although the stereochemical evidence suggests that olefin oxidation occurs by a concerted mechanism, it is clear from the observation of heme alkylation and rearranged products that nonconcerted oxidation pathways are also operative. The oxidation of terminal acetylenes to ketenes by addition of the oxygen to the unsubstituted carbon and to species that alkylate the heme group by addition to the internal carbon also suggests that multiple oxidation pathways are possible. The puzzle that is posed by these mechanistic dichotomies may find a solution in the recently formulated hypothesis of two-state reactivity, in which two energetically similar transition states are obtained, one of which is in a doublet spin state and reacts essentially as if the reaction were concerted, and the second of which is in a quartet spin state and can give rise to products characteristic of nonconcerted reactions (see Chapter 2).

6. Oxidation of Aromatic Rings

The oxidation of an aromatic ring by cytochrome P450 invariably involves oxidation of one of the π-bonds rather than direct insertion of the oxygen into one of the aromatic ring C–H bonds. Thus, benzene oxide has been specifically identified as a product of the oxidation of benzene by liver microsomes[151]. However, benzene oxide and the similarly unstable epoxides expected from the oxidation of other aromatic rings readily undergo heterolytic cleavage of one of the epoxide C–O bonds. This bond cleavage is followed by migration of a hydride from the carbon retaining the oxygen to the adjacent carbocation to give a ketone intermediate. Tautomerization of this ketone yields a phenol product. This sequence of steps is the so-called "NIH-shift" (Figure 6.18)[152]. Key evidence for this mechanism is provided by the finding that the hydrogen atom (H*) at the position that is oxidized migrates to the adjacent carbon, where it is partially retained in the final phenol product. Partial retention of the migrating hydrogen reflects nonstereospecific elimination of one of the two hydrogens in the tautomerization step. This mechanism is widely applicable, but ferryl oxygen transfer to aromatic rings can also proceed via a transient intermediate that undergoes the NIH shift or eliminates a substituent on the tetrahedral carbon before the epoxide is actually formed (*vide infra*). The migrating atom in the NIH shift is usually a hydrogen, but other moieties, notably a halide or an alkyl group, can also shift to the adjacent carbon as the result of the hydroxylation event[152, 153].

The rate of the hydroxylation reaction is not very sensitive to deuterium substitution because the deuterium-sensitive tautomerization step occurs after the rate-limiting enzymatic oxidation. The observation of a small inverse secondary isotope effect (0.83–0.94) for ring hydroxylation of *ortho*- and *para*-xylene is consistent with rate-limiting addition of the ferryl oxygen to the π-bond, as the transition state for the addition reaction requires partial rehybridization of the carbon from the sp^2- to the sp^3-state[154]. The observation of an inverse isotope effect does not support a mechanism in which the rate-determining step is oxidation of the aromatic ring to a π-cation radical, as the secondary isotope effect for such a process should be negligible. The oxidation of cyclopropylbenzene to 1-phenylcyclopropanol and cyclopropylphenols without detectable opening of the cyclopropyl ring also argues against the

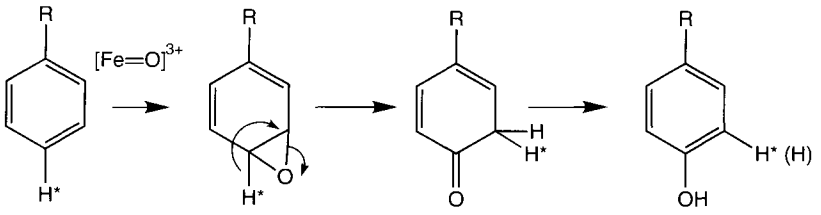


Figure 6.18. The NIH shift involving initial formation of an epoxide metabolite in the oxidation of the aromatic ring by cytochrome P450. The starred hydrogen shows that the hydrogen undergoes a 1,2-shift and then is partially lost in the final tautomerization step.

involvement of a radical cation intermediate in the oxidation of small, unactivated aromatic rings[155].

In some instances, particularly in hydroxylations *meta* to a halide substituent, the hydrogen on the hydroxylated carbon is quantitatively lost (i.e., there is no NIH shift), and a small deuterium kinetic isotope effect is observed[156, 157]. These hydroxylations could result from direct oxygen insertion into the C–H bond, as in a true "hydroxylation" mechanism, but they are more likely to result from oxidation of the aromatic ring without the formation of a discrete epoxide intermediate. Isotope effect studies with deuterated benzenes bearing a variety of substituents have shed some light on this process[158, 159]. A small, normal isotope effect is observed for *meta*-hydroxylation when deuterium is located *meta*- to the halogen in chlorobenzene (k_H/k_D = 1.1–1.3), but a small, inverse isotope effect (k_H/k_D = ~0.95) is observed for *ortho*- and *para*-hydroxylation when the deuterium is at those positions[159]. Simultaneous formation of the two epoxide bonds in a concerted process should be subject to a small, normal isotope effect when either of the two oxidized carbons bears a deuterium atom, although asynchronous formation of the two bonds could give rise to different isotope effects at the two sites. In the limiting situation in which one carbon–oxygen bond is completely formed first, formation of this bond could be followed either by closure to the epoxide or by an *ipso*-substitution mechanism that obviates the epoxide intermediate (*vide infra*).

The cytochrome P450-catalyzed oxidation of pentafluorochlorobenzene to tetrafluorochlorophenol has been proposed to involve ferryl oxygen addition to the fluorine-substituted carbon *para* to the chloride, followed by electron donation from the chloride to eliminate the fluorine as a fluoride ion (Figure 6.19). The resulting positively charged chloronium intermediate can then be reduced to the phenol, possibly by cytochrome P450 reductase, or can undergo hydrolysis to the tetrafluoroquinone[160]. This mechanism is supported by ^{19}F-NMR evidence for the release of fluoride ion and by molecular orbital calculations. A correlation of molecular orbital calculations with the regiochemistry of the oxidation of 1-fluorobenzene, 1,2-difluorobenzene, 1,3-difluorobenzene, 1,2,3-trifluorobenzene, and 1,2,4-trifluorobenzene suggests that the reaction is initiated by direct attack of the electrophilic ferryl oxygen on the aromatic π-system rather than by an initial electron abstraction[161]. More refined local density approximation calculations for the oxidation of benzene and *mono*-fluorobenzene suggest that epoxidation is disfavored vs a direct NIH shift from a tetrahedral oxygen-addition intermediate, and that hydroxylation *para* to the fluorine is favored[162]. In addition to the electronic effect of the halide, a steric interference is observed in the ability of the

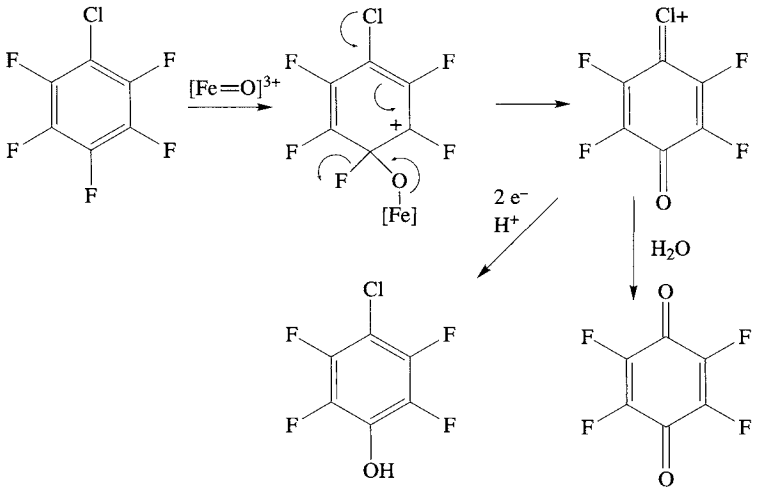


Figure 6.19. Oxidation of a polyhalogenated ring via an *ipso* mechanism that does not involve formation of an epoxide metabolite but rather an addition–elimination reaction that directly yields a quinone product.

enzyme to hydroxylate *ortho* to the halide when it is a bromine or iodine but not fluorine or chlorine[163].

The oxidation by rat liver microsomes of phenols bearing a *para*-$OPhNO_2$, -NO_2, -CN, -CH_2OH, -$COCH_3$, -COPh, -CO_2H, -F, -Cl, or -Br substituent eliminates the *para*-substituent and forms the hydroquinone (Figure 6.20)[164, 165]. Studies with $^{18}O_2$ show that an atom of molecular oxygen is incorporated into one of the two quinone carbonyl groups. These results, and the finding that the *p*-nitrophenoxy group is not eliminated when the phenol hydroxyl is replaced by a methyl ether, suggest that the phenoxy radical generated by one-electron oxidation of the phenol undergoes *ipso*-recombination with the ferryl oxygen to give a tetrahedral intermediate. Direct elimination of the *para*-substituent then gives the quinone. However, it has also been reported that 4-iodoanisole is oxidized to 4-methoxyphenol without the incorporation of label from $H_2{}^{18}O$ or ^2H_2O[166]. This finding argues that the phenol hydroxyl is not absolutely required for the reaction, so the addition can occur via *ipso*-addition without prior formation of the phenoxy radical. In accord with an *ipso*-mechanism, the substituent is eliminated from 4-halophenols as a halide anion, a *para*-CH_2OH group as formaldehyde, and a PhCO-substituent as benzoic acid[164,165].

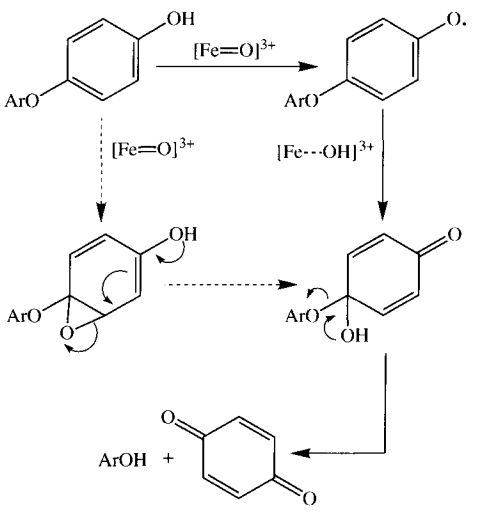


Figure 6.20. Two possible pathways for the direct P450-catalyzed oxidation of a *p*-aryloxy phenol to the quinone, one involving initial formation of a phenoxy radical and the other of an epoxide.

Furthermore, the methyl in 4-methylphenol is not a viable leaving group and this compound is simply oxidized to 4-hydroxy-4-methyl-2,5-cyclohexadiene-1-one[165]. It is to be noted that the regiochemical results do not rule out epoxide formation, as electron donation from the phenolic hydroxyl group would regiospecifically open the epoxide to the same products, but epoxide formation appears an unlikely explanation for the collective results.

Evidence for aniline oxidation without the formation of an epoxide intermediate is provided by the demonstration that an atom of molecular oxygen is incorporated into the quinone oxygen when *p*-ethoxyacetanilide (phenacetin) is oxidized to *N*-acetyl-*p*-benzoquinoneimine[167,168]. This finding requires that the reaction proceeds with cleavage of the bond between the oxygen and the aryl carbon rather than via a conventional *O*-dealkylation mechanism. The most probable mechanism for this reaction is P450-mediated hydrogen abstraction from the nitrogen to give a radical that undergoes *ipso*-recombination with the ferryl oxygen at the carbon bearing the ethoxy moiety (Figure 6.21)[169]. The concurrent formation of 2-hydroxyphenacetin is consistent with this mechanism because high unpaired electron density would be present in the aniline radical on both the *ortho*- and *para*-carbons[170]. The formation of quinoneimines accompanied by the elimination of fluoride anion in the oxidation of 4-fluoroanilines can be explained by a similar mechanism[171].

The oxidation of phenols via HAT from the hydroxyl group (or sequential electron transfer and deprotonation) is supported by data on the oxidation of estradiol and estrone. In accord with a key role for the phenolic hydroxyl group, the predominant *ortho*-hydroxylation of estradiol does not occur when the phenolic hydroxyl is replaced by a methyl ether[172]. Early experiments established that 2-hydroxylation of estradiol occurs without a detectable NIH shift[173]. More recent work has shown that, whereas estrone is converted to both 2- and 4-hydroxyestrone by CYP3A4, conjugation of an additional aromatic ring, as in equilenin and 2-naphthol, leads exclusively to 4-hydroxylation of estrone and 1-hydroxylation of 2-naphthol. In both these reactions, the site that is exclusively hydroxylated is that expected to carry the greatest share of the unpaired electron density if the initial step is

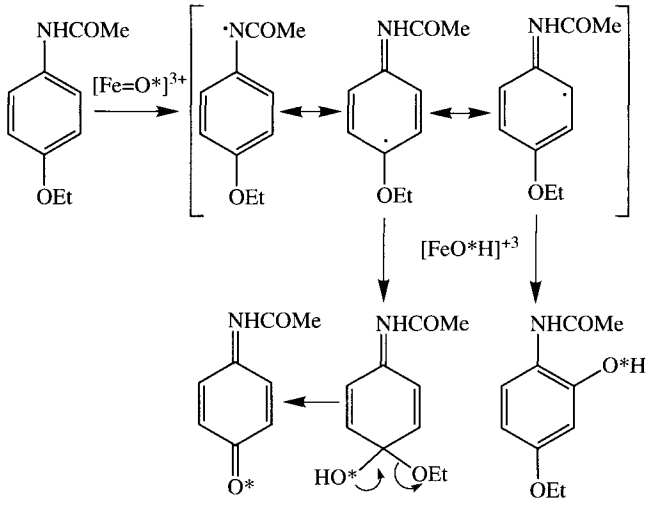


Figure 6.21. Possible mechanisms for the oxidation of phenacetin.

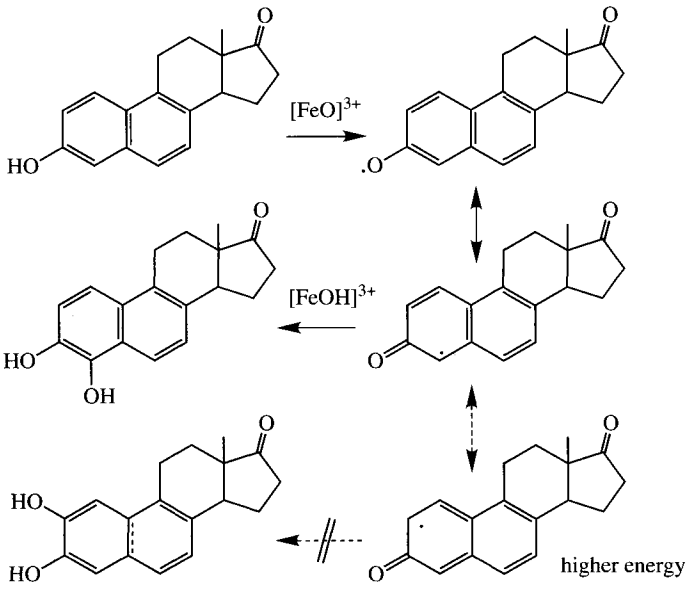


Figure 6.22. The additional aromatic ring in equilenin leads to a change in regiochemistry of hydroxylation of the phenol ring relative to that observed in the oxidation of estrone, possibly due to a change in the localization of the unpaired electron density from the 2- to the 4-position.

formation of the phenoxy radical, followed by recombination with the iron-bound hydroxyl radical (Figure 6.22)[174].

The cytochrome P450-catalyzed formation of phenol radicals is clearly required for the cross-linking of phenol rings catalyzed by a variety of plant cytochrome P450 enzymes, including the enzyme from *Berberis stolonifera* that catalyzes the biosynthesis of dibenzylisoquinoline alkaloids (Figure 6.23)[175, 176], and the enzymes that convert reticuline to salutaridine[177, 178], and autumnaline to isoandrocymbine in colchicine biosynthesis[179].

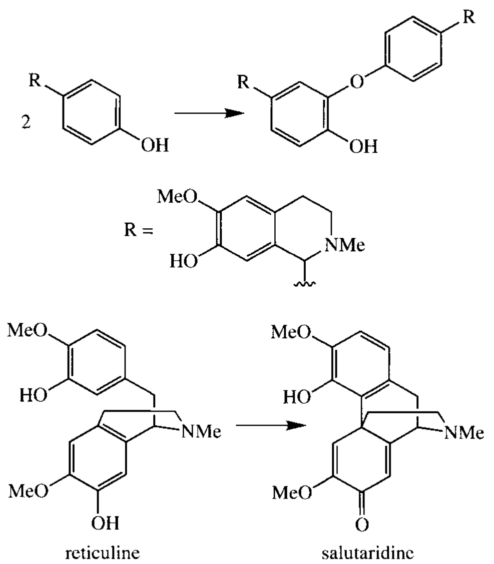


Figure 6.23. The formation of phenoxy radicals is indicated by the coupling products formed in the P450-catalyzed biosynthesis of some alkaloids.

Another interesting example is provided by the therapeutically important antibiotic vancomycin (Figure 6.24), which consists of a crosslinked heptapeptide backbone glycosylated with a disaccharide residue. The phenolic coupling that occurs between the aromatic side chains of the heptapeptide core is believed to be mediated by a P450 enzyme. The vancomycin biosynthetic cluster encodes three highly related P450 enzymes[180] that are suggested by gene knockout studies to be involved in the coupling of residue 4 with resides 2 and 6 via C–O bonds and of residues 5 and 7 via a C–C bond[181]. One of these enzymes ($P450_{OxyB}$) has been cloned and overexpressed in *Escherichia coli* and an X-ray crystal structure obtained. It has a relatively open active site, consistent with a large substrate, but whether the substrate is the free heptapeptide or one bound to a peptidyl carrier domain is unclear[182]. Balhimycin, chloroeremomycin[182, 183], and complestatin[184] are antibiotics structurally related to vancomycin in which analogous C–C and C–O bond formation is believed to be P450 mediated. These reactions, like the

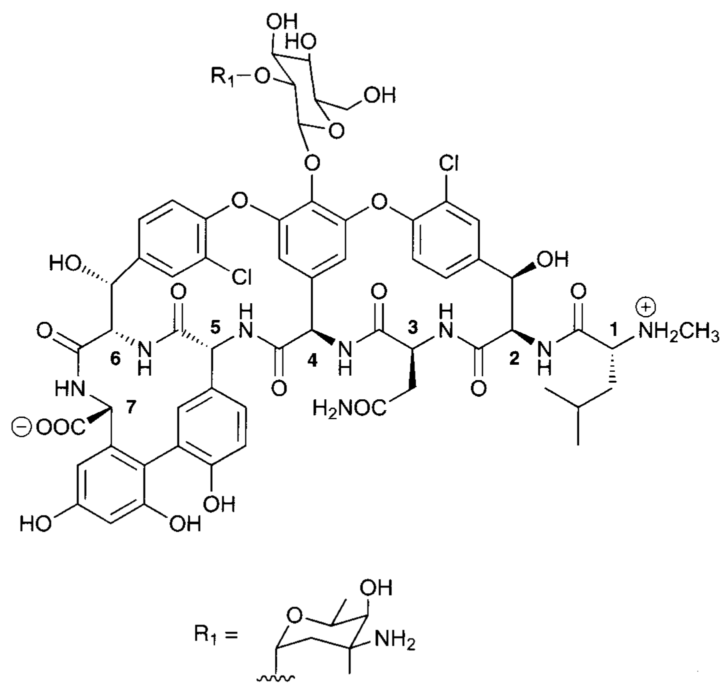


Figure 6.24. The biosynthesis of vancomycin involves a phenoxy radical crosslinking step that is catalyzed by a cytochrome P450 enzyme.

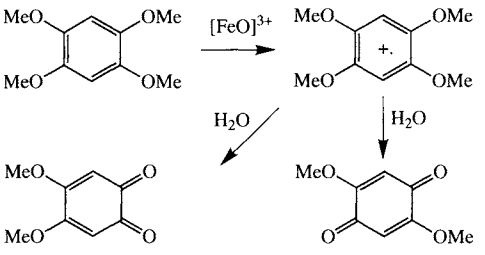


Figure 6.25. The oxidation of a highly oxidizable aromatic probe substituted with multiple electron-donating substituents yields a detectable radical cation product.

peroxidative crosslinking of tyrosine residues, require the coupling of two phenoxy radicals presumably generated by proton-coupled electron transfer from each phenol to the ferryl species. Concurrent formation of the two phenoxy radicals within the confines of the P450 active site should suffice to generate the observed crosslinked products.

The clearest available evidence that aromatic rings can be oxidized to radical cations by cytochrome P450, at least when the ring is substituted with multiple electron-donating groups, is provided by the reported oxidation of 1,2,4,5-tetramethoxybenzene to a radical cation by CYP1A2 (Figure 6.25)[185]. The radical cation was detected by absorption spectroscopy and spin-trapping EPR. The stable metabolites formed in the reaction were 2,5-dimethoxy-1,4-benzoquinone and 4,5-dimethoxy-1,2-benzoquinone, the products expected from hydrolysis of the radical cation. A negligible deuterium isotope effect was observed on formation of the radical cation or products derived from it even though a large isotope effect was seen for simple *O*-dealkylation. The evidence for other aromatic radical cations is less conclusive. In an interesting set of experiments, the products formed in the incubation of 9-methylanthracene and related compounds with the following systems were determined: (a) CYP2B1 in the presence of either NADPH-cytochrome P450 reductase or PhIO, (b) HRP in the presence of H_2O_2 or EtOOH, and (c) a model system consisting of iron tetraphenylporphine and PhIO[186]. Apart from the uninformative 1,2- and 3,4-diols that are formed exclusively with the P450 system, the relevant products were 9-hydroxymethylanthracene, 10-methyl-10-hydroxy-9-anthrone, and anthraquinone[186]. The 9-hydroxymethyl product results from a straightforward carbon hydroxylation, but more complex reactions are required to rationalize the other two products. The incorporation of label from both $H_2{}^{18}O$ and $^{18}O_2$, but not $H_2{}^{18}O_2$, into the ring-oxidized products can best be rationalized by the formation of a radical cation species that combines with water and/or molecular oxygen to give the observed products. However, with cytochrome P450, ^{18}O-label was incorporated only from $^{18}O_2$ and not $H_2{}^{18}O$. The absence of label from water in the products from the P450 system, in view of its incorporation with HRP, is inconsistent with diffusion of a radical cation out of the enzyme active site. Thus, if a radical cation is formed, it occurs as a highly transient intermediate that is immediately trapped by the ferryl oxygen to give the observed products. The results are reminiscent of the report by Ohe, Mashino, and Hirobe that (a) a hydroxymethyl group is eliminated as formaldehyde when the ferryl oxygen adds in an *ipso*-manner to the substituted carbon in a 4-substituted phenol, leading to formation of the 1,4-quinone, and (b) when the substituent is a methyl, the reaction results in addition of the hydroxyl group to the substituted ring carbon with concomitant oxidation of the phenol group to a keto function (Figure 6.20)[164, 165]. As reported by Rizk and Hanzlik, a methoxy group also makes possible this kind of reaction[166]. Thus, mechanisms based on *ipso*-addition of the ferryl oxygen to the aromatic ring are likely to account for the products formed from 9-methylanthracene. A scheme based on that proposed by Anzenbacher *et al.* (Figure 6.26)[186], or a variant of it, readily explains the observed results without requiring a radical cation intermediate. The results do not, however, preclude the existence of nondiffusible radical cations as transient intermediates.

Cavalieri and coworkers, following earlier investigators[187], have championed the hypothesis that the covalent binding of polycyclic aromatic hydrocarbons to DNA is due to radical cations formed from them by the action of cytochrome P450 and/or peroxidase enzymes[188, 189]. They have reported that polycyclic aromatic hydrocarbons with ionization potentials below 7.35 eV can be oxidized to radical cations by peroxidases[190, 191]. Furthermore, the formation of a benzo[a]pyrene-DNA adduct consistent with oxidation of the

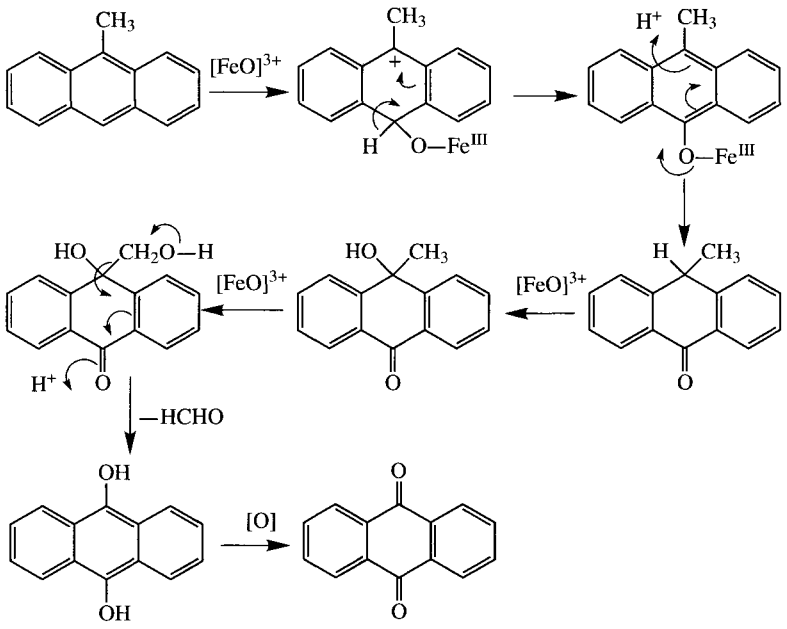


Figure 6.26. Reactions proposed to explain the oxidation of 9-methylanthracene by cytochrome P450.[186]

hydrocarbon to a radical cation by rat liver microsomes and rat skin[192, 193], and the presence of cytochrome P450 in the nuclear membrane[194], support the proposal that cytochrome P450 enzymes may also oxidize polycyclic aromatic hydrocarbons to radical cations[188]. The oxidation of 6-fluorobenzo[a]pyrene by liver microsomes to 6-hydroxybenzo[a]pyrene has been interpreted as evidence for cytochrome P450-catalyzed radical cation formation, although the reaction could arise, as postulated for polyhalogenated benzenes[160], by direct addition of the activated oxygen to the aromatic system[195]. Addition of the ferryl oxygen in such a mechanism would be expected to occur at the 6-position as that is the position most sensitive to electrophilic attack. The evidence for the formation of a diffusible radical cation in the normal (as opposed to peroxide-dependent) cytochrome P450-catalyzed oxidation of polycyclic aromatic hydrocarbons remains inconclusive.

In sum, considerable evidence is now available for the oxidation of aromatic rings not only via the conventional epoxidation pathway, but also by mechanisms that do not involve formation of an epoxide as an intermediate. The non-epoxide mechanisms involve addition of the ferryl oxygen to one carbon of the aromatic ring, producing a tetrahedral intermediate that decays by extrusion of a substituent at that carbon or by electron transfer, resulting in two-electron oxidation of the ring system and/or addition of a second nucleophile from the solution. This type of reaction is particularly favored with aromatic rings such as phenols and anilines that bear electron-donating substituents. The evidence for the formation of radical cations by direct electron abstraction from polycyclic aromatic hydrocarbons remains ambiguous, although the formation of such intermediates is favored by multiple electron-donating substituents.

7. Dehydrogenation Reactions

Cytochrome P450 enzymes catalyze dehydrogenation as well as oxygenation reactions, including the oxidation of saturated to unsaturated hydrocarbons, alcohols to carbonyl compounds, and amines to imines or other unsaturated products. The most extensively investigated of these reactions in terms of mechanism is the desaturation of valproic acid to 2-*n*-propyl-4-pentenoic

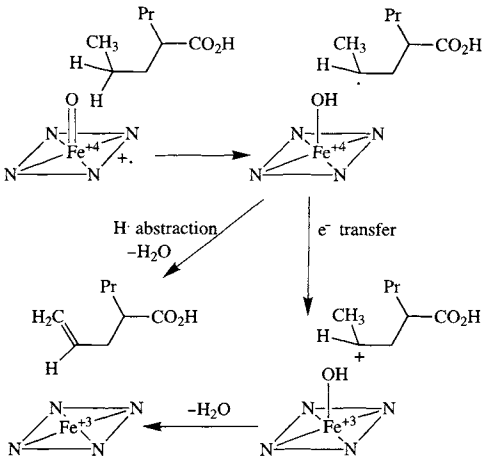


Figure 6.27. Two mechanistic alternatives for the dehydrogenation of valproic acid catalyzed by P450 enzymes.

acid (Figure 6.27)[196–199]. Formation of the $\Delta^{4,5}$-unsaturated product from valproic acid is catalyzed by rat, rabbit, mouse, monkey, and human liver microsomes and by purified CYP2B1, CYP2C9, CYP2A6, CYP3A1, and CYP4B1, but not by CYP3A4 or CYP4A1[196, 197, 200–203]. The $\Delta^{3,4}$ isomer is also formed, in some instances in greater amounts than the $\Delta^{4,5}$ isomer[201]. The 3- and 4-hydroxyvalproic acids are also formed, but these hydroxylated products are not precursors of the unsaturated compounds[196]. Oxidation of the two enantiomers of stereospecifically [3-^{13}C]-labeled valproic acid by cultured hepatocytes shows that the *pro*-(*R*)-side chain is preferentially desaturated[198]. The $\Delta^{2,3}$-unsaturated analogue of valproic acid, 2-*n*-propyl-2(*E*)-pentenoic acid, is also desaturated to give the $\Delta^{2,3}$, $\Delta^{4,5}$-diene, and an asymmetric but related molecule, 2-ethylhexanoic acid, is oxidized to both 2-ethyl-1,6-hexanedioic acid and 2-ethyl-5-hexenoic acid[199, 204].

The intramolecular isotope effects for the oxidation of valproic acid with two deuterium atoms on the C-4 carbon of one of the two propyl side chains by rabbit liver microsomes reveal that 4-hydroxylation (k_H/k_D = 5.05) and $\Delta^{4,5}$-desaturation (k_H/k_D = 5.58) are sensitive to isotopic substitution[197]. In contrast, when the methyl group of one of the side chains is trideuterated, only minor intramolecular isotope effects are observed for 4-hydroxylation (k_H/k_D = 1.09) or $\Delta^{4,5}$-desaturation (k_H/k_D = 1.62). Comparable results have been obtained when the oxidation is mediated by either CYP2B1 or CYP4B1[202]. These results indicate that removal of a hydrogen from C-4 is rate limiting for both 4-hydroxylation and desaturation, whereas loss of a hydrogen from C-5 is not. These results agree well with a mechanism in which removal of a C-4 hydrogen is followed by either oxygen rebound to give the 4-hydroxy product or transfer of a hydrogen from the terminal methyl to the ferryl oxygen to give the olefin product. The hydrogen could be transferred to the ferryl oxygen together with an electron or could be transferred as a proton following transfer of the electron to give a cationic intermediate (Figure 6.27). Analogous mechanisms can be postulated for the finding that CYP3A1 also oxidizes valproic acid to the $\Delta^{3,4}$-unsaturated isomer, except that in this case deuterium isotope experiments suggest that the olefin is obtained equally well by initial oxidation of C-3 (k_H/k_D = 2.00) or C-4 (k_H/k_D = 2.36)[201]. Interestingly, the observation of an isotope effect at only one of the two carbons in an aerobic desaturation process is also found for the nonheme iron-dependent fatty acid desaturases, for which a related mechanism involving a nonheme iron center has been postulated[205].

The ratio of 4-hydroxy to $\Delta^{4,5}$-desaturated metabolites depends on the P450 isoform and is larger for CYP2B1 (37:1) than for CYP4B1 (2:1)[202]. The proportion of the olefin is much higher when the substrate is the $\Delta^{2,3}$-unsaturated valproic acid, a result that is particularly consistent with a mechanism in which the electron is transferred to the ferryl oxygen before the hydrogen[199]. The structural determinants that control whether hydroxylation or desaturation occurs are unknown, but if the Shaik formalism applies (see Chapter 2), it is probable that the desaturation reaction involves the quartet rather than doublet hydroxylation transition state.

The isopropyl group of ezlopitant, which bears a 2-methoxy-5-isopropylbenzylamino group, is oxidized by both CYP3A4 and CYP2D6 to the tertiary alcohol and the desaturated 1-methylvinyl moiety[206]. The alcohol was specifically shown not to be a precursor of the unsaturated product, and a small primary isotope effect was observed when deuterium was placed at the benzylic but not methyl carbons of the isopropyl group. Although not studied in detail, concurrent hydroxylation and

desaturation of an isopropyl group was also observed in the metabolism of α- and β-thujone even though the isopropyl group is not bound to an aromatic or conjugating function[207]. These observations are well accommodated by the mechanistic alternatives proposed for desaturation of valproic acid.

The desaturation of sterols has also been observed. Quantitatively, the most important of these is the P450-mediated Δ^{22}-desaturation in the ergosterol biosynthetic pathway of *Saccharomyces cerevisiae*[208]. The enzyme (CYP61) has been purified and shown to specifically catalyze the Δ^{22}-desaturation without forming hydroxylated sterol products[209, 210]. Related Δ^{22}-desaturases are found in other organisms, including mammals[211, 212]. Sterol desaturation also occurs at other positions. Thus, the CYP2A1-catalyzed oxidation of testosterone yields the 7-hydroxylated, 6-hydroxylated, and $\Delta^{6,7}$-desaturated sterols in a 38:1:1 ratio (Figure 6.28)[213, 214]. As might be expected, a primary intermolecular isotope effect is only observed for 6-hydroxylation and $\Delta^{6,7}$-desaturation when the deuterium is at the allylic C-6 position, although an isotope effect is observed for 7-hydroxylation when the deuterium is at C-7[215]. The formation of 17β-hydroxy-4,6-androstadiene-3-one in this reaction presumably occurs via the mechanism discussed above, although the finding that oxidation of C-6, and not C-7, leads to desaturation again suggests that electron transfer from the free-radical intermediate to the iron to give the allylically stabilized cation may contribute to the emergence of the desaturation pathway.

The past decade has shown that hydrocarbon desaturation is not uncommon but, except in cases such as the biosynthesis of ergosterol, it generally accounts for a minor proportion of the metabolic products. The earliest reported example of P450-mediated hydrocarbon desaturation appears to be the conversion of lindane (1,2,3,4,5,6-hexachlorocyclohexane) to 1,2,3,4,5,6-hexachlorocyclohexene[216], but the known hydrocarbon desaturation reactions now include the $\Delta^{6,7}$-desaturation of androstenedione and deoxycorticosterone by adrenal mitochondria[217], the oxidation of dihydronaphthalene to naphthalene and 7,8-dihydrobenzo[a]pyrene to benzo[a]pyrene[218, 219], the conversion of warfarin to dehydrowarfarin[220], the desaturation of lovostatin and simvastatin to the 6-*exo*-methylene

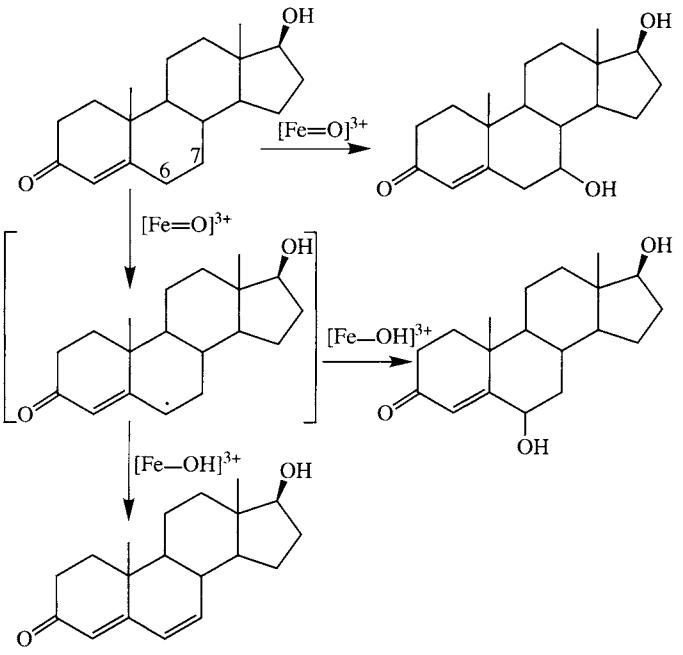


Figure 6.28. Parallel hydroxylation and dehydrogenation of testosterone.

derivatives[221–223], and the formation of 11-dodecenoic acid from lauric acid[224].

The direct desaturation of carbon adjacent to heteroatoms, notably oxygen and nitrogen, has also been observed. These reactions include the desaturation of a tetrahydrofuran ring in the biosynthesis of aflatoxin and sterigmatocystin by a specific P450 enzyme[225], the P450-catalyzed desaturation of flavanones to flavones[226], and the conversion of ethylcarbamate to vinyl carbamate[227]. In some instances, the desaturation may involve the carbon and the heteroatom instead of two carbon atoms. The CYP2B1-catalyzed oxidation of testosterone to androstenedione, which involves oxidation of the 17-hydroxy to a 17-keto function, is possibly such a reaction, because only 5–8% of the keto group oxygen derives from O_2 with testosterone, but 84% with epitestosterone[228]. A similar observation has been made for the P450-catalyzed oxidation of 6-hydroxy- to 6-keto-progesterone by CYP2C13[229]. Thus, either one of the two hydroxyls in a conventional *gem*-diol intermediate is eliminated with high stereoselectivity, or HAT is followed by loss of an electron without actual formation of the *gem*-diol:

$$RR'CHOH + [FeO]^{+3} \rightarrow RR'C \bullet (OH) + [FeOH]^{+3} \rightarrow RR'C{=}O + [Fe]^{+3} + H_2O$$

The dehydrogenation of a carbon next to a nitrogen has been unambiguously demonstrated. Acetaminophen (4-hydroxyacetanilide) is oxidized to the iminoquinone intermediate by a mechanism explicitly shown not to involve hydroxylation of the nitrogen (Figure 6.29)[230]. Other examples are the

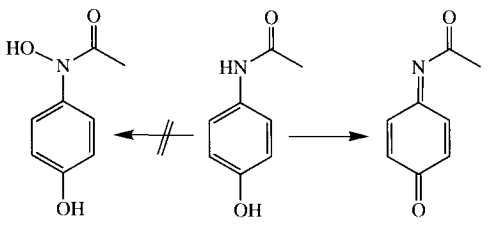


Figure 6.29. Dehydrogenation of acetaminophen to the iminoquinone occurs by a mechanism that does not include the hydroxyamide structure as an actual intermediate, even though the hydroxylamide product is found with related compounds that do not have the *para*-hydroxyl group.

aromatization of 4-alkyl- and 4-aryl-1,4-dihydropyridines[231, 232], the oxidation of 3-methylindole to the highly reactive 3-methyleneindolenine[233], and possibly the conversion of the *N*-ethyl to an *N*-vinyl moiety in the metabolism of tracazolate[234].

8. Carbon–Carbon Bond Cleavage Reactions

The power of P450 enzymes to catalyze oxidative transformations is perhaps nowhere better illustrated than in their ability to catalyze the cleavage of unactivated C–C bonds. Somewhat ironically, these reactions generally form part of biosynthetic pathways and allow organisms to build complex molecules via striking metabolic transformations. However, C–C bond cleavages have also been reported for some degradative/xenobiotic metabolizing enzymes. Additionally, many of these C–C bond cleavage reactions require a sequence of oxidations that are all carried out by the one enzyme. These P450 enzymes thus form a mechanistically fascinating group as they are not only capable of standard oxygen activation and hydroxylation chemistry, but also react through different mechanisms to eventually cleave a C–C bond. The examples below are arranged by the functional group(s) that is (are) adjacent to the C–C bond cleaved, although this group(s) may be introduced by the P450 during the course of oxidation of the original substrate. Excluded from this discussion are reactive compounds specifically designed to undergo cleavage of C–C bonds as mechanistic probes, for example, cyclopropylmethyl containing compounds (Section 3) and the cleavage of C–C bonds as part of the oxidation of an aromatic ring (Section 6).

8.1. Cleavage between Oxygenated Carbons

Diols. One of the best known examples of a P450-catalyzed C–C bond cleavage is carried out by $P450_{scc}$ (CYP11A) which converts cholesterol to pregnenolone and 4-methylpentanal (Figure 6.30). The mechanism by which this P450 effects scission of the C20–C22 bond of cholesterol has been extensively studied. The enzyme is trifunctional, catalyzing three sequential reactions that each consumes

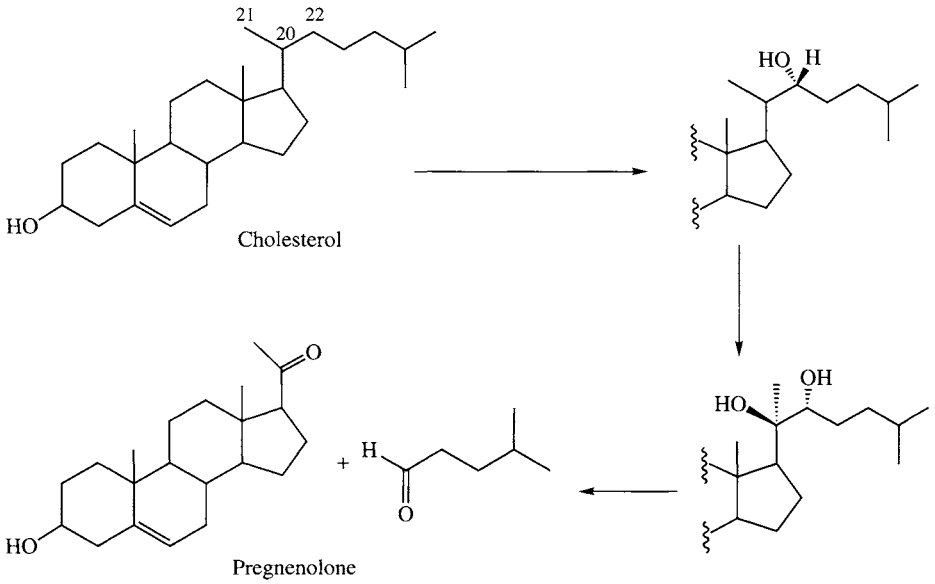


Figure 6.30. The intermediates in the $P450_{scc}$ catalyzed conversion of cholesterol to pregnenolone and 4-methylpentanal.

one molecule of dioxygen and one molecule of NADPH. The first two regio- and stereospecific hydroxylation reactions lead to, respectively, 22(*R*)-hydroxy and 20(*R*), 22(*R*) dihydroxycholesterol. These reactions are unremarkable P450-catalyzed oxidations, proceeding with retention of configuration as expected[235, 236]. The third oxidative transformation leads to cleavage of the C–C bond between the two oxygenated carbons and is of considerable mechanistic interest. The overall transformation is quite efficient as the intermediate hydroxylated cholesterol derivatives are bound up to 300 times more tightly than the parent substrate[237] and the ferrous–dioxygen complex is more stable in each successive turnover[238, 239].

Mechanisms for cleavage of the intermediate diol that involve further oxidation at C-22 are excluded by the fact that the 22(*S*) hydrogen is retained in the 4-methylpentanal produced as a result of C–C bond cleavage[235]. The most likely mechanism is thus one in which one of the hydroxyl moieties is activated in some fashion, followed by decomposition with C–C bond cleavage. The nature of this activation has led to a number of mechanistic proposals. First, a hydrogen may be abstracted from one of the alcohols by the ferryl species to form an alkoxy radical, which decomposes to release one carbonyl fragment and a carbon radical. This radical is then intercepted by the Fe(IV)OH species to yield the second product (Figure 6.31, path B). An alternative mechanism suggests that one of the hydroxyls of the diol intermediate intercepts an activated oxygen species to produce a peroxy complex. Loss of a proton from the adjacent alcohol initiates a heterolytic fragmentation reaction that leads directly to the two carbonyl products (Figure 6.31, path A). Formation of a hydroperoxide may be seen as precedented by the exchange of oxygen between hydrogen peroxide and water via a putative ferryl species in model systems[240]. The chemistry of such a hydroperoxide would also explain nicely the intriguing early observation that (20*S*)-20-(*p*-tolyl)-5-pregnen-3β-ol is cleaved to pregnenolone and presumably phenol by $P450_{scc}$ (Figure 6.32)[241]. This remarkable transformation would be analogous to the well-known formation of acetone and phenol from cumene hydroperoxide under acid catalysis.

Recently another biosynthetic enzyme, $P450_{BioI}$ (CYP107H1) has been shown to cleave an aliphatic chain via a diol intermediate. First found as a gene of unknown function in the biotin biosynthetic operon of *Bacillus subtilis*, $P450_{BioI}$ was implicated

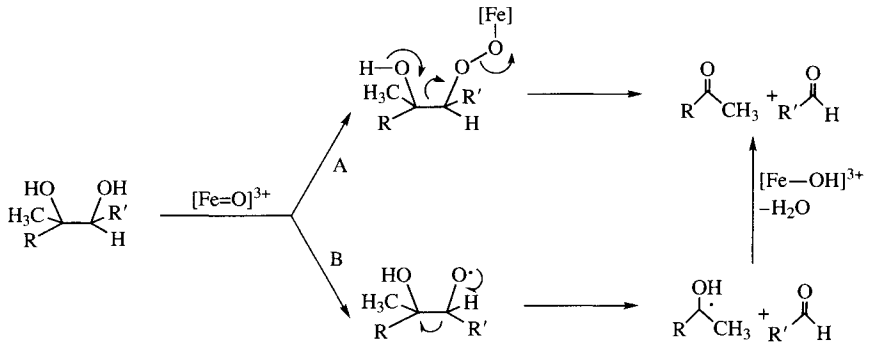


Figure 6.31. Possible mechanisms for the final step in the cholesterol side-chain cleavage reaction, where R = the sterol nucleus and R′ = $CH_2CH_2CH(CH_3)_2$.

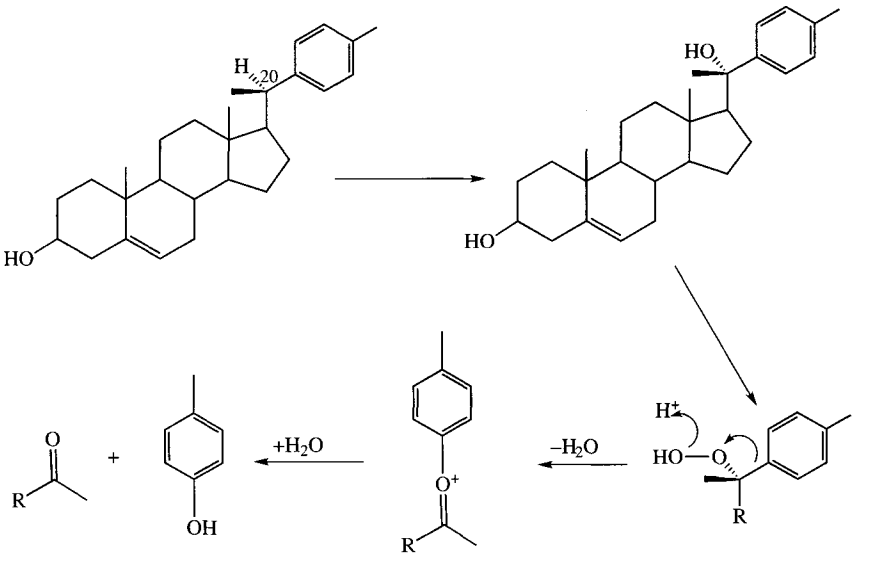


Figure 6.32. A possible mechanism for the $P450_{scc}$ catalyzed conversion of (20*S*)-20-(*p*-tolyl)-5-pregnen-3β-ol to pregnenolone and phenol (R = sterol nucleus).

through analysis of mutants in the formation of a biological equivalent of pimelic acid (heptanedioic acid)[242]. Cloning and overexpression in *E. coli* resulted in isolation of a complex between the P450 and acylated acyl carrier protein (ACP) as well as the P450 alone[243]. It was shown that the acyl moiety of the complex could be cleaved to produce a pimeloyl ACP utilizing a novel flavodoxin as the redox partner. $P450_{Biol}$ was also shown to act on free fatty acids to produce pimelic acid as well as a range of hydroxylated fatty acids[243,244]. Careful analysis of these latter products indicated that a range of hydroxy fatty acids was produced but no ω-oxidation was observed, indicating that production of a long-chain diacid as a pimeloate precursor was not the biological function of this P450[245]. Subsequently, a series of potential intermediates in the C–C bond cleavage reaction were synthesized and incubated with the enzyme[246]. It was shown that pimelic acid production increased when the substrate was changed from the C_{14} fatty acid to the 7-hydroxy derivative with the *threo*-7,8-diol as the best substrate (Figure 6.33). Other derivatives such as the 7- or

8-oxo, 8-hydroxy or *erythro*-7,8 diol gave no increase in pimelic acid formation. It was also found that, as with $P450_{scc}$, the postulated oxygenated intermediates bound much more tightly to the enzyme than the parent substrate[246]. These results are clearly in keeping with a C–C bond cleavage mechanism in which the P450 operates on one face of the extended conformation of the fatty acid chain to produce the *threo*-diol, which is then cleaved to produce two aldehyde fragments. The pimeloyl semialdehyde initially formed is somewhat unstable to aerial oxidation and both it and pimelic acid are seen in the cleavage of the *threo*-7,8-diol. Interestingly, only a small enantioselectivity was seen for the 7-(*S*) alcohol and the derived *threo* diol. This perhaps reflects the fact that the true substrate is an ACP-bound acyl group, making $P450_{BioI}$ one of a growing number of P450s found to act on carrier protein bound substrates[247].

One example of P450 mediated C–C cleavage via a presumed diol intermediate during xenobiotic metabolism has been reported. Olanexidine, an antimicrobial agent, is metabolized mainly to a range of chain shortened carboxylic acids in both rats and dogs, as well as to various other oxygenated metabolites (Figure 6.34)[248, 249]. Dog liver microsome studies indicated that vicinal diol metabolites were further transformed to the C–C bond cleavage products and specific inhibitor studies implicated enzymes of the CYP2D family in all of the oxidative transformations[248]. Interestingly, in contrast to $P450_{scc}$ and $P450_{BioI}$, no diastereoselectivity was observed in the further oxidation of the diols investigated (Figure 6.34)[248]. This may be due to the position of the diol near the terminus of the aliphatic chain such that there is little difference in the energy of binding or oxidation of the conformations accessible to the *erythro* and *threo* isomers. An alternative explanation is that C–C

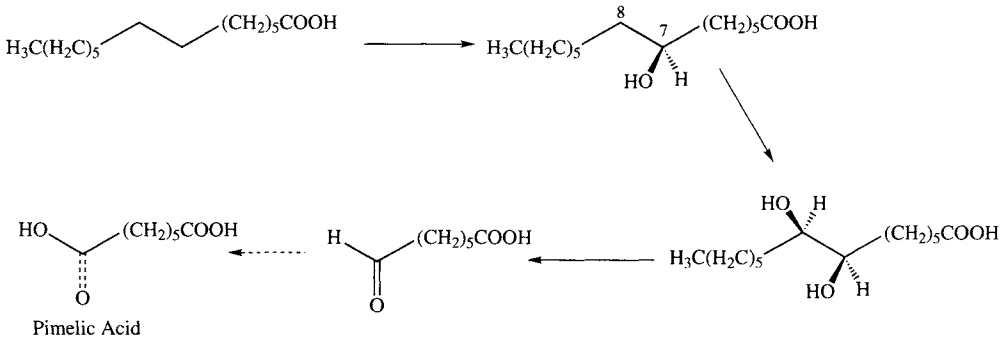


Figure 6.33. The intermediates in the C–C bond cleavage reaction catalyzed by $P450_{BioI}$ that produces pimelic acid from tetradecanoic acid.

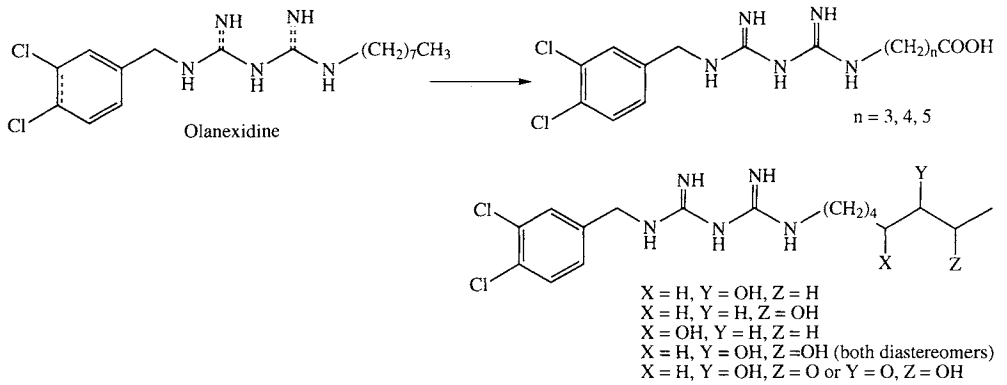


Figure 6.34. Oxygenated metabolites produced by P450-mediated oxidation of olanexidine.

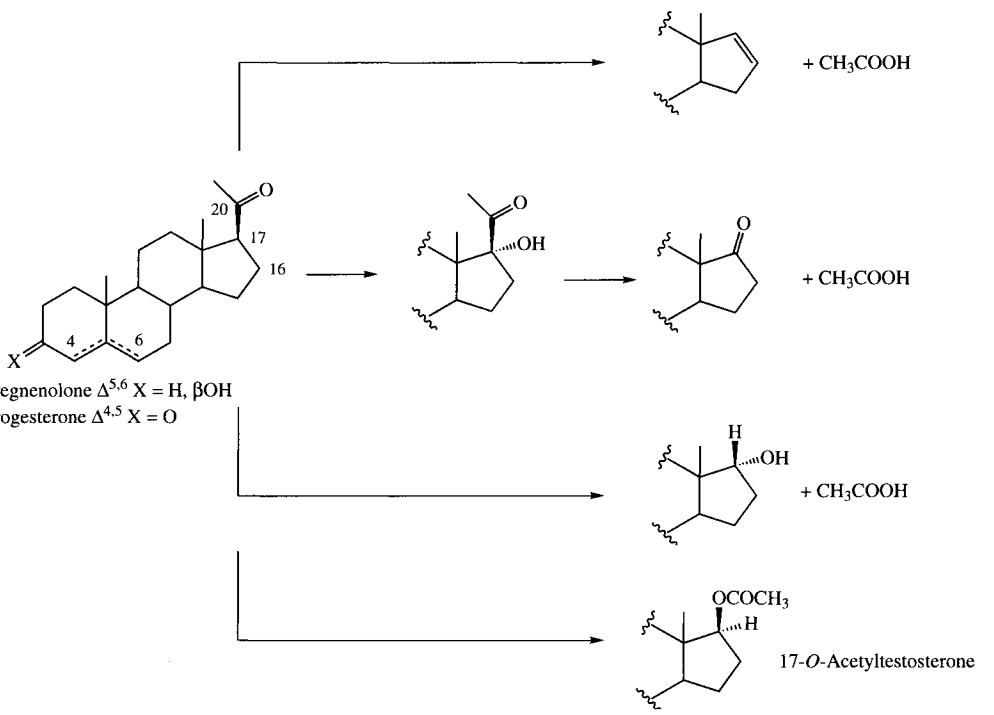


Figure 6.35. Various oxidation products reported to be formed from pregnenolone/progesterone via the action of CYP17A.

bond cleavage does not proceed directly from the diol but rather an α-hydroxy ketone which is also an observed metabolite (cf. CYP17A below). However, the different effects of specific P450 inhibitors on α-hydroxy ketone formation and C–C bond cleavage argue against a precursor product relationship[248]. Final delineation of the pathway awaits studies with purified isoforms but does suggest that C–C bond cleavage may be a significant metabolic pathway for compounds with aliphatic chains.

Keto Alcohols. CYP17A is a remarkable, multifunctional P450 that is primarily responsible for the 17α-hydroxylation of pregnenolone (or progesterone) and the subsequent lysis of the C17–C20 bond to produce dehydroepiandrosterone (or androstendione)[250, 251]. In addition, it catalyzes the cleavage of this same C17–C20 bond in mechanistically distinct ways to yield a number of minor products. These less common pathways lead to the formation of 17α-hydroxyandrost-5-en-3β-ol (note inversion of stereochemistry at C17) and the corresponding $\Delta^{16,17}$-olefin from pregnenolone[252–254] as well as 17-*O*-acetyltestosterone from progesterone (Figure 6.35)[255]. The proposed mechanism for the dominant reaction involves an unremarkable hydroxylation at C17 of the steroid nucleus (Figure 6.36)[256]. This is then followed by an attack of the ferric peroxo moiety on the carbonyl to yield a species that fragments to an alkoxy radical and a one-electron oxidized ferryl species. The alkoxy radical subsequently decomposes to produce acetic acid and a carbon radical that recombines with the ferryl species to yield a *gem*-diol, which dehydrates to the C17 carbonyl of the product. This mechanism is in accord with a wealth of labeling studies and can be modified simply to explain the origin of the other observed products.

Studies with $^{18}O_2$ have demonstrated the incorporation of one atom of ^{18}O into the acetic acid fragment produced upon C17–C20 cleavage of pregnenolone in all of the observed pathways[254, 256, 257]. Additionally, ^{18}O incorporation from $^{18}O_2$ is seen at the C17 position of the steroidal products from pregnenolone bearing an oxygen atom at this position[256]. The minor products from C17–C20 cleavage are proposed to arise from attack

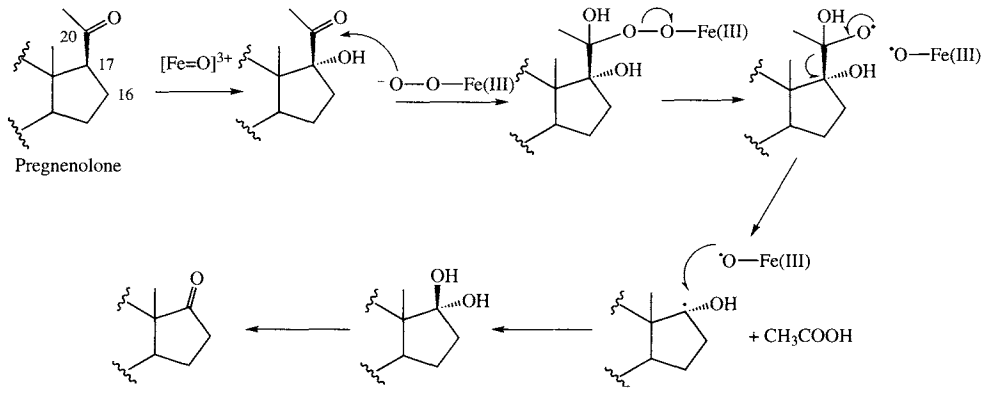


Figure 6.36. Proposed mechanism for the C17–C20 lyase reaction catalyzed by CYP17A. The key steps involve addition of a P450 ferric peroxide species to the C20 carbonyl and subsequent free radical fragmentation of the peroxyhemiacetal.

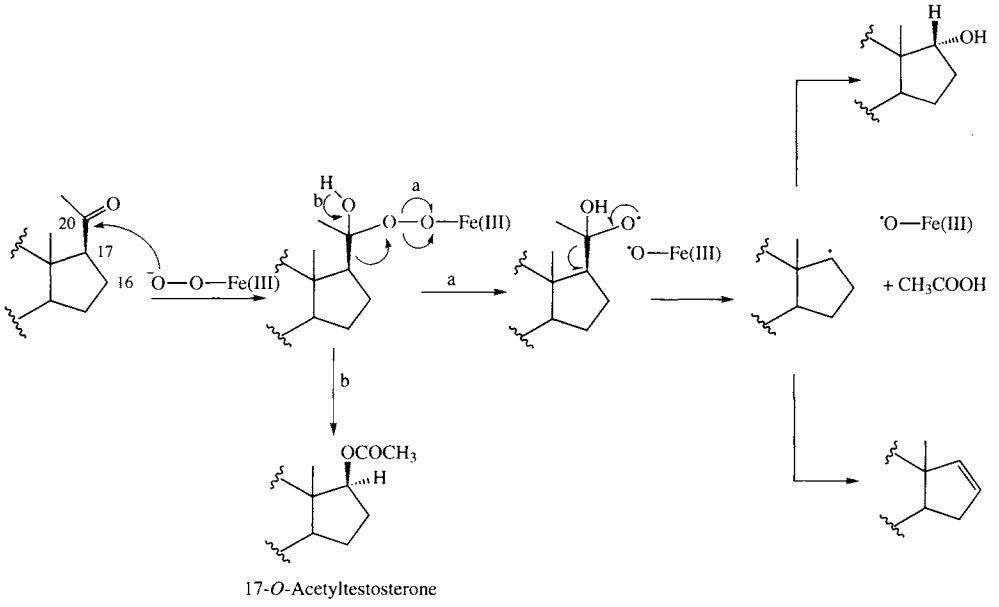


Figure 6.37. Proposed mechanistic manifold to account for the formation of other products in the CYP17A catalyzed oxidation of pregnenolone/progesterone. Ionic decomposition (pathway b) of the peroxyhemiacetal competes with free-radical fragmentation (pathway a) to yield the observed mixture of minor products.

of the ferric peroxo species on the carbonyl prior to any C17 hydroxylation (Figure 6.37). The resultant adduct then fragments to an alkoxy radical which loses acetic acid to produce a C17 radical in a fashion analogous to that proposed for the major pathway. This C17 radical then partitions between direct oxygen rebound to the 17α-hydroxy product and elimination to the $\Delta^{16,17}$-olefin. This latter product may arise via direct hydrogen abstraction or via a single-electron oxidation of the radical to the cation followed by elimination of a proton[256]. Results of CYP17A catalyzed oxidation of substrates bearing deuterium labels at the C16, C17 and the methyl group α to the ketone are in agreement with this mechanism[256]. The 17-*O*-acetyl-testosterone is probably best explained as

the result of a Baeyer–Villiger like decomposition of the peroxo adduct derived initially from an attack on the carbonyl (Figure 6.37)[255]. This indicates that the peroxo adduct may decompose by an ionic mechanism as well as by the radical pathway proposed to explain the other observed products.

The role of the ferric peroxo moiety in the mechanism has been supported by mutagenesis studies in which Thr306 has been replaced by an alanine[258]. This threonine is believed to be the active site residue that directs the delivery of protons required to cleave the O–O bond and form the ferryl species. As expected, its loss results in an approximately 20-fold decrease in the ferryl dependent C17 hydroxylation activity but a much smaller decrease in C17–C20 lyase activity mediated by the ferric peroxo moiety[258]. Experiments involving analysis of the solvent deuterium isotope effect as a function of pH have suggested that the protonation of the ferric peroxide intermediate governs whether the reaction proceeds via a ferryl dependent (17α hydroxylation) or a peroxy adduct (C17–C20 lyase) pathway[259].

The aldehyde corresponding to pregnenolone has also been used as a mechanistic probe with CYP17A as it is reported to undergo exclusive cleavage of the C17–C20 bond to produce formic acid and the 17α alcohol or $\Delta^{16,17}$-olefin[260]. These reactions are believed to proceed via pathways analogous to those proposed for the formation of minor cleavage products of CYP17A catalyzed oxidation of pregnenolone. The more electrophilic carbonyl of the aldehyde favors the direct bond scission pathways by more effectively trapping the ferric peroxide moiety. The aldehyde is not reported to be oxidized to the corresponding acid[260]. This suggests that an ionic cleavage of the proposed peroxy intermediate (Baeyer–Villiger pathway) does not occur to any great extent as hydrogen migration, which would lead to acid formation, is known to be favored for this type of reaction. The experiments with this aldehyde do, however, provide evidence for the bifurcation of a single pathway leading to the two minor products of pregnenolone oxidation (Figure 6.37). Deuteration of the C16α position led to an apparent enrichment in deuteration of the 17α-hydroxy product, suggesting an isotope-induced partitioning away from the $\Delta^{16,17}$-olefin that requires cleavage of the C–D bond[260]. Cleavage of the C–C bond α to an aldehyde is discussed further in Section 8.3.

8.2. Cleavage Alpha to Oxygenated Carbon

Ketones. The CYP17A-mediated cleavage of the C17–C20 bond of pregnenolone (or progesterone) without prior C17 hydroxylation provides the only clearly documented example of cleavage of a C–C bond α to a ketone (Figure 6.37, Section 8.1). The manifold of products formed, however, nicely indicates the variety of mechanistic pathways that might be envisioned. A peroxo adduct from the carbonyl and the ferric peroxide intermediate forms and subsequently decomposes by one of two pathways. A radical mechanism leads to an alkoxy radical that eventually gives C–C bond cleavage to form an alcohol or olefinic product. An ionic mechanism (Baeyer–Villiger) leads to an ester product in which insertion of oxygen has occurred with retention of configuration. It will be of interest to determine whether such pathways might provide the dominant activity of some P450s.

Aldehydes. Cleavage of a C–C bond α to an aldehyde has already been discussed in the context of the CYP17A-catalyzed oxidation of an aldehyde analogue of pregnenolone (Section 8.1). However, such reactions are believed to play a central role in the activities of several other P450s including the important steroid biosynthetic enzymes aromatase (CYP19) and 14α-demethylase (CYP51). It is worth emphasizing that P450-catalyzed aldehyde oxidation does not necessarily result in C–C bond cleavage and that in fact often oxidation to the corresponding carboxylic acid occurs[261]. The factors that govern partitioning between these different modes of oxidation are unknown at present[261].

Aromatase (CYP19), like $P450_{scc}$, plays an essential role in the biosynthesis of steroid hormones. It catalyzes the aromatization of the C_{19} androgen, androstendione to the C_{18} estrogen estrone (Figure 6.38), as well as similar aromatizations of testosterone and 16α-hyroxyandrostendione[262, 263]. These conversions involve three sequential oxidations at the angular C19 methyl group that result in its eventual loss and aromatization of the A-ring of the substrate. Each oxidation requires a molecule of NADPH and of

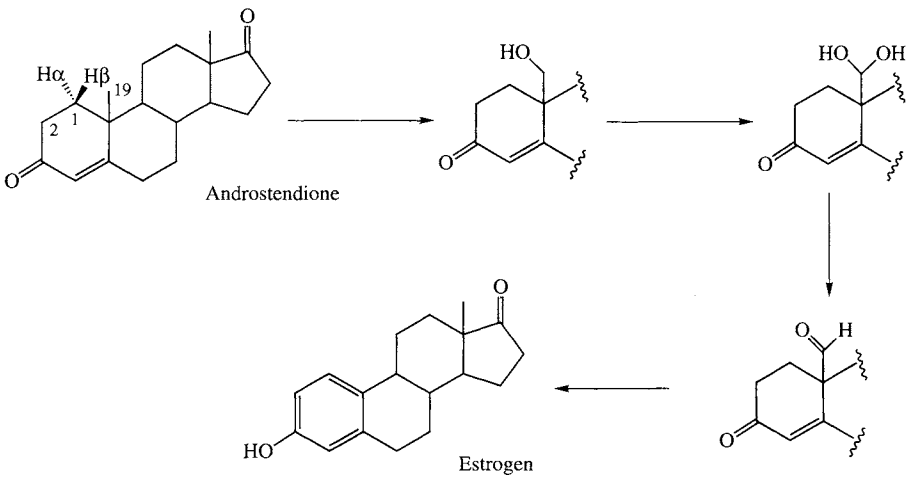


Figure 6.38. Intermediates in the catalytic turnover of aromatase (CYP19).

oxygen[264]. The first two steps appear to be unexceptional P450-catalyzed hydroxylation steps. The initial reaction produces the C19 primary alcohol and proceeds, as expected, with retention of configuration[265, 266], while the second oxidation abstracts the 19-*pro-R* hydrogen to yield a *gem*-diol intermediate[267, 268]. This latter compound is believed to dehydrate to yield the more stable, observed C19 aldehyde. There is an observable tritium isotope effect on the first hydroxylation step[269], but not on the subsequent one with [19-^{3}H] androst-4-ene-3,17-dione or analogues[270, 271]. This is understandable as the first step can discriminate between the hydrogen and tritium atoms on a given methyl group. An isotope effect on the second step, however, which stereospecifically removes the *pro-R* hydrogen, would require the kind of *inter*-molecular effect commonly suppressed in P450 reactions. It is the mechanism of the third oxidative transformation that involves C–C bond cleavage and aromatization that has attracted the most attention. In this reaction, the 1β and 2β hydrogens are lost[272–277] into water and the C19 carbon as formate which contains an oxygen atom from the first and third oxidation steps[278, 279]. A large number of different mechanisms have been proposed to account for this transformation involving the intermediacy of a steroid containing a C19 formyl group and, variously, a 4,5-epoxide[280], a 1β-hydroxyl[276], a 2β-hydroxyl [281, 282], or a C19 peroxide [278, 283] as well as a possible enzymic Schiff base formed from the 3-keto moiety[284]. Several of these intermediates are known to be converted spontaneously[281] or by aromatase[285] into estrone but none of them are currently accepted as lying upon the major pathway for aromatization. This is primarily due to the ^{18}O labeling studies indicating that the third oxygen atom is incorporated into formate[278, 280]. The difficulties in establishing the mechanism are illustrated nicely with the postulated 2β-hydroxy intermediate. This was synthesized and shown to aromatize rapidly in the absence of enzyme[281] and it was also detected in enzymic incubations at low pH (which slows the aromatization reaction)[282]. However, the facts that the 2β-hydroxyl was not incorporated into the released formate[279], and the stereochemistry of loss of hydrogen from C-2 appears to be substrate dependent ruled this compound out as an obligatory intermediate[286, 287]. The currently accepted mechanism[256, 288–290] explains all experimental observations and is supported by model studies[291–293] and analogy with the mechanisms of other P450s such as CYP17A and CYP2B4 (*vide infra*) (Figure 6.39). Thus, the ferric peroxide intermediate is believed to add to the electrophilic aldehyde carbonyl to yield a peroxyhemiacetal. This can fragment to give an alkoxy radical that loses formic acid to produce a C10 radical. Loss of the 1β hydrogen and enolization of the carbonyl is required to produce the aromatized A ring. Recent model studies by Valentine and coworkers provide

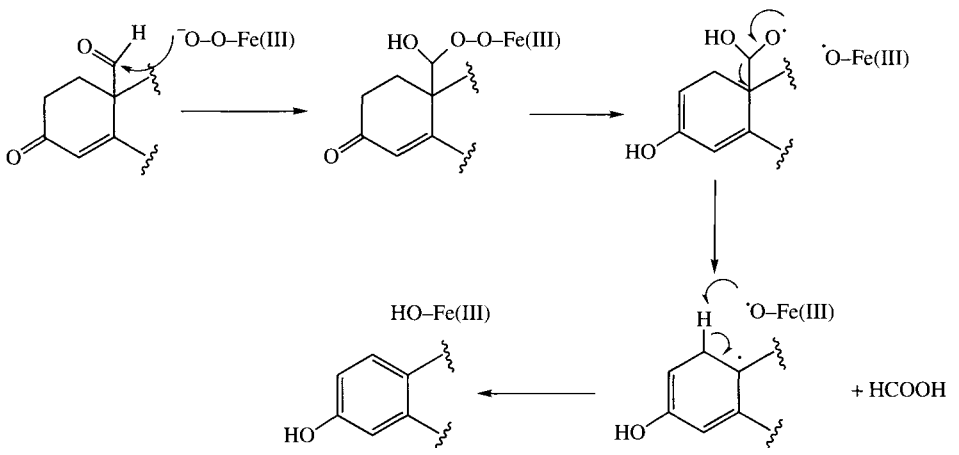
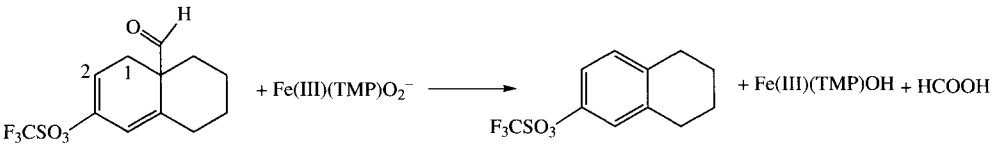


Figure 6.39. The currently accepted mechanism for the final step in the aromatase catalyzed reaction. The timing of enolization of the carbonyl with respect to the addition of the ferric peroxide to the aldehyde and to C–C and O–O bond fission is still uncertain.



TMP = Tetramesitylporphyrin

Figure 6.40. Conversion of enolized analogue of the natural aromatase substrate to the corresponding aromatized compound is catalyzed by a model peroxo ferric porphyrin complex.

strong support for the involvement of the ferric peroxo in the mechanism[291]. They demonstrated that a model peroxo ferric porphyrin complex will quantitatively convert an enolized model of androstendione to the corresponding aromatic compound and formate (Figure 6.40). Reaction of the ferric peroxo model with androstendione itself results in the chemically reasonable epoxidation of the electron deficient C4–C5 double bond. This follows suggestions in the literature that enolization of the C3 carbonyl occurs prior to C19–C10 bond cleavage and additionally activates the 1β hydrogen toward loss[293, 294]. However, whether the chemoselectivity required (C–C bond cleavage vs epoxidation) is achieved enzymatically via prior enolization or by selective positioning of the substrate within the active site remains to be established.

Enzymes of the CYP51 family (sterol 14α-demethylases) catalyze the removal of the 14α-methyl group from a variety of steroidal nuclei with concomitant introduction of a carbon–carbon double bond (Figure 6.41). The archetypal reaction is the loss of the C14 angular methyl group (C32) from lanosterol with formation of a C14–C15 double bond during cholesterol biosynthesis[295, 296]. Once again, this conversion is believed to involve three sequential oxidation steps and proceed initially via an alcohol that is subsequently converted into an aldehyde[297–307]. These steps parallel the first two steps catalyzed by aromatase and are believed to be unexceptional hydroxylation reactions[308–310]. The stereochemical course of the second hydroxylation is unknown, but studies with mechanism-based inhibitors have shown that steroidal 32-*S*-vinylalcohols are transformed to covalent inhibitors, presumably with a C32 carbonyl via a C32 *gem*-diol while the 32-*R* isomers are not oxidized[311]. These results do demonstrate stereospecificity in the oxidation of

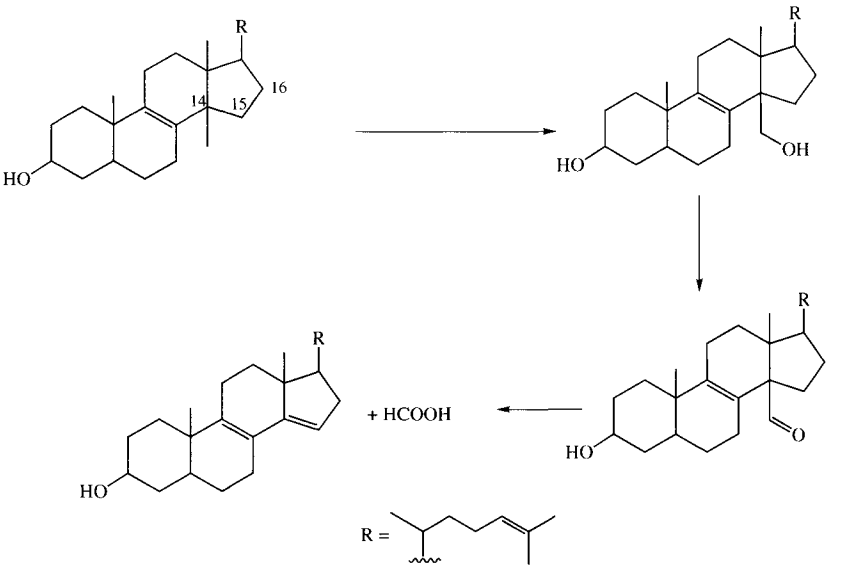


Figure 6.41. The stable intermediates in the 14α-demethylation of lanosterol.

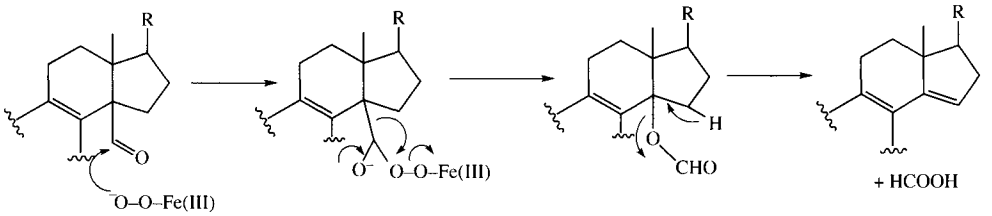


Figure 6.42. A possible mechanism for the final step in the 14α-demethylation of lanosterol that employs the isolated Baeyer–Villiger rearrangement product. Radical decomposition of the peroxyhemiacetal intermediate may also lead to the observed demethylated product.

a C32 alcohol but the deduction that the *proS* hydrogen is removed is more questionable. The 32-*S*-vinyl alcohols that are oxidized have a hydrogen that is stereochemically equivalent to the *proR* hydrogen of the hydroxymethyl intermediate in the demethylation reaction. This would suggest that it is this *proR* hydrogen that is abstracted in the second hydroxylation step catalyzed by CYP51.

The facts on the oxidation of the C32 aldehyde are remarkably similar to those seen for the aromatase catalyzed reaction. The formate moiety that is expelled contains one dioxygen-derived oxygen atom and incorporates one oxygen and one hydrogen atom from the aldehyde precursor[312]. There is also a stereospecific loss of the *syn* 15α hydrogen in the formation of the $\Delta^{14,15}$ double bond[298, 313, 314]. By analogy with the aromatase reaction, the oxidation is proposed to be initiated by addition of the ferric peroxo intermediate to the electrophilic aldehyde to produce a peroxyhemiacetal (Figure 6.42). However, the decomposition of this intermediate is proposed to occur via an ionic Baeyer–Villiger-like mechanism rather than a radical one. The primary reason for this is the isolation and spectroscopic identification of the 14α-formyloxy compound required as an intermediate in the ionic mechanism[315]. It was also demonstrated that the 14α-formyloxy intermediate could be converted into the final product by the enzyme[315]. The mechanism of this elimination has

not been studied in detail but loss of the tertiary allylic formate would be expected to be a facile reaction, subject to standard acid–base catalysis. However, the proposed mechanism invokes an ionic decomposition of a peroxyhemiacetal while those suggested for CYP17 and CYP19 invoke radical pathways. The possibility remains therefore that, as is believed to be the case with CYP17, the Baeyer–Villiger product is formed as a result of a minor pathway while the major route proceeds via simultaneous elimination of C32 and the 15α hydrogen. A unified view of the mechanism of the three P450 families would suggest that this occurs via radical decomposition of the peroxyhemiacetal (cf. Figure 6.39). It is perhaps also possible that the isolated formyloxy compound arises from formate trapping of a C14 cation, the major fate of which would be to undergo elimination with loss of the C15α hydrogen. The cation would derive from a single-electron oxidation of a C14 radical, the intermediate in the radical decomposition of the peroxyhemiacetal. The availability of cloned and overexpressed CYP51 from animals[316, 317], plants[318], fungi[312], and bacteria[319–321] with differing substrate specificity[322] and also of a crystal structure of one of the bacterial enzymes[18] should facilitate complete elucidation of the mechanism of this interesting family of P450s.

Some xenobiotic metabolizing enzymes, particularly CYP2B4, are also reported to be capable of catalyzing the conversion of some aldehydes into the one-carbon diminished alkene and formate[323, 324]. It appears that there is a structural requirement for α or β branching of the aldehyde for the reaction, with alkene formation occurring with compounds such as isobutyraldehyde and 2-methylbutyraldehyde but not with the straight-chain propionaldehyde or valeraldehyde[323]. Although most work has been carried out with CYP2B4, other isoforms such as CYP1A2, 2E1, 2C3, and 3A6 are all reported to catalyze this type of transformation[323]. The extent to which this occurs relative to oxidation of the aldehyde to the corresponding carboxylic acid appears small with a ratio of 50:1 favoring acid formation in the CYP2B4 catalyzed oxidation of 2-phenylpropionaldehyde[325]. However, the reaction has proved very valuable as a model for understanding the mechanism of the C–C bond cleavage reactions of CYP17, 19, and 51 and has played a significant role in the formulation of their mechanisms above. It was shown that the deformylation reaction was supported by P450 reductase/NADPH or H_2O_2 but not cumene hydroperoxide or iodosyl benzene[324]. This concurs with the hypothesis that it is the ferric peroxo species that adds to the aldehyde rather than the ferryl species. Fragmentation of the hydroperoxyhemiacetal then occurs to produce formate and the alkene (Figure 6.43). Significantly, formation of the one-carbon reduced alcohol has also been

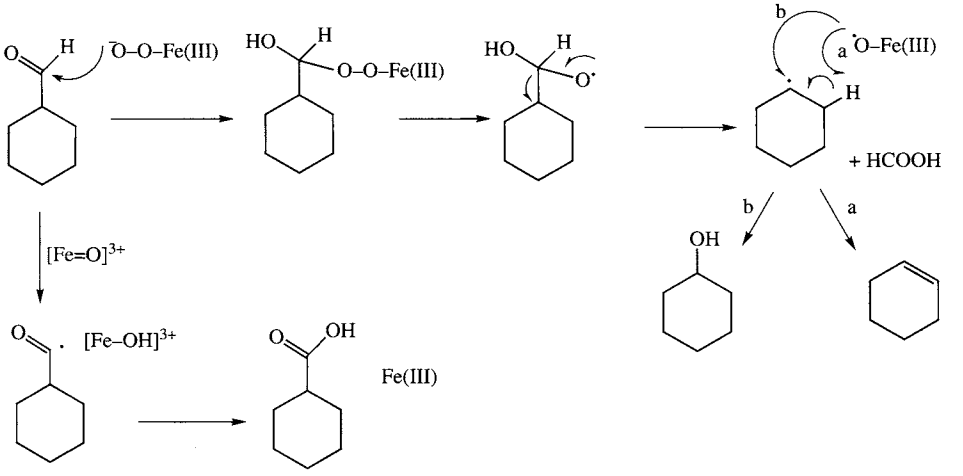


Figure 6.43. The oxidation of cyclohexanecarboxaldehyde by CYP2B4 is believed to proceed via the ferryl species to yield the carboxylic acid and via the ferric peroxo species to yield the deformylated products, cyclohexene and cyclohexanol.

reported, although only in passing![325] This product is analogous to the 17α-hydroxy C_{19} products reported from CYP17A oxidation of pregnenolone and its analogues. It should be noted that while deformylation is thought to involve the ferric peroxo species, oxidation to the acid is believed to proceed via the ferryl species[325].

The relevance of the CYP2B4 catalyzed deformylation reaction as a model for CYP51 is clearly demonstrated by the aromatization of the androstendione analogue 3-oxodecalin-4-ene-10-carboxaldehyde to the corresponding tetrahydronaphthalene (cf. Figure 6.40) with concomitant formate production[326, 327]. Deuterium isotope studies showed that the formyl hydrogen was retained in the formate, that the 1β hydrogen was specifically lost, and that loss of the C2 hydrogen was not stereoselective. These results faithfully reproduce the characteristics of the aromatase catalyzed reaction.

Recently, support for the role of ferric peroxo species in CYP2B4 catalyzed deformylation, and by analogy for the mechanisms of CYP17, -19, and -51, has come from mutagenesis studies[327]. Vaz and Coon reported the effect of replacing Thr302, the residue thought to facilitate O–O bond cleavage in CYP2B4, with alanine. It was expected that this would favor the peroxo pathway and decrease the availability of the ferryl species. In line with these expectations, normal P450-catalyzed reactions, including aldehyde to carboxylic acid oxidation, were suppressed but deformylation to the alkene and alcohol products was significantly enhanced[327]. Evidence for the radical nature of the decomposition of the peroxyhemiacetal has come from examination of the mechanism-based inactivation of P450s that occurs concurrently with aldehyde oxidation[325, 328]. For saturated aldehydes, it was shown that inactivation of CYP2B4 paralleled their ability to undergo a deformylation reaction, suggesting that both of these processes flowed from a common intermediate, the peroxyhemiacetal (Figure 6.43)[325]. It was shown that inactivation of the P450 was due to addition of the carbon radical, formed in a homolytic process, to the γ-*meso* position of the prosthetic heme[328]. Interestingly, although $P450_{BM3}$ is reported to oxidize a variety of aldehydes without detectable deformylation[261], it was demonstrated that a mutant is deactivated by aldehydes when the co-oxidant is H_2O_2[329]. This presumably again occurs through an alkyl radical formed by homolytic decomposition of the peroxyhemiacetal intermediate. An intermediate was detected in this work that was spectroscopically consistent with an isoporphyrin which would be formed upon addition of a carbon radical to the heme cofactor[329]. Finally, it is of note that the ferryl catalyzed oxidation of aldehydes to acids can also cause enzyme inactivation by heme adduct formation, but in this case as predicted for an H abstraction mechanism, an acylated heme is formed[328].

Cytochrome P450s can also interact with aldehydes in a different way to generate the corresponding hydrocarbon and CO_2,[330]. Hydrocarbons are abundant components of cuticular lipids in most insects and can also play a role in their chemical communication. It has been demonstrated that in microsomes derived from the house fly, *Musca domestica*, hydrocarbons are formed from the corresponding aldehyde with concomitant generation of CO_2 and with all the characteristics expected of a P450-mediated reaction:

$$CH_3(CH_2)_8CH{=}CH(CH_2)_{12}CD_2CDO + NADPH + O_2 + H^+ \rightarrow CH_3(CH_2)_8CH{=}CH(CH_2)_{12}CD_3 + NADP^+ + CO_2 + H_2O$$

There is a requirement for NADPH and oxygen and the reaction is inhibited by both CO and an antibody to house fly P450 reductase[330]. Labeling studies showed that deuterium atoms at the C-1, C-2, and C-3 positions were all retained[331]. In addition, active oxygen donors such as hydrogen peroxide, cumene hydroperoxide, and iodosylbenzene all support hydrocarbon production to some extent. The ability of the latter species to support oxidation clearly indicates that the ferric peroxide species is not the active oxidant in this case. On the basis of these results, an unusual mechanism has been proposed[331] and a slightly more conventional version is presented here (Figure 6.44). The first step is the oxidation of the aldehyde to a dioxirane or its resonance form, a carbonyl oxide. Dioxiranes are known to decompose with release of CO_2 and formation of two radicals that can recombine as shown to form a hydrocarbon[332]. Presumably, this recombination would be favored by retention of the fragments within the active site. Complete elucidation of the reaction mechanism awaits identification and purification of the P450 but recent studies have shown this to be a

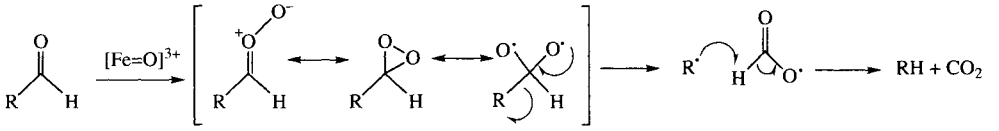


Figure 6.44. Possible mechanism for the P450-catalyzed conversion of an aldehyde into the corresponding hydrocarbon and CO_2 seen in the biosynthesis of insect-derived hydrocarbons.

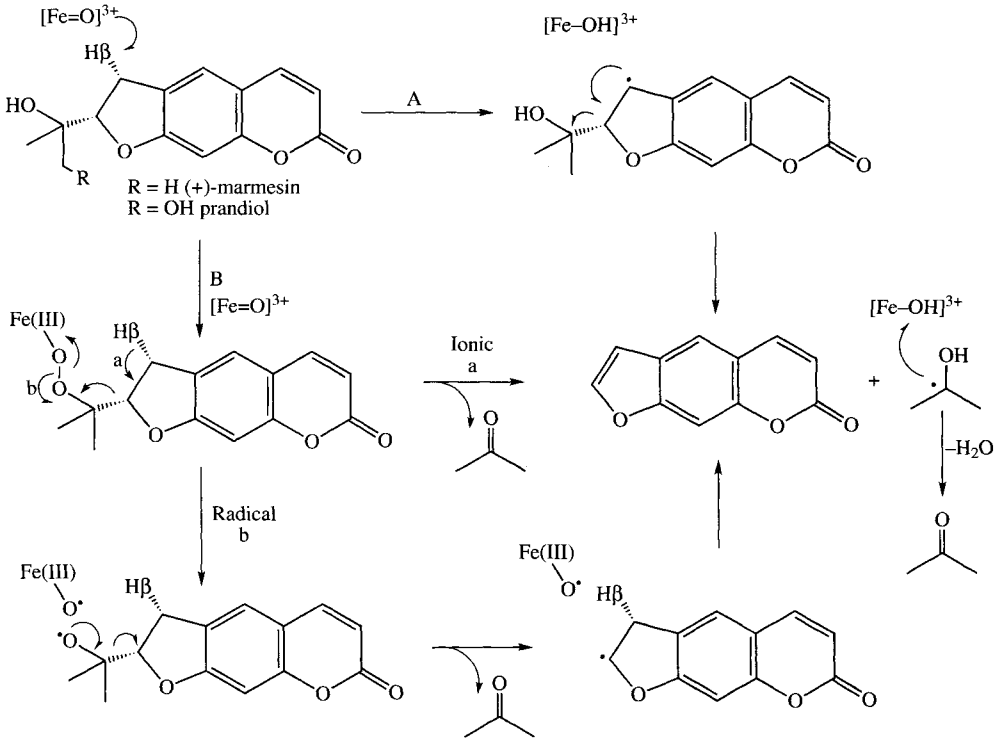


Figure 6.45. Mechanistic possibilities for the P450-catalyzed conversion of (+)-marmesin into psoralen and acetone. Prandiol is known not to be an intermediate in this process.

widespread reaction in insects for the formation of hydrocarbons[333].

Alcohols. Boland and coworkers demonstrated conclusively that a P450 can catalyze the direct fragmentation of an alcohol into an olefin and a carbonyl-containing fragment[69]. They studied the conversion of marmesin to psoralen in microsomes derived from cell cultures of the plant *Ammi majus* (Figure 6.45)[69]. Deuterium labeled precursors allowed them to demonstrate that marmesin was converted into an equimolar mixture of acetone and psoralen, excluding the possibility of other oxygenated intermediates such as the known prandiol (Figure 6.45). The stereochemistry of the elimination was exclusively *syn* and a small isotope effect ($k_H/k_D = 4$) was observed when the abstracted hydrogen was replaced with deuterium. The mechanism proposed (Figure 6.45, pathway A) consists of β-hydrogen atom abstraction, decomposition of the radical intermediate to produce the olefin and an isopropoxyl radical, the latter of which is intercepted by the Fe(IV)OH species[69]. A possible mechanistic alternative (Figure 6.45, pathway B) invokes intermediates analogous to those proposed for the diol cleavage reactions above. In these, it would be the alcohol moiety that is initially

attacked and initiates fragmentation to the observed products.

This type of C–C cleavage reaction, however, appears to be a general and important biosynthetic one in plants and a number of other analogous oxidative C–C bond cleavage reactions seen in bacteria and plants have now been postulated to be P450 catalyzed[334, 335]. In particular, secologanin synthase from *Catharanthus roseus* (CYP72A1) is believed to catalyze the C–C bond cleavage that transforms loganin into secologanin, the final common non-nitrogenous precursor of many plant indole alkaloids (Figure 6.46)[335, 336]. In this case, the reaction involves cleavage of a carbocyclic ring rather than fragmentation of the substrate. This activity was demonstrated *in vitro* with CYP72A1 heterologously expressed in *E. coli* as a fusion with its homologous P450 reductase[336].

A reaction that involves cleavage of the C–C bond α to a phenol occurs in aflatoxin biosynthesis[337]. Aflatoxins are mycotoxins produced by strains in the fungal genus *Aspergillus* and are notable for the complexity of their biogenesis. Genetic evidence suggested that a single P450 was responsible for the transformation of *O*-methylsterigmatocystin to aflatoxin B_1 (Figure 6.47)[338]. A P450 from *Aspergillus parasiticus* was subsequently cloned, heterologously expressed in yeast, and was demonstrated to be capable of catalyzing this remarkable conversion *in vivo*[337]. The first formed 11-hydroxy-*O*-methylsterigmatocystin (Figure 6.47) was also synthesized and shown to be converted to aflatoxin B_1. These experiments interlocked with a wealth of previous results from *in vivo* isotope labeling studies and led to the mechanistic hypothesis shown[337]. After C–C bond cleavage and formation of the proposed hydrolytically unstable lactone, the ensuing decarboxylation, dehydration, rearrangement, and *O*-demethylation reactions are presumed to proceed spontaneously. Two plausible mechanisms for the

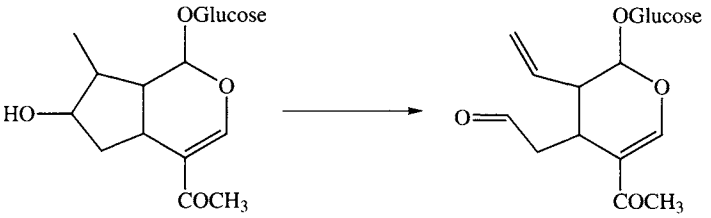
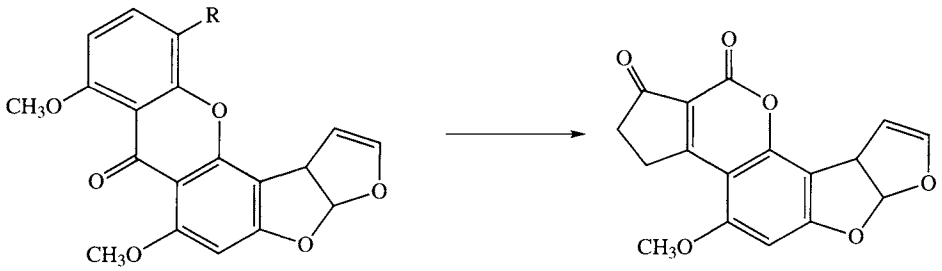


Figure 6.46. Loganin is converted into secologanin via a P450-catalyzed C–C bond cleavage reaction analogous to that seen in psoralen biosynthesis.



R = H *O*-Methylsterigmatocystin
R = OH 11-hydroxy *O*-Methylsterigmatocystin

Figure 6.47. A single cytochrome P450 is responsible for the conversion of *O*-methylsterigmatocystin to aflatoxin B_1 via 11-hydroxy *O*-methylsterigmatocystin.

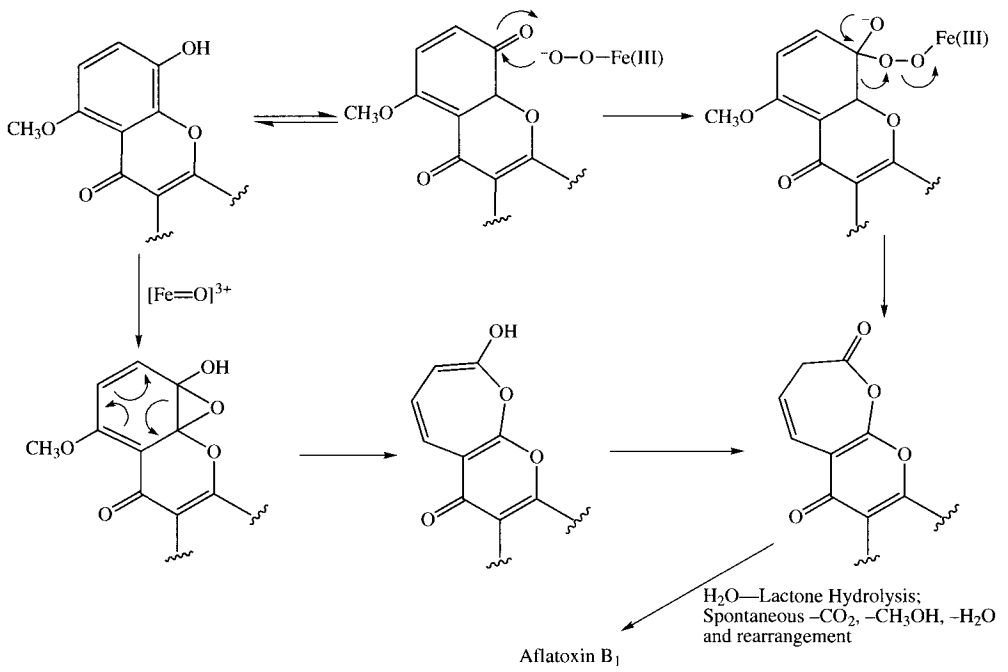


Figure 6.48. Mechanistic proposals for the P450-catalyzed C–C bond cleavage during the biosynthesis of aflatoxin B_1. One possibility involves a Baeyer–Villiger-like reaction of the ferric peroxo species with the keto tautomer of the phenolic substrate while the other proceeds via the epoxide intermediate typical of ferryl catalyzed aromatic oxidations.

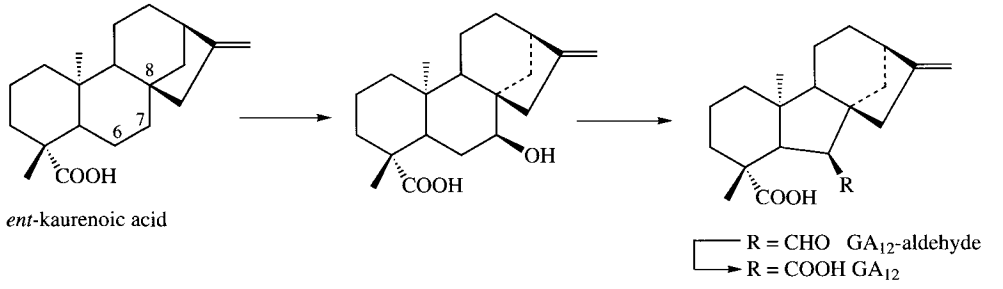


Figure 6.49. CYP88A catalyzes the three oxidative transformations required to convert *ent*-kaurenoic acid into GA_{12}.

C–C bond cleavage process have been proposed (Figure 6.48)[337]. The first involves a Baeyer–Villiger-like oxidation of the keto tautomer of the phenol and the second a rearrangement of the epoxide intermediate in aromatic oxidation. Delineation of the mechanism will require experimentation with purified enzyme, mutants, and substrate analogues.

The gibberellins (GAs) are important plant hormones with remarkably complex structures. Several similarly remarkable multifunctional P450s have been implicated in their biosynthesis in both plants and fungi[339, 340]. CYP88A from *Arabidopsis thaliana* and barley has been shown to catalyze the three oxidative steps required to convert *ent*-kaurenoic acid to GA_{12} (Figure 6.49)[341]. The experiments involved the expression of CYP88A in yeast strains containing *A. thaliana* P450 reductase and monitoring *in vivo* oxidation of potential substrates. The key step in the proposed

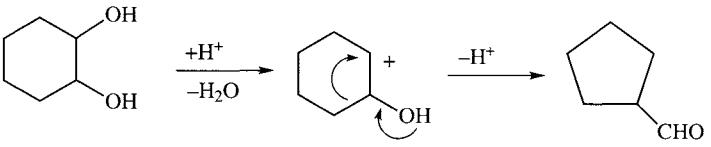


Figure 6.50. Mechanism of a pinacol rearrangement of a diol.

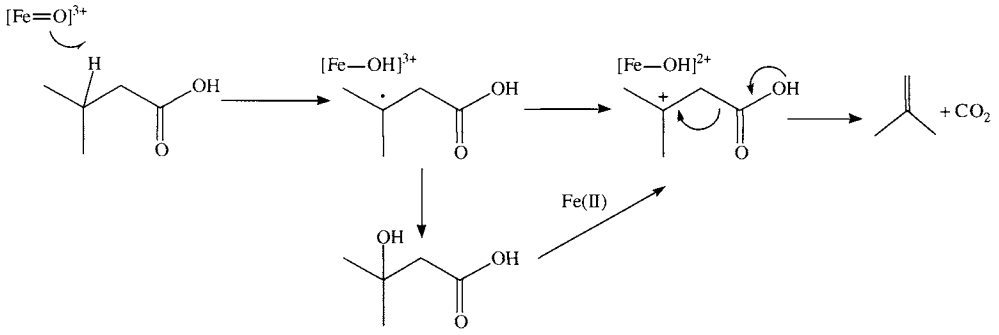


Figure 6.51. Likely mechanisms for the P450-catalyzed conversion of valerate into isobutene and CO_2. A pathway involving direct hydride abstraction has also been proposed but appears less probable.

reaction involves cleavage of a C–C bond α to an alcohol in an oxidative ring contraction to yield an aldehyde (GA_{12}-aldehyde, Figure 6.49)[341]. The mechanism has not been investigated but the process follows the pathway predicted for an α-hydroxy carbocation, such as the intermediate proposed for a pinacol rearrangement of a diol (Figure 6.50). Such a cation could arise from a diol formed by CYP88A catalyzed C6 hydroxylation under the influence of the Lewis acidic heme iron or directly via a SET process from the hydroxylation radical intermediate. Subsequently, a P450 from the fungus *Gibberella fujikuroi* was also shown to catalyze the same pinacol-like transformation, once again by expression and *in vivo* monitoring of putative substrate transformation[342]. In this case, a 6,7-diol was also isolated but was not further transformed via ring contraction, suggesting that such a compound is not an intermediate in this pathway. This single fungal P450 was also proposed to be capable of catalyzing at least *seven* other biosynthetically significant oxidative transformations as well as the three assigned to CYP88A, explaining the various GA metabolites found in *G. fujikuroi*. One of these other transformations is a proposed oxidative cleavage of the vicinal 6,7-diol. Clearly, the results of *in vitro* characterization of the catalytic capabilities of this enzyme will be of great interest.

Acids. Two isolated examples of P450-catalyzed oxidative decarboxylation have appeared in the literature. The first concerns the formation of isobutene from isovalerate by the yeast *Rhodotorula minuta*[343]. A P450 and a homologous reductase were purified and a reconstituted system that produced isobutene from isovalerate was constructed[343]. A large isotope effect upon isobutene formation was found when the β-hydrogen was substituted with deuterium (k_H/k_D = 14), clearly implicating cleavage of this bond in the rate-determining step. It was also found that β-branching appeared to be necessary for alkene formation. A mechanism involving direct hydride abstraction and decarboxylation of the resultant cation was proposed[343]. However, more conventional pathways are also possible in which either (a) hydrogen atom abstraction to give a carbon radical is followed by electron transfer to generate the corresponding carbocation, or (b) the tertiary alcohol is formed but ionizes to the carbocation under the influence of the Lewis acidic heme iron (Figure 6.51). One caveat with this system is that the P450 was subsequently shown to hydroxylate benzoate to 4-hydroxybenzoate as part of phenylalanine catabolism and this latter reaction

appears to be its primary metabolic function[344, 345]. It is also unclear whether other nonvolatile products of isovalerate oxidation are formed in the incubations which were monitored by headspace gas chromatography[343]. Thus, the exact nature of this decarboxylation reaction remains to be established.

Hirobe has reported the P450-catalyzed decarboxylation of carboxylic acids to a one-carbon shorter alcohol[346]. The acid must have an α-carbon bearing either a phenyl group or three substituents. This transformation was originally observed in iron–porphyrin model systems but was subsequently reproduced *in vivo* in rats and in rat liver microsomes for two therapeutic carboxylic acids (Figure 6.52)[346]. Once again, the mechanism has not been investigated but the

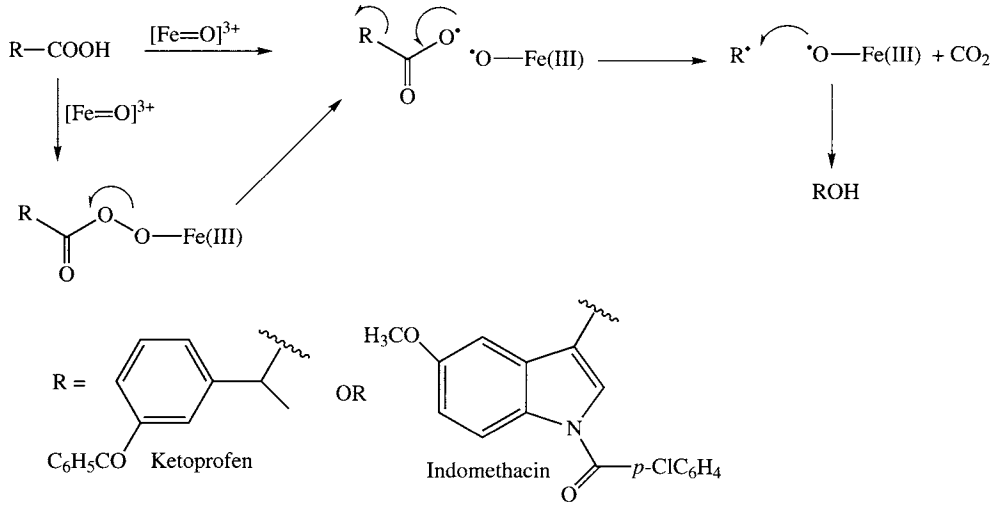


Figure 6.52. Possible pathways for the oxidative decarboxylation of some therapeutic carboxylic acids catalyzed by both P450s and some iron–porphyrin model systems.

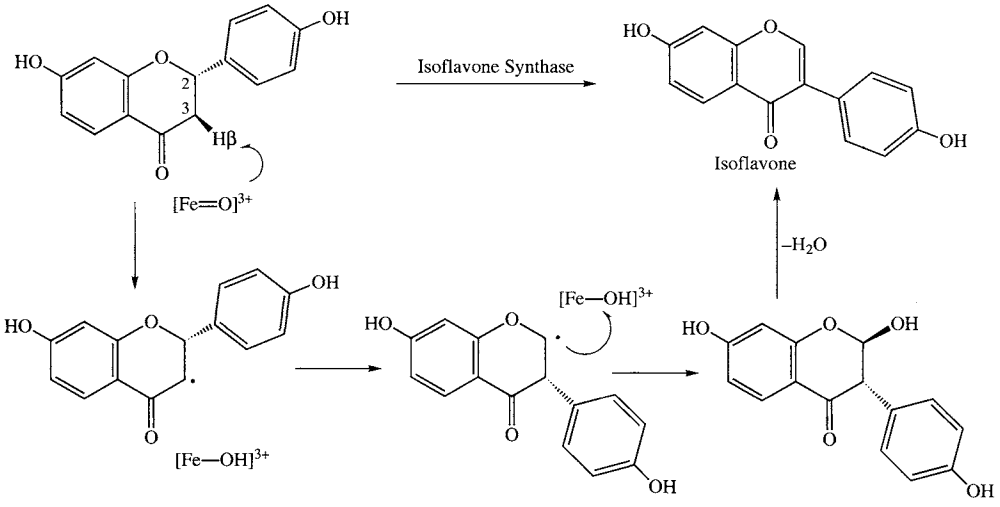


Figure 6.53. Isoflavone synthase (CYP93C) catalyzes the formation of isoflavone from 2*S*-flavone via an oxidative aryl migration. A possible mechanism involving an anchimerically stabilized radical is shown.

proposed decomposition of a carboxyl radical is attractive, especially as this process is known to be quite sensitive to α-substitution. The radical might be produced either directly from the carboxylate by the ferryl species or by decomposition of a peroxyacid initially formed by reaction of the acid and an ironoxo species (Figure 6.52). This latter mechanism would then be analogous to the reported CYP2B4 catalyzed conversion of 2-phenylperacetic acid to CO_2 and benzyl alcohol by homolysis of the O–O bond[347].

Ethers. Isoflavone synthase (CYP93C) catalyzes the formation of isoflavone from 2*S*-flavone via an unusual oxidative aryl migration (Figure 6.53)[348–350]. (This C–C bond cleavage occurs α to an ether and is classified as such here, but it is unclear whether this is a mechanistically significant feature.) Little is known about the reaction, but it is postulated to proceed via 3β HAT to give a carbon radical anchimerically stabilized by the adjacent phenol[348, 351]. Oxygen rebound can then occur at the C2 position to give the unstable 2-hydroxyisoflavone that dehydrates to isoflavone (Figure 6.53). Support for this mechanism is provided by the isolation of the 3β-hydroxyisoflavone as a side product of the reaction[351]. The availability of heterologously overexpressed wild-type protein and site-directed mutants should facilitate investigation of this unusual transformation[351].

8.3. Cleavage Alpha to Carbon Bearing a Nitrogen Atom

Amines. Recently, an example of C–C bond cleavage α to an amine has been reported (Figure 6.54)[352]. Interestingly, this is also a rearrangement reaction and one of the few examples of C–C bond scission catalyzed by non-biosynthetic enzymes. It was found that a variety of tetramethylpiperidine containing compounds were transformed into the corresponding dimethylpyrrolidine derivatives (Figure 6.54). By incubations with recombinant human liver P450s and immuno-inhibition studies, this reaction was shown to be catalyzed by a variety of P450s, with CYP3A4 the major isoform responsible for this transformation. The authors suggest that this is a general metabolic pathway for compounds containing a tetramethylpiperidine moiety as they have also observed similar metabolism in other mammals[352]. The mechanism of the reaction has not been investigated in detail but clearly appears to be a transformation of a secondary amine, formed via *N*-dealkylation if necessary, given the structures of the pyrrolidines produced. The intermediacy of hydroxylamines or the corresponding nitroxyl radical in this reaction has been suggested. One possibility (Figure 6.54) is that the heme iron may promote ionization of a hydroxylamine to an incipient nitrogen cation that rearranges, a pathway comparable to the P450-catalyzed dehydration of oximes to nitriles[353]. Alternatively, it may simply be a rearrangement of the intermediate nitrogen cation radical formed during amine oxidation. This can no longer be stabilized by α-hydrogen elimination and the steric congestion of the surrounding methyl groups may slow the oxygen rebound, allowing rearrangement (Figure 6.54). The piperidine to pyrrolidine rearrangement has precedent in the chemistry of *N*-fluoroamines that undergo the same ring contraction in the presence of a Lewis acid[354]. This latter reaction, however, presumably involves the equivalent of a nitrogen cation, rather than a cation radical species favoring the former pathway. More detailed investigations are required to determine the mechanism of this interesting transformation.

Finally, another of the remarkable multifunctional P450s involved in GA formation in fungi has recently been demonstrated to catalyze the demethylation of an angular carbon along the biosynthetic pathway (Figure 6.55)[355]. The apparently concomitant formation of the lactone with demethylation suggests that a different pathway is followed from that seen in aromatase and 14α-demethylase. It is tempting to speculate that this represents a biosynthetically novel oxidative decarbonylation or decarboxylation reaction in which an alcohol or the corresponding cation is the initial product. This could then be intercepted by the adjacent carboxylate to form the observed lactone (Figure 6.55). Clearly, however, the cytochrome P450s are capable of catalyzing C–C cleavage by a variety of mechanisms and much work remains to understand all the possible permutations of these interesting reactions.

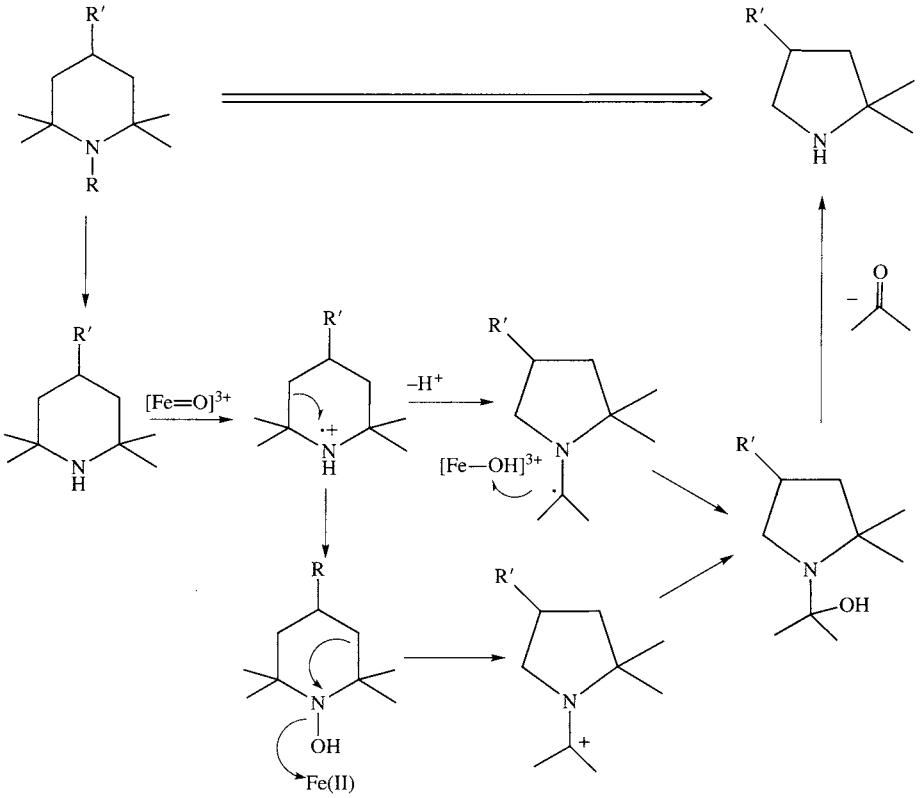


Figure 6.54. A variety of tetramethylpiperidine compounds are converted into the corresponding dimethylpyrrolidine derivatives by a number of xenobiotic metabolizing P450s, particularly CYP3A4. Two mechanistic possibilities for this process are shown. (R = H, R′ = *p*-nitrophenyl-NH- or R = CH_3, R′ = $(C_6H_5)_2HCO$-)

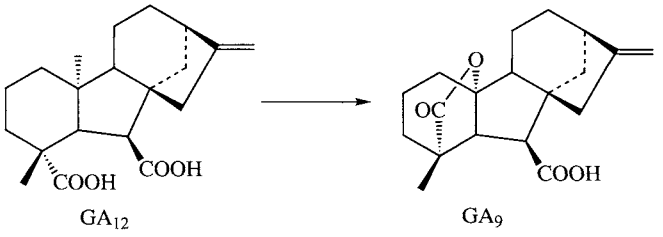


Figure 6.55. Gibberellin 20-oxidase from the fungus *Gibberella fujikuroi* is a multifunctional P450 that catalyzes the angular demethylation of GA_{12} to produce the lactone GA_9.

9. Conclusions

Cytochrome P450 mechanisms continue to surprise and delight, although the field is growing to maturity and the completely unexpected is less frequently encountered. Experimentally, the past few years have seen major progress in characterizing the intermediates that are formed as molecular oxygen is activated to the final oxidizing species. All the intermediates, with the exception of the

critical ferryl species, have now been directly observed by various spectroscopic and crystallographic methods. The ferric peroxo anion has been found to act as the oxidizing agent with a growing range of highly electrophilic substrates. In contrast, the proposed role for the ferric hydroperoxo complex as an electrophilic oxidizing agent remains a matter of debate, as the evidence advanced in support of the proposal is circumstantial and contradictory. Although the ferryl species remains elusive, it is increasingly clear that it plays the predominant role as the oxidizing agent in the P450 catalytic cycle. A second area that has recently received considerable attention is the mechanism of hydrocarbon hydroxylation, the key question being whether the radical rebound mechanism that has held sway for three decades is in fact valid. The contradictory results obtained with radical and cation probes, which have provided most of the new evidence, must be resolved by further experimentation in order for this question to be settled. The development of a two-state model for the catalytic action of P450 enzymes may be one of the most important recent advances in the field, as it provides a ready explanation for a variety of otherwise contradictory data, some of which argues for concerted and some for nonconcerted oxidation mechanisms. No doubt, the next few years will uncover novel aspects of P450 function and will lead to deeper and more sophisticated understanding of the catalytic mechanisms of the amazing family of P450 enzymes.

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7

Inhibition of Cytochrome P450 Enzymes

Maria Almira Correia and Paul R. Ortiz de Montellano

1. Introduction

Three steps in the catalytic cycle of cytochrome P450 (P450, CYP; see Chapters 5 and 6) are particularly vulnerable to inhibition: (a) the binding of substrates, (b) the binding of molecular oxygen subsequent to the first electron transfer, and (c) the catalytic step in which the substrate is actually oxidized. Only inhibitors that act at one of these three steps will be considered in this chapter. Inhibitors that act at other steps in the catalytic cycle, such as agents that interfere with the electron supply to the hemoprotein by accepting electrons directly from P450 reductase[1–3], are not discussed here.

P450 inhibitors can be divided into three mechanistically distinct classes: Agents that (a) bind reversibly, (b) form quasi-irreversible complexes with the heme iron atom, and (c) bind irreversibly to the protein or the heme moiety, or accelerate the degradation and/or oxidative fragmentation of the prosthetic heme. Agents that interfere in the catalytic cycle prior to the actual oxidative event are largely reversible competitive or noncompetitive inhibitors. Those that act during or subsequent to the oxygen transfer step are generally irreversible or quasi-irreversible inhibitors and often fall into the category of mechanism-based (or suicide) inactivators. Extensive lists of P450 inhibitors are available in various reviews[4–12]. This chapter focuses on the mechanisms of inactivation; thus, most of the chapter is devoted to the discussion of agents that require P450 catalysis to fulfill their inhibitory potential. The mechanisms of reversible competitive and noncompetitive inhibitors, despite their practical importance, are relatively straightforward and are discussed more briefly.

2. Reversible Inhibitors

Reversible inhibitors compete with substrates for occupancy of the active site and include agents that (a) bind to hydrophobic regions of the active site, (b) coordinate to the heme iron atom, or (c) enter into specific hydrogen bonding or ionic interactions with active-site residues[4–10]. The first mechanism, in which the inhibitor simply competes for binding to lipophilic domains of the active site, is often responsible for the inhibition observed when two substrates compete for oxidation by a single P450 isoform. A clear example of such an interaction is provided by the mutual *in vitro* and *in vivo* inhibition of benzene and toluene metabolism[13]. This form of inhibition, which is optimal when the inhibitory compound is bound tightly but is a poor substrate, is usually not highly effective but can

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cause physiologically relevant metabolic changes and clinically significant interactions[14].

2.1. Coordination to Ferric Heme

The binding of a strong sixth ligand to the pentacoordinated heme iron atom of a P450 enzyme, or displacement of a weak ligand in a P450 hexacoordinated state by a strong ligand, causes a shift of the iron from the high- to the low-spin state. This shift is characterized by a "type II" binding spectrum with a Soret maximum at 425–435 nm and a trough at 390–405 nm[15–18]. The change in the redox potential of the enzyme associated with this spin state change makes its reduction by P450 reductase more difficult (see Chapter 5)[18, 19]. This change in reduction potential, as much as physical occupation of the sixth coordination site, is responsible for the inhibition associated with the binding of strong iron ligands.

Cyanide and other ionic ligands bind preferentially, albeit weakly, to the ferric state of a P450 enzyme[20, 21]. The three positive charges of the iron are matched in the ferric hemoprotein by the three negative charges of its permanent ligands (i.e., the two porphyrin nitrogens and the thiolate ion), but in the reduced state there is a charge imbalance of three ligand negative charges but only two ferrous iron positive charges. The cyanide ion, a negatively charged species, binds more readily to the neutral (ferric) than the negative (ferrous) enzyme. Indeed, cyanide binds more weakly to ferric P450 than to ferric myoglobin because the P450 thiolate ligand places a higher electron density on the iron than does the imidazole ligand of myoglobin[22]. The chelation of ionic ligands is disfavored, in addition, by the lipophilic nature of the P450 active site[23].

P450 enzymes are inhibited by nitric oxide (NO), a molecule of great interest because of its role in diverse physiological and pathological processes. Inhibition initially involves reversible coordination of the nitrogen to the iron but a subsequent time-dependent, irreversible inactivation of the enzyme by an undefined mechanism has been reported[24–26]. Inhibition by endogenous NO of P450 enzymes involved in endogenous substrate metabolism, including eicosanoid formation and sterol metabolism, may have physiological consequences[27, 28].

2.2. Coordination to Ferrous Heme

In its simplest form, inhibition through coordination to the heme iron is exemplified by carbon monoxide, a neutral ligand that binds exclusively to the ferrous (reduced) form of P450. The binding of carbon monoxide involves donation of electrons from the carbon to the iron through a σ-bond as well as back-donation of electrons from the occupied ferrous iron d-orbitals to the empty antibonding π-orbitals of the ligand[29]. P450 enzymes (P450, CYP) are so named because their carbon monoxide complexes have spectroscopic absorption maxima at approximately 450 nm[30]. Early studies with model ferroporphyrins, which indicated that only those with a thiolate ligand *trans* to the carbon monoxide yielded the 450-nm absorption, provided key evidence for the presence of a thiolate fifth ligand in P450.[31] Inhibition by carbon monoxide is a signature of P450-catalyzed processes, although the sensitivities of different P450 isoforms to carbon monoxide differ[32] and a few P450-catalyzed reactions are resistant to inhibition by carbon monoxide[33–35]. In particular, the sensitivity of aromatase[36, 37] and $P450_{SCC}$[38] to inhibition by carbon monoxide decreases drastically as the enzymes traverse the conformational and ligand states inherent in their multistep catalytic sequences. Among the human liver P450 isoforms, the susceptibility of different families to carbon monoxide inhibition appears to decrease in the order 2D > 2C > 3A[32].

2.3. Heme Coordination and Lipophilic Binding

Agents that simultaneously bind to lipophilic regions of the active site and to the heme iron atom (Figure 7.1) are inherently more effective P450 inhibitors than agents that only exploit one of these binding interactions. The effectiveness of these agents as P450 inhibitors is governed both by their hydrophobic character and the strength of the bond between their heteroatomic lone pair and the heme iron. Agents such as alcohols, ethers, ketones, lactones, and other structures in which an oxygen atom of the ligand coordinates to the iron, or which act by stabilizing the coordination of the distal water ligand, are relatively poorly bound and are

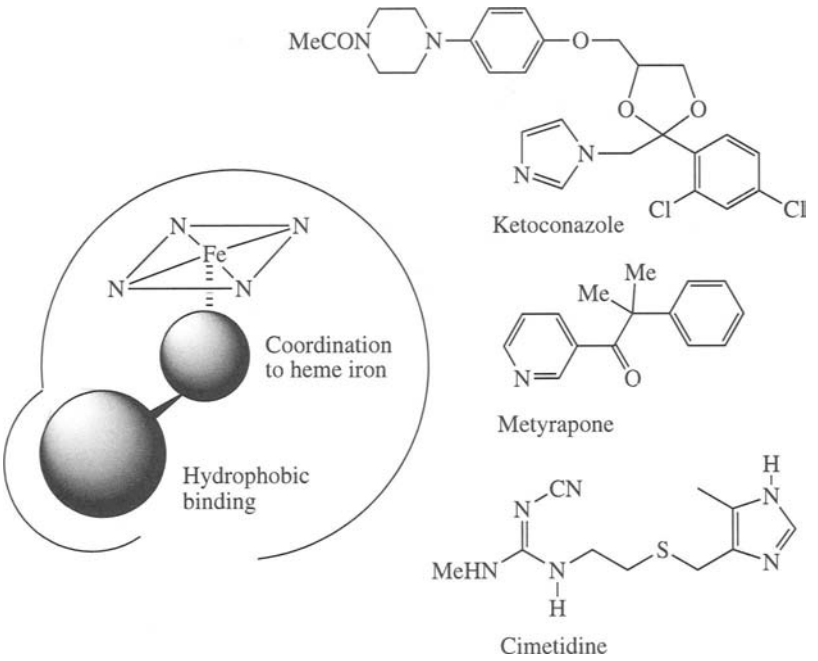


Figure 7.1. Schematic illustration of the binding of an inhibitor to a P450 active site by both coordinating to the heme-iron atom and interacting with the surrounding protein residues. The structures of three agents that inhibit P450 by binding in this manner are shown.

weak inhibitors[17, 38–43]. The Soret band of such complexes is found at approximately 415 nm[14, 17]. In contrast, agents that interact strongly with both the protein and the heme iron atom are often highly effective reversible inhibitors[4–10]. As already noted, the binding of such inhibitors yields a "type II" difference spectrum with a Soret maximum at 430 nm[15, 17, 44, 45]. The structures of these powerful inhibitors usually incorporate nitrogen-containing aliphatic or aromatic functions.

Pyridine, imidazole, and triazole derivatives have proven particularly useful in P450 inhibitors[4]. Metyrapone (Figure 7.1), one of the first P450 inhibitors to be widely employed, first gained prominence as an inhibitor of 11β-hydroxylase, the enzyme that catalyzes the final step in cortisol biosynthesis[46]. This activity led to its use in the diagnosis and treatment of hypercortisolism (Cushing's syndrome) and other hormonal disorders[47]. The determinants of the inhibitory potency of metyrapone and other nitrogen heterocycles are valid for most reversible inhibitors: (a) the intrinsic affinity of the ligand nitrogen electron pair for the heme iron, (b) the degree to which the intrinsic affinity of the ligand for the iron is moderated by steric interactions with substituents on the inhibitor[48, 49], (c) the lipophilicity of the nonligating portion of the inhibitor[23, 50], and, naturally, (d) the congruence between the geometry of the inhibitor and that of the active site. The synergism that results from binding simultaneously through lipophilic interactions and coordination with the heme iron is illustrated by the fact that imidazole and benzene individually are weak inhibitors, but when coupled together as in phenylimidazole they produce a powerful inhibitor[48]. Optimization of these structural features has made possible the therapeutic exploitation of azole compounds such as ketoconazole (Figure 7.1) as antifungal and cancer chemotherapeutic agents[5]. On the other hand, similar features in cimetidine are responsible for the drug–drug interactions that result from the inhibition of the metabolism of co-administered drugs. This

inadvertent side effect instigated the search for non-imidazole containing H2-antagonists devoid of this undesirable feature[40, 51]. Improvement of the potency and particularly specificity of metyrapone, aminoglutethimide, and other classical inhibitors by structural modification continues to be of therapeutic interest. This consideration has led to the development and clinical testing of, for example, pyridylaminoglutethimide, CGS16949A {4-(5,6,7,8-tetra-hydroimidazo[1,5α]pyridin-5-yl) benzonitrile}, CGS18320B bis-(*p*-cyanophenyl) imidazo-1-yl-methane hemisuccinate and R-76713 [6-(4-chlorophenyl)1H (1,2,4 triazol-1-yl)-methyl]-1-methyl-1H-benzotriazole as aromatase inhibitors (see Section 5.2)[5]. Likewise, efforts to improve the properties of ketoconazole have fostered the design and use in antifungal therapy of sterol 14α-demethylase inhibitors such as miconazole, fluconazole, saperconazole, and terconazole (see Section 5.3)[5].

3. Catalysis-Dependent Inhibition

Multiple classes of compounds are now known that undergo P450-catalyzed activation to reactive intermediates that irreversibly or quasi-irreversibly inactivate the enzyme responsible for their activation. This irreversible inactivation by a catalytically generated species is superimposed on the reversible inhibition associated with competitive binding of the parent agent to the ferric enzyme. Mechanism-based[52, 53] (catalysis-dependent) inactivators can be highly enzyme-specific because (a) the compound must first bind reversibly to the enzyme and must satisfy all the constraints imposed on normal substrates of the enzyme, (b) it must be acceptable as a substrate and thus undergo catalytic activation and, finally, (c) the resulting reactive intermediate must irreversibly alter the enzyme and permanently remove it from the catalytic pool. Four general classes of mechanism-based irreversible P450 inactivators are known: (a) agents that bind covalently to the protein, (b) agents that quasi-irreversibly bind to the prosthetic heme iron atom, (c) agents that alkylate or arylate the porphyrin framework of the heme, and (d) agents that degrade the prosthetic heme to products that can, in some cases, themselves modify the protein. However, this mechanistic classification is not rigid, as in the course of its P450, metabolism, a compound may simultaneously partition into two or more of these inhibitory trajectories.

3.1. Covalent Binding to the Protein

Agents that are oxidatively activated and inactivate the enzyme by covalently binding to it include (a) diverse sulfur compounds (e.g., carbon disulfide[54–56], parathion[57, 58], diethyldithiocarbamate[59], isothiocyanates[60], thioureas[61], thiophenes[62], tienilic acid[62, 63], and mercaptosteroids[64–71], (b) halogenated structures such as chloramphenicol[72–75], *N*-monosubstituted dichloroacetamides[76], and *N*-(2-*p*-nitrophenethyl)dichloroacetamide[77], (c) alkyl and aryl olefins and acetylenes[78–84] such as 10-undecynoic acid[78, 83], 10-dodecynoic acid[84], 1-ethynylpyrene[81, 83], 17β-ethynylprogesterone[85, 86], 17α-ethynylestradiol[86–91], gestodene[92], 1- and 2-ethynylnaphthalene[79, 80, 82, 93], 7-ethynylcoumarin (7-EC)[94], mifepristone[95, 96], and secobarbital[97], (d) furanocoumarins such as 8-methoxypsoralen (8-MOP, methoxsalen)[98–106], 6′,7′-dihydroxybergamottin (6′,7′-DHB)[107–109], and the furano-pyridine, L-754,394[110–112] and (e) compounds such as carbamazepine (CBZ) and tamoxifen that are hydroxylated to catechol metabolites[113–116]. 2-Phenylphenanthridinone, which inhibits CYP1A1, is a member of a distinct class of mechanism-based inactivating agents that is thought to bind covalently to the protein[117]. The details of the mechanisms by which a few of these compounds inactivate P450 remain unclear, but much is now known about the mechanisms of activation of many of these inhibitors and, in some instances, about the sites on the P450 enzymes which they covalently modify.

3.1.1. Sulfur and Halogenated Compounds

Incubation of liver microsomes with [^{35}S]parathion results in radiolabeling of the protein but no radiolabeling occurs when the parathion ethyl groups are labeled with ^{14}C[57, 58]. Ninety percent of the ^{35}S-label, bound covalently to the microsomal proteins is immunoprecipitated

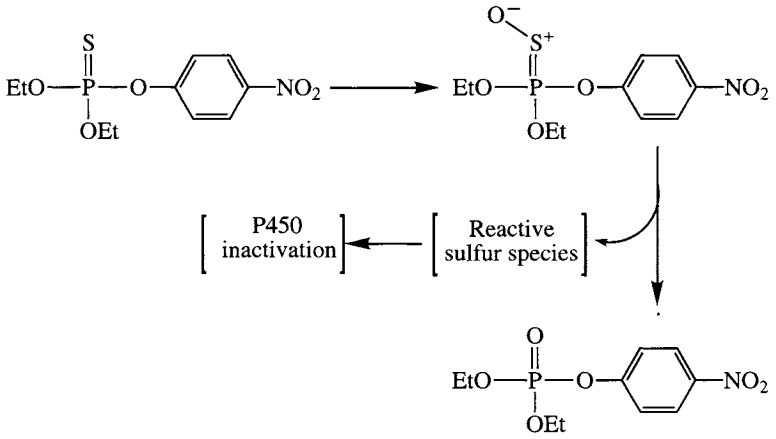


Figure 7.2. The activation of parathion to species that destroy cytochrome P450 is proposed to involve oxidation of the thiophosphonate to reactive sulfur species that bind to protein residues. The reactive species can be envisioned to be a sulfur atom with six electrons or HSOH.

by anti-P450 antibodies. Approximately 75% of the P450 prosthetic heme is degraded to unknown products in incubations with parathion, but ~4 nmol of radiolabeled sulfur are bound covalently to the protein for each nanomole of heme chromophore that is lost. The bulk (50%–75%) of the radiolabeled sulfur is removed from the protein by treatment with cyanide or dithiothreitol, which suggests that most of the sulfur label is present in the form of hydrodisulfides (RSSH), but the enzyme is not reactivated by these treatments. The binding of multiple equivalents of labeled sulfur indicates that catalytic sulfur activation persists despite covalent attachment of the sulfur to the protein until the heme itself is damaged[57] or is released from the protein as a consequence of protein damage. The oxidative mechanism in Figure 7.2 is suggested by (a) the covalent binding of sulfur, (b) the oxidation of sulfur compounds to S-oxides, and (c) the formation of metabolites in which the sulfur is replaced by an oxygen. More recent studies reveal that parathion competitively inhibits certain rat liver P450 enzymes (i.e., CYP2B1 and -2C6) at low concentrations, but at higher concentrations inactivates CYP2A1, -2A2, -2C11, -3A2, and -3A4 (but not CYP2B1 or -2C6) *in vitro* but not *in vivo*[118–121]. Analogous *in vitro* studies with human liver microsomes reveal that CYP3A4 is the principal isoform that is inactivated, CYP2C9 and -1A2 are minor forms that are also inactivated, whereas CYP2E1 is not inactivated[118–121]. While P450 heme destruction unequivocally occurs *in vitro* in incubations of parathion with NADPH-supplemented rat and human liver microsomes or purified recombinant P450 enzymes, the *in vivo* relevance of these findings remains to be established[122]. It appears that the concentrations required for *in vitro* P450 destruction are considerably higher than the concentrations that cause death through inhibition of acetylcholinesterase[122, 123].

Tienilic acid (Figure 7.3), a substituted thiophene, is oxidized by CYP2C9 and -2C10 to a product that irreversibly inactivates these enzymes [the values for half-maximal inactivation (K_I) = 4.3 μM, the maximal rate constant for inactivation (k_{inact}) = 0.22 min^{-1}, and partition ratio = 11.6][62, 63]. Tienilic acid is oxidized by these P450 enzymes to 5-hydroxytienilic acid and a product that is covalently bound to the P450 protein. Covalent labeling of the protein is partially prevented by glutathione (GSH) but GSH does not protect the enzyme from inactivation or loss of the heme chromophore. In the presence of GSH, approximately 0.9 equivalents of label are covalently bound to the protein before catalytic activity is suppressed. The results are explained by the formation of a thiophene sulfoxide that reacts with water to give the isolated metabolite or with a protein nucleophile to inactivate the enzyme[123]. The

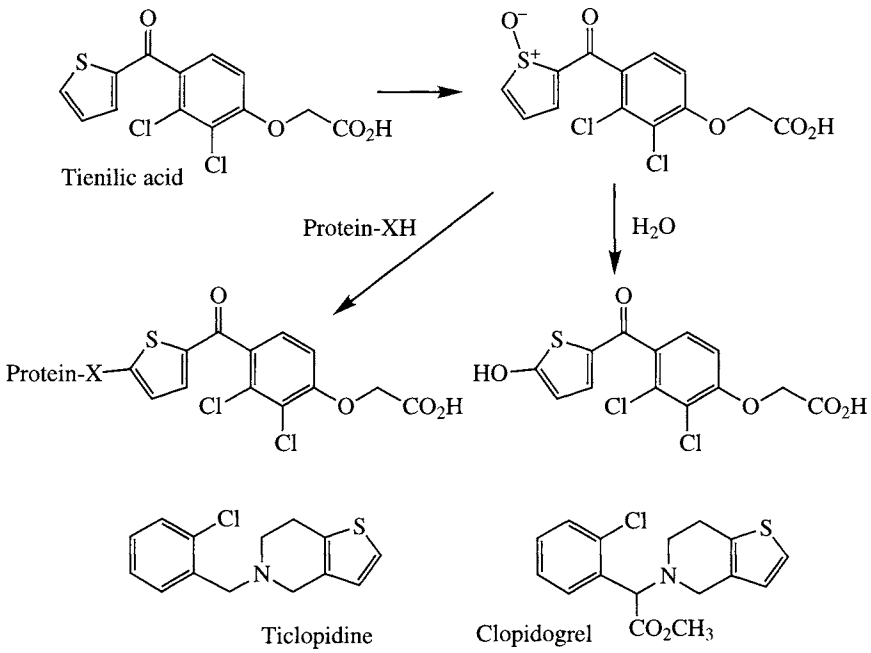


Figure 7.3. The mechanism proposed for P450 bioactivation of the thiophene in tienilic acid to a reactive species that binds covalently to the protein (the protein nucleophile is denoted by Protein-XH). The structures of two other thiophene-containing drugs, ticlopidine and clopidogrel, that might be similarly activated are also shown.

CYP2C9 site that is modified by tienilic acid has not been identified, but HPLC/electrospray mass spectrometric (ESI–MS) analysis of the tienilic acid-modified and native proteins reveals the presence of mono- and diadducted CYP2C9 species with molecular masses of 55,923 ± 1.1 and 56,273 ± 4.4 Da, respectively, which corresponds to mass shifts of 344.4 ± 1.1 and 694 ± 4.2 Da[123]. Diadduct formation is abolished by the inclusion of GSH (10 mM), suggesting that the diadduct may result from modification of a second residue outside the active site. The mass shift in the CYP2C9 monoadduct is consistent with the binding of a monohydroxylated tienilic acid obtained either by thiophene ring oxidation or formation of a sulfoxide that resists dehydration[123].

Ticlopidine (Figure 7.3), another substituted thiophene that is clinically utilized as an anti-platelet aggregation agent, is reportedly the first selective mechanism-based inactivator of CYP2C19[124]. This inactivation apparently occurs during S-oxidation of the thiophene moiety. The failure of GSH (5 mM) to inhibit ticlopidine-mediated CYP2C19 inactivation suggests that it occurs within the active site of the enzyme. The inactivation follows classical mechanism-based inactivation criteria with the following kinetic parameters: $t_{1/2max}$ = 3.4 min, k_{inact} = 3.2 × 10^{-3} s^{-1}, K_I = 97 μM, k_{inact}/K_I = 37 L mol^{-1} s^{-1}, and partition ratio = 26[124]. *In vitro* inhibition studies with functionally competent yeast-expressed recombinant human liver CYP1A2, -2C8, -2C9, -2C18, -2C19, -2D6, and -3A4 yielded IC_{50} (μM) values of 75 ± 15, 100 ± 20, >200, 100 ± 15, 10 ± 5, 10 ± 3, and 50 ± 10, respectively[124]. These findings establish that ticlopidine efficiently inhibits CYP2C19 and -2D6. Furthermore, within the human liver CYP2C subfamily, ticlopidine is a relatively selective mechanism-based inactivator of CYP2C19[124], although it is not quite as potent as tienilic acid is in CYP2C9 inactivation. These findings rationalize the clinical observation that ticlopidine inhibits the clearance of phenytoin, another CYP2C19 substrate[125–128]. However, recent studies with an extensive battery of recombinant human P450 enzymes in Supersomes™ indicate that

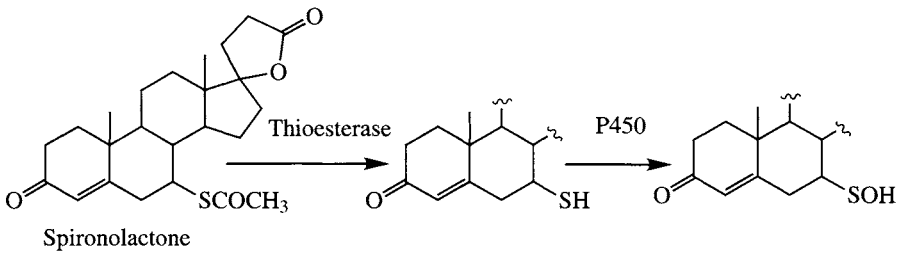


Figure 7.4. The activation of spironolactone to products that inactivate sterol 17α-hydroxylase by covalent attachment to the protein, and hepatic P450 3A by degradation of the heme group, occurs during oxidation of the free thiol group unmasked by the action of a thiolesterase.

CYP2B6 is even more effectively inactivated than CYP2C19 not only by ticlopidine, but also by clopidogrel, a related thienopyridine antiplatelet aggregating agent[129]. These observations suggest that clinically relevant drug–drug interactions with these two agents may occur with substrates of not only CYP2C19 but also CYP2B6 and -2D6.

The aldosterone antagonist spironolactone (Figure 7.4), which is used as a diuretic and antihypertensive[130], inactivates P450 enzymes in both the liver and steroidogenic tissues[64–71], including the adrenal steroid 17α-hydroxylase[64, 65, 70, 71] and members of the hepatic CYP3A and CYP2C subfamilies[67, 68]. Spironolactone-mediated hepatic P450 inactivation requires hydrolysis of the thioester function to give the free thiol that[67] is subsequently oxidized to a species that reacts with the protein and/or the heme[66]. Hepatic P450 inactivation involves fragmentation of the prosthetic heme to products that bind covalently to the protein (see Section 3.4)[66, 67]. In contrast, inactivation of the adrenal P450 appears to result from covalent binding of the thiosteroid itself to the protein[64, 65, 68, 69]. A role for thiol oxidation in enzyme inactivation is supported by the fact that rat hepatic microsomes enriched in CYP3A enzymes oxidize the thiol group (–SH) to the sulfinic ($-SO_2H$), and sulfonic ($-SO_3H$) acids[67], and give rise to a disulfide adduct with GSH[71]. However, formation of the disulfide adduct with GSH is catalyzed, at least in part, by a flavin monooxygenase[71]. Two reactive intermediates that can arise by thiol oxidation are the sulfhydryl radical (–S•) and the sulfenic acid (–SOH), either or both of which could be involved in P450 inactivation. One possibility is that fragmentation of the heme results from its reaction with the sulfhydryl radical, whereas protein modification involves reaction of the sulfenic acid metabolite with amino acid side chains (Figure 7.4).

Three other sulfur-containing drugs are noteworthy in that they are also bioactivated by human liver CYP3A4 and result in inactivation of the enzyme. The first is the oral antidiabetic drug troglitazone (Figure 7.5)[131], which was recently withdrawn from the US market due to its association with clinically severe hepatotoxicity[132, 133]. As revealed by structural analyses of its GSH-adducts, this 2,4-thiazolidinedione is activated by two distinct metabolic routes, one involving oxidation of the substituted chromane ring system to a reactive *o*-quinone methide, and the other involving a novel oxidative cleavage of the thiazolidinedione ring to highly electrophilic α-ketoisocyanate and sulfenic acid intermediates[131].

The second drug is the selective estrogen receptor modulating drug, raloxifene (Figure 7.5)[134], which is used for the treatment of postmenopausal osteoporosis. The *in vitro* bioactivation of raloxifene by human liver microsomal CYP3A4 results in mechanism-based inactivation of this enzyme. The inactivation is attenuated by the CYP3A4-selective inhibitor ketoconazole (10 μM), is quenched minimally (~15%) by GSH (5 mM), and is characterized by K_I, k_{inact}, and partition ratio values of 9.9 μM, 0.16 min^{-1}, and 1.8, respectively[134]. Indeed, raloxifene, albeit a slower CYP3A4 inactivator, is more potent than gestodene, which exhibits K_I, k_{inact} and partition ratio values of 46 μM, 0.39 min^{-1}, and 9, respectively[92]. CYP2D6 is also capable of bioactivating raloxifene at half the rate of CYP3A4, but the CYP2D6 contribution to human liver microsomal raloxifene activation is minimal as judged by

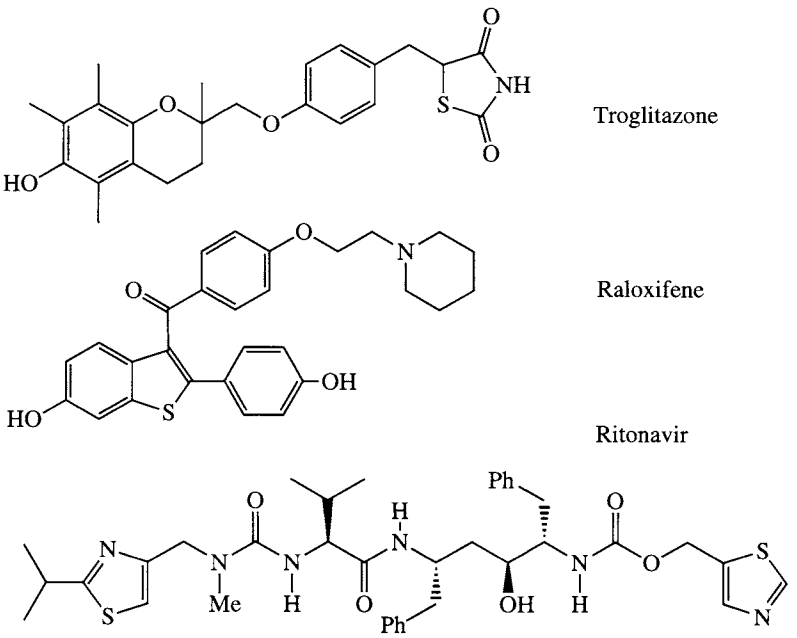


Figure 7.5. Structures of sulfur-containing compounds that inactivate P450 enzymes by mechanisms that have not been elucidated but may involve oxidation of a sulfur atom.

the failure of quinidine to significantly inhibit the process at concentrations known to inhibit CYP2D6[134]. The mechanism of this inactivation has not yet been explored, but could involve oxidative activation of the phenolic or thiophene functions.

Ritonavir (Figure 7.5), an inhibitor of the HIV-1 protease, is another example of a potent, sulfur-based inactivator of CYP3A4/CYP3A5, although it is also a CYP2D6 inhibitor[135–137]. Its inhibitory potency depends on the presence of both the 5-thiazolyl and 2-(1-methylethyl) thiazolyl groups. It has been proposed that it is oxidized by CYP3A to a chemically reactive fragment containing the 2-(1-methylethyl)thiazolyl group that causes enzyme inactivation[137].

Other noteworthy sulfur-containing mechanism-based inactivators include thiocyanates such as phenethyl isothiocyanate[138–140], benzylisothiocyanate[137–143], *tert*-butylisothiocyanate[144]; diallyl sulfide, and diallylsulfone derivatives[145–147], 1,2-dithiole-3-thione[148], oltipraz, and its derivatives[148], sulforaphane[148], and thiosteroids[149].

Chloramphenicol was among the first chlorinated mechanism-based P450 inactivators shown to act through irreversible protein modification[72–75]. Not only does the binding of [^{14}C] chloramphenicol to the apoprotein correlate with the loss of CYP2B1-dependent ethoxycoumarin *O*-deethylase activity, but proteolytic digestion of the inactivated P450 has been shown to yield a single [^{14}C]-modified amino acid[72–75]. Lysine and the chloramphenicol fragment in Figure 7.6 were released by hydrolysis of the modified amino acid. These results indicate that chloramphenicol is converted to an oxamyl chloride intermediate that either acylates a critical lysine, probably at the protein surface, or is hydrolyzed to the oxamic acid. Acylation of the lysine apparently inhibits electron transfer from P450 reductase to the CYP2B1 heme because the inactivated enzyme still catalyzes ethoxycoumarin *O*-deethylation in the presence of cumene hydroperoxide or iodosobenzene[75]. Furthermore, the unimpaired ethoxycoumarin *O*-deethylation supported by activated oxygen donors suggests that chloramphenicol is not covalently bound within the substrate binding-site.

The P450 isoform selectivity of inactivation by chloramphenicol and several of its analogs

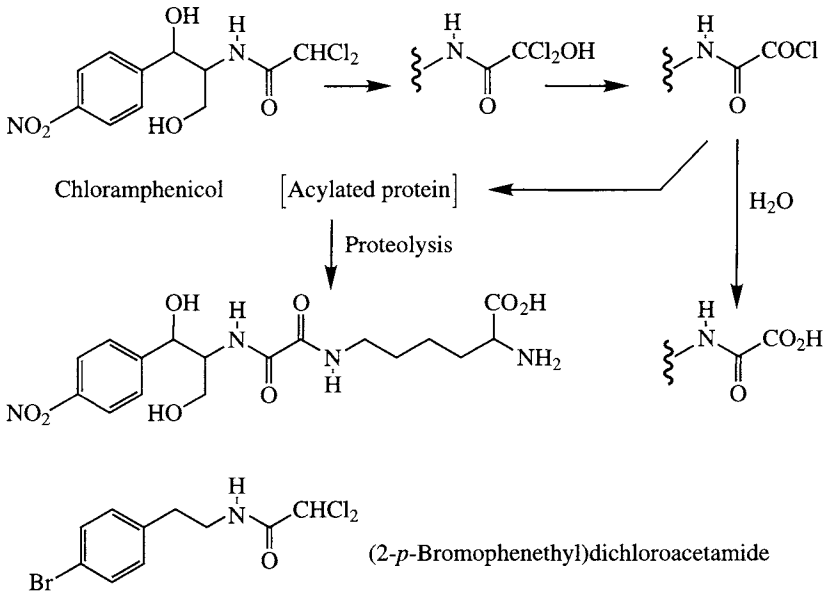


Figure 7.6. The inactivation of P450 by chloramphenicol involves oxidation of the dichloromethyl group to an acyl chloride that reacts with an amino group of the protein. A potent inactivating agent related to chloramphenicol is (2-*p*-bromophenethyl)dichloroacetamide.

has been reported[150]. Using four regiospecific androstenedione hydroxylases as functional markers, chloramphenicol was found to inactivate rat liver microsomal CYP2B1 > -3A > -2C11 > -2A1[150]. The P450 selectivity of the chloramphenicol analogs as mechanism-based inactivators was determined by at the least three structural features: (a) the number of halogen atoms, (b) the presence of a *para*-nitro group on the phenyl ring, and (c) substitutions on the ethyl side chain. Thus the analog *N*-(2-phenethyl)dichloroacetamide reversibly inhibited but did not inactivate CYP3A enzymes, in contrast to *N*-(2-*p*-nitrophenethyl)- and *N*-(1,2-diphenethyl)dichloroacetamide, both of which rapidly did so[150]. The incorporation of a *para*-nitro or -bromo substituent on the phenyl ring, or a second phenyl at the 1- or 2-position of the phenethyl side chain, yielded compounds that selectively inactivated CYP2B1 over CYP2C11, -2C6, or -2A1[77]. Thus, *N*-(2-*p*-bromophenethyl)-dichloroacetamide (Figure 7.6) and *N*-(2-*p*-nitrophenethyl)dichloroacetamide were the two most effective and selective CYP2B1 inactivators both *in vitro* and *in vivo*[77].

21-Chloropregnenolone, 21,21-dichloropregnenolone, and 21,21-dichloroprogesterone have also been probed for their selectivity of P450 inactivation using regiospecific and/or stereoselective progesterone or androstenedione hydroxylases as functional probes[151, 152]. The findings reveal that 21,21-dichloropregnenolone and 21,21-dichloroprogesterone are comparably effective in the inactivation of rat liver microsomal CYP3A enzymes, with k_{inact} values of ≈ 0.1 min^{-1} for both substrate probes, while 21,21-dichloroprogesterone even more efficiently inactivated CYP2C6 (k_{inact} = ~ 0.2 min^{-1})[151]. 21,21-Dichloropregnenolone was also a very efficient mechanism-based inactivator of rabbit liver CYP2C5 but not of rabbit adrenal CYP21[152]. On the other hand, CYP21 was rapidly inactivated by 21,21-dichloroprogesterone[152]. Thus, replacement of a methyl group that is normally oxidized by a P450 enzyme with a dichloromethyl moiety may be a valuable strategy in the design of specific P450 inhibitors.

3.1.2. Olefins and Acetylenes

As discussed in detail below (Section 3.2), compounds containing an olefinic bond, ranging from compounds as simple as ethylene to more complex structures such as allylisopropylacetamide (AIA),

allylisopropylcarbamide (sedormid), aprobarbital, allobarbital, and secobarbital, can inactivate P450 enzymes by *N*-alkylating the porphyrin group of the prosthetic heme[153–156]. However, studies with secobarbital show that it completely inactivates CYP2B1 but only causes partial loss of the heme chromophore[97, 157, 158]. Isolation of the *N*-alkylated porphyrins (see Section 3.3.1) and of the modified CYP2B1 protein reveal that the compound partitions between heme *N*-alkylation, CYP2B1 protein modification, and formation of an epoxide metabolite in a ratio of 0.8 : 0.2 : 59, respectively[97, 157, 158]. The *in situ* presence of the heme adduct in the CYP2B1 active site is spectrally confirmed by its typical ~445 nm absorption maximum,[158] a feature characteristic of iron-complexed *N*-modified porphyrins[159]. The *N*-modified porphyrins have been isolated both as the parent adducts and as the corresponding dimethyl esters and identified by mass spectrometry (MH^+ 816.9 and 845.8 Da, respectively) as adducts of protoporphyrin IX and hydroxysecobarbital (Figure 7.7)[157]. The CYP2B1 peptide modified by the drug has also been isolated and shown to be comprised of residues 277–323, residues that by sequence analogy to $P450_{cam}$ correspond to the distal I helix[157, 160–163]. Similar structural analysis after further digestion of this CYP2B1 peptide has narrowed down the region of secobarbital modification to residues G_{299}-S_{304}[158]. Although the precise residue that is modified remains to be identified, these findings are consistent with modification of the protein within the active site. In view of this, it is not surprising that specific mutations in the CYP2B1 putative substrate-recognition sites (SRS) 2, 4, 5, and 6, but not SRS-1, attenuate secobarbital-mediated inactivation. The SRS-mutations T302S and V363L markedly reduced CYP2B1 heme-modification and a V367A (SRS-5) mutation most markedly impaired protein modification[158].

Terminal acetylenes, like terminal olefins, alkylate the P450 prosthetic heme (see Section 3.3.2), but compounds such as 10-undecynoic acid, 1-ethynylpyrene, 17β-ethynylprogesterone, 17α-ethynylestradiol (EE), and 9- and 2-ethynylnaphthalene have been shown to inactivate P450s primarily by binding covalently to the protein with only partial loss of the heme group[78–83, 90, 91]. Thus, near stoichiometric binding of 2-ethynylnaphthalene and 1-ethynylpyrene to CYP1A1 and -1A2, of EE to CYP3A4, and of 10-undecynoic acid to rat liver CYP4A1 (ω-hydroxylase) has been observed[78–83, 87–91]. The isolation of the terminal acid metabolites from the incubations of 10-undecynoic acid (Figure 7.8) and

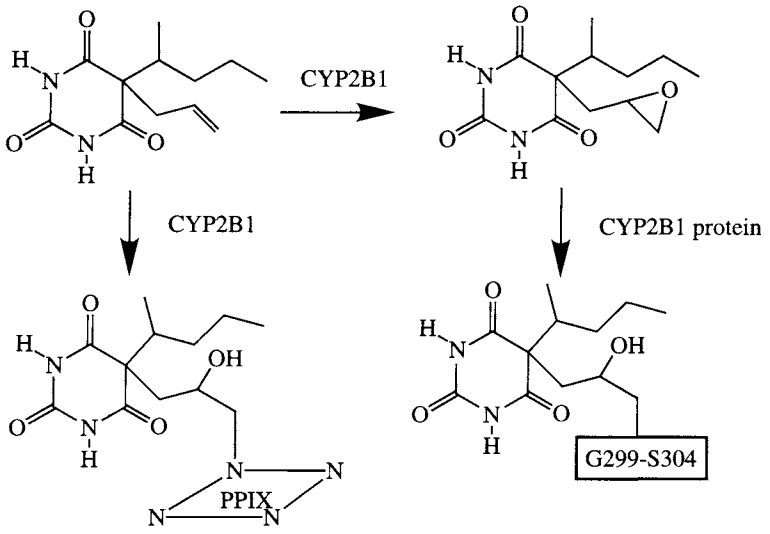


Figure 7.7. The oxidation of secobarbital by CYP2B1 results in both alkylation of one of the residues between Gly299 and Ser304 and *N*-alkylation of the heme group as well as the generation of an epoxide.

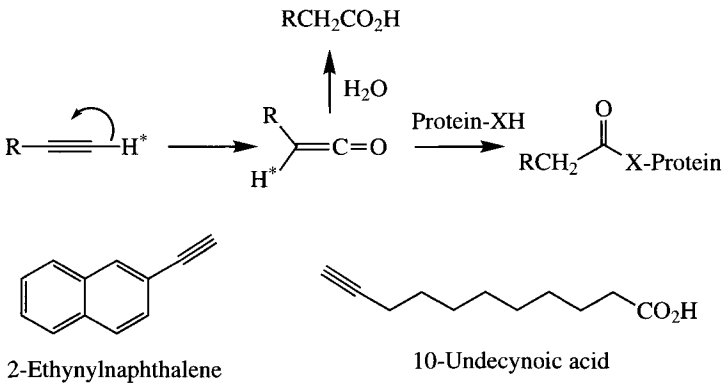


Figure 7.8. The oxidation of terminal acetylenes, and even some internal acetylenes, results in the formation of ketene intermediates that react with water to give the carboxylic acids (see Chapter 6). It appears that the ketenes also react with active-site residues, inactivating the P450 enzyme that forms them. The hydrogen that undergoes a 1,2-migration during the oxidation reaction is indicated by a star. The structures of 2-ethynylnaphthalene and 10-undecynoic acid, both of which inactivate P450 enzymes, at least in part by this mechanism, are shown.

1-ethynylpyrene strongly supports a mechanism in which oxygen transfer from the P450 heme to the terminal carbon of the triple bond triggers migration of the terminal hydrogen to the adjacent carbon (Figure 7.8). The reactive ketene generated by this hydrogen shift mechanism, which is supported by deuterium labeling experiments, either acylates the protein or is hydrolyzed to the carboxylic acid metabolite[83]. The corresponding ketene metabolites have been similarly invoked in the acylation of bovine adrenal CYP21 by 17β-ethynylprogesterone[85, 86] and of CYP1A2, -2B1, and -2B4 by 2-ethynylnaphthalene (Figure 7.8)[80, 82, 93]. In accord with this postulate, CYP2B1 oxidizes 2-ethynylnapthalene to 2-(2-napthyl)acetic acid, presumably via the same ketene intermediate as is postulated to modify the protein. The mechanism-based inactivation of CYP2B1 by 2-ethynylnaphthalene is characterized by a $K_I = 0.08$ μM, $k_{inact} = 0.83\ min^{-1}$, and a partition ratio of approximately 4–5 moles of acid formed per inactivation event[82].

In contrast, CYP2B1-catalyzed addition of the activated oxygen to the internal rather than terminal triple bond carbon of phenylacetylene results in heme *N*-alkylation rather than protein acylation (see below)[83, 164]. Predominant inactivation of CYP2B1 by phenylacetylene via heme alkylation[164] and by 2-ethynylnaphthalene via protein acylation[80, 82, 93] sheds some light on the influence exerted by the fit of the inhibitor within the active site as a determinant of the inactivation mechanism. Both of these terminal aryl acetylenes yield ketene metabolites but only that from 2-ethynylnaphthalene acylates the CYP2B1 protein. Protein acylation by 2-ethynylnaphthalene demonstrates that the failure of phenylacetylene to acylate the CYP2B1 protein is not due to the absence of appropriate nucleophilic residues. Furthermore, the formation of phenylacetic acid in the oxidation of phenylacetylene confirms that the ketene is formed. The differential inactivation of CYP2B1 by 2-ethynylnaphthalene and phenylacetylene suggests, in fact, that: (a) 2-ethynylnaphthalene, unlike phenylacetylene, is bound in a manner that prevents delivery of the ferryl oxygen to the internal carbon and (b) heme alkylation by phenylacetylene is sufficiently efficient relative to protein acylation by the phenylketene metabolite that the enzyme is inactivated before protein acylation becomes significant. These differences presumably stem from differential binding affinities and/or orientations of the two agents within the CYP2B1 active site.

Peptide mapping and amino acid sequence analysis of radiolabeled peptides indicate that 2-ethynylnaphthalene binds to amino acid residues 67–78 and 175–184, respectively, of rat and rabbit CYP1A2[80]. However, the residue that is actually modified and the precise nature of the covalent link to the inhibitor have escaped definition due to the instability of the adducts.

Alignment of the modified peptides with the sequence of $P450_{cam}$ (CYP101) suggests that the CYP1A2 peptides correspond to helices A and D of $P450_{cam}$ (see Chapter 3). Thus, the rat CYP1A2 peptide may include residues from the substrate-binding regions[160–163]. The 2-ethynylnaphthalene-modified peptides from CYP2B1 and CYP2B4 have also been characterized and proposed to be adducts of 2-naphthylacetic acid with a peptide residue[82, 93]. Digestion of CYP2B1 after its oxidation of radiolabeled 2-ethynylnapthalene yields a radiolabeled peptide (ISLLSLFFAGTETSSTTLRYGFLLM) that includes residues 290–314 of the protein. An analogous peptide (E_{273}–M_{314}) is obtained with CYP2B4. The two modified peptides correspond in sequence to the highly conserved distal I helix of $P450_{cam}$ (CYP101) that contacts both the substrate and the heme group[160–163]. The specific residue modified in each peptide has not been identified but several nucleophilic residues, including serine, threonine, and tyrosine, are present. If the protein nucleophile is the hydroxyl group of such a residue, the resulting adduct would be an ester. Indeed, recent studies with a CYP2B4 T302A mutant reveal that the k_{inact} of 2-ethynylnaphthalene is decreased from 0.20 ± 0.05 min^{-1} for the wild type to 0.05 ± 0.01 min^{-1} in the mutant, suggesting that T302 is at least one acylated residue[165].

7-Ethynylcoumarin (7-EC) (Figure 7.9)[94] is a rationally designed mechanism-based inactivator with the ethynyl moiety at the 7-position of coumarin where, by analogy to 7-ethoxycoumarin and 7-ethoxy-4-(trifluoromethyl) coumarin, it should be readily oxidized by CYP2B enzymes[166, 167]. In comparative assays, 7-EC (100 μM) only marginally (15%–20%) inactivated human liver microsomal CYP2A6, but markedly (>90%) inactivated purified CYP2B1[94]. This inactivation left the heme and its thiolate ligand unscathed, as the electronic absorption of the reduced-CO complex of the inactivated enzyme was little affected. The inactivation by 7-EC was unaffected by the presence of nucleophiles such as GSH and NaCN, of the iron-chelator deferoxamine, or of superoxide dismutase or catalase and conformed to all the other established criteria for a mechanism-based inactivation. It exhibited a K_I of 25 ± 2 μM, a k_{inact} at 30°C of 0.39 ± 0.01 min^{-1}, a partition ratio of 25, and a half-life ($t_{1/2}$) of 1.8 min. ESI–LC/MS of the dialysed intact native and 7-EC-inactivated CYP2B1 yielded masses of 55,899 ± 1 Da and 56,084 ± 3 Da, respectively, for the two proteins[94]. This corresponds to a mass difference of 185 Da (0.005% variability in mass assignment), and indicates that the entire 7-EC molecule together with an oxygen atom was covalently bound to CYP2B1 in a 1 : 1 stoichiometry. The precedents set by other arylacetylenes suggest that the protein adduct involves addition of an active-site nucleophilic residue to the activated acetylene. Two mechanisms have been proposed for the acylation: One that is mediated by a ketene metabolite and a second involving attack on a putative oxirene intermediate[94]. In view of the fact that oxirenes are only hypothetical species due their practically barrierless conversion to ketenes, the protein modification is almost certainly mediated by the ketene.

17α-Ethynylestradiol (EE) (Figure 7.9) has long been known to inactivate rat and human liver P450 enzymes in a mechanism-based fashion[87–89]. Its inactivation was shown to result from P450-dependent metabolic activation of its acetylenic moiety to a species that alkylates a heme pyrrole nitrogen. More recently, EE has been shown to also modify the CYP3A4 protein in a reconstituted enzyme system with k_{inact} = 0.04 min^{-1}, K_I = 18 μM, $t_{1/2}$ = 16 min, and a partition ratio of ~50[90]. Loss of activity was paralleled by some loss of CO-dependent chromophore. A net stoichiometry of 1.3 nmol of EE-metabolite bound/nmol CYP3A4 was observed with [^{3}H]EE as the substrate. HPLC analysis yielded several NADPH-dependent [^{3}H]EE-labeled fractions, the predominant one of which eluted at 31 min. ESIMS analysis in the negative ion mode of this peak fraction yielded a mass $(M\text{-}H)^-$ of 479 Da. Although the chemical structure of this EE-related species has not been definitively characterized, the authors believe it to be an EE-adducted monopyrrolic heme fragment[90]. EE thus appears to modify both the heme and protein moieties of CYP3A4, whereas only the heme moieties of CYP2B1 and -2B6 are susceptible to EE-modification.

The monoamine oxidase (MAO) inhibitor deprenyl (Figure 7.9) is a propargylamine whose terminal acetylene reacts irreversibly with the MAO flavin moiety[168]. Deprenyl also inactivates CYP2B1 relatively selectively with K_I = 1.05 μM, k_{inact} = 0.23 min^{-1}, and partition ratio = 2[169]. However, although there is a loss of

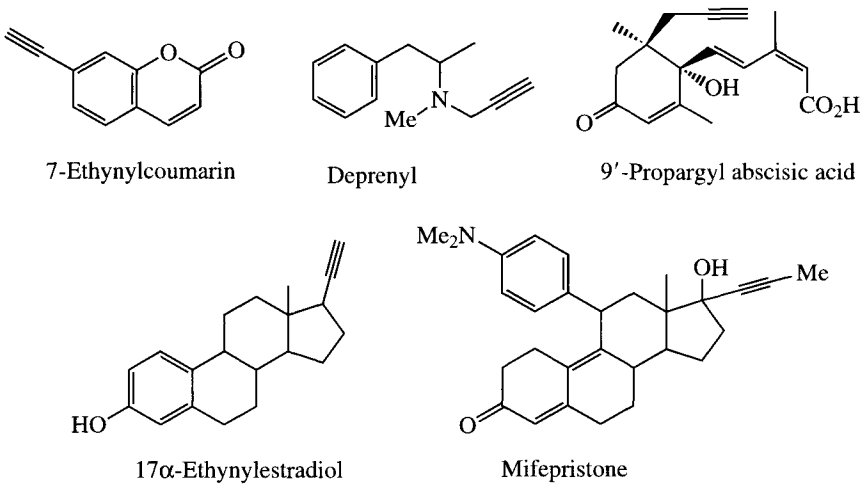


Figure 7.9. The structures of acetylenes that have been shown to inactivate P450 enzymes. In all cases except perhaps that of mifepristone the reactive intermediate appears to arise by oxidation of the triple bond.

spectrally detectable P450 chromophore, no significant change is observed in the 405 nm heme absorbance. The complete loss of CYP2B1 function, however, suggests protein modification following activation of the terminal acetylene. Analogs of deprenyl such as (*R*)-*N*-(2-heptyl)-*N*-methyl-propargylamine and (*R*)-*N*-(2-heptyl)-propargylamine have been tested for their P450 isoform inactivation selectivity[170]. Of these, *(R)*-*N*-(2-heptyl)-propargylamine was the more potent, with K_I = 0.5 μM and k_{inact} = 0.36 min^{-1} [170].

A specific inhibitor of the P450 enzyme that degrades the phytohormone abscisic acid has been constructed by replacing the methyl group of abscisic acid that is normally oxidized with acetylenic moieties[171]. The most active of the inactivating agents is 9′-propargyl abscisic acid (Figure 7.9), which *in vivo* exhibits a greater inhibition of *Arabidopsis thaliana* seed germination than abscisic acid itself.

3.1.3. Other P450 Protein Modifying Inactivators

The recreationally abused psychotomimetic amine phencyclidine [1-(1-phenylcyclohexyl) piperidine; PCP] (Figure 7.10) is another mechanism-based inactivator that inactivates CYP2B1, -2B4, and -2B6 relatively selectively via protein modification[172, 173]. Incubations of radiolabeled PCP with NADPH-supplemented liver microsomal preparations from phenobarbital-pretreated rabbits demonstrated a time- and concentration-dependent loss of ketamine and benzphetamine *N*-demethylase activity as well as irreversible binding of the radiolabeled amine to microsomal protein[174–176]. Inhibition of both of these events by cyanide (NaCN), but not GSH, led to the proposal that α-oxidation yielded a PCP iminium species that was responsible for the inactivation. This iminium derivative could directly bind to proteins, but apparently could also undergo further NADPH-dependent metabolism by P450, resulting in inactivation of those enzymes[176–179]. This second oxidation was proposed to convert the PCP iminium ion to an allyl alcohol via a reactive, electrophilic 2,3-dihydropyridinium intermediate [1-(1-phenylcyclohexyl)-2,3-dihydro-4-pyridone] that reacted irreversibly with the protein[178]. *In vitro* studies with five rabbit liver P450 isoforms confirmed that the inactivation was indeed mechanism-based, highly selective for CYP2B4, and involved parallel loss of the CYP2B4 heme chromophore and function[179]. However, parallel studies of PCP and its iminium metabolite revealed that the latter was less selective than the parent compound and inactivated P450 3b (a constitutive rabbit liver isoform) as well[179]. More recent *in vivo* studies have shown that CYP2D is also markedly (>74%) inactivated by PCP in uninduced

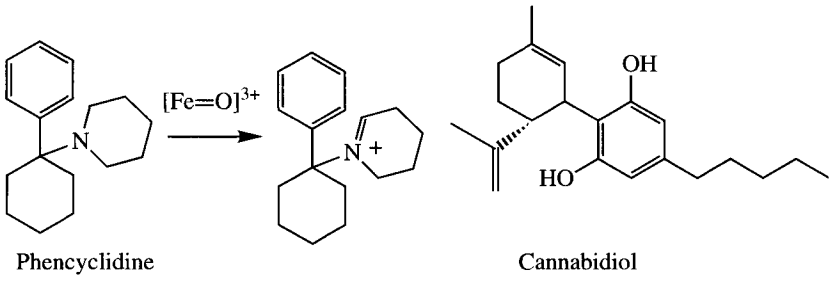


Figure 7.10. Phencyclidine is oxidized to an iminium species whose role as an intermediate in the inactivation of P450 enzymes is unclear. The structure of cannabidiol (CBD), which also inactivates P450 enzymes by an undefined mechanism, is also shown.

rats[180]. Comparative *in vitro* studies of PCP analogs containing a five [PCPY; 1-(1-phenylcyclohexyl) pyrrolidine]- or six [PCHMI; 1-(1-phenylcyclohexyl) hexamethyleneimine]-membered heterocyclic ring indicated that the rates and $t_{1/2}$ of inactivation and heme loss were comparable for PCPY and PCP, both of which were 10-fold higher than those of PCHMI even though the latter was metabolized to a greater extent[181]. No mechanism-based inactivation was observed with either phenylcyclohexylamine or the diethylamino analog of PCP, suggesting that the substituted nitrogen is necessary and must be incorporated into a heterocyclic ring. The observation of prosthetic heme loss and its sensitivity to cyanide ion reinforced the notion that the iminium ion is involved in the PCP-mediated inactivation[181].

HPLC-analyses of heme extracts from PB-pretreated rabbit liver microsomes, incubated with or without PCP, identified PCP-modified heme fractions proposed to be *N*-modified porphyrins, but the nature of these porphyrins was not established[179]. It is therefore unclear whether heme modification or degradation was responsible for the loss of P450 activity or simply coincidental with it. However, a substantial discrepancy in the partition ratio obtained for PCP-mediated functional inactivation and that for heme loss suggested that this enzyme inactivation might also entail protein modification.

Protein modification might indeed be primarily responsible for the PCP-mediated inactivation of CYP2B1 and -2B6, as no loss of heme chromophore in its ferric or CO-bound form was detected[172, 173]. Studies with CYP2B1- and CYP2B6-selective probes (7-ethoxycoumarin *O*-deethylase and 7-ethoxy-4-(trifluoromethyl) coumarin *O*-deethylase, respectively) yielded K_I values of 3.8 and 10 μM, k_{inact} values of 0.12 min^{-1} and 0.01 min^{-1}, respectively, and partition ratios of ~45 for both processes. More importantly, contrary to previous reports, not only was CN (up to 1 mM) incapable of inhibiting either inactivation event, but little inactivation was observed when the synthetic amino enone [1-(1-phenylcyclohexyl)-2,3-dihydro-4-pyridone], the putative reactive electrophilic 2,3-dihydropyridinium intermediate, was used as the inactivating substrate[172, 173, 182]. Studies with [^{3}H]PCP revealed a molar stoichiometry of PCP binding to CYP2B1 and CYP2B6 protein of 4 : 1 and 5.5 : 1, respectively. GSH (10 mM) did not decrease the rate of PCP-mediated inactivation but reduced the binding of [^{3}H]PCP to CYP2B6 protein to the expected 1 : 1 molar stoichiometry. Consistent with these findings, LC–MS analyses of the PCP–P450 adduct in the ESI–LC/MS mode yielded a mass difference of 244 ± 5 Da between PCP-inactivated CYP2B1 and the wild-type protein, confirming the formation of a 1 : 1 PCP–CYP2B1 adduct. Similar studies of PCP-inactivated CYP2B4 gave a mass difference of 261 ± 2 Da, consistent with the binding of one molecule of monohydroxylated PCP to the CYP2B4 protein. Similar analyses of PCP–CYP2B6 adducts were less conclusive due to inconsistent results[173]. Collectively, the recent findings argue for protein modification at the CYP2B active site as the major mode of inactivation. Although a reactive electrophilic inactivating species is involved, the PCP-iminium ion may not be its precursor.

Modification of the P450 protein is also observed with cannabidiol (CBD) (Figure 7.10), a

major constituent of marijuana. CBD inactivates mouse P450 isoforms 2C and 3A via a mechanism that involves stoichiometric covalent binding of the inhibitor and loss of its CBD oxidase activity[183, 184]. Limited structure-activity studies show that a free phenolic group in the resorcinol moiety of CBD is essential for inactivation[183]. Mass spectrometric analyses of the concomitantly generated CBD metabolite trapped as a GSH-adduct led to identification of CBD-hydroxyquinone as the inactivating species[184], a finding consistent with the electrophilic reactivity and P450 inactivating potential of this compound[185]. HPLC-peptide mapping, microEdman degradation, and mass spectrometric analyses of the CBD-modified peptides obtained by proteolytic digestion of CBD-inactivated CYP3A11 identified the peptide region spanning residues A_{344}–K_{379} and G_{426}–K_{454} as the loci of CBD modification[184]. These regions correspond to the CYP3A11 active site SRS-5 in the K-region and the heme-binding Cys_{443}-region/helix L domain[160–163], fulfilling an essential criterion of a mechanism-based inactivation process[184]. Interestingly, both peptides contain Cys residues that could react with the CBD-hydroxyquinone. Analogous studies with CYP3A4 have identified similar protein adducts, albeit at a lower level than those detected with the mouse ortholog (L.M. Bornheim, personal communication). Although Δ^9- and Δ^8-tetrahydrocannabinol (THC), the other major and minor psychoactive components of marijuana, respectively, are efficiently metabolized by CYP3A and -2C, they are not inactivating agents. In contrast, unsaturated Δ^8-THC-enyl and -ynyl analogs that are also present in the complex marijuana extracts have been found to be selective mechanism-based inactivating agents *in vitro* but not *in vivo*[186].

Other classes of compounds that inactivate human and rat liver microsomal P450 enzymes via protein modification are known. One such class is represented by the furanocoumarins, natural constituents of foods such as celery, parsley, figs, parsnips, and grapefruit juice[187]. Among these compounds, the photoactive linear furanocoumarin, 8-MOP (methoxsalen) is a particularly potent P450 inactivator. Evidence that 8-MOP was activated by P450 enzymes to an electrophilic product that bound covalently to the protein[98–102] was obtained from incubations with rodent liver microsomes. Inclusion of cysteine or GSH in these incubations did not prevent P450 inactivation but suppressed nonspecific covalent binding. The residual covalent binding of 8-MOP to microsomal protein was nearly stoichiometric with the loss of microsomal P450[101, 102]. This finding, together with the observation of a type I spectrum in incubations of rat liver microsomes with 8-MOP in the presence but not absence of NADPH[101], implicated an active-site directed inactivation mechanism. Furthermore, because cysteine markedly quenches the covalent binding of the label from [^{3}H]-methoxy- but not [4-^{14}C]-ring-labeled methoxypsoralen, the reactive intermediate is likely to reflect oxidation of the furan ring[102]. This inference is supported by the finding that trioxsalen (trimethylpsoralen), in which the furan ring bears a methyl substituent, does not destroy P450. Methyl-substituted furans, however, can also inactivate P450 enzymes, as illustrated by the inactivation of human liver enzymes observed in incubations with (*R*)-(+)-menthofuran[103].

8-MOP is a potent inactivator of human liver CYP2A6 and rat liver CYP2B1, but also inactivates CYP2B2, -1A, -3A, and -2C11[98, 106, 188]. Of all the furanocoumarins tested, 8-MOP was the most potent inactivator of CYP2B1 with a K_I, k_{inact}, and partition ratio of 2.9 μM, 0.34 min^{-1}, and 1.3, respectively[188]. SDS-PAGE and/or HPLC analyses of the components from incubations of purified CYP2B1 with [^{14}C-]8-MOP indicated that the radiolabel bound to the protein rather than to the heme with a stoichiometry of 0.7 : 1. This [^{14}C-]8-MOP-binding was unaffected by GSH or methoxylamine (MOA), suggesting that covalent binding occurred within the active site where it was inaccessible to external nucleophiles. LC–ESIMS analyses of the [^{14}C-]8-MOP-modified CYP2B1 revealed a mass shift of 237.9 ± 9.6 Da over that of the native enzyme. Analogous studies of 5-MOP- and psoralen-modified CYP2B1 gave mass shifts of 240 ± 6.2, and 204 ± 11.8 Da, respectively[188]. These results indicate that a single psoralen molecule is covalently bound to the protein and are consistent with CYP2B1-catalyzed oxidation of the 8-MOP furan ring to the 8-MOP furanoepoxide (MW, 232.2 Da) followed by reaction of the protein with the epoxide or a cationic species derived from it.

Another notable furanocoumarin, 6′,7′-DHB, that is derived from the processing of grapefruit juice and is one of its common constituents,

is now a well-recognized mechanism-based inactivator of CYP3A4[107–109, 189]. Inactivation of intestinal CYP3A4 after oral grapefruit juice intake has been clinically associated with increased bioavailability of several CYP3A4 drug substrates. Numerous reports exist of clinically relevant adverse drug–drug interactions between grapefruit juice and CYP3A4 substrates, in some instances resulting in withdrawal of the drug in question[189]. Studies with 6′,7′-DHB indicate that it is activated to a reactive species that irreversibly modifies the CYP3A4 protein with $K_I = 59\ \mu M$ and $k_{inact} = 0.16\ min^{-1}$ [107–109], resulting in accelerated hepatic degradation of the protein (see below; Malhotra and Watkins, personal communication).

The furanopyridine L-754,394, *N*-[2(*R*)-hydroxy-1(*S*)-indanyl]-5-[2(*S*)-((1,1-dimethylethyl)amino)carbonyl]-4-[(furo[2,3-*b*]pyridin-5-yl)methylpiperazin-1-yl]-4(*S*)-hydroxy-2(*R*)-(phenylmethyl)pentenamide, is also a potent mechanism-based inactivator of human liver microsomal CYP3A4 but is less selective, as it also inactivates human CYP2D6[110–112, 190]. Its K_I, k_{inact}, and partition ratio for human liver microsomal CYP3A4 are reportedly 7.5 μM, 1.62 min^{-1}, and 1.35, respectively[111]. Structural characterization of the concomitantly generated L-754,394 metabolites revealed that the mechanism of inactivation probably entails CYP3A4-dependent oxidation of the furan ring to the corresponding epoxide and/or γ-ketoenal that binds to the protein within the active site. Accordingly, neither the corresponding dihydrofuran derivative nor the analog that lacks the furan ring is active as a CYP3A4 irreversible inhibitor. Indeed, Tricine-SDS-PAGE, HPLC-peptide mapping, and MALDI-TOF-MS analyses of L-754,394-bound CYP3A4 showed that the inhibitor bound to the I-helix peptide I_{257}–M_{317}[112]. The chemical instability of this adduct under the acidic conditions required for these analyses precluded identification of the active-site residue actually modified. In view of the markedly higher stability of the adducts with GSH, *N*-acetylcysteine (NAC), and MOA, and of *O*-linked heterodimers of the hydroxylated/parent furano- compounds, the instability led the authors to propose an ester linkage between activated L-754,394 and CYP3A4. The authors proposed E307 as the most plausible target[112].

Two other drugs of note that have been reported to inactivate P450 by protein modification are tamoxifen and CBZ. Tamoxifen (*Z*-{1-[4-(2-dimethyl-aminoethoxy)phenyl]-1,2-diphenyl-1-butene}) (Figure 7.11), a nonsteroidal antiestrogen used in hormone-dependent breast cancer chemotherapy, inactivates CYP2B6 (but not CYP1B1 and CYP3A4) with $K_I = 0.9\ \mu M$, $k_{inact} = 0.02\ min^{-1}$, and $t_{1/2} = 34$ min. CYP2D6 and CYP2C9 were also partially (25%) inactivated[114–116, 191]. The ultimate inactivating species remains to be identified, but the sequential metabolism of tamoxifen to 4-hydroxytamoxifen, and then to 3,4-dihydroxytamoxifen-*o*-quinone, a known reactive and carcinogenic species[191], suggested it might be responsible for the inactivation[115]. This proposal has been challenged by studies showing that incubation with 3-hydroxy or 4-hydroxytamoxifen reversibly inhibits CYP3A4 but does not inactivate it[116]. A mechanism-based inactivation by tamoxifen and its *N*-desmethyltamoxifen metabolite (instead of 3- or 4-hydroxytamoxifen) involving the formation of a metabolic intermediate (MI) complex was proposed as an alternative explanation[116].

CBZ (Figure 7.11), a widely used anticonvulsant, is known to be metabolized to several reactive species, including the arene oxide, 9-acridine carboxaldehyde, and an iminoquinone metabolite[192–196]. Clinically, CBZ is often associated with idiosyncratic hypersensitivity syndromes[197–199]. CBZ-treated patients often exhibit anti-CYP3A4 autoantibodies, suggesting that covalent binding of CBZ to CYP3A4 leads to the immunoproteosomal formation of antigenic CBZ-modified peptides[198, 199]. CBZ is indeed an excellent CYP3A4 substrate that is metabolized predominantly to the CBZ 10,11-epoxide, a reaction that has been used as a CYP3A4 functional marker[200]. The incubation of [^{3}H]CBZ with CYP3A4 results in irreversible binding of [^{3}H]CBZ to the protein with a stoichiometry of 1.58 ± 0.15 pmol [^{3}H]CBZ bound/pmol CYP3A4. In the presence of GSH (4 mM) this stoichiometry is reduced to 1.09. However, no concentration (0–1 mM) nor time-dependent CYP3A4 inactivation was detected in these incubations[201]. In the absence of other substrates, CBZ, if anything, protected from NADPH-dependent oxidative uncoupling[201]. Given these findings, one can speculate that the serum anti-CYP3A4 autoantibodies detected in CBZ-dependent hypersensitivity might arise in patients whose CBZ-CYP3A4 adducts remain

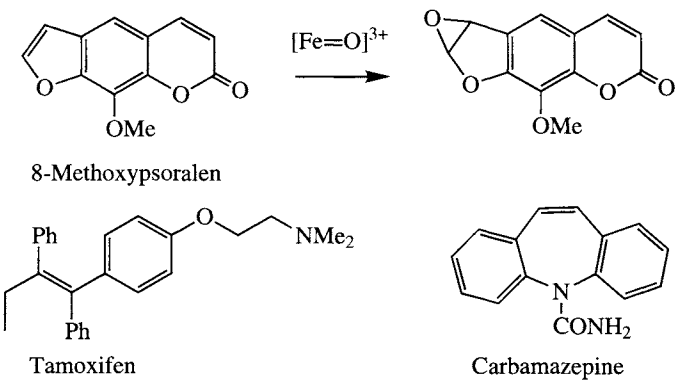


Figure 7.11. 8-Methoxypsoralen (8-MOP) is oxidized to an epoxide, and either the epoxide or a ring-opened product derived from it is responsible for inactivation of P450. The structures of two drugs, tamoxifen and carbamazepine (CBZ), that inactivate P450 by unknown mechanisms are also shown.

stable, possibly because of low intrahepatic GSH levels, and are therefore available for antigenic processing[199, 201].

Collectively, the results convincingly establish that the reactive species formed by P450 enzymes can alkylate, acylate, or otherwise modify the protein skeleton, resulting in the loss of catalytic activity. It is likely, furthermore, that protein modification has gone undetected in some instances where inactivation has been attributed to heme modification. Conversely, the data on parathion and CBZ suggests that heme destruction is required for enzyme inactivation in some instances where protein modification clearly occurs. Furthermore, the fact that CYP2B1 is inactivated by secobarbital by both heme alkylation and protein modification, by phenylacetylene predominantly by heme alkylation, and by 2-ethynylnaphthalene predominantly by protein acylation, underscores the critical role of active-site–substrate interactions in dictating the mode of inactivation. Active-site–inhibitor interactions presumably also explain why *N*-phenyl or *N*-octyl 2,2-dichloroacetamides predominantly inactivate CYP2B1 via protein acylation whereas the corresponding *N*-hexyl, *N*-butyl, or *N*-methyl dichloroacetamides do so via heme destruction[76, 77]. Similar factors presumably also explain the differential modes of inactivation observed with terminal acetylenes (e.g., *N*-alkylation of CYP2B1 by phenylacetylene vs protein acylation of CYP4A1 by 10-undecynoic acid)[78, 83].

3.2. Quasi-Irreversible Coordination to the Prosthetic Heme

This section focuses on inhibitors that are transformed by P450 enzymes into metabolic intermediate (MI) products that coordinate so tightly to the heme iron atom that they can be displaced only under unique experimental conditions. The two major classes of these inhibitors are compounds with a dioxymethylene function and nitrogen compounds, usually amines that are converted *in situ* to nitroso metabolites. A related mechanism is also partially responsible for the inhibition of P450 by 1,1-disubstituted hydrazines and acyl hydrazines. The anaerobic reductive coordination of halocarbons to the heme iron atom is discussed in Section 3.4 because the reaction is linked to destruction of the heme.

3.2.1. Methylenedioxy Compounds

P450 enzymes oxidize aryl and alkyl methylenedioxy compounds, some of which are used as insecticide synergists[202, 203], to species that coordinate tightly to their heme iron atom[204]. The time, NADPH, oxygen, and concentration dependence of the reaction, as well as the finding that NADPH and oxygen can be replaced by cumene hydroperoxide, confirm that the inhibitory species is unmasked by the catalytic action of the P450

enzymes[202, 205, 206]. The resulting ferrous complex is characterized by a difference absorption spectrum with maxima at 427 and 455 nm, whereas the ferric complex exhibits a single absorption maximum at 437 nm[202, 207]. The ferrous peaks at 427 and 455 nm are due to distinct complexes, although their structural interrelationship is obscure[204]. The ferrous complex can be isolated intact from animals treated with isosafrole, demonstrating its stability, but the less stable ferric complex can be disrupted by incubation with lipophilic compounds with concomitant regeneration of the catalytically active enzyme[208, 209]. The ferrous complex is unaffected by incubation with lipophilic compounds but can be disrupted by irradiation at 400–500 nm[210, 211]. Structure activity studies of 4-alkoxy-1,2-methylenedioxybenzene reveal that the size and lipophilicity of the alkoxy group is an important determinant of the complex stability: alkyl chains of 1–3 carbons yield unstable complexes whereas those with longer alkyl groups are stable[212, 213]. As in the case of reversible inhibitors (Section 2.3), the ferrous complex is stabilized by concurrent binding interactions of the ligand with the lipophilic active site[213]. The ferrous to ferric transition weakens the complex, indicating that the activated species, like carbon monoxide, only strongly coordinates to the ferrous iron.

The above results are consistent with the catalysis-dependent generation of a carbene–iron complex (Figure 7.12). The synthesis and characterization of model carbene complexes provides supporting evidence for a carbene complex[214, 215]. The structural analogy between a carbene and carbon monoxide provides a ready explanation for the unusual 455-nm absorption maximum of the complexes. As already noted, a different complex is responsible for the absorption maximum at

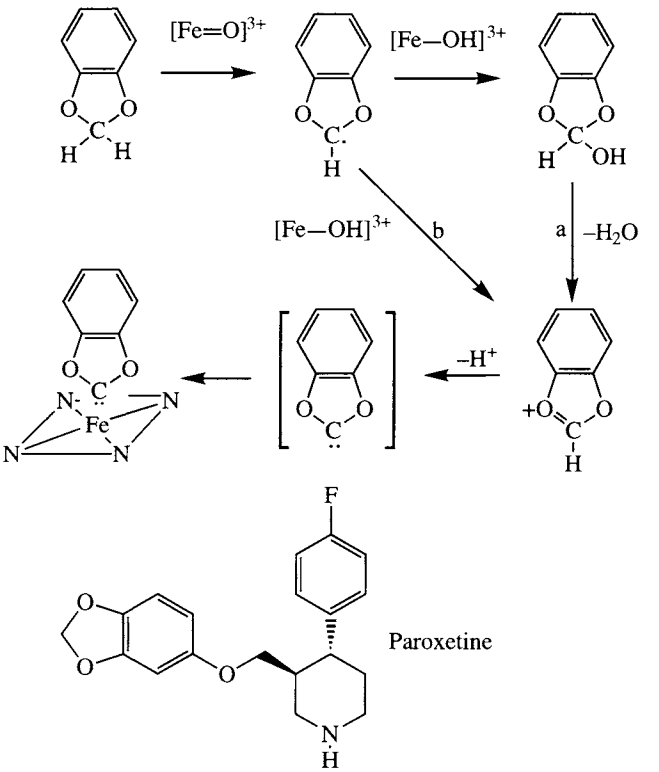


Figure 7.12. The quasi-irreversible inactivation of P450 enzymes by methylenedioxy compounds involves oxidation of the methylene bridge to a species that forms a tight, but reversible, complex with the heme iron atom. The coordinating species is probably a carbene, as shown. Paroxetine is an example of a drug that inhibits P450 by this mechanism.

427 nm, perhaps a carbene complex in which the *trans* ligand, as in P420, is not a thiolate[216]. A carbene complex also provides a ready rationale for the incorporation of oxygen from the medium into the carbon monoxide metabolite formed from the dioxymethylene bridge carbon (see below), and the observation that carbon monoxide formation is enhanced by electron-withdrawing substituents[217]. Water addition to the iron-coordinated carbene produces an iron-coordinated anion that should readily decompose into the observed catechol and carbon monoxide metabolites. A different but undefined mechanism is required to explain the incorporation of an atom of molecular oxygen into a fraction of the carbon monoxide[217].

The link between the dioxymethylene function and P450 inhibition, the requirement for catalytic activation of the inhibitor, and the fact that the dioxymethylene group is oxidized implicate this function in the inhibitory events. An inhibitory role has been postulated for free radical[218], carbocation[219], and carbanion[210] intermediates, but formation of the carbene from the bridge-hydroxylated metabolite or from its radical precursor is most consistent with the results (Figure 7.12). Substituents other than an alkoxy group on the dioxymethylene group suppress complex formation[203, 204, 220]. The retention of activity with an alkoxy substituent is understandable because *O*-dealkylation of the substituent provides an independent route to the bridge-hydroxylated precursor of the carbene[211]. The oxidation of aryldioxymethylenes to catechols, carbon monoxide, carbon dioxide, and formic acid is consistent with hydroxylation of the methylenedioxy bridge[203, 217, 221–223], as is the observation that deuterium substitution on the dioxymethylene carbon decreases the rate of formation of carbon monoxide (k_H/k_D = 1.7–2.0). The observation of a similar isotope effect on the insecticide synergizing *in vivo* activity of these compounds clearly links the formation of carbon monoxide with complex formation and P450 inhibition[224].

Three mechanisms can be envisioned for oxidation of the dioxymethylene bridge to the iron-coordinated carbene. In one mechanism, elimination of a molecule of water after hydroxylation of the dioxymethylene bridge yields an acidic oxonium ion that upon deprotonation gives the carbene (Figure 7.12, path a). In a second mechanism, formation of the oxonium species could precede formation of the bridge-hydroxylated metabolite if the radical formed in the hydroxylation reaction is oxidized by the ferryl species before the oxygen rebound occurs (Figure 7.12, path b). Finally, the same radical intermediate could bind to the iron of the $[Fe–OH]^{3+}$ catalytic intermediate[214]. Deprotonation and intramolecular transfer of the oxygen from the iron to the carbon would give the bridge-hydroxylated metabolite that could then decompose to the carbene complex as in the first mechanism.

Whatever the precise mechanism, *in vitro* experiments with purified CYP2D6 indicate that the formation of MI complexes with a telltale spectroscopic signature at 456 nm may be responsible for the clinical reports of potent CYP2D6 inhibition by paroxetine (Figure 7.12), a serotonin reuptake inhibitor[225–231]. The formation of a carbene complex is supported by the fact that paroxetine is metabolized by CYP2D6 via demethylenation of the methylenedioxy group to a catechol and formic acid[228, 232]. K_I and k_{inact} values of 6.6 ± 2.7 μM and 0.25 ± 0.09 min^{-1}, respectively, have been calculated for the paroxetine-mediated inhibition of human liver microsomal CYP2D6-dependent dextromethorphan *O*-demethylation[231].

Additional methylenedioxyphenyl compounds have been synthesized and their human isoform selectivity as mechanism-based inactivators evaluated[233]. Their inactivating potential depends on the side-chain structure, with bulky side chains such as 1,4-benzothiazine inactivating some P450 enzymes but not others[233].

3.2.2. Amines

Alkyl and aromatic amines, including the MAO inhibitor clorgyline[234], and a number of clinically useful amine antibiotics such as troleandomycin (TAO) (Figure 7.13) and erythromycin, belong to a second large class of agents that form quasi-irreversible (MI) P450 complexes[4, 235–240]. These amines are oxidized to intermediates that coordinate tightly to the ferrous heme and give rise to a spectrum with an absorbance maximum at 445–455 nm[235]. Complex formation requires a primary amine but secondary and tertiary amines, as in the case of TAO, can give P450 complexes if they are first *N*-dealkylated to the primary amines. The complexes from aromatic amines differ from

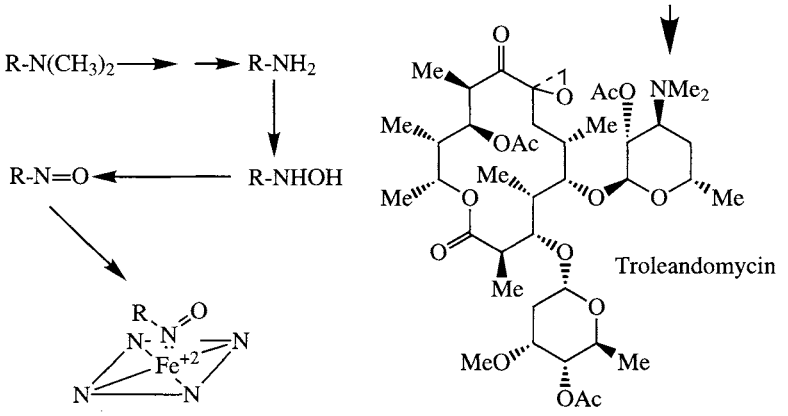


Figure 7.13. The spectroscopically detectable metabolic intermediate (MI) heme complexes formed when some primary amines are oxidized by P450 enzymes involve oxidation of the nitrogen to a nitroso species that coordinates to the iron. The primary amine function can be unmasked by *N*-demethylation reactions, as is the case in the inhibition of CYP3A enzymes by troleandomycin (TAO) (AcO– in the structure represents $CH_3CO_2^-$). The arrow shows the nitrogen that is involved in the reaction in TAO.

those from alkyl amines in that they are unstable to reduction by dithionite[238]. The normal competitive inhibition associated with the binding of amines does not, of course, depend on catalytic oxidation of the inhibitor, but catalytic activation is required for formation of the tight, quasi-irreversible complexes[234, 238, 239]. It is likely that the primary amines are first hydroxylated because the same complexes are obtained with the corresponding hydroxylamines[240], but the coordination requires further oxidation and thus involves a function beyond the hydroxylamine[238, 240]. In fact, the moiety that chelates to the iron appears to be the nitroso function obtained by two-electron oxidation of the hydroxylamine (Figure 7.13)[239–241]. As hydroxylamines readily autooxidize, the final oxidative step may not always require catalytic participation of the enzyme[242]. The coordination of a nitroso function is consistent with the observation that apparently identical complexes are obtained by reduction of nitro compounds[243]. The crystal structure of a complex between a nitroso compound and a model iron porphyrin shows, as expected, that the nitrogen rather than the oxygen of the nitroso group is bound to the iron[214].

It is noteworthy that the *in vivo* complexation of TAO to the heme of CYP3A enzymes stabilizes them and prolongs their half-lives in hepatocytes[244–246]. A consequence of this is that the concentration of the protein in the cell increases, an example of "induction" through protein stabilization. It remains unclear whether the protein levels are elevated because of a substrate-induced conformational stabilization or because the formation of a heme complex suppresses normal damage to the protein associated with the reactive O_2 species produced through uncoupled turnover of the enzyme. This latter possibility is the most likely, given that inhibition of P450 reductase[247, 248] or conditional deletion of the reductase[249], which suppresses catalytic turnover, also results in enzyme stabilization.

3.2.3. 1,1-Disubstituted and Acyl Hydrazines

P450 enzymes oxidize 1,1-disubstituted, but not monosubstituted, hydrazines (see Section 3.3.4) to products that coordinate tightly to the heme iron atom. The complexes, which are characterized by a ferric absorption maximum at ~438 nm and a ferrous maximum at 449 nm, are formed in a time-, NADPH-, and oxygen-dependent manner[250]. The oxidation of isoniazid and other acyl hydrazines by liver microsomes yields a transient complex with a similar absorption maximum at 449 nm[251, 252]. However, the isoniazid complex dissociates on addition of

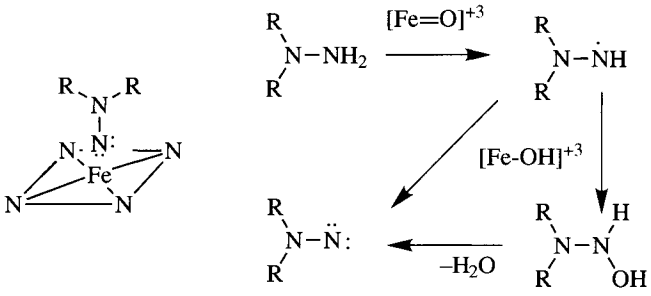


Figure 7.14. The nitrene–iron structure proposed for the complexes formed during the metabolism of 1,1-dialkylhydrazines and possible mechanisms for formation of the nitrene.

ferricyanide and thus is only stable in the ferrous state[253]. Model studies indicate that 1,1-dialkylhydrazines are oxidized to disubstituted nitrenes that form end-on complexes with the iron of metalloporphyrins. The nitrene complexes formed in the reactions of 1-amino-2,2,6,6-tetramethylpiperidine and several iron tetraarylporphyrins have been characterized by NMR, Mössbauer, and X-ray methods[254, 255]. The P450 complexes generated during the metabolism of 1,1-disubstituted hydrazines, and possibly acyl hydrazines, are therefore likely to be aminonitrene–iron complexes (Figure 7.14). Oxidation of the dialkylhydrazines to aminonitrenes is easily rationalized by initial hydroxylation of the hydrazine or, more probably, by stepwise electron removal from the hydrazine (Figure 7.14).

3.3. Covalent Binding to the Prosthetic Heme

P450 is often irreversibly inactivated via covalent attachment of the catalytically activated inhibitor, or a fragment of it, to the heme group. A heme alkylation mechanism has been unambiguously demonstrated, in many instances, by evidence of equivalent activity and heme loss and the isolation and structural characterization of the modified hemes. It must be noted that in the absence of explicit evidence for heme adduct formation, an equimolar loss of enzyme content and heme does not unambiguously establish that heme alkylation is responsible for enzyme inactivation because alternative mechanisms exist for the catalysis-dependent destruction of the heme (see Section 3.4). It is also possible for a heme adduct to be generated that is either reversible or too unstable to be isolated. Unfortunately, the quantitative correlation of heme adduct formation with enzyme inactivation is technically difficult. Without such data, it is difficult to exclude the possibility that the enzyme is also inactivated by mechanisms such as protein modification even when heme alkylation is conclusively demonstrated.

3.3.1. Terminal Olefins

The P450-catalyzed epoxidation of terminal olefins is often associated with *N*-alkylation of its prosthetic heme and inactivation of the enzyme (see Figure 7.7)[153, 156, 256]. Early studies with 2-isopropyl-4-pentenamide (AIA) and 5-allyl-substituted barbiturates such as secobarbital[153, 154], established that the oxidative metabolism of homoallylic amides results in: (a) comparable loss of P450 and heme content, (b) the accumulation of "green pigments" identified as abnormal porphyrins, and (c) derangement of the heme biosynthetic pathway.

The only structural requirement for prosthetic heme alkylation by olefins is a monosubstituted double bond. Accordingly, ethylene, but not ethane, is able to destroy the P450 heme while 3-hexene, cyclohexene, and 2-methyl-l-heptene are inactive[153, 156]. Even monosubstituted olefins fail to inactivate the enzyme if they are not substrates for the enzyme, if the double bond is not the site of catalytic oxidation, or if the double bond is a part a of a conjugated system[156]. Thus, the oxidation of styrene by a model iron porphyrin showed that heme alkylation only occured once in

ten thousand turnovers[256], in contrast to the ratio of less than 300 turnovers per alkylation even that is commonly observed with unconjugated terminal olefins[7]. These observations suggest that alkylation of the heme by olefins is compromised by steric constraints and/or by the presence of substituents that can delocalize charge or electron density from the double bond.

Spectroscopic methods have unambiguously established the structures of *N*-alkylated porphyrins isolated from the livers of rats treated with diverse olefins, including ethylene, propene, octene, fluroxene, 2,2-diethyl-4-pentenamide, 2-isopropyl-4-pentenamide, and vinyl fluoride[7, 257–260]. Analogous products are probably formed in the inactivation of P450 by other olefins, such as in the inactivation of CYP2E1 by the garlic components diallyl sulfide and diallylsulfone[261–264], but the resulting adducts have not been isolated. The terminal carbon of the double bond is bound to a porphyrin nitrogen and the internal carbon of the olefin bears a hydroxyl group in the structures of all the olefin adducts determined so far (Figures 7.7 and 7.15[154, 258]). The oxygen in the ethylene and 2-isopropyl-4-pentenamide adducts, which has been shown by ^{18}O studies to derive from molecular oxygen, is presumed to be the catalytically activated oxygen[257, 258]. The structure of the adduct is consistent with addition of the porphyrin nitrogen to the epoxide metabolite of the olefin, but this possibility is precluded by the following findings: (a) the enzyme is refractory to inactivation by the epoxides of olefins that destroy the enzyme[153, 156, 258], (b) *cis*-addition of the nitrogen and oxygen to the double bond is inconsistent with the *trans* sterochemistry expected for the addition of a nucleophile to an epoxide[258], (c) the nitrogen reacts with the terminal rather than internal carbon of vinyl ethers although the internal (oxygen-substituted) carbon is more reactive in the corresponding epoxides[259], and (d) the pyrrole nitrogens are weak nucleophiles and do not react with epoxides even under harsh chemical conditions. These considerations and the requirement for enzyme turnover indicate that catalytic oxygen transfer to the double bond initiates enzyme inactivation but it does not result from reaction with the epoxide metabolite.

Ethylene, propene, and octene, all linear olefins, only detectably alkylate pyrrole ring D of the prosthetic heme of the phenobarbital-inducible rat liver P450 enzymes (Figure 7.15), but heme alkylation by the more "globular" olefins 2-isopropyl-4-pentenamide and 2,2-diethyl-4-pentenamide is less regiospecific[257, 260]. The regiochemistry and stereochemistry of heme alkylation by *trans*-[1-^{2}H]-1-octene has established that the olefin stereochemistry is preserved

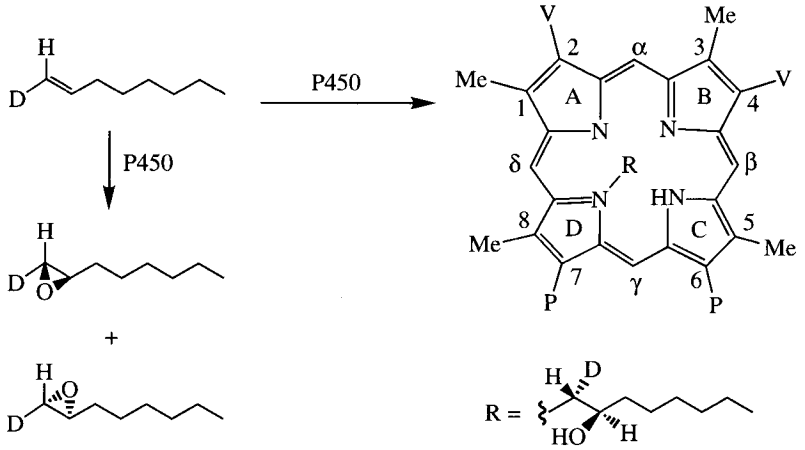


Figure 7.15. The oxidation of *trans*-1-[1-^{2}H]octene by rat liver microsomes yields both the epoxide metabolite and the indicated *N*-alkyl heme adduct, the structure of which has been unambiguously established. The heme substituents are: Me=CH_3, V=CH=CH_2, P=$CH_2CH_2CO_2H$. The peripheral pyrrole carbons and the *meso* carbons of the porphyrin are labeled.

during heme alkylation. Furthermore, heme alkylation only occurs when the oxygen is delivered to the *re* face of the double bond even though stereochemical analysis of the epoxide metabolite shows that the oxygen is delivered almost equally to *both* faces of the π-bond[258]. P450 heme alkylation is thus a highly regio- and stereospecific process.

Chemical models have successfully reproduced autocatalytic heme alkylation and have confirmed and extended the mechanistic information provided by enzymatic studies[262-269]. Thus, iron porphyrins can oxidize terminal olefins to species that alkylate the porphyrin nitrogens and give the same types of adducts as are obtained biologically[262-265]. In the models, as in the biological process, the oxygen is added to the olefin from the same side as the porphyrin nitrogen, the nitrogen is not alkylated by epoxide or aldehyde metabolites, and the reaction is subject to steric interference by substituents on the olefin[262-264]. However, in the model systems, heme alkylation can occur with disubstituted olefins, and binding of the porphyrin nitrogen to the internal carbon and the hydroxyl group to the terminal carbon of monosubstituted olefins occurs to a limited extent[262-266]. As a case in point, spectroscopic studies suggest that an *N*-alkylated porphyrin is transiently formed in the oxidation of norbornene by an iron porphyrin[267, 268]. The finding that formation of the adduct appears to be reversible led to the suggestion that this reversibility may mask the biological formation of secondary *N*-alkyl adducts[266, 269, 270]. Reversible *N*-alkyl adduct formation has not been generally detected with P450, although evidence for the reversible *N*-alkylation of the heme of a CYP2E1 T303A mutant by *tert*-butyl acetylene has recently been reported[271]. On the other hand, it has been known for some time that the catalytic oxidation of terminal olefins by chloroperoxidase and H_2O_2 results in reversible *N*-alkylation of its heme group, with up to 80% recovery of the enzyme activity over several hours at 25°C[272].

Several mechanisms can be envisaged for heme alkylation that are consistent with the experimental data, none of which involves a concerted transfer of the oxygen to the π-bond. Subsequent to possible formation of a charge transfer complex between the ferryl species and the olefin π-bond, addition of the oxygen to the π-bond could give a transient carbon radical that alkylates the heme, closes to the epoxide, or transfers the unpaired electron to the heme to give a cation that alkylates the heme. The partitioning between metabolite formation and heme alkylation may be determined, in part, by the regiochemistry (i.e., inner or outer carbon) of oxygen addition to the π-bond. The P450-catalyzed oxidation of olefins can also be explained by initial addition of the oxoiron complex to the π-bond to give one of the two possible metallacyclobutane intermediates. The ratio of epoxide formation to heme alkylation might then reflect the relative proportion of the metallacyclobutane with the oxygen bound to the internal carbon vs that with the oxygen bound to the terminal carbon. However, the heme alkylation details, the parameters that govern partitioning between epoxidation and heme alkylation, and the relationship between the mechanism of heme alkylation vs epoxide formation remain to be clarified. The recent formulation by Shaik of a two-state oxidation mechanism, in which spin state pairing of electrons in the transition state determines whether oxygen transfer follows a virtually concerted pathway or occurs stepwise, provides a highly attractive rationale for the observation of both heme alkylation and epoxide formation pathways in the turnover of a single substrate[273]. The two-state hypothesis is treated in detail in Chapter 2 and in less detail in Chapter 6.

3.3.2. Acetylenes

P450-catalyzed oxidation of terminal acetylenes to substituted acetic acids (Chapter 6) is more prone to result in heme alkylation than the oxidation of terminal olefins. The structure–activity relationships for the acetylene reaction are similar to those for terminal olefins, except that there are fewer instances in which the reaction does not result in enzyme inactivation. For example, P450 is inactivated by phenylacetylene but not detectably by styrene[164], and P450 is inactivated by internal acetylenes, albeit without heme adduct formation, but not by internal olefins[156, 274]. Catalytic oxidation of the acetylenic function is required for enzyme inactivation and terminal acetylenes give heme adducts analogous to those obtained with terminal olefins[258, 259]. The salient difference in the adducts obtained with acetylenes and olefins

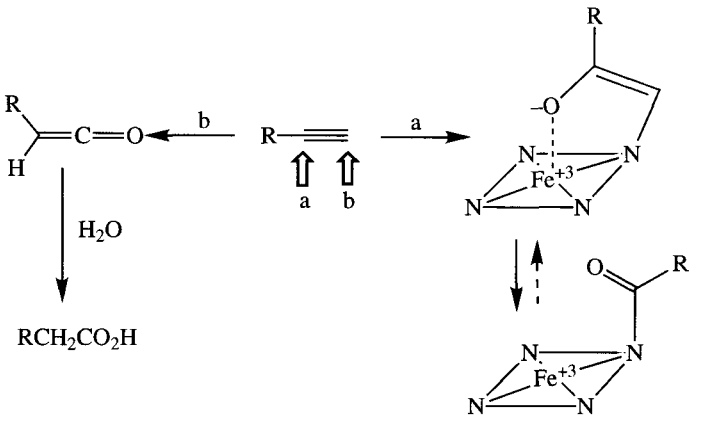


Figure 7.16. The P450-catalyzed oxidation of a terminal acetylene partitions between formation of the ketene and heme alkylation. Which of these events occur is determined by the carbon to which the activated oxygen is added: addition to the internal carbon (a) results in heme alkylation, and to the terminal carbon (b) yields the ketene. In the absence of the iron, the enol adduct tautomerizes to the ketone.

is that addition of the hydroxyl group and the porphyrin nitrogen across the triple bond produces an *enol* that eventually tautomerizes to a ketone. A second difference is that in the adducts of phenobarbital-inducible rat liver P450 enzymes the alkylation occurs almost exclusively on the nitrogen of pyrrole ring A whereas linear olefins primarily alkylate the nitrogen of pyrrole ring D. A topographical rationale has been provided to explain this difference in alkylation regiochemistry.

The mechanisms proposed for P450 inactivation by terminal olefins can be applied to inactivation by terminal acetylenes if one keeps in mind that all the reaction intermediates bear an additional double bond. Triple bond oxidation is furthermore unique in that the carbon to which the oxygen is *initially* bound is revealed by the products, whereas this information is lost in olefin epoxidation because the oxygen is bound to both carbons in the epoxide metabolite. Thus, in all the heme adducts with acetylenes that have been sufficiently well characterized, the oxygen is bound to the internal carbon, whereas all the metabolites are derived from the ketene obtained by addition of the oxygen to the terminal carbon. As already mentioned, the ketene or a closely related species can also inactivate the enzyme by reacting with the protein[78, 81]. This difference in oxygen addition regiochemistry, in conjunction with the observation of a large isotope effect on metabolite formation but not heme alkylation in the metabolism of terminally deuterated phenylacetylene, indicates that the commitment to either metabolite formation or heme alkylation occurs as the oxygen transfer step is initiated (Figure 7.16)[164]. The factors that determine to which triple bond carbon the oxygen is transferred, and therefore whether heme or protein modification occurs, are unclear. However, the observation that 1-ethynylpyrene and phenylacetylene inactivate CYP1A2 by, respectively, protein and heme modification demonstrates that the reaction regiochemistry is governed by substrate–enzyme interactions and not simply by intrinsic differences between P450 enzymes[83]. Indeed, such specific substrate/inactivator-active sit fit may account for the relatively selective inactivation of CYP3A4 by gestodene (K_I = 46 μM, k_{inact} = 0.4 min^{-1}, and partition ratio of ~9)[92].

To gain insight into these reactivity determinants, a series of aryl and arylalkyl acetylenes varying in the size and shape of the aromatic ring system, the placement of the carbon–carbon triple bond, the length of the alkyl side chains, and/or the presence of a terminal hydrogen or methyl group have been examined as inhibitors/inactivators of human and rat liver CYP1A1, -1A2, and -2B1/2B2[79–83, 275]. The findings reveal that all these features can influence the potency, type, and selectivity of P450 inhibition. Accordingly, the arylacetylenic compounds with the larger pyrene, phenanthrene, or biphenyl rings

appear to inactivate the CYP1A isoforms, whereas 2-ethynylnaphthalene, 4-phenyl-1-butyne, 1-phenyl-1-propyne, and 5-phenyl-1-pentyne are selective CYP2B1 inactivators[79–83, 275]. On the other hand, the 9-ethynyl- and 9-propynylphenanthrene isomers reversibly inhibit the CYP1A isoforms, but are among the most effective mechanism-based inactivators of CYP2B1/2B2[275].

The length of the alkyl side chain was an important reactivity determinant among the arylalkyl acetylenes[275]. Thus, while phenylacetylene is a reversible CYP2B1/2B2 inhibitor, analogues with three or four methylene groups (5-phenyl-1-pentyne and 6-phenyl-1-hexyne, respectively) are among the most potent prototype CYP2B1/2B2 inactivators. Replacement of the terminal hydrogen with a methyl, giving disubstituted acetylenes, results in reduced CYP2B and increased CYP1A inactivation. Thus, 2-(1-propynyl)phenanthrene, 4-ethynylbiphenyl, and 4-(1-propynyl)biphenyl are very effective inactivators of both rat liver CYP1A1 and -1A2, whereas 1-(1-propynyl)pyrene, 2-ethynylphenanthrene, 3-ethynylphenanthrene, 3-(1-propynyl) phenanthrene, 2-(1-propynyl)naphthalene, and 6-phenyl-2-hexyne are effective inactivators of CYP1A1 but not CYP1A2[275]. Furthermore, replacement of the terminal acetylenic hydrogen with a methyl enhanced the mechanism-based inactivation of both CYP1A1 and -1A2, or converted a reversible inhibitor into an effective inactivator, as exemplified by 1-ethynylpyrene and 1-(1-propynyl)pyrene, 2-ethynylphenanthrene and 2-propynylphenanthrene, 3-ethynylphenanthrene and 3-propynylphenanthrene, and 6-phenyl-1-hexyne and 6-phenyl-2-hexyne[275]. Indeed, 2-propynylphenanthrene and 4-propynylbiphenyl (4PBi) are among the more selective inhibitors of rat liver CYP1A and human liver CYP1A2 enzymes.

In contrast, 4PBi fails to inactivate human liver microsomal CYP2E1, -2C9/10, -3A4 or -2C19. The identification of 2-biphenylylpropionic acid from the CYP1A1- and -1A2-catalyzed metabolism of 4PBi links this mechanism-based inactivation with that of terminal acetylenes, as it involves a 1,2-shift of the terminal methyl to give a ketene intermediate[275]. The importance of the 1,2 methyl shift and the resulting ketene in P450 inactivation by internal acetylenes such as 4PBi is underscored by the finding that P450 enzymes such as CYP2B1, which do not oxidize 4PBi to 2-biphenylylpropionic acid, are refractory to inactivation.

There are other documented examples of mechanism-based P450 inactivation by methyl-substituted (i.e., internal) acetylenes[84, 94]. A clinically relevant internal acetylene that potently and selectively inactivates human liver CYP3A4 is the antiprogestin drug mifepristone [RU486; (11β,17β)-11-[4-(dimethylamino)-phenyl]-17-hydroxy-17-(1-propynyl)-estra-4,9-dien-3-one] (Figure 7.9)[95, 276], a drug used for medical abortion in the first trimester of pregnancy[277]. K_I and k_{inact} values of 4.7 μM and 0.089 min^{-1} place mifepristone among the most potent CYP3A4 inactivators[95]. Although the activities of CYP1A, -2B, and -2D6 enzymes were also inhibited *in vitro*, this inhibition, unlike that of CYP3A4 and -3A2, was reversed when mifepristone was removed by dialysis. Inactivation with [^{3}H]mifepristone showed that the drug binds covalently to the CYP3A4 protein with a stoichiometry of 1.02 ± 0.15 mol per mol of protein[95]. *In vitro* studies with CYP3A4 and -3A5, the other major adult human liver CYP3A isoform, indicate that the latter, although capable of metabolizing the drug, is not subject to mifepristone-mediated inactivation[96]. Mifepristone may be a useful probe with which to distinguish these two CYP3A isoforms. The acetylenic moiety of mifepristone is thought to also be activated to a ketene, although the expected propionic acid metabolites have not been detected with either enzyme. However, LC-MS of mifepristone metabolites revealed that although both enzymes generate the *N,N'*-didemethylated and *N*-monodemethylated products, only CYP3A4 hydroxylates the terminal methyl group. Thus, the susceptibility of CYP3A4 but not CYP3A5 to inactivation may be due to the ability of the first but not the second to oxidize the acetylenic moiety of mifepristone[96]. Although not considered in the publications, it is very possible that the inactivation observed with mifepristone does not reflect oxidation of the triple bond at all but rather oxidation of the terminal methyl to an aldehyde, giving an α,β-unsaturated aldehyde that adds to the protein as a Michael acceptor.

Not surprisingly, the acetylenic function has been exploited in the design and synthesis of P450 isoform-selective or -specific irreversible inhibitors, including inhibitors of $P450_{scc}$, aromatase, prostaglandin ω-hydroxylase[278], and the

P450 enzymes that oxidize saturated fatty acids, arachidonic acid, and leukotriene B_4 (Section 5.5). It may also play a role in the alterations of oxidative metabolism observed in individuals treated with ethynyl sterols such as gestodene and 17α-EE[87–92].

Strategies to convert selective P450 substrates to suicide inactivators by the incorporation of a suitable activatable function at the position oxidized are not always successful. For instance, the introduction of an acetylenic moiety into the chemical template *N*-(3,5-dichloro-4-pyridyl)-3-(cyclopentyloxy)-4-methoxybenzamide (DCMB), a CYP2B6 functional marker, to yield *N*-(3,5-dichloro-4-pyridyl)-4-methoxy-3-(prop-2-ynyloxy)benzamide gave a mechanism-based agent that, based on a correlation of activity and ferrous–CO chromophore loss, probably inactivated CYP2B6 via heme modification[279]. However, this inactivation was not very selective as other human liver CYP2C isoforms were also inactivated, indicating that the catalytic selectivity for CYP2B6 resides in the *O*-alkyl chain of the parent DCMB molecule.

3.3.3. Dihydropyridines and Dihydroquinolines

The administration of 3,5-bis(carbethoxy)-2,4,6-trimethyl-1,4-dihydropyridine (DDC)[280–284] perturbs heme biosynthesis and causes a loss of hepatic P450 content, both of which have been traced to *N*-methylation of the P450 prosthetic heme[285–290]. Substitution of the dihydropyridine at position 4 with a primary, unconjugated moiety (methyl, ethyl, propyl, *sec*-butyl, nonyl), but not an aryl (phenyl), secondary (isopropyl), or conjugated (benzyl) group results in *N*-alkylation of the heme[287, 291–294]. 4-Aryl-substituted dihydropyridines do not inactivate the enzyme, whereas those bearing secondary or conjugated substituents inactivate the enzyme but do not yield detectable *N*-alkyl heme adducts[291–294]. Dihydropyridines with simple 4-alkyl groups *N*-alkylate the heme of certain P450 isoforms, but inactivation of others occurs by a mechanism that appears to involve heme degradation to fragments that irreversibly modify the protein (Section 3.4).

The mechanisms of enzyme inactivation and heme destruction by analogs that do not give identifiable heme adducts remain unclear, but the mechanism of analogues that alkylate the heme nitrogen is understood better. The adducts consist of protoporphyrin IX with the 4-alkyl group of the parent substrate covalently attached to one of its nitrogen atoms (Figure 7.17)[285, 286, 288, 289]. Different nitrogens are alkylated in different P450 enzymes[290, 295], and the dihydropyridines cause isoform-selective inactivation[295, 296]. The catalytic turnover of 4-alkyl-1,4-dihydropyridines thus leads to transfer of the 4-alkyl group to a nitrogen of the heme. The following observations further elucidate the nature of the enzyme inactivation: (a) the dihydropyridines are oxidized to the pyridines with partial loss of 4-alkyl but not 4-aryl groups[293, 294], (b) the *N*-methyl or *N*-ethyl derivatives of 4-alkyldihydropyridines still inactivate P450 enzymes but inactivation may follow *N*-dealkylation because with those substrates *N*-dealkylation is faster than dihydropyridine aromatization[293], (c) no primary isotope effect is observed on enzyme inactivation when the hydrogen at position 4 is replaced by deuterium[293], (d) the heme adducts that are formed are chiral and therefore are generated within the active site[297], and (e) free radicals have been detected with a spin trap in incubations of a 4-ethyldihydropyridine with hepatic microsomes[286]. However, studies with deferoxamine-washed microsomes suggest metal-catalyzed oxidation of the dihydropyridine accounts for most if not all of the spin-trapped radical[298]. In any case, as neither GSH nor the radical trap prevent enzyme inactivation, the radicals detected in the medium appear not to be involved in heme alkylation. Conversely, if radicals are formed within the active site, they are not readily detected in the medium. In view of these results, it is highly likely that electron abstraction from the dihydropyridine produces a radical cation that aromatizes either by extruding the 4-alkyl group as a radical or by directly transferring the alkyl group to the heme. Although the 4-alkyl group may be directly trapped by the porphyrin nitrogen, model studies suggest that it first adds to the iron to form an alkyl–iron complex from which it migrates to the porphyrin nitrogen. The absence of detectable heme adducts in the P450 inactivation by the 4-isopropyl and 4-benzyl analogs is consistent with such a mechanism, not only because the iron–nitrogen shift is sensitive to steric effects[299], but also because the more oxidizable secondary or benzylic moieties may be converted

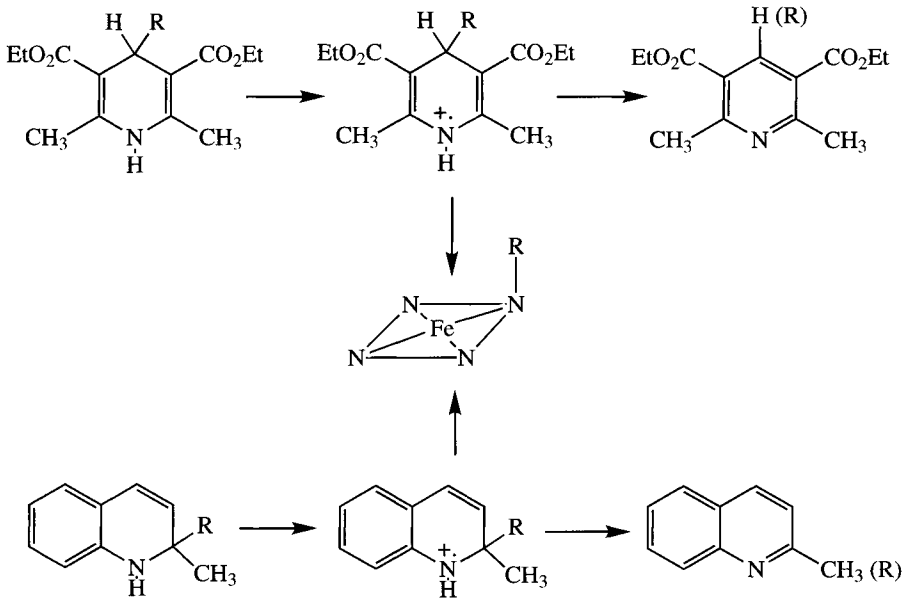


Figure 7.17. Oxidation of 4-alkyl-1,4-dihydropyridines and 2,2-dialkyl-1,2-dihydroquinolines is proposed to yield cation radical intermediates that aromatize by either losing a second electron and a proton or an alkyl radical that adds to a nitrogen of the prosthetic heme group.

to the corresponding cations by electron loss to the iron in preference to undergoing the oxidative iron–nitrogen shift.

The precedent set by the P450-catalyzed oxidation of dihydropyridines to radical cations that aromatize by radical extrusion has led to the observation of comparable processes in related structures. Thus, 2,2-dialkyl-1,2-dihydroquinolines are also oxidized by P450 enzymes to species that *N*-alkylate the heme and inactivate the enzymes. In these reactions, the 2-alkyl substituent of the dihydroquinoline is bound to a nitrogen of protoporphyrin IX, presumably by a mechanism similar to that proposed for the 4-alkyldihydropyridines (Figure 7.17)[300].

3.3.4. Alkyl- and Arylhydrazines and Hydrazones

The P450-destructive mechanism of phenelzine, an alkylhydrazine, is relatively well defined. It causes an approximately equimolar loss of enzyme and heme when incubated with hepatic microsomes[301], and these losses are accompanied by the generation of a heme adduct identified as *N*-(2-phenylethyl)protoporphyrin IX (Figure 7.18)[302]. The generation of products in a microsomal system that implicate the 2-phenylethyl radical as a central MI suggests a role for the 2-phenylethyl radical in this enzyme inactivation[303]. Spin-trapping experiments confirm that the 2-phenylethyl radical is generated in the incubations but the bulk of the radical that is spin trapped is formed by transition metal, rather than P450-catalyzed reactions[302, 304]. It appears from these results that phenelzine is converted to the 2-phenylethyl radical within the P450 active site where it is captured by the heme to give the *N*-(2-phenylethyl) adduct (Figure 7.18). The 2-phenylethyl radical could react directly with the porphyrin nitrogens, but by analogy to the reactions of hemoproteins with arylhydrazines (see below) it is likely that the alkyl radical is trapped by reaction with the heme iron to give an unstable alkyl–iron complex that subsequently rearranges to the isolated *N*-alkyl heme adduct.

A complex with an absorbance maximum at 480 nm is generated in the reaction of P450 with phenylhydrazines and *N*-phenylhydrazones.

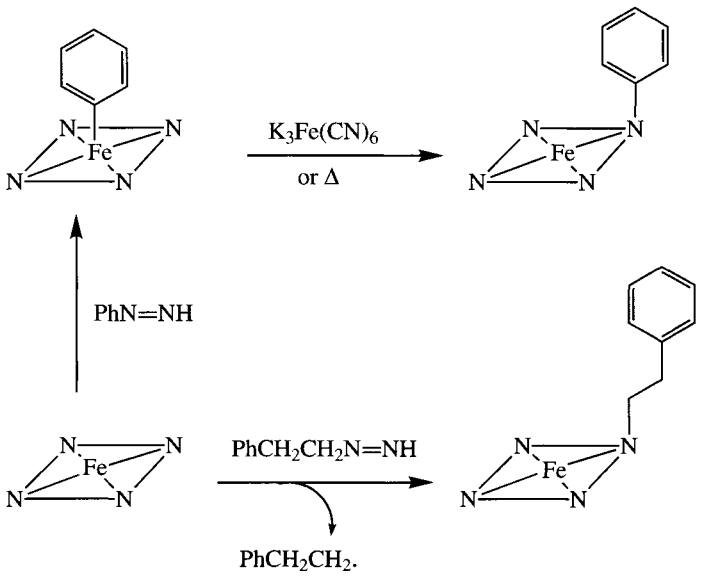


Figure 7.18. The oxidation of phenelzine ($PhCH_2CH_2NHNH_2$) and phenylhydrazine ($PhNHNH_2$) by P450 produces carbon radical products that bind to the prosthetic heme group. In the case of phenylhydrazine, the phenyl radical binds first to the iron atom to give a complex that subsequently rearranges under oxidative conditions to the *N*-phenyl adduct. It is not known if phenelzine initially forms a similar but less stable carbon–iron intermediate.

Formation of this complex inactivates the enzyme and precedes irreversible destruction of its prosthetic heme[305–308]. The reactions of myoglobin, hemoglobin, and catalase with phenylhydrazine yield a similar complex, in these cases with an absorbance maximum at ~430 nm due to the difference between the proximal thiolate and histidine ligation[309–313]. As revealed by X-ray crystallography, the myoglobin and CYP101 ($P450_{cam}$) structures are σ-aryl–iron complexes with the phenyl group bound end-on to the iron (Figure 7.18)[308, 314]. Extraction of the heme complex from CYP101 under oxidative conditions, yields a roughly equal mixture of the four possible *N*-phenylprotoporphyrin IX regioisomers, as found with the complexes from myoglobin, hemoglobin, and catalase[308, 315]. The intact phenyl–iron heme complex, which has been characterized by absorption and NMR spectroscopy, is obtained if the prosthetic group is extracted under *anaerobic* conditions[308, 312]. Exposure of the anaerobically extracted phenyl–iron heme complex to oxygen or other oxidizing agents under acidic conditions results in migration of the phenyl from the iron to the porphyrin nitrogens (Figure 7.18)[312]. This migration is sensitive to steric effects because the aryl moiety in aryl-iron complexes obtained from *ortho*-substituted phenylhydrazines does not undergo the oxidative shift[299]. Arylhydrazines, including phenyl-, 2-naphthyl-, and *p*-biphenylhydrazine, are known to be P450 substrates[316]. Migration of the phenyl group from the iron to the porphyrin nitrogens can be induced to occur within the undenatured active site by the addition of ferricyanide to the intact P450 complexes[317–319]. The distribution of the four *N*-aryl protoporphyrin IX regioisomers produced by this method reflects the active-site topology and varies widely from enzyme to enzyme. The migration of aryl groups within the active sites of P450 enzymes provides a tool for the determination of their active-site topology because the regioselectivity of the migration is controlled by the degree to which the active site is sterically unencumbered above each of the four pyrrole ring nitrogens[316–322]. The *in situ* shift of the aryl moiety from the iron to the porphyrin nitrogens occurs readily with most, but not all, of the P450 iron–aryl complexes, but does not occur with the complexes of proteins

such as myoglobin that have an imidazole as the fifth iron ligand.

The likelihood that alkyl radicals, like their aryl counterparts, bind to the iron before shifting to the nitrogen is supported by the observation that the type II complexes formed between alkyldiazenes and P450 in the absence of oxygen are converted, in the presence of limited amounts of oxygen, to complexes with an absorption maximum at ~480 nm characteristic of iron–carbon σ-bonded complexes[306, 323]. Furthermore, alkyl diazene-iron tetraphenylporphyrin complexes can be prepared under anaerobic conditions[324]. However, the alkyl–iron complexes are much less stable and less well characterized than the aryl–iron complexes and their involvement in heme *N*-alkylation reactions remains to be demonstrated.

In addition, some aryl hydrazines, notably dihydralazine, also inactivate P450 via protein modification in a mechanism-based process[325]. Thus, dihydralazine has been shown to inactivate rat liver microsomal CYP1A2, -2C11 and -3A, but not CYP2B1 or -1A1[325], and also inactivates human liver CYP1A2 and -3A4, but not CYP2C9[326]. This inactivation appears to involve irreversible binding of a reactive metabolite to the P450 protein in a process that is not affected by co-incubation with GSH (5 mM)[326]. The generation of irreversible dihydralazine–protein adducts *in vivo* and their subsequent immunoproteasomal processing into antigenic P450 peptides could possibly account for the detection of CYP1A2-reactive anti-liver microsomal (anti-LM) autoantibodies, in the sera of patients with dihydralazine-induced immunoallergic hepatitis[327, 328].

3.3.5. Other N–N Functions

The P450 heme is *N*-alkylated or *N*-arylated by reactive intermediates formed when it oxidizes 1-aminoaryltriazoles, 2,3-bis(carbethoxy)-2,3-diazabicyclo[2.2.0]hex-5-ene, and the sydnones.

The oxidation of 1-aminobenzotriazole (ABT) by chemical reagents yields benzyne, an exceedingly reactive species, and two molecules of nitrogen[329]. The finding that benzyne, or its equivalent, is bound across two of the nitrogens of the prosthetic heme isolated from inactivated P450 enzymes suggests that the enzyme-catalyzed oxidation of ABT follows the same reaction trajectory[330, 331]. The benzyne may add directly to the two nitrogens, generating an *N,N*-bridged species that autooxidizes to the isolated bridged porphyrin, or may first bind to the iron and a nitrogen of the heme and subsequently rearrange to the *N,N*-bridged species (Figure 7.19). Introduction of small substituents on the phenyl ring or on the exocyclic nitrogen of ABT, or replacement of the phenyl framework with alternative aryl moieties, does not impair destructive activity[331–333]. It is not known whether the oxidation of ABT to benzyne proceeds via hydroxylation of the exocyclic nitrogen or electron abstraction to give a radical or radical cation (Figure 7.20), but a notable similarity exists between the activation mechanism proposed for ABT and other 1,1-disubstituted hydrazines (Figure 7.14).

ABT inactivates a wide variety of P450 enzymes without detectable toxic effects[332–338]. Thus, ABT administration to guinea-pigs inactivates both adrenal steroidogenic- and xenobiotic-metabolizing P450 isoforms[337, 338]. The inactivation of steroidogenic enzymes is apparently indirect and due to the generation of an extra-adrenal ABT metabolite(s), as these guinea-pig adrenal P450 isoforms (unlike their hepatic and adrenal xenobiotic metabolizing counterparts) are not susceptible to direct ABT-mediated inactivation in reconstituted *in vitro* systems[337, 338]. P450 isoform and tissue selectivity is conveyed by placing substituents on the exocyclic nitrogen of the aminotriazole function[332, 333, 339–341]. Furthermore, it is noteworthy that certain ABT analogs [*N*-benzyl-, *N*-(α-methylbenzyl)-, or *N*-(α-ethylbenzyl)-1-ABT] also inactivate phenobarbital-inducible hepatic P450 enzymes (other than the CYP2B4/CYP2B1 orthologs) via the formation of MI complexes rather than by heme modification[342]. Overall, ABT is a highly effective agent for the *in vivo* inactivation of a variety of P450 enzymes in plants[343, 344], insects[345], and animals[334–342, 346–348].

Cyclobutadiene, which can be envisioned as a rectangular structure with a singlet electronic state or a square structure with a triplet electronic state, is formed upon chemical oxidation of 2,3-diazabicyclo[2.2.0]hex-5-ene[349]. Bis(carbethoxy)-2,3-diazabicyclo-[2.2.0]hex-5-ene (DDBCH), a precursor of the above compound, is a mechanism-based irreversible inhibitor of P450 that exploits the basic reactivity of the parent bicyclic system[350]. The bis(carbethoxy) derivative was employed for the enzymatic studies because the

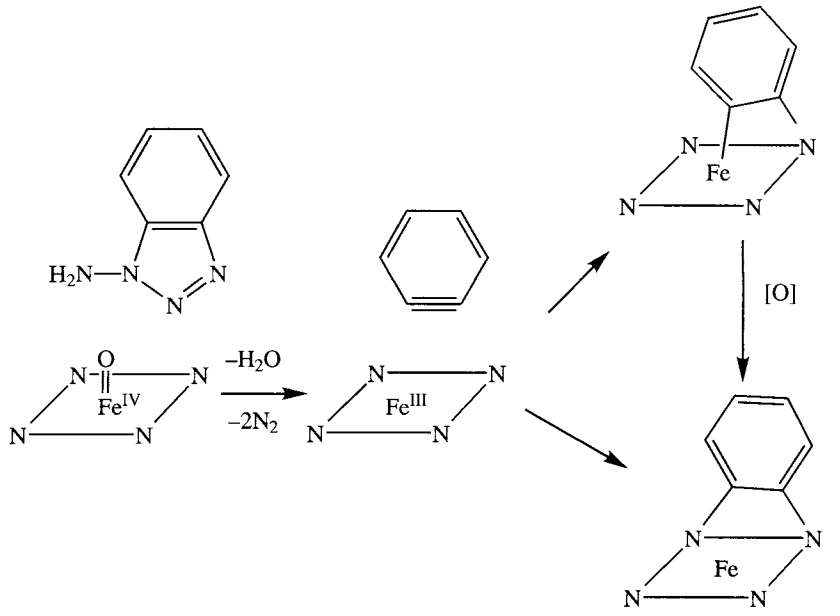


Figure 7.19. Two alternative mechanisms for addition of the benzyne released from 1-aminobenzotriazole (ABT) to the heme. The heme porphyrin framework is represented by a square of nitrogens, each of which represents one of the four nitrogens of the porphyrin.

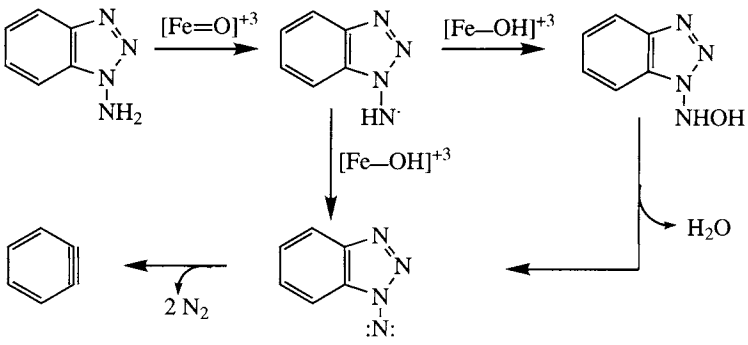


Figure 7.20. Mechanistic alternatives for the P450-catalyzed generation of benzyne from 1-aminobenzotriazole.

parent bicylic hydrazine autooxidizes too readily to be biologically useful. The heme of the P450 enzyme is converted into the *N*-2-cyclobutenyl derivative during the inactivation reaction (Figure 7.21). The secondary, allylic, carbon–nitrogen bond in this adduct makes it much less stable than other adducts, which bear the primary, unactivated, *N*-alkyl groups. The failure of internal olefins and acetylenes to detectably alkylate the prosthetic heme suggests, in fact, that secondary carbons are generally too sterically encumbered to react with the heme moiety. Although the precise nature of the reactive species remains undefined, the generation of the 2-cyclobutenyl adduct implies the involvement of cyclobutadiene itself, or of a closely related

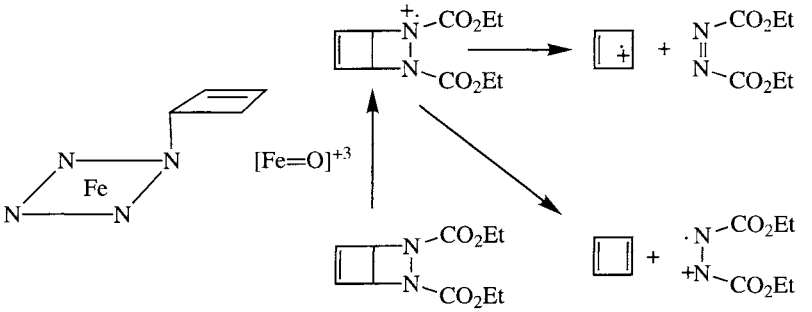


Figure 7.21. Possible mechanisms for the oxidative generation of cyclobutadienoid species that alkylate the prosthetic heme group of P450. The heme of P450 is abbreviated as indicated in Figure 7.19.

species, in heme alkylation. The structure of the adduct is readily rationalized if cyclobutadiene adds to a porphyrin nitrogen to give a transient, probably anionic intermediate, that is neutralized by a proton from the medium. The transient intermediate could be stabilized by formation of a carbon–iron bond with the carbon that is eventually protonated. Electron abstraction from DDBCH can lead to the observed adduct by pathways that depend on whether the cyclobutadiene is generated as a neutral, cationic, or anionic species (Figure 7.21).

The accumulation of a fluorescent hepatic pigment in dogs and rats administered a sydnone derivative[351] led to the discovery that the sydnone is catalytically activated by P450 enzymes to a species that alkylates the prosthetic heme[352]. The heme adduct isolated from rats treated with the sydnone has been identified as *N*-vinylprotoporphyrin IX (Figure 7.22). This finding suggests that the sydnone is first activated by hydroxylation of the electron-rich zwitterionic carbon, followed by ring opening and elimination of the carboxylic fragment to give the diazo species (Figure 7.22). A similar mechanism explains the oxidation of sydnones by simple chemical reagents[353]. The diazoalkane then reacts with the heme, possibly via an initial carbene complex, to give a nitrogen–iron bridged intermediate. The formation of just such a bridged nitrogen–iron complex has been observed in model porphyrin systems[354, 355]. The negative charge on the carbon in the bridged intermediate finally eliminates the thiophenyl moiety and generates the *N*-vinyl adduct[352]. The mechanism is further clarified by the fact that P450 inactivation by 3-(2-phenylethyl)-4-methylsydnone produces both *N*-(2-phenylethyl)- and *N*-(2-phenylethenyl) protoporphyrin IX[356]. These results are most consistent with oxidation of the sydnone to the (2-phenylethyl)diazonium cation that reacts with the heme in two different ways. In one mechanism, deprotonation of the diazonium intermediate, as discussed above, results in a carbene-like addition. In the absence of a β-leaving group, the resulting carbanion intermediate is oxidized to a cation that is deprotonated to introduce the double bond into the *N*-alkyl group[356]. In the second mechanism, reduction of the diazo intermediate prior to deprotonation yields a phenyldiazenyl radical that is trapped by a porphyrin nitrogen atom in the same manner as the 2-phenylethyl radical produced by the oxidation of phenylethyldiazene (Figure 7.18)[302]. The validity of this mechanism is strengthened by the fact that the 2-phenylethyl adduct obtained from the 1,1-dideuterated substrate retains both deuteriums and therefore arises by a mechanism that does not involve deprotonation of the diazo intermediate[356].

The *in vivo* administration of diethylnitrosamine (Et_2N–N=O) to mice reportedly generates an alkylated porphyrin that was tentatively identified by mass spectrometry as *N*-(2-hydroxyethyl)protoporphyrin IX[357]. *In vitro* studies with rabbit liver microsomes and purified P450 enzymes have independently shown that P450 oxidizes diethylnitrosamine to ethylene[358]. Although not actually demonstrated, the proposed *N*-(2-hydroxyethyl) porphyrin adduct is likely to be derived from P450 enzymes inactivated by the ethylene metabolically generated from diethylnitrosamine (see Section 3.3.1).

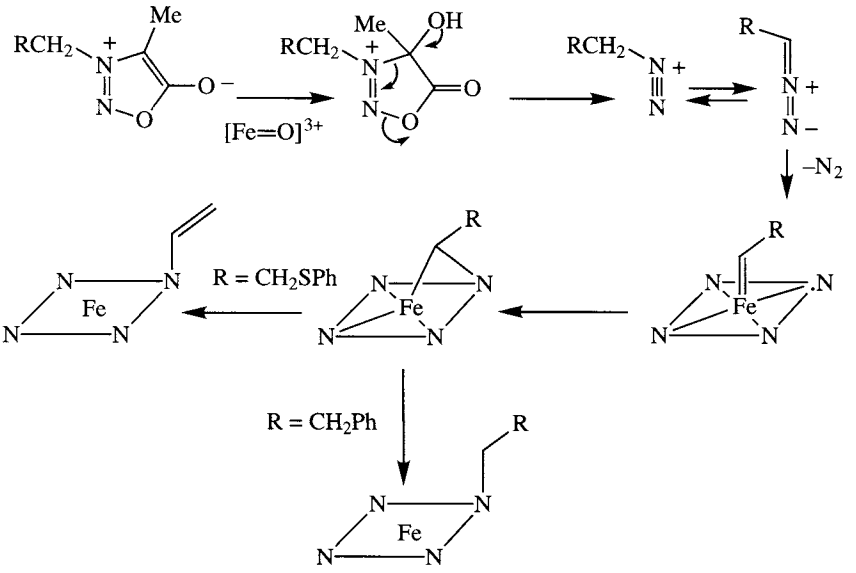


Figure 7.22. Mechanism proposed for the oxidation of sydnones to reactive intermediates that add to the prosthetic heme group of P450. The structures of the *N*-alkylporphyrins isolated from rats treated with the 3-(2-phenylthioethyl)- and 3-(2-phenylethyl) sydnones are shown.

3.3.6. Other Functionalities

The herbicide 1-[4-(3-acetyl-2,4,6-trimethylphenyl)-2,6-cyclohexanedionyl]-*O*-ethyl propionaldehyde oxime (ATMP) (Figure 7.23) causes hepatic protoporphyria in the mouse, albeit not in other species, and this derangement of the porphyrin biosynthetic pathway appears to be linked to P450 inactivation via a heme modification mechanism[359]. A pigment tentatively identified as *N*-methylprotoporphyrin IX by HPLC analysis has been isolated from mice treated with ATMP, and its formation has been shown to be decreased by pretreatment with the P450 inhibitors SKF 525A and piperonyl butoxide, consistent with a P450-dependent process[359]. Replacement of the ethyl on the oxime carbon with a propyl group suppresses the porphyrinogenic activity of the analog, presumably by preventing heme alkylation. Nothing further is known about the mechanism by which ATMP mediates the P450-dependent formation of an *N*-alkyl (possibly *N*-methyl) protoporphyrin IX adduct.

Treatment of mice with griseofulvin (Figure 7.23), an agent long known to cause hepatic porphyrias, has been reported to cause the catalysis-dependent destruction of P450 and the formation of a green pigment[360]. In order to characterize the pigment, the *N*-alkyl group was transferred to an amine in a copper-mediated reaction and the alkyl amine was analyzed by mass spectrometry. The results suggested that *N*-methylprotoporphyrin IX was a minor product and a porphyrin with most of the griseofulvin structure bound to the nitrogen of pyrrole ring C of the heme was the major product[361–363]. The NMR spectrum of the latter adduct confirms that it is an *N*-alkylated porphyrin with the griseofulvin structure attached to the nitrogen of either pyrrole ring C or D[364]. The process appears to be species-specific because, at most, only very low levels of pigment have been observed with rats or chicken embryos[365–367]. It is difficult to postulate a mechanism for the formation of *N*-methyl heme from griseofulvin, particularly as an identical pigment is reportedly present in lower amounts in the livers of control mice[365, 366]. The presence of an endogenous *N*-alkylporphyrin in mice would clearly be of high interest, but more definitive evidence on its origin is required before the significance of these results can be evaluated. It is to be

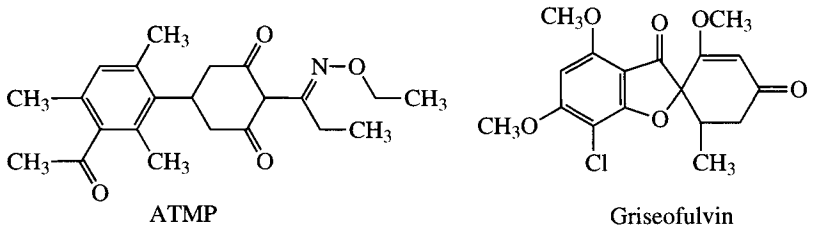


Figure 7.23. The structures of two compounds that cause P450 inactivation. In the case of griseofulvin, the inactivation appears to involve heme *N*-alkylation, but the detailed mechanism of inactivation by ATMP is not known.

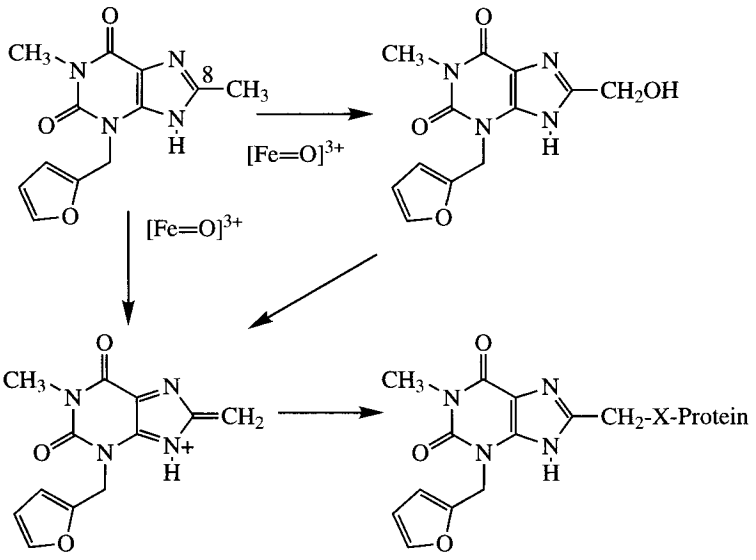


Figure 7.24. The oxidation of furafylline is proposed to yield a chemically reactive intermediate that binds to a protein residue (denoted by Protein-X).

noted that a mechanism is also not obvious for attachment of the griseofulvin structure to the porphyrin nitrogen via a mechanism-based process.

Furafylline (Figure 7.24), a potent selective inhibitor of human CYP1A2, causes time- and NADPH-dependent inactivation of this enzyme[368, 369]. CYP1A1, -2A6, -2B6, -2C9, -2C19, -2D6, -3A4, and -2E1 are not similarly inactivated, although the evidence suggests that other enzymes can be inhibited[369]. Clinical studies confirm that furafylline can almost completely suppress *in vivo* human CYP1A2 function[370, 371]. This loss of function is paralleled by a similar loss of the heme chromophore, with K_I = 23 μM and k_{inact} = 0.87 min^{-1}, and a partition ratio of 3–6 substrate molecules oxidized per enzyme molecule inactivated[368]. Oxidation of the C-8 methyl in the inactivation process is suggested by the fact that deuterium substitution on the C-8 methyl gives an isotope effect of ~2.0 on k_{inact} but not on K_I, and the observation that inactivation activity is suppressed when the methyl is removed[368].

Recent mechanistic studies confirm that the oxidation of the 8-methyl of furafylline yields the corresponding 8′-carbinols and/or results in covalent binding of the agent to the CYP1A2 protein (Figure 7.24)[372]. This result suggests that the oxidation of furafylline converts it to a two-electron oxidized electrophilic intermediate such as the exocyclic 8-methyleneimidazolenine or

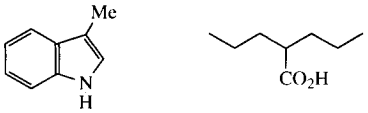
imidazomethide, and that this intermediate is trapped by a nucleophilic side chain within the CYP1A2 active site[372]. Consistent with this, neither GSH nor cyanide significantly impair either covalent binding or inactivation. Although the identity of the alkylated active-site residue remains undetermined, studies of furafylline docked in a homology model of the CYP1A2 active site suggest that the interactions of the furan moiety with the protein indeed favor oxidation of the 8-methyl group[372–374]. Ancillary evidence that the 8-methyl and not the furan moiety of furafylline is critical for CYP1A2 inactivation is provided by comparable findings (k_{inact} = 0.89 min^{-1}, partition ratio = 7.6, equivalent covalent binding to CYP1A2) with cyclohexylline, in which the furan is replaced by a cyclohexyl[372]. Supportive evidence for the proposal that the imidazole N^7-hydrogen is lost during CYP1A2 inactivation is provided by the inactivity of the corresponding N^7-methylated analogs as CYP1A2 inactivators[372].

Analogous P450-catalyzed dehydrogenations have been invoked in the mechanism-based inactivation of select P450 isoforms by the pulmonary toxin 3-methylindole[375], the anticonvulsant valproic acid (see Chapter 6)[376], and the leukotriene inhibitor zafirlukast (Figure 7.25)[377].

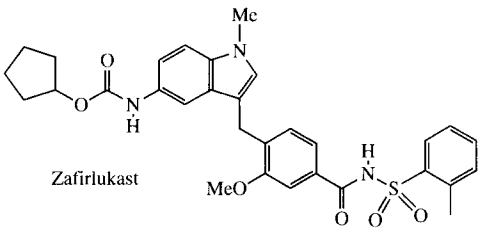
The mechanism of P450 inactivation is unclear for some classes of agents. As an example, CYP2E1 is inactivated by 3-amino-1,2,4-triazole in a time- and NADPH-dependent manner but the inactivation is not associated with covalent binding of the radiolabeled agent to the protein, the formation of P420, or loss of the heme[378]. Similarly, the inactivation of CYP3A4 by delavirdine (1-[3-[(1-methylethyl)amino]-2-pyridinyl]-4-[[5-[(methylsulfonyl)amino]-1H-indol-2-yl] carbonyl]-piperazine), a potent inhibitor of HIV-1 reverse transcriptase, also reportedly adheres to the criteria for a mechanism-based inactivation, but the chemical details of the process remain to be elucidated[379].

3.4. Modification of the P450 Protein by Heme Fragments

During the catalytic oxidation of some substrates, certain P450 enzymes (i.e. CYP3A, CYP2E) undergo a mechanism-based inactivation process in which fragments of the heme are irreversibly bound to the protein. Examples of such inactivators include CCl_4[380–382], spironolactone (see Figure 7.4)[67, 68], 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine (DDEP), and its 4-isopropyl and 4-isobutyl analogs (see also Figure 7.17)[383–385]. The features that predispose an enzyme to cross-linking of heme fragments to the protein remain unclear. Studies with the above substrates suggest that the generation of free radical products is important, but per se is not sufficient because not all the P450 enzymes that produce radicals undergo such inactivation. Thus,



3-Methylindole Valproic acid



Zafirlukast

Figure 7.25. Structures of three agents that cause irreversible inactivation of cytochrome P450.

during the one-electron oxidation of DDEP, CYP2C6 and -2C11 undergo heme *N*-ethylation, whereas the CYP3A enzymes predominantly incur cross-linking of heme fragments to the protein[293, 384]. Furthermore, studies with DDEP and its analogs with a secondary carbon attached to the 4-position (4-isopropyl and 4-isobutyl) reveal that the reaction outcome is largely dictated by the P450 active-site structure rather than by the inability of the inactivator to *N*-alkylate the heme[385]. Thus, DDEP, which can form *N*-ethyl porphyrins, and the 4-isopropyl and 4-isobutyl analogs that cannot, exhibit comparable extents of heme destruction and heme fragment cross-linking. Cross-linking of heme fragments to the protein is thus not merely the result of a defective or inefficient heme *N*-alkylation process. The fact that spironolactone inactivates hepatic CYP3A enzymes via heme fragment cross-linking[66, 67] but inactivates adrenal P450 enzymes by direct protein modification[70] further demonstrates that the binding of heme fragments to the protein is isoform-specific. Conceivably, the propensity of the CYP3A enzymes to undergo heme fragment cross-linking is related to their unusually large and, given their ability to accommodate large substrates such as cyclosporin, macrolide antibiotics, and FK506, as well as small substrates such as DDEP, highly flexible active sites. Their active sites may therefore exhibit an unusual degree of substrate mobility and/or water content (Chapter 10). Regardless of the mechanism, it is clear that CYP3A active sites are particularly susceptible to inactivation by heme fragment cross-linking. A related process is mediated by peroxides such as H_2O_2 and cumene hydroperoxide that partially degrade the prosthetic heme to soluble monopyrrole and dipyrrole fragments[383, 386–388]. In these reactions, the bulk of the heme is fragmented to products that irreversibly bind to the protein[382–391].

Myoglobin and hemoglobin have been employed as models in efforts to elucidate this unusual heme degradation process, but it now appears that these hemoproteins are not good models for the P450 reaction[392–395]. The H_2O_2-mediated oxidation of myoglobin results in the covalent binding of its heme via either the α or β-*meso* carbon or one of the vinyl groups to Tyr103[392]. In contrast, the reduction of CCl_4 or $CBrCl_3$ by myoglobin results in covalent attachment of the heme via one of its vinyl groups to His93[394, 395]. In both of these processes, as well as during the hemoglobin-mediated reduction of $CBrCl_3$[396] the cross-linked heme retains its Soret absorption maximum (at ~405 nm) and is thus bound to the protein without substantial structural disruption of its chromophore. Furthermore, γ-*meso* alkylated heme adducts without appreciable heme-protein cross-linking are observed during the myoglobin-mediated oxidative metabolism of alkylhydrazines[393]. In contrast, the cross-linking of heme fragments to protein observed in the P450 reactions with H_2O_2, cumene hydroperoxide, DDEP, and spironolactone involves complete loss of the heme chromophore and therefore major structural disruption of the heme skeleton[66, 383, 386–391]. This heme degradation also occurs if the cumene hydroperoxide-mediated inactivation is carried out under anaerobic conditions, albeit at a considerably slower rate, implying a role for molecular O_2 in this process[389, 390].

Attempts to elucidate this process have focused on cumene hydroperoxide-inactivated [^{14}C]-heme-labeled CYP3A23, -3A4, and -2B1[389, 390]. Proteolytic digestion with lysyl endopeptidase-C of the [^{14}C]-heme-modified P450enzymes, coupled with HPLC-mapping of the [^{14}C]-heme-modified peptides, Tricine-SDS-PAGE, electroblotting, microEdman degradation/amino acid sequencing, and electrospray ionization mass spectrometry (ESIMS), have located the specific sites modified by the heme fragments within the active sites of the P450 enzymes[389, 390]. Specifically, the labeled peptide in CYP3A23 encompasses residues 287–330, and in CYP2B1 residues 434–466. Sequence alignment of CYP3A23 and -2B1 with bacterial CYP101, -102, -107 and -108 reveal that the [^{14}C]-heme-fragment-modified peptide in CYP3A23 corresponds to the bacterial I-helix[160–163]. This domain contains the conserved Thr, which in the crystal structure of CYP101 is known to interact both with the substrate and the heme-bound O_2 and to be part of the active site (Chapter 3)[160–163]. On the other hand, the labeled CYP2B1 region corresponds to the bacterial L-helix that provides the conserved Cys thiolate ligand, and thus is also within the active site. However, until recently, the structure of the attached heme-derived fragments remained uncharacterized, largely because of their highly labile nature under the experimental conditions

required for isolation and structural analysis of the modified P450 peptides.

Optimization of the methodology combined with the larger peptide amounts available through the use of recombinant [^{14}C]-heme-labeled CYP3A4, enabled the structural characterization not only of the CYP3A4 peptide targets, but also of the protein modifying heme-fragments[390]. The combined structural analyses identified three major heme-modified CYP3A4 peptides comprised of residues 354–371, 372–386, and 429–450. Sequence alignments and homology modeling of CYP3A4 reveal that the 354–371 and 429–450 peptides correspond to the K-region and helix L/Cys region respectively, of P450. Several residues in these peptides are within 5 Å of the heme and thus within striking distance. Differential LC–ESMS analyses of the native and heme-modified peptide fragments provided molecular masses of ≈302, 314, and 197 for the heme-modifying species, corresponding to the deformylated and formylated A–D/B–C ring dipyrroles and the monopyrrole 2-formyl hematinic acid[390]. The precise amino acid residues modified in these peptides remain to be identified. Nevertheless, these findings suggest that the peroxidative inactivation of CYP3A4 (and presumably other P450 enzymes) involves rupture of its tetrapyrrolic skeleton along its α-γ and/or β–δ axes to yield reactive heme fragments that modify residues in their immediate proximity.

It is instructive that in this process, HCOOH (rather than CO) is the major product, and that together with minor amounts of CO and CO_2, it stoichiometrically accounts for the oxidative loss of two heme *meso*-carbons[386, 387, 397–399]. In contrast, peroxidative heme degradation in model systems yields considerably larger quantities of soluble dipyrrolic species [hydroxylated and nonhydroxylated propentdyopents and HCOOH] as major products[387, 397–400]. Approximately 15%–20% of the prosthetic heme loss after NADPH-induced oxidative uncoupling can be traced to soluble mono- and dipyrrolic products in incubations of purified CYP2B1[386]. However, this fraction drops to ~2.5% (comprised largely of hematinic acid, with traces of methylvinylmaleimide and propentdyopents) in CYP3A-enriched rat liver microsomal incubations with DDEP or cumene hydroperoxide[383]. Accordingly, in liver microsomal or purified P450 incubations, the bulk of the heme-derived species appear to irreversibly modify the CYP3A proteins.

The chemical nature of the heme fragment–protein adduct remains to be elucidated. In principle, it could entail a Schiff-base between a protein NH_2-group and the formyl group of a hydroxydipyrrolic fragment. Alternatively, it could involve attack by a suitable nucleophilic protein moiety on the 2-formylated dipyrrole. The possibility of a protein adduct with the heme vinyl also exists, even though no vinyl-modified dipyrrolic species have been detected in model heme degradation systems.

Both *in vivo* and *in vitro*, cross-linking of heme fragments to the CYP2E1 and -3A proteins targets them for proteasomal degradation by the 20*S* or ubiquitin-dependent 26*S* species[383, 401–405]. Low basal levels of microsomal heme fragment-cross-linked P450 proteins detected after [^{14}C]labeling of the P450 heme moiety *in vivo* indicate that P450 heme-modification probably occurs physiologically, possibly as a result of futile oxidative cycling of the enzymes. Furthermore, the extent of this cross-linking is increased considerably after CYP3A is induced by dexamethasone and phenobarbital[385, 406]. Since this endogenous post-translational modification targets the P450 proteins for proteolytic degradation, it could serve as a determinant of their normal physiological turnover. Not surprisingly, suppression of P450 futile oxidative cycling by blocking the P450 heme iron with TAO or isosafrole, or interrupting the electron flow through chemical or genetic impairment of P450 reductase, results in protein stabilization and consequent "induction" of CYP1A2, -2E1, and -3A[246–249].

3.5. Other Modes of P450 Heme Degradation and Protein Denaturation

The inactivation of CYP2B4 by aldehydes such as citral (an α,β unsaturated terpenoid aldehyde), and other aromatic aldehydes (cinnamaldehyde, benzaldehyde, and 3-phenylpropionaldehyde) is accompanied by bleaching of the heme chromophore that is not prevented by catalase, superoxide dismutase, epoxide hydrolase, GSH, or ascorbic acid[407, 408]. The corresponding k_{inact} values revealed that saturated aldehydes are generally

more inhibitory than their α,β unsaturated counterparts, and primary aldehydes are more potent inactivators than the structurally related secondary and tertiary aldehydes[408]. Studies with wild-type CYP2B4 and its T302A mutant, including measurements of the deuterium isotope effects, rates of inactivation, and rates of product formation suggest that aldehyde-mediated CYP2B4 inactivation involves deformylation of the aldehyde. In the inactivation of CYP2B4 by 3-phenylpropionaldehyde, a heme adduct is formed with a molecular weight equal to that of native heme plus 104 mass units, in agreement with loss of the carbonyl group from the original aldehyde. P450 inactivation by aldehydes has been proposed to involve homolytic cleavage of a peroxyhemiacetal intermediate to yield formic acid and an alkyl radical that adds to the heme moiety[408]. The heme adduct obtained in similar reactions of the F87G mutant of CYP108 (P450BM3) has been fully characterized by NMR and has been shown to involve addition of the decarbonylated substrate radical specifically to the γ-*meso* position of the heme group (Figure 7.26)[409]. The resulting heme-modified enzyme could be reduced in the presence of NADPH and lauric acid but was not able to actually oxidize the lauric acid.

Interestingly, incubation of CYP2B4 with artificial oxidants and aldehydes yielded different heme adducts. 3-Phenylpropionaldehyde yielded an adduct with a mass equal to that of native heme plus a phenylethyl group (Figure 7.26). The adduct was proposed to involve reaction of the hydroperoxy catalytic intermediate with the aldehyde to give a peroxyhemiacetal that fragmented to yield an alkyl radical[410]. In contrast, with *m*-chloroperbenzoic acid, 3-phenylpropionaldehyde yielded a phenylpropionyl-modified heme adduct purportedly generated from the reaction of the heme with the corresponding carbonyl radical (Figure 7.27). In this reaction, homolytic oxygen–oxygen bond cleavage of *m*-chloroperbenzoic acid itself also generated a chlorobenzoyloxy–heme adduct[410].

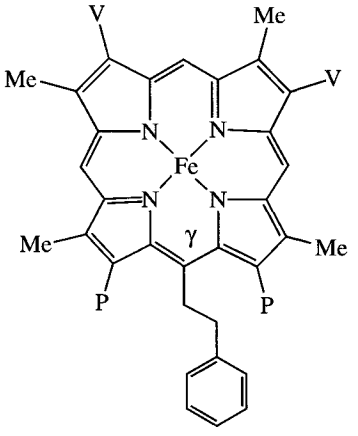


Figure 7.26. Structure of the heme adduct isolated from P450 inactivated by 3-phenylpropionaldehyde.

Similar studies with *trans*-4-hydroxy-2-nonenal (HNE, a cytotoxic byproduct of biological membrane lipid peroxidation), indicate that it is also metabolically activated by CYP2B1 and -2B4 to a reactive species that binds irreversibly to their prosthetic heme[411]. Unlike the mechanism-based inactivation by aromatic aldehydes, structural analyses of the corresponding heme adduct (MW 770) revealed that the reaction proceeds without deformylation and involves an acyl carbon radical that partitions between addition to the heme and formation of the carboxylic acid[411]. Together these findings suggest that the P450-mediated metabolic activation of aldehydes is a versatile process wherein the enzyme may be inactivated via mechanistically diverse heme modifications.

It is to be noted that P450 enzymes are sometimes inactivated by mechanisms that involve destruction of the prosthetic heme without the detectable formation of heme adducts. In some instances, these reactions result in binding of heme fragments to the protein (Section 3.4), but in most instances the incidence of heme-protein cross-linking has not been investigated. The destructive mechanisms of most peroxides[386–388], halocarbons (CCl_4)[380, 381], internal acetylenes (3-hexyne)[274], allenes (1,1-dimethylallene)[412], cyclopropylamines (*N*-methyl-*N*-benzylcyclopropylamine)[413, 414], benzothiadiazoles such as 5,6-dichloro-1,2,3-benzothiadiazole[415], and methyl thieno[3.2-*d*][1,2,3]-thidiazole-6-carboxylate[416], phenolic compounds such as the anti-inflammatory drug diclofenac[417, 418], rhapontigenin[419], and resveratrol[420], the HIV-1 reverse transcriptase inhibitor delavirdine[379, 421], and the D4 dopamine receptor antagonist SCH66712[422] (Figure 7.28) remain poorly characterized. Hypothetical mechanisms can be formulated

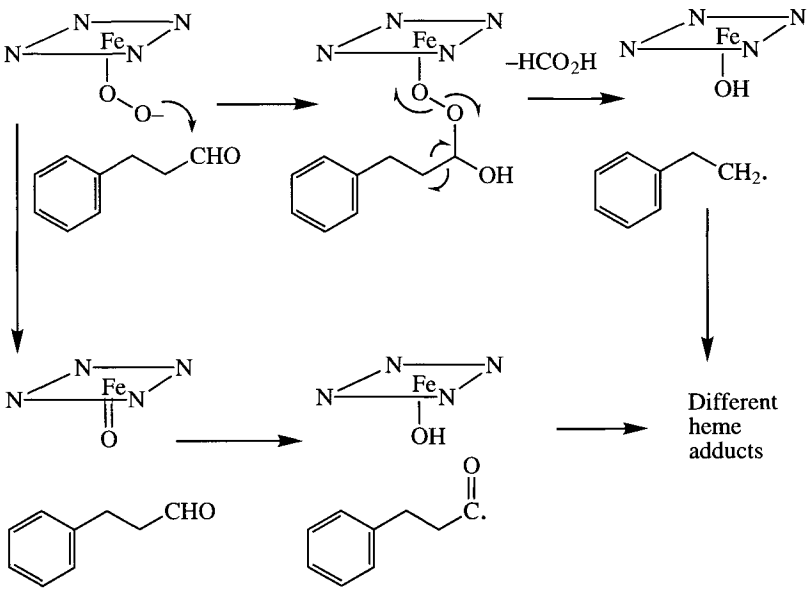


Figure 7.27. The inactivation of P450 enzymes by aldehydes appears to involve free radical intermediates, one of which retains the carbonyl group and one which does not. These radicals add to the heme group.

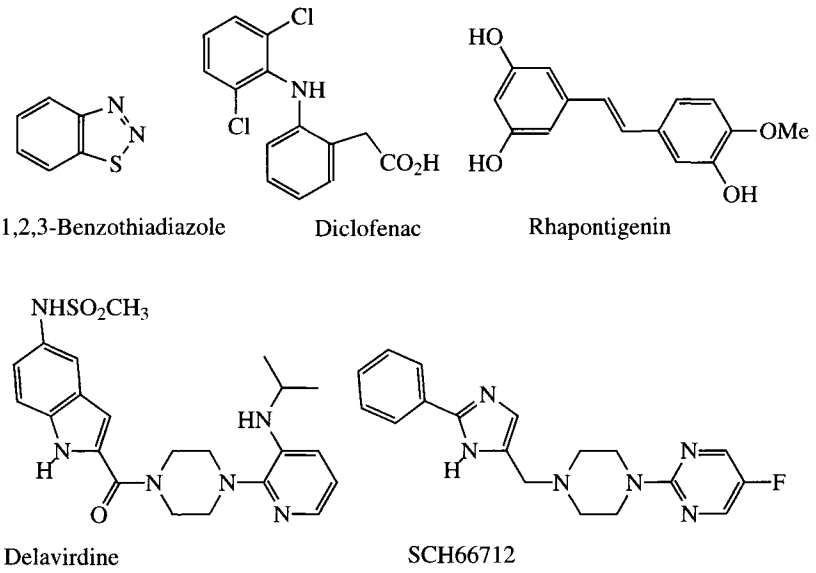


Figure 7.28. Structures of other classes of P450 mechanism-based inactivating agents.

for these reactions but experimental evidence to support the mechanisms is not available.

The P450 destruction mediated by halocarbons was once believed to stem from the secondary action of the lipid peroxides that are concomitantly formed, but it is now evident that substances like CCl_4 can destroy the heme group directly[380, 381, 423–426]. The associated cross-linking of heme fragments to the protein suggests that a radical species ($CCl_3^{\bullet}$ or $CCl_3O_2^{\bullet}$) may be responsible for heme destruction[381]. On the other hand, the catalytic reduction of halocarbons, including CCl_4, produces semistable complexes with Soret maxima in the 450–500 nm range[425–431]. Studies with model iron porphyrins, including the detailed characterization of a dichlorocarbene–metalloporphyrin complex[432], suggest that the long-wavelength Soret bands are due to ferrous halocarbene–heme complexes. This hypothesis is supported by the finding that carbon monoxide is formed in the reductive metabolism of CCl_4 by P450. This reaction is likely to occur by a mechanism similar to that for the generation of carbon monoxide from methylenedioxyphenyl complexes (Section 3.2.1)[433]. In fact, porphyrin dichlorocarbene–iron complexes do react with water to give carbon monoxide and with primary amines to give isonitriles[433, 434]. Studies with halothane suggest that it is also possible to form complexes in which the halocarbon is σ-bonded to ferric heme iron atom[435–437].

The links between formation of an iron–alkyl complex and irreversible destruction of the heme moiety have not been forged, but model studies with diaryl- and carbethoxy-substituted carbene complexes suggest that the halogenated carbenes may shift to form a bond with a nitrogen of the porphyrin[438–441]. The resulting *N*-haloalkyl adduct are likely to undergo water-dependent hydrolysis and might therefore not be detected by the methods used to isolate other *N*-alkyl porphyrins. However, the formation of alternative reactive species that attack the protein or the heme cannot be ruled out.

High (1–5 mM) concentrations of indomethacin and other nonsteroidal anti-inflammatory agents reportedly denature P450 enzymes because of their surfactant properties[442]. The loss of P450 content seen when indomethacin is added to liver microsomes is paralleled by essentially stoichiometric appearance of a P420 peak. Although it is likely that other agents cause P450 denaturation, it is unlikely that the process is physiologically relevant because of the high drug concentrations that are required.

4. P450 Enzyme Specificity

The isoform-specific inhibition of P450 enzymes is a promising avenue for the development of therapeutic, insecticidal, and herbicidal agents, as well as for investigation of the structures, mechanisms, and biological roles of individual P450 enzymes. The biosynthetic P450 enzymes have been the primary focus of efforts to develop isoform-specific P450 inhibitors because (a) they are better targets for specific inhibitors because of their high substrate specificity and (b) there is high practical utility for such inhibitors. In contrast, the broad, overlapping, specificities of xenobiotic metabolizing P450 isoforms makes the design of isoform-specific rather than -selective inhibitors more difficult[443, 444]. Selective inhibitors of P450 enzymes, as illustrated by the amphetamines[235, 240], TAO[236, 237,244–246], secobarbital[97, 157], gestodene[92], furafylline[368, 372], 1-ethynylpyrene[81, 83], and 2,3′,4,5′-tetramethoxystilbene[445] are fairly common (see Appendix). Caution is required in evaluating claims of inhibitor selectivity or specificity, as they are limited by the range of P450 enzymes actually examined. Only in the case where an inhibitor has been tested with all the known P450 isoforms in an organism can it be truly said to be specific, at least in that organism. The claim for specificity of inhibitors tested against only two or three isoforms is necessarily limited.

5. Inhibitors of Biosynthetic Enzymes

Several comprehensive review articles have discussed the potential clinical relevance and applications of inhibitors of biosynthetic P450 enzymes[5, 446–448]. The following discussion will therefore be limited to an illustration of the strategies employed in the design and development of the currently available and/or prospective inhibitors and their mechanistic diversity.

5.1. $P450_{scc}$

A single P450 enzyme ($P450_{scc}$, CYP11A) catalyzes the three oxidative steps required to cleave the side chain of cholesterol. A rational approach to the development of $P450_{scc}$ inhibitors was based on the incorporation of amino[449–452] and thiol[453] functions on the cholesterol side chain at positions that favor their coordination to the prosthetic heme iron (Figure 7.29), yielding potent reversible inhibitors (K_I = 25–700 nM). Thus, replacement of the first hydroxyl group catalytically inserted into the cholesterol side chain with an amine function yields (22*R*)-22-aminocholesterol, one of the most potent $P450_{scc}$ inhibitors[452]. The stereochemistry of this insertion is critical for inhibition since (22*S*)-22-aminocholesterol, binds to $P450_{scc}$ ~1,000 times more weakly (K_I = 13 μM) even though the amino function is located on the correct carbon.

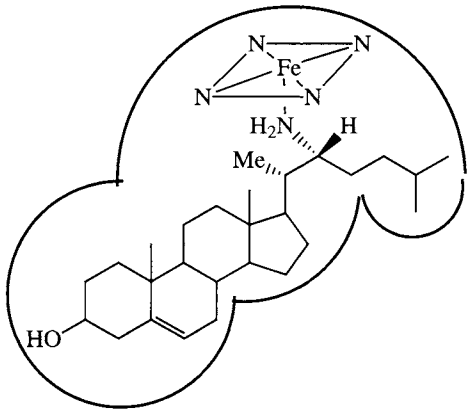


Figure 7.29. Reversible inhibitors of biosynthetic enzymes are usually constructed by incorporating a nitrogen or other coordinating atom into a lipophilic structure with high affinity for the protein active site (see Figure 7.1). An inhibitor of $P450_{scc}$ was thus obtained by placing an amino group at the position normally occupied by the first hydroxyl group added to the cholesterol side chain by $P450_{scc}$.

Various mechanism-based, irreversible inhibitors of $P450_{scc}$, such as analogs of pregnenediol with an acetylenic group grafted into their side chain, have been developed (Figure 7.30)[454–456]. Although this enzyme inactivation results in destruction of the heme chromophore, no alkylated heme was detected. Replacement of the side-chain carbons beyond C-23 in 20-hydroxycholesterol by a trimethylsilyl group also results in a $P450_{scc}$ mechanism-based inactivator (Figure 7.30)[457]. Model studies have demonstrated that 1-substituted 3-trimethylsilyl-1-propanol is oxidized by chemical reagents to ethylene, the trimethylsilyl radical, and an aldehyde[458]:

$$RCH(OH)CH_2CH_2Si(CH_3)_3 \rightarrow RCHO + CH_2{=}CH_2 + (CH_3)_3Si^{\bullet}$$

If the chemical model is relevant, $P450_{scc}$ may be inactivated by reaction of the enzyme with the trimethylsilyl radical or, less likely, from oxidation of the ethylene produced in the initial catalytic turnover. An interesting variant of a mechanism-based inhibitor is provided by (20*S*)-22-*nor*-22-thiacholesterol, in which the sulfur that replaces the carbon at position 22 is oxidized by $P450_{scc}$ to a sulfoxide that is a potent but not an irreversible inhibitor of the enzyme[457, 459, 460].

5.2. Aromatase

Aromatase, through a three-step catalytic transformation, controls the conversion of androgens to estrogens. Competitive and mechanism-based inhibitors of aromatase have been clinically exploited in the treatment of estrogen-dependent

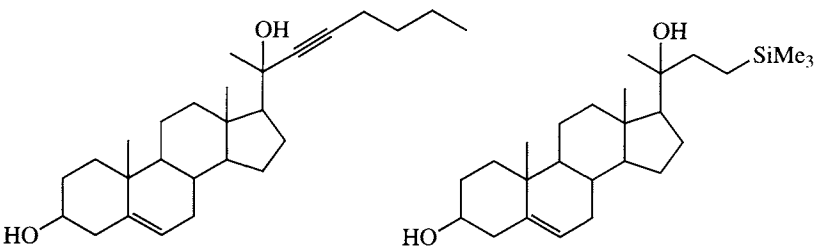


Figure 7.30. Two mechanism-based inhibitors of cytochrome $P450_{scc}$.

mammary tumors[5, 461–466] and benign prostatic hyperplasia[467, 468], and have some promise in the control of coronary heart disease[469]. Indeed, some of the more promising newer agents are in clinical trials[463–466]. Aminoglutethimide, an inhibitor of aromatase, has been used to treat hormone-dependent metastatic breast carcinoma, but its poor specificity and the incidence of side effects, primarily from inhibition of $P450_{scc}$, has compromised its utility[470–472]. Replacement of the aminophenyl group in aminoglutethimide (Figure 7.31) by a pyridine moiety affords [pyridoaminoglutethimide (3-ethyl-3-(4-pyridyl) piperidine-2,6-dione)], an agent that inhibits aromatase but not $P450_{scc}$[472, 473]. The enhanced specificity for aromatase may reflect a differential positioning of the pyridyl nitrogen within the two active sites that enables it to coordinate with the heme of aromatase but not of $P450_{scc}$. Interestingly, an aminoglutethimide analog with a nitrogen at the opposite end is a more potent $P450_{scc}$ inhibitor than glutethimide but has little or no activity against aromatase[473]. These findings suggest that the 19-methyl and carbon 22 of the sterol side chain are separated by a distance roughly equal to the length of the aminoglutethimide structure. It is therefore tempting to speculate that aminoglutethimide binds within the active sites of $P450_{scc}$ and $P450_{arom}$ in approximately the same orientation as the normal sterol substrate, and that the location of the nitrogen in the two inhibitors dictates their differential enzyme selectivity.

Potent inhibitors have also been developed that use an imidazole or related functions to coordinate to the aromatase iron atom. The most promising of these for the treatment of breast cancer are fadrozole, {4-(5,6,7,8-tetrahydroimidazo[1,5α]-pyridin-5-yl)benzonitrile monochloride} (CGS16949; Figure 7.31), and its congener [bis-(*p*-cyanophenyl)-imidazo-1-yl-methane hemisuccinate][474–478]. Both of these agents selectively inhibit aromatase rather than of $P450_{scc}$, P45021, or P45011B[475, 478]. Fadrozole does inhibit aldosterone production (18-hydroxylase activity) in rats, but this is much less pronounced with its congener[474]. Phase I clinical trials of fadrozole indicated that it is a potent inhibitor of estrogen biosynthesis in postmenopausal women with advanced breast cancer[474–478]. In addition to being well tolerated by patients, even at the maximally effective dose, it did not significantly alter cortisol, androstenedione, testosterone or aldosterone levels[474–478].

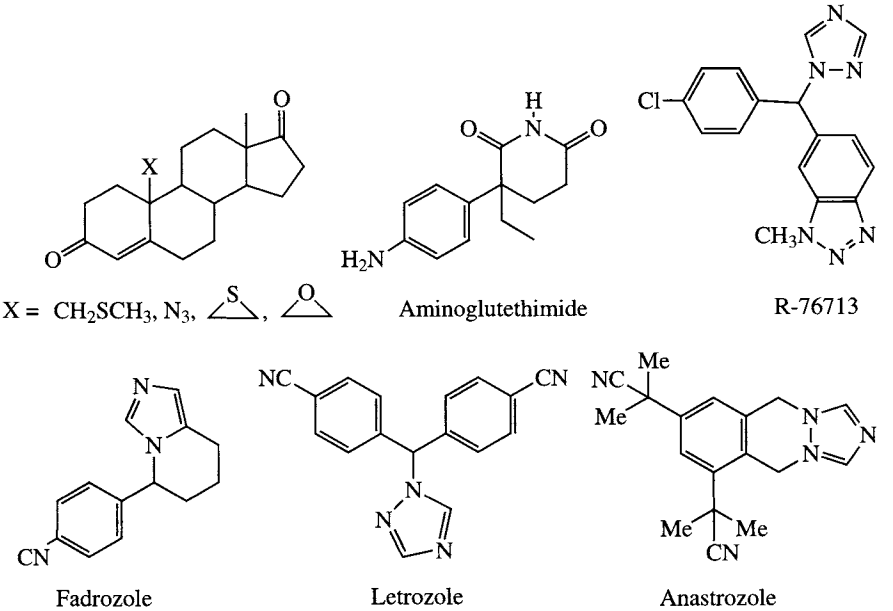


Figure 7.31. Structures of some competitive inhibitors of aromatase. In each of these compounds, a heteroatom is placed so that it can coordinate to the heme iron atom.

However, fadrozole may now be surpassed by letrozole (CGS20267 or Femara; Figure 7.31), an advanced nonsteroidal aromatase inhibitor, which appears to be more potent and effective than fadrozole in the treatment of postmenopausal women with advanced breast cancer[479, 480].

6[(4-Chlorophenyl)(1H-1,2,4-triazol-1-yl) methyl]-1-methyl-1H-benzotriazole, (R76713; Figure 7.31), is a relatively selective and very potent inhibitor of human placental aromatase[481–483]. Its (+) enantiomer has a lower IC_{50} value than the (−) enantiomer when assayed against human placental aromatase and exhibits no appreciable inhibition of other steroidogenic enzymes or liver microsomal P450 enzymes at concentrations up to 1,000-times the aromatase IC_{50}[482, 483].

Several other nonsteroidal compounds have been developed as novel and selective aromatase inhibitors, including 4-(4′-aminobenzyl)-2-oxazolidinones[484], 7-(alpha-azolylbenzyl)-1H-indoles and indolines of which 1-ethyl-7-[(imidazol-1-yl) (4-chlorophenyl)methyl]-1H-indole 12c exhibited the most promising potency[485], 4-imidazolylflavans[486], and anastrozole (Arimidex; Figure 7.31)[487]. Of these anastrozole has recently been approved in the United States and several other countries for the adjuvant treatment of postmenopausal women with hormone receptor-positive early breast cancer[488, 489].

Powerful competitive steroid inhibitors of aromatase have been synthesized by replacing the C-19 methyl with sulfur- or nitrogen-containing functions that can coordinate to the heme iron (e.g., Figure 7.31)[490–495]. The 19-methyl substituents include the following: CH_3SCH_2[490–492], $CH_3SCH_2CH_2$[493], $HSCH_2$[491, 494], $RSSCH_2$ (Org-30958, R = ethyl is best)[494], SH[491], NH_2, and NH_2CH_2[496]. Oxygen (oxiranyl), sulfur (thiiranyl), and nitrogen (aziridinyl) three-membered rings have also been used to replace the 19-methyl group (Figure 7.32)[495–501], the best of the resulting steroids exhibiting K_I values in the 1 nM range. The epoxide, episulfide, and aziridine functions inhibit the enzyme by stereoselective coordination of the heteroatom to the iron atom but, despite their reactivity, apparently do not inactivate the enzyme. However, the steroids in which the 19-methyl group is replaced by a sulfhydryl or $HSCH_2$ group (Figure 7.32) are irreversible mechanism-based inhibitors rather than simple competitive inhibitors[491].

The strategies developed for the inactivation of hepatic P450 enzymes have also been exploited

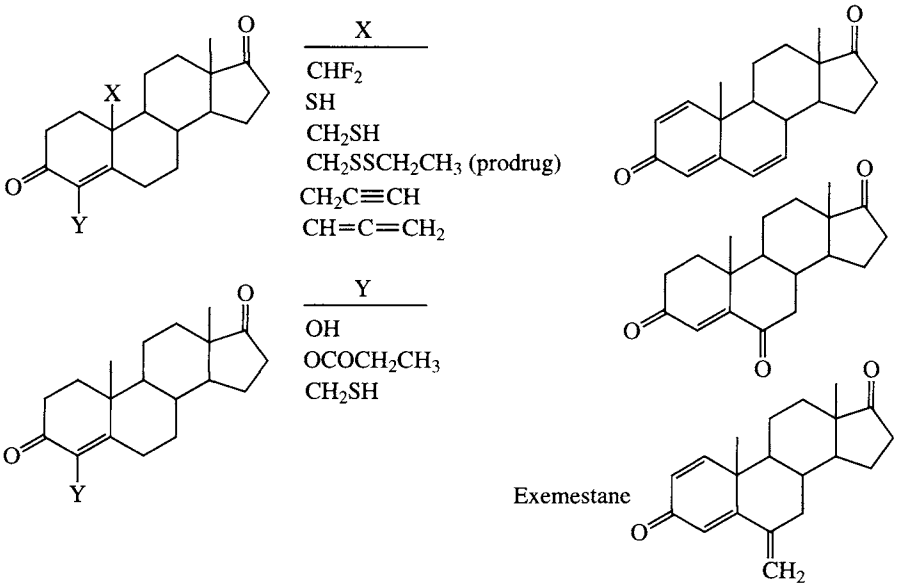


Figure 7.32. Structures of a selection of mechanism-based inactivators of aromatase.

in the design and synthesis of mechanism-based aromatase inactivators. Substitution of the normally hydroxylated methyl group (C-19) with a propargylic or allenic moiety (Figure 7.32) converts the sterol into an irreversible aromatase inhibitor[502–507]. The details of aromatase inactivation by these acetylenic and allenic agents remain unclear, but it is likely that they are activated to intermediates that react with either the heme or the protein (see Sections 3.1 and 3.3.2). Replacement of the C-19 methyl with a difluoromethyl also yields a mechanism-based inactivator that must be activated by C-19 hydroxylation (Figure 7.32)[506–511] as tritium release from the tritium-labeled difluoromethyl derivative is required for enzyme inactivation[512]. It is likely that the difluoromethylalcohol thus produced decomposes to the acyl fluoride that irreversibly binds to a protein nucleophile.

The 19-substituted analog of androst-4-ene-3, 17 dione steroid inhibitors, Org-30958 [19-(ethyldithio)androst-4-ene-3,17-dione], has been assessed in Phase I clinical trials for estrogen-dependent breast cancer chemotherapy[494]. The ethyldithio substitution apparently renders the steroid more stable extracellularly than the free thiol Org-30365 (19-mercapto-androst-4-ene-3, 17-dione), resulting *in vivo* in animal models in an 8-fold greater aromatase inhibitory activity than either 4-OHA or SH-489. Its *in vivo* potency requires intracellular reduction of the disulfide to release the 19-mercapto analog Org-30365, a more potent mechanism-based human placental aromatase inactivator[468] than 4-OHA or SH489[494].

Clinically effective mechanism-based aromatase inactivators can also be obtained by introducing substituents at the 4- or 6-positions of the sterol skeleton. 4-Acetoxy- and 4-hydroxy-4-androstene-3,17-dione (4-OHA) (Figure 7.32) irreversibly inactivate placental aromatase by catalysis-dependent mechanisms involving the 19-methyl[513, 514]. A possible mechanism for inhibition of aromatase by the 4-substituted analogs, as illustrated by 4-OHA, is shown in Figure 7.33. 4-OHA is used for the treatment of estrogen-dependent breast cancer[462, 515]. Of a series of $\Delta^{1,2}$, $\Delta^{4,5}$, and $\Delta^{6,7}$ analogs evaluated as prospective aromatase inhibitors in preclinical trials, FC 24928 (4-aminoandrostan-1,4,6-triene-3,17-dione) is the most promising candidate because it inactivates human placental aromatase activity as potently as 4-OHA and FCE-24304 (6-methylene-androstan-1,4,-diene-3,17-dione) but, unlike both these compounds, it has little intrinsic androgenic activity and does not affect 5α-reductase or $P450_{scc}$[516–518].

Conjugation of the 4-hydroxyandrostene nucleus as in 1,4,6 androstatriene-3,17-dione (ATD), conveys aromatase inhibitory and marked tumor regression activities (~80%)[519, 520]. On the other hand, the introduction of a C_1-methyl into 1,4-androstadiene-3,17-dione as in Atamestane (1-methylandrosta-1,4-diene-3,17-dione, SH-489), apparently enhances its affinity (K_I ~2 nM vs K_I of 29 nM for 4-OHA) for the human placental aromatase while slowing its inactivation of the enzyme, thereby reducing the production of estrogenic products[518, 521]. The compound along with its 1,2 methylene-substituted congeners has been evaluated in Phase I clinical trials for possible therapy of estrogen-dependent conditions such as breast cancer and benign prostatic hypertrophy. Additional steroidal agents explored for their aromatase suicide inactivation include androst-5-ene-7,17-dione and its 19-hydroxy derivative[522].

Turnover-dependent irreversible inactivation of the enzyme via protein modification is also achieved by introducing a 6-keto group into the steroid skeleton (Figure 7.33)[523–525]. Monitoring the $^3H:^{14}C$ ratio in studies with the C-19 double-labeled inhibitor indicates that the C-19 methyl, one of the C-19 hydrogens and, from a separate double label experiment, the 1β-hydrogen, are retained in the covalently bound species[525]. These findings do not define the underlying inactivation mechanism but appear to exclude the involvement of C-19 demethylation and aromatization, although normal aromatization is possible because 6-oxoestrone and 6-oxoestradiol are concurrently formed.

Exemestane, 6-methylene-androsta-1,4-diene-3,17-dione (Figure 7.32), is an aromatase inhibitor with an IC_{50} for inhibition of human placental aromatase comparable to that of 4-OHA[516, 517]. The K_I (nM) and $t_{1/2}$ (min) values for the inactivation processes were 26 ± 1.4 and 29.0 ± 7.5, and 13.9 ± 0.7 and 2.1 ± 0.2, for Exemestane and 4-OHA, respectively. In spite of its relatively slow inactivation of aromatase, Exemestane is a more potent agent in experimental animals[516], and also much more effectively causes regression of

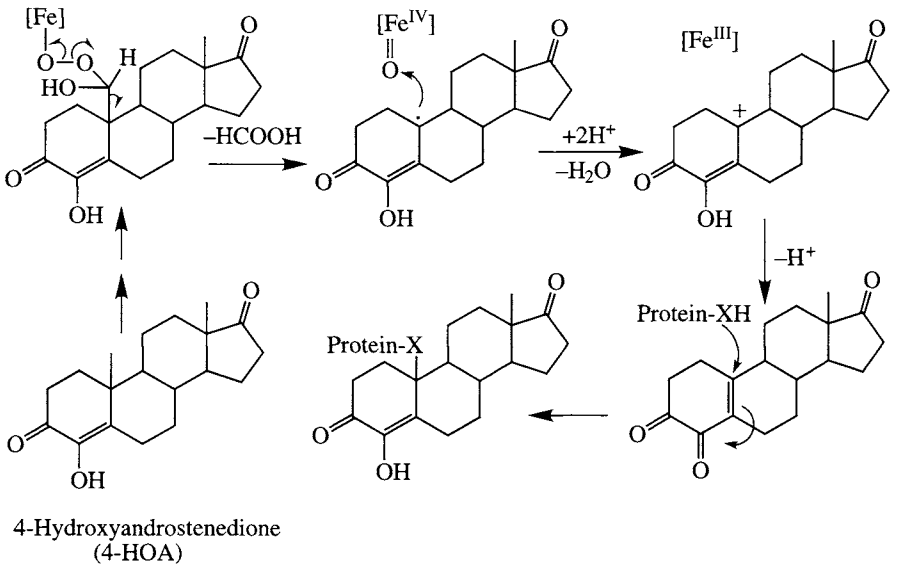


Figure 7.33. A possible mechanism for the inactivation of aromatase by 4-OHA.

chemically induced mammary tumors in rat than MDL18962 or SH-489[518]. Because of its oral effectiveness and potent irreversible aromatase inhibition, Exemestane has been recently approved and introduced into the global market under the name Aromasin[526–531].

MDL18,962 10-(2-propynyl)estr-4-ene-3,17-dione is one of the most potent of the mechanism-based inactivators of aromatase, with K_I values of the order of 3–4 nM, and $t_{1/2}$ of 9 and 6 min, respectively, for the human and baboon placental aromatases[503, 504, 506, 508, 532]. Phase I clinical trials and preclinical findings indicate that MDL18,962 is indeed effective in lowering estrogen levels[533].

The time-dependent inactivation of aromatase by 10-hydroperoxy-4-estrene-3,17-dione, reportedly a mechanism-based inactivator of aromatase[534, 535], is inhibited by NADPH or an alternative substrate and is partially reversed by dithiothreitol. Other high affinity 10-substituted analogs, such as the mechanism-based inhibitor 10-β-mercaptoestr-4-ene-3,17-dione, and the competitive inhibitors (19*R*)-10-oxiranyl and 10-thiiranyl-estr-4-ene-3,17-diones and 10-β,-$MeSCH_2$-estr-4-ene-3,17-dione, have been reported[491, 492, 494, 536, 537]. C-10, C-2 hydroxyethyl bridged steroids synthesized as stable carbon analogs of the 2β-hydroxylated 19-oxoandrostenedione, a putative intermediate in the aromatization reaction, have been found to be potent competitive inhibitors of the human placental aromatase[538]. In contrast, related halohydrin analogs are potent mechanism-based aromatase inhibitors[538].

Finally, 4β,5β-epoxyandrostenedione as well as its 19-hydroxy and 19-oxo derivatives have been examined as aromatase inhibitors. The epoxides were found to be weak competitive inhibitors, whereas the 19-hydroxy and 19-oxo derivatives were largely ineffective[539].

5.3. Lanosterol 14-Demethylation

The 14-demethylation of lanosterol is a key step in the biosynthesis of cholesterol. The preferential inhibition of the P450 enzyme that catalyzes this reaction by a number of substituted imidazoles, pyridines, pyrimidines, and other lipophilic heterocycles has been exploited in the construction of clinically important antifungal agents[540–542]. The antifungal action following the inhibition of 14-demethylase is thought to result from the accumulation of 14-methyl sterols in the membranes of susceptible fungi that cause deleterious changes in their membrane permeability[543–545].

Miconazole (Figure 7.34), fluconazole (Figure 7.34), ketoconazole (Figure 7.1), and

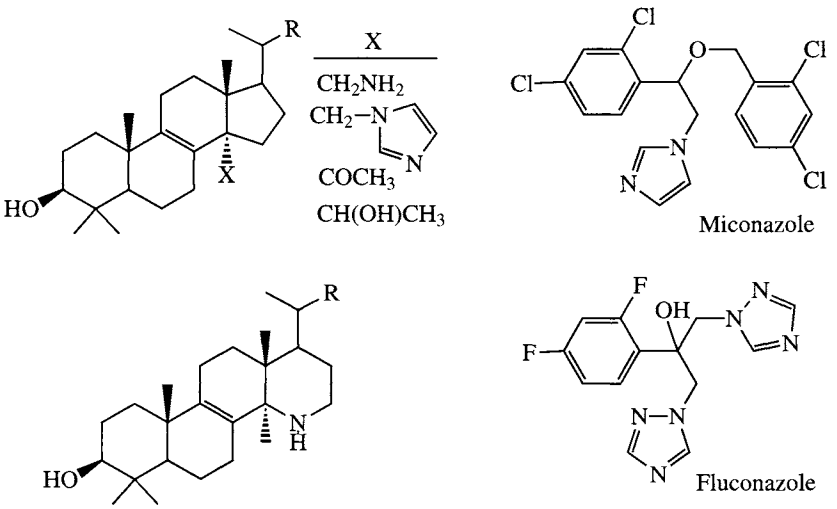


Figure 7.34. Competitive inhibitors of lanosterol 14-demethylase. The group R on the sterols is either $C(CH_3)CH_2CH_2CH_2C(CH_3)_2$ or a close variant. Fluconazole and miconazole are clinically employed as antifungal agents.

related azole structures inhibit the fungal 14-demethylase activity at extremely low (nM) concentrations[546, 547], but only inhibit the 14-demethylase activity of the mammalian host at higher (up to 100 μM) concentrations[548, 549]. Low doses of ketoconazole, however, appear to inhibit not only the C_{17-20} lyase, but also human hepatic CYP3A4, and thus this agent is not very clinically useful[550, 551]. As expected, the substituted imidazoles and other nitrogen-based heterocyclic antifungal agents coordinate with the P450 heme iron to yield typical type II binding spectra[552].

Potent isoprenoid-containing imidazole antifungal agents, such as AFK-108 (1-[2-(2,4-dichlorophenyl)-2-((2*E*)-3,7-dimethylocta-2,6-dienyloxy) ethyl]-1H-imidazole), have also been developed whose geranyl moiety specifically interacts with the sterol side-chain recognition region within the 14α-demethylase active site. Unfortunately, this compound and its farnesyl derivative inhibited not only the rat liver counterpart but also other hepatic drug metabolizing P450 enzymes, placing their practical utility in question[553].

Structural considerations have also prompted the development of 14α-methyl-15-aza-D-homosterols (Figure 7.34), which exhibit relatively high *in vitro* fungistatic potencies and high fungicidal activities as well as respectable *in vivo* efficacies in a murine model of candidiasis[554]. Introduction of an oxime functionality (=NOH) at position 15 of the sterol group, also results in an inhibitor with a very similar geometry of the nitrogen as that in the 15-azahomosterols[555]. Similarly, 7-oxo-24,25-dihydrolanosterols, which uncouple the catalytic turnover of CYP51 by competitively blocking transfer of a second electron to the oxyferrocomplex, have also been considered as prospective inhibitors[556]. Furthermore, 15-, 32-, and 15,21-oxylanosterol analogs have been examined as mechanism-based inactivators of lanosterol demethylase. Of these (32*S*)-vinyllanost-8-en-3β,32-diol is a potent time-dependent inactivator ($k_{inact}/K_I = 0.36\ min^{-1}\ \mu M^{-1}$), while the (32*R*)-vinyllanost-8-en-3β,32-diol functions solely as a competitive demethylase inhibitor[557]. Replacement of the 15-hydrogen normally lost during catalysis by a fluorine yields 15α-fluorolanost-7-en-3β-ol which is oxidized by the enzyme to the 14-aldehyde but proceeds no further due to the fluorine substitution[558]. The compound is therefore a "prodrug" inhibitor that requires metabolic activation of its latent form rather than a true mechanism-based inactivating agent.

Steroid analogs with amino and imidazole functions attached to the 14-methyl carbon have

been developed as inhibitors of lanosterol 14α-demethylation (Figure 7.34)[559]. Steroids with a 14-(1-hydroxyethyl), 14-(1-oxoethyl), or 14-carboxyl group are competitive inhibitors of the enzyme (Figure 7.34)[560–562]. Lanosterol analogs in which the 14α-methyl group has been replaced by a vinyl, ethynyl, allyl, propargyl, 1-hydroxypropargyl, 1-ketopropargyl, difluoromethyl, epoxide, or episulfide moiety have been synthesized as potential mechanism-based inhibitors of lanosterol 14α-demethylase[562–565]. Although the ethynyl sterols are clearly mechanism-based inactivators of the enzyme[565], most of these compounds act as simple competitive inhibitors (e.g., the epoxide and episulfide analogs). Finally, because of their cholesterol lowering potential, 14α-demethylase inhibitors have also been considered as hypolipidemic agents[566].

5.4. Other Biosynthetic Sterol Hydroxylases

A current strategy for the development of new drugs for the treatment of prostatic cancer is the development of inhibitors of CYP17-dependent androgen biosynthesis. Thus, as in the case of other steroidogenic enzymes, an intense search has been undertaken for reversible and irreversible CYP17 inhibitors[567]. The resulting agents include substituted imidazoles, pyridines, pyrimidines, and other lipophilic heterocycles as the enabling moiety[567–581]. Of these, abiraterone (17-(3-pyridyl)androsta-5,16-dien-3β-ol), a potent inhibitor (IC_{50}, 4 nM) of the hydroxylase activity of human cytochrome CYP17, has been employed clinically[571]. Its potency is apparently due to activation of the 16,17-double bond that is required for irreversible binding of these pyridyl steroids to CYP17[571]. Analogs such as [17-(5-pyrimidyl)androsta-5,16-diene-3β-ol] and its 3-acetyl derivative, which are even more potent CYP17 inhibitors in rats than abiraterone, are particularly promising[580]. With testicular microsomal CYP17, these compounds apparently exhibit the dual characteristics of a type II binding spectrum and noncompetitive inhibition[581].

Another mechanism-based inactivator of both the 17-hydroxylase and C-17/C-20 lyase activities of CYP17 is 17β-(cyclopropylamino)-androst-5-en-3β-ol[582]. This compound reportedly does not inhibit $P450_{scc}$ or the sterol 21-hydroxylase. In contrast, sterols with a 17-difluoromethyl group selectively inactivate the C-21 hydroxylase, whereas sterols with a 17-dichloromethyl, -vinyl, or -ethynyl function inactivate both the C17- and C21-hydroxylases[86]. More recently, 20-fluoro-17(20)-pregnenolone derivatives were designed as enol mimics of pregnenolone. All of the targeted, novel fluoroolefins were found to be potent inhibitors of C-17(20) lyase[579].

Similar strategies have been used to develop inhibitors that target the C-18 hydroxylation involved in the biosynthesis of aldosterone[583–586]. Thus, aldosterone analogs with C-18 iodomethyl, chloromethyl, allyl, propargyl, vinyl, and methylthiomethyl functionalities have been synthesized and some have been found to irreversibly inactivate the enzyme. An active site-directed 18-acetylenic deoxycorticosterone [21-hydroxy-13(-2-)propynyl)-18-nor-preg-4-ene-3,20-dione, MDL19,347] is a promising inactivator of the rat and rhesus monkey adrenal corticosterone 18-hydroxylase ($K_I \approx 38$ nM; $t_{1/2}$, 4.6 min) that also effectively reduces plasma aldosterone levels *in vivo*[586]. This compound appears to be a relatively selective inhibitor of aldosterone biosynthesis, as it fails to inhibit the 11β-hydroxylation of corticosterone and DOC[586]. Rationally developed inhibitors such as these may be useful in the management of conditions such as hypertension, hypokalemia, and edema that are associated with hyperaldosteronism.

5.5. Fatty Acid and Leukotriene Monooxygenases

Microsomal P450 enzymes of the CYP2C and 4A subfamilies in tissues such as the liver, lung, kidney, peripheral vasculature, and polymorphonuclear leukocyte oxidize a variety of fatty acids, including arachidonic acid and its derivatives, to physiologically active metabolites. Some of these metabolites are well recognized as biologically important regulators of renal, pulmonary, and cardiac function and vascular tone[587–595]. Two general classes of such vasoactive metabolites are known to be produced in vascular and extravascular tissues: EETs generated by CYP2C epoxygenases, and HETEs products hydroxylated at the ω- and ω-1 positions

by CYP4A[590–596]. Furthermore, with the exception of 20-HETE, all the other arachidonic acid products occur as stereo- and regioisomers that vary considerably in their biological activities and potencies[592, 593]. Thus, 5,6-, 8,9-, 11,12-, and 14,15-EETs are potent vasodilators, specially in various capillary beds, that act through activation of K^+-channels in vascular smooth muscle cells. In contrast, 12(*R*)- and 20-HETEs are potent vasoconstrictors. 20-HETE is a particularly potent cerebral and renal microvessel vasoconstrictor, as well as a mediator of other important physiological processes (reviewed in Chapter 11). The altered production of EETs and 20-HETE in various genetic and experimental models of pathological diseases has led to their consideration as plausible causative factors. Thus, not surprisingly, the P450 enzymes responsible for their biosynthesis have been singled out as key targets not only for defining the pathological roles of these metabolites, but also as possible sites for pharmacological intervention in the treatment of these conditions[592–598]. In this context, both the previously existing and new agents have been exploited as reversible or irreversible inhibitors of these enzymes.

Accordingly, two reversible inhibitors of CYP4A enzymes have been assessed as probes of 20-HETE involvement in various physiological processes with varying degrees of success: the not so selective antifungal agent miconazole (Section 2.3)[599–601], and the rationally designed and thus more selective, *N*-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS; Figure 7.35) and its acid analog, 12,12-dibromo-dodec-11-enoic (DBDD)[602–608]. DDMS has been found to be an effective acute and chronic blocker of 20-HETE formation *in vivo* in rats[602–604]. More recently an even more potent and selective inhibitor of 20-HETE formation, HET0016 (*N*-hydroxy-*N'*-(4-butyl-2-methylphenyl)formamidine) (Figure 7.35) has been discovered through combinatorial chemistry, with an IC_{50} of 35 ± 4 nM[608]. Because of its high selectivity, it holds considerable promise as a probe of 20-HETE dependent function.

Several mechanism-based irreversible inhibitors of P450-dependent arachidonic acid metabolism have also been developed. For instance, in 11-dodecynoic acid, the terminal acetylenic analog of lauric acid, and 10-undecynoic acid have been shown to inactivate hepatic CYP4A lauric acid ω-hydroxylases while minimally altering the spectrophotometrically detectable hepatic P450 content or function[78, 609, 610].

The high susceptibility of these acetylenic fatty acid inactivators to β-oxidation, however, compromises their *in vivo* utility and prompted the development of 10-undecynyl sulfate (10-SUYS), the acetylenic analog of 10-undecynoic acid in which the carboxyl group is replaced by a sulfate (Figure 7.35)[610]. The sulfate is not susceptible to β-oxidation and thus prolongs the biological lifetime, reduces the toxicity, and enables the use of sulfate agent as an *in vivo* mechanism-based

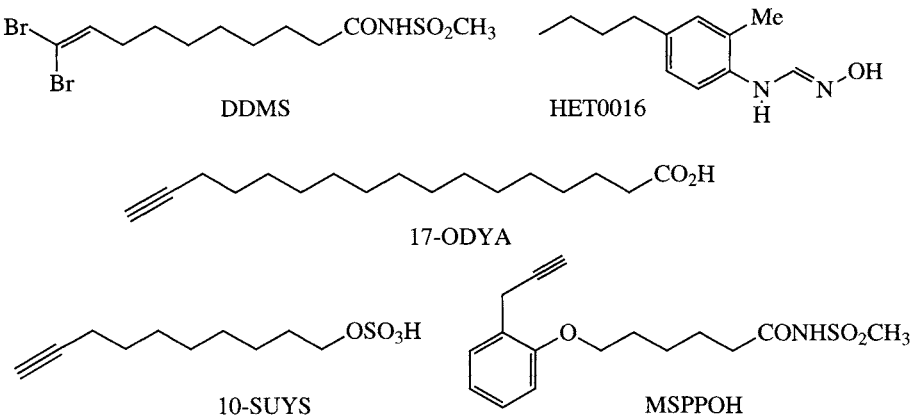


Figure 7.35. Reversible and mechanism-based inhibitors of enzymes involved in the metabolism of fatty acids and related endogenous substrates.

CYP4A inactivator[610]. Administration of a single dose of 10-SUYS, a potent and selective mechanism-based CYP4A inactivator, acutely reduced the mean arterial blood pressure as well as the urinary 20-HETE excretion in spontaneously hypertensive rats, consistent with the inactivation of renal 20-HETE formation[611]. These findings thus suggest that 20-HETE could play an important role in blood pressure regulation in hypertensive states and that the inhibition of its synthesis in these conditions may be of therapeutic benefit[611].

CYP4A and CYP2C isoforms are also inactivated by the acetylenic fatty acid analog, 17-octadecynoic acid (17-ODYA; Figure 7.35), in a mechanism-based process[612–614]. 17-ODYA has been particularly useful *in vivo* to probe the involvement of these metabolites in physiological and/or pathological processes[597, 598, 603, 605–609, 612–618]. Its use has helped elucidate the specific role of 20-HETE in the myogenic activation and hypoxic dilation of skeletal muscle resistance arteries as well as the vasodilatory effects of NO in the renal microcirculation[603, 606, 607, 618].

The acetylenic fatty acids 15-hexadecynoic (15-HDYA) and 17-ODYA have also been explored as modulators of leukotriene B_4 (LTB_4), an important and clinically relevant inflammatory mediator, and its physiologically active ω-hydroxylated metabolite[619–622]. Both 15-HDYA and 17-ODYA inactivated the polymorphonuclear leukocytic LTB_4 ω-hydroxylase in whole cells and cell lysates[622]. In contrast, the shorter-chain acid, 10-undecynoic acid was much less effective, while the saturated analogs of 15-HDYA and 17-ODYA were inactive. 15-HDYA and 17-ODYA also inactivate pulmonary prostaglandin ω-hydroxylases[623].

The mechanism-based inactivator 1-ABT (Section 3.3.5), which is not very selective and inactivates multiple P450 enzymes, including those responsible for the synthesis of both EETs and 20-HETE, has also been used, alone or in conjunction with other inhibitors, to assess the role of these metabolites in skeletal muscle angiogenesis induced by electrical stimulation as well as in the renal and vasoconstrictor actions of angiotensin II[348, 602, 608, 624].

Mechanism-based inhibitors of EET formation have also been used as probes of the physiological roles of these metabolites. One such agent is *N*-methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide (MSPPOH; Figure 7.35)[605, 625–627].

Finally, since fatty acid hydroxylations at the ω, ω-1, ω-2, ω-3, and ω-4 positions also occur in plants and bacteria[628, 629], acetylenic fatty acids have also been employed to examine whether these hydroxylases are mechanistically similar to their mammalian counterparts and/or to identify the role of specific plant and bacterial P450 enzymes[629, 630]. Thus, midchain and terminal acetylenes such as 10-dodecynoic acid have been used as probes of plant lauric acid ω−hydroxylases[84, 629]. Similarly, 17-ODYA has been shown to inactivate $P450_{BM-3}$ (CYP108), an enzyme that hydroxylates fatty acids at positions other than the terminal (ω) carbon, through a heme alkylation mechanism[630].

6. Summary

Our knowledge of the structure and function of P450 enzymes has greatly expanded over the past three decades, as has our appreciation of the chemical functionalities that they accept as substrates and of the moieties that can inhibit or terminate their catalytic action. As a result, multiple routes are now available for the inhibition or inactivation of P450 enzymes that target either the protein and/or the heme. This knowledge has been exploited in the design and construction of both reversible and irreversible inhibitors of increasing potency, specificity, and potential practical utility. One area of continuing growth is that of reversible inhibitors that, by simultaneously conforming to the predominantly lipophilic contours of the P450 active site and providing a nitrogen that coordinates to the heme iron atom, have achieved enhanced potency and specificity. These agents include the clinically relevant azole antifungal and cancer chemotherapeutic drugs (Section 5).

The high promise of irreversible P450 inactivating agents has not yet culminated in agents of large-scale practical utility. Progress in the field has continued to provide instruction on approaches for the design of mechanism-based inhibitors that inactivate a P450 enzyme with high specificity and negligible release of reactive metabolites into the medium, whether the inactivation involves modification of the protein or the

heme. High specificity is provided by the requirement not only for binding within the P450 active site but also for the more rigorous test of compatibility with catalytic turnover by the target enzyme. Potency results from the irreversible nature of the inhibition, which means that relatively high concentrations of the agent need not be maintained throughout the period of inhibition. However, the involvement of reactive intermediates such as radicals and ketenes in the inactivation requires that the inactivation process be highly efficient and occur without leakage of reactive functions into the medium. As our increased understanding of mechanism-based inhibition makes this feasible, this remains an attractive and highly promising approach to novel therapeutic agents and other commercial applications.

On the negative side, the inhibition of P450 enzymes has increasingly been recognized as a key locus of clinically relevant drug–drug interactions and other pharmacological effects. One needs only to consider the consequences of the lethal inhibition of CYP3A4 in patients treated with agents like terfenadine to appreciate the general concern in the pharmaceutical industry with the subject of P450 inhibition. Our growing understanding of the structural and chemical basis of P450 inhibition should greatly improve our ability to rapidly elucidate their nature when and if they do occur and, of course, to avoid their occurrence in the later stages of drug design.

Finally, on a more heuristic level, the past few years have led to the development of an increasing repertoire of isoform-selective inhibitors that can be deployed in basic studies of the physiological function of individual P450 enzymes. One area to which such inhibitors have contributed is to elucidation of the specific roles of the products of P450-catalyzed arachidonic acid oxidation. A toolbox of highly isoform-specific inhibitors covering the full range of human and rodent P450 enzymes, each with pharmacokinetic properties and a low toxicological profile that makes it suitable for *in vivo* use, would be a panacea but at this time is nothing more than a possibility.

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8

Induction of Cytochrome P450 Enzymes

Susanne N. Williams, Elizabeth Dunham, and Christopher A. Bradfield

1. Introduction

1.1. Cytochrome P450 Enzymes and the Adaptive Response

Organisms are constantly exposed to an ever-changing spectrum of foreign chemicals or xenobiotics. In response to this challenge, adaptive mechanisms have evolved in higher eukaryotes that allow them to detect an insulting agent and accordingly increase its metabolism and clearance. Commonly, the front line in this metabolic defense is the cytochrome P450 monooxygenases (CYPs). These enzymes catalyze the first step in the metabolism of lipophilic xenobiotics to more water-soluble compounds that can be readily excreted. A common feature of the CYPs is that exposure to a xenobiotic substrate often results in increased expression of the CYP enzyme(s) capable of its metabolism. This adaptive response, known as induction, is a tightly regulated process that is controlled primarily at the level of transcription. Regulating the expression of CYPs in a manner that is sensitive to xenobiotic exposure allows the cell to increase the levels of the necessary CYP enzymes only as needed to facilitate elimination of a toxicant.

1.2. Overview of Nuclear Receptors

The adaptive response to xenobiotics is orchestrated in the cell by a subset of receptors that act primarily in the nucleus. For this chapter, we will employ a liberal definition of nuclear receptor (NR) that includes all signaling molecules that function as ligand-binding transcription factors that bind to specific DNA enhancer sequences and upregulate the transcription of CYP genes. Two classes of NRs will be discussed in this chapter. Members of the nuclear hormone receptor (NHR) superfamily to be reviewed include the constitutive androstane receptor (CAR), the pregnane X-receptor (PXR), and the peroxisome proliferator activated receptors (PPARs). A single member of the PAS superfamily, the aryl hydrocarbon receptor (AHR), will also be discussed.

The NHRs have a modular structure characterized by an N-terminal ligand-independent AF-1 transactivation domain (TAD), a highly conserved DNA binding domain (DBD) containing two zinc finger motifs, and a ligand binding domain (LBD) that contains a ligand-dependent AF-2 TAD in its C-terminal portion[1]. The CAR and the PXR play major roles in the induction of the CYP2 and CYP3

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genes, while PPARα mediates the upregulation of the CYP4 family[2]. The PXR, CAR, and PPARα are similar in that they form heterodimers with the retinoid X receptor (RXR) to bind response elements that contain two copies of the core sequence AG(G/T)TCA arranged as everted repeats (ER) or direct repeats (DR) separated by varying numbers of nucleotide spacers[2]. These response elements are commonly designated by the type of repeat, followed by the nucleotide spacer number; for example, a direct repeat separated by four nucleotides is termed "DR4." The binding specificity and the transactivation potential of different NHR/RXR heterodimers can be determined by the spacer number, the nucleotide sequence in the half-sites, and often by sequences 5′ of the half-sites[1].

The nuclear receptor known as the aryl hydrocarbon receptor (AHR) is a member of the PAS superfamily of proteins and regulates the CYP1 genes[3, 4]. The PAS domain was named for the first three proteins in which it was recognized: *P*ER, *A*RNT, and *S*IM[5]. Most members of the PAS superfamily, including the AHR, have an N-terminal basic helix-loop-helix (bHLH) domain adjacent to the PAS domain and a carboxy terminal domain that influences transcription[6]. The AHR binds to specific response elements upstream of the CYP1 genes containing the core sequence TNGCGTG to induce gene expression.

A common characteristic of NRs is that they interact with coactivators and corepressors. In general, in the absence of ligand or when bound by antagonist, NRs exist in a complex with corepressors, such as SMRT or NcoR, which inhibit transcriptional activity through recruitment of histone deacetylases or other mechanisms[7, 8]. Activation of the NR by agonist binding or phosphorylation causes a conformational change in the receptor that results in the dissociation of corepressors and the recruitment of coactivators, such as SRC-1 and CBP, which interact with NRs through conserved LXXLL motifs[7–9]. Coactivators can increase the rate of gene transcription through chromatin remodeling or by interacting with components of the basic transcriptional machinery to increase the number of functional basal promoter complexes[7, 8]. The final composition and activity of the recruited multiprotein transcriptional complex is dependent on both promoter and enhancer sequences, as well as on the specific ligand bound to the NR.

Our goal for this chapter is to provide an overview of the discoveries that have occurred over the last 10 years in the area of NR-mediated induction of CYP enzymes by xenobiotics. Our current understanding of the mechanism of signal transduction of each receptor will be presented, and we will highlight some areas where further investigation is needed. Along the way, we will also touch on emerging physiological roles of some NRs. While we hope to provide a basic working knowledge of NR-mediated signal transduction, the breadth of the topic prevents us from discussing in depth many of the more detailed aspects of NR signaling pathways. For those desiring more information on specific topics, readers are referred to relevant reviews.

2. The Pregnane X Receptor

2.1. Introduction

The CYP3A enzymes are the most abundant cytochrome P450s in human liver and are responsible for the metabolism of endogenous steroids and numerous xenobiotics[10]. The main isoform in humans, CYP3A4, is estimated to be responsible for the metabolism of more than 50% of the currently used drugs and is considered central in many clinically important drug interactions[11]. Due to the importance of the CYP3A enzymes, the mechanisms of CYP3A induction are of special interest and have been an area of intense research.

A series of discoveries over many years have led to our current understanding of CYP3A induction. Early studies demonstrated that the administration of certain steroids to rats caused greatly enhanced transcription of CYP3A genes in the liver and small intestine. Induction was seen after treatment with the potent glucocorticoid dexamethasone and, paradoxically, also with the synthetic antiglucocorticoid pregnenolone 16α-carbonitrile (PCN)[12–14]. The response of CYP3A to glucocorticoids was distinct from a classical glucocorticoid receptor (GR)-dependent response with respect to both the time course of induction and the dose of dexamethasone required, as well as the rank order of the potency of various steroids[15, 16]. Analysis of the promoter region of the CYP3A23 gene revealed conserved enhancer elements similar to those recognized by NRs;

however, further experimentation showed that the elements were not bound by the GR[17, 18]. It was postulated that the induction of CYP3A23 involved a novel NR acting by a mechanism distinct from that of the classical GR pathway.

2.2. The PXR

The long-standing paradox of CYP3A induction by both GR agonists and antagonists was explained after a novel orphan NR was cloned and characterized[19]. Initial experiments to identify ligands of the orphan receptor demonstrated that it could be activated by many compounds, including dexamethasone, 6,16α-dimethyl pregnenolone, and PCN. The unusual pharmacology of the orphan receptor, specifically its activation by glucocorticoids (dexamethasone) and antiglucocorticoids (RU486 and PCN), strongly suggested that it was the unknown mediator of CYP3A induction observed in earlier studies. Further experimentation proved this to be the case and the receptor was named the pregnane X receptor (PXR) because of its strong activation by natural and synthetic pregnanes.

After the identification of PXR in the mouse, orthologues of the receptor were identified in many other species including human, rabbit, and rat[20–22]. The human orthologue was named steroid and xenobiotic receptor (SXR) and also pregnane activated receptor (PAR)[20, 21]. For simplicity, we will use the name PXR to refer to all orthologues. A comparison of PXR amino acid sequences among different mammalian species shows that while the DBD is highly conserved (>90% identity), the LBD displays much more variability (~80% identity)[23]. In all species, PXR is highly expressed in the liver and to a lesser extent in the small intestine[19, 23]. The PXR expression profile matches that observed for induction of CYP3A enzymes and provides further evidence for the idea that PXR is the master regulator of CYP3A expression.

2.3. PXR Ligands and Species Differences

The PXR is a very promiscuous, low affinity receptor that is activated by a wide array of structurally diverse compounds[22]. Crystal structures of the human PXR (hPXR) LBD, both with and without agonist, have been useful in understanding the receptor's ability to accommodate ligands of various structures and sizes[24]. The crystal structure of the hPXR LBD in the absence of ligand revealed a hydrophobic ligand binding pocket that is larger than that of most NHRs; furthermore, a unique flexible loop found adjacent to the ligand-binding cavity likely contributes to the ability of the PXR to bind both small and large ligands[24]. When the LBD was cocrystallized with a hPXR ligand, SR12813, it was discovered that the ligand could dock into the ligand binding pocket in three different orientations, each with a distinct pattern of hydrogen bonding and van der Waals contacts[24]. Thus, unlike many NHRs, the PXR can bind a variety of hydrophobic ligands in multiple binding orientations.

The ligand-binding specificity of the PXR is markedly different among species[22]. For example, PCN is a strong activator of rat and mouse PXR, but has little effect on rabbit or human PXR. Conversely, rifampicin, phenobarbital, and SR12813 activate both rabbit and human PXR, but have little effect on rodent PXR. These species differences are due to differences in the amino acid sequence of the LBD of the receptor. Using the crystal structure of SR12813 bound to hPXR, four polar residues in the LBD that interacted with SR12813 were identified that were different from the corresponding amino acids in the mouse PXR (mPXR)[24]. When the residues in the mPXR were mutated to the amino acids found in the human receptor, the mutant mPXR was no longer responsive to the rodent-specific inducer PCN but rather to the human-specific agonist SR12813[24]. The dependence of specific ligand binding on PXR amino acid sequence has also been demonstrated *in vivo*. A PXR-null mouse that has been "humanized" by integrating an albumin-SXR (hPXR) transgene is responsive to the PXR ligands rifampicin and PB, but is no longer responsive to PCN[25].

2.4. Activation of Transcription

Analyses of PXR target gene promoters revealed that the receptor can upregulate transcription by binding as a heterodimer with RXR to several different motifs, including DR3, DR4, and ER6 elements (Figure 8.1)[10]. The human CYP3A4

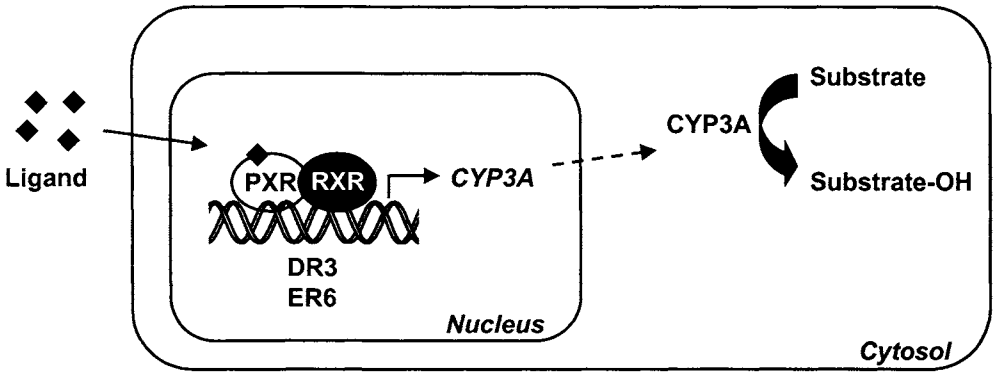

Figure 8.1. A model of the transcriptional regulation of CYP3A expression by PXR. The PXR binds as a heterodimer with RXR to response elements in the promoter of CYP3A and other target genes. Binding of ligand to the PXR results in increased CYP3A enzyme activity, which in turn increases the hydroxylation of substrates such as steroids, bile acids, and drugs.

gene contains a proximal ER6 response element and a distal xenobiotic response element module (referred to as XREM) consisting of both an imperfect DR3 and an ER6 element[26]. Although PXR-mediated transactivation can be conferred by the proximal ER6 element alone, maximal induction of the CYP3A4 gene requires both the ER6 and XREM motifs[26]. The PXR can also activate the transcription of a number of CYP2B genes, which are classically thought of as target genes for the nuclear receptor, CAR[27–29]. Interestingly, the PXR has been demonstrated to upregulate CYP2B expression by binding to the same DR4 elements upstream of the CYP2B gene to which CAR binds[27–30]. The reciprocal is also true in that CAR can bind to response elements in the CYP3A genes to induce gene expression[27, 28, 31]. These findings and others have made it increasingly clear that CAR and PXR serve broad and often overlapping functions[32, 33].

In addition to PXR and CAR, other NRs are also involved in the regulation of CYP3A expression. For example, activation of the GR by dexamethasone increases the expression of both PXR and CAR through glucocorticoid response elements (GREs) in their promoters, and this can increase the expression of CYP3A[34–36]. The transcription factor hepatocyte nuclear factor-4 (HNF-4) seems to play an important role in CYP3A expression as well. It has been shown that binding of HNF-4 to the promoter of CYP3A23 is necessary to maintain both its constitutive expression and its induction by dexamethasone[37]. Similarly, binding of HNF-4α to a specific *cis*-acting element in the CYP3A4 gene promoter was found to be necessary for transactivation of gene expression by PXR or CAR[38]. Moreover, HNF-4α-null mice express neither PXR nor CAR, indicating that expression of these receptors is regulated by HNF-4α[39]. Given the complexity of emerging cross talk pathways among receptors, it is likely that other NRs may be implicated in CYP3A regulation in the future.

2.5. Mouse Models

An important advancement in the PXR field came with the generation of a PXR-null mouse model[25, 40]. Data obtained using these mice have confirmed that the PXR plays a major role in regulating CYP3A gene expression and in xenobiotic metabolism. Mice that lack PXR do not induce CYP3A in response to PCN or other PXR-specific ligands and exhibit altered metabolism of xenobiotics that are CYP3A substrates[25, 40]. The exact role of the PXR in maintaining the constitutive expression of CYP3A remains unclear as the two independently derived PXR-null models display either unchanged or increased levels of basal CYP3A[25, 40].

Mouse models have also been used to demonstrate a role for PXR in regulating the levels of toxic bile acids. It had been previously established

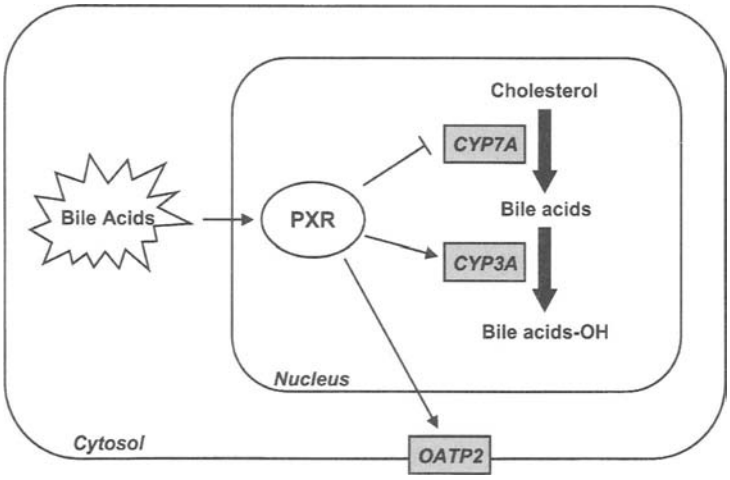

Figure 8.2. An overview of the PXR's involvement in regulation of bile acid metabolism. Bile acids, such as LCA can bind and activate the PXR to regulate hepatic gene expression. The PXR negatively regulates the expression of CYP7A, which catalyzes the rate-limiting step in the conversion of cholesterol to bile acids. Conversely, the PXR upregulates the expression of CYP3A and OATP2, which are involved in the metabolism and transport of bile acids, respectively. This coordinate regulation of genes results in the increased clearance of toxic bile acids from the hepatocyte.

that treatment of rats with PCN decreased the expression of CYP7A1, the enzyme that catalyzes the rate limiting step in the synthesis of bile acids from cholesterol[41]. To examine whether the PXR played a role in the repression of CYP7A1, a PXR-null mouse model was utilized[40]. It was demonstrated that the PXR mediated not only the repression of CYP7A1 by PCN, but also its basal expression. Additionally, the organic anion transporter polypeptide 2 (OATP2), a bile acid transporter, was found to be induced by PCN in wild-type animals but not in PXR-null mice. When bile acids were examined for their ability to activate the PXR, it was found that a secondary bile acid, lithocholic acid (LCA), was an efficacious activator of both mouse and human PXR[40]. In a parallel study utilizing the humanized PXR mouse model, bile acids such as LCA were identified as PXR ligands that could induce CYP3A expression and it was shown that CYP3A catalyzed the hydroxylation and detoxification of bile acids[42]. Administration of LCA to mice results in severe hepatotoxicity. Both studies demonstrated that PXR-null mice were resistant to LCA toxicity and furthermore, that sustained activation of the PXR protected against LCA-induced hepatotoxicity in wild-type mice[40, 42]. Collectively, these findings demonstrate that a regulatory loop exists by which elevated concentrations of bile acids activate the PXR to block new bile acid biosynthesis and to induce the metabolism and excretion of existing bile acids (Figure 8.2).

2.6. Future Research

The finding that CAR binds to many of the same response elements as the PXR and that these two receptors share many ligands and target genes has made it clear that the net effect of a xenobiotic on CYP3A gene expression will often depend on more than one receptor pathway. Identifying all of the NRs involved in the regulation of CYP3A will be necessary in the future. In addition, the promoters of many suspected PXR target genes, including CYP7A and OATP2, have not yet been characterized. Since the expression of CYP3A is coordinately regulated by PXR, CAR, and other NRs, it seems likely that other PXR target genes are regulated in a similar fashion. The analyses of regulatory regions in novel genes may provide additional insight as to how the PXR interacts with other NRs at response elements to regulate gene expression. In the future, continued analyses

of the cross talk that occurs among the PXR and other NRs will be an exciting area of research that will eventually provide the details necessary to understand how the PXR works with other receptors to form a master regulatory circuit that controls CYP3A expression.

3. The Constitutive Androstane Receptor

3.1. Introduction

In early studies it was observed that treatment of rats with phenobarbital (PB) caused a marked proliferation of the liver and endoplasmic reticulum, an increase in DNA synthesis, and increased activities of drug- and steroid hormone-metabolizing enzymes[43, 44]. PB is now considered the prototype for a large group of structurally diverse, lipophilic chemicals that induce a similar spectrum of effects. PB and PB-type chemicals induce the expression of numerous cytochrome P450 genes, including genes in the CYP1A, CYP2B, CYP2C, and CYP3A subfamilies[45, 46]. Of these, the CYP2B subfamily is most effectively induced and will be discussed here as a paradigm.

The coordinate induction of hepatic enzymes by PB has long been recognized to require direct activation of transcription[47]. While evidence was suggestive of a receptor-mediated process, studies aimed at identifying a PB-binding receptor were hindered for years by lack of an appropriate model system. A significant advance in understanding PB-induced gene expression came with the characterization of a regulatory element in the CYP2B genes. Using transgenic mice containing rat CYP2B2 promoter constructs of different lengths, it was determined that PB-responsiveness was due to regulatory regions at least ~1 kb upstream of the CYP2B2 core promoter region[48]. Experiments in primary cultures of rat hepatocytes identified a 163 bp fragment ~2.3 kb upstream of the CYP2B2 gene that conferred PB-responsive activity and this enhancer was termed the PB response element (PBRE)[49]. The responsiveness to PB conferred by the PBRE was eventually refined to a core 50 bp element that contained three distinct DNA-binding motifs[50]. Later, a similar 51 bp enhancer was characterized in the mouse CYP2B10 gene and was termed the PB responsive enhancer module (PBREM)[51, 52]. Sequence analysis revealed that the PBREM contained two DR4 motifs, commonly referred to as NR-binding sites one and two (NR1 and NR2), which flanked a nuclear factor 1 (NF1)-binding site[31, 52].

3.2. The Nuclear Receptor CAR

A search for the receptors capable of binding to PBREM ensued in the hopes of identifying the elusive "PB receptor." Findings from two laboratories were incorporated to eventually identify the NR that could bind PBREM in response to PB. In one experimental approach, proteins that could bind the NR1 sequence of the PBREM were isolated from PB-treated mouse liver nuclear extracts using DNA affinity chromatography[53]. When the proteins were analyzed using electromobility shift assays with the NR1 element and various NR antibodies, it was found that the NR1-nuclear protein complex contained RXRα. A search of the literature revealed that a separate laboratory had previously identified a liver-enriched orphan NR that could function as a heterodimer with RXRα to bind a retinoic acid receptor element (RARE), which contains a DR5[54, 55]. The orphan receptor had originally been identified as a "constitutively active receptor," or CAR, based on findings that the receptor could activate transcription from a RARE without the addition of exogenous ligand[55]. Based on these earlier findings, the unidentified NR binding to NR1 of the PBREM was postulated to be CAR. Further experimentation using primary mouse hepatocytes and whole animals proved this to be the case and, furthermore, suggested that CAR could mediate the induction of CYP2B by PB[31, 52, 53, 56]. Contrary to findings in early studies that used transfected cell lines, it was later demonstrated in primary hepatocytes and *in vivo* that CAR is sequestered in the cytosol in untreated cells and that its nuclear translocation is dependent on treatment with PB or PB-type chemicals[56, 57].

3.3. Mediators of CAR Activity

Although PB treatment induces the nuclear translocation and transcriptional activity of CAR, results from ligand-binding assays have indicated that neither PB nor known PB metabolites are

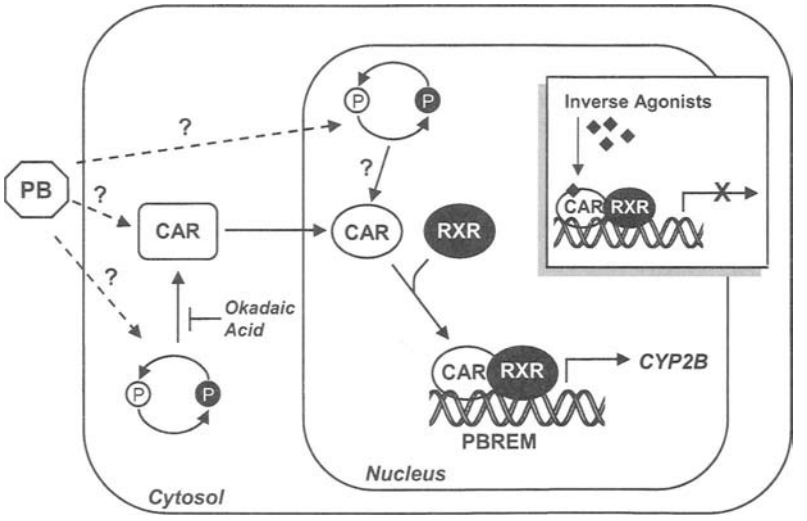

Figure 8.3. A model of CAR-mediated induction of CYP2B expression by PB. Inactive CAR normally resides in the cytoplasm. PB acts on unknown cellular targets to induce the nuclear accumulation of CAR. In the nucleus, activated CAR heterodimerizes with RXR to bind response elements in the promoter of CYP2B and other target genes to induce gene expression. Phosphorylation events are thought to be important in regulating the CAR signaling pathway. *Inset:* In the absence of PB and other CAR activators, inverse agonists can bind CAR and repress its transcriptional activity.

bona fide CAR ligands. So how might PB function to activate CAR? Because of CAR's apparent constitutive activity, it has been postulated that subcellular localization may be a major determinant of receptor activity. In this regard, treatment with okadaic acid, a phosphatase inhibitor, has been shown to block the PB-induced nuclear translocation of CAR[56]. These findings suggest that the localization of CAR in the cell may be regulated by phosphorylation events. Thus, PB may activate CAR-mediated gene transcription by altering the phosphorylation status of the receptor or related cellular targets, resulting in CAR's translocation to the nuclear compartment (Figure 8.3). Additional phosphorylation events in the nucleus have also been postulated to be important[58].

Ligands that bind directly to CAR have also been identified. Initially, in a search for CAR activators, it was discovered that the constitutive activity of CAR could be repressed by androstanes, which are testosterone metabolites[59]. As a result, CAR is now known as the "constitutive androstane receptor." Androstanes bind CAR directly to repress its transcriptional activity and have been termed "inverse agonists"[32, 59]. While androstanes are effective mouse CAR (mCAR) inverse agonists, they have little effect on human CAR (hCAR). Also, it is important to note that supraphysiological concentrations of androstanes are required to repress mCAR-mediated gene expression; thus, androstanes are not likely the physiological ligand of CAR. Interestingly, pharmacological concentrations of several endogenous steroids have also been demonstrated to activate (estrogens) or repress (androgens, progesterone) mCAR activity while having little effect on hCAR activity[58]. Thus far, the only identified steroidal compound that exerts activity toward hCAR is the progesterone metabolite 5β-pregnane-3,20-dione, which at pharmacological concentrations can directly bind the receptor and increase its activity above the constitutive level[32]. Collectively, these findings raise the intriguing possibility that a yet unidentified physiological steroid may function as an endogenous CAR ligand to either activate or repress activity.

Direct-binding xenobiotic ligands of CAR have also been identified and many of these show marked species specificity as well. For example, the planar hydrocarbon 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene binds directly to mCAR and is

the strongest agonist identified to date, but it apparently lacks activity toward hCAR[60]. Conversely, an agonist selective for hCAR, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime, has recently been identified[61]. Moreover, the antifungal agent clotrimazole is a potent inverse agonist of hCAR while it has little or no activity toward mCAR[32]. Other CAR xenobiotic activators that have been reported include PCBs, chlorinated pesticides such as DDT, and methoxychlor[31, 32].

3.4. Activation of Transcription

The fact that both PB and direct-binding ligands can regulate CAR suggests that there are multiple mechanisms for the regulation of CAR activity. While important differences likely exist in the cellular targets affected by receptor agonists compared to PB, agonists of CAR are similar to PB in that they induce the nuclear translocation and binding of CAR to DNA. A model of how PB may induce CYP2B expression through the CAR pathway is shown in Figure 8.3. PB interacts with unknown cellular targets to likely alter the phosphorylation status of CAR and induce its translocation to the nucleus. The receptor may undergo further modifications before binding as a heterodimer with RXR to PBREM to induce CYP2B expression. The PBREM is highly conserved in rat, mouse, and human CYP2B genes. The NR1 site seems to serve as the major CAR-binding site and is critical for CAR transactivation of CYP2B genes[53]. Once bound to PBREM, the final effect of CAR regulators on gene expression seems to be determined by the ability of CAR to recruit coactivators to the transcriptional complex. In this regard, it has been demonstrated that CAR can interact with a number of coregulators, including SMRT, SRC-1, and GRIP-1[59, 62, 63].

In addition to the DR4 elements in PBREM, CAR can bind to a variety of DNA motifs including DR3 elements, DR5 motifs (e.g., those found in RARE), and ER6 motifs[31, 54, 60]. These response elements are the same as those recognized by PXR and, not surprisingly, CAR and the PXR share many overlapping target genes[46]. Indeed, it has been demonstrated that CAR transactivates the CYP3A genes by binding to the same response element that serves as the PXR-binding site[27, 28, 31].

Aside from the PXR, other NRs are also important in CYP2B expression. As mentioned earlier, HNF-4α is critical for CAR expression, as HNF-4α-null mice express neither PXR nor CAR[39]. Both the GR and HNF4-α can bind to elements in the CAR promoter to regulate the level of CAR expression, which in turn can influence the expression of CYP2B and likely other CAR target genes[36, 38]. The study of interactions of NRs with the CAR pathway is a relatively new area of investigation and roles for other NRs in CAR-mediated CYP expression are likely to be identified in the future.

3.5. Mouse Models

The generation of mice null at the CAR locus has recently been reported[64]. Mice lacking CAR are resistant to many of the toxic effects of PB, including hepatomegaly and increased DNA synthesis, confirming that CAR mediates these toxic phenotypes[64]. In addition, studies using this model have confirmed that CAR is essential in mice for the induction of the CYP2B genes by PB[64]. The CAR-null model has been invaluable in identifying novel PB-inducible genes that are regulated by CAR. The analysis of over 8,500 genes using DNA microarray technology was recently performed to examine PB-induced hepatic gene expression in CAR-null mice compared to wild-type mice[65]. Findings from this study demonstrate that CAR mediates the PB-inducible expression of numerous hepatic genes, both negatively and positively. After PB treatment, the expression of more than 70 genes was found to be dependent on CAR, while 60 genes were regulated in a CAR-independent manner. About half of the CAR-dependent genes encoded xenobiotic metabolizing enzymes (XMEs), highlighting the importance of this receptor in protecting organisms against xenobiotic exposure. Interestingly, some CAR-dependent genes were downregulated in response to PB and were found to encode proteins that play roles in basic liver function, fatty acid metabolism, and signal transduction. These findings provide evidence for the idea that CAR is not only important in regulating the expression of XMES, but also that it plays an important physiological role as well.

Using a combination of both PXR- and CAR-null mice, the ability of CAR and PXR to share

response elements and induce the same target genes has been demonstrated *in vivo*. For example, treatment of PXR-null mice with PB results in the induction of CYP3A and this has been shown to occur through CAR binding to the CYP3A promoter[31, 45]. Similarly, treatment of CAR-null mice with the mPXR ligand dexamethasone results in CYP2B induction through the binding of PXR to PBREM[46]. Through studies such as these, the relative contribution of each of these receptors on CYP gene expression has begun to be explored.

A "humanized" mouse model that expresses hCAR rather than mCAR in the liver has recently been engineered[66]. Given the fact that the effect of xenobiotics on CAR activity differs significantly among species, this model should prove useful in evaluating the relevance of toxic responses. For example, once the toxicity of an agent is determined to be dependent on mCAR using the CAR-null mouse model, the humanized mice can be used to evaluate whether the toxic response can also be mediated by hCAR. Recent studies employing this approach have implicated CAR in the hepatotoxicity of acetaminophen in humans[66]. It was found that acetaminophen at high doses activates hCAR and induces the expression of CYP1A2 and CYP3A, which are the enzymes that catalyze the rate-limiting step in the formation of toxic acetaminophen metabolites. These findings have identified CAR as a possible therapeutic target in cases of acetaminophen overdose[66]. In addition, a separate study using humanized mice and CAR-null mice demonstrated that CAR plays a role in protecting the body from elevated bilirubin levels by inducing the expression of enzymes involved in bilirubin clearance[67].

3.6. Future Directions

The identification of PBREM and CAR, have led to major advances in understanding how PB and PB-like chemicals regulate gene expression However, many unanswered questions remain. Exactly how PB interacts with the CAR signaling pathway to induce gene expression is still unclear. Moreover, it is not known if CAR agonists mediate gene expression by mechanisms similar to or distinct from those of PB. In addition, further investigation into how phosphorylation is involved in regulating the CAR pathway is important. The dependence of CAR signaling on phosphorylation may represent a model of activation that could be applicable to the other xenobiotic receptors. It is not known whether physiologically relevant endogenous agonists and inverse agonists exist. If identified, these endogenous CAR ligands will offer clues as to what role CAR plays in normal physiology. Compared to the PXR, CAR seems to bind to a more limited spectrum of steroidal compounds and xenobiotics. Solving the crystal structure of CAR's LBD will allow for the investigation of how ligand specificity is determined between these two receptors and may provide information useful in the evaluation of their separate but overlapping roles in regulating gene expression. Finally, the most challenging area of research for the future will be in understanding how CAR, the PXR, and other NRs interact to regulate CYP gene expression.

4. The Peroxisome Proliferator Activated Receptor α

4.1. Introduction

Peroxisome proliferators (PPs) are a group of structurally dissimilar chemicals that cause a similar spectrum of effects including a proliferation of peroxisomes in the hepatocyte, liver hyperplasia, and an increase in the expression of numerous enzymes involved in fatty acid oxidation[68]. The enzymes upregulated by PPs include a large number of enzymes involved in the β-oxidation of fatty acids and the CYP4A enzymes, which are important in the ω-oxidation of many medium and long-chain fatty acids[68, 69]. In the body, fatty acids are oxidized to produce energy when other substrates are not available, such as during times of fasting or starvation[70].

The ability of PPs to cause the rapid, coordinate transcriptional upregulation of gene expression in a tissue-specific manner suggested that PPs acted through a NR-mediated mechanism[71]. This proved to be the case when a screen for novel NHRs identified a mouse cDNA encoding an orphan receptor that could be activated by known PPs, such as the drug clofibrate[72]. The receptor was named the peroxisome proliferator activated receptor, or PPAR[72]. In later studies, the rat homologue of the PPAR

was identified and it was found that the receptor could not only be activated by PPs, but also by endogenous fatty acids[73].

4.2. PPAR Isoforms

The PPAR that was originally cloned from mouse is now known as the alpha isoform, or PPARα. This designation arose after the identification of two additional distinct PPAR isoforms, termed PPARβ (also referred to as δ) and PPARγ The three PPAR isoforms are encoded by three separate genes and have been identified in many species including human, rat, and rabbit[68]. The three PPAR isoforms play distinct roles and display tissue specific expression patterns[74]. The PPARα is highly expressed in the liver and kidney and plays a major role in regulating the catabolism of fatty acids. Not surprisingly, the CYP4A enzymes are coexpressed with PPARα in these tissues[75]. The PPARγ gene actually gives rise to two gene products, PPARγ1 and PPARγ2, through differential promoter usage. The PPARγ2 isoform is highly expressed in adipose tissue and mediates adipogenesis and lipid storage; however, PPARγ1, which is expressed more broadly and at lower levels, can also induce adipogenesis[76]. The PPARβ is ubiquitously expressed and while the exact physiological function of this isoform is still unclear, recent findings have suggested that this isoform modulates the activity of both PPARα and PPARγ[77]. Since PPARβ and PPARγ do not seem to regulate the expression of CYP4A or any other P450 enzyme, these isoforms will not be discussed to any great extent.

4.3. PPARα Ligands

While the quantitative effects of agonist binding on the activity of PPARα seem to be species-specific, the spectrum of ligands that can activate the PPARα across species is similar. Clofibrate, originally recognized for its ability to increase both the number and size of peroxisomes when administered to rats, is considered the prototype for a class of drugs called fibrates, which are all potent PPARα ligands[78, 79]. The fibrate drugs are widely used today as lipid lowering agents in humans. Other synthetic ligands of the PPARα include the industrial plastisizer mono (2-ethylhexyl) phthalate, trichloroacetic acid, and the pesticide DTT[80]. Interestingly, these xenobiotics induce the expression of CYP4A even though this enzyme does not seem to play a role in their metabolism.

Many of the endogenous fatty acids that are metabolized by CYP4A are also PPARα ligands. These include an array of saturated and unsaturated very long-chain fatty acids, such as linoleic acid, palmitic acid, and arachidonic acid[73, 81]. Moreover, findings using acyl-CoA oxidase (AOX)-null mice suggest that the acyl-CoA derivatives of very long-chain fatty acids are most likely endogenous PPARα ligands. In mice with a disrupted AOX gene, acyl-CoA derivatives accumulate to high levels and the animals display a phenotype similar to that seen after treatment of rodents with synthetic PPs[82]. Some eicosanoids and eicosanoid metabolites that are important mediators of inflammation, including leukotriene B_4 and prostaglandins, are PPARα ligands[83, 84]. These arachidonic acid derivatives can be metabolized by CYP4A to compounds that are inactive in terms of mediating the inflammatory response[75]. In light of the role that PPARα plays in the induction of CYP4A, it is not surprising that mice lacking PPARα have been demonstrated to display a prolonged inflammatory response[85].

4.4. Activation of Transcription

The experimental drug Wy14643, an acetic acid derivative of clofibrate, is a potent PPARα agonist and was instrumental in elucidating the signaling pathway of PPARα. The PPARα binds as a heterodimer with RXR to DNA motifs termed peroxisome proliferator response elements (PPREs) (Figure 8.4)[86]. The core PPRE sequence was initially identified as an imperfect DR1 motif by analyzing the promoter of the AOX gene[87]. Unlike the PXR and CAR, PPARα can form heterodimers with either ligand-free or 9-*cis* retinoic acid-bound RXR, and ligand binding to either RXR or PPARα can activate gene expression through PPREs[88, 89]. Other NRs can also bind to PPREs and competition for binding has been observed among the three PPAR isoforms as well as HNF-1, thyroid receptor, and RXR/RXR dimers. Depending on the NR complex bound to the PPRE, the transcription of a target gene can be either activated or repressed. Studies have shown

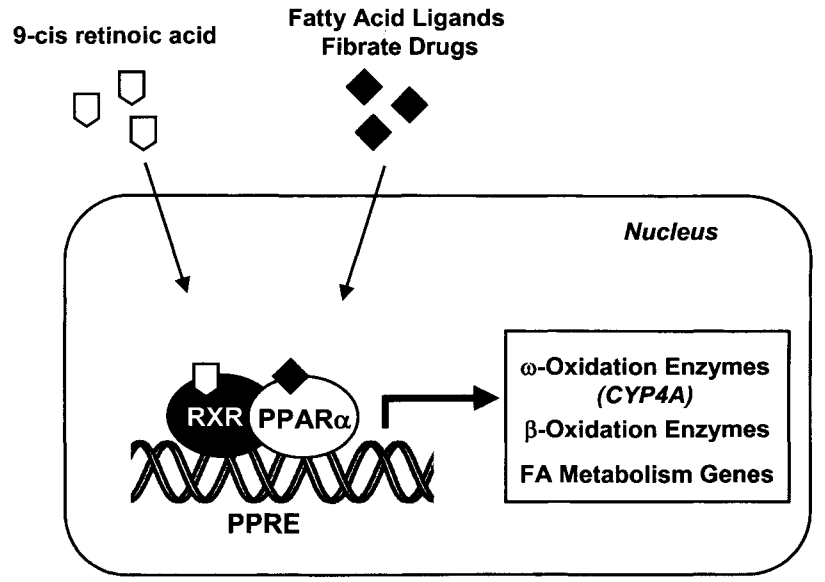

Figure 8.4. A model of the transcriptional regulation of gene expression by PPARα. The PPARα binds as a heterodimer with RXR to PPREs upstream of target genes. Ligand binding to either the RXR or PPAR activates the transcriptional complex resulting in the induction of numerous genes involved in fatty acid oxidation and metabolism, such as CYP4A.

that the specificity of a PPRE for NR binding is determined not only by the sequence of the DR1 element, but also by the sequence immediately 5′ of the PPRE[90, 91].

As with other NRs, transcriptional regulation by the liganded PPAR involves the interaction of many cofactors that form distinctive multiprotein complexes. In addition to several common coregulators that have been shown to interact with the PPAR (e.g., SRC-1, CBP, SMRT), coactivators that seem to be PPAR-specific have also been identified including PPAR binding protein (PBP) and PPAR interacting protein (PRIP). Interestingly, a transcriptionally active PPARα-interacting cofactor (PRIC) complex from rat liver nuclear extracts was recently isolated and was found to contain over 25 different proteins, including PBP, PRIP, CBP, and a novel coactivator PRIC285[92]. The composition of this PRIC complex may provide an insight into the basis for differences in tissue and species sensitivity to PPs.

The crystal structures of the PPARα LBD bound to an agonist with a coactivator motif from SRC-1 and, alternatively, bound to an antagonist with a corepressor motif from SMRT have recently been resolved[93, 94]. It was found that agonists cause the recruitment of coactivators by interacting with the ligand-dependent activation helix (AF-2) to maintain it in an active conformation. In this active conformation, the AF-2 helix can bind tightly to LXXLL motifs in coactivators and stabilize coactivator binding into a hydrophobic cleft that is formed in the receptor[93]. In the unliganded state, the PPARα is preferentially bound to corepressors. The crystal structures reveal that corepressor motifs bind to a hydrophobic groove in the receptor and prevent the AF-2 helix from interacting with coactivators. The binding of antagonists further stabilizes the inactive conformation of the receptor by altering the position of a residue in the AF-2 helix that is critical to agonist binding. These studies have demonstrated how ligands can promote basic structural changes in the PPARα to mediate its interaction with coregulators, and hence, its transcriptional activity. Results of these studies have also allowed for the realization that NRs can distinguish coactivators from corepressors by the length of their conserved interaction motifs. Importantly, these findings have also resulted in a model of

receptor repression that can likely be applied to many NRs[94].

4.5. Species Differences

Quantitatively, the response of humans and rodents to PPs has been found to differ dramatically. Humans are indeed responsive to PPs in regard to their ability to reduce serum lipids *in vivo*, a response known to be mediated by PPARα in rodents[95]. However, chronic exposure of rats and mice to PPs causes a dramatic peroxisome proliferation response in the liver and eventually leads to liver tumors. These PP-induced toxicities have not been observed in humans even though the fibrate drugs have long been used at high doses in humans to lower triglyceride and cholesterol levels[71, 96]. Several mechanisms have been postulated to play a role in the seemingly refractory nature of humans to PP toxicities. Different expression levels of PPARα and the existence of a splice variant of PPARα in humans that may negatively regulate PPARα have been suggested to play a role[97, 98]. Recently, the human PPARα transgene was introduced by an adenoviral approach into PPARα-null mice and was found to be as effective as the mouse PPARα in transcriptionally activating PPARα target genes under *in vivo* conditions[99]. The findings of this study demonstrate that the human PPARα is fully competent to induce PP-induced pleiotropic responses in the context of mouse liver[99]. Thus, other factors in the human liver environment are likely important in PPARα function and in determining the PP response in humans. Competition between PPARα and other NRs for binding to RXR or coactivators has been postulated to play a role in species differences[80]. Moreover, differences in the sequence of PPREs and surrounding sequences in target genes exist between humans and rodents, and it is not known exactly how this affects PPARα transactivation potential *in vivo*. The analysis of changes in global gene expression in wild-type and null animals in response to PPs has been performed using DNA microarrays and this approach may eventually allow for a better understanding of how the PPARα mediates the toxic response to PPs in rodents[100].

4.6. Mouse Models

A PPARα-null mouse model has been generated and the animals are viable and fertile[101]. While exhibiting no detectable gross phenotype in the fed state, experiments using null mice have demonstrated that the PPARα plays an important role in the hepatic response to fasting. Unlike wild-type mice, fasted PPARα null animals do not upregulate the expression of fatty acid oxidation enzymes, including CYP4A, and they exhibit hypoglycemia, hypoketonemia, and elevated plasma levels of free fatty acids[102]. These findings and others have demonstrated that PPARα plays a central role in maintaining lipid homeostasis.

The PPARα-null mice do not display the typical toxic responses after exposure to PPs. Studies have shown that treatment of null mice with PPs does not induce liver hyperplasia, peroxisome proliferation, or hepatocarcinogenesis, confirming that PPARα is the mediator of these PP-induced toxic responses[80, 101]. Moreover, these findings suggest that the PPARβ and PPARγ isoforms do not play a critical role in these PP-induced toxicities. The induction of CYP4A and many other fatty acid oxidation enzymes in response to PPs is also absent in mice lacking PPARα confirming that it mediates the induction of these enzymes. Interestingly, while PPARα-null mice have lost the CYP4A induction response, basal levels of CYP4A are not affected, indicating that other NRs control the constitutive expression of CYP4A[102].

Studies using rodents have also demonstrated that a normal AOX gene is necessary for proper physiological regulation of the PPARα[82]. The AOX gene encodes an enzyme critical in the β-oxidation of certain very long-chain fatty acid acyl-CoA metabolites[68]. Targeted disruption of the AOX gene in mice results in sustained PPARα activation, leading to profound peroxisome proliferation and increased levels of PPARα target genes, such as the CYP4A genes[82]. These findings suggest that acyl-CoA metabolites, and possibly other unmetabolized oxidase substrates, are endogenous ligands of the PPARα and that AOX is critical in metabolizing these ligands *in vivo*.

4.7. Future Directions

Over the last 10 years, great strides have been made in understanding the biology of the PPARα. The synthesis of specific and potent PPARα agonists have made it possible to examine the mechanism of signal transduction of the PPARα. Furthermore, the resolution of the crystal structures

of PPARα bound to ligands and coregulators has resulted in a model of how agonists and antagonists alter the conformation of NRs to mediate coregulator binding. In the future, a more complete understanding at the molecular level is needed as to how PPAR/coregulator complexes interact with other proteins to modulate gene expression in a species- and tissue-specific fashion.

The generation of a PPARα-null mouse has been critical in establishing a major role for PPARα in lipid homeostasis and has confirmed the role of PPARα in PP-induced toxicity in rodents. However, many questions remain concerning differences between mice and humans in regard to the PPARα pathway. The basis for species differences in the response to PPs is unclear and it is not known what role differences in the expression of CYP4A and other PPARα target genes play in mediating this response. In the future, generation of a "humanized" PPARα mouse, such as those available for the NRs, PXR and CAR, will be useful for long-term studies to investigate species differences and to allow for the more accurate extrapolation of findings to human risk assessment when evaluating PP-induced toxicities.

5. The Aryl Hydrocarbon Receptor

5.1. Introduction

Nearly 50 years ago, it was noted that rats exposed to 3-methylcholanthrene (3-MC) displayed a marked increase in metabolic capacity toward that substrate and other polycyclic aromatic hydrocarbons (PAHs)[103]. This enhanced metabolic activity was referred to as "aryl hydrocarbon hydroxylase" (AHH) based on the ability of these enzymes to efficiently hydroxylate aromatic hydrocarbons[104]. It is now known that AHH activity is the collective activities of the CYP1A1, CYP1A2, and CYP1B1 enzymes.

Over the next 30 years, two lines of evidence led to the identification of the AHR, the protein that functioned as the PAH sensor and regulated AHH activity. The first indications that such a receptor existed came from genetic studies of inbred mouse strains. Early studies demonstrated that C57BL/6 mice were much more responsive than DBA mice to the PAH-induced upregulation of AHH activity[105]. Using classical genetic approaches, the locus responsible for the AHH inducibility phenotype was shown to segregate in a simple autosomal dominant fashion. This locus was termed the "*Ah*" locus because of its ability to mediate responsiveness to *a*ryl *h*ydrocarbons[106, 107]. The allele found in the more responsive C57BL/6 strain was designated as Ah^b while the allele that conferred decreased responsiveness in DBA mice was termed Ah^d[108].

The second line of evidence came from pharmacological studies using an extremely potent inducer of AHH activity, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or "dioxin")[109]. Using radiolabeled TCDD, a receptor in mouse liver cytosol was identified that bound this ligand with high affinity and in a saturable and reversible manner[110, 111]. The proof that this TCDD-binding site was in fact the AHR was 3-fold. First, it was found that receptor isolated from mice harboring the responsive Ah^b allele bound ligands with higher affinity than did receptor isolated from mice harboring the less responsive Ah^d allele[105, 106, 112, 113]. Second, competitive binding studies with various dioxin congeners revealed that binding affinities correlated with their potency as inducers of AHH activity[114–116]. The last line of evidence was biochemical in nature. In the absence of ligand, the receptor was found in the cytosolic fraction of cell extracts; however, the binding site/receptor was found in the nuclear fraction after exposure to ligand[117]. Thus, genetic, biochemical, and pharmacological evidence demonstrated that the *Ah* locus encoded the AHR and this protein was the mediator of AHH induction.

5.2. The AHR

It was many years before the AHR was cloned and characterized. Attempts to purify the receptor were initially hampered by its low cellular concentration and relative instability. The development of a photoaffinity ligand, 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin, was the essential step that allowed the eventual purification of the AHR[118, 119]. Once the receptor was purified, a partial amino acid sequence was obtained and this lead to the cloning of the AHR cDNA from mouse liver[120, 121]. The deduced amino acid sequence revealed that the AHR was

as a member of the PAS superfamily of proteins[120, 121]. The AHR was found to be most similar in amino acid sequence to the AHR nuclear translocator (ARNT). Interestingly, ARNT had been cloned only a year before in a screen to identify gene products that were important in AHR signaling in a mouse hepatoma cell line[122]. One mutant cell line that was deficient in signaling expressed normal amounts of AHR, but the cells did not upregulate AHH activity after agonist exposure. A human genomic DNA fragment that rescued the mutant phenotype was found to contain the ARNT gene product. Further experimentation demonstrated that the corresponding ARNT protein was required to direct the activated AHR to specific regulatory elements upstream of target genes such as CYP1A1[123]. The structural similarities of the AHR and ARNT were recognized and it was postulated that the proteins might be dimerization partners. This proved to be the case, making AHR and ARNT the first PAS protein heterodimer to be shown to have physiological relevance[123, 124].

The overall structural organization of the AHR is typical of most members of the PAS superfamily of proteins. The N-terminus of the AHR contains a bHLH domain that is important in dimerization and subsequent positioning of the basic regions of the proteins such that they can bind to specific DNA enhancer motifs[125–127]. As in most PAS proteins, the bHLH region is found immediately N-terminal to the PAS domain. The 250–300 amino acids comprising the PAS domain contain two highly degenerate repeats, termed "A" and "B" repeats[6]. The PAS domain of the AHR harbors the LBD, a dimerization surface for binding to ARNT, and an interaction surface for chaperones such as Hsp90 and ARA9 (also called AIP, or XAP2)[6, 128, 129]. The region of the AHR important in ligand binding and chaperone binding overlaps the PAS B repeat[125, 130]. The C-terminus of the AHR encodes a hypervariable TAD[125].

The AHR is expressed in many cell types and tissues with high levels of expression found in placenta, lung, thymus, and liver. The expression profile of the AHR is in good agreement with the expression of PAH-target genes. However, the expression of CYP1 genes is fairly tissue specific, with CYP1A2 primarily found in the liver, CYP1A1 highly expressed in epithelial cells throughout the body, and CYP1B1 found in mesenchymal cells[131, 132]. This indicates that factors other than AHR expression level are involved in the tissue specific expression of these CYP1 genes.

5.3. AHR Ligands

Putative orthologues of the AHR have been identified in numerous higher eukaryotes, including nematodes, insects, fish, birds, and mammals. Striking differences in molecular weight of the AHR are observed among various species, and even in different strains of laboratory mice. This difference is mostly due to differences in the length of the C-terminus and results from different stop codon usage. Despite differences in receptor size, the vertebrate AHR signaling pathway is highly conserved across species and the induction of CYP1 gene expression is observed in all species[133, 134]. Importantly, significant species and strain differences have been observed in ligand-binding affinities. It seems that changes in specific amino acid residues in the LBD may be responsible for these differences. For example, the *Ah* alleles found in C57BL/6 and DBA mice exhibit a 10-fold difference in ligand binding and this arises, at least in part, from an alanine to valine substitution at amino acid 375[135, 136]. Moreover, the human AHR has the same mutation at the corresponding amino acid and is similar to the Ah^d allele in that it binds the ligand with 10-fold less affinity compared with the Ah^b allele[124]. Since the crystal structure of the AHR has not been solved, the identification of amino acids important in ligand binding has relied upon the examination of ligand-binding affinities of AHRs with different amino acid mutations.

The most extensively studied agonists are the halogenated aromatic hydrocarbons such as TCDD, polychlorinated biphenyls, and polychlorinated dibenzofurans as well as PAHs such as benzo[a]pyrene and 3-MC[3]. One of the highest affinity ligands of the AHR and the most potent inducer of CYP1A1 expression is TCDD. As the result of this ligand–receptor interaction, exposure to TCDD produces a wide variety of toxic effects that are species- and tissue-specific[3]. The response to TCDD is due to the fact that TCDD has a remarkably high affinity for the AHR (on the order of 10^{-12} M, K_D) and that this ligand is

resistant to metabolism. The toxic endpoints are dependent on the AHR and are thought to arise from long-term alterations in AHR-mediated gene expression, but it is still unclear if TCDD toxicity involves the transcriptional upregulation of CYP1A genes. The discussion of TCDD here will focus primarily on its use as a prototype agonist of the AHR and the mechanism by which it acts as an inducer of the CYP1 genes.

Apart from xenobiotics, it is assumed the AHR recognizes some endogenous ligand. While some endogenous compounds, such as heme degradation products, have been shown to bind and activate the AHR, no compound has been convincingly demonstrated to be the bona fide "endogenous AHR ligand"[137]. Naturally occurring AHR ligands have been found in teas, fruits, vegetables, and herbal supplements and include polyphenolic compounds such as flavonoids, indoles, and various carotinoids. The continued identification and analysis of these naturally occurring ligands may provide insight that could lead to the identification of an endogenous AHR ligand in the future, or to the identity of the environmental stresses that have led to the evolutionary conservation of the receptor system.

5.4. Activation of Transcription

While it has long been recognized that the expression of CYP1 genes is regulated at the level of transcription, it took many years to develop our current understanding of how the AHR mediates upregulation of gene transcription in response to xenobiotics. An overview of the mechanism of AHR-mediated gene expression is shown in Figure 8.5. In the absence of ligand, the AHR is found in a cytosolic complex with two molecules of Hsp90, an immunophilin-like chaperone protein known as ARA9 and the chaperone p23[138–141]. The Hsp90 chaperone is a necessary component of the AHR pathway and seems to anchor the receptor in the cytosol as well as hold the protein in a high affinity ligand-binding conformation[142–145]. The ARA9 protein has been shown to increase the amount of properly folded AHR in the cytoplasm, while the chaperone p23 has been suggested to play a role in regulating ligand responsiveness and receptor translocation[145, 146].

The signal transduction pathway of the AHR is well characterized and analogous to that of many NHRs, described above. Ligand binding to the

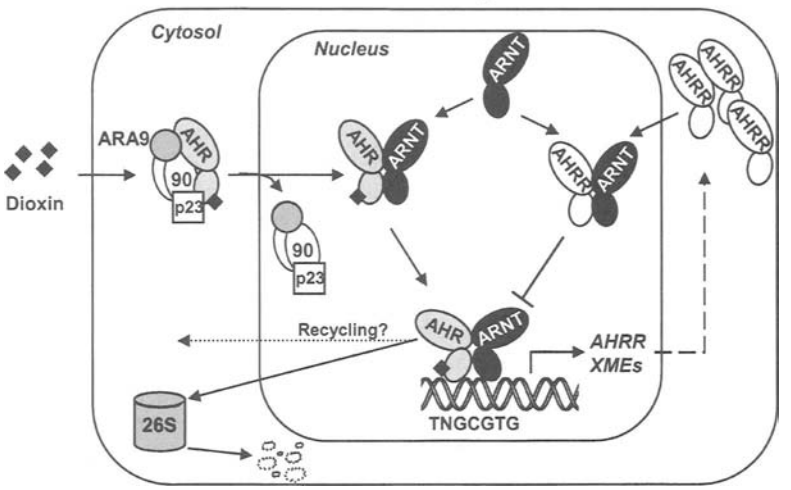

Figure 8.5. A model of AHR signal transduction. The AHR normally resides in the cytoplasm with the chaperones Hsp90, ARA9, and p23. Upon ligand binding, the AHR translocates to the nucleus where it exchanges its chaperones for ARNT. The AHR/ARNT heterodimer binds to dioxin response elements (DREs) to activate the transcription of downstream target genes, including the AHRR and XMEs, such as CYP1A. The ligand-activated AHR is exported from the nucleus and degraded through a proteosome pathway, or may undergo recycling within the cytoplasm. The AHRR protein, a negative regulator, can compete with the AHR for dimerization with ARNT resulting in inhibition of AHR-mediated gene expression.

cytosolic AHR induces a conformational change and nuclear translocation of the receptor. In the nucleus, the AHR sheds some of its associated chaperones and binds to its partner ARNT[147–150]. The resulting AHR/ARNT heterodimers bind to specific enhancers in DNA to alter DNA conformation and increase the transcription of target genes[151]. The enhancers, made up of the consensus sequence 5′-TNGCGTG-3′, were first characterized in the mouse CYP1A1 gene and have been called "dioxin responsive elements" (DREs), "xenobiotic responsive elements" (XREs), or "AH-responsive elements" (AHREs)[152–155]. For the remainder of this chapter, we shall refer to the enhancers as DREs. In addition to nucleotides in the core DRE, sequences outside of the DRE can modulate the binding affinity of AHR/ARNT to DNA and appear to be important determinants of AHR-mediated gene expression[155–157].

Functional DREs have been identified upstream of numerous AHR-inducible genes, many of which encode XMEs. These genes are collectively referred to as the *Ah* gene battery and include CYP1A1, CYP1A2, CYP1B1, NQO1 (NADPH : quinone oxidoreductase), ALDH3A1 (an aldehyde dehydrogenase), UGT1A6 (a UDP glucuronosyl transferase), and GSTYa (a glutathione *S*-transferase)[158]. The coordinate upregulation of these enzymes results in the enhanced metabolism of most inducers to hydrophilic compounds that can be more easily excreted from the body. Thus, the AHR plays an integral role in mediating the adaptive response to PAHs and related environmental chemicals.

Another interesting aspect of the AHR pathway is that prolonged agonist exposure results in the attenuation of signaling through the AHR. One mechanism by which this occurs is mediated through the AHR repressor protein (AHRR)[159, 160]. The AHRR is structurally similar to the AHR, except it lacks the PAS B-domain and its C-terminus functions as a transcriptional repressor. Because of these features, the AHRR can dimerize with ARNT in a manner that is independent of agonist. This heterodimer can bind to DREs to repress target gene transcription[159, 161]. The expression of the AHRR gene is controlled by a DRE and its transcription is upregulated upon exposure of the cell to AHR ligands. Another way the cell attenuates agonist-induced AHR signaling is by targeting ligand-bound AHR for degradation through the ubiquitin/proteosome pathway[162, 163].

The ARNT protein serves as a dimerization partner not only for the AHR, but also for other PAS proteins, such as the hypoxia inducible factors (HIF1α, HIF2α, HIF3α). When in a complex with ARNT, these various heterodimers mediate the upregulation of various genes important in dealing with cellular hypoxia[164]. It has been postulated that competition among PAS proteins for the limited pool of ARNT could be an important mechanism of transcriptional regulation. Some studies have found that activation of the HIF1α pathway can interfere with AHR-mediated induction of CYP1A1[165–167]. However, others have reported that simultaneous activation of both the HIF1α and the AHR pathways caused no changes in the expression level of any AHR or HIF1α target genes, suggesting ARNT is not a limiting factor[168]. These conflicting results may be due to differences in cell type and/or experimental conditions. Although cross talk between the AHR and HIF1α pathways seems to occur under certain conditions *in vitro*, it remains to be proven that competition for ARNT occurs *in vivo* and what, if any, effect this has on CYP1 gene expression or TCDD toxicity.

5.5. Mouse Models

Targeted disruption of the *Ah* locus in mice has been achieved by a number of laboratories. As expected, AHR-null mice fail to upregulate CYP1A1, CYP1A2, and other members of the *Ah* battery in response to AHR agonists[169, 170]. Furthermore, AHR-null mice are resistant to TCDD- and PAH-induced toxicity, confirming that the AHR is the mediator of dioxin toxicity[171, 172]. The AHR-null mouse models have also provided evidence for a physiological role for this receptor. These mice have defects in vascular development, display decreased fertility, and have overall decreased body weight compared to wild-type mice. Thus, in addition to mediating TCDD toxicity and the adaptive response to PAHs and other chemicals, the AHR clearly plays an important role in development. Such an observation supports the hypothesis that the AHR has an unknown endogenous ligand.

5.6. Future Directions

Significant advances have been made in many areas of AHR biology, especially in understanding

the AHR signal transduction mechanism in response to xenobiotics. However, the lack of a three-dimensional structure for the receptor has hindered the investigation of mechanisms underlying species-specific responses to certain ligands, such as TCDD. Also, it is not understood how ligand binding to the AHR alters its conformation to induce nuclear translocation. Determination of the crystal structure of the AHR will greatly facilitate the investigation of these and other aspects of AHR research. In spite of the questions remaining, the AHR signaling pathway has been a useful model to provide a broad understanding of the biological roles of PAS proteins. Yet, how the PAS domain mediates protein–protein interactions is still not fully understood. More in depth examination of the interactions between AHR and ARNT in the future should prove helpful in the identification and characterization of PAS domain function. Finally, although we know the AHR plays an important physiological role in development, the mechanism by which the AHR mediates these processes is not clear. For example, we do not know if AHR signaling during development is similar or different from the pathway by which AHR regulates xenobiotic metabolism. The ultimate identification of an endogenous AHR ligand will shed light on the physiological role of the AHR.

6. Conclusions

Over the last decade, great strides have been made in understanding the roles that the nuclear receptors PXR, CAR, PPARα, and AHR play in the induction of CYP genes. The ability of xenobiotics to bind and activate NRs to induce the expression of the CYP enzymes involved in their metabolism provides a mechanism by which an organism can mount an adaptive response to its changing chemical environment. The identification of endogenous ligands for some NRs indicates that these receptors play important roles in regulating CYP levels during physiological processes as well. It has become clear that the expression of many CYP genes is dependent on more than one NR. Recent studies have demonstrated that NRs often share xenobiotic ligands, response elements, and target CYP genes. The existence of multiple xenobiotic receptors with broad and sometimes overlapping functions likely increases the ability of an organism to detect and respond to a wide range of chemicals. The challenge for the future will be to understand how the NRs participate in a complex network to regulate CYP gene expression and to mediate the physiological response to xenobiotics.

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9

Hormonal Regulation of Liver Cytochrome P450 Enzymes

David J. Waxman and Thomas K.H. Chang

1. Introduction

Sex differences in hepatic drug metabolism have been known for more than 30 years, based on the early studies of Kato, Conney, Gillette, and others[1–3]. In rats and certain other species, the rate of drug metabolism is often several-fold higher in males as compared to females as revealed by *in vivo* pharmacokinetics and as demonstrated *in vitro* by assaying prototypic phase I cytochrome P450 drug substrates, such as ethylmorphine, benzo[*a*]pyrene, or hexobarbital using isolated liver microsome preparations (Figure 9.1). This sex-dependence of P450 metabolism is most striking in the rat, where sex differences in metabolic rates can be as high as 5-fold with some drug substrates. This finding was initially unexpected, because measurements of total liver P450 content indicated that the overall P450 levels in rat liver tissue are only ~20% higher in males compared to females (Figure 9.1). Research carried out during the 1980s resolved this discrepancy with the finding that there are multiple liver-expressed P450 enzymes[7, 8], each of which is encoded by a separate gene, and only some of which are expressed in a sex-dependent manner.

Based on the recently published human genome sequence, we now know that there are 57 functional human cytochrome P450 genes, grouped into 17 distinct gene families[9]. Many of these P450 enzymes catalyze the biosynthesis of physiologically important endogenous substances, such as steroid hormones, whereas others are primarily involved in metabolism of environmental chemicals and other xenobiotics, notably drugs. From the perspective of foreign compound metabolism, the most important cytochrome P450 ("CYP") genes are those in the *CYP1*, *CYP2*, and *CYP3* families. These three families encompass ~15–20 different cytochrome P450 enzymes, and collectively carry out essentially all of the phase I cytochrome P450 metabolic reactions in mammalian liver[10]. As individual liver P450 enzymes were purified and characterized, and subsequently, when their genes were cloned from multiple species, it became apparent that in certain species, such as the rat and mouse, a subset of these drug-metabolizing liver P450s is expressed in a sex-dependent fashion and subject to endocrine control[11]. Human liver P450 enzyme levels and their associated drug metabolism activities may also be determined, in part, by age, sex, and hormone status[12–17]. Studies of the underlying mechanisms governing the endocrine regulation of rat liver P450 enzymes may thus be of general importance for our understanding of the hormonal

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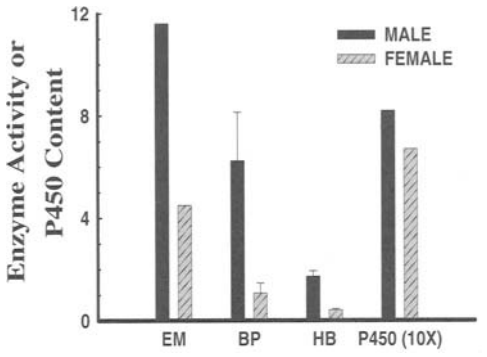

Figure 9.1. Sex-differences in rat hepatic microsomal drug metabolism. Data shown are based on enzyme assays in rat liver microsomes using the three indicated xenobiotic substrates: ethylmorphine (EM)[4], benzo[*a*]pyrene (BP)[5], and hexobarbital (HB)[6]. Ethylmorphine *N*-demethylase and benzo[*a*]pyrene hydroxylase activities are expressed as nmol product formed per minute per mg microsomal protein, whereas hexobarbital hydroxylase activity is expressed as nmol product formed per 30 min per g liver. Also shown is hepatic microsomal total cytochrome P450 content, which is expressed as nmol per mg microsomal protein (values multiplied by 10)[4]. The data are shown as mean ± SD for 4 or 5 rats, except for ethylmorphine *N*-demethylase and total P450 which are based on a pool of 6 livers.

regulation of liver-expressed genes in both rodent models and in man. Endocrine-regulated steroid hydroxylase P450s contribute in an important way to foreign compound metabolism in the liver, and studies of their hormonal control may shed light on the underlying basis for the influence of hormone status on a broad range of P450-catalyzed drug metabolism and carcinogen activation reactions.

This chapter reviews studies leading to the identification of the key endocrine regulatory factors and the underlying mechanisms through which these factors operate to control the expression of liver cytochrome P450 enzymes. Primary emphasis is given to studies on the hormonally regulated P450s expressed in rat liver, the best-studied model system. Also discussed are environmental and pathophysiological factors that can perturb hormonal status and the impact of these factors on the expression of sex-dependent cytochromes P450.

2. Steroid Hormones as Substrates for Sex-Dependent Liver P450s

The precise physiological function of the endocrine-regulated rodent liver P450s is unclear; however, steroid hormones are metabolized by liver P450 enzymes with a higher degree of regiospecificity and stereoselectivity than many foreign compound substrates[11], suggesting that these endogenous lipophiles serve as physiological P450 substrates. The physiological requirements with respect to steroid hormone hydroxylation differ between the sexes, and not surprisingly, several steroid hydroxylase liver P450s are expressed in a sex-dependent manner[11,18]. Rat P450 enzymes CYP2C11 and CYP2C12 are prototypic examples of sex-specific liver P450 enzymes, and they have been a major focus of studies of the underlying endocrine factors, as well as the cellular and molecular regulatory mechanisms that govern sex-specific liver gene expression. CYP2C11 is the major male-specific androgen 16α- and 2α-hydroxylase in adult rat liver, and is induced at puberty in males but not females[19, 20] under the influence of neonatal androgenic imprinting (programming)[21]. By contrast, the steroid sulfate 15β-hydroxylase CYP2C12 is expressed in a female-specific manner in adult rats[21, 22].

3. Developmental Regulation of Sex-Dependent Rat Liver P450s

Multiple rat liver cytochrome P450 enzymes are expressed in a sex-dependent manner, subject to complex developmental regulation and endocrine control (Table 9.1). CYP2C11, the major male-specific androgen 2α- and 16α-hydroxylase of adult liver, is not expressed in immature rats but is induced dramatically at puberty (beginning at 4–5 weeks of age) in male but not female rat liver[19, 20]. A similar developmental profile characterizes three other male-specific rat liver cytochromes P450, CYP2A2 (refs [27], [36]), CYP2C13 (refs [28], [37]), and CYP3A18 (refs [25], [38]). By contrast, another

Table 9.1. Hormonal Regulation of Sex-Dependent Rat Liver P450 Enzymes

CYP enzyme[a]	Testosterone hydroxylase activities[b]	Hormonal regulation[c]	
		Androgenic imprinting[d]	Thyroid hormone[e]
I. Male-specific			
2A2	15α	++	+/−
2C11	2α, 16α	++	+/−
2C13	6β[f], 15α	++	ND
3A2	6β, 2β	++	−−
3A18	6β, 2β, 15β, 16α	ND	ND
4A2	(see footnote *g*)	ND	−
II. Female-specific			
2C12	15β[h]	−−	+/−
III. Female-predominant[i]			
2A1	7α	ND	−
2C7	16α	ND	++
3A9	6β	ND	ND
5α-reductase	—	−−	++

[a]P450 gene designations are based on the systematic nomenclature of ref. [23]. Table is modified from ref. [24].
[b]The major sites of testosterone hydroxylation catalyzed by the individual P450 proteins are shown. Testosterone metabolites specific to the P450's activity in rat liver microsomal incubations are underlined. Based on refs [8], [11], [21], [25], [26] and references therein.
[c]"++" indicates a positive effect on adult enzyme expression, while "−−" indicates a suppressive effect. "−" indicates a lesser degree of suppression, while "+/−" indicates no major effect. ND—not determined in a definitive manner.
[d]For further details see refs [27]–[29].
[e]Based on refs [30]–[34].
[f]Purified CYP2C13 exhibits high testosterone hydroxylase activity in a reconstituted enzyme system, but this enzyme makes only marginal contributions to liver microsomal testosterone hydroxylation[35].
[g]CYP4A2 catalyzes fatty acid ω-hydroxylation, but does not catalyze testosterone hydroxylation.
[h]15β-hydroxylation of steroid sulfates. CYP2C12 also catalyzes weak testosterone 15α- and 1α-hydroxylase activities.
[i]Liver expression of these enzymes is readily detectable in both male and female rats, but at a 3–10-fold higher level in females as compared to males.

adult male-specific liver P450, the steroid 6β-hydroxylase CYP3A2, is expressed in prepubertal rat liver at similar levels in both sexes, but is selectively suppressed at puberty in females[21, 39–41]. The steroid sulfate 15β-hydroxylase CYP2C12 is expressed at a moderate level in both male and female rats at 3–4 weeks of age. Beginning at puberty, however, CYP2C12 levels are further increased in females while they are fully suppressed in males[21, 22]. Several other female-predominant liver enzymes have also been shown to increase at puberty in adult female rats. These include: CYP2C7 (refs [37], [42]), which catalyzes retinoic acid 4-hydroxylation[43]; CYP3A9 (ref. [44]), which catalyzes steroid 6β-hydroxylation[26]; and steroid 5α-reductase, which is not a cytochrome P450 enzyme but plays an important role in steroid metabolism in adult female rats[21, 45]. Finally, CYP2A1 is a female-predominant steroid 7α-hydroxylase that is expressed in both sexes shortly after birth, but is suppressed at puberty to a greater extent in male than in female rat liver[21, 30, 46] (Table 9.1). Each of these sex-dependent P450 enzymes is primarily expressed in liver, although low-level extrahepatic expression may occur in some cases[47–49]. Studies of liver P450 expression during senescence have revealed a general loss of gender-dependent enzyme expression that reflects a decrease in male P450 levels and an increase in expression of female-predominant P450 enzymes

in aging male rats[50, 51]. This appears to be related to the age-dependent reduction in the secretion of growth hormone (GH) releasing factor and the changes in plasma profile of GH[52], which is a major regulator in the sex-dependent expression of liver CYP enzymes (see Section 4.2).

4. Hormonal Control of Liver P450 Expression

4.1. Regulation by Gonadal Hormones

Initial studies on the endocrine regulation of the liver P450 enzymes demonstrated that the gonadal steroids play an important role in regulating enzyme expression, but only indirectly. Gonadal steroids do not act directly on the liver to confer the sex-dependent pattern of hepatic steroid and drug metabolism and P450 expression, but rather, act indirectly via the hypothalamus, which regulates the pituitary gland and its secretion of the polypeptide hormone, GH (see Section 4.2).

4.1.1. Testosterone

4.1.1.1. Distinct effects of neonatal androgen and adult androgen. Gonadal hormones play an essential role in determining the expression of the major sex-specific rat liver P450 forms at adulthood. In the case of testosterone, there are two distinct postnatal developmental periods of hormone production, neonatal and postpubertal, and each period makes a distinct contribution to the expression of the sex-dependent liver P450s at adulthood. Castration of male rats at birth eliminates both periods of androgen production, and this in turn abolishes the normal adult expression of each of the male-specific P450s: CYP2A2 (ref. [27]), CYP2C11 (refs [20], [21], [29], [37], [53]), CYP2C13 (ref. [28]), and CYP3A2 (refs [21], [29], [53]). CYP2C13 mRNA levels[28] are also abolished in birth-castrated rats, indicating that enzyme expression is regulated at a pretranslational step. Treatment of birth-castrated male rats with testosterone during the neonatal period leads to a partial restoration of the expression of these male-specific P450 forms at adulthood[21, 27, 28]. A brief period of neonatal androgen exposure is thus sufficient to "imprint" or irreversibly program the male rat to express these P450 enzymes later on in adult life. These effects of neonatal androgen on male-specific P450 enzymes are very similar to the androgenic imprinting effects observed in earlier studies of liver microsomal steroid hydroxylase activities[45, 54, 55], several of which can be associated with specific liver P450 forms[11].

However, neonatal testosterone given to birth-castrated male rats only partially restores CYP2C11 (refs [21], [53]) or CYP2C13 (ref. [28]) to normal adult male levels, indicating that neonatal androgen alone is insufficient for full adult expression of these male-specific P450s. Consistent with this observation, the combination of neonatal androgen treatment with adult androgen exposure results in complete restoration of normal adult male expression of the male-specific P450s (ref. [28]). Moreover, testosterone treatment of adult male rats that were castrated either neonatally or prepubertally, can substantially increase the expression of CYP2C11 (refs [29], [53], [56], [57]) and CYP2C13 (ref. [28]). However, in contrast to the irreversible imprinting effects of neonatal androgen treatment, the effects of adult androgen exposure are likely to be reversible, as evidenced by the partial loss of CYP2C11 in male rats castrated at adulthood[20, 21] and by the reversal of this loss by the synthetic androgen methyltrienolone[58]. Similarly, the continued presence of testosterone at adulthood is also required to maintain normal adult expression of CYP3A2, since castration at 90 days of age reduces hepatic CYP3A2 mRNA levels by >80%, but this can be restored by subsequent administration of testosterone to the adult rat[59]. Thus, while neonatal testosterone imprints the rat for expression of the male-specific P450 enzymes beginning at puberty, a time when the demand for P450-dependent liver steroid metabolism is increased, the additional presence of androgen during the pubertal and postpubertal periods is required to maintain full enzyme expression during adult life[60, 61].

4.1.1.2. Testosterone suppression of female enzymes. In contrast to the positive regulation by testosterone of the male-specific enzymes, testosterone suppresses expression of the female-specific CYP2C12 as well as the female-predominant enzymes CYP2A1 and

steroid 5α-reductase. Hepatic CYP2C12 content is reduced in intact, adult female rats exposed chronically to testosterone[29] or to the synthetic androgen methyltrienolone[22]. Similarly, treatment of neonatally or prepubertally ovariectomized rats with testosterone, either neonatally or pubertally, results in a major decrease in microsomal steroid 5α-reductase activity[29, 60]. Birth castration of male rats increases the adult levels of hepatic CYP2A1, but testosterone administration to these animals re-masculinizes (i.e., decreases) the levels of this P450 (ref. [62]). Androgens thus exert a suppressive effect on liver CYP2A1 expression. Studies of the effect of testosterone on the expression of the female-predominant CYP2C7 are inconclusive[56, 63].

4.1.1.3. Mechanisms of testosterone regulation. The mechanisms by which neonatal testosterone imprints the expression of liver P450 enzymes during adulthood are only partially understood. Testosterone's primary effects on liver P450 profiles are mediated by the hypothalamic–pituitary axis[64] and its control of the sex-dependent pattern of pituitary GH secretion[65, 66]. Consistent with this conclusion, testosterone has only minor effects on liver enzyme profiles in hypophysectomized rats in most[67] but not all[68, 69] instances. As discussed in Section 4.2, pituitary GH secretory patterns play a key role in regulating the expression of the sex-dependent P450 forms.

4.1.2. Estrogen

Whereas testosterone has a major positive regulatory influence on the male-specific P450 forms, estrogen plays a somewhat lesser role in the expression of the female-specific and the female-predominant liver P450 enzymes. Ovariectomy at birth reduces, but does not abolish, expression of hepatic CYP2C7, CYP2C12, and steroid 5α-reductase in adult female rats[21, 29, 56] and normal adult enzyme levels can be restored by estrogen replacement. Ovariectomy during adulthood[70] or neonatal administration of an estrogen receptor antagonist, tamoxifen[71], reduces hepatic CYP3A9 expression in adult female rats. The reduced expression of CYP3A9 in ovariectomized rats can be restored by estrogen[70]. By contrast, estradiol suppresses hepatic CYP2C11 in both intact and castrated male rats[29, 56]. However, the absence of CYP2C11 in adult female rats is not due to a negative effect of estrogen. Thus, ovariectomy alone does not lead to CYP2C11 expression in female rats[21, 29, 56]. In male rats, the suppression of CYP2C11 by estradiol may be irreversible, as demonstrated by the major loss of this P450 in adult male rats exposed to estradiol during the neonatal period or at puberty. However, this effect is not a consequence of a direct action of estradiol on the liver, since estradiol does not impact on hepatic CYP2C11 levels in hypophysectomized rats[68]. Rather, the effects of estradiol on liver P450 expression involve the hypothalamo–pituitary axis[64, 72], and most likely result from an estrogen-dependent increase in the interpeak baseline levels of plasma GH[64, 72]. This effect of estradiol may be sufficient to alter the sex-specific effects of the GH secretory pattern since, as discussed in greater detail below, recognition of a "masculine" GH pulse by hepatocytes requires an obligatory recovery period during which there is no plasma GH and hence no stimulation of hepatocyte GH receptors (GHRs)[73]. In addition, estrogen may antagonize the induction of CYP2C11 by testosterone as suggested by the absence of androgen imprinting of this P450 in intact female rats treated with neonatal or pubertal testosterone[56, 57]. Indeed, the stimulatory effect of testosterone on pulsatile GH secretion can be blocked by the presence of intact ovaries in female rats[66]. Interestingly, prepubertal treatment of intact (i.e., non-ovariectomized) female rats with tamoxifen enhances the induction of CYP2C11 and CYP3A2 protein expression by pubertal and postpubertal androgen[99]. The precise neuroendocrine mechanisms responsible for the antagonistic effects of estrogen on androgen imprinting remain to be elucidated.

4.2. Regulation by Growth Hormone

4.2.1. Sex-Dependent GH Secretory Profiles

In many species, the pituitary gland secretes GH into plasma in a highly regulated temporal fashion that differs between males and females. This sex-dependent secretion of GH is most striking in rodents[74–77], but key features are conserved in humans[78–82]. In the rat, GH is secreted by the pituitary gland in adult males in an intermittent, or

pulsatile, manner that is characterized by high peaks of hormone in plasma (150–200 ng/ml) each 3.5–4 hr followed by a period of very low or undetectable circulating GH (<1–2 ng/ml) (Figure 9.2(A)). By contrast, in the adult female rat, GH is secreted more frequently (multiple pituitary secretory events per hour) and in a manner such that the plasma GH pulses overlap and the

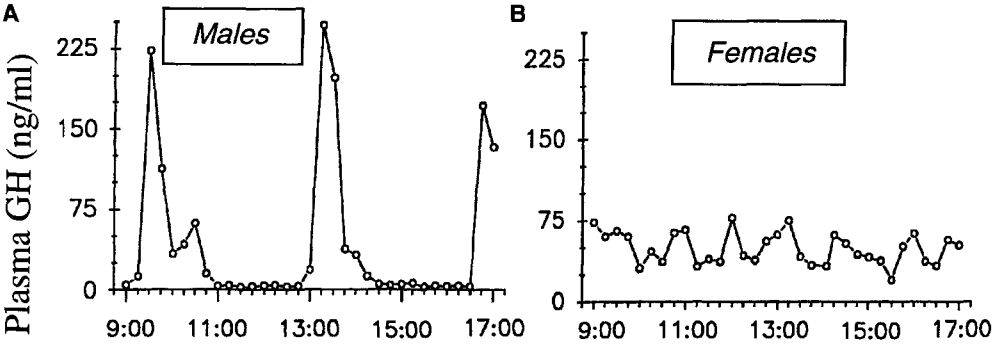

Figure 9.2. Sex-dependence of plasma GH profiles in adult rats. Shown are plasma GH profiles measured during the course of an 8 hr day in unrestrained and unstressed male (panel A) and female rats (panel B). Data shown are from ref. [73].

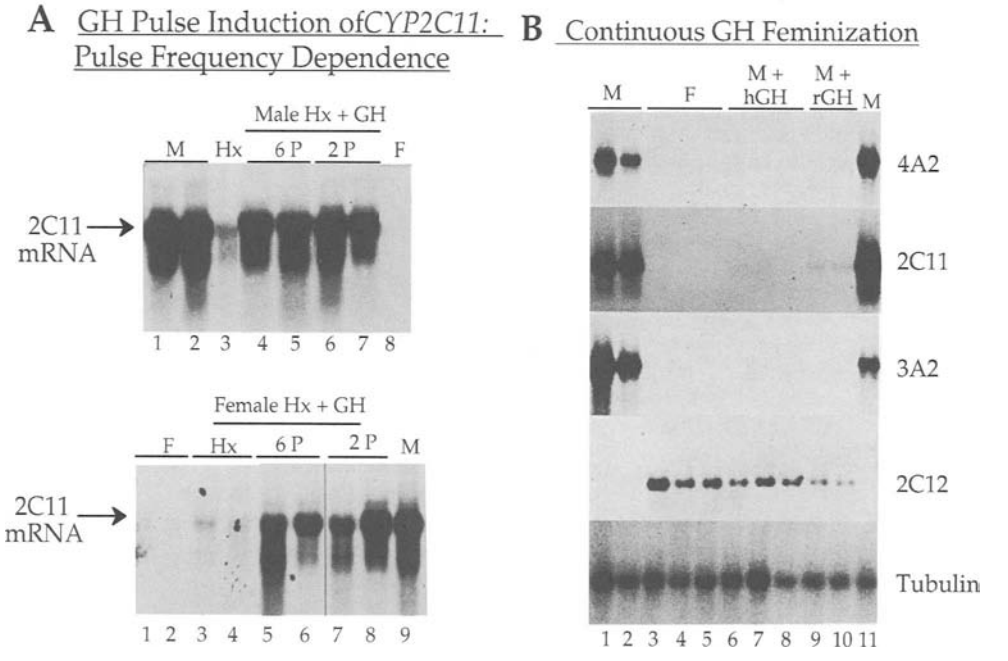

Figure 9.3. Role of GH secretory profiles in the expression of sex-dependent rat hepatic CYP mRNAs. Shown are Northern blots probed with oligonucleotide probes specific to each of the indicated CYP RNAs. Panel A shows the male (M) specificity of CYP2C11, its absence in hypophysectomized rats (Hx) and its induced expression in either male or female (F) hypophysectomized rats given either 2 or 6 pulses (P) of GH/day for 7 days. Data based on ref. [73]. Panel B shows the effects of continuous rat (r) or human (h) GH infusion in male rats (lanes 6–10) on the expression of CYPs 4A2, 2C11, and 3A2 (all male-specific; lanes 1, 2, 11 vs lanes 3–5), as well as the induction of CYP2C12. Tubulin RNA is shown as a loading control. Data based on ref. [32].

hormone is continually present in circulation at significant levels (~15–40 ng/ml) at nearly all times[67] (Figure 9.2 (B)). Hypophysectomy and GH replacement experiments carried out in several laboratories have demonstrated that these sex-dependent plasma GH profiles are, in turn, responsible for establishing and for maintaining the sex-dependent patterns of liver P450 gene expression in rats[20, 21, 27, 73, 83] and mice[84, 85] (for earlier reviews, see refs [24], [86]). Clinical studies in humans also demonstrate a role for GH[87–91] and its sex-dependent plasma secretory patterns[92] in regulating P450-dependent drug metabolism.

Studies in the rat model reveal three distinct responses of liver P450s to plasma GH profiles (Figure 9.3).

(1) Continuous plasma GH, a characteristic of adult female rats, stimulates expression of female specific enzymes, such as CYP2C12 and steroid 5α-reductase[22, 62], and female-dominant liver enzymes, such as CYP2A1, CYP2C7, and CYP3A9 (refs [31], [62], [93], [94]). Hepatic levels of CYP2C12 and steroid 5α-reductase are undetectable in hypophysectomized female rats but can be restored to near-normal female level by continuous GH replacement[62, 95–97]. This restoration can be achieved with as little as 12–25% of the physiological levels of GH[97]. Higher levels of GH are required to induce expression of CYP2C12 and steroid 5α-reductase in hypophysectomized male rats[98].

(2) Intermittent plasma GH pulses, which are characteristic of adult male rats, induce expression of the male-specific liver enzyme CYP2C11 (Figure 9.3(A)) and its associated testosterone 2α-hydroxylase activity[20, 60, 73, 83]. The stimulatory effects of intermittent GH stimulation on this "class I" male P450 enzyme can be distinguished from the effects of GH pulses on a larger, second group ("class II") male-specific liver P450s (CYP2A2, CYP2C13, CYP3A2, CYP3A18, and CYP4A2). In contrast to the class I CYP2C11, class II male P450s are not obligatorily dependent on GH pulses, as judged by their high level of expression in the absence of GH, as shown in hypophysectomized rats of both sexes[27, 28, 38, 41, 97, 98, 100, 101]. Nevertheless, liver expression of the class II enzymes CYP2A2 and CYP3A2 is induced when intermittent GH pulses are given to adult male rats that are depleted of circulating GH by neonatal monosodium glutamate (MSG) treatment[100].

(3) Continuous GH exposure exerts major negative regulatory effects on liver P450 enzyme expression, as revealed by the marked suppression of each of the class I and class II male-specific rat P450s following continuous GH treatment of intact male rats (Figure 9.3(B)). In some cases this effect can be achieved at fairly low GH levels, corresponding to only 3–12% of the physiological GH levels present in intact female rats[98]. The high level expression of class II P450 mRNAs seen in the absence of GH pulses, that is, in hypophysectomized male rats, is also suppressed by continuous GH, indicating that continuous GH actively suppresses P450 gene expression, and does not simply abolish the stimulatory pulsatile plasma

Table 9.2. Response of Sex-Specific Rat CYPs to GH

	Intact rats			Hypophysectomized rats				MSG-treated rats	
CYP	F	M	M + GH_{cont}	F	M	M + GH_{int}	M + GH_{cont}	M	M + GH_{int}
CYP2C11[a] (Male class I)	−	++	−	−	−	++	−	−	++
CYP2A2[b] (Male class II)	−	++	−	++	++	++	+/−	−	++
CYPC12[c] (Female-specific)	++	−	++	−	−	−	++	−	−

F, female; M, male; GH_{cont}, continuous GH; GH_{int}, intermittent (pulsatile) GH.
"++" indicates a positive effect, "−" indicates a suppressive effect, or absence of expression, and "+/−" indicates no major effect.
[a]Data are based on refs [20], [73], [97], [98], [100], [101], [104]–[110].
[b]Data are based on refs [27], [97], [98], [100], [101], [104], [109]–[111].
[c]Data are based on refs [95], [97], [98], [104], [107], [109]–[111].

GH pattern. GH suppression is also a key determinant of the lower responsiveness of female rats to phenobarbital induction of CYP2B1 (refs [102], [103]), and probably also the lower responsiveness of the females to the induction of CYP4A enzymes by peroxisome proliferators such as clofibrate[32].

The response of the class II male P450 genes to hypophysectomy of female rats, which derepresses, that is, increases liver enzyme levels to near-normal intact male liver levels, demonstrates that the class II male liver P450s are subject to negative pituitary regulation in female rat liver, where their expression is strongly repressed by the near continuous pattern of plasma GH exposure. These patterns of hormonal regulation are summarized in Table 9.2, which presents the responses of representative sex-specific liver CYPs to continuous and intermittent GH treatment applied to intact, hypophysectomized and neonatal MSG-treated rats.

4.2.2. Transcriptional Effects of GH on *CYP* Genes

GH regulates liver P450 steady-state mRNA levels (e.g., Figure 9.3) in parallel with P450 protein and P450 enzyme activity levels, all but ruling out major regulation by translational and post-translational mechanisms, such as GH regulation of P450 protein turnover. Induction of CYP2C12 mRNA by continuous GH requires ongoing protein synthesis[112], suggesting either an indirect induction mechanism or a requirement for one or more protein components that may have a short half-life. Analysis of liver nuclear RNA pools demonstrates that unprocessed, nuclear CYP2C11 and CYP2C12 RNAs respond to circulating GH profiles in a manner that is indistinguishable from the corresponding mature, cytoplasmic mRNAs[113]. Consequently, transport of CYP2C11 and CYP2C12 mRNA to the cytoplasm, and cytoplasmic P450 mRNA stability are unlikely to be important GH-regulated control points for sex-specific P450 expression. Moreover, nuclear run-on transcription analyses have established that GH regulates the sex-specific expression of the *CYP2C11* and *CYP2C12* genes at the level of transcript initiation[113, 114]. Transcription is also the major step for regulation of the male class II CYP2A2 and CYP2C13 mRNAs[113, 114], whose male-specific expression is primarily a consequence of the suppressive effects of continuous GH exposure in adult female rats[27]. Thus, transcription initiation is the key step at which the three distinct effects of GH outlined in Section 4.2.1 are operative: stimulation of 2C11 expression by pulsatile GH, suppression of both class I and class II male-specific P450s by continuous GH, and stimulation of CYP2C12 expression by continuous GH[113].

Consistent with the finding that GH regulates sex-dependent liver CYPs by transcriptional mechanisms, the 5′-flanking DNA segments of the *CYP2C11* (ref. [115]) and *CYP2C12* genes[116] both contain specific DNA sequences that interact in a sex-dependent and GH-regulated manner with DNA-binding proteins (putative transcription factors) that are differentially expressed in male vs female rat liver nuclei[113, 117]. These DNA sequences are hypothesized to include GH response elements that contribute to the sex-specific transcription of the *CYP2C11* and *CYP2C12* genes. Two negative regulatory elements ("silencer elements") were also identified in the CYP2C11 promoter; however, their significance with respect to GH regulation and sex-specific P450 expression is as yet unclear[118]. Functional studies of the sex-specific CYP promoters and their interactions with liver-enriched and GH-responsive transcription factors are discussed below.

4.2.3. Cellular Mechanisms of GH Signaling

The cellular mechanisms whereby pituitary GH secretory profiles differentially regulate expression of the sex-dependent liver P450s are only partially understood. GH can act directly on the hepatocyte to regulate liver P450 expression, as demonstrated by the responsiveness of primary rat hepatocyte cultures to continuous GH-stimulated expression of CYP2C12 mRNA; however, these effects do not involve IGF-I, a mediator of several of GH's physiological effects on extrahepatic tissues[112, 119]. Discrimination by the hepatocyte between male and female plasma GH profiles is likely to occur at the cell surface, where a higher level of GHRs (see below) is found in female as compared to male rats[120]. This sex difference in

cell surface GHR abundance may, at least in part, be due to differential effects of intermittent vs continuous GH stimulation[121] and could play a role in the activation of distinct intracellular signaling pathways by chronic (female) as compared to intermittent (male) GH stimulation.

4.2.3.1. Significance of GH pulse frequency. Studies have been carried out to determine which of the three descriptive features of a GH pulse—namely, GH pulse duration, GH pulse height, and GH pulse frequency—is required for proper recognition of a GH pulse as "masculine." Direct measurement of the actual plasma GH profiles achieved when GH is administered to hypophysectomized rats by twice daily s.c. GH injection (i.e., the intermittent GH replacement protocol most commonly used to stimulate *CYP2C11* expression) has revealed broad peaks of circulating GH, which last as long as 5–6 hr[73]. These sustained GH "pulses" are effective in stimulating expression of the male-specific *CYP2C11*, provided that they are not administered in close succession. It is thus apparent that physiological GH pulse duration (<2 hr) is not required to elicit a male CYP response. Studies carried out in GH-deficient rat models (either dwarf rats or rats depleted of adult circulating GH by neonatal MSG treatment) demonstrate that GH pulse height is also not a critical factor for stimulation of *CYP2C11* expression[104, 122]. This finding can be understood in terms of the K_d of the GH–GHR complex, which at 10^{-10} M (~2 ng/ml)[123], is only 1% of the peak plasma hormone level in adult male rats. In contrast, GH pulse frequency is a critical determinant for GH stimulation of a male pattern of liver P450 expression, as shown in hypophysectomized rats given physiological replacement doses of GH for 7 days by intermittent intravenous injections at frequencies of 2, 4, 6, or 7 times per day[73]. Analysis of liver CYP2C11 levels in these rats revealed a normal male pattern of liver *CYP2C11* gene expression in response to 6 GH pulses per day (which approximates the normal male plasma GH pulse frequency), as well as in response to GH pulses given at lower frequencies, 2 or 4 times per day (e.g., Figure 9.3). However, hypophysectomized rats are not masculinized by 7 daily GH pulses, indicating that the hepatocyte does not recognize the pulse as "masculine" if GH pulsation becomes too frequent. Hepatocytes thus require a minimum GH off time (~2.5 hr in the hypophysectomized rat model used in these studies), which implies the need for an obligatory recovery period to effectively stimulate *CYP2C11* expression. This condition is not met in the case of hepatocytes exposed to GH continuously (female hormone profile). This recovery period may serve to reset the cellular signaling apparatus, for example, by replenishing GHRs at the cell surface (see below).

4.2.3.2. Role of GH receptor (GHR). The effects of GH on hepatocytes and other responsive cells are transduced by GHR, a 620 amino acid cell surface transmembrane protein[123] belonging to the cytokine receptor superfamily[124]. GHR is comprised of a 246 amino acid extracellular domain that binds GH, a single transmembrane segment, and a 350 amino acid intracellular domain that participates in the intracellular signaling events stimulated by GH[123, 125]. X-ray crystallographic and other studies establish that a single molecule of GH binds in a stepwise manner to *two* GHR molecules to yield receptor dimers: $GH + GHR \rightarrow GH\text{–}GHR \xrightarrow{+GHR} GH\text{–}(GHR)_2$ [126, 127] (Figure 9.4).

The four helix bundle protein GH is proposed to initially bind to a single receptor molecule by contacts that involve amino acids comprising GH's Site 1, followed by binding of a second molecule of GHR, which interacts with Site 2 on the GH molecule to give a heterotrimeric GH–$(GHR)_2$ complex. GH-induced receptor dimerization is reversible, and the equilibrium may be shifted in favor of monomer formation in the continued presence of excess GH: $GH\text{–}(GHR)_2 \xrightarrow{GH} 2GH\text{–}GHR$. Other, more recent studies suggest that GH may bind to, and thereby activate a preformed receptor dimer at the cell surface[129, 130]. Independent of whether the receptor dimer is preformed, however, it is clear that the conformational changes that accompany formation of the active GH–$(GHR)_2$ complex are necessary, and probably sufficient, for stimulation of GH-induced intracellular signaling events[128] (Figure 9.4). Although GHR dimerization is thus required for most GH responses, some GH responses might not require receptor dimerization[131] and indeed, might be mediated by monomeric GH–GHR complexes. Conceivably, the distinct patterns of liver P450 gene expression induced by continuous plasma GH (female GH pattern; *CYP2C12* expression) as compared to

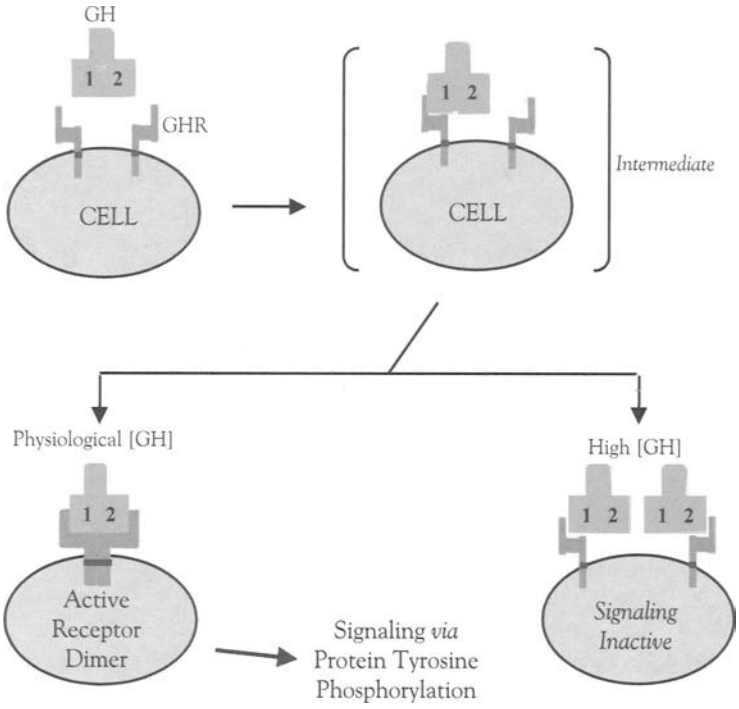

Figure 9.4. Activation of GHR by GH-induced sequential dimerization mechanism. GHR is shown localized in the plasma membrane, and sites 1 and 2 of the GH molecule (see text) are as indicated. The active receptor dimer dominates at physiological GH concentrations. Model based on data presented in ref. [128].

pulsatile GH (male GH pattern; *CYP2C11* expression) might, in part, arise from distinct GH signaling pathways perhaps stimulated by monomeric (GH–GHR) as compared to dimeric (GH–$(GHR)_2$) hormone–receptor complexes. GH mutants and analogs that bind GHR without effecting functional receptor dimerization[127–129] could be used to test this hypothesis.

In intact male rat liver, GHR internalizes to an intracellular compartment coincident with its stimulation by plasma GH pulses, and then reappears at the cell surface at the time of the next hormone pulse[132, 133]. Other studies suggest that GHR undergoes endocytosis constitutively, that is, in a ligand-independent manner. GHR internalization involves coated vesicles and ultimately takes the receptor to lysosomes for degradation. GHR endocytosis and degradation both require (a) an intact ubiquitin conjugation system, which targets a specific 10 amino acid-long cytoplasmic GHR tail sequence, and (b) 26S proteasome activity, as evidenced by the inhibitory effects of the proteasome inhibitor MG132. Interestingly, although cellular ubiquitination activity is required for receptor endocytosis, GHR itself does not need to undergo ubiquitination, as shown using a mutant GHR devoid of its cytoplasmic lysine residue targets for ubiquitination[134, 135]. Thus, the ubiquitin–proteasome system is a major regulator of intracellular GHR trafficking.

4.2.4. Role of STAT5b in Sex-Dependent *CYP* Expression

4.2.4.1. GH signaling pathways involving STAT transcription factors. How does GH impart sex-dependent transcriptional regulation to liver P450 genes? To answer this question, we may consider the following hypotheses: (a) that cell surface GHRs can discriminate between the two temporally distinct patterns of plasma GH stimulation, and (b) the receptors can then transduce this information to the nucleus, where both pulse and

continuous GH-stimulated transcriptional events occur. Presumably, GH-activated GHR accomplishes this by activating two distinct pathways of intracellular signaling, one in response to GH pulses and the other in response to continuous GH stimulation (Figure 9.5). Studies of GH-induced signal transduction pathways[136–138] have highlighted the importance of the GH-bound receptor dimer in activating JAK2, a GHR-associated tyrosine kinase that initiates several downstream pathways of intracellular protein tyrosine phosphorylation (Figure 9.4). Other studies have shown that a distinct pattern of nuclear protein tyrosine phosphorylation is induced in rat liver when the effects of the male pulsatile plasma GH hormone profile are compared to those of the female pattern of continuous GH stimulation[139]. This led to the discovery that an intracellular signaling protein and transcription factor, termed STAT5b, is present in its nuclear, tyrosine phosphorylated form at a substantially higher level in male rat liver than in female rat liver[139]. STATs are latent cytoplasmic transcription factors that are activated by tyrosine phosphorylation induced by a variety of cytokines and growth factors, and were first discovered as signal mediators that carry transcription signals into the nucleus in the interferon signaling pathway[140].

In hypophysectomized rat liver, where there is no endogenous GH signaling, there is little or no tyrosine phosphorylated STAT5b protein in the nucleus; essentially all of the STAT5b protein is found in the cytosolic fraction, where it resides in a

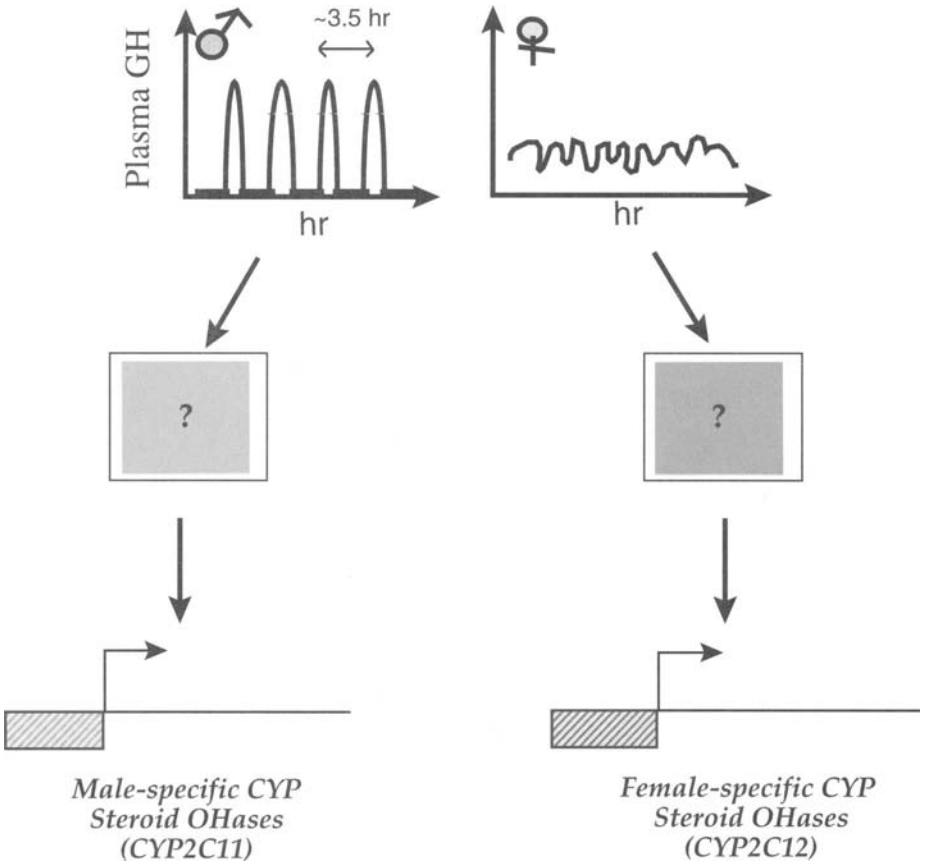

Figure 9.5. Distinct intracellular signaling pathways induced by GH are proposed to be activated by plasma GH pulses, leading to male-specific CYP expression (left), and by continuous GH stimulation, leading to female-specific CYP expression (right).

latent, inactive (non-tyrosine phosphorylated) form. However, when a hypophysectomized rat is injected with a single pulse of GH, STAT5b protein appears in the nucleus in its active, tyrosine phosphorylated state within 10–15 min[139, 141]. This tyrosine phosphorylation reaction occurs on STAT5b tyrosine residue 699, enabling two STAT5b molecules to dimerize via mutual interactions between the phosphotyrosine residue on one STAT5b molecule and the SH2 domain (a protein module that recognizes and binds specifically to phosphotyrosine residues) on a second STAT5b molecule. The STAT5b–STAT5b dimer that is thus formed quickly enters the nucleus, where it binds with high affinity to DNA sites upstream of genes that are transcriptionally activated in response to the initial GH stimulus (Figure 9.6).

STAT5b is not present in the nucleus at all times in male rat liver. Rather, STAT5b is repeatedly activated in concert with the onset of each male plasma GH pulse. It thus undergoes repeated cycles of translocation from the cytoplasm into the nucleus, and then back out to the cytoplasm[139, 142]. For example, if the liver is excised from a rat killed at the peak of a plasma GH pulse, then STAT5b is tyrosine phosphorylated and localized to the nucleus, whereas if the liver is excised from a rat killed at a time point between successive plasma GH pulses, STAT5b is inactive and cytoplasmic. Moreover, in female rats there is generally a much lower level of active, nuclear STAT5b protein (~5–10% that of the peak male liver level)[143]. This close temporal linkage between plasma GH pattern and the state of liver STAT5b activation has been confirmed in intact male rats killed at times shown to be specifically associated with spontaneous peaks or troughs of the plasma GH rhythm[144]. The key difference between male and female rat liver is that STAT5b is repeatedly, and efficiently, activated by plasma GH pulses in the case of the

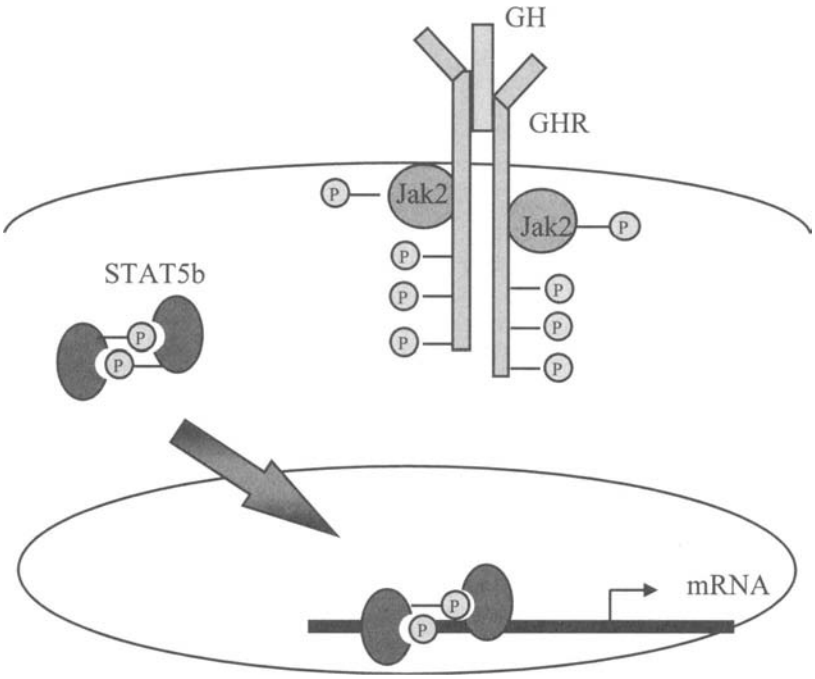

Figure 9.6. Role of complex formed by GH, GH receptor, and the tyrosine kinase JAK2 in activation of STAT5b by tyrosine phosphorylation. JAK2 tyrosine phosphorylates itself and multiple tyrosines on the cytoplasmic tail of GHR. Several of these sites serve to recruit STAT5b to the receptor–kinase complex. STAT5b is then tyrosine phosphorylated, whereupon it dimerizes, translocates to the nucleus, and binds to DNA regulatory elements upstream of target genes.

males, but is inefficiently activated by the more continuous GH profile of the females. The relatively weak STAT5b activation pathway in female rat liver appears to reflect a partial desensitization of this signaling pathway in response to the chronic presence of GH in plasma (Figure 9.7)[145].

4.2.4.2. STAT5b gene knockout mouse model. The proposal that STAT5b is a critical factor in mediating GH-regulated liver P450 gene expression is strongly supported by studies carried out in mice that are deficient in STAT5b (STAT5b knockout mouse model)[146] (see ref. [147] for a review). Disruption of the *STAT5b* gene results in two striking phenotypes (Figure 9.8). These phenotypes are seen in STAT5b-deficient males but not in corresponding females. First, there is a global loss of GH-regulated, male-specific liver gene expression, including male-specific P450

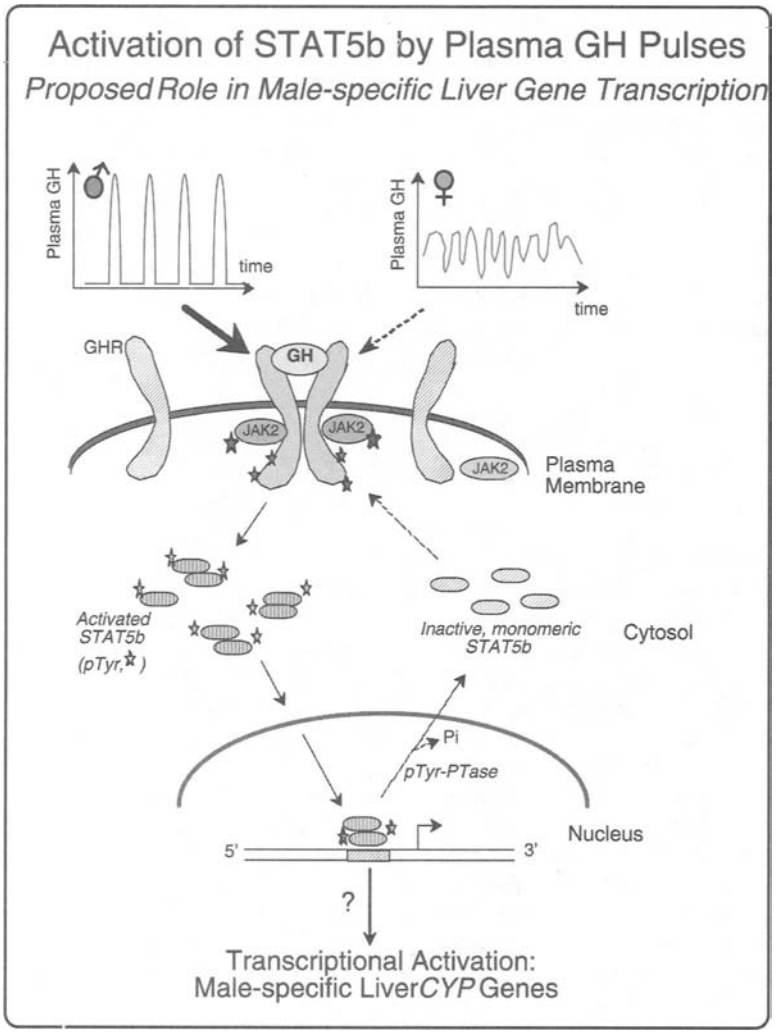

Figure 9.7. Proposed cycle for STAT5b activation and deactivation in response to sexually dimorphic plasma GH profiles. GH pulses, but not continuous GH, induce robust STAT5b tyrosine phosphorylation (pTyr). Nuclear STAT5b is dephosphorylated by a tyrosine phosphatase and then returned to the cytoplasm, where it may undergo a subsequent round of activation/deactivation.

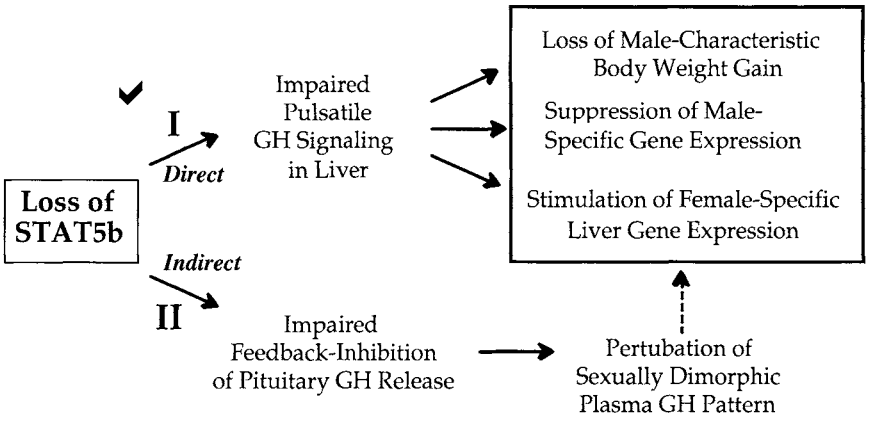

Figure 9.8. Impact of STAT5b loss in knockout (KO) mice. Shown in the *box* at right are the major effects of STAT5b deficiency. These effects all are a direct result of the loss of GH-induced liver STAT5b signaling (model I), rather than an indirect response to impaired feedback-inhibition of pituitary GH release (model II), as demonstrated by the lack of responsiveness of hypophysectomized STAT5b-deficient male mice to GH pulses[148].

gene expression. Thus, in the absence of STAT5b, the liver does not express the male-specific P450s. Moreover, the expression of female-specific, GH-regulated liver P450s is increased to near-normal female levels in STAT5-deficient male mice. Thus, the overall pattern of sexually dimorphic liver gene expression is critically dependent on the presence of STAT5b. The elevated expression in STAT5b-deficient males of female-specific P450s indicates that STAT5b can serve as a negative regulator of female-specific liver P450s, in addition to its positive regulatory effects on male-specific P450 genes.

A second phenotype exhibited by STAT5b-deficient mice is that the male pubertal growth spurt is absent[146]. This growth deficiency does not emerge until the beginning of puberty, and is not seen in STAT5b-deficient females. A male-enhanced pubertal growth spurt is characteristic of all mammals, including humans. In the case of rodents, the pubertal growth spurt is augmented by the strong growth stimulatory effects of the male pattern of pulsatile GH secretion, which explains why this growth response is enhanced in the males. The two major phenotypes that characterize STAT5b knockout mice are also seen in STAT5a/STAT5b double knockout mice[149] but are not seen in mice where the disruption is limited to the STAT5a gene[146, 150], whose protein-coding sequence is ~90% identical to that of STAT5b[151]. Hypophysectomy and GH pulse replacement studies establish that both major phenotypes of STAT5b-deficient mice are a direct response to the loss of STAT5b-dependent GH signaling in the liver, as opposed to indirect effects of the loss of STAT5b on the overall pattern of GH secretion by the pituitary gland[148] (Figure 9.8).

4.2.4.3. Interaction of GH-responsive CYP promoters with GH-activated STAT5b. The strong, repeated pulses of nuclear GH-activated STAT5b that occur in adult male rats have been proposed to induce binding of STAT5b directly to STAT5 response elements found in promoters of STAT5 target genes, which may include sex-dependent P450 genes, and thereby mediate GH pulse-stimulated gene transcription[139]. Consistent with this hypothesis, STAT5 response elements matching the consensus sequence TTC–NNN–GAA have been found upstream of several male-specific rat liver P450 genes, including CYPs 2C11, 2A2, and 4A2 (ref. [152]). GH-stimulated CYP promoter–luciferase reporter activity has been demonstrated using the corresponding isolated STAT5 response elements, although the magnitude of the GH- and STAT5b-dependent gene induction is small, generally only ~2–3-fold[152, 153]. Moreover, although pulsatile STAT5b signaling is first seen in young male rats at ~5 weeks of age, when liver CYP2C11 expression is first detected, precocious activation of

STAT5b, achieved in 3-week-old male rats given pulsatile GH injections, does not lead to precocious *CYP2C11* gene induction[142]. These and other findings suggest that STAT5b may in part act in an *indirect* manner, by regulating the transcriptional activity of liver-enriched transcription factors (HNFs, hepatocyte nuclear factors) that cooperate with STAT5b to control the expression of sexually-dimorphic liver P450 genes[152, 154–156].

4.2.4.4. Interactions between STAT5b and liver transcription factors regulating sex-specific CYPs. Liver-enriched transcription factors are key determinants of the liver-specific expression of many hepatic P450s (ref. [157]), including sex-dependent P450s. Functional analysis of sex-specific liver CYP proximal promoters has led to the identification of the liver transcription factors HNF1α and HNF3β as strong *trans*-activators of CYP2C11 (ref. [152]). By contrast, HNF3β and HNF6 serve as strong *trans*-activators of CYP2C12 (refs [155], [158]), with synergistic interactions between these two liver transcription factors leading to an overall ~300-fold activation of CYP2C12 promoter activity[155]. Other studies indicate a role for C/EBPα[112, 159] and HNF4[156] in the regulation of *CYP2C12* transcription. Interestingly, two of the factors regulating *CYP2C12* expression, HNF6 and HNF3β, require GH for maximal expression in liver, with HNF6 being expressed at 2–3-fold higher levels in female compared to male rat liver[154]. This sex difference in HNF6 levels is too small to account for the >50-fold higher level of CYP2C12 in female compared to male liver. Similarly, the STAT5b-responsiveness of male CYP promoter-derived regulatory elements[152, 153] is too weak to account for the high degree of male-specificity (>100-fold) that characterizes male-specific CYP expression. Accordingly, additional factors and GH regulatory mechanisms are likely to be required for the sex-specific pattern of gene expression seen *in vivo*.

One such mechanism may involve an as yet uncharacterized "regulator of sex-limitation" ("Rsl"), which has been defined genetically and appears to repress certain GH-regulated, sex-dependent liver CYP genes in female mouse liver in a manner that is independent of GH action[160]. Another possible mechanism for GH-regulated sex-specific CYP expression involves interactions between STAT5b and HNFs. For example, studies of the CYP2C12 promoter have shown that STAT5b can substantially block the induction of promoter activity by HNF3β and HNF6 (ref. [152]). This inhibitory action of male GH pulse-activated STAT5b on HNF3β- and HNF6-stimulated *CYP2C12* transcription is consistent with the de-repression (i.e., upregulation) of certain female-specific, GH-regulated mouse P450 genes seen in male STAT5b-deficient mice[146, 150]. The synergistic interaction between HNF6 and HNF3β in stimulating CYP2C12 promoter activity illustrates how small differences in the levels of these factors in liver cells *in vivo* (cf. the 2–3-fold higher expression of HNF6 in females) might lead to much larger differences in *CYP2C12* gene expression, particularly when coupled to the inhibitory action of GH pulse-activated STAT5b in males. Other studies point to additional complexities, based on the potential of STAT5b to upregulate *CYP2C12* gene transcription via an upstream pair of STAT5 response elements[156], and the potential role of an inhibitory, COOH-terminal truncated STAT5 form[161].

4.2.4.5. Downregulation of hepatic STAT5b signaling. Other issues relating to GH and the STAT5b signaling pathway that are of current research interest include how the cycle of STAT5b activation is turned off at the conclusion of each GH pulse, and how STAT5b is subsequently returned to the cytoplasm in an inactive form, where it apparently waits for ~2–2.5 hr until it can be re-activated by the next pulse of GH (Figure 9.7)[162]. Another important question is why robust activation of the STAT5b signaling pathway is not achieved in female liver. The answer to these questions may, in part, involve an intriguing family of inhibitory proteins, referred to as SOCS and CIS proteins, which turn off signals to various hormones and cytokines, including GH[163, 164]. In the case of GH signaling, SOCS proteins bind to the GHR–JAK2 tyrosine kinase complex, enabling them to inhibit GH signaling by a complex series of interrelated mechanisms[165]. One of these inhibitory factors, CIS, may be induced to a higher level by the continuous (female) GH pattern than by the pulsatile (male) GH pattern. This inhibitor of STAT5b signaling has been implicated in the downregulation of GH-induced STAT5b signaling in liver cells exposed to the female GH pattern[165].

4.3. Regulation by Thyroid Hormone

4.3.1. Cytochromes P450

Although GH is the major regulator of specific liver P450s, thyroid hormone also plays a critical role. The major thyroid hormones, T3 and T4, positively regulate some[31, 46] but not all[30] of the female-predominant liver P450 enzymes, while they negatively regulate several of the male-specific enzymes[33, 34] (Table 9.1). These effects of thyroid hormone are operative at the level of mRNA expression, and are independent of the indirect effects that thyroid hormone has on liver P450 levels as a consequence of its effects on liver GHRs[166] and its stimulation of GH gene transcription and GH secretion by the pituitary[167]. Molecular studies of these effects of thyroid hormone have not been carried out.

4.3.2. NADPH-Cytochrome P450 Reductase

Thyroid hormone is also required for full expression of NADPH-cytochrome P450 reductase, a flavoenzyme that catalyzes electron transfer to all microsomal P450s. P450 reductase is an obligatory, and often rate-limiting electron-transfer protein that participates in all microsomal P450-catalyzed drug oxidation and steroid hydroxylase reactions[168, 169]. This thyroid hormone dependence of P450 reductase enzyme expression is evidenced by the major decrease (>80% reduction) in liver microsomal P450 reductase activity and P450 reductase mRNA levels that occurs following hypophysectomy[170] or in response to methimazole-induced hypothyroidism[171]. It is further supported by the reversal of this activity loss when thyroxine (T4), but not GH or other pituitary-dependent factors, is given at a physiological replacement dose[170, 171]. Restoration of liver P450 reductase activity *in vivo* by T4 replacement also effects a substantial increase in liver microsomal P450 steroid hydroxylase activities. A similar effect can be achieved when liver microsomes isolated from hypophysectomized rats are supplemented with exogenous, purified P450 reductase, which preferentially stimulates steroid hydroxylation catalyzed by microsomes prepared from thyroid-deficient animals[170]. The induction of rat hepatic P450 reductase by thyroid hormone in rats occurs by transcriptional[172] and post-transcriptional mechanisms[173] and appears to involve enhanced protein stability in hyperthyroid rat liver[174]. P450 reductase levels are also modulated by thyroid hormone status in several extrahepatic tissues[171]. Conceivably, interindividual differences in P450 reductase levels may occur in response to physiological or pathophysiological differences in circulating thyroid hormone levels and could be an important contributory factor to individual differences in P450 reductase/cytochrome P450-catalyzed carcinogen metabolism and carcinogen activation reactions.

5. Alteration of Liver P450 Expression by Hormonal Perturbation

As discussed earlier in this chapter, many, although not all, liver P450s are under hormone regulatory controls. An individual's circulating hormonal profile can, however, be altered under certain situations, including drug therapy, exposure to chemicals found in the environment, and disease states such as diabetes and liver cirrhosis. The resultant changes in circulating hormone levels or alterations in hormone secretory dynamics could, therefore, influence the expression of specific liver P450s. The following sections describe some of the factors that are known to cause hormonal perturbation and discuss the impact of these changes on liver P450 expression and on P450-dependent drug and xenobiotic metabolism and toxicity.

5.1. Modulation by Drugs

The anticancer drugs cisplatin[175, 176], cyclophosphamide[177, 178], and ifosfamide[178] alter the profile of P450 enzyme expression in liver and perhaps other tissues, at least in part due to the hormonal perturbations that these cytotoxic agents induce. Treatment of adult male rats with a single dose of cisplatin depletes serum androgen, and this effect persists for up to 28 days after drug administration[175]. Serum androgen depletion by cisplatin is associated with a feminization of hepatic liver enzyme expression. Thus, cisplatin-treated male rats have elevated levels of the female-predominant

CYP2A1, CYP2C7, and steroid 5α-reductase, but have reduced levels of the male-specific CYP2A2, CYP2C11, and CYP3A2 (refs [175], [176]). The effects of cisplatin on androgen levels may result from the drug's action on the testes[179, 180]; however, effects on the hypothalamus are also suggested to contribute, both to the observed depletion of circulating testosterone and the resultant alteration in liver P450 expression[175]. Cisplatin treatment of adult female rats severely decreases circulating estradiol levels and significantly reduces the expression of the estrogen-dependent CYP2A21, CYP2C7, and CYP2C12 (ref. [176]).

Serum testosterone is also depleted in adult male rats treated with cyclophosphamide[177, 178, 181] or ifosfamide[178] and this depletion is associated with feminization of liver enzyme profiles[177, 178] in a manner similar to that produced by cisplatin. While endogenous androgen secretion can be stimulated in cyclophosphamide-treated rats by the luteinizing hormone analog chorionic gonadotropin, the resultant increase in serum testosterone does not reverse the loss of hepatic *CYP2C11* expression[177]. This observation is analogous to the earlier finding that the suppression of CYP2C11 by 3,4,5,3',4',5'-hexachlorobiphenyl[182] is not causally related to the associated depletion of serum testosterone[183]. Consequently, modulation of liver enzyme expression by cyclophosphamide may involve action at the hypothalamic–pituitary axis, which establishes the sex-dependent plasma GH profile that in turn dictates the expression of CYP2C11 and other sex-dependent liver P450 enzymes, as discussed earlier in this chapter. CYP2C11 can also be suppressed by other mechanisms, as demonstrated by the finding that CYP2C11 levels are suppressed by the anticancer drug 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU; lomustine) without affecting circulating testosterone levels[184]. Conceivably, CCNU may act directly on the hypothalamic–pituitary axis to alter key signaling elements in the ultradian rhythm of circulating GH.

Other drugs that suppress hepatic CYP2C11 and CYP3A2 levels include chloramphenicol[185] and cyclosporine[186, 187]. The effects of chloramphenicol are strain-specific, occurring in Sprague–Dawley rats but not in Fischer 344 rats. Moreover, this suppression is accompanied by a modest reduction in plasma levels of thyroxine but not testosterone[185]. GH does not appear to play a role in the suppression of CYP2C11 and CYP3A2 by cyclosporine, a drug that does not alter the plasma GH peak amplitude, number, or duration[188]. Phenobarbital[19, 189, 190], dexamethasone[191], and 5-fluorouracil[192] also reduce hepatic *CYP2C11* expression, but the mechanism(s) by which these effects occur have not been elucidated.

5.2. Modulation by Polycyclic Aromatic Hydrocarbons

Exposure of adult male rats to polycyclic aromatic hydrocarbons, including 3-methylcholanthrene (3MC)[182, 190, 193], 2,3,7,8-tetrachlorodibenzo-*p*-dioxin[194], anthracene, benz(*a*)anthracene, dibenz(*a,c*)anthracene, dibenz(*a,h*)anthracene, and 7,12-dimethylbenz(*a*)anthracene[195], leads to decreases in hepatic CYP2C11 protein and activity levels. In the case of 3MC, this suppression reflects a decrease in the rate of *CYP2C11* transcription[196]. The hormonal mechanisms by which polycyclic aromatic hydrocarbons modulate *CYP2C11* expression are not known, however, 3MC[182] and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin[197] have been reported to decrease serum testosterone levels. 3MC may interfere with the stimulation of *CYP2C11* expression by GH[198], but in a manner that does not involve STAT5b[199]. Interestingly, the extent of *CYP2C11* suppression by polycyclic aromatic hydrocarbons is correlated with Ah receptor-binding affinity and Ah receptor transformation potency[195]. However, it is not clear whether the Ah receptor plays a role in the transcriptional suppression of *CYP2C11* by polycyclic aromatic hydrocarbons[200].

5.3. Modulation by Pathophysiological State

5.3.1. Diabetes

Uncontrolled insulin-dependent diabetes is accompanied not only by defective carbohydrate metabolism, which results in hyperglycemia, hyperlipidemia, and hyperketonemia, but it is also associated with hormonal perturbation, including a reduction in circulating testosterone[201–203], thyroid hormone, and plasma GH[204, 205]. As described earlier in this chapter, these hormones regulate many liver P450 enzymes, either directly or indirectly. Accordingly, the diabetic state is

associated with profound changes in the levels of various hepatic P450 enzymes. Whereas diabetes leads to induction of several rat liver P450 forms, including CYP2A1 (refs [205], [206]), CYP2B1 (refs [207], [208]), CYP2C7 (ref. [206]), CYP2E1 (refs [209]–[212]), CYP4A2 (ref. [206]), and CYP4A3 (ref. [206]), it suppresses CYP2A2 (ref. [205]), CYP2C11 (refs [205], [207], [208], [212]), and CYP2C13 (ref. [205]). Changes in the levels of some of these liver P450s (e.g., CYP2C11 and CYP2E1) have been shown at the mRNA level[207, 208, 213, 214] and are reversed by insulin replacement.

The profile of GH secretion in the diabetic male rat is altered so as to resemble the pattern found in the normal female rat[204]. The induction of CYP2A1 and CYP2C7 in diabetic male rats can therefore be explained, at least in part, as a response to the more continuous pattern of GH secretion, which stimulates expression of these P450 forms[27, 31, 63, 215]. In contrast, this pattern of GH secretion reduces CYP2A2 and CYP2C13 levels because continuous GH administration suppresses these two P450s[27, 28, 116]. *CYP2C11* expression is obligatorily dependent on the intermittent male pattern of plasma GH secretion[73]. Therefore, the more continuous secretion of GH in diabetic male rats[204] would be expected to suppress this P450. In the case of CYP2B1, GH pulse height is the suppressive signal[102] and accordingly, the reduction in GH peak concentration in diabetic male rats[205] leads to increases in CYP2B1 levels[207, 208]. A GH-independent mechanism is likely to contribute to some of the other effects of diabetes on liver P450 expression. GH, independent of its plasma profile, is suppressive toward hepatic CYP2E1 (ref. [62]), but the levels of this P450 are substantially elevated in both diabetic male and female rats[205, 216, 217]. CYP2E1 induction in diabetes has been attributed to increased plasma concentrations of ketone bodies[210, 218]. Recently, a role of hypoinsulinemia and hyperglucagonemia was proposed for the suppression of *CYP2C11* in diabetes, based on the finding that treatment of cultured rat hepatocytes with glucagon decreases *CYP2C11* expression in a cAMP-dependent manner and this decrease can be reversed by insulin administration[219].

5.3.2. Liver Cirrhosis

Gonadal abnormalities occur in liver cirrhosis. Adult male rats fed a chronic choline-deficient diet to induce cirrhosis have enhanced serum estradiol concentrations[220] and reduced testicular weight[221] and serum testosterone levels[220]. In association with the perturbation in hormonal status is a major decline in hepatic CYP2C11 content[220], and this decline is not accompanied by induction of steroid 5α-reductase activity[222]. The suppression of hepatic *CYP2C11* is also evident in other models of liver cirrhosis, including bile duct ligation[223, 224] and carbon tetrachloride-induced cirrhosis[223, 224]. Whether the alteration in serum steroid hormone levels is directly or indirectly responsible for the apparent demasculinization of liver P450 remains to be determined.

5.4. Modulation by Ethanol and Dietary Factors

Adult male rats administered ethanol by a total enteral nutrition system have reduced hepatic CYP2C11 and CYP3A2 levels, whereas their CYP2A1 activity is unaltered[225]. The same ethanol treatment alters the dynamics of plasma GH secretion by decreasing the GH pulse amplitude and increasing the GH pulse frequency. The increased frequency of GH pulses thus explains the reduced expression of *CYP2C11* after chronic ethanol intake because hepatocytes require a minimum "off time" in order to express the male-pattern of GH secretion that stimulates *CYP2C11* expression[73]. In another study, chronic intragastric infusion of ethanol-containing diets resulted in suppression of *CYP3A2* while substantially increasing the expression of *CYP3A9* in adult male rats[226].

Dietary vitamin-A deficiency reduces hepatic CYP2C11 (refs [227]–[229]) and CYP4A2 levels[229], while inducing steroid 5α-reductase activity in adult male rats[230]. These effects, which are accompanied by a reduction in plasma testosterone levels, can be restored by the inclusion of all-*trans*-retinoic acid in the diet[228] or by exogenous administration of androgen[229, 231]. Interestingly, twice daily subcutaneous administration of GH, which is known to induce *CYP2C11* expression in hypophysectomized male rats[73], is not effective in restoring the levels of CYP2C11 or CYP4A2 in male rats fed a vitamin A-deficient diet[229].

Dietary trace minerals can also alter liver expression of sex-dependent P450 enzymes. Prepubertal male rats fed a zinc-deficient diet

during the pubertal period have depleted serum androgen levels and a feminized pattern of hepatic mRNA expression, as evidenced by a reduction in CYP2C11, CYP3A2, and CYP3A18 and by an elevation in CYP2C12 and CYP3A9 (ref. [232]). However, the precise neuroendocrine mechanisms responsible for the feminization of hepatic P450 enzyme expression by dietary zinc deficiency remain to be elucidated.

Finally, caloric restriction and food deprivation have been shown to modulate hepatic expression of sex-dependent liver steroid-metabolizing enzymes, including CYP2C11 (ref. [233]) which is suppressed, and CYP3A9 (ref. [234]) and steroid 5α-reductase[233], which are induced. These are situations in which glucagon levels are high and insulin levels are low, analogous to the diabetic state (see Section 5.3.1).

5.5. Impact on Drug Metabolism and Procarcinogen Activation

As discussed in Section 5.1, the anticancer drug cisplatin provides an example of a foreign chemical that depletes serum testosterone and consequently feminizes the expression of liver P450s in adult male rats. This type of alteration in the profile of liver P450 enzymes could have important pharmacological consequences, as suggested by the finding that cisplatin suppression of *CYP2C11* decreases liver P450-catalyzed activation of anticancer prodrugs, such as cyclophosphamide[176, 235, 236] and ifosfamide[236] when assayed in isolated microsomal systems. Liver P450 activation of these latter two drugs is required for their anticancer drug activity[237], and CYP2C11 contributes significantly to this metabolic pathway in male rat liver[235, 236]. Clinical studies indicate that cisplatin may exert effects on circulating hormone levels in human cancer patients that are similar, though not identical, to those seen in rats[238]. If these hormone perturbations in turn alter P450 enzyme levels in human liver, this could have an impact on drug–drug interactions in patients given cisplatin in combination with anticancer P450 prodrugs such as cyclophosphamide.

As discussed above, drug metabolism in diabetic rats can be altered as a consequence of perturbations of GH secretory patterns and the resultant changes in the expression of multiple liver P450 enzymes. Accordingly, diabetes is associated with a decrease in P450-mediated *in vitro* hepatic metabolism of imipramine[203, 239], lidocaine[203], codeine, and chlorpromazine[240]. In addition, alteration of liver P450 expression in diabetes is postulated to be responsible for the enhanced *in vitro* metabolic activation of certain chemical carcinogens, including Try-P-1(3-amino-1,4-dimethyl,5H-pyrido(4,5-b)indole) and Try-P-2(3-amino-1-methyl-5H-pyrido(4,3-b) indole)[241]. These examples demonstrate the potential for alterations in liver P450 expression that potentially lead to reduced drug metabolism and enhanced procarcinogen activation. Further investigations will be necessary to determine the extent to which these events occur in humans and to evaluate their true pharmacological and toxicological significance.

6. Conclusion

Sex-dependent liver P450 enzymes are subject to complex patterns and multiple mechanisms of hormonal control. Plasma GH pulsatility, acting via transcriptional mechanisms, is a key regulator of liver-expressed, sex-dependent P450 enzymes and their associated steroid hydroxylation and xenobiotic metabolism activities. This results in the sex differences in metabolism, pharmacokinetics, and toxicity of various xenobiotics that are seen in rodents and other species, including humans. The pulsatile plasma GH profile characteristic of adult male rats repeatedly stimulates tyrosine phosphorylation that directly activates the transcription factor STAT5b. This triggers translocation of STAT5b into the nucleus, where it is proposed to modulate transcription of sex-dependent liver P450 genes through both direct and indirect mechanisms. Continuous or near-continuous exposure to GH, as occurs in the female rodent liver, can downregulate liver STAT5b signaling. As a consequence, active nuclear STAT5b protein is generally found at a low level in adult female rat liver. Thyroid hormones are also important endocrine regulators of liver metabolic function that can act directly, by influencing the expression of individual P450 enzymes, as well as indirectly, via their effects on NADPH-P450 reductase gene expression.

Gonadal hormones also play a role, but their effects on liver P450 activity are largely indirect and are mediated by the hypothalamic–pituitary axis and its control of plasma GH levels. Sex differences in GH secretory dynamics also occur in humans and can be modulated by gonadal hormones. This sexually dimorphic GH secretion may contribute to the apparent sex differences in the levels of certain human P450s and in human P450-catalyzed drug metabolism and pharmacokinetics. Finally, diverse factors, including many drugs and other xenochemicals, pathophysiological state, and dietary factors can affect hormone secretion and the hepatic expression of sex-dependent P450s, which in turn may impact drug metabolism and procarcinogen activation.

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10

Human Cytochrome P450 Enzymes

F. Peter Guengerich

1. Background and History of Development of the Field

Much of P450 research has always been done with the view of application to humans, even when done with experimental animals and microorganisms. Research with the human P450s has been done in several stages, and clinical pharmacology has utilized the knowledge at each point in its development.

Aside from *in vivo* experiments with drugs, human P450 work in the late 1960s and 1970s was done with tissue samples, primarily biopsies. Some data on metabolic patterns and rates were collected[1]. In the late 1970s, several groups began to purify P450s from human liver microsomes. The first purified proteins, selected because of their abundance and ease of purification, were probably what are recognized now as P450 3A and 2C subfamily proteins[2–4]. Efforts were shifted to purifying individual P450s on the basis of catalytic activities with the evidence that in some cases a single P450 could be identified in this way; for example, the enzyme now known as P450 2D6 was found to be under monogenic control[5]. The approach is technically demanding because of the need to do separations in the presence of detergents and then remove them from individual chromatography fractions prior to analysis of catalytic activity. Nevertheless, human P450s 1A1 (ref. [6]), 1A2 (ref. [7]), 2A6 (ref. [8]), 2C9 (ref. [9]), 2D6 (refs [7], [10], [11]), and 3A4 (ref. [12]), were isolated in this general manner. Another general approach that was used was purification from tissue on the basis of immunochemical cross-reactivity with animal P450s[13–15].

With the development of recombinant DNA technology, cDNAs for many of the human P450s were cloned in the 1980s[16]. In the late 1980s, methods came into use for the heterologous expression of P450s, first in mammalian and yeast systems, and then in baculovirus and (by the mid-1990s) in bacterial systems[17–20]. In the late 1980s, the nomenclature system developed by Nebert[21] was applied and allowed individual human and other P450s to be discussed on the basis of their sequences. (For a guide to some of the earlier nomenclature, see ref. [22].)

By the early 1990s, much of the interest in P450 research had shifted to the human P450 enzymes because of the availability of systems for handling these. In particular, studies in the areas of drug metabolism and chemical toxicology/carcinogenesis were facilitated by the knowledge that a relatively small number of the P450s account for a large fraction of the metabolism of the drugs and other chemicals of interest. In the pharmaceutical industry, the roles of the major hepatic P450s are extensively studied in developing predictions

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about bioavailability, drug–drug interactions, and toxicity. Since the publication of the chapter on human P450s in the last edition of this book[23], apparently all of the remaining human P450 genes have been identified, and the number (57) appears to be complete because of more general knowledge about the human genome (html://drnelson.utmem.edu/CytochromeP450.html). Much of the progress since 1995 has involved extrahepatic P450s, many with roles in the processing of "endobiotic" chemicals, for example, sterols and vitamins.

The list of the 57 human P450s is presented in Table 10.1, along with available knowledge about

Table 10.1. Human P450s

P450	Tissue sites	Subcellular localization[a]	Typical reaction[b]
1A1	Lung, several extrahepatic sites, peripheral blood cells	ER	Benzo[*a*]pyrene 3-hydroxylation
1A2	Liver	ER	Caffeine N^3-demethylation
1B1	Many extrahepatic sites, including lung and kidney	ER	17β-Estradiol 4-hydroxylation
2A6	Liver, lung, and several extrahepatic sites	ER	Coumarin 7-hydroxylation
2A7		ER	
2A13	Nasal tissue	ER	Activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)
2B6	Liver, lung	ER	(*S*)-Mephenytoin *N*-demethylation
2C8	Liver	ER	Taxol 6α-hydroxylation
2C9	Liver	ER	Tobutamine methyl hydroxylation
2C18	Liver	ER	
2C19	Liver	ER	(*S*)-Mephenytoin 4′-hydroxylation
2D6	Liver	ER[c]	Debrisoquine 4-hydroxylation
2E1	Liver, lung, other tissues	ER	Chlorzoxazone 6-hydroxylation
2F1	Lung	ER	3-Methylindole activation
2J2	Lung	ER	Arachidonic acid oxidations
2R1			
2S1	Lung	ER	
2U1			
2W1			
3A4	Liver, small intestine	ER	Testosterone 6β-hydroxylation
3A5	Liver, lung	ER	Testosterone 6 β-hydroxylation
3A7	Fetal liver	ER	Testosterone 6 β-hydroxylation
3A43	(mRNA detected in gonads)	(ER)	
4A11	Liver	ER	Fatty acid ω-hydroxylation
4A22		ER	
4B1	Lung	ER	Lauric acid ω-hydroxylation
4F2	Liver	ER	Leukotriene B_4 ω-hydroxylation
4F3	Neutrophils	ER	Leukotriene B_4 ω-hydroxylation
4F8	Seminal vesicles	ER	Prostaglandin ω-2 hydroxylation

Table 10.1. Continued

P450	Tissue sites	Subcellular localization[a]	Typical reaction[b]
4F11	Liver	ER	
4F12	Liver	ER	Arachidonic acid ω-,ω-2-hydroxylation
4F22			
4V2			
4X1			
4Z1			
5A1	Platelets	ER	Thromboxane A_2 synthase reaction
7A1	Liver	ER	Cholesterol 7α-hydroxylation
7B1	Brain	ER	Dehydroepiandrosterone 7α-hydroxylation
8A1	Aorta, others	ER	Prostacyclin synthase reaction
8B1	Liver	ER	7α-hydroxyprogesterone 12-hydroxylation (?)
11A1	Adrenals, other steroidogenic tissues	Mit	Cholesterol side-chain cleavage
11B1	Adrenals	Mit	11-Deoxycortisol 11-hydroxylation
11B2	Adrenals	Mit	Corticosterone 18-hydroxylation
17A1	Steroidogenic tissues	ER	Steroid 17α-hydroxylation
19A1	Steroidogenic tissues, adipose, brain	ER	Androgen aromatization
20A1			
21A2	Steroidogenic tissues	ER	17-Hydroxyprogesterone 21-hydroxylation
24A1	Kidney	Mit	25-Hydroxyvitamin D_3 24-hydroxylation
26A1	Several	ER	Retinoic acid 4-hydroxylation
26B1	Brain	ER	Retinoic acid 4-hydroxylation
26C1		(ER?)	
27A1	Liver	Mit	Sterol 27-hydroxylation
27B1	Kidney	Mit	Vitamin D_3 1-hydroxylation
27C1			
39A1	Liver (?)	ER	24-Hydroxycholesterol 7-hydroxylase
46A1	Brain	ER	Cholesterol 24-hydroxylation (?)
51A1	Liver, testes	ER	Lanosterol 14α-demethylation

[a]ER = endoplasmic reticulum (microsomal), Mit = mitochondria.
[b]If known.
[c]Mainly ER, some detected in mitochondria.

sites of tissue expression, subcellular localization, and a typical reaction. In the 1995 edition[23], the list included 31 human P450s, and 2 of these have been dropped as apparent cloning artifacts (2C10, 2C17), leaving 29. Thus, the list was only half complete at that time. It should be emphasized that we still have little knowledge about the roles of some of these P450s beyond the genomic information. Of the 57, most that have been examined appear to be expressed primarily in the endoplasmic reticulum and only 6 are located exclusively in mitochondria. (However, work in animal models by Avadhani has clearly demonstrated the import of what have been generally considered microsomal P450s into mitochondria[24, 25]. The basis of this transport appears to be signals in the N-terminal region[26]. Limited information is available about this phenomenon with human P450s;

recent collaborative experiments with Avadhani's group indicate that many human liver samples contain immunochemically detectable and catalytically active P450 2D6 in mitochondrial as well as microsomal fractions [N. Avadhani and F.P. Guengerich, unpublished results].) In many cases, no direct information is available because protein studies have not been done, although most of these "orphan" P450s are predicted to reside primarily in the endoplasmic reticulum.

Of the P450s with known catalytic activities, 14 are clearly involved in steroidogenesis, 4 are involved in what appear to be important aspects of metabolism of vitamins (vitamins A and D), 5 are involved in eicosanoid metabolism, 4 appear to have fatty acids as their substrates, and 15 catalyze transformation of xenobiotic chemicals (Table 10.2). Some of these categories should be considered tentative. This classification accounts for only 42 of the 57 P450s. Many of the xenobiotic-metabolizing P450s can also catalyze oxidation of steroids and fatty acids, but these functions do not appear to be critical to homeostasis (e.g., testosterone 6β-hydroxylation by P450 3A4, lauric acid 11-hydroxylation by P450 2E1, possibly 17β-estradiol 4-hydroxylation by P450 1B1). Most of the steroid-oxidizing enzymes are critical, and the levels of these P450s are relatively invariable among individuals, in contrast to the xenobiotic-metabolizing P450s, which vary considerably (Figures 10.1 and 10.2). Deficiencies in the expression or catalytic activities of most of the P450s involved in steroid metabolism lead to serious diseases (Table 10.3) (included in this list[29] are P450s 1B1 [true function unknown] and 24A1 [vitamin D hydroxylation]). In one case, a high level of P450 activity (P450 19) can be a problem (estrogen formation in estrogen-dependent tumors) and this P450 is a target for therapeutic attenuation[30]. The 15 identified xenobiotic-metabolizing P450 are mainly in the families 1–3, and levels of these vary considerably in humans (Figures 10.1 and 10.2). Studies with transgenic (knockout) mice do not indicate critical function associated with the apparent orthologs in the absence of xenobiotic challenge[31]. Analysis of the lists of drugs in which individual human P450s are involved[32] indicates that ~75% of the drugs can be oxidized by 3 P450s (3A4, 2D6, 2C9), and a set of 6–7 P450s will account for 90–95% of all drug metabolism (Figure 10.3)[33]. Similar numbers of P450s are involved in the metabolism of chemical carcinogens, although the pattern shifts from that of Figure 10.3, with P450s 2C19 and 2D6 being replaced by P450s 1A1, 1B1, 2A6, and 2E1 (Table 10.4). The relative contributions of these xenobiotic-metabolizing P450s are, to some extent, a function of the relative abundance (Table 10.5, Figure 10.4), although there are some important exceptions. Further, the

Table 10.2. Classification of Human P450s based on Major Substrate Class[a]

Sterols	Xenobiotics	Fatty acids	Eicosanoids	Vitamins	Unknown
1B1	1A1	2J2	4F2	24	2A7
7A1	1A2	4A11	4F3	26A1	2R1
7B1	2A6	4B1	4F8	26B1	2S1
8B1	2A13	4F12	5A1	27B1	2U1
11A1	2B6		8A1		2W1
11B1	2C8				3A43
11B2	2C9				4A22
17	2C18				4F11
19	2C19				4F22
21A2	2D6				4V2
27A1	2E1				4X1
39	2F1				4Z1
46	3A4				20
51	3A5				26C1
	3A7				27C1

[a]As pointed out in the text, this classification is somewhat arbitrary, for example, P450s 1B1 and 27A1 could be grouped in two different categories.

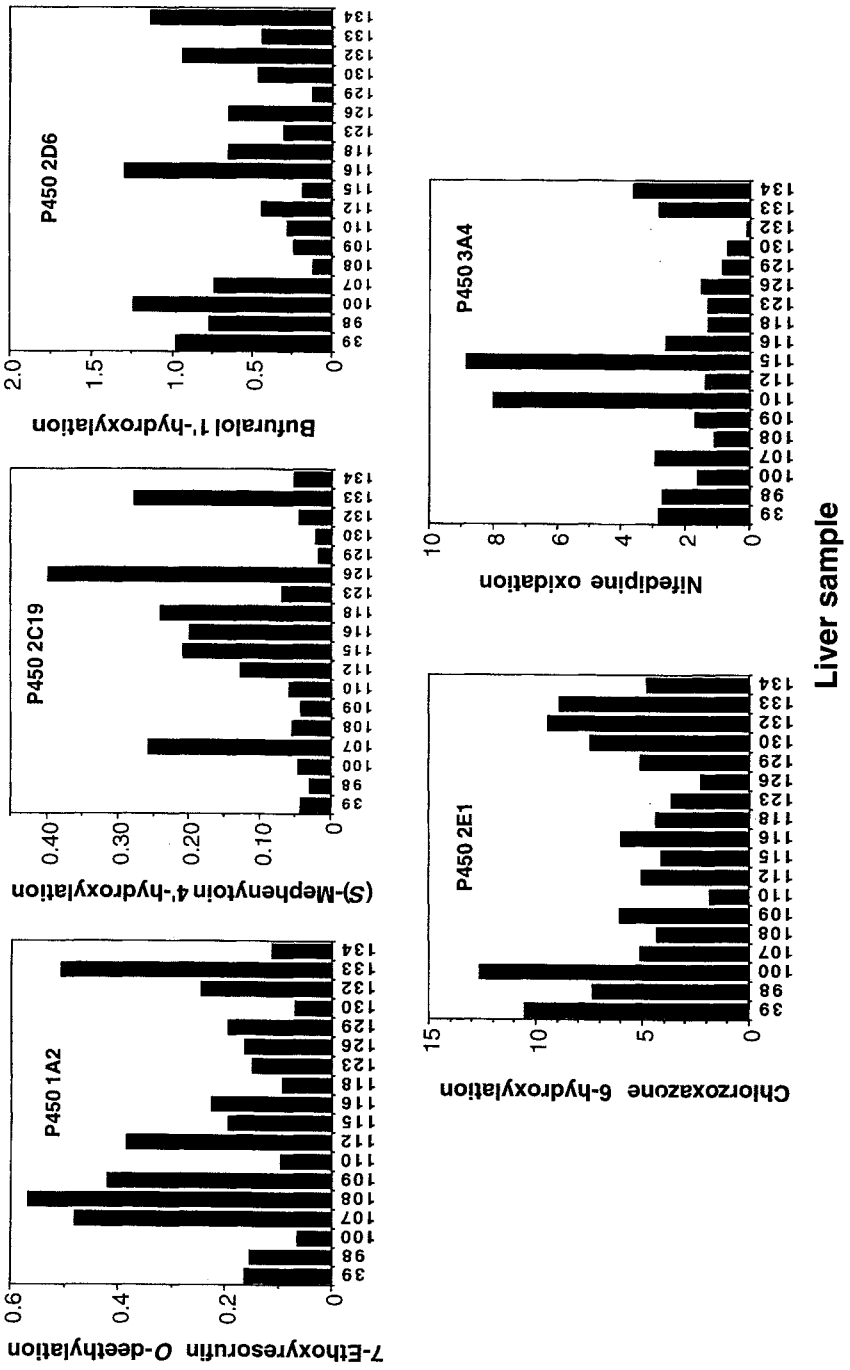

Figure 10.1. Variation in levels of five P450s in 18 human liver samples. Individual P450s and catalytic activities are indicated on each chart[27]. Sample number refers to a code from this laboratory.

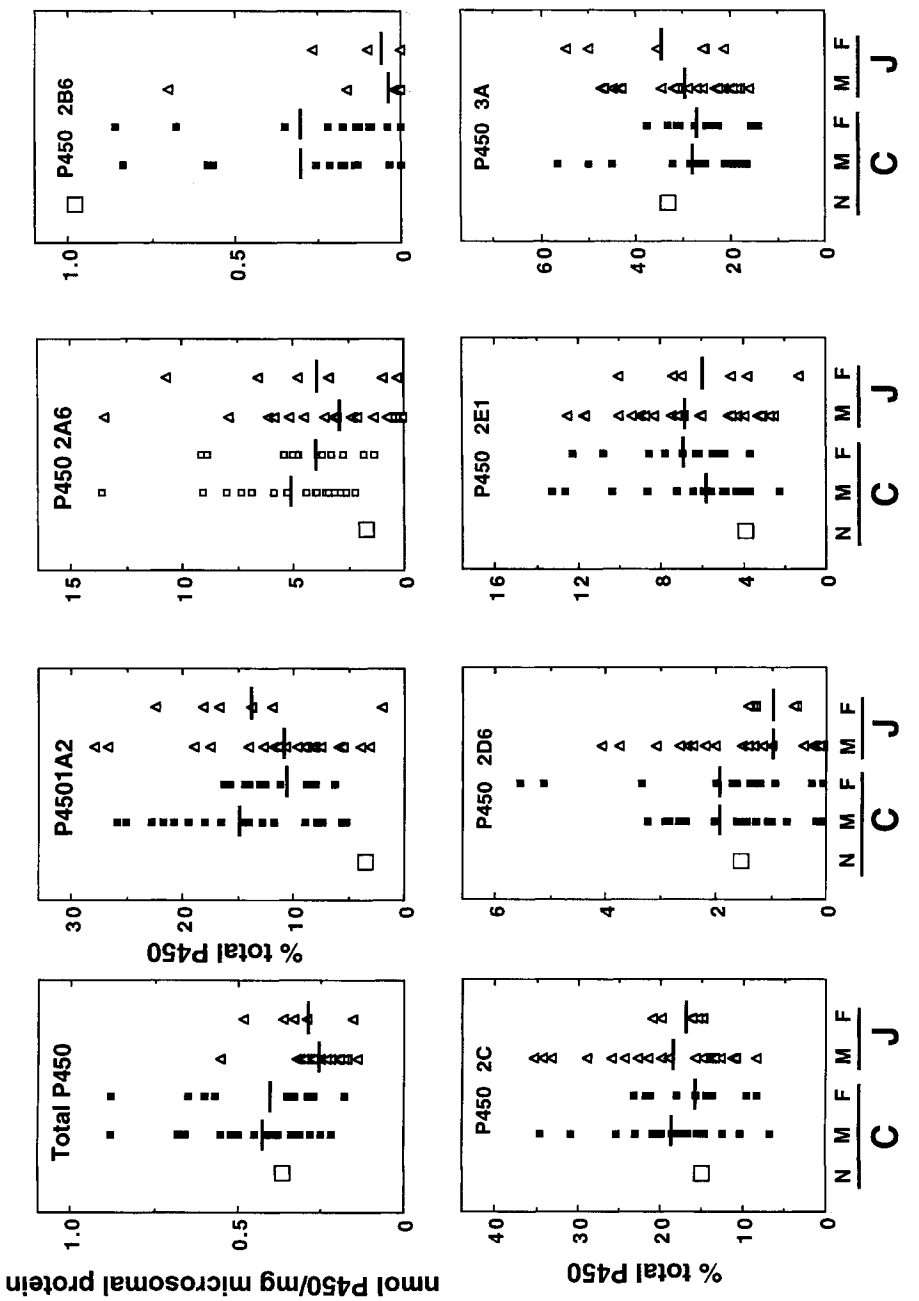

Figure 10.2. Comparison of some immunochemically determined levels of individual P450s and catalytic activities in human liver microsomes. Results from samples obtained from Caucasian (C) and Japanese (J) males (M), females (F), and a single neonatal (Japanese) sample (N) are shown[28]. The vertical axis is nmol P450/mg protein in the "Total P450" chart and percentage of total P450 in all other cases. Horizontal bars indicate mean values.

Table 10.3. Diseases Associated with Mutations in *CYP* Genes[29]

Gene	Disorder
CYP1B1	Primary congenital glaucoma (buphthalmos)
CYP4A, 4B[a]	Defects in salt metabolism, water balance leading to arterial hypertension
CYP5A1, 8A1	Defects leading to clotting and inflammatory disorders, coronary artery disease, and pulmonary hypertension
CYP7A1	Hypercholesterolemia, resistance to statin drugs
CYP7B1	Severe hyperoxysterolemia and neonatal liver disease
CYP11A1	Lipoid adrenal hyperplasia; occasional congenital adrenal hyperplasia (CAH)
CYP11B1	Occasional CAH
CYP11B2	Corticosterone methyloxidase deficiency type I, or type II; occasional CAH
CYP11B1, 11B2	Chimeric enzymes causing glucocorticoid-remediable aldosteronism; occasional CAH
CYP17A1	Mineralocorticoid excess syndromes, glucocorticoid, and sex hormone deficiencies; association with increased risk of prostate cancer and benign prostatic hypertrophy; occasional CAH
CYP19A1	Loss of function: virilization of females, hypervirilization of males, occasional CAH; gain of function: gynecomastia in young males
CYP21A2	>90% of all CAH
CYP24A1[a]	Hypervitaminosis D
CYP27A1	Cerebrotendinous xanthomatosis
CYP27B1	Vitamin D-dependent rickets type I

[a]Strong evidence of disease in animal models but not yet in clinical studies.

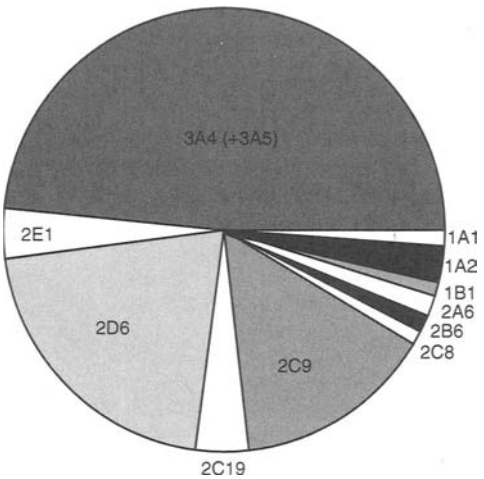

Figure 10.3. Estimated contributions of individual human P450s to the metabolism of all drugs, based upon literature available (adapted from Evans and Relling)[33].

patterns reported for human liver (Table 10.5, Figures 10.1, 10.2, and 10.4) do not necessarily apply in other tissues, some of which may be targets for carcinogens and other toxicants[37].

2. General Issues of Variability and Polymorphism

Variability in patterns of drug metabolism has been recognized for some time, even before the discovery of P450s. For instance, the phenomenon of pharmacogenetic variation had been identified by the 1950s[38, 39] and the early work of Remmer[40] showed the influence of barbiturates upon drug metabolism. Further, a number of congenital defects in steroid metabolism were known and some could be attributed to alterations in specific hydroxylations[41]. Much of the subsequent work on inducibility has been done in experimental animal models[42] and later, cell culture.

In the 1960s and 1970s, a number of accounts appeared describing variations in rates of metabolism of drugs in human liver biopsy samples[1]. The first characterization of a monogenic variability in a human drug-metabolizing P450 was the work of Smith with debrisoquine[5], which was paralleled by the work of Dengler and Eichelbaum on sparteine[43]. This polymorphism was first described in the context of extensive metabolizers (EMs) and poor metabolizers (PMs) (Figure 10.5). These

Table 10.4. Some Human P450 Enzymes involved in the Activation of Carcinogens[34] (See also Table 10.8)

P450 1A1	P450 1A2	P450 2A6	P450 2E1	P450 3A4
Benzo[*a*]pyrene and other polycyclic hydrocarbons 2-Amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP)	PhIP 2-Amino-6-methyl-dipyrido[1,2-*a*:3,2′-*d*]-imidazole (Glu P-1) 2-Aminodipyrido- [1,2-*a*:3,2′-*d*] imidazole (Glu P-2) 2-Amino-3-methylimidazo-[4,5-*f*] quinoline (IQ) 2-Amino-3,5-dimethyl-imidazo[4,5-*f*] quinoline (MeIQ) 2-Amino-3,8-dimethyl-imidazo[4,5-*f*]quinoline (MeIQx) 3-Amino-1-methyl-5*H*-pyrido [4,3-*b*]indole (Trp P-2) 4-Aminobiphenyl 2-Naphthylamine 2-Aminofluorene 2-Acetylaminofluorene	*N,N*-Dimethylnitrosamine (DMN) *N,N*-Diethylnitrosamine (DEN) NNK 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butano (NNAL) Nornitrosonicotine (NNN) 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	Benzene Styrene Acrylonitrile Vinyl carbamate Vinyl chloride Vinyl bromide Ethyl carbamate Trichlorethylene Carbon tetrachloride Chloroform DMN DEN NNK NNAL NNN Butadiene	Aflatoxin B_1 Aflatoxin G_1 Sterigmatocystin 7,8-Dihydroxy-7,8-dihydrobenzo(*a*)pyrene and some other polycyclic hydrocarbons 17β-Estradiol 6-Aminochrysene Senecionine 4,4′-Methylene-*bis* (2-chloroaniline) (MOCA) *tris*(2,3-Dibromopropyl) phosphate

Table 10.5. Contents of Liver Microsomal P450 Enzymes in Japanese and Caucasian Populations[28]

	n	Total P450 (spectral assay)	P450 (pmol P450/mg protein [% of total P450])							Total of these 7 P450s (immunochemical sum)
			1A2	2A6	2B6	2C9	2D6	2E1	3A4	
Total	72	309 ± 175 (100)	37 ± 24 (13)	13 ± 13 (4.0)	0.68 ± 1.4 (0.15)	55 ± 28 (20)	4.5 ± 2.9 (1.7)	20 ± 13 (6.6)	87 ± 53 (29)	217 ± 107 (73)
Japanese	40	233 ± 102 (100)	26 ± 20 (12)	6.5 ± 7.3 (2.8)	0.14 ± 0.62 (0.03)	46 ± 23 (21)	3.0 ± 1.9 (1.4)	15 ± 9 (6.4)	72 ± 44 (30)	168 ± 80 (74)
Caucasian	32	406 ± 199 (100)	50 ± 22 (14)	21 ± 14 (5.6)	1.4 ± 1.8 (0.29)	68 ± 29 (18)	6.4 ± 2.8 (1.9)	26 ± 14 (6.9)	106 ± 58 (27)	277 ± 106 (73)

Total P450 contents in liver microsomes were determined spectrally and individual forms of P450 were assayed immunochemically. All values are the means and standard deviations. Parentheses indicate relative contents (% of total P450) of individual P450 forms.

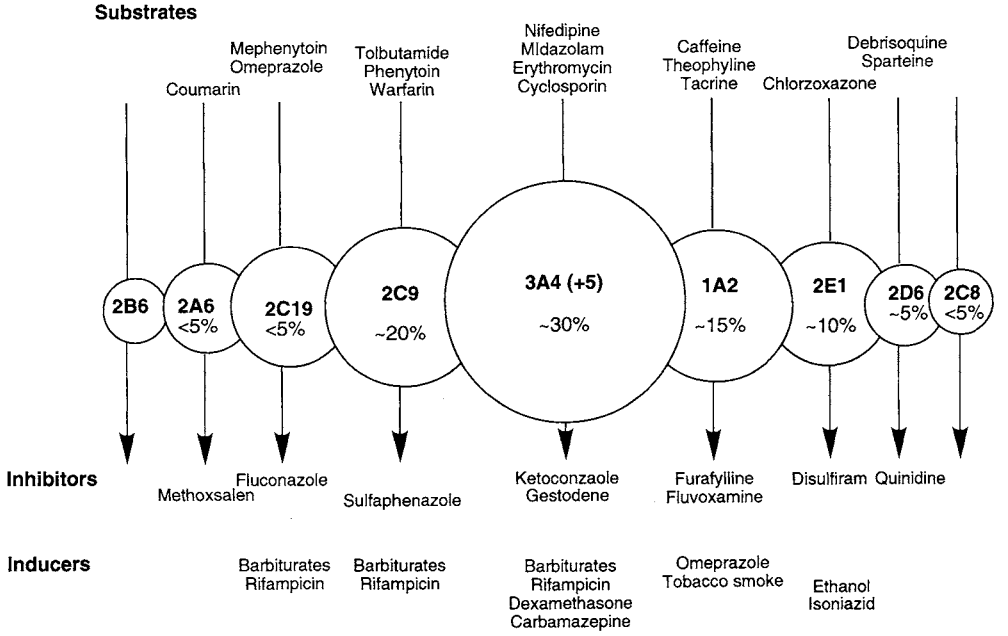

Figure 10.4. A summary of major human P450s involved in drug metabolism, including major substrates, inhibitors, and inducers (adapted from Breimer)[35, 36]. The sizes of the circles indicate the approximate mean percentages of the total hepatic P450 attributed to each P450 (See also Figure 10.1 and Table 10.5). The overlap of the circles is to make the point that overlap of catalytic action is often observed, although the overlap does not necessarily refer to the indicated substrates (or inhibitors).

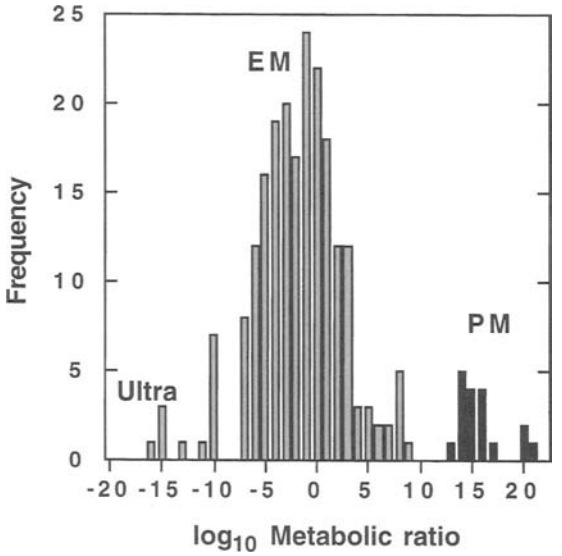

Figure 10.5. Frequency distribution histogram of (*in vivo*) debrisoquine 4-hydroxylation in a Caucasian population[44]. The metabolic ratio is the ratio of debrisoquine/4-hydroxydebrisoquine in the urine of individuals who were administered debrisoquine (10 mg free base) 8 hr previously. The groups are designated PM (poor metabolizers, solid bars) and EM (extensive metabolizers, gray bars). The group labeled "Ultra" is from retrospective research[45] and probably represents gene duplication.

polymorphisms were first studied at the level of phenotype, that is, pharmacokinetics and in some cases unusual responses to drugs due to reduced metabolism[46]. The area of pharmacogenetics (now also known as—or expanded to—"pharmacogenomics") was facilitated by the identification of the P450 enzymes involved in the drug metabolism phenotypes, and particularly by the development of molecular biology, which allows the precise characterization of genetic differences between individuals. The majority of the allelic differences are single nucleotide polymorphisms (SNPs), or single base changes. As anticipated from previous knowledge of pharmacoethnicity, many of these SNPs and polymorphisms show racial linkage. (A polymorphism is generally defined as a 1% frequency of an allelic variant in a population; below this frequency, the terms "rare genetic trait" or "rare allele" are applied or, in the case of a very detrimental allele, a mutant or "inborn error of metabolism.")

The debrisoquine polymorphism is now understood in terms of P450 2D6 and has been a prototype for research in this area. The characterization of the gene[47] led to a basic understanding of the PM phenotype. The incidence of the PM phenotype is about 7% in most Northern European populations, with different phenotypic incidence (and SNPs) in other racial groups[44, 48–50]. More than 70 allelic variants are now known, and 98% of the PMs in Northern European populations can be accounted for by four variant alleles[49, 51]. A nomenclature system has been set up for P450 alleles (using the suffixes *1, *2, *3, ...) and is maintained by Oscarson at http://www.imm.ki.se/Cypalleles/.

Several allelic variants clearly lead to the PM phenotype, for a variety of reasons. A relatively rare case is a gene deletion (*5)[52]. The most common (Caucasian) PM phenotype is an SNP that leads to aberrant RNA splicing (i.e., in splice site) and no mRNA or protein. Other alleles involve partial deletions, frameshifts, and coding for protein with either intrinsically low catalytic activity or instability (reduced halflife). These general patterns have been seen in other P450s (and other genes). In addition to the EM and PM phenotypes, there is also an "ultrarapid metabolizer" phenotype, due to gene duplication. A Swedish family has been identified with 13 gene copies, leading to 13 times more enzyme[45]. The level of hepatic P450 2D6 and a parameter of *in vivo* debrisoquine metabolism (the urinary metabolic ratio = urinary debrisoquine/4-hydroxydebrisoquine) vary $\sim 10^4$ fold among people (Figure 10.5). With P450 2D6, and several other P450s, the alleles describing the high and low levels of metabolism have been described, but the kinetic parameters for many of the alleles have not been determined by heterologous expression and measurement of catalytic activity. This is still the general case with most of the human P450s. P450 2D6 is regulated by a hepatic nuclear factor (HNF) element[53], but is not considered to be inducible by xenobiotics. With many other P450s, there is regulation and variability due to noncoding region SNPs, levels of inducers consumed, and interactions between P450s and transporters, such as *P*-glycoprotein[54, 55], may influence the phenotype.

Although the level of P450 2D6 may have a dramatic effect on the metabolism of certain drugs

Table 10.6. Some Major Inducers of Human P450 Enzymes

Class of inducers	Some sources	Example	P450s induced[a]
Ah ligands	Tobacco, broiled meat, accidental exposures	Polychlorinated biphenyls	1A1, 1A2
Barbiturates and similar compounds	Drugs, some polyhalogenated biphenyls, DDT	Diphenylhydantoin	2C, 3A4
PXR ligands	Some steroids and antibiotics, other drugs	Rifampicin	3A4
P450 2E1 inducers	Ethanol, isomiazid	Ethanol	2E1

[a]Based on *in vivo* responses.

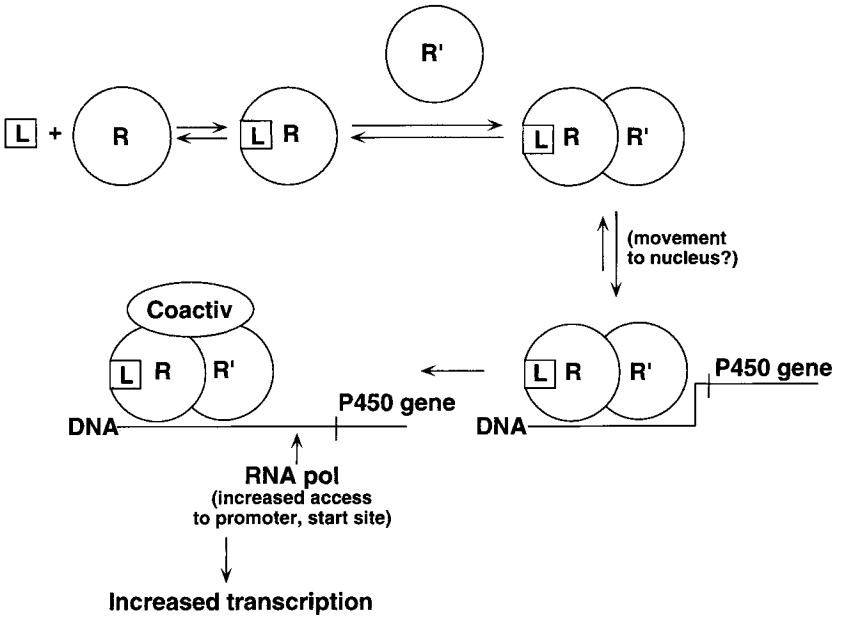

Figure 10.6. Generalized model for regulation of P450 genes by induction. L = ligand, R = receptor, R' = partner protein for heterodimer of R, Coactiv = coactivator, RNA pol = RNA polymerase.

(Figure 10.5), no other biological changes have been reported in PMs. This appears to be the general case for many of the hepatic P450s primarily involved in the metabolism of xenobiotics, and few observable physiological effects have been reported in transgenic mice in which these genes have been deleted[31]. As pointed out earlier, however, deficiencies in some of the steroid-hydroxylating P450s can be very debilitating or lethal[41]. In general, the variation in the levels of these "more critical" P450s is limited in most of the population, compared to the xenobiotic-metabolizing P450s in which an order of magnitude variation is not unusual[28].

The influence of inducers on the expression of each P450 will be mentioned later (Table 10.6, Figure 10.6). It should be pointed out that several of the P450s can be downregulated by cytokines, and the result has practical significance in the impairment of drug metabolism in individuals with colds or flu, or who have received vaccinations[56]. Another general point to make is that, in contrast to some animal models (see Chapter 8)[57], human P450 expression shows little if any gender differences. When developmental differences are seen in humans, they tend to be relatively soon after birth (e.g., P450 3A4, 3A7, see refs [58], [59]), and changes in expression seen in the elderly have not been very dramatic[60–62].

3. Approaches to Defining Catalytic Specificity of Human P450s

Knowledge of the roles of individual P450s in specific reactions is critical in the application of P450 biochemistry to practical issues in drug metabolism. Originally some of the P450s were purified on the basis of their catalytic activities toward certain specific drugs[7, 9, 11, 12], but even in those cases, there are the issues of the extent of contribution of that form and the involvement of that P450 in other reactions, particularly with new drugs. Identification of the individual P450s contributing to the metabolism of a new drug candidate is routinely done in the pharmaceutical industry. This information is usually required by the Food and Drug Administration at the time of

application. Identifying P450s involved in oxidation is important in predicting drug–drug interactions and the extent of variation in bioavailability. In general, it is desirable to develop drugs for which several P450s have a contribution to metabolism. Drug candidates that are metabolized exclusively by a highly polymorphic P450 (e.g., 2D6, 2C19) are usually dropped from further development.

A combination of methods involving the use of human tissues and recombinant human P450s is usually used to identify P450s involved in a particular reaction, using an approach outlined earlier[23, 34]. A combination of the following methods is usually done, not necessarily in a particular order. Lu[63] has recently reviewed these approaches.

3.1. Inhibitors

The reaction is demonstrated in NADPH-fortified human liver microsomes (if the reaction of interest is restricted to another tissue, then this tissue would be used instead). The effects of selective inhibitors on the reaction are examined. A list of some of the inhibitors that have been used is presented elsewhere in this volume by Correia (Chapter 7)[64, 65].

The choice of concentration parameters is important in this and some other approaches. Ideally the effect of the substrate concentration on the rate of catalytic activity should be determined in the absence of inhibitor to determine V_{max} and K_m parameters. If this information is available, the inhibition experiments are best done with a concentration of substrate at or below the K_m, in order to observe the effect of the inhibitor on the ratio V_{max}/K_m, which is the parameter usually most relevant to human drug metabolism. If the V_{max} and K_m information is not available, an alternative is to select a substrate concentration near that expected for the *in vivo* plasma concentration ($C_{p,max}$ or less).

With regard to inhibitor concentration, ideally a range of concentrations would be used. However, if a single concentration of the diagnostic inhibitor is used, it must be selected on the basis of previous literature because nonselective effects are often observed. For instance, α-naphthoflavone (αNF) (5,6-benzoflavone) can inhibit P450s other than 1A2 at high concentrations[66] and azoles inhibit many P450s at higher concentrations[64, 65]. Use of a titration approach (concentration dependence) has merit[63].

Another general issue is the selection of a protein concentration. Microsomal proteins can bind drugs in a nonselective manner and effectively lower the free concentration of substrate or inhibitor[67, 68], which can influence the interpretation of results. Another point is that the concentration of the P450 of interest should be less than that of the drug and the inhibitor, in order for the basic assumptions about steady-state kinetics to apply (and for the reaction to remain linear during the incubation time, although some of the inhibitors are mechanism-based and the loss of activity will be time dependent, requiring preincubation). A corollary of these latter points, which also apply to the other approaches that follow, is that having a very sensitive assay method is very desirable. Thus, methods such as HPLC/fluorescence and particularly HPLC/ mass spectrometry have gained popularity.

Finally, the choice of an organic solvent is an issue. Ideally the substrate should be dissolved in H_2O or very little organic solvent, but this may not be possible with many drugs. Several examinations of the effects of individual solvents on human P450s have been published[69, 70].

In principle, the extent of inhibition of a reaction by a P450-selective inhibitor indicates the fraction of that reaction attributable to that P450. For instance, if a 1 μM concentration of quinidine (a P450 2D6 inhibitor) inhibits 50% of a reaction, then 50% of that reaction may be attributed to P450 2D6. If one desires a more global view than within a single liver sample, then a pooled set of microsomes (e.g., from 10 samples, balanced on the basis of liver weight or protein) may be used for the inhibition assays. However, if one desires to examine the differences among individuals in terms of the contribution of a P450, then doing several experiments with individual liver samples is the approach to use.

3.2. Correlations

Another approach with a set of human tissue microsomal samples is to measure the new reaction of interest in each and attempt correlation with rates of marker activities (for individual P450s). Lists are also published in this volume in Chapter 7 by Correia[65] and elsewhere[71].

Correlation can be done by plotting the specific activity for the new reaction vs the marker reaction (Figure 10.7). In principle, the correlation coefficient r^2 estimates the fraction of the variance attributable to the relationship between the two activities, that is, the fraction of the activity catalyzed by the particular enzyme (assuming that all of the marker activity is catalyzed by this enzyme). In some cases, excellent correlations have been reported[72, 73]. An alternative method of analysis is the Spearman rank plot, which has some deficiencies but avoids the overweighting of unusually high or low values[74].

Although the approach works well when high correlation coefficients are generated, the method is less useful when several P450s contribute to a reaction, that is, $r^2 < 0.4$. The results should, in all cases, be considered in the context of results obtained with other approaches.

3.3. Antibody Inhibition

The points raised in the Section 3.1, Inhibitors, apply to antibodies as well. Antibodies are used to inhibit activities in human liver (or other tissue) microsomes and are of several general types: (a) polyclonal antibodies raised against purified animal P450s, (b) polyclonal antibodies raised against purified human P450s, (c) monoclonal antibodies raised against purified human P450s, (d) polyclonal antibodies raised against peptide fragments of P450s, and (e) antibody phage display library antibodies selected for recognition of individual P450s.

At this time, almost all antibodies raised against intact P450s have been generated using recombinant P450s (or against peptides), in contrast to early work in the field with P450s isolated from liver. Another point to make is that not all antibodies inhibit catalytic activity. Further, specificity in one immunochemical assay (e.g., electrophoretic/immunoblotting) does not necessarily implicate specificity in another (immunoinhibition).

Three points should be made in designing immunoinhibition experiments. (a) The concentration of antibody should be varied and increased to the point where the extent of inhibition is constant. (b) A nonimmune antibody should be used as a control, using the same concentrations as with the antibody raised against the P450. (c) The antibody should be shown not to inhibit a reaction known to be attributable to other P450s. Immunoglobulin G fractions are generally preferred in that they produce less nonspecific inhibition than crude preparations such as sera. Polyclonal antibodies can vary in their specificity and titer from one animal to another and from one bleed to another, so constant properties cannot necessarily be assumed. In principle, monoclonal antibodies and antibodies eluted from phage display libraries should not vary, although this has not always been the case with monoclonals.

In general, antibodies are often selective for individual P450 subfamilies, for example, 1A vs 1B vs 2A vs 2B vs 2C, etc., but cross-reaction among families can be detected, and in some cases the (P450) sites of cross-reactivity have been identified[75]. Achieving selectivity among individual P450 subfamily members (e.g., P450 3A4 vs 3A5 vs 3A7) is more difficult. With polyclonal antibodies, this can be achieved by cross-adsorption[76]; with monoclonals and phage display libraries, this can be done by selection. The point should be made that any selectivity demonstrated among classes of animal P450s (e.g., rat P450 families) cannot be assumed to carry over to human P450s.

Antipeptide antibodies have become popular in recent years and have two major advantages: (a) peptides can be synthesized and readily purified by HPLC, avoiding the need to express and rigorously purify P450 proteins (although demonstration of purity by HPLC, capillary electrophoresis, and mass spectrometry is still in order), and (b) peptides can be selected for use as antigens by sequence comparisons, favoring specific regions.

Phage display antibody libraries are relatively new and have been used in a few P450 applications to date (D.S. Keeney personal communication). These have a number of advantages, including potential selectivity due to the large number of potential antibodies in libraries, the ability to avoid animal protocols, the immediate availability of libraries (as opposed to waiting on animals to develop antibodies), the consistency of reproduction of the proteins propagated in bacterial systems, and the ability to include a second "epitope tag" for recovery, etc.

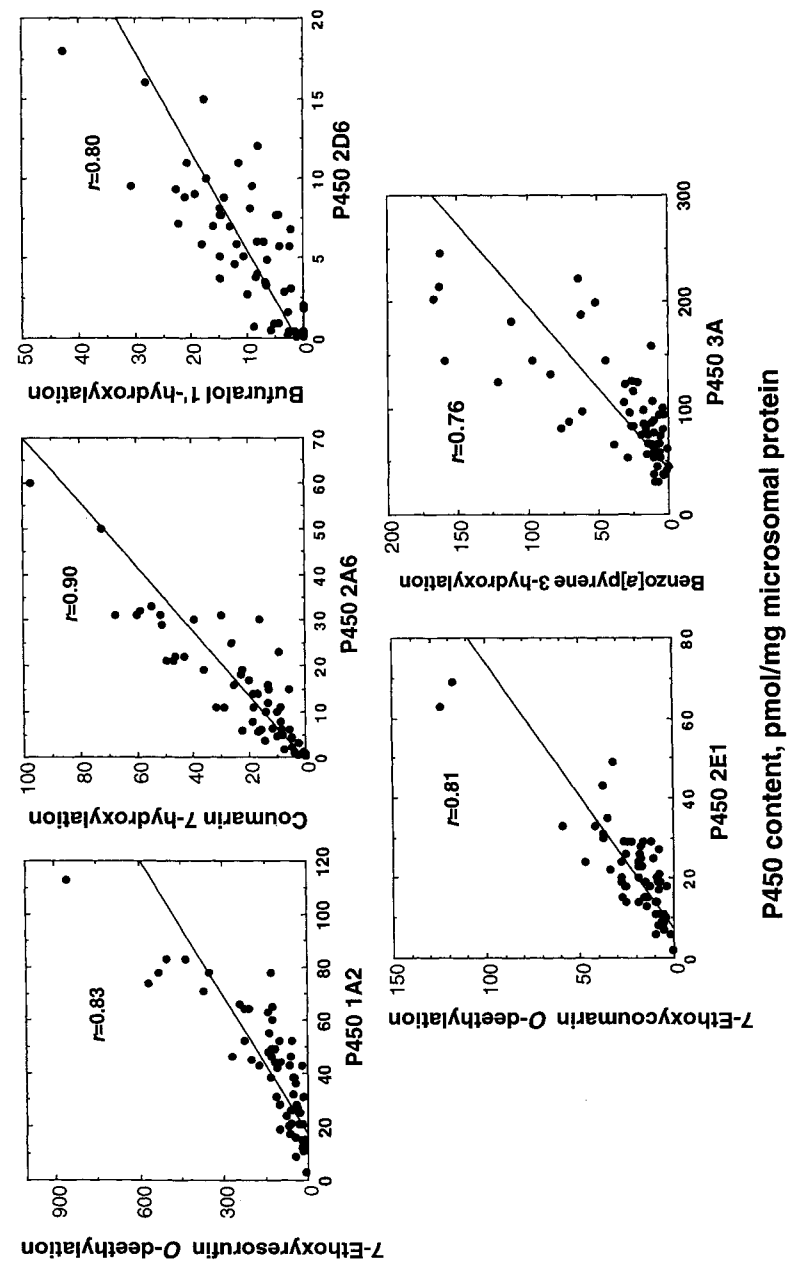

Figure 10.7. Correlation of catalytic activities with immunochemically determined levels of five P450s in human liver microsomes. The correlation coefficients (r) were determined using linear regression analysis[28].

3.4. Demonstration of Reaction with Recombinant P450

In early work in this field, this point would have been the demonstration of the reaction of interest with an enzyme purified from tissue. Today P450 proteins are generally produced in recombinant systems and seldom purified from tissue sources. In routine practice in the pharmaceutical industry, new reactions are examined with a battery of the major recombinant human (liver) P450s, many of which are available from commercial sources. Systems used for expression include bacteria, yeast, baculovirus (-infected insect cells), and mammalian cells. The P450s need not be purified for these comparisons but must have suitable provision for NADPH-P450 reductase in a crude system (and cytochrome b_5 [b_5] in certain cases).

Usually activity results obtained with several of the major P450s are compared to each other and to those obtained with tissue microsomes, in order to put the work in context. Ideally assays are done at several substrate concentrations and the parameters k_{cat} (V_{max}) and k_{cat}/K_m are obtained. These values should be normalized on the basis of P450 concentration, in that any values based on milligram protein for the expression system cannot be used for comparisons with tissue microsomes. In principle, the k_{cat} (total P450 basis) should be at least as high for the recombinant reaction as for the tissue microsomes. A more realistic way to make a comparison is to immunoquantify the amount of the particular P450 in the tissue microsomes and then use this value in correcting the microsomal k_{cat} for comparison with the recombinant system. The matter of scaling these parameters to generate predicted microsomal (or *in vivo*) rates from *in vitro* experiments with recombinant enzymes is not trivial, but a number of efforts have been made[77–80].

4. Relevance of P450s in *In Vivo* Drug Metabolism

P450s are the major enzymes involved in human drug metabolism. In looking at the fraction of the number of drugs processed by "Phase I" enzymes (Figure 10.3), P450s account for ≥80% (and the number is even higher if one moves the esterase and epoxide hydrolase reactions to the Phase II group because they are not involved in redox reactions). Constructing a figure of this type can be somewhat misleading in that the contribution of each P450 is more difficult to evaluate *in vivo* than *in vitro* (for a more original tabulation, see ref. [32]). The large contributions of P450s 3A(4) and 2C9 are driven to a large extent by the high levels of expression of these two enzymes in human liver (and small intestine) and to their broad substrate specificity. The charts do not necessarily reflect all drugs currently in development. A current tendency has been the development of larger molecules as drug candidates, in order to achieve target specificity and affinity, and a general axiom is that these are more readily accommodated by P450s 3A(4) and 2C9. In recent years, pharmaceutical companies have tried to avoid developing drug candidates that are substrates (or inhibitors) for the highly polymorphic P450s 2D6 and 2C19. With all of these caveats in hand, the allocation of the chart in Figure 10.3 is probably a good estimate and may not change considerably in the near future. However, a point to be made here is that the metabolism of many drugs is a function not only of P450s but also of other enzymes and, as recognized more in recent years, transporters that alter the concentrations of drugs within cells. A discussion of drug transporters is outside the scope of this chapter, and the reader is referred elsewhere[81].

The subjects of P450 regulation and polymorphism (or mutation in some cases) have already been mentioned, and will be treated again, with individual P450s. At this point, some general practical considerations will be discussed. If one considers the total concentration of P450 in liver samples from different healthy individuals (on a milligram protein basis), most individuals fall within a range of ~3-fold[1]. However, when individual "drug-metabolizing" P450s (e.g., families 1, 2, 3) are considered, the variation is considerable, with 5–10-fold being common and 40-fold not unusual, for example, P450 1A2 (ref. [73]). With P450 1A2, a similar variability (40-fold) is seen in *in vivo* caffeine pharmacokinetics[82]. With highly polymorphic enzymes, the variability in the same *in vivo* pharmacokinetic parameters can be as much as 10^4-fold (Figure 10.5).

Two examples of studies of the variability among individuals are presented in Figure 10.5

(Caucasians) and Figure 10.2 (Caucasian and Japanese). Gender has not been shown to have a major influence on levels of expression of the major xenobiotic-metabolizing P450s, and inter-gender pharmacokinetic differences are probably due to other influences on bioavailability or volume of distribution[55]. Racial differences exist due to allelic variations, which may influence either levels of expression or the inherent catalytic activity of the P450s [ref. [49]). Some apparent racial differences are seen here (Figure 10.2) and have also been reported in *in vivo* studies (e.g., 3A4 (ref. [83]), 2E1 (ref. [84])). Controlling diets is an issue in many *in vivo* studies of this type, and *in vitro* studies can also be affected. In general, the differences in activities of a given P450 between races are much less than within a race (e.g., Figure 10.2). Finally, the point made above should be noted that the levels of the P450s involved in steroid metabolism (e.g., families 11, 17, 19, 21) vary considerably less than do the xenobiotic-metabolizing P450s (families 1, 2, 3), probably due to their well-defined roles in regulation of physiological processes.

Many chemicals are capable of inducing P450s, as clearly demonstrated in animals and with cell culture systems[85]. *In vivo* induction experiments with humans are not as readily done as with animals, but ample evidence for P450 induction is available, going back to the barbiturate observations of Remmer in the 1950s[40]. A list of some established P450 inducers is presented in Table 10.6. This list is rather conservative in that only information is included from studies in which *in vivo* evidence has been obtained. Many of the studies have involved pharmacokinetics, but some "moderately invasive" studies have involved direct measurement of proteins, mRNA, or enzyme activities in peripheral blood cells or small intestinal biopsies; liver biopsy data is rare. Table 10.6 could probably be expanded considerably if all information from *in vitro* studies were included, for example, P450s 1B1 and 2S1 are probably inducible by Ah ligands[86, 87]. The major problem in demonstrating human P450 induction *in vivo* is the lack of diagnostic pharmacokinetic parameters for many of P450s.

The clinical influence of differences in P450 activity can be rationalized using the scheme of Figure 10.8. In this model example, the drug doses have been developed with the EMs as the general

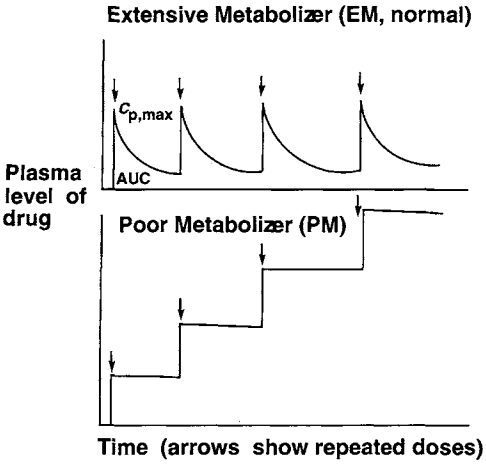

Figure 10.8. Significance of low metabolism of a drug by P450s (or other enzymes). A "typical" pattern is seen in the upper panel (EM), where the plasma level of the drug is maintained in a certain range when a particular repetitive dose is prescribed. Unusually slow metabolism (lower panel, PM) results in an elevated plasma level of the drug. $C_{p,max}$ = maximum plasma concentration; AUC = area under the curve.

population of interest. The plasma concentration rises to a peak ($C_{p,max}$) following the first dose and then decreases to a lower level prior to the next dose. With subsequent doses, the plasma concentration remains within this region and yields the desired pharmacological effect. Without prior knowledge about a problem with this drug, the PM (lower panel of Figure 10.8) would be administered the same doses. Very limited metabolism would occur between doses, and the plasma concentration of the drug (and presumably the concentration of the drug in the target tissue) will rise to an unexpectedly high level, with an attendant increase in the area under the curve (AUC). The simplest effect would be an exaggerated (and probably undesirable) pharmacological response. One can also imagine a situation in which metabolism is more rapid than expected in the typical patient, for example, due to gene amplification or enzyme induction. In this case, $C_{p,max}$ and AUC would be smaller than in the case of the EM (Figure 10.8, upper panel), and decreased drug efficacy would be expected.

Some practical situations follow. With regard to polymorphisms, several are known that can render some drugs dangerous due to toxicity

(e.g., perhexiline, leading to peripheral neuropathy due to lack of metabolism by P450 2D6 (ref. [88]) or can alter the recommended dose (e.g., warfarin/P450 2C9 (refs [89–91]) and omeprazole/P450 2C19 (refs [92], [93]). Drug interactions are a serious problem, and pharmacokinetic interactions have several molecular bases. One is enzyme induction, which usually results in decreased bioavailability. The decreased bioavailability of a drug can be the result of induction by that same drug or by another drug. A classic example is the decreased bioavailability of the oral contraceptive 17α-ethynylestradiol following treatment of individuals with rifampicin, barbiturates, or St. John's wort and consequent P450 3A4 induction[72, 94, 95]. Another aspect of drug–drug interactions involves P450 inhibition. The inhibition can be of a competitive nature, that is, two substrates competing for a limiting amount of a P450 or a *bona fide* inhibitor (no enzymatic transformation) competing with substrates. An example here is the antihistamine terfenadine, the metabolism of which is inhibited by the P450 3A4 inhibitors, erythromycin and ketoconazole. Another major type of P450 inhibition is "mechanism-based" (or "suicide") inactivation, in which oxidation of a substrate destroys the P450 (refs [64], [96]). An example here is the inactivation of P450 3A4 by bergamottin and other flavones found in grapefruit juice[97–100].

In the above cases, the effects have been discussed only in terms of altered bioavailability; that is, with increased clearance of 17α-ethynylestradiol, unexpected menstruation and pregnancies have resulted[95, 101, 102]. Some of the drug interaction problems can be more complex, even when the analysis is restricted to pharmacokinetic aspects. For instance, in the example mentioned above, terfenadine can be considered a prodrug[103]; in most individuals, the P450 oxidation (followed by further oxidation) yields fexofenadine, the circulating form of the drug. Low levels of P450 3A4 activity (due to inhibition or other reasons) cause the accumulation of the parent (prodrug) terfenadine to toxic levels that can cause arrhythmia[103, 104]. Another possibility is that blocking a primary route of metabolism of a drug may favor secondary pathways that lead to toxicity, for example, blocking phenacetin *O*-deethylation (P450 1A2) can lead to deacetylation, *N*-oxygenation, and methemoglobinemia[105]. Although a good example is not available, it is possible that blocking the oxidation of one drug by a P450 could cause it to accumulate and behave as an inhibitor toward another. A potential example would be decreasing the P450 3A4-catalyzed oxidation of quinidine and having the accumulated drug inhibit P450 2D6 (ref. [106]). P450 induction could result not only in decreased oral availability but also in the enhanced bioactivation of chemicals. This is a general concern with potential carcinogens, as discussed in the next section of this chapter, and one of the reasons why regulatory agencies have concerns about P450 1A inducers.

The phenomenon of P450 stimulation has been studied in some detail *in vitro*[107]. By stimulation we mean the enhancement of P450 catalytic activity by the direct addition of another compound, outside of a cellular environment in which gene regulation is involved. Some aspects of P450 stimulation will be treated under the topic of P450 3A4 (Section 6.20.4), with which much of the work has been done. An open question is whether such behavior occurs in humans. At least four pieces of evidence suggest that such behavior is possible: (a) cooperativity has been reported in hepatocyte cultures[108], (b) an early experiment with neonatal mice (individual P450s unknown) by Conney's group indicated the immediate enhancement of an activity by flavones[109]; (c) the work of Slattery and Nelson with rats showed an interaction between caffeine and acetaminophen that implies such behavior[110]; and (d) quinidine enhanced the *in vivo* oxidation of diclofenac in monkeys, in a manner consistent with *in vitro* human work[111]. If stimulation does occur *in vivo*, it is a phenomenon that has been very difficult to predict (even *in vitro*), and in the case of P450 3A4 substrates, the situation would probably be further complicated by issues involving *P*-glycoprotein behavior (and *P*-glycoprotein also shows cooperativity of its own[112]).

In the process of drug development, there are three guiding principles to dealing with P450 metabolism, aside from details of each specific case: (a) use *in vitro* screening to delete compounds that will have poor bioavailability (i.e., rapid *in vitro* oxidation); (b) use *in vitro* screens to avoid obvious problems of toxicity, induction, and inhibition; and (c) seek drug candidates in which the metabolism is the result of several different enzymes and not dependent upon a single one,

particularly a highly polymorphic P450 (or another highly polymorphic enzyme).

5. Relevance of P450s in Toxicology and Cancer Risk

Historically much of the attention given to P450s has come from the interest in cancer, going back to some of the first demonstrations of redox reactions in the metabolism of chemical carcinogens[113] and the inducibility of P450s by carcinogens[42]. The interest in P450s was also extended to chemical toxicities other than cancer with the demonstration of bioactivation of compounds such as the drug acetaminophen[114] and the insecticide parathion[115, 116]. Many studies have been done with P450 animal models, particularly using P450 inducers and inhibitors and genetically modified mice, either naturally occurring or transgenic. These studies provide strong evidence that alterations in the activities of P450s can modify the sensitivity of mice to various chemicals. For instance, the *Ah* locus (which controls P450s 1A1, 1A2, and 1B1 as well as some Phase II enzymes) can modify the sensitivity in Ah receptor-deficient mice, depending upon the chemical *and* the organ site[117]. Effects of specific P450 knockouts have been reported in transgenic mice as well, for example, prevention of acetaminophen toxicity by deleting P450 2E1 (ref. [118]) and of 7, 12-dimethylbenz[*a*]anthracene-induced lymphomas by deleting P450 1B1 (ref. [119]).

Despite the strong evidence for effects of variability of P450 on chemical toxicity and cancer risk in animals and the knowledge that human P450 levels vary considerably (Figures 10.1, 10.2, 10.5, and 10.7), demonstrating relationships with human disease has been difficult. In the 1960s, the demonstration of the inducibility of aryl hydrocarbon hydroxylase (thought to be what is now known as P450 1A1) by Nebert and Gelboin[120] led to more investigations with human samples, particularly peripheral blood cells. The work of Shaw and Kellerman[121, 122] suggested that the inducibility of aryl hydrocarbon hydroxylase (now recognized as P450 1A1 and 1B1 under these conditions) is correlated with susceptibility of smokers to lung cancer. In the early work, this apparently genetic variability was trimodal. Subsequently, this phenomenon has proven difficult to study, in part due to technical difficulties in the earlier phases of the work[123]. Many of the early problems have been circumvented with the ability to measure mRNA expression and the access to DNA sequences. While evidence for correlation of P450 1A1 mRNA expression with lung cancer incidence has been obtained[124], an unresolved issue is the nature of any genetic variability. In contrast to the situation seen in mouse models[125], the allelic variations in the human *Ah* receptor (which has apparently considerably lower affinity for many of the ligands of interest than the mouse receptor[126]) do not appear to account for interindividual levels of inducibility of P450 1A1 (refs [127], [128]). Kawajiri's laboratory has presented epidemiological evidence for association of lung cancer incidence with an *Msp*I polymorphism of P450 1A1 (ref. [129]). However, these results, from studies done with Japanese, have not been reproducible in Caucasians (refs [130–132]). Further, the heterologously expressed human P450 1A1 allelic variant (V462I) showed only a relatively small change in oxidation of the prototype polycyclic aromatic hydrocarbon carcinogen benzo[*a*]pyrene diolepoxide[133, 134]. A recent explanation to the quandary comes from the work of Kamataki's group, who have shown that P450 1B1, not P450 1A1, is the major P450 responsible for the aryl hydrocarbon hydroxylation activity in lymphocytes and that it is P450 1B1 expression that shows the classic trimodality, not P450 1A1, (ref. [135]).

Today the field is such that the search for roles of a particular P450 in human disease follows a route similar to that just discussed for P450 1A1, that is, the identification of SNPs is a basis for epidemiological associations with various maladies. This approach is commonly applied to the possible roles of P450s in cancers at various organ sites. The general concept is also utilized for other diseases and is the major basis for the Environmental Genome Project of the National Institute of Environmental Health Sciences (which includes many other gene candidates in addition to P450s)[136]. The positive aspects of this strategy are that we have an extensive knowledge base of allelic variations of P450s (e.g., http://www.imm.ki.se/CYPalleles/), sophisticated and very sensitive biological tools, and the potential to noninvasively analyze large populations, at least in the case of some diseases and P450s. On the negative side, the ability to rapidly screen for associations without serious thought about chemical exposure

levels has lead to many studies with little or only marginal biological plausibility. Many association studies have been difficult to repeat. An example in point is the reported association of attenuated lung cancer risk (of smokers) with the P450 2D6 PM phenotype. Although the initial reports were quite exciting[137], subsequent studies yielded variable results, and meta analysis has not supported an association[138]; moreover, no real experimental support for a biological association was ever found[139]. A recent review by Vineis[140] concludes that the risks of cancer due to genetics are considerably less than those associated with smoking or other environmental factors.

What associations of P450 have been adequately demonstrated? The list below is short and not intended to necessarily be totally inclusive, but emphasizes some of the more positive associations found to date. (The absence of several of the steroid-oxidizing P450s is known to be debilitating [Table 10.3], but these are not treated here; see the sections on individual P450s and ref. [29].) The possible association between P450 1A1 and lung cancer has already been discussed above; a confounding factor may be expression of P450 1B1. Truncation of P450 1B1 is associated with glaucoma, for unknown reasons[141]; this defect has not been seen in the P450 1B1-knockout mice[31, 119]. Allelic variants in P450 1B1 do not appear to have major effects in the oxidation of carcinogens[142]; some differences in cancer risk have been reported in the epidemiology literature[143, 144]. P450 1A2 activity has been reported to be associated with colon cancer incidence, when the factors of *N*-acetyltransferase and well-done meat intake are considered[145]; an association has plausibility in the activation of heterocyclic amines by P450 1A2 (ref. [146]). One of the strongest associations reported to date involves that of P450 2A6 with lung cancer; the association is driven by the data obtained with individuals with the gene deletion[147]. A relationship is plausible due to the demonstrated ability of P450 2A6 to activate *N*-nitrosamines (Table 10.4), and possibly via the decreased smoke intake of null-type individuals due to impaired metabolism of nicotine[148] (see Section 6.4.6). Although many epidemiological studies have been done with SNPs of P450 2E1, any putative changes in P450 2E1 phenotype have not been validated with *in vivo* assays and must be considered suspect[149].

In the process of drug development, the induction of P450 1 and P450 2B enzymes (in animals or in human cell or reporter assays) has often been considered an issue for potential toxicity[150, 151]. The concern about induction is that the rodents may be likely to develop liver or other tumors in cancer bioassays with these compounds, and any association between these inductions and human cancer is not established; for example, epileptics with long-term exposure to barbiturates and hydantoins have not been found to have more cancer[152]. Likewise, the induction of P450 4A is an indicator of peroxisomal proliferation, a phenomenon associated with rodent liver tumors but probably not human[153]. Thus, induction of rodent P450s has been shown to be a means of identifying types of potential rodent toxicity[154], some of which may be relevant to humans, but should not be used as evidence for adverse roles of these agents in humans.

6. Individual Human P450 Enzymes

Each of the 57 human P450s will be covered here. Clearly much more information is available about some than others. Points to be covered with each, when possible, include sites of expression and relative abundance, polymorphism and inducibility, substrates and reactions, knowledge of important residues and active site characteristics, inhibitors, and clinical issues.

6.1. P450 1A1

6.1.1. Sites of Expression and Abundance

The gene has seven exons, and the cDNA region is ~70% identical to that of the closest relative, P450 1A2. P450 1A1 is expressed in fetal liver but not at appreciable levels in adult liver[155–157]. P450 1A1 can be induced in primary human hepatocyte cultures[158]. The dominance of P450 1A2 over 1A1 *in vivo* may be due to preferential induction of P450 1A2 > 1A1 at low doses of inducers (a phenomenon established in rats[159]) or the presence of factors in liver that are not preserved in hepatocyte cultures.

P450 1A1 is expressed in human lung and has been partially purified[6]. A recent estimate of a median level of P450 in human lung is 6.5 pmol/mg microsomal protein ($n = 7$) and 16 pmol/mg microsomal protein for smokers ($n = 18$)[160]. The variation in levels of P450 1A1 is very high (>100-fold)[6, 160], as suggested from earlier work in which only benzo[*a*]pyrene hydroxylation was used as an indicator[161].

P450 1A1 is also expressed in placenta[162] and peripheral blood cells (lymphocytes, monocytes)[163], and these tissues have been used in many studies. Expression (at least at the mRNA level) has been reported in a number of other extrahepatic tissues including pancreas, thymus, prostate, small intestine, colon, uterus, and mammary gland[164].

6.1.2. Regulation and Polymorphism

Polymorphism in the inducibility of benzo[*a*] pyrene hydroxylation activity has attracted considerable interest following the reports of Shaw and Kellerman[121, 122] that the induction in lymphocytes of smokers can be associated with susceptibility to lung cancer. The link to lung cancer has been studied extensively but few general conclusions can be reached. Smoking clearly induces levels of lung P450 1A1 (refs [124], [160], [165]). Some epidemiological investigations link the *2A (*Msp*I) and *2B (I462V) polymorphisms to lung cancer incidence in Japanese[129], but this association has not been reproducible in other studies with Caucasians[130, 131]. These two alleles are in linkage disequilibrium[132]. Two studies with recombinant human P450 1A1 have not shown a major difference in any catalytic activities due to the substitution at codon 462 (refs [133], [134]). Although there is a general consensus that phenotypic variation in the inducibility of P450 1A1 is observed, extensive searches have not associated the inducibility with any known polymorphisms in the P450 1A1, Ah receptor, or arylhydrocarbon nuclear translocator (ARNT) genes[166, 167].

The induction of P450 1A1 has been studied extensively and is discussed elsewhere in this book[168]. Briefly, the Ah receptor resides in the cytosol and, when activated by binding of an appropriate agonist, loses the accessory protein Hsp90 and dimerizes with the ARNT protein, moving to the nucleus and interacting with an XRE element to initiate transcription (Figure 10.6, with R = Ah receptor), R^1 = ARNT, and L = TCDD or other inducers. A number of details regarding this scheme remain to be elucidated, such as roles of coactivators, whether an endogenous ligand exists and if so what it is, etc. The list of inducers reported from *in vitro* studies includes TCDD and is quite long. The list of compounds for which *in vivo* evidence of induction is more limited, but it is generally accepted that it includes cigarette smoke, heterocyclic amines, polychlorinated biphenyls[169], and some drugs (e.g., omeprazole[170]).

6.1.3. Substrates and Reactions

This enzyme was first explored in the context of an aryl hydrocarbon hydroxylase, using fluorescence assays that measured primarily the 3-hydroxylation of benzo[*a*]pyrene[120]. (It should be noted that the fluorescence assay also detects other fluorescent products, for example, 9-hydroxybenzo[*a*]pyrene, and that other P450s also catalyze the 3-hydroxylation reaction, for example, P450 2C9 in human liver[171].) Another classic model reaction used for P450 1A1 is 7-ethoxyresorufin *O*-deethylation[172, 173]. Human P450 1A1 oxidizes benzo[*a*]pyrene to a variety of products[174, 175]. Many other polycyclic hydrocarbons are substrates for P450 1A1 and have been studied extensively[176, 177]. Some heterocyclic and aromatic amines can also be activated by P450 1A1 (ref. [178]). P450 1A1 does not appear to play a major role in the metabolism of many drugs, possibly because of its locations of expression.

6.1.4. Knowledge about Active Site

Relatively little is known about the active site of P450 1A1. Early work on pharmacophore models for rat P450 1A1 was done by Jerina's group[179]. The early modeling of substrates and inhibitors suggested that P450 1A1 ligands were relatively planar. Some homology modeling has been done by Lewis[180], although little has been done with site-directed mutagenesis on the roles of individual amino acids. The lack of effect of interchanging Val and Leu at position 462 has already been mentioned[133, 134].

6.1.5. Inhibitors

Despite the long interest in this enzyme, the list of inhibitors is relatively short, and many inhibitors show overlap with P450s 1A2 and 1B1 (ref. [181]). For instance, αNF is often used as an inhibitor but is more effective against P450 1A2 (refs [181], [182]). Another inhibitor is ellipticine[64]. 1-(1′-Propynyl)pyrene and 2-(1-propynyl) phenanthrene were found to be selective P450 1A1 inhibitors when compared with human P450s 1A2 and 1B1 (ref. [181]).

6.1.6. Clinical Issues

Due to a rather limited role of P450 1A1 in drug metabolism, there are no real pharmacokinetic issues. The issue with P450 1A1 is induction and a possible role in chemical carcinogenesis. Work with animal models shows that P450 1A1 inducers can be co-carcinogens[85, 117]. Thus, regulatory agencies tend to look unfavorably at induction of P450 1A1 by potential drugs in animal models. However, the point should be made that there is presently little experimental or epidemiological evidence to support this hypothesis, and Ah inducers can afford protection from cancer in some animal models[117].

6.2. P450 1A2

6.2.1. Sites of Expression and Abundance

As mentioned earlier, human P450s 1A1 and 1A2 both have seven exons and 70% sequence identity in their coding regions. Both these genes show similar patterns of regulation by the Ah receptor system, but P450 1A2 is essentially expressed only in the liver[164], probably due to the involvement of HNF in its regulation (*vide infra*). Several lines of evidence indicate that the level of expression is substantial (Figures 10.1 and 10.2, Table 10.5), ~10–15% of the total P450, on the average, with levels varying ~40-fold among individuals (Figure 10.4).

Occasional reports cite mRNA expression in some extrahepatic tissues, for example, colon[183]. Extensive searches have not found expression in human lung[164].

6.2.2. Regulation and Polymorphism

The variability and inducibility of P450 1A2 have been recognized for some time, indirectly, going back to studies on phenacetin metabolism by Conney and his associates[184]. The characterization of P450 1A2 ("$P450_{PA}$") as the low K_m phenacetin *O*-deethylase[7] led to some interpretation of the earlier results. P450 1A2 was shown to be the caffeine N^3-demethylase[73], and the 40-fold variation in levels of liver P450 1A2 is reflected in the 40-fold variation in some *in vivo* parameters of caffeine metabolism[82], some of Vesells's earlier work on the metabolism of antipyrine in twins suggests a role for genetic polymorphism in P450 1A2 activity[185], and a more recent twin study confirms the strong genetic component of caffeine demethylation[186]. However, elucidating details of any functional polymorphism has been difficult.

At least 13 allelic variants of P450 1A2 are now known[50]. Of these, at least five have changes in the coding sequences that cause amino acid changes. Recent work in this laboratory with the expressed coding region variants indicates that most do not differ more than 2-fold in their kinetic parameters for several assays (phenacetin *O*-deethylation and *N*-hydroxylation of heterocyclic amines), although one of the variants (R431W) did not express holoprotein in *Escherichia coli*[186a]. Some (*1C and *1F) have been proposed to modify levels of expression[50]. However, the basis for a polymorphism is not clear. One view is that the variability is not genetic, based upon the lack of modality breaks in analysis of some *in vivo* parameters of caffeine metabolism[187].

One complication with genetic polymorphism, as with P450 1A1 (*vide supra*), is the inducibility. Because of the availability of markers of hepatic P450 1A2 function (phenacetin is no longer used but caffeine and theophylline are), demonstrating *in vivo* changes in P450 is relatively easy to do and the effects are consistently seen, at least quantitatively. The mechanism of induction appears to be similar to that of P450 1A1 (Figure 10.6), with expression restricted to the liver because of the need for HNF (ref. [188]). An interesting observation made recently in mice is that the inducer 3-methylcholanthrene causes a persistent induction (of P450 1A1) in liver, lasting beyond the time suggested by pharmacokinetic expectations[189].

One interpretation is that a P450 1A2-generated metabolite is involved. Further details and any relevance to humans remain to be established. With animal P450 1A2, one mechanism of induction involves protein stabilization, for example, by isosafrole-derived products[190]. Whether or not this mechanism is relevant in humans is unknown. Reported inducers include cigarette smoking, charbroiled food (presumably via polycyclic hydrocarbons and heterocyclic amines), cruciferous vegetables, vigorous exercise[191], and the drug omeprazole (actually a metabolite)[192].

6.2.3. Substrates and Reactions

P450 1A2 has been expressed in a number of systems and is used in analyses of catalytic selectivity. Of the P450s, this has one of the highest levels of expression in bacterial systems[193, 194].

The list of drug substrates is long[32], and only a few of the more well-known reactions are listed in Table 10.7.

Many carcinogens are substrates, particularly aromatic and heterocyclic amines (Table 10.4). Other carcinogens shown to be substrates include polycyclic hydrocarbons, nitropolycyclic hydrocarbons, and some *N*-nitrosamines[202].

The only major endogenous substrates are 17β-estradiol and estrone (2-hydroxylation). The physiological relevance of this reaction is unknown, particularly because of the wide variation in levels of P450 1A2 (this reaction is also catalyzed by other P450s, e.g., 3A4 (ref. [203])). Induction of P450 1A2 and 2-hydroxylation has been proposed as a means of preventing oxidation of 17β-estradiol to the potentially more reactive 4- and 16α-hydroxy products[204, 205].

Table 10.7. Some Drug Substrates for Human P450 1A2[a]

Drug[a]	References
Acetaminophen (3')	195
Antipyrine (4,3-methyl)	196
Bufuralol (1,4)	197
Caffeine (3)	73
Clozapine	49
Olanzapine	49
Ondansetron (7,8)	198
Phenacetin	7
Tacrine	199, 200
Theophylline (1,3,8)	201

[a]See also Rendic[32].

6.2.4. Knowledge about Active Site

Considerable site-directed mutagenesis work has been done with rat P450 1A2 (refs [206–208]) but relatively little with human P450 1A2. Some pharmacophore[209] and homology[180, 210] modeling work has been reported.

An approach was developed in this laboratory for doing random mutagenesis of P450 1A2, utilizing changes in the rates of formation of mutagenic hydroxylamines[211]. Changing Glu226 (to Ile or Gln) had the effect of increasing rates of phenacetin *O*-deethylation 8-fold (effect on k_{cat})[212]. Also of interest is a 100-fold decrease of activity seen in mutation of the neighboring Phe225 (to Ile or Tyr)[212]. The mutation of Asp320 (to Ala) also decreased activity[213]. Kinetic deuterium isotope effect studies suggest little change in rate-limiting steps of the *O*-dealkylation reaction over a wide range of activities with these mutants, and the mutations do not affect ground-state substrate binding or rates of P450 reduction[213]. The effects of these substitutions have not been interpreted in terms of specific roles.

The issue of cooperativity will be discussed later under P450 3A4. Cooperativity has not been reported for human P450 1A2 but behavior of the rabbit ortholog has been interpreted in the context of multiple, overlapping binding sites[214].

6.2.5. Inhibitors

Several human P450 1A2 inhibitors are known from clinical work, including furafylline (mechanism based)[215] and fuvoxamine. αNF is a commercially available and strong inhibitor of human P450 1A2 (K_i ~6 nM)[181] for *in vitro* work. A number of polycyclic acetylenes are potent inhibitors of P450 1A2 (ref. [181]). With rat P450 1A2, 2,3,7,8-tetrachloro[*p*]dibenzodioxin and some polyhalogenated biphenyls are strong inhibitors, but these studies have not been extended to human P450 1A2 (ref. [216]).

6.2.6. Clinical Issues

Some drug interactions have been reported. An older example is that of low activity toward phenacetin favoring a potentially toxic secondary pathway, deacetylation followed by quinoneimine formation and methemoglobinemia[105]. Furafylline was a drug candidate but was never developed because of its strong P450 1A2 inhibition and interference with caffeine metabolism[217]. High levels of P450 1A2 activity have also been associated with ineffectiveness of theophylline therapy (for asthma)[218, 219].

The other concern about P450 1A2 is the same discussed earlier for P450 1A1, the co-carcinogenic effect. In this regard, there is some epidemiological evidence that high P450 1A2 activity (measured as *in vivo* caffeine metabolism) is associated with enhanced risk of colon cancer, although the effect was not seen in the absence of high *N*-acetyltransferase activity and high consumption of charbroiled meat[145].

6.3. P450 1B1

6.3.1. Sites of Expression and Abundance

P450 1B1 was originally discovered in keratinocyte cultures in a search for new dioxin-inducible genes[86] and in work on adrenals in animal models[220]. In contrast to P450 1A1 and 1A2 (seven exons), the P450 1B1 gene has only three exons and is located on chromosome 2 instead of 15 (ref. [221]). Although most of the detailed studies of tissue-specific expression have been done at the mRNA level and not protein, strong responses are seen in fetal kidney, heart, and brain, in that order[178]. In adults (human), there is little detectable expression in liver, but there is more detectable expression in kidney, spleen, thymus, prostate, lung, ovary, small intestine, colon, uterus, and mammary gland[178]. Many of these tissues are of particular interest because of the tumors that develop there. Immunochemical staining of P450 1B1 has been reported in a variety of malignant tumors[222].

The level of expression (of the protein) in human lung has been estimated to be at the level of ~1 pmol/mg microsomal protein in nonsmokers and 2–4 pmol/mg microsomal protein in smokers, the levels are of an order of magnitude lower than for P450 1A1 (ref. [160]). These low values may explain the lack of immunostaining in (nontumor) tissues reported by Murray *et al.*[222] Specific values for levels of expression in tissues other than lung have not been published. Recently Chang *et al.*[223] found traces of P450 1B1 mRNA in human liver using real-time PCR, but the protein was undetectable within the limit of sensitivity.

6.3.2. Regulation and Polymorphism

Levels of P450 1B1 in human lung vary by at least one order of magnitude[160]. An interesting observation is that a termination variant of P450 1B1 is strongly associated with glaucoma[141, 224]. A similar phenotype has not been seen in P450 1B1-knockout mice[119]. Other polymorphisms of (human) P450 1B1 are known and are predominant in a set of haplotypes involving four variations Arg/Gly 48, Ala/Ser 119, Val/Leu 432, and Asn/Ser 453. Assays involving the metabolism of 17β-estradiol and polycyclic hydrocarbons by recombinant P450 1B1 variants show some variations but have not been particularly dramatic (reviewed by Shimada *et al.*[142]).

In vitro experiments show the inducibility of P450 1B1 in patterns expected for an *Ah*-responsive gene, which is the way in which the gene was found[86]. Unlike P450 1A1 and particularly 1A2 (*vide supra*), there is limited direct evidence for inducibility of P450 1B1 *in vivo* because of the low, extrahepatic expression and the lack of a diagnostic probe drug. Although the expression of P450 1B1 is driven by the *Ah* system, additional factors must be involved because of the known tissue and cell line selectivity of expression. For instance, major differences are seen between HepG2, MCF-7, and ACHN cells (of liver, breast, and kidney tumor origins, respectively)[221]. With the information available today, one would expect the gene to be induced (in extrahepatic tissues) by the compounds that induce P450s 1A1 and 1A2.

6.3.3. Substrates and Reactions

Human P450 1B1, like P450 1A1, has never been purified from tissue and all of our information has come from protein expressed in heterologous systems. 7-Ethoxyresorufin *O*-deethylation

can be used as a model reaction[225]. The catalytic activity of P450 1B1 is intermediate between P450s 1A1 and 1A2 (ref. [181]). Some other model reactions can be used as well[225].

Much of the interest in P450 1B1 has been because of its ability to activate a broad spectrum of chemical carcinogens, including polycyclic hydrocarbons and their oxygenated derivatives, heterocyclic amines, aromatic amines, and nitropolycyclic hydrocarbons[178] (Table 10.8). Of particular interest is the observation that human P450 1B1 is at least as active as P450 1A1 in the conversion of the classic carcinogen benzo[*a*]pyrene to the 7,8-dihydrodiol, the first step in the formation of the diol epoxide[230]. In general, it would appear from the available information that the rodent P450 1B1 enzymes have similar catalytic specificity as human P450 toward carcinogens, from the available information[231]. If this is a valid view, then the observation that P450 1B1-knockout mice do not form tumors from 7,12-dimethylbenz[*a*]anthracene is of particular importance[119].

One of the interesting findings with human P450 1B1 is that this enzyme is an efficient catalyst of 17β-estradiol hydroxylation and that the pattern is for 4- > 2-hydroxylation[203, 228, 232]. This pattern is the opposite of that seen for P450s 1A2 and 3A4 (2- > 4-hydroxylation)[203, 233] and is of significance because 4-hydroxyestradiol is chemically more reactive with oxygen and more likely to oxidize (to *O*-quinone) and bind DNA[234]. Thus, 4-hydroxyestrogens are considered to be candidates for causing estrogen-dependent tumors[235]. The available information indicates that mouse P450 1B1 does not catalyze estrogen hydroxylation[221, 231], providing a potentially important difference with the human enzyme. This apparent lack of conservation of selectivity has relevance in the use of mouse (and rat) models in some of the biology, for example, the human glaucoma mentioned earlier[141, 224].

Table 10.8. Carcinogens Activated by Human P450 1B1

Substrate	References	Substrate	References
Polycyclic aromatic hydrocarbons		IQ	178
Benzo[*a*]pyrene	181	Trp-P1	178
Benzo[*a*]pyrene-4,5-diol	178	Trp-P2	178
(+) Benzo[*a*]pyrene-7,8-diol	178	PhIP	178
(−) Benzo[*a*]pyrene-7,8-diol	178	*Aromatic amines*	
Dibenzo[*a,l*]pyrene	226	2-Aminoanthracene	178
Dibenzo[*a,l*]pyrene-11,12-diol	178	2-Aminofluorene	178
Benz[*a*]anthracene	181	4-Aminobiphenyl	178
Benz[*a*]anthracene-1,2-diol	178	3-Methoxy-4-aminoazobenzene	178
Benz[*a*]anthracene-*cis*-5,6-diol	178	*O*-Aminoazotoluene	178
7,12-Dimethylbenz[*a*]anthracene	178	6-Aminochrysene	178
7,12-Dimethylbenz[*a*]anthracene-3,4-diol	178	*Nitropolycyclic hydrocarbons*	
Benzo[*c*]phenanthrene-3,4-diol	178	1-Nitropyrene	227
Fluoranthene-2,3-diol	178	2-Nitropyrene	178
Benzo[*b*]fluoranthene-9,10-diol	178	6-Nitrochrysene	178
Chrysene-1,2-diol	178	2-Nitrofluoranthene	227
5-Methylchrysene	226	3-Nitrofluoranthene	227
5-Methylchrysene-1,2-diol	178	6-Nitrobenzo[*a*]pyrene	227
5,6-Dimethylchrysene-1,2-diol	178	1,8-Dinitropyrene	227
Benzo[*g*]chrysene-11,12-diol	178	1-Aminopyrene	227
6-Aminochrysene-1,2-diol	178	*Estrogens*	
Heterocyclic amines		17β-Estradiol	228
MeIQ	178	Estrone	229
MeIQx	178		

6.3.4. Knowledge of Active Site

Very little knowledge about the active site is available. The general pattern of catalytic specificity, with similarity to P450 1A1 and 1A2, would argue for some similarity. The effects of the allelic variants are probably not strong enough to be of much use in understanding the effects of those residues[142]. Some homology modeling has been done[236].

6.3.5. Inhibitors

αNF is a strong inhibitor, as in the case of P450 1A2 (ref. [181]). Some acetylenes developed by Alworth's group have been found to selectively inhibit P450 1B1 (at least relative to P450s 1A1 and 1A2), including 2-ethynylpyrene[181]. A potential drawback to these compounds is that they are rapidly oxidized by P450 1B1.

Resveratrol is a polyphenol found in red grapes and has been of interest in the context of its potential to inhibit cancer[237]. This compound is a noncompetitive inhibitor of P450 1B1, with a K_i value of 23 μM in model systems[238] (with selectivity toward P450 1A1). Recently, Potter *et al.*[239] reported that P450 1B1 oxidizes resveratrol to the known anticancer agent piceatannol, a tyrosine kinase inhibitor. Further studies showed that the natural product rhapontigenin is a low K_i inhibitor of P450 1A1 (ref. [240]). A series of methoxy-substituted *trans* stilbene compounds of the resveratrol/rhapontigenin family were prepared and tested: of these, 2,4,3′,5′-tetramethoxystilbene was found to be a strong and selective competitive inhibitor of P450 1B1 (K_i = 3 nM) and resisted demethylation[240].

6.3.6. Clinical Issues

No issues regarding drug interactions have been raised. As with the P450 1A subfamily enzymes, an issue is that induction of P450 1B1 might increase the activation of procarcinogens. This issue may be real, although presently there is no epidemiological evidence to support such a relationship. Although the coding region polymorphisms have only indicated a limited potential for contribution to cancer (*vide supra*), the recent evidence for trimodal induction[135] is certainly of interest (see Section 5, *vide supra*), particularly in light of the number of carcinogens that P450 1B1 activates (Table 10.8). The issue of oxidation of estrogens to reactive products is one worth considering, in light of the experimental evidence supporting a link with cancer in estrogen-dependent tumors. Another matter that has not been addressed is the possible metabolism of the various estrogens in postmenopausal hormone treatments (e.g., Premarin® by P450 1B1, e.g., see refs 234 and 241 regarding DNA adducts formed by some of these estrogens).

6.4. P450 2A6

6.4.1. Sites of Expression and Abundance

P450 2A6 (formerly termed IIA3 and 2A3[22]) was purified from human liver microsomes[8] and a cDNA was isolated from a human liver library[242]. The protein is expressed at medium to low levels in liver (Table 10.5, Figure 10.2). In one study, the fraction of total human liver P450 attributed to P450 2A6 ranged from <0.2% to 13% among individual samples, with a mean of ~4%[28]. P450 2A6 was not found in placenta (full term)[243].

P450 2A6 is also expressed in other tissues, particularly in the nasopharyngeal region. Expression has been detected in nasal mucosa, trachea, lung[244], and esophageal mucosa[245]. These sites of expression are of interest regarding certain cancers. In liver cancers, overexpression of P450 2A6 protein was associated with chronic inflammation and cirrhosis[246].

6.4.2. Regulation and Polymorphism

The regulation of P450 2A6 expression has been studied in primary cultures of human hepatocytes. Expression (mRNA, protein) is inducible by rifampicin[247] and phenobarbital[248] and, to a lesser extent, clofibrate, cobalt, griseofulvin, and pyrazole[248]. The nuclear receptor HNF-4 is involved in the expression of cultured hepatocytes[249].

Many polymorphisms (≥11) are known for the *CYP2A6* gene[50]. These include a splice variant (*12) in which *CYP2A7* exons are included and the protein has lost catalytic activity[250, 251]. Another SNP polymorphism (*2), recognized earlier, is the L160H change which yields very low catalytic activity[252]. Also of interest is a gene deletion (*4).

The incidence of these polymorphisms is racially linked[50]. P450 2A6 is involved in nicotine oxidation, and in 1998, Tyndale and her associates reported that individuals with low P450 2A6 activity smoke less and might have lower cancer risk[148]. This proposal seems reasonable but the findings have been questioned. General agreement exists that defective P450 2A6 genes cause reduced nicotine metabolism (the presumed basis for reduced smoking)[253–255]. Several reports conclude that deficient P450 2A6 reduces smoking[256–259] and also lung cancer[147, 260, 261] in smokers. The latter hypothesis has biological plausibility because many carcinogens from tobacco are activated by P450 2A6 (Table 10.4 and *vide infra*). However, other studies have not revealed any relationship between *CYP2A6* genotype and smoking; cancer is also controversial[262–265]. Some of the discrepancies may be racial[266] but even this is unclear[267]. Some problems are attributed to technical shortcomings in genotype analyses[268] and a definite relationship is still lacking[268] in Caucasians, but is more likely in Asians[269], where the incidence of gene deletion is higher.

6.4.3. Substrates and Reactions

The most characteristic and specific reaction of P450 2A6 is coumarin 7-hydroxylation[8, 242]. Coumarin 7-hydroxylation has also been used as an *in vivo* diagnostic assay[270–272].

One issue with P450 2A6 is whether b_5 is required for optimal catalytic activity. Soucek[273] demonstrated that a 1:1 ratio of b_5 to P450 was optimal in coumarin 7-hydroxylation catalyzed by the purified recombinant enzyme. The effect of b_5 on catalytic selectivity has not been evaluated in all reports on P450 2A6.

Coumarin 7-hydroxylation can be used *in vivo* with humans as a phenotypic assay. An alternative procedure is to administer caffeine to individuals and determine the conversion of 1,7-dimethylxanthine to 1,7-dimethyluric acid, a reaction catalyzed by P450 2A6 (ref. [274]).

Some industrial chemicals are substrates for oxidation by P450 2A6, including alkoxyethers (used as fuel additives, e.g., *tert*-butyl methyl ether)[275] and the vinyl monomer 1,3-butadiene, a cancer suspect[276].

Some drugs are also substrates, including (+) *cis*-3,5-dimethyl-2-(3-pyridyl)thiazolidin-4-one (SM-12502)[277, 278] and tegafur[279, 280], which is converted to 5-fluorouracil. Halothane is reductively converted to a free radical by P450 2A6, which can yield at least two products and initiate lipid peroxidation[281].

Some of the catalytic selectivity of P450 2A6 overlaps with that of P450 2E1 (*vide infra*). One area in which the overlap has been noted is in the oxidation of nitrosamines. P450 2A6 preferentially catalyzes the oxidation (and activation) of *N*-nitro- sodiethylamine, in contrast to P450 2E1 which preferentially oxidizes *N*-nitrosodimethylamine[282, 283]. P450 2A6 is also involved in the oxidation of many tobacco-specific nitrosamines, including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)[283–286]. P450 2A6 appears to be the major human P450 involved in the activation of *N*-nitroso-benzylmethylamine[287], *N*-nitrosodipropylamine, *N*-nitrosobutylamine, *N*-nitrosophenyl- methylamine, and *N*-nitrosonornicotine[288]. Fujita and Kamataki[289] studied the bacterial mutagenicity of a number of tobacco-specific *N*-nitrosamines and concluded that P450 2A6 is the major human enzyme involved in the activation of all examined.

P450 2A6 is also involved in the metabolism of nicotine (*vide supra*). P450 2A6 is the main catalyst in the oxidation of nicotine to cotinine[290–292]. P450 2A6 is also involved in the 3′-hydroxylation of cotinine[293]. In addition, P450 2A6 catalyzes 2′-hydroxylation of nicotine, yielding a precursor of a lung carcinogen[294].

P450 2A6 can also *N*-demethylate hexamethylphosphoramide[295].

Several forms of human P450 catalyze the 3-hydroxylation of indole[296], and the product dimerizes to indigo. P450 2A6 was the most active human P450 identified for this activity and could also catalyze several oxidations of indole[296]. Mutants of P450 2A6 generated from a randomized library were shown to catalyze the oxidation of several substituted indoles to generate variously colored indigos and indirubins[297].

6.4.4. Knowledge about Active Site

Relatively little site-directed mutagenesis work has been done on P450 2A6 (although the rodent P450 2A enzymes were an early target for the approach)[298]. Some information has been gleaned

from naturally occurring SNPs. *In vivo* studies are consistent with a shift from coumarin 7-hydroxylation to 3-hydroxylation associated with the L160H allele[252], although this phenomenon has not been verified *in vitro*. The substitution R128Q yields a protein with half the content of heme, but the inability to bind carbon monoxide (to ferrous iron) (plus a loss of 98% of the coumarin 7-hydroxylation activity)[299].

Lewis has published several homology models of P450 2A6 (refs [300–302]) and also attempted to rationalize the pattern of nicotine oxidation using molecular orbital calculations[303].

6.4.5. Inhibitors

Several selective inhibitors of P450 2A6 are known. Diethyldithiocarbamate appears to be a mechanism-based inactivator, although the inactivation has not been extensively characterized[283]. Diethyldithiocarbamate and its oxidized form, disulfiram, also inhibit P450 2E1 (ref. [304]). *In vivo* single-dose treatment of people with disulfiram inhibits P450 2E1 but not P450 2A6 (ref. [305]). The vegetable watercress, a source of phenethyl isothiocyanate, did not inhibit P450 2A6 *in vivo*[306].

A number of chemicals have been tested as inhibitors of P450 2A6 in human liver microsomes[307]. Of these, the most selective and potent inhibitors appear to be 8-methoxypsoralen, tranylcypromine, and tryptamine, with K_i values ~1 μM[307–309]. The inhibition by the natural product 8-methoxypsoralen (in many foods) is mechanism based[310]. 8-Methoxypsoralen (methoxysalen) inhibits P450 2A6 *in vivo*[305] and has also been reported to decrease nicotine metabolism in smokers[311]. Both of the inhibitors 8- and 5-methoxypsoralen were covalently bound to P450 2A6 during incubation with NADPH[312]. Menthofuran, another natural product, is also a mechanism-based inactivator of P450 2A6 (ref. [313]).

Isoniazid has been reported to be a weak mechanism-based inactivator of P450 2A6 (ref. [314]).

6.4.6. Clinical Issues

As indicated in Section 6.4.2, the major issue regarding P450 2A6 polymorphisms is the effects on lung and esophageal cancers and smoking habits, which have good epidemiology in Asians[260] but remain controversial in Caucasians[266, 268, 269, 315, 316].

Some drugs are P450 substrates, although the relative contribution of P450 2A6 is still so small (Figure 10.3) that P450 2A6 reactions are generally not included in screens.

P450 2A6 expression has been reported to be induced during infection by (carcinogenic) liver flukes[317] and downregulated during infection by hepatitis A virus[318].

6.5. P450 2A7

The situation involving the *CYP2A7* gene is complex, and sometimes this has even been erroneously referred to as a pseudogene[50]. Two pseudogenes (*CYP2A7PTX* and *CYP2A7PCX*) are known. The P450 2A7 mRNA transcript is produced in human liver, at roughly the same level as that for P450 2A6 (ref. [250], [319]). Gonzalez's laboratory had isolated cDNA clones now recognized as 2A6, the 2D6 variant L160H, and 2A7, and expressed all three in HepG2 cells[242]. Of the three, only the "wild type" P450 2A6 incorporated heme. Others have also expressed P450 2A7 in heterologous systems but not reported any evidence of a catalytically active P450 2A7 holoprotein[250]. Whether or not a functional P450 2A7 is transcribed from the mRNA in human tissues is still unclear, and nothing can be said about catalytic activity.

Gene conversion events between the *CYP2A6* and *CYP2A7* genes have been reported, yielding chimeric proteins in humans[250, 251, 320]. These proteins have some of the coumarin 7-hydroxylation conferred by the 2A6 component[251].

6.6. P450 2A13

6.6.1. Sites of Expression and Abundance

The *CYP2A13* gene has been recognized for some time[321]. The gene is expressed in human liver[295, 322] and several other extrahepatic tissues, including nasal mucosa, lung, trachea, brain, mammary gland, prostate, testis, and uterus[295]. The highest level seems to be in nasal mucosa[295], which is of interest in the context of tobacco-related cancers because of some of the catalytic activities toward nitrosamine substrates (*vide infra*).

6.6.2. Regulation and Polymorphism

Little is known about the regulation and inducibility of the *CYP2A13* gene. Several variant alleles have been identified, including one in the coding region (R257C) with somewhat less activity toward NNK[323].

6.6.3. Substrates and Reactions

Recombinant P450 2A13 has much lower coumarin 7-hydroxylation activity than does P450 2A6, but coumarin is also converted to the 3,4-epoxide[324]. P450 2A13 also catalyzes several reactions at rates as high or higher than P450 2A6, including 2,6-dichlorobenzonitrile activation, *N*-nitrosodiethylamine *N*-deethylation, hexamethylphosphoramide *N*-demethylation, *N,N*-dimethylaniline *N*-demethylation, 2′-methoxyacetophenone *O*-demethylation, *N*-nitrosomethylphenylamine *N*-demethylation, and the activation of NNK[295]. The latter reaction is of particular interest with regard to tobacco-related cancer because of the localization of expression of this P450 in nasal mucosa.

6.6.4. Knowledge about Active Site

No information is presently available beyond an effect of the R257C variant allele[323].

6.6.5. Inhibitors

No inhibitors have been reported.

6.6.6. Clinical Issues

P450 2A13 probably does not make a major contribution to the metabolism of drugs. The major interest in P450 2A13 involves a possible role in chemical carcinogenesis[295].

6.7. P450 2B6

6.7.1. Sites of Expression and Abundance

P450 2B6 is expressed primarily in liver, and the protein has been partially purified[325]. The protein has also been detected in human lung[326].

Much of the early work with P450s in experimental animals was focused on the phenobarbital-inducible enzymes now recognized to be in the 2B subfamily[327, 328] and a general expectation was that similar P450s would be prominent in human liver (and further suggested by immunochemical studies[3] and early cloning work[329]). However, the major P450 in human liver (and small intestine) proved to be P450 3A4 (Figures 10.2 and 10.3). The mean level of P450 2B6 in human liver has been somewhat controversial. One of the problems has been antibody specificity. Antibodies raised against rat P450 2B1 have not been very specific[325]; unfortunately many papers in this area show only limited sections of gels or actually show major cross-reactive material. The results tend to fall into two groups. One set reports levels very low to 80 pmol P450 2B6 per milligram protein[330–332]. Another set of reports range from near zero levels to 28 pmol P450 2B6/mg microsomal protein[325, 333–336]. However, the mean values differ considerably in both the former and latter groups. While some of the discrepancy may be attributable to the differences in liver samples, the main difference is probably with the antibodies used and cross-reactivity with other proteins, as well as error inherent in other aspects of immunochemical methods. Our own work is in line with the lower set of estimates of expression levels (mean ~1% of total P450, with values rarely exceeding 5% even in samples from individuals administered inducers)[336]. This level is an order of magnitude less than for P450 3A4 (Figures 10.2 and 10.4).

6.7.2. Regulation and Polymorphism

Until recently, the mechanisms of induction by barbiturates had been rather vague in humans and experimental animals. Studies with HepG2 cells (derived from hepatocytes) show the role of the constitutive androstane receptor (CAR), a member of the steroid receptor superfamily, and its interaction with the phenobarbital-responsive enhancer module (PBREM) in the region between −1733 and −1683 bp in the 5′ flanking region[337]. Other work with HepG2 cells has implicated the liver-selective transcription factor C/EBPα[338]. Kliewer's group[339] also demonstrated the involvement of another previously orphan receptor, pregnane X receptor (PXR), in binding to PBREM in primary

human hepatocytes to induce P450 2B6. PXR is active only when ligand-activated but CAR apparently acts without an added ligand; both CAR and PXR heterodimerize with (liganded) RXR[340]. "Cross-talk" also exists at the PBREM site with the vitamin D receptor as well as CAR and PXR[341, 342]. The levels of CAR and PXR mRNA in individual human livers are correlated with the level of P450 2B6 mRNA[343]. The regulation of P450 2B6 has considerable similarity to that of P450 3A4 (*vide infra*), with some differences. Several recent findings provide some further insight into the mechanism, although several questions persist. CAR does have ligand-activated effects and 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime has been identified as an agonist[344]. A novel distal enhancer regulated by PXR and CAR has been identified in the *CYP2B6* gene[345].

Alternative splicing in the *CYP2B6* gene was already identified in 1990[346], with use of a cryptic exon within introns 3 and a splice site acceptor within exon 4. Extensive polymorphism (mainly SNPs) has been identified in the *CYP2B6* gene[50, 347, 348]. Several of the mutants appear to yield attenuated levels of protein and catalytic activity[348]. Some evidence for enhanced catalytic activity of a P450 2B6 SNP variant (N172H) has been reported (~2-fold) and attributed to the homotropic activation seen in 7-ethoxycoumarin *O*-deethylation[349].

6.7.3. Substrates and Reactions

Many reactions have now been demonstrated to be catalyzed by recombinant P450 2B6, mirroring the early research in the P450 field with rat P450 2B1 and rabbit P450 2B4 (refs [350–353]). However, this information must be considered in the context of the amount of P450 2B6 present in liver and intestine, particularly in comparison with P450 3A4 (*vide supra*). One estimate has been made that P450 2B6 is involved in ~3% of drug metabolism reactions (Figure 10.3).

Lists of P450 2B6 substrates have been published elsewhere, for example, refs 32 and 354, and will not be reiterated here. One of the drugs to which P450 2B6 apparently makes a significant contribution is cyclophosphamide[355, 356]. Some other reactions attributed to P450 2B6 involve anesthetics, e.g., ketamine *N*-demethylation[357] and propofol hydroxylation[358]. The *N*-demethylation of (*S*)-mephenytoin has been used as a marker of P450 2B6 *in vitro* (microsomes)[336, 359, 360]. However, a valid *in vivo* probe for P450 2B6 is still lacking[354, 360, 361].

As with animal P450 2B enzymes, P450 2B6 can also oxidize some environmental pollutants[362].

Nonhyperbolic kinetics have been reported for some P450 2B6-catalyzed reactions but these have not been extensively characterized[354].

6.7.4. Knowledge about Active Site

Several homology models of P450 2B6 have been published[363, 364], including one using molecular dynamics[365].

Relatively little site-directed mutagenesis has been done with P450 2B6. Halpert's laboratory modified 10 residues and measured some activities, although most of the changes were ≤2-fold[366].

Recently, Halpert's laboratory has solved a crystal structure of a derivative of the related rabbit P450 2B4 (ref. [366a]), which should be of relevance in understanding P450 2B6.

6.7.5. Inhibitors

Lists of the reported inhibitors of P450 2B6 have been compiled by Rendic[32]. Orphenadine had been utilized in some work with microsomes but does not appear to be particularly selective[361, 367]. More recently 2-isopropenyl-2-methyladamantane and 3-isopropenyl-3-methyldiamantane have been reported as selective inhibitors of P450 2B6 (ref. [368]). Triethylenethiophosphoramide has also been reported to be a selective inhibitor of P450 2B6 (ref. [369]).

The oral contraceptive 17α-ethynylestradiol is a mechanism-based inactivator of P450 2B6 and modifies the (apo)protein[370], but the *in vivo* relevance of the inhibition has not been established.

6.7.6. Clinical Issues

No real clinical issues have been identified yet, primarily because of the difficulty in identifying reactions catalyzed *in vivo* due to the lack of specific inhibitors and the overlapping regulatory mechanisms with other enzymes. Some pharmaceutical companies have begun to include P450

2B6 in their *in vitro* screens for individual P450s with potential roles, however.

The phenomenon of barbiturate-like enzyme induction is still an issue in drug development, however. The point is not only drug interactions, but particularly the prospect of tumor promotion in rodent cancer bioassays, which is probably unrelated to the P450 induction[152].

6.8. P450 2C8

The P450s in the 2C subfamily have been of interest for some time. In retrospect, some of the first human P450 preparations purified were probably P450 2C9 (refs [3], [4]). A major impetus for research in this field was the observed genetic polymorphism in (*S*)-mephenytoin 4′-hydroxylation[371, 372], which led to efforts at purification. Purified proteins had some catalytic activity toward mephenytoin[9], but subsequent *in vivo* pharmacokinetic[373] and heterologous expression experiments[374] demonstrated a distinction between tolbutamide and (*S*)-mephenytoin hydroxylation. Genomic analysis indicated the complexity of the *CYP2C* gene subfamily[375]. Subsequently the subfamily was characterized in terms of four P450s: 2C8, 2C9, 2C18, and 2C19 (ref. [376]). P450 2C19 is the polymorphic (*S*)-mephenytoin 4′-hydroxylase[377, 378]; P450 2C9 is involved in a considerable number of drug oxidations (Figure 10.3). Two previous entries, 2C10 and 2C17, are considered allelic variants of other genes or other artifacts and have been deleted (Table 10.1)[379].

6.8.1. Sites of Expression and Abundance

P450 2C8 was first purified from human liver[9]; the enzyme is known to be expressed in liver and kidney[380]. The available data indicate that the level of expression of P450 2C8 is relatively low in liver but may be one of the more substantial P450s in the kidney. Other sites of P450 2C8 (mRNA) include adrenal gland, brain, uterus, mammary gland, ovary, and duodenum[381].

6.8.2. Regulation and Polymorphism

The level of P450 2C8 expression in human liver varies at least 20-fold[382]. Rifampicin induces P450 2C8 in hepatocyte culture[247]. The enzyme appears to be inducible by barbiturates[383] but a PBREM was not found in the 5′ untranslated region of the gene[381].

Several polymorphisms have been reported and studied[380, 382, 384]. Two coding region polymorphisms involve the amino acid substitutions I264M and K399R, with the latter appearing in a haplotype with R139K (ref. [382]). Polymorphisms upstream of the coding region are also known[382]. The metabolism of taxol (paclitaxel) is decreased with the *3 allele (K399R/R139K haplotype), but the extent of the decrease has been variable in different studies, ranging from 90%[380] to 25%[382, 384]. The *1C polymorphism appears to be associated with some attenuation of the mean level of expression[382].

6.8.3. Substrates and Reactions

P450 2C8 does not appear to have the general significance of P450 2C9 (or 2C19) in drug metabolism. An important substrate is taxol (paclitaxel)(6α-hydroxylation)[66, 385]. Another substrate for P450 2C8 is all-*trans* retinoic acid[386]. P450 2C8 also contributes to the oxidation of troglitazone[387] and verapamil, rosiglitazone, cerivastatin, amiodarone, dapsone, and amodiaquine (reviewed in refs [32, 382]).

In general, P450 2C8 has relatively low catalytic activity toward the known substrates of P450s 2C9 and 2C19. However, Mansuy's laboratory has recently synthesized a sulfaphenazole derivative toward which all of the human P450 2C subfamily P450s have activity[388].

6.8.4. Knowledge about Active Site

Some of the knowledge about the catalytic selectivity of various P450 2C enzymes can be interpreted in terms of the active site. The rabbit P450 2C5 structure provides some possible insight in this area. Recently, the groups of Johnson and Mansuy[389] have obtained a three-dimensional structure with a common P450 2C subfamily substrate[388] bound. Two binding modes were observed, one of which corresponds to the observed oxidation[389]. Very recently Johnson's group has obtained a crystal structure of a slightly modified P450 2C8 (ref. [389a]).

6.8.5. Inhibitors

In contrast to P450 2C9, sulfaphenazole is not a strong inhibitor of P450 2C8. Mansuy's group has synthesized some sulfaphenazole-based selective inhibitors of individual P450 2C enzymes, including P450 2C8 (refs [390], [391]). The early work on paclitaxel metabolism suggests that high concentrations of the natural flavonoids naringenin, quercitin, and kaempferol and the synthetic αNF inhibit[66], but little *in vivo* inhibition would be expected.

6.8.6. Clinical Issues

Induction and inhibition of P450 2C8 are not particular issues at this point. Although P450 2C8 may play a prominent role in the hepatic and renal oxidation of arachidonic acid and retinoic acid, no disease etiology has been implicated at this point. The most serious issue is probably any impact on the disposition of the cancer chemotherapeutic agent paclitaxel. Polymorphisms may have some effect on *in vivo* 6α-hydroxylation[380, 382], although any influence may be modulated in part by the contribution of P450 3A4 to other reactions[66].

6.9. P450 2C9

In retrospect, many of the observations regarding *in vivo* metabolism of barbiturates[40, 392] are some of the first reports on what is known as P450 2C9. P450 2C9 is one of the major enzymes involved in drug metabolism (Figure 10.3). Retrospectively some of the first purified human liver P450s can now be recognized as P450 2C9 (refs [3], [4]). The protein purified with some mephenytoin 4′-hydroxylation activity (MP-1) is also P450 2C9 (ref. [9]), and the cDNA corresponds[393]. Proteins were also purified from liver on the basis of their oxidation of tolbutamide[373] and hexobarbital[394, 395]. The human P450 2C subfamily is complex[375] and characterization of individual members was not achieved without heterologous expression and careful analysis of catalytic activities[374, 396]. A transcript designated as P450 2C10 from this laboratory had only two apparent coding region changes (Cys358 and Asp417) from the *CYP2C9*1* allele, one of which (reported initially as Cys358) was subsequently shown to result from a sequencing error[375]. This is now thought to be an allelic variant of P450 2C9, although since no evidence for the Asp417 mutation has been found in population studies, no allele designation has been made; the original assignment had been based on the now unexplained distinct 3′ noncoding sequence[375]. Most of the literature dealing with P450 2C10 can be interpreted as 2C9.

6.9.1. Sites of Expression and Abundance

P450 2C9 is primarily a hepatic P450. The level of expression is probably the highest, on the average, except for P450 3A4 (Figures 10.1 and 10.4; Table 10.5)[28]. All P450 2C enzymes are absent in fetal liver, including P450 2C9 (ref. [393]), and levels rise quickly in the first month after birth[397]. Pharmacokinetic experiments with accepted P450 2C9 substrates indicate that the level of hepatic P450 2C9 does not change with age, at least to 68 years[398].

P450 2C9 is also expressed in the small intestine[399].

6.9.2. Regulation and Polymorphism

Early work with human hepatocytes showed induction of P450 2C9 by barbiturates and rifampicin[400], consistent with earlier *in vivo* work on the induction of barbiturate metabolism[392]. Subsequent studies have shown that P450 2C9 is the only P450 2C subfamily enzyme expressed at a significant level in untreated hepatocytes and that expression is induced by rifampicin, dexamethasone, and phenobarbital[383, 401]. The induction involves a glucocorticoid receptor, CAR, and PXR, with CAR and PXR apparently competing at the same site[402].

Recently, evidence for action of CAR at an additional site has been presented[403]. It should be emphasized that the action of CAR is somewhat different than other receptors from the steroid receptor superfamily, in that it may be enhanced in the absence of a bound ligand and some of the control is at the level of nuclear translocation[404]. Other factors involved are HNF-4 (ref. [405]) and C/EBPα (ref. [338]), accounting at least in part for hepatic localization.

The genetic polymorphism of P450 2C9 has been studied extensively and has clinical

significance, although P450 2C9 probably does not have a critical function in normal physiology. Tolbutamide metabolism had been reported to display polymorphism[406], which was an impetus to purify the protein catalyzing the hydroxylation[373]. A 6-base deletion in the coding region lowered catalytic activity in a recombinant enzyme[407]. A number of P450 2C9 SNPs have been identified[408] and their racial linkage has been explored[409].

P450 2C9 polymorphism has been reviewed recently[410, 411] and the reader is referred to these reviews and to the website http://www.imm.ki.se/Cypalleles/ for more details. Of some interest, in addition to the *2 and *3 alleles with generally lower catalytic activity, is the *5 allele (of higher frequency in Africans) with lower catalytic activity[412]. Some of the SNPs occur in the 5′-flanking region and attenuate the expression of P450 2C9 (ref. [413]). Also of interest is an unusual phenomenon in which the *CYP2C18* exon 1-like locus is fused with combinations of exons and introns from *CYP2C9* to yield chimeric RNA transcripts[414]. Finally, linkage between *CYP2C8* and *CYP2C9* genetic polymorphisms has been reported[415].

6.9.3. Substrates and Reactions

P450 2C9 is one of the major P450s involved in drug metabolism (Figures 10.3 and 10.4). Some aspects of substrate specificity have been reviewed by Miners and Birkett[416]. A more extensive recent compendium of substrates has been developed by Rendic[32].

One of the early substrates examined was phenytoin, which undergoes 4-hydroxylation[9]. P450s 2C19 and 2C18 (R. Kinobe and E.M.J. Gillam, personal communication) can also catalyze this reaction but P450 2C9 is the major catalyst[417].

Recently, Mansuy's group has used the P450 2C9 inhibitor sulfaphenazole to build a substrate common to all four P450 2C subfamily enzymes[388].

Some compounds normally in body are oxidized by P450 2C9, including linoleic acid (epoxidation)[418] and vitamin A (all-*trans*-retinoic acid, 4-hydroxylation)[419], although the physiological significance is unknown.

Several reactions have been used as *in vivo* probes, including tolbutamide, warfarin, flurbiprofen, and losartan[420].

One substrate of recent interest is celocoxib, a cyclooxygenase (COX)-2 inhibitor (Celebrex®). P450 2C9 is the major catalyst of oxidation, and polymorphisms affect the *in vivo* pharmacokinetic parameters[421, 422].

Several aspects of P450 2C9 reactions are of concern regarding interpretation of results, at least in *in vivo* research. One issue is the effect of solvents on catalytic activity[423]. A concentration of 1% (v/v) CH_3CN markedly inhibited the catalytic activity of P450 2C9 (ref. [423]). Another issue is the enhancement of most reactions by b_5 (ref. [424]). Further work also showed that apo-b_5 (devoid of heme) was as effective as b_5 (ref. [425]), arguing against a need for electron transfer. Other work showed that even other P450s could enhance the rates of some P450 2C9 reactions, even though those P450s did not catalyze the reactions themselves[424]. These results are reminiscent of some of the interactions of rabbit P450s 1A2 and 2B4 reported by Backes[426] and are still unexplained.

Other work with P450 2C9 has provided evidence for cooperativity in some reactions, although the area has not been as developed as for P450 3A4 (*vide infra*). Dapsone and some analogs enhance the binding and 4-hydroxylation of diclofenac[427, 428]. However, the activity of P450 2C9 toward dapsone is unaffected by diclofenac, in a situation similar to that of P450 3A4, aflatoxin B_1, and αNF[429]. The interpretation that P450 2C9 uses two binding sites in these interactions is probably valid[428], although (as with P450 3A4) the mechanism remains to be elucidated (including the exact nature of the binding).

6.9.4. Knowledge about Active Site

The point should be made before detailed considerations of site-directed mutagenesis, etc., that changes in particular residues of P450 2C9 yield markedly different effects depending on the substrate and reaction under consideration. For instance, the polymorphism *3 (I359L), which appears to be very conservative, changed catalytic efficiencies of different reactions by factors of 3–27-fold (*in vitro*)[430]. Although the *2 and *3 polymorphisms cause considerable changes with some substrates, diclofenac metabolism is not altered[431], consistent with the *in vitro* findings.

With the above caveats, roles of a number of amino acids have been examined with several reactions, although extrapolation to more reactions

requires caution. Arg97 and Arg98 affected activity toward diclofenac in a yeast recombinant system[432]; in contrast, mutation of Arg97 ablated hemoprotein expression in a bacterial system (E.M.J. Gillam, personal communication). Mutation of Lys72 failed to affect affinity for ibuprofen or diclofenac (E.M.J. Gillam, personal communication). Asp293 has been shown to have a relatively general structural role, possibly by bonding to a partner amino acid or amide[433]. Studies with coumarins suggested two sites, one for Π-stacking of aromatic rings and an ionic binding site for organic anions[434]; many P450 2C9 ligands have an anionic charge[435, 436].

P450 2C9 was converted into an enzyme with (*S*)-mephenytoin 4′-hydroxylation activity (i.e., P450 2C19-like) with a relatively small number of changes (I99H, S220P, P221T, S286N, V292A, F295L). Comparisons with the crystal structure of rabbit P450 2C5 suggests that most of these residues are unlikely to directly contact the substrate but probably influence packing of substrate-binding sites and substrate-access channels[437]. Conversely, P450 2C19 could be transformed to an enzyme with warfarin hydroxylation activity similar to that of P450 2C9 (and also sulfaphenazole binding) with the changes N286S, I289N, and E241K (ref. [438]). Other work identified roles of residues 292, 295, and 399 plus residues 231–288 (substrate-binding sequence [SRS] 3) as important in P450 2C9 activities[439]. Mansuy's laboratory identified residues 476, 365, and 114 as being important in diclofenac and sulfaphenazole binding and in inactivation by tienilic acid[440]. Phe114 is proposed to be involved in Π-stacking[440] perhaps serving the role proposed in the coumarin studies mentioned earlier[434]. It might be speculated that Phe120 in P450 2D6 could serve a similar role in that enzyme (*vide infra*)[441].

Several models of P450 2C9 have been published[436, 442–445]. Some of these take experimental binding studies into consideration in their formulation while others are only based on homology. Of interest is the recent work with rabbit P450 2C5 using P450 2C9 ligands, showing multiple substrate-binding modes[389].

A crystal structure of P450 2C9 with bound warfarin has been published recently[445a]. Obviously no information is available regarding ligand interactions either. Very recently Johnson's group has also announced a P450 2C9 crystal structure[446].

6.9.5. Inhibitors

Sulfaphenazole has been recognized as a highly selective competitive inhibitor of P450 2C9 for some time[447] and has relatively poor affinity for other P450 2C subfamily enzymes[390]. Mansuy's group has examined some other similar compounds as ligands and inhibitors[389, 435].

Other inhibitors have been reported, although some have relatively poor affinity[448, 449], including several warfarin analogs[450]. For a more extensive compilation of inhibitors, see Rendic[32].

Tienilic acid is a mechanism-based inactivator of P450 2C9 (ref. [451]). The mechanism involves *S*-oxygenation, and the unstable product reacts with P450 2C9 (ref. [452]). Subsequently, autoimmune antibodies develop in some patients that recognize unmodified P450 2C9 (ref. [451]). Exactly how (or if) this process is related to the hepatitis seen in some individuals who used tienilic acid is still unclear[453], but the phenomenon has raised concerns about whether such processes might be associated with other drugs that covalently modify proteins and could lead to idiosyncratic drug reaction in patients, one of the major concerns today for safety assessment in drug development. Structure–activity relationships have been reported on thiophenes other than tienilic acid[436].

6.9.6. Clinical Issues

The major issue regarding P450 2C9 is its role in drug development because of the sizeable fraction of drugs oxidized by this enzyme (Figure 10.3)[32]. Although the polymorphism is not as dramatic as with P450 2C19 or P450 2D6 (*vide infra*), it can be an issue in drug interactions and safety.

A general issue with P450 2C9, because of its relatively high abundance (Figures 10.1 and 10.4), is its role in reducing bioavailability. However, estimating *in vivo* pharmacokinetic properties from *in vitro* data is still not trivial. Houston has reviewed the issue with P450 2C9 recently[454].

Goldstein[455] has reviewed the clinical relevance of genetic polymorphisms in the P450 2C subfamily. One of the most relevant involves warfarin,

which has a relatively low therapeutic index[455]. (*R*)-Warfarin is oxidized by P450 1A2 (6- and 8-hydroxy) and P450 3A4 (10-hydroxy), and (*S*)-warfarin is oxidized primarily by P450 2C9 (7-hydroxy)[456, 457]. The metabolism of (*S*)-warfarin is competitively inhibited by (*R*)-warfarin, but the converse is not the case[458]. The hydroxylation of (*S*)-warfarin by P450 2C9 (ref. [459]) is an issue because of reduced catalytic efficiency by the *2 and *3 variants[89, 460, 461]. The differences are manifested in altered toxicity of warfarin (hemorrhaging) at a given dose and in an altered optimal dose of warfarin[89–91, 462, 463]. The issue extends to the analog acenocoumarol[464]. The principles of physiologically based pharmacokinetic modeling have been applied to the variation of warfarin risk in individuals with different genotypes/phenotypes[465]; this effort may serve as a paradigm for other efforts to convert *in vitro* data on P450 variability into estimates of risk.

Tolbutamide hydroxylation is another example of a manifestation of *in vitro* knowledge about P450 2C9 in clinical pharmacology[466–468]. In one sense, this is rather logical because the *in vitro* work with tolbutamide[373] was developed from *in vivo* findings[406].

In other clinically relevant research involving P450 2C9, the genotype has been reported to predict the blood pressure response to the drug irbesartan[469], a relative to the P450 2C9 substrate (and the prodrug losartan)[470, 471]. Although P450 2C9 is involved in the metabolism of diclofenac, no relationship of the genotype with the cases of diclofenac-induced hepatitis was observed[472].

The final issue about P450 2C9 is possible relevance to cancer risk. Some carcinogens are substrates (e.g., benzo[*a*]pyrene[171]) although many of the reactions are probably detoxications. *CYP2C9* SNPs have been analyzed in relation to colorectal cancer. An association was found in one study[473], but not a subsequent one[474]. In another study, no association of *CYP2C9* SNPs was found with lung cancer[475].

6.10. P450 2C18

6.10.1. Sites of Expression and Abundance

Of the four human P450 2C subfamily members, the level of hepatic expression appears to be lowest for P450 2C18, at both the mRNA[376, 476] and protein[477] levels. However, expression in lung and skin appears to be significant[244, 478, 479].

6.10.2. Regulation and Polymorphism

The variability in levels of expression of P450 2C18 in human liver is difficult to assess because of the very low levels (<2.5 pmol/mg microsomal protein)[477]. The extent of variability in other tissues is not known.

Rae *et al.*[247] reported that P450 2C18 was not inducible by rifampicin in human hepatocytes, in contrast to P450s 2C8 and 2C9.

Polymorphisms in the *CYP2C18* gene have been reported[480], but the effects on expression and catalytic activities are not well characterized. One possible polymorphism has an exon 5 deletion[481].

6.10.3. Substrates and Reactions

P450 2C18 has low catalytic activity in tolbutamide methyl hydroxylation[481]. Limited activity toward drugs has been shown, and P450 2C18 probably does not make much contribution in general drug disposition, in part because of low expression levels. P450 2C18 is active in phenytoin metabolism, having an enzyme efficiency (k_{cat}/K_m) for 4-hydroxylation comparable to P450 2C9, and being more active in the bioactivation to a reactive intermediate (R. Kinobe and E.M.J. Gillam, personal communication).

Minoletti *et al.*[476] studied a series of derivatives of tienilic acid and characterized an aroylthiophene, 3-[2,3-dichloro-4-(2-thenoyl)phenoxy]propan-1-ol, as a selective substrate for 5-hydroxylation by P450 2C18 (k_{cat} = 125 min^{-1}, K_m = 9 μM).

6.10.4. Knowledge about Active Site

Information about the active site of P450 2C18 is relatively limited beyond the substrates cited above[476], the interaction of other P450 2C proteins with general 2C substrates[388] and inhibitors[390], and inferences from the rabbit P450 2C5 structures[389]. At least one homology model has been published[482].

6.10.5. Inhibitors

P450 2C18 is not appreciably inhibited by sulfaphenazole. Mansuy's group has published on

some synthetic inhibitors (sulfaphenazole derivatives) that can be used *in vitro*[390, 391].

6.10.6. Clinical Issues

The limited expression and repertoire of catalytic activity of P450 2C18 preclude consideration of clinical issues at this point in time.

6.11. P450 2C19

Interest in P450 2C19 developed from the discovery of the polymorphic metabolism of the *S*-isomer of mephenytoin, the first major polymorphism to be studied following P450 2D6 (refs [371], [372]). Initial work led to the purification of an enzyme with some (*S*)-mephenytoin 4′-hydroxylation activity[9]. Exactly how this and other gene products from the complex P450 2C family[375, 393] were involved was unclear[483, 484]. Although there were some indications that the hexobarbital 3′-hydroxylase (P450 2C9) was the enzyme of investigation[395, 485], expression of P450 2C9 cDNA[393] in yeast yielded a protein with activity towards tolbutamide but not (*S*)-mephenytoin[374, 396]. P450 2C18 had also been suggested to be the enzyme[376].

Wrighton[377] compared (*S*)-mephenytoin 4′-hydroxylation activity in different liver samples with a protein gel band recognized by anti-rat P450 2B1 and correlated this with P450 2C19, a sequence which had been reported earlier. Subsequently, Goldstein *et al.*[378] expressed several P450 2C subfamily cDNAs in yeast and identified P450 2C19 as having the highest activity.

6.11.1. Sites of Expression and Abundance

Apparently significant expression only occurs in the liver. As with all other P450s examined to date, there appears to be no gender difference[486]. P450 2C19 is a relatively minor P450 in its abundance, probably accounting for <5% of total P450 even in EM liver samples (Figure 10.4).

P450 2C19 and (*S*)-mephenytoin 4′-hydroxylation activity were not detected in fetal liver samples[393].

6.11.2. Regulation and Polymorphism

In vivo work had shown that the enzyme was inducible by rifampicin[487]. Thus, this P450 differed from P450 2D6 in that it was both polymorphic and inducible. Analysis of the regulatory system has not been extensive, but studies with human hepatocytes have demonstrated induction of P450 2C19 mRNA by rifampicin, dexamethasone, and phenobarbital[401].

The polymorphism is now relatively well understood. The incidence of the PM phenotype in Caucasians is generally 3–5% but the incidence in Asians is ~20%[48]. On some Pacific islands, the incidence is as high as 75%[488, 489]. The major defect in Caucasians and Japanese was first identified in an exon 5 mutation that leads to an aberrant splice site and yields a truncated protein[490]. Other polymorphisms are collected at the website http://www.imm.ki.se/CYPalleles/. These are rather diverse and include a mutation of the initiation codon[491] and altered enzymatic properties[488].

6.11.3. Substrates and Reactions

(*S*)-Mephenytoin 4′-hydroxylation is the classic reaction attributed to P450 2C19. Early studies on the basis of the polymorphism of tolbutamide hydroxylation suggested that the same enzyme might be responsible for both activities[373], but *in vivo* work[373] and heterologous expression studies[374] distinguished the two activities. Nevertheless, recombinant P450 2C19 has now been shown to have some tolbutamide hydroxylation activity[492].

Extensive lists of reports of P450 2C19 reactions have been published by Rendic[32] and only a few will be mentioned. The scope of P450 2C19 in drug metabolism is relatively restricted (Figure 10.3). One drug of particular interest is the ulcer drug omeprazole (and related compounds), because individuals with low enzyme activity show a better response to treatment for ulcers[92, 93]. Some of the early variations seen in warfarin metabolism[493] can be explained by the finding that P450 2C19 catalyzes the 8-hydroxylation of (*R*)-warfarin[494]. 18-Methoxycoronaridine is *O*-demethylated by P450 2C19[495]. P450 2C19 is responsible for the 5- and 5′-hydroxylation of thalidomide, an older drug notorious for teratogenic effects that has been "rediscovered"[496]. Whether the polymorphism was related to the birth defects is unclear.

P450 2C19 also oxidizes steroids, including progesterone 21-hydroxylation and testosterone 17-oxidation[497]. Finally, the organphosphate insecticide diazinon is activated in human liver by P450 2C19 (ref. [498]).

6.11.4. Knowledge about Active Site

As with other P450 2C subfamily enzymes, P450 2C19 activities are usually stimulated by b_5 (ref. [425]). In this case, stimulation is not dependent on heme in the b_5 so electron transfer cannot be involved[425].

Homology models of P450 2C19 have been published[302, 444].

Goldstein's group did chimeric analysis and then site-directed mutagenesis on P450 2C9 to convert it to a protein with P450 2C19-characteristic omeprazole hydroxylation activity[499]. Only three changes were needed to achieve the activity of wild-type P450 2C19: I99H, S200P, and P221T. However, at least three different mutations were needed to convert P450 2C9 to an enzyme with (*S*)-mephenytoin 4′-hydroxylation activity, even to a catalytic efficiency one third of wild-type P450 2C19 (ref. [437]). In an opposite experiment, P450 2C19 was converted to a P450 2C9-like warfarin hydroxylase with high sensitivity to sulfaphenazole[438]. Residues 286 and 289 appear to be important. However, these residues may exert an indirect influence by adjusting the active site or substrate-access channels[437].

6.11.5. Inhibitors

Relatively little has been published concerning P450 2C19 inhibitors, although screening may be done in some pharmaceutical companies. Recently, Mansuy's group has developed some P450-selective inhibitors for the 2C subfamily enzymes, including P450 2C19 (refs [390], [391]).

6.11.6. Clinical Issues

The issue is the polymorphism, particularly for drugs marketed in Asian populations. At least eight alleles have been associated with the PM phenotype[489]. Desta *et al.*[489] have reviewed some of the drugs for which the 2C19 phenotype is a problem.

Most pharmaceutical companies and regulatory agencies discourage development of a P450 2C19 substrate because of potential problems for PM individuals. However, several studies indicate that PM patients may have more effective therapy (for ulcers) with omeprazole and related compounds[489, 500–503].

As with many polymorphisms, epidemiology studies have been done to explore risks to diseases in the absence of information about etiology, substrates, etc. Some of the reports include suggestion of more hepatocellular cancer in PMs[504] and lack of association of leukemia with polymorphism[505]. Other possible relationships have been explored but evidence for any associations is limited at this time[489].

6.12. P450 2D6

P450 2D6 is one of the main enzymes involved in drug metabolism (Figure 10.3). It was the first "xenobiotic-metabolizing" P450 recognized to be under monogenic regulation[5].

6.12.1. Sites of Expression and Abundance

P450 2D6 is expressed mainly in liver and was first purified from liver microsomes[7, 10]. In the average person, P450 2D6 accounts for ~5% of total P450 (with wide variation)[28]. However, this enzyme is involved in the oxidation of ~25% of all drugs oxidized by P450s (Figure 10.3).

Developmental studies show little P450 2D6 in fetal liver and a rapid increase in protein shortly after birth, yielding a peak accumulation in newborns and decline in adulthood[506].

P450 2D6 is also expressed at low levels in lung (bronchial mucosa and lung parenchyma)[507].

Another site of P450 2D6 expression is brain, with localization in large principal neurons[508]. Higher levels of brain expression have been reported in alcoholics[509].

6.12.2. Regulation and Polymorphism

All information available indicates that P450 2D6 is not inducible. Some factors are known to be involved in constitutive expression, including C/EBPα[338] and HNF-4α[53].

The wide variability in the activity of P450 2D6 is attributed to genetic variability (Figure 10.5). Reduced ability to metabolize the drug debrisoquine was first noted (personally) by Smith in a drug trial. Subsequent work led to the report of polymorphic hydroxylation of debrisoquine[5], including a phenotypic hypotensive response[510]. Racial differences were first noted with Africans[44]. The phenomenon of polymorphic debrisoquine hydroxylation[511] was also reported for sparteine oxidation[43, 512]. Purification of the P450 2D6 enzyme[7, 10, 11] was followed by Gonzalez's cloning of the gene[47] and identification of some of the genetic defects as mRNA splicing variants[513].

Today more than 70 alleles of P450 are known and have been classified with a nomenclature system[51]. Systems for genotyping have become relatively powerful[514] and the "intermediate metabolizer" phenotype has been characterized[515]. The most significant decreases in activity for P450 2D6 alleles, aside from mRNA splicing problems and gene deletion[52], are considered to result from less stable proteins[516], although low activity P450 2D6 variant proteins have also been reported[517, 518]. Some of the allelic differences are present as haplotypes[519].

In addition to the "poor" and "intermediate" metabolizer phenotypes, an "ultrarapid" metabolizer phenotype was identified in early work (Figure 10.5). Ingelman-Sundberg's group identified the basis for this as a gene duplication, with up to 13 copies present in some individuals[45]. The main form of this phenomenon is a haplotype resulting from gene duplication[45, 520]. The amplification appears to result from unequal segregation and extrachromosomal replication of the acentric DNA[521]. As many as 7% of Caucasians show some of this effect, and the incidence is even higher in some Ethiopian and Middle Eastern populations[522].

6.12.3. Substrates and Reactions

Since the original work with debrisoquine[5], many substrates and reactions have been reported

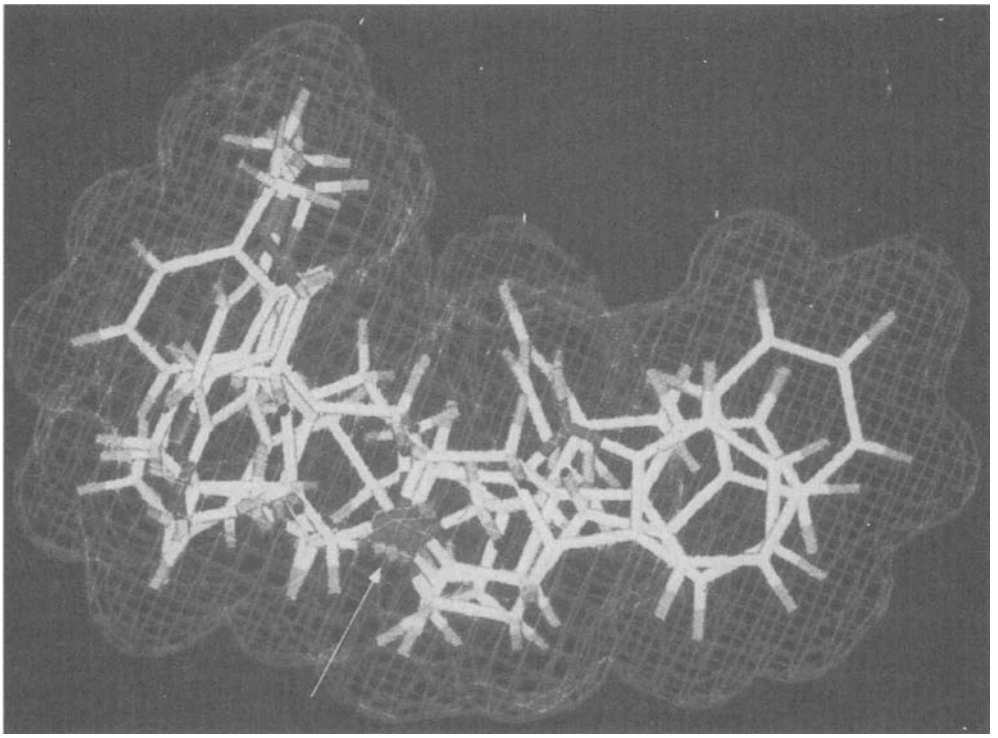

Figure 10.9. A pharmacophore model for the active site of P450 2D6 (ref. [527]). Inhibitors are overlaid to keep the nitrogen atoms (marked with arrow) in a fixed position.

for P450 2D6. In some cases, the role of P450 2D6 is very dominant *in vivo* and the clinical manifestations of genetic polymorphism are important and even deadly[510, 523]. An extensive list of P450 2D6 substrates has been published recently by Rendic[32].

P450 2D6 catalyzes many of the basic kinds of oxidative reactions of P450s, for example, aliphatic and aromatic hydroxylations, heteroatom dealkylations, etc[524]. In early work in this laboratory[525], the observation was made that most of the substrates contained a basic nitrogen atom situated ~5 Å away from the site of oxidation, possibly due to a specific anionic charge in P450 2D6. Subsequently, more detailed pharmacophore models have been developed[526–529] (Figure 10.9). All of these are based on the premise that a basic nitrogen atom in the molecule interacts (coulombic bond) with an acidic amino acid in P450 2D6, usually Asp301 in most studies. (Recent work shows a role for Glu216, however, *vide infra*.)

The use of these models requires some caveats. Although the pK_a of the substrate has been proposed to have a dominant influence[530], work in this laboratory has shown that the intrinsic pK_a of a substrate can be altered in the active site of P450 2D6 (ref. [531]). Another issue is that some compounds with a single amine nitrogen undergo *N*-dealkylation, for example, deprenyl[532], which cannot be rationalized with an amine-oxidation site interatomic distance of 5–7 Å. Some substrates devoid of basic nitrogen (and any nitrogen) have been reported, including steroids[533, 534]. Spirosulfonamide and several analogs are devoid of basic nitrogen and have been shown to be good substrates and ligands for P450 2D6 (ref. [535]) (Figure 10.10).

A large fraction of the population is devoid of active P450 2D6 but appears to function well. This

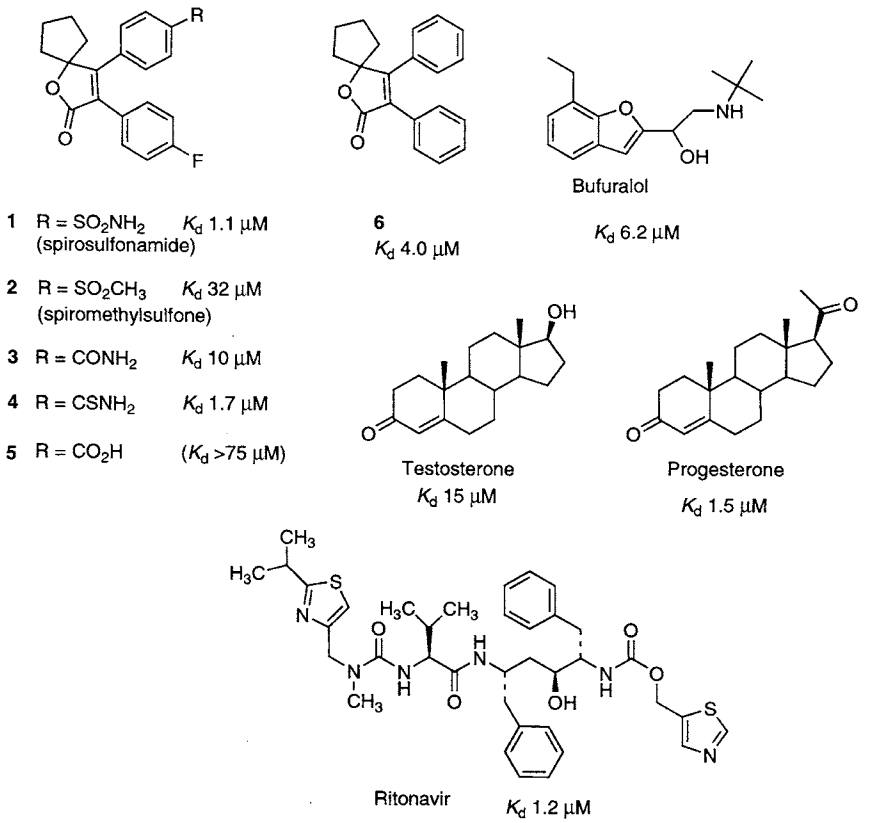

Figure 10.10. Analogs of spirosulfonamide and other P450 2D6 ligands. K_d values were estimated by spectral titrations[535].

information may be interpreted to mean that P450 2D6 has no "physiological" substrate. Nevertheless, some reactions may be catalyzed by P450 2D6 and yield physiological responses that yield less than obvious changes. For instance, overexpression of human P450 2D6 in transgenic mice produces a somewhat lethargic phenotype (F.J. Gonzalez, personal communication). Tryptamine has been proposed as a physiological substrate in one study[536] but discounted in another[537]. Proposed physiological reactions catalyzed by P450 2D6 are the *O*-demethylations of 5-methoxytryptamine, 5-methoxy-*N*,*N*-dimethyltryptamine, and pinoline (6-methoxy-1,2,3,4-tetrahydro-β-carboline)[537, 538]. Whether significant catalytic is seen at the low concentrations that occur *in vivo* and what the effect is remains to be established.

6.12.4. Knowledge about Active Site

The active site of P450 2D6 has been the subject of considerable interest, probably because of the relevance to issues in the pharmaceutical industry. Some residues have been identified as being important, and many homology and pharmacophore models have been published[526–529, 539–545] (Figure 10.9).

The original clone reported by Gonzalez[47] had Met at position 374 but this now appears to be an artifact and the correct residue is Val[546, 547]. This residue appears to be in the active site and affects activity.

In 1995, Ellis *et al.*[548] found that mutation of Asp301 to neutral residues reduced catalytic activity toward several substrates and concluded that this acidic residue was involved in docking amine substrates through coulombic interaction. Subsequently, all models published until recently have been based on this view. A caveat about the reduction in the catalytic activity of the Asp301 mutants is that heme incorporation is diminished (and is completely abolished when basic residues are substituted)[549]. Further, as indicated earlier, some P450 2D6 substrates (e.g., spirosulfonamide) are devoid of basic nitrogen but the hydroxylations are still attenuated by mutation at Asp301 (ref. [535]). Subsequent work in this laboratory showed that the oxidations of basic amine substrates (and their binding) are dependent upon Glu216 (Asp216 is also effective)[441], a result independently reported by Wolf's group[550].

A list of P450 2D6 residues postulated to form the active site includes at least Asp100, Trp316, Pro371 (ref. [539]), Pro103, Ile106, Thr107, Leu110, Pro114, Ser116, Ala122, Asp301, Ser304, Ala305, Thr309, Val370, Gly373, Val374, and Phe483 (refs [542], [551]), Phe120, Glu216 (refs [441], [541], [543], [544], [550], [552], [553]), and Gln117, Leu121, Leu213, Phe219, and Phe481 (ref. [543]). Only six of these residues have been examined experimentally to date. The effects of Asp301 have already been mentioned, with caveats about general changes in the protein[441, 549]. Changing Val374 to Met also has an effect[546, 547]. Mutation at Asp100 or Ser304 has been reported to have little effect, if any[548, 554]. Mutation of Phe483 to Ile produced some alteration of the pattern of testosterone oxidation by P450 2D6 (ref. [551]). A change in Phe481 yielded a 10-fold lower catalytic efficiency (k_{cat}/K_m) toward some substrates but not others[555]. The effects of Glu216 have already been mentioned[441, 550] and seem to be restricted largely to the basic amines[441]. Recent models of the P450 2D6 active site (Figure 10.11) suggest that both Asp301 and Glu216 are within bonding distance of amine substrates[441, 545]. Another suggestion from the more recent models[441, 544] is that one role of Asp301 is to use amide hydrogen bonds to establish the juxtaposition of Phe120, which may be involved in hydrophobic bonds with substrates. Site-directed mutagenesis experiments with this residue are currently in progress (F.P. Guengerich and E.M.J. Gillam, unpublished results).

The work cited above brings up the point that certain mutations may alter activity toward some substrates but not others (e.g., Phe481 (ref. [555]), Glu216 (ref. [441])). Similar behavior is seen with some of the natural allelic variants of P450 2D6 as well[556].

Modi *et al.*[557] reported differences in product profiles of P450 2D6 reactions supported with artificial oxygene surrogates and NADPH-P450 reductase, and interpreted these as evidence for an allosteric influence of the reductase. Subsequent experiments in this laboratory did not support this conclusion and are in accord with some differences in the chemical mechanisms for the oxygen surrogates[558].

Detailed experiments have been done on the *O*-demethylation of 3- and 4-methoxyphenethylamine by P450 2D6 (ref. [559]). Analysis of kinetic deuterium isotope effects, kinetic simulation, and

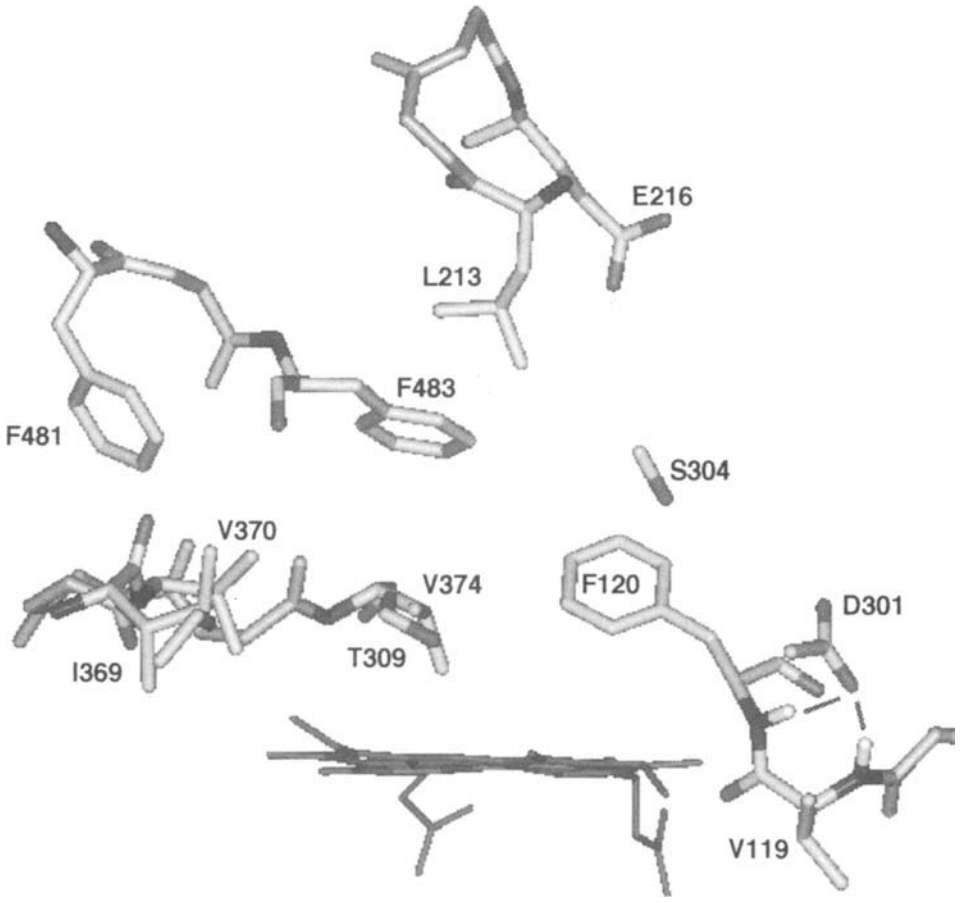

Figure 10.11. Model of some residues in the P450 2D6 active site[441]. Views of the substrate-binding cavity in the P450 2D6 homology model are shown with only the relevant residues, plus the heme, I-helix backbone, and other areas of peptide backbone shown. Hydrogens are not shown except for the amide hydrogens of residues 119 and 120 hypothesized to hydrogen bond to the carboxylate oxygen atoms of Asp301. Side view of the active site from the perspective of the I-helix: I-helix residues have been cut away excepting the side chains of residues 309, 301, and 313 shown at the front of the view.

other experiments yield evidence that both late steps in O_2 activation *and* C–H bond breaking contribute to k_{cat}. The exact meaning of K_m is still not defined with this and most P450 reactions. Some of the P450 2D6 allelic variants show no changes in k_{cat} for certain reactions but do show K_m differences[560]; these are probably more complex than simple "affinity" for the substrate.

6.12.5. Inhibitors

Many inhibitors of P450 2D6 have been reported; for a compilation of the literature, see Rendic[32]. Inhibition of P450 2D6 is an undesirable issue in drug development, and most pharmaceutical companies have screening programs in place.

The most established inhibitor of P450 2D6 is quinidine[561]. The K_i is ~50 nM and inhibition is competitive. Interestingly, quinidine is not a substrate for P450 2D6 (refs [106], [559]).

Mechanism-based inactivation of P450 2D6 is known, for example, 5-fluoro-2-[4-[(2-phenyl-1*H*-imidazoyl-5-yl)methyl]-1-piperazinyl]pyrimidine (SCH66712)[562]. In the case of this compound, covalent binding to protein was detected but the position of attachment has not been identified.

6.12.6. Clinical Issues

The clinical issues regarding P450 2D6 are considerable due to the large variation in the genetics in the population (Figures 10.2 and 10.5), and the contribution of P450 2D6 in the total scheme of drug metabolism (Figure 10.3). Individuals seem to be rather tolerant of the wide variability in expression with many marketed drugs, probably because of generally wide therapeutic windows selected for in the basic process of drug development. However, P450 2D6 PMs can be at considerable risk when they encounter certain drugs, as first observed by Smith[5, 510]. The problem is seen with drugs having a relatively narrow therapeutic index, for example, debrisoquine[5], phenformin[563], captopril[564]. The effects of P450 2D6 deficiency are seen not only in short-term treatments but also in long-term therapy[565]. The issue of ineffectiveness of drugs that are very rapidly metabolized by "ultrarapid" metabolizers is an issue (Figure 10.5). Modeling of the variability is still an issue[566] and may be a function of particular drugs. The issue of whether genotyping/phenotyping is economical has been considered, particularly in the case of neuroactive and antipsychotic drugs[567, 568]. The overlap between P450 2D6 substrates and neuroactive drugs is also an issue in drug development, largely due to the overlap of these two groups of compounds[569].

Another issue with P450 2D6 is the relevance of the polymorphism to cancer risks. In 1984, Idle[137] reported an association of lower risk of lung cancer (in smokers) with the P450 2D6 PM phenotype. These epidemiology results were reproduced in some studies[570], but not others[138]. Attempts were made to resolve the discrepancies on the basis of levels of smoking[571]. Although some expression of P450 2D6 is detectable in lung[507], no clear role for P450 2D6 in carcinogen activation could be established, even with crude tobacco smoke fractions[139]. The issue of whether lung cancer is associated with P450 2D6 was not resolved by changing analyses from phenotyping to genotyping. The generally accepted epidemiological conclusion today is that P450 2D6 is not related to lung cancer[138, 572–575].

Other epidemiology studies have suggested relationships of P450 2D6 with other cancers[576, 577], but these findings have not been scrutinized as much as the lung cancer hypothesis.

Another disease in which P450 2D6 has been proposed to play a role, on the basis of epidemiology, is Parkinson's disease[578]. Contradictory findings have been reported[579, 580]. Although a hypothesis has been raised that induction of P450 2D6 by smoking might explain some discrepancies[581], this proposal lacks biological plausibility in light of the known refractory response of P450 2D6 to induction.

A final issue is that of autoantigens. Autoantigens (LKM1) that recognize P450 2D6 have been known for some time[582, 583]. These antibodies are associated with some cases of hepatitis. The exact mechanism of how they arise is still unclear, as is the relationship with hepatitis. The antibodies may arise by molecular mimicry[584] or they may result from P450 2D6 translocation to the outer plasma membrane[585, 586]. These "LKM1" antibodies may serve as diagnostic tools for particular types of hepatitis[587, 588], but causal relationships have never been demonstrated.

6.13. P450 2E1

The mixed-function oxidation of ethanol was reported nearly 40 years ago[589]. The view that ethanol could be a P450 substrate was not readily accepted because of the hydrophilic nature of the molecule, but Lieber's group characterized the enzyme in rat liver[590, 591]. Collaborative work with Levin led to the isolation of the P450 ("j"), which was also found to be inducible by isoniazid[592]. Human P450 2E1 was purified by Wrighton *et al.*[593], and Gonzalez's group characterized the human gene[594].

6.13.1. Sites of Expression and Abundance

The greatest concentration is in the liver, and P450 2E1 is a moderately abundant P450 (Figure 10.4). The inter-individual variation is an order of magnitude (Figure 10.2)[28, 595]. A racial difference exists, with Japanese samples having mean expression levels less than Caucasians (Figure 10.2)[84].

P450 2E1 is reported not to be present in fetal liver but appears within a few hours after birth,

regardless of the gestational age[596]. The activity increases during the first year of childhood, and transcriptional regulation due to hypermethylation has been proposed.

P450 2E1 is expressed in many extrahepatic sites including lung[326], esophagus, small intestine[597], brain[598, 599], nasal mucosa[600], and pancreas[601] (some of the evidence is extrapolated from rat work but not necessarily extended to humans).

P450 2E1 is found mainly in the endoplasmic reticulum. With heterologous expression in bacteria, (rabbit) P450 2E1 is membrane bound and catalytically active even when amino acids 3–29 are deleted[17, 602]. The same bacterial localization was seen with human P450 2E1 from which 21 N-terminal residues were deleted[603]. However, P450 2E1 can show some unusual localization in mammalian systems. Ingelman-Sundberg's group deleted residues 2–29 of rat P450 2E1 and demonstrated the presence of a shattered fragment in the mitochondria of a mouse hepatoma cell line[604]. Avadhani's group found P450 2E1 intact in rat liver mitochondria and reported that it could couple these with adrenodoxin and adrenodoxin reductase there with full catalytic activity[605]. Subsequent work demonstrated a cryptic mitochondrial targeting signal at positions 21–31 that was activated by cyclic AMP-dependent phosphorylation of Ser129 (ref. [26]). Neve *et al.*[606] found that the charge of the *N*-terminus of (rat) P450 2E1 was such that a part is directed to either the lumen of the endoplasmic reticulum or the outside of the plasma membrane. The relevance of these localizations to human tissues is still unknown but likely.

6.13.2. Regulation and Polymorphism

Early work in experimental animals was focused on the induction of P450 2E1 in rat liver[590]. Subsequently, many other chemicals, including isoniazid and some solvents, were shown to induce P450 2E1 (ref. [607]). It is also of interest to note that some of the common polycyclic hydrocarbons and other inducers of P450 1 family enzymes attenuated the level of P450 2E1 (ref. [607]). The regulation of P450 2E1 has come to be recognized to be relatively complex, involving transcriptional activation, mRNA stabilization, increased mRNA translation efficiency, and decreased protein degradation[608].

HNF-1 is reported to regulate *CYP2E1* gene transcription[609]. Obesity and diabetes are known to modulate P450 2E1 in rat models. In rat hepatocyte cell culture, insulin attenuated mRNA levels and glucagon or dibutyryl cyclic AMP elevated mRNA, with the latter effect downregulated by a protein kinase A inhibitor[610]. mRNA levels are also selectively attenuated in mice or cell culture (relative to other P450s) by interleukin-6[611], interleukin-4[612], or interleukin-1β or tumor necrosis factor (TNF)α[613]. Multiple mechanisms have been invoked, including kinase pathways, control of HNF-1α function, and regulation of other transcription factors.

Evidence for control at the level of mRNA stability and enhanced translation efficiency has been presented by Novak[614, 615]. The 3′-region of the gene appears to be important in stability. The relevance of this rat model to human P450 2E1 is still unknown.

Another mechanism, generally well accepted although not completely understood, involves protein stabilization by substrate. Rat studies (*in vivo*) showed that ~1/2 of P450 2E1 was lost in 1 hr, and a ubiquitin-linked pathway was invoked[616]. Similar findings were also reported for human P450 2E1 in HepG2 cells[617]. An attempt has been made to estimate the halflife of P450 2E1 in humans *in vivo* using chlorzoxazone pharmacokinetics and a P450 2E1 inhibitor[618]. The halflife was estimated at 50 ± 19 hr, but this approach may not be sensitive enough to detect a short-lived P450 2E1 pool. The relevance of substrate stabilization of P450 2E1 to *in vivo* parameters has been addressed by Thummel and Slattery[619].

P450 2E1 is polymorphic and, because of the nature of many of the substrates, many efforts have been made to determine the relevance of SNPs and other polymorphisms to disease and risk of injury. For a current update on *CYP2E1* polymorphisms, see http://www.imm.ki.se/Cypalleles/. A polymorphism in the 5′ flanking region was suggested to be related to the binding of a transcription factor and related to alcohol intake[50, 620]. A number of other polymorphisms have been identified[50, 621, 622]. However, the evidence to date indicates that these polymorphisms do not seem to have much significance in terms of their effects on *in vitro* or *in vivo* activity of P450 2E1 (refs [84], [621], [623–625]).

6.13.3. Substrates and Reactions

P450 2E1 was originally characterized as an ethanol-oxidizing enzyme. P450 2E1 can oxidize some compounds that are present in the body, including acetone and possibly other ketones involved in certain physiological syndromes (fasting, diabetes)[626]. Transgenic P450 2E1-knockout mice appear to be relatively normal, although the blood acetone levels become much higher (than in wild-type mice) after fasting[627].

The role that P450 2E1 plays in ethanol metabolism has been debated for many years[628]. What seems to be the general consensus is that alcohol dehydrogenase is the main enzyme involved in ethanol oxidation. P450 2E1 may make a contribution at very high ethanol concentrations or in individuals with low levels of alcohol dehydrogenase activity. P450 2E1-knockout mice have blood ethanol levels not significantly different from wild-type animals after administration of ethanol[629]. Acetaldehyde, the product of ethanol oxidation, is also oxidized to acetic acid by rat and human P450 2E1 (ref. [630–632]).

The oxidation of 4-nitrophenol to 4-nitrocatechol has been used as an *in vitro* marker of human P450 2E1 (ref. [633]). Chlorzoxazone 6-hydroxylation was demonstrated to be a relatively specific reaction catalyzed by human P450 2E1; other enzymes (e.g., P450 1A1) can catalyze the reaction but with poor catalytic efficiency[634, 635]. Chlorzoxazone is a relatively innocuous muscle relaxant and the assay can be used *in vivo* to estimate hepatic P450 2E1 function noninvasively[84, 625].

One group of substrates of interest is *N*-nitrosamines, which are carcinogens at many sites and can be formed by chemical reactions within the body (e.g., stomach acid)[636]. Early research on the activation of *N*-nitrosodimethylamine (*N*,*N*-dimethylnitrosamine) indicated biphasic kinetics of the activating *N*-demethylation reaction and the possible contribution of multiple P450s and possibly other enzymes[637, 638]. The enzyme involved in the "low K_m" reaction was shown to be P450 2E1 in rat and human liver[639, 640]. An *in vivo* role of P450 2E1 has been confirmed in rats[641]. However, P450 2A6 has a significant share of the role of activation of some more complex nitrosamines, even *N*-nitrosodiethylamine[282, 283].

P450 2E1 has been shown to be a major P450 involved in the oxidation of a number of low molecular weight cancer suspects including not only nitrosamines but also benzene, styrene, CCl_4, $CHCl_3$, CH_2Cl_2, CH_3Cl, CH_3CCl_3, 1,2-dichloropropane, ethylene dichloride, ethylene dibromide, vinyl chloride, vinyl bromide, acrylonitrile, vinyl carbamate, ethyl carbamate, and trichloroethylene[304]. The oxidations by P450 2E1 all have relevance to the activation and detoxication of these compounds and their risk assessment[304, 642]. Another substrate is the gasoline additive methyl *tert*-butyl ether[643]. A role of P450 2E1 has been shown in the activation of some of these chemicals in knockout mice[644, 645].

Another substrate for human P450 2E1 is lauric acid, which undergoes 11-hydroxylation[646, 647]. The physiological relevance of this reaction is unknown. Indole is oxidized by P450 2E1 (3-hydroxylation, generating indigo) as well as by other P450s, particularly P450 2A6 and 2C19 (refs [296], [648]). The relevance of this reaction to the urinary excretion of indigoids[649] is still unclear.

Relatively few drugs are oxidized by P450 2E1 (Figure 10.3). Chlorzoxazone is one[634]. Halogenated anesthetics are often metabolized by P450 2E1, including halothane[650] and isoflurane[651].

For more lists of substrates, see Rendic[32].

6.13.4. Knowledge about Active Site

One of the issues in P450 2E1 reactions is the need for b_5, first demonstrated with the rat enzyme[639] and also the human enzyme[640, 652]; the involvement also exists in microsomes[653]. b_5 also augments P450 2E1 activity in bacterial expression systems[425, 654]. In contrast to several of the P450s, apo-b_5 (minus heme) does not function, arguing for a "classic" role of electron donation in enhancement of catalysis[425, 655].

A number of homology models of human P450 2E1 have been published, based upon bacterial P450s and rabbit P450 2C5 (refs [302], [656], [657]). One of the difficulties in dealing with models for the low molecular weight substrates is that many of these compounds have very little in the way of features to bond to, other than hydrophobic residues or halogens. Utilizing these models for both very small substrates (e.g., ethanol, CH_3Cl) and larger, more conventional ones (e.g., chlorzoxazone, lauric acid) is an issue, unless only parts of the larger substrates are inserted. One problem is

that inherent binding affinities are generally unknown and spin-state changes have not been very useful with P450 2E1 (refs [652], [658]).

Mathematical models have also been developed for rates of oxidation by P450 2E1 (refs [659], [660]). In essence, these are based on chemical reactivity at individual substrate atom sites. In both of the cited examples[659, 660], the models were used for relatively small sets of related compounds and may have some utility. An inherent problem in more extended sets is the difficulty in interpretation of the parameters k_{cat} and K_m. Thus, the rate-limiting step may not be related to hydrogen abstraction or a similar chemical step involving the substrate (*vide infra*).

Keefer *et al.*[661] reported a kinetic deuterium isotope effect on the carcinogenicity of *N*-nitrosodimethylamine in rat liver. Subsequent work with rat and liver microsomes indicated that the effect of the deuterium substitution was expressed in the parameter K_m but not k_{cat} (V_{max})[662], and that isotope effects on K_i were seen when deuterated *N*-nitrosodimethylamine was used as a competitive inhibitor of other P450 2E1 reactions[663]. These results were of interest in that deuterium substitution would not be expected to modify the affinity of a substrate for an enzyme. Studies with deuterated and tritiated ethanol in this laboratory also indicated an isotope effect on the oxidation of both ethanol and acetaldehyde by recombinant human P450 2E1, manifested mainly in K_m [632, 652]. The results are understood in the context of a reaction sequence where burst kinetics are observed, that is, the first reaction cycle is much faster (~400 min^{-1}) than the subsequent ones, which control k_{cat}. Pulse-chase experiments suggest that little of the acetaldehyde (or its hydrated form $CH_3CH(OH)_2$) dissociates, due to kinetic phenomena. Neither ethanol, acetaldehyde, nor acetic acid has much affinity for P450 2E1. The rate-limiting step occurs after product formation (for both ethanol and acetaldehyde oxidations), but is not product release *per se*. This view of the reaction sequence may apply to some P450 2E1 reactions but not others. Recent work in this laboratory with both P450s 2E1 and 2A6 has shown a kinetic isotope effect primarily on K_m, for the *N*-dealkylation of *N*-nitrosodimethylamine but not *N*-nitrosodiethylamine[664], and the kinetic mechanisms remain to be further elaborated. An interesting point of the deuterated ethanol work is that the intermolecular isotope effect is expressed in the K_m parameter, which includes the C–H bond-breaking step. k_{cat} is governed largely by an enzyme physical step after oxidation of substrate. In this system, the K_m term contains k_{cat} as a variable[632, 652].

A final point involves a report of the kinetics of CO binding to human P450 2E1 following flash photolysis[658]. The kinetics appeared to be monophasic and the rate was decreased in the presence of (400 mM) ethanol. One interpretation of the results is that binding of the substrate makes P450 2E1 more rigid[658].

6.13.5. Inhibitors

As mentioned earlier, many low molecular weight solvents are substrates for P450 2E1. These are also inhibitors of P450 2E1 (refs [69], [70]). Such inhibition is a problem in that historically many insoluble P450 substrates have been added to enzymes using final solvent concentrations of 1% (v/v), which is often ~100 mM. Thus, care is needed in analyses. It is possible to dilute many of the P450 2E1 low molecular weight substrates directly in water to add them to incubations, for example, methylene chloride has a solubility of ~100 mM in H_2O[665].

Some of the alcohol and aldehyde dehydrogenase inhibitors are also inhibitors of P450 2E1, making interpretations of *in vivo* ethanol metabolism studies difficult. 4-Methylpyrazole is an excellent inhibitor[202, 666] and probably one of the best choices for *in vitro* experiments at this time. 3-Amino-1,2,4-triazole[667] and diethyldithiocarbamate[304] are mechanism-based inactivators. The latter is of interest in that the oxidized form, disulfiram (Antabuse®), is an aldehyde dehydrogenase inhibitor used in patients in alcohol aversion therapy. Many of the early animal and human studies on interactions of ethanol and disulfiram with various chemicals can now be rationalized in the context of P450 2E1 (refs [668], [669]).

A number of compounds of natural origin have also been examined as P450 2E1 inhibitors, many of which are derived from vegetables such as onions, garlic, and cruciferous vegetables[670, 671].

6.13.6. Clinical Issues

The major clinical issues involve the role of P450 2E1 in the oxidation of certain drugs, alcoholism, oxidative stress, and risk from cancer.

As pointed out earlier, the most generally accepted noninvasive human assay involves 6-hydroxylation of the muscle relaxant chlorzoxazone[625, 634]. Studies with humans show little effect of diabetes[625, 672], but an effect of body weight/obesity[672, 673]. As mentioned before, genotype has shown little impact on the *in vivo* parameters to date[84, 673].

Another issue is drug metabolism and toxicity. Acetaminophen overdose remains a major cause of liver failure in the United States. Several P450s are involved in the oxidation to the reactive iminoquinone[195]. Studies with P450 2E1 knockout mice indicate that P450 2E1 is a major determinant of acetaminophen toxicity, because the toxicity was considerably attenuated in null animals[118].

P450 2E1-null mice have the same blood ethanol levels as wild-type animals after ethanol dosing[629] suggesting that P450 2E1 activity is not a major factor in ethanol metabolism, at least in mice. The situation regarding a role for P450 2E1 in alcohol-induced liver injury in other models is unclear, with some reports suggesting a link[674, 675] and others not[629, 676]. Autoantibodies against P450 2E1 have been reported in alcoholics[677] and attributed to hydroxyethyl radicals[678] (which may arise from lipid peroxidation processes rather than as intermediates in P450-catalyzed oxidation, *vide supra*). P450 2E1 is also a major autoantigen associated with halothane hepatitis, a rather idiosyncratic response[679]. As with other autoimmunities involving P450s, causal associations remain to be demonstrated[453].

Another issue is the contribution of P450 2E1 to oxidative stress. Ingleman-Sundberg reported that P450 2E1 contributed ~20% of the NADPH-dependent lipid peroxidation in rat liver microsomes (and 45% in microsomes prepared from rats treated with acetone to induce P450 2E1)[680]. Transfection of human P450 2E1 into a rat hepatic stellate cell culture system yielded elevated production of reactive species[681]. Cederbaum[682] has reviewed studies on the relationship of oxidative stress to P450 in liver cell models. The exact relevance to liver injury and alcohol-induced disease requires more investigation.

Many studies have been reported on the relationship of *CYP2E1* polymorphisms to risk of diseases. Benzene poisoning in Chinese workers showed some changes in risk with one genotype but only in smokers[683]. With regard to cancers, the results appear to be very mixed. An early report suggested a link of lung cancer with a polymorphism[684], but since then the results have been mixed for cancers of the lung[685–690], oral cavity[691, 692], and stomach[693]. In most of these cases, it should be emphasized that there is little information about exposure and the only relevant etiology is probably tobacco-derived nitrosamines. In a study of workers exposed to vinyl chloride (a P450 2E1 substrate[304]), some association was found between P450 2E1 polymorphisms and p53 mutations[694]. However, it should be emphasized again that the relevance of *CYP2E1* polymorphisms to known P450 2E1 reactions is unclear, particularly *in vivo*[673], and it is difficult to define roles of these genetic polymorphisms in cancer risk; overall P450 2E1 expression due to environmental influences may have a role but is more difficult to establish.

6.14. P450 2F1

This is primarily a lung P450. In 1990, Nhamburo *et al.*[695] cloned the cDNA from a human lung library. The level of expression appears to be relatively low, as judged by the mRNA abundance. The apparent orthologs 2F2 and 2F3 have been studied in mouse and goat lung, respectively.

P450 2F1 has been expressed in heterologous systems. Catalytic activity was observed for 7-ethoxy- and -propoxycoumarin *O*-dealkylation and 7-pentoxyresorufin *O*-dependentylation. The enzyme showed modest activation of the lung toxin and (potential drug candidate) 4-ipomeanol[696]. However, the ability of P450 2F1 to activate the potential lung toxicants 3-methylindole, naphthalene[697], and styrene[698] is more impressive (the activation of 3-methylindole appears to involve initial desaturation[697]).

The basis for the selective expression of P450 2F1 in lung is unknown. Recently, Carr *et al.*[699] isolated the *CYP2F1* gene. Using luciferase-based constructs, they identified a specific promoter element that binds a protein in the −152 to −182 5′ region. This protein is termed a lung specific factor (LSF).

6.15. P450 2J2

The P450 2J2 cDNA was first isolated from a human liver library but was found to be most highly expressed in heart[700]. Expression (mRNA)

has also been found in kidney and muscle[700], lung[701], and the gastrointestinal tract[702].

Zeldin's group has done most of the work on this P450, including the initial cloning and analysis of tissue expression. Incubation of a recombinant P450 2J2 (plus reductase and NADPH) with arachidonic acid yielded all four epoxides, that is, epoxyeicosatetraenoic acids (EETs)[700]. These EETs were found in heart tissue, and the stereochemistry of the recombinant P450 2J2 products was found to match that of the compounds isolated from tissue. A number of physiological functions have been postulated for the EETs, reviewed elsewhere[703].

The extent of human variability of expression of P450 2J2 has not been reported. However, Zeldin's group has sequenced *CYP2J2* genes and found a number of SNPs[704]. One was in the promoter region, eight were exonic regions, five were in introns, and four were in the 3′-untranslated region. Only four of the SNPs resulted in amino acid changes. These allelic variants were expressed in a baculovirus system; all had activity toward arachidonic and linoleic acids within a 2-fold level of wild-type P450 2J2, with the N404Y variant showing only 10% catalytic activity (all assays only done at a 100 μM substrate concentration), although some qualitative changes in products were seen with the I192N substitution. The physiological relevance of these substitutions is presently unknown.

6.16. P450 2R1

The only information available is the presence of the *CYP2R1* gene in the human genome[705].

6.17. P450 2S1

This gene was identified by searching databases by Rylander *et al.*[706] mRNA and protein blotting work indicate highest expression in trachea, lung (and fetal lung), stomach, small intestine, and spleen. Expression was also relatively abundant (mRNA level) in colon, appendix, liver, kidney, thymus, substantia nigra, peripheral leukocytes, and placenta. Absolute levels of abundance are unknown.

Rivera *et al.*[87] demonstrated that both mouse and human P450 2S1 mRNA transcripts are inducible by TCDD in cell culture.

No other information is presently available about P450 2S1.

6.18. P450 2U1

As with some of the other human P450 genes, the only information presently available is the identification of the *CYP2U1* gene in the human genome[705].

6.19. P450 2W1

No information is available at this time except for the existence of the *CYP2W1* gene in the human genome[705].

6.20. P450 3A4

P450 3A4 is the most abundant P450 in the body (e.g., Figures 10.2 and 10.4) and has a dominant role in drug metabolism (Figure 10.3). Some of the earliest preparations of human P450 (refs [3], [4]) were retrospectively found to be P450 3A4. Two approaches led to an extensive characterization. Watkins *et al.*[13] isolated a P450 from human liver using the criterion of immunochemical cross-reactivity with what is now recognized as a rat 3A subfamily P450; this laboratory isolated an enzyme from human livers that catalyzed the oxidation of the hypotensive dihydropyridine drug nifedipine[12]. cDNA cloning yielded sequences corresponding to *CYP3A3* (ref. [707]) and *CYP3A4* (ref. [708]). (The former differed from *CYP3A4* at 14 sites and could be considered a rare allele, although it has not been reported again[709–711] and originally came from the same single-liver cDNA library as the *CYP3A4* clone; *CYP3A3* has been dropped from the nomenclature and earlier references to this should probably be considered to indicate P450 3A4.)

Subsequently, studies with microsomes, antibodies, and purified P450 3A4 quickly indicated that nifedipine was not the only substrate; other substrates included other dihydropyridines[712], steroids[12, 713], quinidine[106], the oral contraceptive 17α-ethynylestradiol[72], and the carcinogen aflatoxin B_1 (ref. [714]). With more studies and the application of recombinant systems, the repertoire of substrates expanded rapidly[715].

6.20.1. Sites of Expression and Abundance

P450 3A4 is the most abundant P450 in human liver and in the small intestine. The average fraction of the total P450 in liver accounted for by P450 3A4 is ~25–30%[28] (Figures 10.2 and 10.4); in the small intestine, the fraction attributed to P450 3A4 is even higher. A study with the selective inhibitor gestodene, which destroys P450 3A4, indicates that P450 3A4 can constitute 60% of the total hepatic P450 (ref. [716]).

P450 3A4 is also expressed in some extrahepatic tissues, including lung[244, 717], stomach, colon[244], and adrenal (weak)[718]. P450 3A4 does not appear to be expressed in kidney, prostate, testis, or thymus, but other 3A subfamily P450s are[718]. The literature is mixed on whether expression occurs in peripheral blood lymphocytes or not[718, 719].

A gender difference in P450 3A4 expression does not appear to occur[28] and apparent pharmacokinetic differences may be attributable to *P*-glycoprotein, not P450 3A4 (ref. [55]). In fetal liver, P450 3A7 is the most abundant form and P450 3A4 expression is very low[58, 720]. P450 3A4 expression increases rapidly after birth and reaches 50% of adult levels between 6 and 12 months of age[720]. Although many general regulatory concerns have been expressed about additional safety margins for children with drugs and other chemicals, the evidence in this case indicates that P450 3A4 activity levels in infants are slightly higher than in adults[720].

P450 3A4 is expressed in some tumors, although the literature is mixed as to reports of levels lower and higher than the surrounding tissue[721–723].

6.20.2. Regulation and Polymorphism

The *CYP3A4* gene is at chromosome 7q22.1 (ref. [724]). Although 3A subfamily enzymes were long known to be inducible in animals[725] and considerable literature existed on the *in vivo* induction of many activities by barbiturates and macrolide antibiotics (e.g., rifampicin)[40], early demonstrations of inducibility were indirect but some progress was made[13]. A general correlation between enzymes and mRNA levels could be shown in human livers[707, 709]. Defining the mechanism of regulation was difficult[726], to some extent because of difficulty in finding appropriately responsive cells to utilize the *CYP3A4* gene and vector constructs derived from it. Guzelian's laboratory reported that the source of liver cells was a greater issue than the *CYP3A* regulatory region in comparing interspecies differences in *CYP3A* gene regulation[727], and this result can now be rationalized in the context of new knowledge about receptors (*vide infra*).

Although most *CYP3A* subfamily genes are inducible by dexamethasone, the classic glucocorticoid receptor was shown not to be involved in rat liver[728]. In early 1998, Maurel reported that the macrolide antibiotic rifampicin acted as a nonsteroid ligand and agonist of the human glucocorticoid receptor, providing a possible mechanism for regulation and a difference with the rodent systems[729]. The interpretation of these conclusions was questioned by Ray *et al.*[730].

Shortly thereafter, Kliewer's group characterized the human homolog of a mouse receptor (PXR) that bound steroids and interacted with *CYP3A* subfamily genes in the manner expected for a major regulatory influence[731, 732] (some literature also refers to the human PXR as "SXR"). This member of the steroid receptor family "orphan" group interacted with barbiturates, steroids (including dexamethasone), statin drugs, macrolide antibiotics, and some organochlorine pesticides[732, 733].

Knowledge of the PXR and its cognate binding site has led to the development of PXR receptor and reporter assays to screen for P450 3A4 induction with new drug candidates[734–736]. The discovery of the PXR receptor suggested that alleles of this receptor might be responsible for the variable inducibility in different individuals. However, the SNPs found to date have not been found to control P450 3A4 induction[737]. The regulation of *CYP3A4* expression is more complicated than simple loading of activated PXR (e.g., Figure 10.6), as suggested by Kliewer's early work showing the roles of coactivators[731, 732]. However, the glucocorticoid-mediated induction of P450 3A4 is mediated by elements in addition to the now-canonical PXR site[738, 739]. Some compounds (e.g., ketoconazole) suppress *CYP3A4* gene expression, apparently via binding to the PXR and interaction with "co-repressors" (NCoR, SMRT)[740]. CAR (see Section 6.7.2) appears to interact with the *CYP3A4* gene at the PXR site and induce[741]. Further, there is

evidence that 1α,25-dihydroxyvitamin D_3 (see Section 6.53) also controls the transcription of P450 3A4 (ref. [742]). This effect is mediated through the vitamin D receptor[342], which has similarity to PXR and CAR in the steroid receptor superfamily. Kinases have been shown to modulate the induction of P450 3A4 via the vitamin D receptor in Caco-2 cells[743].

Other factors also contribute to P450 3A4 regulation. Among these are C/EPPα and DBP[744] and HNF-4α (ref. [745]). Interleukin-6 has been reported to downregulate P450 3A4 through translational induction of the repressive C/EBPβ-LIP protein[746]. Thus, the transcriptional regulation of P450 3A4 expression centers on PXR but involves many other aspects.

Another aspect of P450 3A4 regulation involves degradation. Troleandomycin, erthyromycin, and some related amine macrolide antibiotics form "metabolite complexes" (C-nitroso : iron, R–N= O : Fe) and inactive protein accumulates[747, 748]. These studies have relevance to *in vivo* P450 3A4 inhibition by these drugs.

P450 3A4 appears to be degraded by a ubiquitin-linked pathway[221]. Correia's group also reported that protein kinase C modified P450 3A4 at Thr264 and Ser420; the relevance of these phosphorylations to ubiquitin-linked degradation is yet unknown[749].

The issue of polymorphism is considered in the context of attempts to explain the population variability in P450 3A4 activity, which does not show true modality in its distribution[750]. A number of SNPs and other polymorphisms have been identified, but they have not shown much relationship to catalytic activities yet[751–757].

6.20.3. Substrates and Reactions

Analysis of the catalytic activity of P450 3A4 and other 3A subfamily enzymes is not always easy to assess because of nuances about the effects of the membranes and other proteins, as discussed in Section 6.20.4. Wrighton has examined P450s 3A4, 3A5, and 3A7 under identical conditions and concluded that P450 3A4 is generally more catalytically active than 3A4 or 3A7 toward all substrates examined[758].

P450 3A4 contributes to the metabolism of ~50% of the drugs on the market or under development (Figure 10.3). For an extensive list, see Rendic[32]. Many of these are important drugs such as lovastatin (Mevacor®) and other statins[759], the prostate hypertrophy inhibitor finasteride (Proscar®/Propecia®)[760], the immune suppressant cyclosporin[761, 762], protease inhibitors such as indinavir[763], and sildenafil (Viagra®)[764].

In the course of these reactions, P450 3A4 catalyzes examples of some atypical reactions[524] including desaturation[759], oxidative carboxylic acid ester cleavage[765], and oxidation of a nitrile to an amide[766]. An unexpected reaction encountered in this laboratory was the oxidation of alkylphenyl ether non-ionic detergents, which have been commonly used in enzyme purifications[328] and also have some medical and industrial applications[767]. Methylene hydroxylations yield hemiacetals, which break down to shorten the chains[767].

One of the classic (and fastest) reactions catalyzed by P450 3A4 is testosterone 6β-hydroxylation[12]. However, the physiological significance of this and other (P450 3A4-catalyzed) steroid hydroxylations[713] is unclear. The significance of P450 3A4 in physiology may be questioned, given its variability (Figure 10.1 and Table 10.5). However, some contributions are possible and may be suggested in recent work. Cholesterol is oxidized by P450 3A4 to 4β-hydroxycholesterol, a major circulating oxysterol[768, 769]. P450 3A4 also catalyzes the 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol[770, 771]. The product is a potent PXR agonist, and this system might function as an autoregulatory pathway (i.e., excess triol activates PXR and P450 3A4, which reduces the level of triol[772]).

P450 3A4 also functions in the metabolism of cancer chemotherapeutic drugs. In addition, attention has been given to activations of drugs and chemical carcinogens. P450 3A4 activates the estrogen receptor antagonist tamoxifen to produce DNA adducts[773]. Another example of carcinogen activation involves aflatoxin B_1, which undergoes both a detoxicating 3α-hydroxylation and formation of the highly mutagenic 8, 9-*exo*-epoxide[714, 774, 775]. Some other carcinogen substrates of P450 3A4 are listed in Table 10.4.

One of the issues with P450 3A4 is which reaction provides the most appropriate index of activity, both *in vitro* and *in vivo*. Historically nifedipine oxidation and testosterone 6β-hydroxylation were among the first activities identified[12] and are still used *in vitro*[71]. Midazolam 1′-hydroxylation has also been used[71], in part because of its acceptance for *in vivo* assays.

Some higher throughput fluorescence assays have also been developed and gained commercial appeal[776, 777]. One issue regarding these and also several other P450 3A4 reactions is that they show variable effects of added chemicals, that is, one compound may inhibit a certain P450 3A4 reaction but stimulate another. Chauret *et al.*[778] reported a fluorescence reaction that behaves in a very similar way to testosterone 6β-hydroxylation. Houston has examined the behavior of P450 3A4 probe substrates *in vitro* and grouped them into two categories. Although all of these reactions are catalyzed by P450 3A4, they are categorized into two groups by their behavior in the presence of other compounds, as mentioned above[779]. One group includes testosterone, cyclosporin, and erythromycin. The second includes midazolam, triazolam, dextromethorphan, and diazepam. Terfenadine fits in either group and nifedipine seemed to have properties unique from both groups[779].

The ambivalence about the variability of probe drugs is even worse for *in vivo* human experiments than *in vitro*, as one might expect. A number of reactions have been used including nifedipine oxidation[780], erythromycin *N*-demethylation[781], lidocaine oxidation[782], dapsone *N*-hydroxylation[783], midazolam 1′-hydroxylation[784], and quinine 3′-hydroxylation[785]. In most cases, the test drug is administered orally for convenience, except for some uses of erythromycin and midazolam (i.v.). The ratio of urinary 6β-hydroxycortisol to cortisol has also been used to assess P450 3A4 function[786]. Many of the assays reflect the activity of P450 3A4 in the small intestine, particularly with the drugs administered orally. The erythromycin breath test (exhaled CO_2 produced from the HCHO released in the reaction) is generally used to estimate hepatic P450 3A4 and has been used as an aid in selecting cyclosporin doses for liver transplant patients[787]. The lack of correlation of these indicators is still a problem in the practical analysis of drug interactions[788–790]. Some of the discrepancies are probably inherent in the nature of P450 3A4 itself (i.e., see *in vitro* assays, *vide supra*). Other issues involve the lack of coordinate regulation of hepatic and intestinal P450 3A4 (ref. [791]) and the activity of *P*-glycoprotein[792], which shows some overlap in regulation patterns with P450 3A4 (ref. [793]) and influence the availability of substrates to P450 3A4 in both small intestine and liver.

6.20.4. Knowledge about Active Site

Because of the importance of this enzyme in drug metabolism (Figure 10.3), many efforts have dealt with developing a better understanding of its function and there is hope that intelligent predictions can be made regarding activities toward new drug candidates. However, as already alluded to, P450 3A4 has some unusual properties and a number of important issues have not been resolved.

In the early purifications of P450 3A4 (ref. [12]), reconstitution conditions were difficult to optimize and showed some unexplained variations, which was also the case with recombinant enzyme[715, 794]. A major factor was the composition of the lipid/detergent environment[795, 796]. Another issue was the NADPH-P450 reductase. P450 3A4 expressed in yeast showed poor coupling with yeast NADPH-P450 reductase[715], although P450 2C9 had coupled well[374]. Pompon developed a yeast system in which yeast NADPH-P450 reductase and b_5 were eliminated and replaced by the human counterparts, enabling P450 3A4 function[797]. Studies with purified P450 3A4 and NADPH-P450 reductase have shown that P450 3A4 reduction is generally slow in the absence of substrate and greatly enhanced by substrate[798].

The role of b_5 in P450 3A4 reaction is a somewhat complex subject. Some reactions of purified P450 3A4 are stimulated by b_5 but others are not[799, 800]. With reactions that are influenced by b_5 (e.g., nifedipine oxidation and testosterone 6β-hydroxylation), a role for b_5 could be demonstrated in human liver microsomes using antibodies[801]; stimulation by b_5 can also be demonstrated with P450 3A4 heterologously expressed in bacterial membranes[425, 802], although the presence of b_5 does not seem as critical for function as with the purified systems[803, 804]. Other considerations suggest that the amount of b_5 in heterologous expression systems should not be an issue in the development of "relative activity factors" for extrapolations from hepatic systems[805]. The mechanism of stimulation of P450 3A4 activities by b_5 is still not clear. Pompon used a membrane system and interpreted the results in the context of a "classical"[655, 806] transfer of an electron to the FeO_2^{2+} complex[807]. However, the activities of purified P450 3A4 were stimulated by apo-b_5 (devoid of heme) as effectively as by b_5 (ref. [808]), and similar effects have now been

reported for several other P450s (refs [425], [809], [810]). Although an alternate mechanism involving rapid transfer of P450 3A4 heme to apo-b_5 has been proposed[811], evidence ruling out this mechanism has been presented[812].

A number of models of the P450 3A4 protein have been presented[301, 813–815], most of which are based on homology modeling. At the time of this proof (April 2004), the Astex company has publicly announced a crystal structure for P450 3A4, presumably in the absence of ligands, but the information is proprietary and no details have been released.

A number of site-directed mutagenesis studies on the possible roles of individual residues have been published. Phe304[816] and Ala305 (ref. [817]), in the putative I-helix, are proposed to control access to the catalytic center. Phe304 was also implicated in the partitioning of aflatoxin B_1 oxidation (between 3α-hydroxylation and 8,9-*exo*-epoxidation)[818]. A role for Asn206 was also proposed in the work with aflatoxin B_1 (ref. [818]). Leu211 is also postulated to control the size of the active site[819].

Another issue about considerations of predicting sites and rates of P450 3A4 reactions deals with models based on chemical reactivity. The concept has been proposed that P450 3A4 has a relatively open active site and that reactions are influenced largely by the chemical lability of C–H bonds[820], and some commercial software systems have utilized such concepts. This approach to P450 catalysis may have some utility, and substrate linear free energy relationships have been exploited in our own research with rat P450 2B1 (ref. [821]). However, there are some concerns about exactly how appropriate the predictions are beyond specific sets of substrates. Knowledge of rate-limiting steps in P450 3A4 reactions is still rather rudimentary, in part because of some of the various complications discussed here. The rate of transfer of the first electron from NADPH-P450 reductase is slow in the absence of substrate, but quite rapid in the presence of substrate and an equimolar concentration of NADPH-P450 reductase[798]. In liver microsomes, the testosterone-stimulated rate of P450 reduction appears to be faster than the overall rate of 6β-hydroxylation[798], but conclusions are complicated by the inability to observe only the P450 3A4 component of the reduction. Although some apparent (FeO) intermediate complexes do appear to accumulate in P450 3A4 reactions[822], these are not well characterized yet. One approach to analysis of rate-limiting components of P450 (and other enzyme) reactions is the use of kinetic deuterium isotope effects[823, 824]. Interpretation of kinetic isotope effects in enzymatic reactions is a complex subject, but a simple interpretation of a significant intermolecular noncompetitive hydrogen isotope effect is that C–H bond-breaking is at least partially rate limiting[825]. Despite the interest in P450 3A4, relatively few kinetic deuterium isotope effect studies have been reported. Obach[826] reported an isotope effect of only 1.3 on the hydroxylation of the drug ezlopitant, although details regarding k_{cat} and K_m are lacking and further interpretation of this result is difficult. Work in this laboratory with 6-d_2-testosterone has somewhat surprisingly shown a low competitive isotope effect ($^{D}V$and $^{D}(V/K)$ ~3, J.A. Krauser and F.P. Guengerich, unpublished results). Testosterone 6β-hydroxylation is one of the fastest reactions catalyzed by P450 3A4 and one might expect less masking of an isotope effect in a system in which other steps are known to be occurring efficiently.

In some of the previous sections, compounds have been mentioned that stimulate the catalytic activities of P450s after direct addition to the enzyme, as opposed to regulation of genes in cells or animals. This process is referred to as "stimulation" (as opposed to induction). The phenomenon has been recognized for some time with P450s (refs [107], [109], [827]). Conney's group demonstrated the activation of benzo[*a*]pyrene 3-hydroxylation and aflatoxin B_1 activation by αNF in human liver microsomes[828, 829]. Johnson also demonstrated the enhancement of human liver microsomal 17β-estradiol 2-hydroxylation by αNF[830]. Subsequent work in this laboratory provided evidence that P450 3A4 was the human liver P450 most stimulated by αNF[146, 714]. P450 3A4 is the P450 about which most of the discussion about cooperative behavior has been given, although some reports of cooperativity have appeared regarding P450s 1A2, 2B6, and 2C9 (Sections 6.7.4 and 6.9.4). In addition, P450 2C19 may show stimulation by some compounds (R. Kinobe, B.D. Hammock, and E.M.J. Gillam, personal communication).

In considering cooperativity, two types will be described, using a convention we[429, 831] have adapted from Kuby[824]. "Homotropic" cooperativity refers to nonhyperbolic phenomena (either

steady-state reaction kinetics or binding) seen with the addition of a single compound to an enzyme. The most typical type is a sigmoidal, or "S-shaped" curve, which when analyzed by a Hill plot ($v = V \cdot S^n/[S + S^{50}]$) yields a value for $n > 1$ (S^{50} is an approximation of the usual K_m), or "positive cooperativity." Examples of "negative cooperativity" are less common but documented ($n < 1$) as in a case with rabbit P450 1A2 (ref. [214]). The other phenomenon is "heterotropic cooperativity," described above as "stimulation," where two compounds are added to an enzyme and one enhances the catalytic action of the enzyme on the other. In some cases, both homotropic and heterotropic cooperativity can be operative[429].

Homotropic cooperativity was seen in the oxidation of aflatoxin B_1 by P450 3A4 (ref. [832]). The previously reported stimulation of P450 3A4 activities by αNF[714] was not seen for some reactions, and the *N*-oxygenation of 4, 4′-methylene-*bis*(2-chloroaniline) was inhibited[833]. Subsequently, studies with aflatoxin B_1 oxidation showed that 3α-hydroxylation was inhibited and 8,9-(*exo*)-epoxidation was stimulated by αNF[429, 774]. Aflatoxin B_1 (or an analog) did not modify the 5,6-epoxidation of αNF, however. Interestingly, the positive cooperativity seen in Hill plots for the oxidation of aflatoxin B_1 (for both reactions) was eliminated in the presence of αNF[429]. The values for *n* in the Hill plots (2.1–2.3) are probably the highest for any P450 apparent cooperativity reported to date. Most are much lower. One technical issue of particular note is that those reactions that proceed too far at low substrate concentrations will show apparently low rates (due to substrate depletion or product inhibition) and artificial sigmoidicity can be created.

Many seemingly unusual P450 3A4 reactions and patterns have been reported (*vide supra*). P450 3A4-catalyzed testosterone 6β-hydroxylation and erythromycin *N*-demethylation are not very competitive[834]. Hydroxylation of meloxicam is stimulated by another substrate, quinidine[835]. Lu and his group showed that inhibition patterns for several known P450 3A4 reactions were substrate dependent[836]. Similar discrepancies were reported by Weinkers' laboratory[837]. The (mechanism-based?) inactivation of P450 3A4 by diclofenac was stimulated by the substrate quinidine[838]. One interpretation of some of the results is that a single active site accommodates two (or more substrates), and many of the data can be fit to a model with this much freedom (basically Michaelis–Menten expression with two values each for k_{cat} and K_m, or insert of proportionality factors before the parameters)[839–843].

Halpert's laboratory has changed a number of the amino acids in P450 3A4, based mainly upon homology modeling, and found that several can alter the homotropic and the heterotropic cooperativity. These residues include Ala305 (ref. [844]), Leu211, Asp214 (ref. [845]), Ser119, Ala370, Ile301 (ref. [846]), and possibly some others as well[847]. A general conclusion from much of this work is that two or possibly three ligands co-occupy the binding site and alter each other's juxtaposition to generate some of the observed effects. One problem with much of the work in this field is that actual binding phenomena are not necessarily investigated. However, binding has been analyzed in some of the work[831, 845] and shown to exhibit homotropic and heterotropic cooperativity. Further, combinations of binding and inhibition results obtained with several ligands in this laboratory were consistent with a scheme in which three ligand subdomains exist in the overall binding site of P450 3A4 (ref. [831]), in agreement with the current hypotheses of Halpert[846]. More evidence consistent with such a model is available from a fluorescence study by Atkins and Weinkers[848], in which pyrene–pyrene stacking spectra were observed. This work provides some of the stronger evidence to support the "multiple-substrate site" model.

A crystal structure of bacterial P450 107A1 has been solved with two ligand molecules present[849]. The binding titration shows homotropic cooperativity[849] and also some heterotropic cooperativity[850]. Because the redox partner of P450 107A1 is not known, obtaining reasonable catalytic activity is difficult and the relevance to P450 3A4 is still not definite[850].

Another aspect and possibly another solution to the issue comes from work by Friedman using flash photolysis kinetics (of CO rebinding after photodissociation from ferrous P450 3A4). The kinetics were multiphasic and were selectively altered by the presence of different substrates[851]. Heterotropic effects were observed with benzo[*a*]-pyrene and αNF[852]. The interpretation of the results is that different substrates differentially modulate these kinetics by (a) changing the P450

conformation to alter the rate, and/or (b) steric effects (of ligands) that reduce rates[853]. Both effects are possible, although the enhancement of rates in some cases[851] argues against the generality of the latter explanation and in favor of multiple conformations for P450 3A4 bound to various ligands. The concept advanced is that some ligands act as allosteric factors to "switch" P450 3A4 conformations[854]. Some possibly relevant work has been done by Anzenbacherová *et al.*[855], who did pressure studies on P450 3A4 and found that the compressibility of P450 3A4 was less than that of bacterial P450 102; the compressibility was modified by the ligand troleandomycin (TAO). The concept of preexisting multiple conformers of P450 3A4 is an explanation for the flash photolysis work[851–854] and has support in newer nonclassical approaches to general protein chemistry[856–858]. This view differs from the more general static "lock-and-key" view of enzyme/substrate complexes and the induced-fit theory in which enzymes are "shaped" by their substrates. The basic concept is protein dynamics present an ensemble of structures of an enzyme in solution and different ligands bind to individual states depending upon their complementation[856–858]. Another consideration in this discussion, somewhat related, is that there is good evidence that P450 conformations change during the course of the catalytic cycle[859], and evidence has already been presented that different forms of P450 3A4 can differ in their binding of a ligand (e.g., ferric and ferrous)[831].

Where does all of the work in this area to date leave us? A recent review by Atkins *et al.*[860] summarizes much of the work in more detail and presents a cogent analysis. Summarizing and expanding on this, there are several major possibilities to explain the observed cooperativity of P450 (and the other P450s showing this behavior), which are not necessarily exclusive: (a) a "classic" allosteric model with binding of effectors at a site that then regulates the conformation of substrate binding, (b) a relatively rigid P450 with a large active site that can accommodate 2–3 ligands, with the results depending on the chemical interactions of the two ligands with each other and with P450 residues; and (c) a series of preexisting conformations of P450 3A4 that selectively interact with individual ligands[856–858]. A general concept of induced fit is related to the third possibility, as in the phenomena already mentioned that different protein conformations exist throughout the catalytic cycle, can differ in affinities and substrate orientation, and may not be in rapid equilibria. Many steady-state kinetic schemes have been proposed but, in considering the possible origins[824, 861], can never be considered unique and do not provide mechanistic answers. The availability of a series of three-dimensional X-ray structures of P450 3A4 with various ligands would provide insight into the conformational rigidity of P450 3A4 and the number of modes of binding. Another possible set of experiments involves restraining conformational changes through engineering (e.g., with reversible disulfide bonds) and examination of the effects. Another concern, already expressed here, is that most of the studies in this field have avoided measuring binding, with some exceptions[831, 845, 848]. Some attempts have been made to directly quantify P450 3A4-ligand interactions (e.g., dialysis and equivalent methods), but the technical problems associated with equilibrium binding (e.g., insolubility and nonspecific components) are not trivial. At this time *a priori* prediction of cooperativity is not really possible, except perhaps in extension of chemical classes already covered. The lack of ability to predict P450 3A4 cooperative ligand interactions indicates a deficiency in being able to predict all ligand interactions.

Is the cooperativity of P450 3A4 relevant to any practical issues? Atkins[862] has discussed the general implications of the issue to toxicology, although conclusions are speculative because the function of P450 3A4 can be good, bad, or not really selected for anyway. Some evidence for an interaction between caffeine and acetaminophen in rat models is suggestive of a heterotropic interaction[110, 863]. Dextromethorphan studies in primary hepatocytes also show cooperativity[108]. Cooperativity in human hepatocyte systems has also been reported for oxidation of midazolam and warfarin[864]. Cooperativity is a possible mechanism for a drug interaction between felbamate and carbamazepine[865]. One of the strongest cases involves an *in vivo* study on the enhancement of diclofenac in monkeys by quinidine[111, 864], which apparently cannot be attributed to induction. In summary, there is some evidence for *in vivo* P450 3A4 cooperativity, but at this time, the issue is generally considered to be less of a problem than enzyme induction or inhibition.

6.20.5. Inhibitors

Inhibition of P450 3A4 is a major issue in the pharmaceutical industry because of a number of important drug–drug interactions. One example of a problem leading to recall of a drug is that of terfenadine[103, 104, 866].

Erythromycin and ketoconazole are two of the most established inhibitors of P450 3A4, based on clinical experience. Ketoconazole, used at ~1 μM, is probably the best established P450 3A4 inhibitor for *in vitro* use[64]. Another P450 inhibitor is TAO[867], which also has clinical implications. TAO has been used as a diagnostic *in vitro* inhibitor of P450 3A4, although its mode of action (activation to a nitroso that complexes P450 iron) requires time for the inhibition to occur.

A compendium of P450 3A4 inhibitors has been compiled by Rendic[32]. Only a few other specific examples of P450 inhibitors will be mentioned here.

One issue is the inhibition of P450 3A4 by grapefruit juice, first reported by Bailey[868]. The effect was rather specific for grapefruit and a few other citrus fruits (not orange), and warning labels now include this contraindication for many drugs[869]. Naringenin has some effect[870], but the most active principles appear to be the furanocoumarins bergamottin and 6′,7′-dihydroxybergamottin, which behave as mechanism-based inactivators to destroy intestinal P450 3A4 (refs [99], [100]). The magnitude of the effect of the interaction varies with drugs, with some of the statins, buspirone, terfenadine, astemizole, and amiodarone reported to show the greatest interactions[869].

Many of the HIV protease inhibitors are also potent inhibitors of P450 3A4 as well as substrates in some cases[871]. Because of the variety of drugs that AIDS patients use, the potential for interactions is considerable.

The effects of some herbal medicines on P450 3A4 have already been mentioned. In addition to P450 3A4 induction (e.g., St. John's wort), some of these materials also contain inhibitors. For instance, kava-kava extracts produce kavapyrones that inhibit P450 3A4 (ref. [872]).

Oral contraceptives contain acetylenes and can be mechanistic inactivators of P450 3A4. Inactivation has been demonstrated for 17α-ethynylestradiol, the major estrogenic component of oral contraceptives[72, 873], and several of the progestogenic components, particularly gestodene[716]. Because of the very low doses of these contraceptives that are used today, the effects might be expected to be small[874] although some *in vivo* effects have been reported[875, 876].

Finally, some chemicals and also oxidants have been shown to cause the covalent crosslinking of heme to apo-P450 (ref. [877]). Correia's group has characterized the products of the destruction of P450 3A4 with cumene hydroperoxide; the information is consistent with a dipyrrolic fragment of heme bound to a fragment of the protein[878].

6.20.6. Clinical Issues

The major issues involving P450 3A4 in drug development and clinical use are related to the role of the enzyme in drug disposition, particularly bioavailability and drug–drug interactions due to induction or inhibition[879, 880]. High enzyme activity toward a drug will reduce bioavailability, and variations in levels of P450 3A4 can cause clinical problems when the therapeutic window is narrow. For instance, low cyclosporin levels will not prevent organ rejection during transplant but high levels cause renal toxicity, so adjustment of the dose can be very useful[881]. Terfenadine has a relatively wide window for use but a few serious problems were encountered[104, 882]. Renwick has considered population models of P450 3A4 variability and concluded that there is more inter-individual variability from the oral route than i.v., which is not surprising in light of the previous discussion of the intestinal contribution to drug metabolism. A "default factor" for adults of 3.2-fold is presented, but a factor of 12(-fold) was calculated to be needed to cover 99% of the neonates as well[883].

The effect of disease on P450 3A4 has been considered. P450 3A4 expression appears to be decreased as a result of liver cirrhosis or cancer[595, 721, 884]. P450 3A4 levels were also decreased in celiac disease and reversed by a change in diet[885].

The interactions of herbal medicines with P450 3A4 have already been mentioned and are one of the worst problems with these mixtures[886]. One of the most studied issues is St. John's wort, which induces P450 3A4 as an agonist of the PXR receptor[887, 888]. The induction of P450 3A4 by St. John's wort has been responsible for the loss of the effectiveness of oral contraceptives[95, 889]. The resulting pregnancies

are the result of contraceptive failure due to more rapid elimination of 17α-ethynylestradiol, a phenomenon previously reported for P450 3A4 induction by rifampicin and barbiturates[72, 94, 101].

P450 3A4 is also of some interest regarding cancer, regarding exogenous carcinogens, drugs used to treat cancer, and metabolism of steroids or other compounds that may affect cancer risk or response to chemotherapy. Some chemical carcinogens activated by P450 3A4 are shown in Table 10.4. The activation and detoxication of aflatoxin B_1 have already been discussed in the context of 3α-hydroxylation (to aflatoxin Q_1) and formation of the highly reaction *exo*-8,9-epoxide[714, 774]. However, aflatoxin B_1 is a hepatocarcinogen and must reach the liver to cause damage. In a rat model, induction of rat P450 led to an increase in small intestinal DNA adducts, suggesting that activation of aflatoxin B_1 at this site constitutes a detoxication process, in that these cells are rapidly sloughed and do not progress to tumors[890].

CYP3A4 genotypes have been reported to be related to leukemias caused by prior treatment with epipodophyllotoxin[891]. P450 3A4 expression, measured at the mRNA level, has shown an inverse correlation with response of breast cancer patients to docetaxel, presumably due to changes in bioavailability[892]. However, no relationships were found for any *CYP3A4* genotypes in therapy-related myeloid malignancies[893]. One of the more controversial issues involves whether *CYP3A4* genotypes are linked with prostate cancer, with reports for and against an association[894–899]. The point should be made that strong evidence for a change in an accepted P450 3A4 phenotype has not been made in many of these cases.

6.21. P450 3A5

P450 3A5 has 85% sequence identity with P450 3A4 and, although generally accepted to have less importance than P450 3A4, is of interest because of its polymorphic and racial distribution and possible relevance to clinical issues with P450 3A subfamily reactions.

6.21.1. Sites of Expression and Abundance

P450 3A5 ("Hlp3") was first purified from human adult liver and found to be polymorphically expressed[900]. Gonzalez found a liver sample apparently expressing only P450 3A5 and not 3A4, and used this to clone the cDNA[901].

P450 3A5 expression has been reported in liver, small intestine, kidney, lung prostate, adrenal gland, and pituitary[718, 902–904]. Some researchers have reported expression of P450 3A5 in peripheral blood cells (and *not* P450 3A4)[905] but others have not[718].

P450 3A5 is expressed in fetal liver, in contrast to P450 3A4, but in a polymorphic manner[906]. The overall expression of P450 3A5 (mRNA) as a part of all P450 3A subfamily transcripts has been estimated at 2%[718]. However, only about 25% of Caucasians express P450 3A5, and when it is present, the level is usually less than that of P450 3A4. However, a few individuals have been identified in which P450 3A5 is the predominant P450 3A subfamily enzyme. The variability in expression levels has been linked to a polymorphism (*vide infra*).

6.21.2. Regulation and Polymorphism

The regulation of *CYP3A5* gene seems to be similar to that of *CYP3A4*, although P450 3A5 does not seem as inducible. The fetal/adult selectivity of P450 3A4/3A7 is not seen with P450 3A5 (ref. [906]).

Maurel[907] reported genomic clones and found a CATA box (not TATA) in the promoter. The responses to glucocorticoids are probably explained by the PXR system[908]. A general conclusion has been reached that P450s 3A4 and 3A5 are co-regulated in the liver and intestine, in terms of transcriptional control[909], although other factors may alter the expression[791].

The polymorphic variation of P450 3A5 has been studied and several variants have now been identified (http://www.imm.ki.se/Cypalleles/)[757]. Alternate splicing is a very common phenomenon with *CYP3A5*, with ~50% of liver samples showing this (E.G. Schuetz, personal communication). Most Caucasians with low P450 3A5 protein expression have the *3 allele with an inserted intron[757, 910]. Interesting, the representation of the *1 allele is much higher in Africans and they express active P450 3A5. Other alleles are known, including changes in the 5′-regulatory region where transcription factors bind[911].

The *in vivo* consequences of 3A5 polymorphism are not clear. For instance, Huang found no

significant effect of the *3 polymorphism on midazolam pharmacokinetics[912].

6.21.3. Substrates and Reactions

Since the discovery of P450 3A5, the catalytic selectivity has been known to be similar to that of P450 3A4 (ref. [900]), and subsequent comparisons with P450 3A4 confirmed this view[913]. However, a general problem with P450 3A subfamily enzymes is that they are sensitive to the membrane environment and many reactions of P450 3A5 (but not all) are stimulated by b_5 (refs [425], [800]). In a few cases, the selectivity of P450 3A5 for different oxidation sites appears to differ from that of P450 3A4, for example, aflatoxin B_1 3α-hydroxylation vs 8,9-epoxidation[800, 818].

Recently, as noted above, Wrighton reported an extensive comparison of many reactions by recombinant P450s 3A4, 3A5, 3A7 under identical reconstitution conditions and concluded that P450 3A5 had equal or reduced activity compared to P450 3A4 in all cases[758].

6.21.4. Knowledge about Active Site

Because of the similarity of reactions of P450s 3A4 and 3A5, homology models for P450 3A4 are probably about as valid for P450 3A5. The availability of the Astex P450 3A4 three-dimensional structure should improve the understanding of P450 3A5 as well. Relatively little site-directed mutagenesis of P450 3A5 has been done, but one study of note is the effort by Correia and Halpert to utilize the differences in reactions with aflatoxin B_1 (refs [774], [800]) to probe the effects of changing residues in the putative active site[818].

6.21.5. Inhibitors

In general, the P450 3A4 inhibitors also inhibit P450 3A5. For instance, ketoconazole and fluconazole inhibited both P450s 3A4 and 3A5 (ref. [914]). The mechanism-based inactivator gestodene[716] also inhibits P450 3A5 (ref. [913]).

6.21.6. Clinical Issues

At this point, the significance of the wide variability in P450 3A5 is difficult to assess. As mentioned previously, Huang[912] found no significant effect of the *3 allele on midazolam pharmacokinetics in Chinese individuals. However, it is possible that the extrahepatic expression[718] may influence the course of particular drugs and other chemicals.

6.22. P450 3A7

Early work in the field of human P450 research was done by Kamataki and his associates with fetal samples which led to the purification of a P450 termed HFLa, now known as P450 3A7 (refs [915], [916]). Early research established that this was a major P450 in fetal liver (not in adult liver), and that the enzyme could catalyze several reactions[916].

6.22.1. Sites of Expression and Abundance

Early work established that P450 3A7 is the major P450 present in fetal liver[916] and is also present in other fetal tissues including kidney, adrenal, and lung[917]. Further work by Kamataki's group showed the existence of some immunochemically detectable P450 3A7 in gynecologic tumors and in human placenta, but interestingly not in cynomologous monkey placenta[918]. Guzelian's group also reported P450 3A7 protein in human placenta and endometrium, with elevation in the latter site during pregnancy or during the secretory phase of the menstrual cycle[919]. Subsequently, Sarkar *et al.*[920] reported 10-fold greater expression of P450 3A7 in endometrium in the proliferative rather than the secretory phase. Hakkola *et al.*[921] reported some expression of P450 3A7 mRNA in some first trimester placentas but not in full-term placenta[243].

With regard to development in fetal tissues, Juchau's group found expression of P450 3A7 in early fetal tissue (50–60 days)[922]. Schuetz *et al.*[923] found P450 3A7 mRNA in all fetal liver samples analyzed and also reported its presence in one half of adult liver samples. However, the issue may be the level of expression because Kamataki's group[58] had reported the fetal > adult selectivity. De Wildt *et al.*[720] also found fetal specificity and only very low levels of P450 3A7 in adults. P450 3A7 expression was high during embryonic and

fetal life, and decreased rapidly during the first week of life. Similar findings were reported by Hakkola *et al.*[906] Also, the variability of P450 3A7 expression was 5-fold in fetal tissue (and 77-fold in mRNA). In another report[924], P450 3A7 also disappeared rapidly after infancy.

6.22.2. Regulation and Polymorphism

As in the case of P450 3A4, relatively little solid evidence is available regarding the functional relevance of coding region SNPs. However, the regulation of this gene is complex, as one might expect after considering the temporal patterns of expression during development that were discussed earlier.

Kamataki's group published the cDNA[925] and genomic[926] sequences, which are similar to those of P450 3A4. However, more identity (~90%) is seen in the coding region than elsewhere[907, 926]. Recent work by Koch *et al.*[718] re-established that P450 3A7 only accounted for <2% of all P450 expression in adult human liver; a bimodality of P450 3A7 expression was seen, however. P450 3A4 and 3A7 constructs were expressed in various cell lines by Ourlin *et al.*[744], who showed differential responses to C/EBPα and DBP. As in the case with P450 3A4, P450 3A7 was inducible by rifampicin in cell culture[927]. P450 3A7 has a functional PXR element[928], as does P450 3A4 (*vide supra*), explaining the rifampicin response. Thus, one would expect fetal P450 3A7 induction by the usual P450 3A4 inducers.

Bertilsson *et al.*[929] have also reported a distal xenobiotic response enhancer module (XREM) in the *CYP3A7* gene. An $3A7_{\kappa}B$ element in *CYP3A7* is inactive in *CYP3A4* (ref. [930]), and this element has recently been shown to respond to p53 (T. Kamataki, personal communication). *CYP3A7* expression is regulated by Sp1, Sp3, HNF-3β, and upstream stimulatory factor (USF) 1. Far upstream (~11 kb) there are HNF-1 and HNF-4 and USF1 elements, which differ from the *CYP3A4* gene. Exactly how these and other sequence differences are involved in the rapid onset of P450 3A4, and decrease in P450 3A7 shortly after birth[59] is still not totally clear. Recent work in Kamataki's group suggests that after birth, CEB/Pα recruits an uncharacterized protein factor to squelch the $3A7_{\kappa}B$ site (T. Kamataki, personal communication).

Some interesting variants of *CYP3A7* genes have been reported. An mRNA species was found that contains exons 2 and 13 of a nearby *CYP3A* pseudogene spliced at the 3 end[931]. The *CYP3A7* 1C* allele is unusual in the sense that a part of the *CYP3A4* promoter replaces the corresponding region of *CYP3A7* (ER6 motif) and thus confers high levels of expression to *CYP3A7*1C* (ref. [932]).

6.22.3. Substrates and Reactions

Early studies with P450 3A7 purified from fetal liver established that testosterone 6β-hydroxylation is catalyzed by this enzyme[933]. Another early study indicated 16α-hydroxylation of dehydroepiandrosterone (DHEA) 3-sulfate[934]. These activities were later verified with the use of recombinant P450 3A7 (ref. [935]).

In general, P450 3A7 has catalytic activities rather similar to P450 3A4 and 3A5 (refs [936], 937]). Activation of aflatoxin B_1 (refs [938–940]) and heterocyclic amines[938] has been observed in various recombinant and transgenic systems, including transgenic mice[941]. Retinoic acid 4-hydroxylation by P450 3A7 has also been reported[942]. Wrighton's laboratory has done an extensive comparison of catalytic activities and concluded that rates for P450 3A7 are generally considerably lower for P450 3A7 than for P450 3A4 or 3A5 under similar conditions[758].

6.22.4. Knowledge about Active Site

Much less has been done with P450 3A7 than with P450s 3A4 and 3A5. Because the catalytic selectivity of P450 3A7 is similar to P450s 3A4 and 3A5, those models are probably about as applicable. One point of interest is the work of Kamataki's group showing that the substitution T485P improved holoprotein expression in *E. coli*[943].

6.22.5. Inhibitors

Inhibitors have not been studied extensively, but presumably all inhibitors of P450 3A4 are effective with P450 3A7, for example, ketoconazole, troleandomycin, etc.

6.22.6. Clinical Issues

The general point has already been made that P450 3A7 is the major human fetal P450 and, therefore, makes a major contribution to drug metabolism in the fetus. Thus, many, if not most, of the considerations regarding drug interactions etc. with P450 3A4 should be considered with respect to the fetus during pregnancy.

Another potentially important aspect is a report that P450 3A7 expression increases in hepatocellular carcinoma[944], possibly as a part of dedifferentiation.

6.23. P450 3A43

In 2001, three groups reported the characterization of a fourth member of the 3A subfamily, P450 3A43 (refs [945–947]). The sequence identity with the other 3A subfamily proteins is 71–76%. Expression could be detected in liver, kidney, pancreas, and prostate. Rifampicin was reported to induce P450 3A43 in human liver[945]. The level of expression in liver was generally agreed to be very low (~0.1% of P450 3A4).

Heterologous expression was achieved in bacteria[946] but not any of several eukaryotic systems[947]. The recombinant protein had only very low testosterone 6β-hydroxylation activity[946].

No information is available about polymorphisms, although the transcripts appear very prone to splicing[945]. There is a general agreement that P450 3A43 makes little contribution to drug metabolism, but specialized roles in extrahepatic tissues may be possible.

6.24. P450 4A11

6.24.1. Sites of Expression and Abundance

P450 4A11 cDNAs were first cloned from human kidney cDNA libraries using rodent P450 4A probes[948–950]. P450 4A11 is now known to be the major lauric acid ω-hydroxylase in human liver and kidney[951, 952], a fact of some historical interest in the sense that this was the activity first utilized in the separation and reconstitution of (rabbit) P450 (ref. [953]). The exact level of expression in these tissues is unknown; P450 4A11 expression has also been reported in human keratinocytes[954]. In cell cultures, P450 4A11 expression has been observed in primary cultures of human kidney proximal tubular cells[955] and HepG2 cells[956].

A gene originally assigned as *CYP4A11* by Kawashima's group[957] has now been assigned as that corresponding to *CYP4A22* and replaced by the *CYP4A11* gene corresponding to the P450 4A11 cDNA[952].

6.24.2. Regulation and Polymorphism

The levels of hepatic P450 4A11 vary ~10-fold among humans[951, 956]. To date no polymorphisms have been reported (April 2004; http://www.imm.ki.se/CYPalleles/). The regulation of P450 4A11 expression is not well understood but has relevance in consideration of the peroxisome proliferation system and any relevance to cancer. Induction of P450 4A11 by peroxisome proliferators has not been seen in primary human hepatocytes cultures, although P450 4A11 expression is readily detected[958]. In confluent HepG2 cell cultures, P450 4A11 is induced (independently) by peroxisome proliferators (e.g., Wy 14643) and dexamethasone[956]. The relevance of these results to the induction *in vivo* and the observed variability in expression is unknown.

6.24.3. Substrates and Reactions

P450 4A11 is the major lauric acid ω-hydroxylase[950, 951, 957, 959, 960]. The enzyme also catalyzes ω- and some ω-1 hydroxylation of myristic and palmitic acids[957, 960]. Some papers have reported arachidonic acid hydroxylation[952, 961, 962], but most studies have not associated this activity with P450 4A11 at any appreciable level[950, 957, 960]. Oliw's group has reported some hydroxylation of prostaglandin H_2 and analogs by P450 4A11 (ref. [963]).

6.24.4. Knowledge about Active Site

Some information is available about the active site from the catalytic selectivity among fatty acid substrates[960]. Some homology models have been presented[301, 964].

The interesting observation was made that the L131F mutant catalyzes only ω-1 hydroxylation and not ω-hydroxylation of lauric acid[959]. Residue 131 also controlled access to substituted imidazole inhibitors. Interestingly, some of the results on binding of imidazoles provide evidence that the ferric enzyme undergoes a conformational change that depends on both reduction of the iron and the presence of both NADPH-P450 reductase and NADPH[959].

Another interesting observation is that P450 4A enzymes, including P450 4A11, show at least partial covalent heme attachment[965]. Covalent heme binding involves a conserved I-helix glutamic acid (apparently unique in the P450 4A subfamily) and covalent heme binding occurs via an ester bond to the heme 5-methyl group, mediated by an autocatalytic process[965]. The extent of effect of this modification on catalytic activity is difficult to define, although with animal P450 4A enzymes, there appears to be some effect.

6.24.5. Inhibitors

Substituted imidazoles have been used as inhibitors *in vitro*[959]. Presumably some acetylenic fatty acids might be inhibitors but no studies have been reported.

6.24.6. Clinical Relevance

The significance of P450 4A11 is not very clear. Apparently individuals can vary in their expression levels by an order of magnitude[956]. Further, the ω-hydroxylation of medium chain fatty acids occurs, but its relevance is generally considered not to be as important as in the case of long chain fatty acids.

6.25. P450 4A22

Relatively little is known about P450 4A22. The originally reported *CYP4A11* gene[957] was subsequently shown to be *CYP4A22* (ref. [952]), but the cDNA and protein have not been reported. The similarity of the two genes is 95%.

Johnson's laboratory[956] has reported that P450 4A22 is expressed at lower levels than P450 4A11 in human liver, as well as kidney[952]. There was no correlation of expression levels of P450 4A11 and 4A22 in human liver[956]. P450 4A22 expression could not be observed in HepG2 cells or PPARα-overexpressing cells[956].

6.26. P450 4B1

6.26.1. Sites of Expression and Abundance

P450 4B1 was cloned by Nhamburo *et al.*[966] from a human lung cDNA library. P450 4B1 expression has also been reported (in addition to lung) in kidney, bladder[967], breast[968], and prostate[969]. Expression has also been reported in bladder and breast tumors[967]. Definitive information about the level of expression of P450 4B1 is not available.

6.26.2. Regulation and Polymorphism

The extent of variability of P450 4B1 expression is considerable, at least in bladder where the variation is two orders of magnitude[967]. Several SNPs have been reported, including one resulting in a premature truncation[970].

No evidence for the inducibility of human 4B1 has been presented.

6.26.3. Substrates and Reactions

Direct information about the catalytic specificity of human P450 4B1 has been difficult to obtain because of problems in heterologous expression. Following the initial cDNA cloning, expression in a baculovirus system was unsuccessful and only yielded inactive cytochrome P420 (ref. [971]). The substitution S427P allowed for expression, and ω-hydroxylation of lauric acid could be demonstrated. However, information about the native human enzyme has not been available (the S427P mutant does not occur naturally[970]).

Imaoka *et al.*[972] found that functional P450 4B1could be successfully expressed as a fusion protein with NADPH-P450 reductase. They were also successful in developing transgenic mice in which functional P450 was expressed in liver. The authors postulate that expression in the presence of auxiliary proteins (NADPH-P450 reductase, b_5) may stabilize P450 4B1 (ref. [972]). With these systems, it was possible to demonstrate that

P450 4B1 catalyzes the *N*-hydroxylation of 2-aminofluorene and ω-hydroxylation of lauric acid, as expected from studies with rabbit P450 4B1. If other results from work with animal P450 4B1 enzymes also carry over to the human enzymes, one might expect the reaction 4-ipomeanol activation (epoxidation?), 3-methoxy-4-aminoazobenzene *N*-hydroxylation, 2-aminoanthracene *N*-hydroxylation, valproic acid hydroxylation (and desaturation?), and dehydrogenation of 3-methylindole[970].

6.26.4. Knowledge about Active Site

Because of the paucity of information about catalytic selectivity (*vide supra*), little can be said about the active site of human P450 4B1. Some kinetic hydrogen isotope effect work with *rabbit* P450 4B1 suggests that the active site is more restricted than that of P450 2B1 (ref. [973]). Another interesting observation with rabbit P450 4B1 is the covalent linking of the heme to the protein[974], a phenomenon observed with several of the P450 4 family proteins[965, 975]. Whether this binding is seen in human P450 4B1 is unknown.

6.26.5. Inhibitors

No inhibitors of human P450 4B1 have been reported.

6.26.6. Clinical Issues

There are two issues with P450 4B1. First, the enzyme has been shown to activate carcinogens, for example, 2-aminofluorene, and could be a risk factor in bladder cancer[967]. The level of P450 4B1 in tumorous tissue was not higher than in the surrounding tissue, levels of bladder P450 4B1 were higher in tumor patients than in controls[967].

The other aspect is the use of P450 4B1 as a means of drug delivery. Rabbit P450 4B1 has been utilized as an experimental transgenic activation system in the activation of 4-ipomeanol and 2-aminoanthracene, to date only in cell culture models[976].

6.27. P450 4F2

The Kusunose laboratory reported the cloning of a human liver cDNA corresponding to the leukotriene B_4 ω-hydroxylase[977]. The site of expression was distinct from P450 4F3, which is restricted to polymorphonuclear leukocytes. P450 4F2 is found not only in liver but in several extrahepatic tissues, however[978], including kidney (S2 and S3 segments of proximal tubules, in cortex and outer medulla). The extent of variation of P450 4F2 in human liver was ~5-fold[979].

P450 4F2 catalyzes ω-hydroxylation of several lipids, including leukotriene B_4 (refs [979], [980]), arachidonic acid[962], 6-*trans*-leukotriene B_4, lipoxin A_4, 8-hydroxyeicosatetraenoic acid, 12-hydroxyeicosatetraenoic acid, and 12-hydroxystearic acid[981]. The physiological relevance of some of these reactions is of interest but the effects of variability of P450 4F2 have not been demonstrated. Part of the interest lies in the fact that leukotriene B_4 is a potent proinflammatory agent[978, 979].

6.28. P450 4F3

In 1993, Kikuta *et al.*[982] cloned a P450 now known as P450 4F3 from a human leukocyte cDNA library. The protein was expressed in a yeast vector system and was shown to catalyze leukotriene B_4 ω-hydroxylation. The K_m (0.7 μM) was much lower than that reported for P450 4F2 for this reaction (although the k_{cat} and k_{cat}/K_m values have not been carefully compared)[978].

The gene was cloned in 1998[983]. Interestingly, the *CYP4F3* gene has been shown to use tissue-specific splicing and alternate promoters. A 4F3A form contains exon 4 (but not 3) and is expressed in neutrophils; a 4F3B form contains exon 3 (but not 4) and is expressed in fetal and adult liver and kidney, trachea, and gastrointestinal tract[984, 985]. The K_m of the 4F3B (liver) form was 26-fold higher than for the 4F3A (neutrophil) form[984], although the significance of this report is qualified by the absence of k_{cat} or k_{cat}/K_m parameters. Further studies by Soberman's group have shown that the substitution of exon 3 changes the catalytic selectivity from leukotriene B_4 to arachidonic acid (ω-hydroxylation in both cases)[985]. Again, the usefulness of the observation is limited by the lack of kinetic parameters. The relevance of the preferential localization[985] and altered catalytic selectivity are presently unknown but there is potential clinical relevance in light of the known physiological action of both leukotriene B_4 and 20-hydroxyeicosatetraenoic acid.

6.29. P450 4F8

Bylund *et al.*[986] used degenerate PCR primers and isolated a cDNA from human seminal vesicles, denoted P450 4F8. This P450 was shown to be a 19-hydroxylase with prostaglandin endoperoxides[986, 987] Recombinant P450 4F8 catalyzed the ω-2 hydroxylation of arachidonic acid and three stable prostaglandin H_2 analogs but prostaglandins D_2, E_1, E_2, and $F_{2\alpha}$ and leukotriene B_4 were poor substrates[987]. (19*R*)-Hydroxy prostaglandins E_1 and E_2 are the main prostaglandins of human seminal fluid. Bylund *et al.*[987] propose that ω-2 hydroxylation of prostaglandins H_1 and H_2 by P450 4F8 occurs in seminal vesicles, and that isomerization to (19*R*)-hydroxy prostaglandin E is the result of action of prostaglandin E synthase.

Further investigations by Bylund and Oliw[988] have demonstrated the expression of P450 4F8 protein in human epidermis, hair follicles, sweat glands, corneal epithelium, proximal renal tubules, and epithelial linings of the gut and urinary tract. P450 4F8 was shown to be upregulated (mRNA and protein) in the epidermis in psoriasis[988]. The exact physiological role of P450 4F8 is unclear, although 19-hydroxy prostaglandins do have several biological activities[988].

6.30. P450 4F11

P450 4F11 is another member of the *CYP4A* gene cluster found on chromosome 19[989]. Expression has been demonstrated primarily in liver, with some expression also in kidney, heart, and skeletal muscle. No other information is presently available, although it might be expected to be capable of leukotriene hydroxylation based upon its similarity to other P450 4F enzymes.

6.31. P450 4F12

P450 4F12 was originally cloned from human liver[990] and small intestine[991] cDNA libraries. Expression has been demonstrated in liver, kidney, colon, small intestine, and heart[990, 991]. Actual levels of abundance are unknown, although this would appear to be a minor P450.

Two groups have expressed P450 4A12 in yeast. Catalytic activities include the hydroxylation of arachidonic acid at carbons 18 (ref. [990]) and 20 (ref. [991]), hydroxylation of the antihistamine ebastine[992], and ω-oxidation of leukotriene B_4 (refs [990], [991]), and ω-hydroxylation of some prostaglandins and prostaglandin analogs[990].

No further information is yet available about the relevance of this enzyme in physiological or clinical situations.

6.32. P450 4F22

No information is available except the existence of the *CYP4F22* gene in the human genome[705].

6.33. P450 4V2

No further information is available except for the existence of the human *CYP4V2* gene[705].

6.34. P450 4X1

Relatively little is known beyond the existence of the human *CYP4X1* gene except for one recent paper on rat P450 4X1 (ref. [993]). mRNA expression was highly selective in brain (cortex, hippocampus, cerebellum, brainstem). The rat protein was expressed in yeast but has not been examined for catalytic activity.

6.35. P450 4Z1

The only information available is the existence of the *CYP4Z1* gene in the human genome[705].

6.36. P450 5A1

P450 5A1 is the classification of thromboxane synthase, which converts prostaglandin H_2 to thromboxane (Figure 10.12). Thromboxane causes vasoconstriction and platelet aggregation, which are of considerable interest.

6.36.1. Sites of Expression and Abundance

P450 5A1 is expressed in platelets and also erythroleukemia cells[995]. The enzyme is also found in human monocytes[996], leukocytes[997], and kidney interstitial dendritic reticulum cells

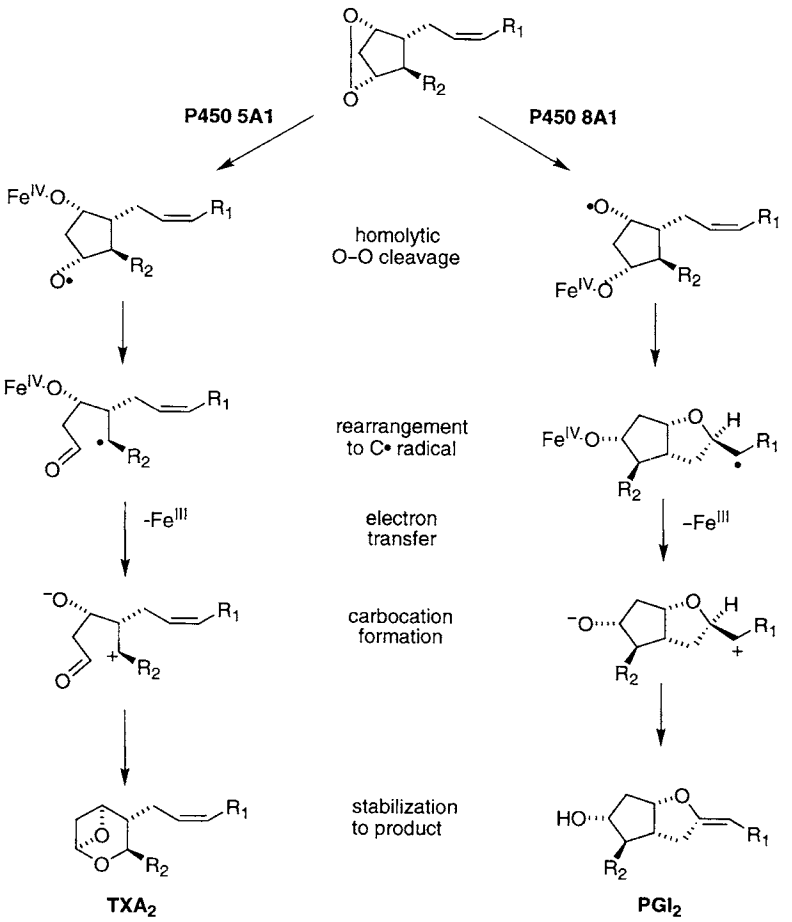

Figure 10.12. Rearrangement of prostaglandin H_2 to prostacyclin (PGI_2) by P450 8A1 and thromboxane (TXA_2) by P450 5A1 (ref. [994]).

surrounding the tubules[998]. Some expression is also seen in lung and liver[996].

6.36.2. Regulation and Polymorphism

As one might expect from its function, P450 5A1 is a highly regulated system. Dexamethasone induces P450 5A1 in human monocytes[996]. Phorbol esters also induce P450 5A1 (e.g., 12-*O*-tetradecanoylphorbol-13-acetate) in human erythroleukemia cells[999]. Patients with systemic sclerosis showed 6-fold enhanced levels of leukocyte P450 5A1 (ref. [997]).

Promoter analysis indicates a 39-bp core promoter, containing TATA and initiator elements that control transcription. Binding of the transcription factor NF-E2 is critical for both alteration of the nucleosomal structure and activation of the P450 5A1 promoter[1000].

Chevalier *et al.*[1001] identified 11 polymorphic variants in the *CYP5A1* gene, including 8 missense changes in the coding region. The effects of these changes have not been reported yet.

6.36.3. Substrates and Reactions

The thromboxane synthase reaction has been known for many years but was associated with a P450 by Ullrich and his associates, first in spectral studies[1002] and then by purification[1003]. With the purified enzyme or one expressed in a baculovirus system[1004], prostaglandin H_2 was

converted to thromboxane A_2 and 12-hydroxyheptatrienoic acid (HHT) plus malondialdehyde, in equimolar amounts[1005]. Prostaglandin G_2 was transformed to malondialdehyde and the corresponding 15- and 12-hydroperoxy products. Prostaglandin H_1 was enzymatically transformed into 12(*L*)-hydroxy-8, 10-heptadecadienoic acid, and prostaglandin H_3 yielded thromboxane B_3 and 12(*L*)-hydroxy-5,8,10,14-heptadecatetraenoic acid[1005] (Figure 10.12).

These are all rearrangement reactions, not involving input of O_2 or electrons from pyridine nucleotides. The reaction of the "oxygen-surrogate" iodosylbenzene with a P450 5A1-containing preparation and the stable prostaglandin H_2 analog 15(*S*)-hydroxy-11α,9α-epoxymethano-5(*Z*), 13(*E*)-prostadienoic acid (U46619) yielded three oxidation products (that could also be formed in a similar system using rat liver microsomes)[1006]. These and other studies led Hecker and Ullrich[1007] to propose a mechanism involving homolytic cleavage of the prostaglandin endoperoxide (with the Fe^{IV} bonded to one oxygen and the other oxygen bearing a radical), transfer of the radical to a carbon, further electron transfer to generate Fe^{III} plus a carbocation, and collapse of the *bis*-ionic structure to yield thromboxane A_2 (ref. [994]). Fragmentation competes with the electron transfer step to also yield malondialdehyde and heptatrienoic acid[994].

6.36.4. Knowledge about Active Site

Relatively little is known about the active site of P450 5A1 beyond the information about the reactions presented above. As indicated, the protein does not bind NADPH-P450 reductase. Presumably, the active site is rather specific, although iodosylbenzene could be utilized as an oxygen surrogate.

6.36.5. Inhibitors

Thromboxane synthase inhibitors have been a matter of interest for many years because of their potential use in preventing plugs of platelets, and efforts at development preceded the characterization of the enzyme as a P450 (refs [1008–1010]). Many of these inhibitors have a basic nitrogen atom that binds to the P450 5A1 heme[1011]. The search for inhibitors as drug candidates has continued[1012].

6.36.6. Clinical Issues

As indicated earlier, platelet aggregation due to the produced thromboxanes is important but overproduction can yield plugs, so control of homeostasis is desirable.

6.37. P450 7A1

P450 7A1 catalyzes cholesterol 7α-hydroxylation, the rate-limiting step in bile acid synthesis. Much has been done in several animal models, including purification of the enzyme from rabbit and rat liver[1013, 1014]. The enzyme was partially purified from human liver[1015] and the cDNA was cloned by several groups in 1990[1016–1018].

6.37.1. Sites of Expression

Apparently the only site of P450 7A1 expression is the liver. The *CYP7A1* gene is on chromosome 8q11–q12 and contains recognition sequences for a number of liver-specific transcription factors[1019–1021].

The level of the enzyme in liver appears to be similar to some of the low-to-moderately abundant xenobiotic-metabolizing enzymes in liver.

6.37.2. Regulation and Polymorphism

The regulation of the *CYP7A1* gene is very complex, as might be expected from the important physiological role this enzyme plays.

P450 7A1 activity has long been known to be upregulated by dietary cholesterol in most animal models[1017], although there are some exceptions[1022]. Feeding rats the competitive inhibitor 7-oxocholesterol led to reduced bile acid synthesis (due to inhibition) and a compensatory increase in P450 7A1 synthesis[1023]. Chiang[1024] identified a bile acid-responsive element in the *CYP7A1* promoter.

Studies with P450 7A1-knockout mice show that this reaction (cholesterol 7α-hydroxylation) is essential for proper absorption of dietary lipids and fat-soluble vitamins in newborn mice, but not for maintenance of cholesterol and lipid levels[1025].

The mice exhibit a complex phenotype with abnormal lipid excretion, skin pathologies, and behavioral irregularities. The cholesterol levels were not altered. Interestingly, vitamin D_3 and E levels were low to undetectable.

A new era in the regulation of P450 7A1 began with reports of the involvement of some of the orphan steroid receptors. The proximal promoter region interacts with LXRα. The oxysterols 24(*S*)-hydroxycholesterol and 24(*S*)-epoxycholesterol activate LXRα (and LXRβ)[1026]. Further, mice devoid of LXRα fail to induce *CYP7A1* transcription[1027]. Two other proteins, FXR and CPF, are also involved[1028–1030]. Chenodeoxycholate, a bile acid derived from cholesterol, interacts with FXR to suppress *CYP7A1* transcription[1031]. However, the action of FXR has been reported to be indirect[1031]. PXR binds lithocholic acid and downregulates *CYP7A1* (ref. [1032]). Thus, cholesterol metabolites control their synthesis in the liver through feedback suppression of *CYP7A1* (ref. [1028]). Hylemon[1033] has concluded that the dominant factor is LXRα. CPF binds to the promoter (as a monomer) and leads to *CYP7A1* transcription[1030].

Other studies have addressed the role of PPARα in P450 7A1 downregulation[1034]. However, differences exist between humans and mice gene responses have been observed, with the mouse gene showing an enhanced response to ligands because of an additional binding site[1035] (further, humans have much less PPARα than rodents[1036]). Chiang[1037] analyzed the PPARα response and provided evidence that the downregulation by PPARα-agonist complex is due to competition with HNF-4 for the DR-1 sequence.

The regulation of P450 7A1 by other factors has been considered. Downregulation by TNFα has been interpreted in the context of MEKK1, an upstream nitrogen-activated protein kinase, affecting HNF-4 (ref. [1038]). The same mechanism may be involved in the repression by endotoxin and interleukin-1 (ref. [1039]). A novel *CYP7A1* site appears to be involved in the repression of *CYP7A1* by thyroid hormone (T_3)[1040]. Studies with rats indicate differences in the regulation of P450 7A1 and P450 27A1, a sterol 27-hydroxylase[1041]. Human *CYP7A1* expression is also repressed by insulin and phorbol esters[1042]. Estrogen (100 μg/kg/week) increased hepatic cholesterol 7α-hydroxylation 2.7-fold in ovariectomized baboons[1043]. Retinoic acid increased (rat) *CYP7A1* expression in a reporter assay[1044].

In addition to the mouse *CYP7A1* knockouts, work has been done with overexpression in mice[1045, 1046]. The mice did not exhibit altered cholesterol levels[1046]. The lack of an LXR element in a region (−56 to −49) of the human promoter may dictate some of the differences seen in mouse and human models. With regard to humans, one study of biopsy samples from gallstone patients led to the conclusion that there was no correlation between levels of total bile acids and P450 7A1 activity[1047]. A correlation was seen with levels of chenodeoxycholic acid.

A long-standing observation from rodent studies is the apparent circadian rhythm of P450 7A1 (ref. [1048]). This phenomenon has been suggested to be indicative of a short halflife of the enzyme[1049, 1050]. The phenomenon has also been reported in nonhuman primates[1051]. The circadian rhythm can be demonstrated at the level of actual P450 7A1 in rats[1052]. The molecular mechanism of the rhythm is still not clear. One aspect is the instability of P450 7A1 in microsomes (*in vitro*), with a $t_{1/2}$ of ~1–2 hr in humans and rats[1053]. Alternatively, the mRNA has a short $t_{1/2}$ and the circadian rhythm can be seen at the mRNA level[1054]. Another unresolved aspect of P450 7A1 research is the issue of phosphorylation, postulated early in the field[1055]. *In vitro* experiments with microsomes show some effects of various treatments[1056, 1057]. More recent work with microsomes and recombinant proteins also shows effects[1058], although the *in vivo* significance is yet unclear.

Polymorphisms in the coding and noncoding regions of the *CYP7A1* gene are known[1059]. Some have been associated with clinical changes[1060], but others have not[1061].

6.37.3. Substrates and Reactions

The classic reaction of P450 7A1 is cholesterol 7α-hydroxylation[18], and esterified cholesterol is not a substrate[1062]. However, recent experiments have established that the enzyme also catalyzes the 7α-hydroxylation of 24-hydroxycholesterol, with preference for the (*S*)-isomer[1063]. 7α-Hydroxylation (with recombinant human P450 7A1) was observed with 20(*S*)-hydroxycholesterol, 25-hydroxycholesterol, and

27-hydroxycholesterol[1064]. The relevance of the activity toward 25(*S*)-hydroxycholesterol is unknown compared to P450 39 (ref. [1065]).

6.37.4. Knowledge about Active Site

Relatively little has been done with site-directed mutagenesis or modeling. As indicated (*vide supra*), the enzyme only catalyzes 7α-hydroxylation but is not very sensitive to side-chain hydroxyls.

The region 214–227 has been postulated to interact with the membrane and to serve as a substrate-access channel[1066]. Mutations in this region yielded some changes in kinetic parameters toward cholesterol.

6.37.5. Inhibitors

Limited information about inhibitors is available. As indicated earlier, 7-oxocholesterol is a (n) (competitive) inhibitor[1023].

6.37.6. Clinical Issues

P450 7A1 has been a topic of considerable interest in the areas of hepatology and gastroenterology.

The hypersecretion of cholesterol in obesity does not appear to be due to reduced 7α-hydroxylation[1067]. Coffee terpenes (e.g., cafestol) inhibit P450 7A1 and also raise cholesterol levels[1068], although it is not clear that the two phenomena are linked. The complex regulation of P450 7A1 makes interpretation of some experiments difficult. Overexpression of P450 7A1 in HepG2 cells increased bile acid synthesis but led to decreased hydroxymethylglutarate (HMG) CoA reductase activity (rate-limiting step in cholesterol biosynthesis)[1069].

Alterations in P450 7A1 were not seen in hypo- or hyperthyroidism[1070].

A 10-week old child with a stop-codon mutation and lacking P450 7A1 presented with severe cholestasis, cirrhosis, and liver synthetic failure[1060]. A frameshift leading to (homozygous) lack of P450 7A1 was associated with high low-density lipoprotein (LDL) cholesterol, but not total cholesterol[1071]. Heterozygotes were also hyperlipidemic. However, Beigneux *et al.*[1072] have discussed some of the caveats associated with interpretation of results of family and experimental studies with P450 7A1.

6.38. P450 7B1

Almost all of the work with P450 7B1 is from rodent models and application to the human *CYP7B1* gene system is by inference. A P450 7B1 transcript was first characterized in a rat (brain) hippocampus cDNA library[1073]. A heterologously expressed protein was shown to catalyze the 7α-hydroxylation of the steroids DHEA and pregnenolone[1074]. Expression has also been reported in liver and kidney[1073, 1075]. Disruption of the mouse *CYP7B1* gene yielded animals that were viable and apparently normal, but *ex vivo* 7α-hydroxylation of DHEA and 25-hydroxycholesterol was blocked in brain, spleen, thymus, heart, lung, prostate, uterus, and mammary gland[1075].

Although extrapolation to humans has not been reported, P450 7B1 is considered to be a neurosteroid hydroxylase and have a potentially important role[1065, 1075]. Functional polymorphisms in the human *CYP7B1* gene have not been reported, but have been postulated to lead to severe hypercholesterolemia and neonatal liver disease[29].

6.39. P450 8A1

Prostacyclin (prostaglandin I_2) has strong vasodilation and anti-aggregation effects on platelets, and the imbalance of prostacyclin and thromboxane A_2 (product of P450 5A1) is a factor in several diseases, for example, myocardial infarction, stroke, atherosclerosis[1076, 1077]. The reaction yielding prostacyclin from prostaglandin H_2 is another "internal" oxygen transfer, without the input of O_2 and electrons from NADPH (Figure 10.12), and the involvement of a P450 was not immediately obvious. Ullrich hypothesized P450 involvement on the basis of spectral interaction studies[1078]. DeWitt and Smith[1079] used a monoclonal antibody to purify catalytically active prostacyclin synthase from bovine aorta and demonstrated a P450 Fe^{2+}•CO spectrum. Subsequently, P450 8A1 was cloned from bovine endothelial cells[1080].

6.39.1. Sites of Expression and Abundance

Human P450 8A1 was cloned from aorta endothelial cells by Tanabe's group[1077]. The mRNA is widely expressed in human tissues, including ovary, heart, skeletal muscle, lung, prostate[1077], and umbilical vein[1081]. More recent work has shown some localization in the brain, including neurons[1082, 1083]. Another site of expression is fallopian tubes, with expression in luminal epithelia, tubal smooth muscle, vascular endothelial cells, and vascular smooth muscle cells[1084].

6.39.2. Regulation and Polymorphism

P450 8A1 is constitutively expressed in human endothelial cells[1081]. The human *CYP8A1* gene (chromosome 20) has 10 exons[1085–1087] and has consensus sequences for Sp1, AP-2, an interferon-γ response element, GATA $NF_{\kappa}B$, a CACCC box, glucocorticoid receptor, and a shear stress responsive element (GAGACC)[1085]. Whether or not all of these are functional and how they interact to maintain constitutive expression is not well understood yet.

Polymorphisms have been of interest because of disease relevance. The coding sequence contains at least five alleles[1088]. In the 5′-region, these are polymorphisms involving a variable number of tandem repeats (VNTR) that affect transcription, as demonstrated in reporter systems *in vitro*[1089]. At least nine of these allelic variants are known[1089]. An association between this VNTR polymorphism and cerebral infarction has been reported[1090].

A SNP in exon 8 has been reported to be linked to myocardial infarction, although no amino acid change occurs[1091]. However, the VNTR polymorphism does not appear to be related to essential hypertension[1092], nor does the 5′-flanking region SNP T192G (ref. [1093]). However, a novel splicing variation leading to skipping of exon 9 has been linked to hypertension[1094].

6.39.3. Substrates and Reactions

P450 8A1 has a very limited catalytic specificity, functioning only as the prostacyclin synthase (Figure 10.12). Prostaglandins G_2, H_2, 13(*S*)-hydroxy H_2, 15-keto H_2, and H_3 are isomerized to the corresponding prostacyclins[1007]. Spectral binding studies with 9,11-epoxymethano prostaglandins F_2 and $F_{2\alpha}$ lead to the view that the binding juxtaposition is the key determinant in distinguishing the courses of catalysis by P450s 5A1 and 8A1 (ref. [1007]). A mechanism consistent with available data has been proposed (Figure 10.12)[994, 1007].

6.39.4. Knowledge about Active Site

Mutagenesis of Cys441 (heme binding Cys) or Glu347 or Arg350 (EXXR motif) abolished catalytic activity, suggesting that the placement of these residues is correct[1095]. Other site-directed mutagenesis studies suggest roles of Ile67, Val76, Leu384, Pro355, Glu360, and Asp364, which have been suggested in models[1096]. However, the level of residual activity was 5–10% and only a single substrate concentration was used; another caveat is that the expression work was done in COS cells and the level of expression of holoprotein was not measured.

Other work has been on membrane topology, and antibody studies indicate that P450 8A1 is mainly exposed on the cytoplasmic site of the endoplasmic reticulum with a single transmembrane anchor[1097, 1098]. The (unstable) substrate, prostaglandin H_2, is produced in the lumen and apparently passes through the membrane to reach P450 8A1. Antibodies raised to the peptides of the putative substrate channel (66–75 and 95–116) interact only after membrane solubilization, implying that the substrate-access channel is very near the membrane[1099].

6.39.5. Inhibitors

Relatively little interest has been shown in the development of drugs that inhibit P450 8A1 because inhibition is generally considered to be deleterious. Phenylbutazone has been reported to inhibit[1100].

P450 8A1 is slowly inactivated during the normal reaction itself, apparently by one of the reactive intermediates in the catalytic cycle (Figure 10.12)[1101]. A $k_{inactivation}$ of 0.06 s^{-1} was reported[1101].

Peroxynitrite is a powerful inhibitor of P450 8A1, with a reported K_i of 50 nM[1102]. Peroxynitrite is formed by the chemical reaction

of NO$^{\bullet}$ and $O_2^{-\bullet}$ (ref. [1103]). The mechanism is believed to involve tyrosine nitration[1104], and recently Tyr430 has been implicated as the site of nitration[1105].

6.39.6. Clinical Issues

As mentioned earlier, prostacyclin is a powerful vasodilator and inhibits platelet adhesion and undesired cell growth. Although this view may be overly simplistic, prostacyclins are a counterbalance to thromboxanes in a "yin–yang" relationship. Thus, the action of P450 8A1 balances that of P450 5A1.

Decreased expression of P450 8A1 has been reported in severe pulmonary hypertension[1106]. With regard to general cardiovascular disease, a study of Japanese subjects associated the VNTR polymorphism with hypertension (odds ratio 1.9)[1107]. Individuals with 3–4 repeats had less promoter activity and higher risk. In experimental studies, the overexpression of P450 8A1 in transgenic mice protected against the development of hypoxic pulmonary hypertension[1108]. In another study, the expression of human P450 8A1 in the carotid arteries of rats after arterial balloon injury (using a virus) led to increased synthesis of prostacyclin and to reduced neointimal formation[1109].

P450 8A1 also has relevance in cancer treatment. Transfection of colon adenocarcinoma cells with P450 8A1 led to slower growth and reduced vascular development following inoculation into syngeneic mice[1110].

Finally, antibodies in the sera of some patients with hypersensitivity reactions to phenytoin and carbamazepine recognize rat P450 3A1 but not human P450 3A (ref. [1111]). The antisera also recognize peptides derived from P450s 8A1 and 5A1, although relationships of etiology and causality are unclear.

6.40. P450 8B1

P450 8B1 is a sterol 12α-hydroxylase expressed in the liver. The human *CYP8B1* gene was characterized on the basis of the rabbit and mouse orthologs[1112]. Of interest is the finding that this gene is devoid of introns, unique for this gene among the P450 family[1112].

Regulation of the gene is of interest, in that P450 8B1 catalyzes the synthesis of cholic acid and controls the ratio of cholic acid to chenodeoxycholic acid in the bile[1113]. HNF-4α activates human *CYP8B1* expression in HepG2 cells[1113]. Bile acids and farnesoid X receptor (FXR) downregulate HNFα expression. Inflammation in liver cells causes increased synthesis of α_1-antitrypsin, a serum protease inhibitor, and in a derived peptide (C-36). C-36 appears to interact with the α_1-fetoprotein transcription factor (FTF) site in the human *CYP8B1* promoter, inducing a conformational change to lower DNA binding ability, and suppressing the transcription of the *CYP8B1* (and *CYP7A1*) genes[1114, 1115]. HNFα could overcome the inhibitory effects of FTF and bile acids[1115]. Thus, regulation of P450 8B1 is involved in bile acid feedback inhibition.

6.41. P450 11A1

P450 11A1 is the enzyme involved in the initiation of steroid synthesis (Figures 10.13 and 10.14). It catalyzes the conversion of cholesterol to pregnenolone by side-chain cleavage and has been referred to in the older literature as $P450_{scc}$ or cholesterol desmolase. The enzyme was purified from bovine adrenal cortex mitochondria[1116]. The human gene was cloned by Omura and Fujii-Kuriyama in 1987[1117] and includes nine exons. Of historical significance is the fact that this P450 only contains a single cysteine and further establishes the position of the heme thiolate peptide in P450s, extending the work on the location from the crystal structure of bacterial P450 101 (ref. [1118]).

6.41.1. Sites of Expression

P450 11A1 is found primarily in steroidogenic tissues, that is, adrenal cortex and gonads, including ovary (corpus luteum[1119, 1120] and theca interna cells[1121] and others[1122]). Of interest are recent reports of P450 11A1 in brain[1123–1126] and pancreas[1127].

P450 11A1 is one of the few P450s localized in the mitochondria (Table 10.1 and Figure 10.14). Studies with the bovine enzyme demonstrated that

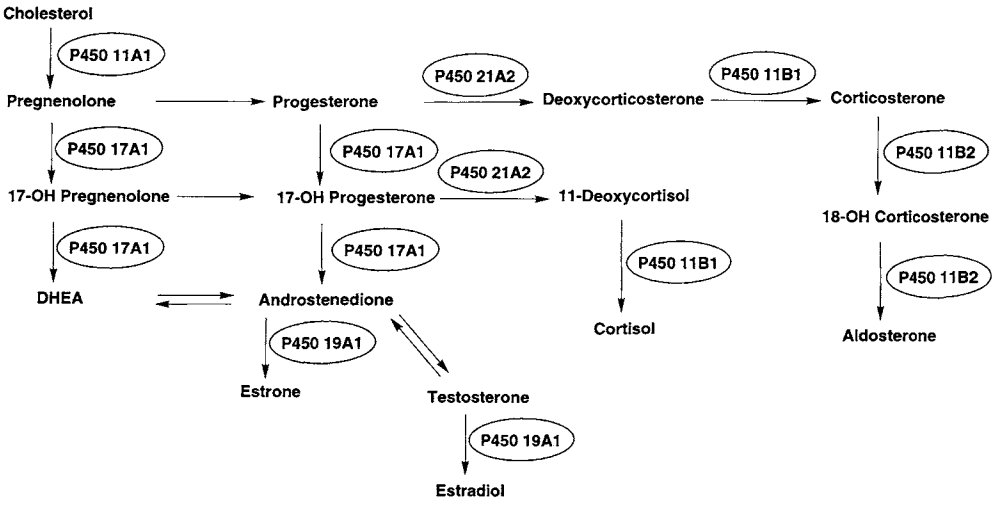

Figure 10.13. A view of the metabolic pathway of steroidogenesis and the major P450s involved[29].

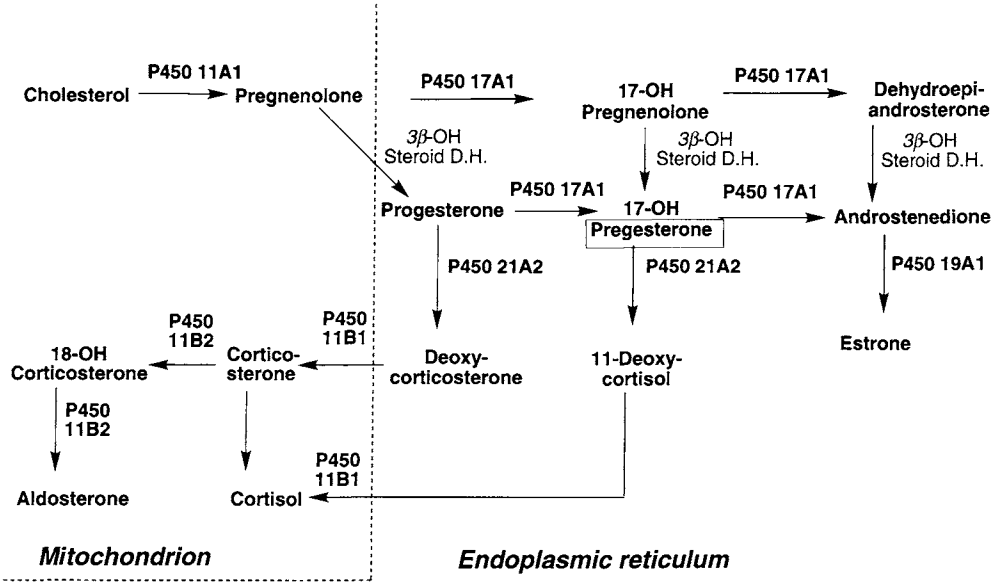

Figure 10.14. Overview of steroidogenic pathway and cellular compartmentalization[23].

P450 11A1, synthesized on ribosomes in the cytosol, is imported into mitochondria without processing of the amino terminal extension peptide[1128]. The protein moves to the mitochondrial inner membrane and is then cleaved to yield the mature form[1128]. Alteration of the basic amino acid residues of the *N*-terminus resulted in less efficient mitochondrial import[1129]. Miller's group constructed vectors that could be used to direct P450 11A1 to the endoplasmic reticulum and found that the enzyme was inactive[1130]. The membrane environment was concluded to be more important in modulating catalytic function than the electron transfer partners.

6.41.2. Regulation and Polymorphism

The regulation of P450 11A1 is relatively complex, as might be expected for the initial step in steroid formation[1122]. Moreover, the system must be able to respond to signals in many different tissues. Much of our understanding of the regulation of P450 11A1 expression is based on studies with *CYP11A1* genes of experimental animals and reinvestigated with human *CYP11A1*.

P450 11A1 has long been known to be regulated by ACTH and cyclic AMP. In the bovine *CYP11A1* gene two Sp1-binding sites mediate cyclic AMP transcription through the protein kinase A signaling pathway, utilizing the rather ubiquitous transcription factor Sp1 (ref. [1131]). The steroidogenic factor 1 (SF1) activates *CYP11A1* transcription through interaction with protein factors upstream[1122]. An upstream CREB-binding region and an AP-1 site are also involved in the cyclic AMP response. Sp3 can also be involved[1132]. The TATA box drives cell type-specific cyclic AMP-dependent transcription[1133]. SF-1 also interacts with Sp1 (refs [1134–1136]). Thus, the regulation of the human *CYP11A1* gene involves all the above factors plus an AdE element[1122]. More recently, the expression of the human gene has been shown to involve the zinc finger protein TreP-132, interacting with both CBP/p300 (ref. [1137]) and SF-1 (ref. [1138]). Also, salt-inducible kinase (SIK) represses cyclic AMP-dependent protein kinase-mediated activation through the CREB basic leucine zipper domain[1139].

Other recent work with human placenta show that activating protein-2 (AP-2) assumes the role of SF-1 by binding to an overlapping promoter element[1140].

Mutations are also found in *CYP11A1* and can cause congenital adrenal insufficiency. Arg353 was found to be critical in a study with an afflicted patient[1141].

6.41.3. Substrates and Reaction

P450 11A1 appears to be quite specific in using cholesterol as a substrate. The reaction proceeds in a three-step sequence, with generation of (22*R*)-20α, 22-dihydroxycholesterol as an intermediate[1142]. Oxidative cleavage of the diol to pregnenolone and 4-methylpentanal (isocaproic aldehyde) completes the overall reaction. The mechanism of the last step is not completely clear, but some proposals have been presented[1143–1145].

The rate of electron transfer from adrenodoxin is important and appears to be the rate-limiting step for the enzyme in human placenta[1146]. The redox potential of adrenodoxin can be varied by site-directed mutagenesis, but had little effect on rates of electron transfer, consistent with the view that other factors such as protein–protein interactions are more important than the intrinsic thermodynamics[1147]. When P450s 11A1 and 11B1 are expressed together in cells, they can compete for reducing equivalents from adrenodoxin[1148]; exactly how important the competition is in tissues is unclear. Another report indicates interaction of P450 11A1 with and enhancement by b_5 (ref. [1149]) although the relevance is unclear because of the compartmental separation of P450 11A1 (mitochondria) and b_5 (endoplasmic reticulum) (Figure 10.14).

6.41.4. Knowledge about Active Site

Knowledge about this enzyme is still relatively limited. Studies with bovine P450 11A1 indicated the significance of Lys377 and Lys381 in adrenodoxin binding[1150]. As indicated earlier, a mutation at Arg353 was found to attenuate the function of P450 11A1 in a patient[1141]. Site-directed mutagenesis of human P450 11A1 (in *E. coli*) indicated that Ile462 had some effect on kinetic parameters[1151].

6.41.5. Inhibitors

A number of inhibitors of P450 11A1 have been reported, although some were studied only with the bovine enzyme[1152, 1153], including some acetylenic mechanism-based inactivators[1154]. With regard to the human enzyme, there is some potential for use of inhibitors in treatment of prostatic cancer, and prodrug forms of aminoglutethimide have been examined[1155].

Anti-convulsants have been reported to inhibit P450 11A1, but the interaction is not strong[1156].

6.41.6. Clinical Issues

Two major issues are of interest. Because of the nature of P450 11A1 in initiating steroidogenesis, deficient P450 11A1 can lead to (congenital) adrenal hyperplasia[29]. Rabbit and mouse models show the effects[1157, 1158]. *CYP11A1*-null mice die shortly after birth, but can be rescued by steroid injection[1158]. ACTH levels become very high due to lack of feedback regulation by glucocorticoids. Male null mice are feminized with female external genitalia and underdeveloped male accessory sex organs. These manifestations resemble various human steroid deficiency syndromes.

Another issue is autoantibodies to P450 11A1 (and also P450 17A1) in patients with autoimmune polyglandular syndrome types I and II, and Addison's disease[1159, 1160]. As with other P450s recognized by autoantibodies, causal relationships between immunity and disease are unclear.

6.42. P450 11B1

P450s 11B1 and 11B2 differ in only 32 residues. P450 11B1 catalyzes the 11β-hydroxylation of deoxycortisol to yield cortisol, which is the main glucocorticoid in the body. Deficiencies in the enzyme are known, causing congenital adrenal hyperplasia[29, 1161].

6.42.1. Sites of Expression

P450 11B1 is expressed in the adrenal cortex, specifically the *zona fasciculate/reticularis*[1161]. In rats, some expression has been detected in brain but the relevance is not clear.

P450 11B1 is synthesized in the cytosol and directed to the mitochondria with a 24-residue *N*-terminal targeting sequence (where this is lost after entry). As with the other four (exclusively) mitochondrial P450s (Table 10.1 and Figure 10.14), P450 11B1 receives electrons from adrenodoxin instead of NADPH-P450 reductase.

The characterization of the *CYP11B* gene has developed considerably in recent years. Much of the early research in this field was done with bovine adrenal glands because of the need for large amounts of material, and the bovine P450 11B protein has the function that P450 11B1 (11-hydroxylation) and the P450 11B2 (11-hydroxylation, 18-hydroxylation, and oxidation of the 18-alcohol to an aldehyde) have in most other species, including humans[1162]. The two human genes (*CYP11B1*, *CYP11B2*) were characterized and clearly shown in both to be essential[1163–1166].

P450 11B1 expression has also been deleted in human fetal adrenal gland, particularly in the "fetal zone" (as opposed to neocortex)[1167].

6.42.2. Regulation and Polymorphism

Much of the background on regulation of P450 11B1 came from studies with the bovine gene, which responds to ACTH and has six *cis*-acting regulatory elements[1168]. The protein (Ad4BP) that binds to one of these (Ad4) is a member of the steroid hormone receptor superfamily[1169]. Other studies by Omura[1170] indicated the cooperative nature of these elements in transcription. Work with rat *CYP11B1* showed that ACTH stimulates transcription by changing composition in AP-1 factors (Fos, Jun)[1171].

The human gene also has a cyclic AMP response element (CRE)[1172]. The Ad1 element bound CRE-binding protein, activating transcription factor-1 (ATF-1), and ATF-2. Steroidogenic factor-1 (SF-1) interacted at the Ad4 site (−242/−234) and was required for transcription[1172, 1173], which contrasts with the lack of response of *CYP11B2*.

Many mutations are known because of the relationship of the gene with congenital adrenal hyperplasia[1161]. These include a 5-base duplication[1174] and clusters of mutations in exons 6–8 (ref. [1175]). The high similarity and proximity of the *CYP11B1* and *CYP11B2* genes appear to lead to mutant generated by unequal crossover and inactive chimeric product[1176–1179]. Splice donor site mutations are also known[1180].

6.42.3. Substrates and Reactions

As indicated previously, the only substrate for P450 11B1 is deoxycortisol, which undergoes

11β-hydroxylation to yield cortisol (Figures 10.13 and 10.14).

6.42.4. Knowledge about Active Site

One of the concerns about studies on the function of particular residues in site-directed mutagenesis is that expressions in some cellular systems lead to competition between P450s 11A1 and 11B1 for (adrenodoxin) reducing equivalents in cellular systems[1148]. Another issue is that human P450s 11B1 and 11B2 have been difficult to express in bacteria, so that most experiments have relied on mammalian cells (*Schizosaccharomyces pombe* has provided some success[1161]). Nevertheless, much information about function has been obtained from patients' samples[1161].

The close similarity of P450s 11B1 and 11B2 (and their reactions) has also facilitated studies. Making the changes S288G and V320A yielded an enzyme with both P450 11B1 and 11B2 activities[1181]. Changes at positions 147 (refs [1182], [1183]), and 301/355 (ref. [1184]) have also had the same effect.

Homology models of P450 11B1 have also been published[1161, 1185, 1186], although the effects of all of the mutants known to alter function have not been systematically rationalized.

6.42.5. Inhibitors

Compared with some of the other steroidogenic P450s, there is some reason to develop P450 11B1 inhibitors. High levels of cortisol are associated with Cushing's syndrome[1161]. Cellular expression systems have been set up to assay for inhibitors, using measurements of concentrations of steroids[1187, 1188].

18-Vinylprogesterone and 18-ethynylprogesterone have been reported to be mechanism-based inactivators of bovine P450 11B, but apparently have not been tested with human P450 11B1 (ref. [1189]).

6.42.6. Clinical Issues

As indicated previously, the main issue with P450 11B1 is the impaired synthesis of cortisol and congenital adrenal hyperplasia, characterized by hypertension and signs of androgen excess[1190, 1191]. Overproduction of glucocorticoids, which could have any of several causes including overactive P450 11B1, is associated with Cushing's syndrome[1161].

6.43. P450 11B2

P450 11B2 is highly related to P450 11B1 (*vide supra*) and has a somewhat similar function. P450 11B2 catalyzes the 11β-hydroxylation of 11-deoxycorticosterone followed by 18-hydroxylation and 2-electron oxidation of the 18-alcohol to an aldehyde (Figures 10.13 and 10.14). Changes in the gene can lead to corticosterone methyloxidase deficiency and hyperaldosteronism[29, 1165, 1192, 1193]. In the older literature, this P450 is sometimes termed as "$P450_{aldo}$."

6.43.1. Sites of Expression

P450 11B2 is expressed in the adrenal cortex (zona glomerulosa) and is involved in the synthesis of aldosterone (the 11β-hydroxy, 19-aldehyde product). It is a mitochondrial P450, as are the other 11 family P450s. The cDNA was first cloned from an adrenal tumor of a patient suffering from primary aldosteronism[1194]. Another early study showed higher levels of P450 11B2 in aldosterone-secreting tumors[1195].

There is some evidence for the synthesis of aldosterone outside of the adrenals, and Li *et al.*[1196] reported expression of P450 11B2 in hepatic stellate cells of liver; the activation of these cells is a key event in liver fibrogenesis.

6.43.2. Regulation and Polymorphism

Some of the research on regulation overlaps that presented for *CYP11B1* (*vide supra*). A CRE/Ad1 element and ATF-1 (and ATF-2?) play a role with both *CYP11B1* and *CYP11B2* (ref. [1197]). However, SF-1 does not appear to regulate *CYP11B2*, in contrast with *CYP11B1* (ref. [1173]). Many aspects of regulation remain to be further investigated, including the mechanisms of the observed Ca^{2+} and cyclic AMP signaling[1198] and the effects of kinase inhibitors[1199, 1200].

As in the case of *CYP11B1*, many mutations have now been defined from clinical studies. The "crossovers" between P450s 11B1 and 11B2 yield inactive P450 11B2, as well as P450 11B1 (refs [1178], [1179], [1201], [1202]). Other mutations in *CYP11B2* were associated with corticosterone methyloxidase I and II deficiency[1192, 1193, 1203]. Polymorphisms in *CYP11B2* have also been linked to idiopathic hyperaldosteronism, a condition characterized by autonomous production of aldosterone and arterial hypertension[1204]. A polymorphism in the promoter region of *CYP11B2* (−344 TK) has been associated with predisposition to essential hypertension[1205].

6.43.3. Substrates and Reactions

P450 11B2 catalyzes the three-step conversion of 11-deoxycorticosterone to aldosterone, with 11β-hydroxylation, 18-hydroxylation, and 2-electron oxidation of the 18-carbinol (Figures 10.13 and 10.14). No other substrates are known. Information about the processivity of the human enzyme (i.e., extent of release of intermediate products) is not available at this time.

6.43.4. Knowledge about Active Site

Some studies with the closely related P450 11B1 have already been mentioned. Bernhardt's laboratory found that changes only at positions 320 and 335 conferred some 18-hydroxylation activity to P450 11B1 (ref. [1184]). Homology modeling has also been done[1161, 1186]. In other site-directed mutagenesis work, residues that differed among species were changed and residues 112, 147, and 152 were found to have effects[1206]. Modeling suggested an indirect effect of residue 147 and that residue 112 might be in the substrate-access channel.

6.43.5. Inhibitors

Elevated aldosterone levels can be detrimental and some interest exists in targeting P450 11B2. A yeast system has been developed that can be used for screening for inhibitors[1207].

The literature contains one older report of the use of an acetylenic P450 19 inhibitor to inhibit the activity of what may have been P450 11B1 (ref. [1208]), although that paper reported that the enzyme used catalyzed 11β-, 18-, and 19-hydroxylation.

6.43.6. Clinical Issues

The issues of congenital adrenal hyperplasia and Types I and II corticosterone methyloxidase deficiency in individuals with attenuated P450 11B2 activity have already been mentioned. The other issue also mentioned is elevated aldosterone. Several studies have reported an association between polymorphisms and essential hypertension, although the measurements of aldosterone excretion are still lacking in some studies[1209]. Other studies show association of the –344C allele with increased left ventricular size[1210–1212]. The hypertension association has been seen in several studies[1209, 1210, 1213, 1214], but not in a Japanese study[1215].

6.44. P450 17A1

17-Hydroxylation and the 17, 20-lyase reaction ("desmolase") are two important reactions in steroid biosynthesis (Figures 10.13 and 10.14). Cloning of a cDNA which, when expressed, yielded both activities that established the role of what is now known as human P450 17A1 (previously termed $P450_{17\alpha}$, etc.)[1216]. The gene[1217] showed similarity to *CYP21A1*. The demonstration of both catalytic activities in a single protein established work previously done with purified hog protein[1218]. The two activities have long been known to be regulated by b_5 (refs. [1219], [1220]), and aspects of this duality of function still remain unclear.

6.44.1. Sites of Expression

Human P450 17A is expressed in steroidogenic tissues, including adrenals and gonads. The enzyme has also been reported in fetal kidney, thymus, and spleen[1221]. The enzyme has also been found in human (adult) heart[1222] and adipose tissue[1223].

P450 17A1 is a microsomal enzyme. A proline rich region in the *N*-terminus has been found to be important for efficient folding, but not for subsequent maintenance of the folded structure[1224].

6.44.2. Regulation and Polymorphism

As with the other steroidogenic P450s, the regulation of the *CYP17A1* gene is relatively complex. Induction of P450 17A1 has long been known to be cyclic AMP-mediated and the induction is suppressed by testosterone (mouse model)[1225], and a cyclic AMP response region was mapped in porcine Leydig cells[1226].

With the human *CYP17A1* gene, the homeodomain protein Pbx1 was shown to interact with protein kinase A in the cyclic AMP-dependent regulation (at −250/−241)[1227]. Further analysis showed interaction at a cyclic AMP-related site (−80/−40) by SF-1 (ref. [1228]). Further, interactions were shown for Sp1 and Sp3 (−227/−184) and NF-1C (−107/−85 and −178/−152)[1229]. SF-1 (*vide supra*) also interacts with $p54^{nrb}$, NonO, and protein-associated splicing factor[1230]. The ACTH/cyclic AMP response is dependent upon phosphatase activity, as well as kinase activity[1231, 1232]. The cyclic AMP-dependent protein kinase enhances transcription via MKP-1 activation, involving phosphorylation of SF-1 (ref. [1233]).

Polymorphisms of *CYP17A1* are known, but most of the attention has been given to mutations that result in serious defects in patients[1234]. Most of the mutations have been SNPs in the coding region[1234–1236], but others include a 2-bp deletion yielding a frameshift and premature stop codon[1237], a 4-bp duplication changing the *C*-terminal 28 amino acids[1238], and a 5′-splice site mutation[1239]. Some of the patients presenting with symptoms yielded P450 17A1 that, upon heterologous expression, retained 17-hydroxylation but not 17,20-lyase activity[1240, 1241]. Mutations of the latter type led Auchus[1242] to propose a model in which neutralization of positive charges in the redox partner binding surface of P450 17A may block the lyase activity but not 17-hydroxylation.

6.44.3. Substrates and Reactions

The generally accepted reactions of P450 17A are the 17α-hydroxylation of pregnenolone to 17-hydroxypregnenolone and of progesterone to 17-hydroxyprogesterone. 17-Hydroxypregnenolone is also oxidized to DHEA in the lyase reaction (Figures 10.13 and 10.14)[1241, 1243]. The mechanism of the lyase reaction is not completely established, but mechanisms have been proposed using analogs[1244]. Lieberman[1245] has proposed alternative reactions, although the favored pathway involves what would be a very unstable diradical. No other substrates are known presently, other than pregnenolone and progesterone and possibly closely related analogs. Very recently, Soucy *et al.*[1246] have provided evidence that human P450 17A1 also converts pregnenolone into 5,16-androstadien-3β-ol, a "16-ene synthase" reaction (without intermediate formation of an alcohol).

A key to research on the protein was the development of a robust *E. coli* expression system by Waterman's group in 1991[19]. Further work on the differential effects of b_5 on individual catalytic activities has been reported[1247]. The ratio of b_5 to P450 is high in testis and this phenomenon might regulate the two activities of P450 17A1. Miller's group has proposed that phosphorylation of Ser and Thr residues in P450 17A1 may alternatively influence the two activities[1248], although any experimental evidence in support of this hypothesis is still very limited[1248, 1249].

A second b_5 gene has been identified recently, and this protein also has the same stimulatory effect on lyase activity[1250]. Auchus *et al.*[809] also demonstrated that the same stimulatory effect of b_5 could be obtained with apo-b_5, arguing against the requirement for electron transfer. P450 17A enzymes from other species vary in their ability to catalyze the 17,20-lyase reaction, and comparisons of the rat and human enzymes also led to the conclusion that selective enhancement of the lyase reaction was not due to changes in electron transfer[1251].

The concertedness of the P450 17A1 lyase reaction has been examined, and both the studies both reached the conclusion that much of the 17α-hydroxypregnenolone dissociates[1252, 1253]. In one of the studies[1252], the authors concluded that the off-rate was an important factor in determining the balance between 17-hydroxypregnenolone and DHEA. Exactly how b_5 would control this rate, which was modeled to be rather slow (2.6–29 min^{-1}), is unclear, unless the effect is on the protein conformation. However, a classic burst kinetic experiment was not done in the cited work and the hypothesis remains to be addressed in more detail.

6.44.4. Knowledge about Active Site

Much of the information about the significance of active site residues comes from the analysis of mutations in patients presenting with diseases (see Section 6.44.2.). The changes H373L (ref. [1254]) and P409R (ref. [1255]) led to a loss of heme incorporation. Mutation at Thr306, possibly involved in protonation of Fe–OO^- or O–O cleavage, impaired 17α-hydroxylation more than the lyase reaction[1256]. However, the change R346A selectively abolished lyase activity[1257], as did F417C (ref. [1258]). Mutations at Lys83, Arg347, Arg358, and Arg449 produced proteins that were refractory to b_5 stimulation and attenuated in lyase activity[1259–1261]. Of these, only R347H and R358Q have been found in patients[1262]. Some mutants found in patients do cause the loss of both 17-hydroxylation and the lyase reaction, however[1263, 1264].

Some animal P450 17A1 enzymes have different ratios of 17-hydroxylation/lyase activity and efforts have been made to use these properties to define more elements controlling the latter steps, although the results have been limited to date[1265, 1266].

Several homology models of human P450 17A1 have been published and some of the mutagenesis results can be interpreted[1185, 1267–1271].

6.44.5. Inhibitors

Inhibitors of P450 17A1 have been studied for some time. Interestingly, ketoconazole inhibits lyase activity but not 17-hydroxylation activity[1272]. 7α-Thiospirolactone is a mechanism-based inhibitor of (guinea pig) P450 17A1 (ref. [1273]).

A number of steroidal inhibitors have been studied, primarily with the goal of treating cancers[1274–1278]. The enantiomer of progesterone (*ent*-progesterone) is reported to be a competitive inhibitor of P450 17A (K_i = 0.2 μM)[1279].

Nonsteroidal inhibitors have also been studied[1280, 1281].

Molecular modeling (Section 6.44.4) has also been applied to searches for inhibitors[1271, 1282]. Other approaches utilize P450 17A expressed in *E. coli* to screen for P450 17A inhibition in medium-to-high throughput systems[1283, 1284].

6.44.6. Clinical Issues

P450 17A1 has a central role in human steroid metabolism because of its role in regulating steroid flux (Figures 10.13 and 10.14). Perturbations lead to problems in adrenarche, aging, and polycistric ovary syndrome[1241, 1285]. Some of the more serious mutations have been mentioned already; another is a case of pseudohermaphroditism due to lack of lyase activity[1286].

Some of the other possible disease conditions or risks are being studied in relationship to less serious polymorphisms. In most of these cases, the relationships are more difficult to establish in the serious diseases. A possible link of *CYP17A1* polymorphism has been made with rheumatoid arthritis[1287]. Little influence of polymorphism was seen on age of menarche[1288]. However, a link was made between a particular polymorphism and the prediction to use hormone replacement therapy (i.e., postmenopausal estrogen therapy)[1289]. No association was found with polycistronic ovarian syndrome in a study with an SNP at the regulatory Sp1 site[1290].

Much attention has been given to the possibility of a link between *CYP17A1* allelic SNPs and breast cancer risk[1291]. The epidemiology results are mixed at best[1292–1295] and a conclusion in favor of a relationship cannot be made at this time[1296–1298].

Some positive epidemiology has been presented for a relationship with prostate cancer[1299], although probably not a strong one[1300]. Some positive results have also been reported for endometrial cancer and *CYP17A1* alleles[1301].

As with some other P450s, circulating antibodies to P450 17A are seen in some autoimmune diseases, for example, autoimmune polyglandular syndrome and Addison's disease[1159, 1302], but no causal relationship has been demonstrated.

6.45. P450 19A1

P450 19A1 is the classic "aromatase," often known by that name in endocrinology. This enzyme oxidizes the androgens androstandione and testosterone to the estrogens estrone and 17β-estradiol, respectively (Figure 10.15). This process is very important in normal physiology and also a target for inhibition in some tumors.

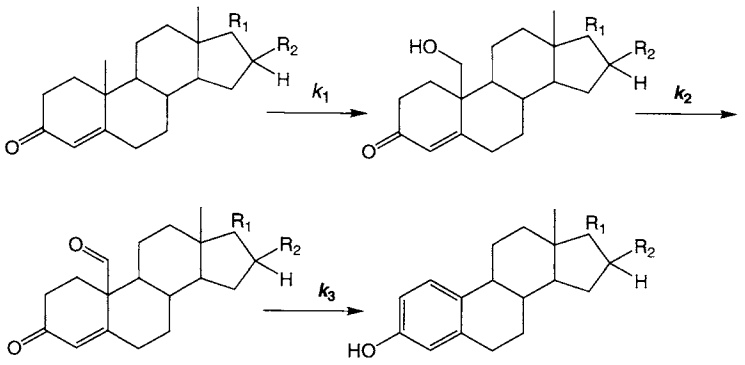

Figure 10.15. Aromatization reactions catalyzed by P450 19. The three distinct steps are shown, with the possible substrates: Testosterone, R_1: –OH, R_2: H; androstenedione, R_1: =O, R_2: –OH; 16-hydroxytestosterone, R_1=R_2=–OH.

6.45.1. Sites of Expression

Estrogens have a number of functions and not only feminization. Sites of (human) expression include the ovaries, testes, placenta, fetal (but not adult) liver, adipose tissue, chondrocytes and osteoblasts of bone, vasculature smooth muscle, and several sites in brain, including parts of the hypothalamus, limbic system, and cerebral cortex[1303]. As discussed later, regulatory mechanisms differ considerably in these tissues. P450 19A1 is also expressed in some tumors, particularly those derived from these tissues.

The actions of androgens and estrogens in the gonadal tissues are fairly well understood, but less is known in the brain. Androgens and androgen-derived estrogens regulate complementary and interacting genes in many neural networks[1304].

6.45.2. Regulation and Polymorphism

The regulation of the *CYP19A1* gene is quite complex, primarily because of the use of four tissue-selective promoters[1303, 1305]. Either the I.1, I.4, I.f, or I.6 sequence is read as exon I and spliced into the mRNA, depending upon the tissue. However, exon I does not code for the protein, so the P450 19A1 enzyme is always the same.

In preovulatory follicles and corpora lutea of human ovary, the 5′-untranslated region of P450 19A1 transcripts is encoded by exon IIa (ref. [1306]). The major operatives here are CRE and SF-1 elements[1303].

In adipose tissue, the promoter from exon I.4 is utilized[1303]. The same exon is utilized in bone and skin[1303], and in leiomyoma tissue derived from myometrium[1307]. This system is regulated with Sp1, a glucocorticoid regulatory element, STAT3, and possibly PPARγ[1303, 1308]. Pre-adipocytes also involve regulation with liver receptor homolog-1 (LRH-1)[1309].

In placenta exon I.1, an 89 kb upstream element is utilized[1303]. This is a strong promoter and involves C/EBP-β[1303]. A strong positive enhancer element between −42 and −501 is present[1310]. The possibility exists that vitamin D receptor/RXRα heterodimers and PPARγ may have effects[1303].

Regulation in bone uses exon I.6 (ref. [1303]). The study of regulation in bone is less extensive than in other sites, and 1,25-dihydroxycholecalciferol, interleukins, TNFα, and TGF-β_1 have had stimulatory activity.

Regulation in brain uses exon I.f and has also not been as extensively studied[1303]. P450 19A1 does seem to be upregulated by androgens.

Regulation in fetal liver involves exon I.4, as with adipose tissue[1303]. The same pattern appears to apply in skin fibroblasts and intestine.

In cancer cells, alternate regulatory pathways are utilized[1303].

A number of polymorphisms have been reported in the *CYP19A1* gene. These have been studied in relationship to breast but without convincing relationships (*vide infra*); also, there was no relationship with breast density[1311].

The number of cases of serious P450 19A1 deficiency is apparently only about ten, including two adult males[1303].

6.45.3. Substrates and Reactions

The reaction involves three steps and has been the subject of considerable mechanistic interest (Figure 10.15). Androstanedione is converted to estrone, testosterone to 17β-estradiol, and 16-hydroxy DHEA to estriol. The first two steps are relatively straightforward, for example, $RCH_3 \rightarrow RCH_2OH \rightarrow CHO$ (at C19). The third step was difficult to rationalize with "classic" FeO^{3+} chemistry, and there has been general acceptance of a $FeOO^-$-based mechanism originally developed by Robinson[1312] and Akhtar[1313], and further developed in models by Coon and Vaz[1314].

The possibility of utilization of DHEA as a substrate for estrone synthesis has been proposed but not addressed directly[1315].

6.45.4. Knowledge about Active Site

One of the historic problems in studying structure–function relationships in P450 19A1 has been the availability of expression systems. Recently two *E. coli* systems have been developed[1316, 1317].

Site-directed mutagenesis has been done using mammalian and insect cell-based systems, and models have been in existence for some time[1318]. More recent modeling work[1319] has been done, with an emphasis on docking of inhibitors in SRS-1.

6.45.5. Inhibitors

P450 19A1 inhibitors have long been of interest for treatment of estrogen-dependent cancers in a variety of tissues[30, 1320]. Today, the process has reached the stage of "3rd-generation" inhibitors[1321], moving beyond early drugs such as aminoglutethimide[1322]. The newer inhibitors are more effective in lowering the body limit of estrogens[1323]. One example of a newer drug is exemestane, a mechanism-based inactivator currently in Phase III clinical trials and being compared with the estrogen receptor antagonist tamoxifen[1322–1325]. The inactivation appears to involve generation of a Michael acceptor in the active site[1322].

Another report suggests generation of inhibitory Michael agents from prostaglandin J_2, but detailed characterization has not been done[1326].

Other nonsteroidal inhibitors of P450 19A1 are also under consideration[1327].

Breast cancer is the major target area for P450 19A1 inhibition, but other cancers are also under investigation[1328].

The point has been made by Simpson *et al.*[1303] that a future goal of P450 19A1 inhibition should be tissue selectivity. The diverse role of P450 19A1 in different tissues might indicate that generalized inhibition of estrogen synthesis may be less than desirable. Targeted inhibition of P450 19A1 could, in principle, be achieved by (a) selective targeting of inhibitors of P450 19A1 catalysis to tumors/individual organs or (b) targeted down-regulation of P450 19A1 synthesis in selected areas.

6.45.6. Clinical Issues

Several clinical issues have already been alluded to. The first issue is congenital aromatase deficiency. Serious cases in adults appear to be relatively rare[1303, 1329] and have been treated with estrogen replacement therapy[1330]. However, some children are considered to have attenuated P450 19A1 activity[1331].

Studies with P450 19A1 knockout mice show expected reproductive and sexual phenotypes and also adipose and bone phenotypes[1329, 1332], as well as a sociosexual behavior phenotype[1333].

The issue of using P450 19A1 inhibitors in the treatment of a variety of estrogen-dependent tumors has already been presented. In addition, there is consideration of the use of inhibitors for breast cancer prevention in high-risk individuals[1334].

A number of studies have been made on the relationship of *CYP19A1* polymorphisms with breast cancer, but the evidence has not shown a change in risk[1311, 1335]. No strong association was seen for endometriosis either[1336].

6.46. P450 20A1

At the time of writing this chapter, the only available information was the existence of the *CYP20* gene in the human genome[705]. The gene

appeared to be vertebrate specific and had been speculated to be involved in development.

6.47. P450 21A2

P450 21A2 is the enzyme involved in the 21-hydroxylation of progesterone and 17-hydroxyprogesterone, yielding deoxycorticosterone and 11-deoxycortisol from the two substrates, respectively (Figures 10.13 and 10.14). The 21-hydroxylation reaction is an important step in the synthesis of glucocorticoids and mineralcorticoids, and deficiencies lead to "salt-wasting syndrome," if not treated, and to congenital adrenal hyperplasia in the worst cases.

6.47.1. Sites of Expression

The major site of expression is the adrenal cortex. This reaction has been known for some time, and many of the early biochemical studies were done with bovine adrenals because of the need for large amounts of tissue[1337].

Low amounts of P450 21A2 have been reported in human lymphocytes[1338] and brain[1339]. Any specific function in these tissues is unknown at this time.

6.47.2. Regulation and Polymorphism

The regulation of P450 21A2 has some similarity to that of P450 17A1, in that both are regulated by ACTH. The cyclic AMP responsive sequence in the 5′-flanking region[1340] uses adrenal-specific protein factor and an Ad4-like sequence[1341]. One issue in the regulation of the *CYP21A2* gene is the neighboring homologous but nonfunctional *CYP21A1* pseudogene, which can compete for transcription factors and other regulatory proteins[1342]. In other work, protein kinases A and C and Ca^{2+} were found to regulate *CYP21A2* gene expression in a human cortical cell line[1343].

Another interesting aspect of the regulation of the *CYP21A2* gene is that it is located very close to the major histocompatibility locus, 2.3 kb downstream from the *C4* gene. Transcriptional regulatory elements for the *CYP21A2* gene lie within intron 35 kb of the *C4* gene[1344].

Steroid 21-hydroxylase deficiency is the most common cause of congenital adrenal hyperplasia, and many mutations are now known to be associated with the disease. Many are the result of recombination with the related pseudogene[1345, 1346]. Some are in the coding region[1346–1348] and the 5′-flanking region[1349]. The incidence of carriers of congenital adrenal hyperplasia is 1–2% in the population, and many deleterious mutations have now been identified[1350–1357].

6.47.3. Substrates and Reactions

The only known substrates are progesterone and 17-hydroxyprogesterone, which are hydroxylated only at the 21-position (Figures 10.13 and 10.14).

6.47.4. Knowledge about Active Site

Homology models have been reported[1185, 1358]. The amount of site-directed mutagenesis has been limited, but the disease has yielded many locations for loss of function because the severity of the disease is (inversely) correlated to the residual 21-hydroxylation activity. Many of the mutants could be rationalized in the context of a homology model[1358], although some associated with disease are more subtle (e.g., E380D).

6.47.5. Inhibitors

Relatively little has been published about inhibitors. Detrimental effects of spironolactone have been attributed to inhibition of 21-hydroxylation[1359], although further details with this P450 are lacking. Recently, Auchus[1279] reported that the enantiomeric form of progesterone (*ent*-progesterone) is a competitive inhibitor of P450 21A2 (although not as effective as with P450 17).

6.47.6. Clinical Issues

As mentioned earlier, the incidence of defects is relatively frequent and the ability to form cortisol is a problem. At least 56 different mutations have been identified[1351]. Patients who cannot synthesize sufficient aldosterone may lose sodium balance and can develop a fatal "salt-wasting" syndrome. Treatment involves administration of mineralocorticoids and glucocorticoids. Females with severe, classic P450 21A2 deficiency are

exposed to excess androgens prenatally and born with virilized external genitalia, but prenatal diagnosis permits prenatal treatment of affected females[1350]. Experimental research is being done on gene therapy to transfer active *CYP21A2* genes; work done on mice suggests feasibility[1360].

6.48. P450 24A1

The next three P450s (24A1, 27A1, 27B1) are involved in vitamin D metabolism (Figure 10.16). All three are mitochrondrial and receive electrons from the iron sulfur protein adrenodoxin (via the flavoprotein adrenodoxin reductase) (Table 10.1).

6.48.1. Sites of Expression and Abundance

The 24-hydroxylation of 25-hydroxyvitamin D_3 has long been known to occur in the kidney mitochondrial membrane[1361]. Following the purification of a rat P450 with this activity[1362], cDNA clones for chicken[1363] and human[1364] homologs were obtained.

The enzyme is expressed in both proximal and distal kidney tubules[1365], but has also been found in human nonsmall cell lung carcinomas[1366]. This would appear to be a relatively low abundance P450. Expression has also been reported in human keratinocytes[1367, 1368], colon carcinoma cells[1369], and prostatic cancer cells[1370].

6.48.2. Regulation and Polymorphism

The regulation of the *CYP24A1* gene appears to be complex, although some phenomena observed in animal models have not been examined in as much detail in humans. The activity has long been known to be inducible by vitamin D, perhaps to relieve the cells of an overload, and a vitamin D receptor element has been found in the 5′-region of the *CYP24A1* gene[1371, 1372]. Parathyroid hormone and cyclic AMP both enhance induction by the vitamin D receptor[1365].

In human keratinocytes, P450 24A1 mRNA was also elevated by 1α,25-dihydroxyvitamin D_3 (ref. [1367]). Studies with rat systems indicate that this response is also mediated by vitamin D response elements and that two of these (VDRE-1, VDRE-2) operate synergistically[1373]. A functional Ras-dependent Ets-binding site is located downstream from the proximal VDRE site and was critical; the model indicates transcriptional cooperation between Ras-activated Ets proteins and the vitamin D receptor–RXR complex in mediating 1α,25-dihydroxyvitamin D action on the P450 24A1 promoter[1374]. The YY1 transcription factor has been reported to repress 1α,25-dihydroxyvitamin D_3-induced transcription in cell culture[1375]. The isoflavone genistein was reported to block the transcription of the *CYP24A1* gene in human prostatic cancer cells and this block could be relieved with the histone deacetylase inhibitor trichostatin A[1370]. Finally, the earlier results with Ets proteins (*vide supra*) have been expanded to show distinct roles of the MAP kinases

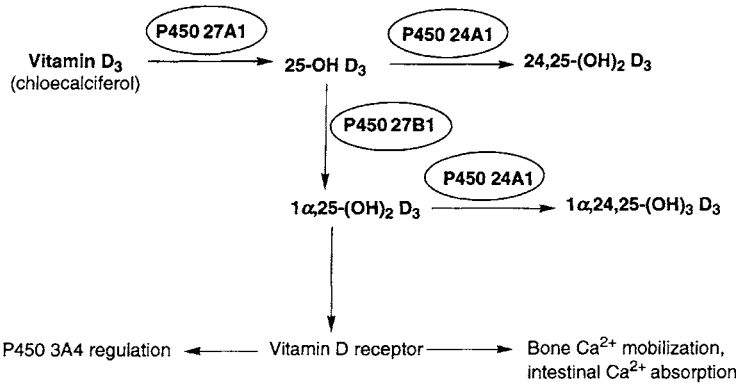

Figure 10.16. Overview of P450s involved in key steps of vitamin D activation[29].

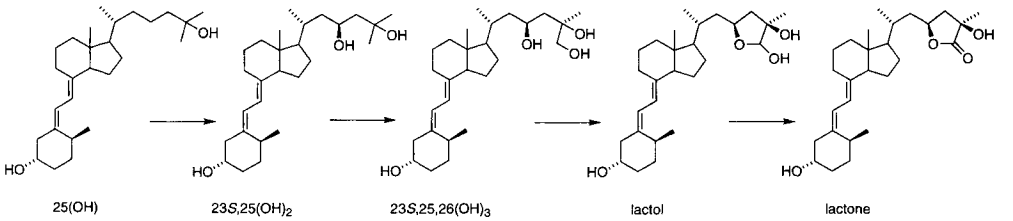

Figure 10.17. C-23 hydroxylation pathway for 25-hydroxyvitamin D_3 (25(OH)) oxidation catalyzed by P450 24A1 (ref. [1378]).

ERK1/ERK2 and ERK5 (ref. [1376]). Induction of P450 24A1 by 1α,25-dihydroxyvitamin D_3 involves Ets-1 phosphorylation at Thr38, but 1α,25-dihydroxyvitamin D_3 stimulation of ERK1/ERK2 required RXRα phosphorylation on Ser260 (ref. [1376]).

Polymorphisms in P450 24A1 have apparently not been reported.

6.48.3. Substrates and Reactions

Both 25-hydroxyvitamin D_3 and 1α,25-dihydroxyvitamin D_3 are substrates for 24-hydroxylation (Figure 10.16), with the latter being the preferred substrate[1377]. However, human P450 24A1 can also catalyze other side-chain reactions (Figure 10.17). Studies with side-chain-fluorinated vitamin D analogs also provide evidence for some flexibility of this side chain in allowing P450 24A1 to oxidize[1380, 1381]. Rat P450 24A1 differs from the human ortholog in taking 1α,25-dihydroxyvitamin D_3 on to calcitroic acid instead of the products shown in Figure 10.17[1382–1384].

6.48.4. Knowledge about Active Site

Limited site-directed mutagenesis has been done with P450 24A1. Expression studies using *E. coli* indicate species conservation in the residues 245–253 (F-helix), and the mutants M246F and F249T showed changes in the ratio of 24-hydroxylation to other pathways[1384].

Studies by Jones' group[1385] with analogs indicate that the site of hydroxylation by P450 24A1 depends on the distance from the ring to the site (23 or 24), and not by the distance beyond this. Schuster's group has developed pharmacophore models[1386].

6.48.5. Inhibitors

Inhibition of P450 24A1 is of some interest in the context of raising levels of active vitamin D metabolites. Schuster[1368, 1386, 1387] has identified some inhibitors that differ in their selectivity between P450 24A1 and P450 27B1 and have sub-μM IC_{50} values.

6.48.6. Clinical Issues

The scheme presented in Figure 10.16 depicts P450 24A1 as an enzyme involved in deactivating the activated form of vitamin D. The possibility has been considered that defects in P450 24A1 might lead to hypervitaminosis D[29]. An overactive P450 24A1 could lead to vitamin D deficiency. Henry[1388] has reviewed the role of P450 24A1 and made comparisons to other "multistep" P450 enzymes. The possibility is raised that P450 24 could serve to generate products with their own biological activities, with P450 24 thus being involved in an anabolic pathway. Recently, transgenic rats overexpressing (rat) P450 24A1 were found to have *low* plasma levels of 24,25-dihydroxy vitamin D_3[1389], which was unexpected. Further, the transgenic rats developed albuminuria and hyperlipidemia shortly after weaning, and later developed atherosclerotic lesions in the aorta. These results raise the possibility that P450 24A1 is involved in functions other than vitamin D metabolism[1389].

6.49. P450 26A1

Retinoic acid is a vitamin A derivative that plays an important role in gene regulation and development. The metabolism of retinoic acid has been a matter of interest for some time, and

the oxidation of retinoic acid by some hepatic P450s has been demonstrated[386, 942, 1390]. However, the relevance of these transformations to retinoic acid homeostasis in target tissues is not clear. White *et al.*[1391] probed a panel of mRNAs from mammalian cell lines with a cDNA from a zebrafish P450 shown to be involved in retinoic acid-inducible retinoic acid oxidation and characterized P450 26A1 (ref. [1391]). The heterologously expressed enzyme converted all-*trans*-retinoic acid to the 4-hydroxy-, and 4-oxo-, and 18-hydroxy products. The turnover numbers are unknown because the amount of P450 was not quantified, but the enzyme is clearly able to catalyze the oxidation of sub-μM additions of all-*trans*-retinoic acid[1391]. Apparently other retinoic acid isomers are not substrates.

The enzyme is expressed in cell lines derived from several different tissues (kidney, lung, liver, breast)[1391]. P450 26A1 has also been shown to be expressed in human fetal liver and brain tissues[1392, 1393], with a pattern differing from P450 26B1.

P450 26A1 (and 26B1, *vide infra*) may function to protect fetal brain and possibly other tissues from excess retinoic acid, which is known to be teratogenic, by metabolism. The interplay between P450s 26A1 and 26B1, and the significance of changes in their levels to normal tissue function is not yet known.

6.50. P450 26B1

The *CYP26B1* gene has 44% sequence identity with *CYP26A1*; it was found by searches of genomic databases[1394]. The gene expression pattern for P450 26B1 is different from that of P450 26A1 in mice[1395] and also in human fetal brain[1396]. The localization of P450 26B1 in adult human brain differs from that of P450 26A1, with a higher level of P450 26B1 mRNA in the cerebellum[1396]. Exactly how critical this enzyme is in brain function is unknown[1392]. The catalytic specificity is very similar to that of P450 26A1, acting only on all-*trans* retinoic acid and catalyzing the formation of 4-hydroxy-, 4-oxo-, and 18-hydroxy (*trans*) retinoic acid[1392].

As with P450 26A1, further studies are needed to define the presence of any defects in the gene and what the consequences might be.

6.51. P450 26C1

The only information presently available about the *CYP26C1* gene is its existence in the human genome[705]. Any suggestion of a role in retinoid metabolism is only speculatory at this time.

6.52. P450 27A1

This is a mitochondrial enzyme that was characterized on the basis of two rather divergent catalytic activities, the 25-hydroxylation of vitamin D_3 (Figure 10.16) and the oxidation of cholesterol at the C27 position (Figure 10.18). Thus, the enzyme bridges between hormone (vitamin D) and oxysterol pathways, and the clinical relevance of P450 27A1 is considerable.

6.52.1. Sites of Expression and Abundance

The enzyme is localized in liver mitochondria. Confusion existed in the early literature because some animal species have liver microsomal vitamin D_3 25-hydroxylases (e.g., hog liver and kidney P450 2D25, (refs [1398], [1399])), but not humans[1400]. The rat and human liver mitochrondrial P450 27A1 recombinant enzymes were clearly shown to catalyze both vitamin D_3 25-hydroxylation and the 27-hydroxylation of the side chains of cholesterol and several derivatives[1401, 1402].

Expression, at least at the mRNA level, has also been reported in leukocytes[1403], skin fibroblasts[1404], kidney[1405] (and fetal liver and kidney[1405]), and the arterial wall[1406].

6.52.2. Regulation and Induction

In a "normal" human population, the variation in the steady-state P450 27A1 mRNA level was reported to be ~25-fold, compared with 60-fold for P450 7A1 in the same study[1041]. However, at least two polymorphisms (≥1% incidence, no dramatic effect) and 42 mutations (rare alleles, usually debilitating) are known[1404, 1407]. Truncation mutations are known[1403], as well as splice variants[1408]. Defects in the *CYP27A1* gene are associated with a condition known as cerebrotendinous xanthomatosis (CTX), a rare, autosomal recessive disorder characterized by accumulation of

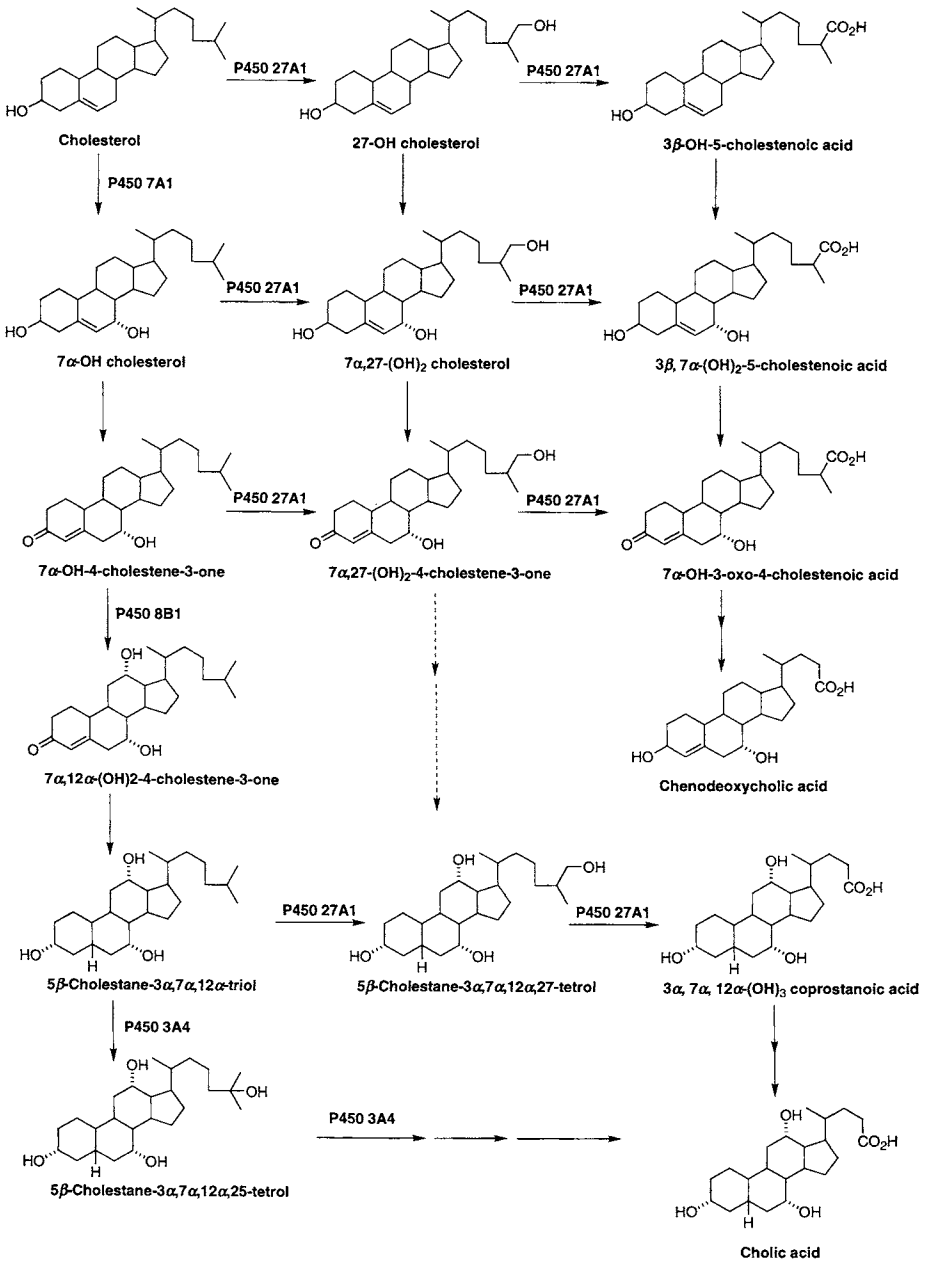

Figure 10.18. Bile acid synthesis from cholesterol[1397]. The steps shown with dashed arrows are tentative.

cholestanol and cholesterol in many tissues. The clinical manifestations include tendon xanthoma, premature cataracts, juvenile atherosclerosis, and a progressive neurological syndrome involving mental retardation, cerebellar atoxia, pyramidal tract signs, myelopathy, and peripheral neuropathy[29, 1407].

Several aspects of regulation of the *CYP27A1* gene have been studied. In rats, the enzyme can be induced by gonadotropin[1409]. A hamster model

showed downregulation of the gene in cholestatic liver[1397], although human P450 27A1 (used in HepG2 cells) was not subject to negative feedback regulation[1041].

6.52.3. Substrates and Reactions

Expanding on previous discussion, P450 27A1 catalyzes the 25-hydroxylation of vitamin D_3 (Figure 10.16), 1α-hydroxyvitamin D_3, vitamin D_2, and 1α-hydroxyvitamin D_2, and also the 27-hydroxylation of cholesterol and several derivatives (Figure 10.18)[1410, 1411]. The cholesterol alcohols are further oxidized by the enzyme to aldehydes and then carboxylic acids[1412]. The available information suggests release of the intermediates in the pathway[1412]. The regioselectivity of the enzyme is considered to be a function of the distance of the hydroxylation site to the end of the side chain[1385].

More detailed analysis of the vitamin D_3 reaction has been with *E. coli* recombinant P450 27A1, with evidence for the following products (from vitamin D_3): 25-hydroxy, 26-hydroxy, 27-hydroxy, 24*R*-25-dihydroxy, 1α,25-dihydroxy, 25, 26-dihydroxy, 25-,27-dihydroxy, 27-oxo, and an unidentified dehydrogenated product[1402, 1413].

6.52.4. Knowledge about Active Site

Some information about the roles of amino acids can be inferred from the knowledge of alleles involved in CTX; many of these proteins were unstable when attempts were made at heterologous expression[1414]. Other work by Pikuleva *et al.*[1415] with the putative F and G helices has shown differences due to substitution of Phe207, Ile211, Phe215, Trp235, and Tyr238. Interestingly, the I211K and F215K mutations affected the regioselectivity and enabled the enzyme to catalyze C–C bond cleavage. Further work with mutants in this region led to weaker association of P450 27A1 with the membrane, and some of the nonconservative changes yielded impaired catalytic activity[1416].

Human P450 27A1 can be contrasted with porcine P450 2D25, which also catalyzes vitamin D_3 25-hydroxylation. The only human P450 2D subfamily enzyme which does not have activity toward vitamin D is P450 2D6. Further, changing a set of residues of porcine 2D25 to their counterparts in (human) P450 2D6 abolished the activity toward vitamin D_3[1417].

6.52.5. Inhibitors

Apparently little specific work has been done on inhibition of this enzyme. Inhibition of this enzyme by a drug would probably be undesirable.

6.52.6. Clinical Issues

Low serum 25-hydroxyvitamin D_3 concentrations have been reported in a variety of other medical conditions and are considered to be potential problems[1418]. Although CTX is linked with defective P450 27A1 (ref. [29]), there are a number of enigmas about the etiology. A heterozygote showed frontal lobe dementia and abnormal cholesterol metabolism[1419]. Compound heterozygous mutations have also been reported to cause a variation of CTX[1407].

Björkhem has recently reviewed the issue of whether oxysterols (e.g., hydroxycholesterol) control cholesterol homeostasis[1420]. Studies with rodents and cultured cells have not been very clear to date. For instance, disruption of the mouse *CYP27A1* gene yielded reduced bile acid synthesis but apparently caused no change in levels of cholesterol or 1α,25-dihydroxyvitamin D_3[1421]. P450 27A1 is constitutively expressed in the normal artery wall and is substantially upregulated in atherosclerosis, and the possibility has been raised that P450 27A1 may be a protective mechanism for removing cholesterol[1406]. Further, immune complexes and IFN-γ decreased P450 27A1 expression in human aortic endothelial cells, peripheral blood mononuclear cells, monocytes-derived macrophages, and a human monocytoid cell line, suggesting downregulation of P450 27A1 to maintain cholesterol homeostasis in the arterial wall[1422].

In *Cyp27A1*$^{-/-}$ mice, a dramatic increase in the level of P450 3A enzymes is seen; some sterols accumulate and induce via the mouse PXR system[1423]. P450 3A4 has some side-chain hydroxylation activities toward cholesterol-derived sterols[771]. However, elevated P450 3A4 activities were not increased in CTX[771], indicating a difference in the murine and human systems. Recently, Escher *et al.*[1424] have reported that cholesterol

efflux in CHOP cells is enhanced by (heterologous) expression of human P450 27A1, and the authors suggest this as part of a protective system against atherosclerosis. The basis is probably the ability of 27-hydroxycholesterol to act as an endogenous ligand for the liver X receptor in cholesterol-loaded cells[1425].

In considering the general question of whether oxysterols (e.g., 27-hydroxycholesterol) control cholesterol homeostasis, the hypothesis is still open and the rodent data are not totally clear here. Björkhem[1041] has made the point that humans lacking P450 27A1 have normal circulating levels of cholesterol.

6.53. P450 27B1

As discussed earlier, vitamin D is an important hormone. A critical step in activation is 1α-hydroxylation[1426] (Figure 10.16). Early work established the P450 nature of the enzyme, localized in kidney mitochondria[1427]. Subsequent work demonstrated that the 1α- and 24-hydroxylation activities could be attributed to different enzymes[1428, 1429]. Some early work had suggested that the 1α- and 25-hydroxylation activities were associated with the same enzyme[1430], but later work showed that these activities were due to P450 27B1 and 27A1, respectively.

6.53.1. Sites of Expression and Abundance

The cloning of the human cDNA for what is now known as P450 27B1 established the kidney mitochondrial P450 (27B1) as the vitamin D_3 1α-hydroxylase[1431]. The gene has nine exons and spans only 5 kb[1432].

P450 27B1 is expressed in many parts of the human kidney, including the distal convoluted tubule, the cortical and medullary part of the collecting ducts, and the papillary epithelia[1433]. Lower expression was observed along the thick ascending limb of the loop of Henle and Bowman's capsule. Some weaker expression was observed in glomeruli or vascular structures. In normal humans, the distal nephron is the predominant site of expression[1433].

P450 27B1 is also expressed in many extrarenal sites (human) where it is involved in vitamin D-related activities, including skin (basal keratinocytes, hair follicles), lymph nodes (granulomata), colon (epithelial cells and parasympathetic ganglia), pancreas (islets), adrenal medulla, brain (cerebellum and cerebral cortex)[1434], placenta (decidual and trophoblastic cells)[1434–1436], cervix[1437], and parathyroid glands[1438]. Thus, P450 27B1 may be an intracrine modulator of vitamin D function in peripheral tissues[1434]. The expression of P450 27B1 was elevated in parathyroid adenomas, but attenuated in carcinomas, relative to normal parathyroid tissue[1438].

6.53.2. Regulation and Polymorphism

Although the *CYP27B1* gene is only 5 kb in size, the regulation is quite complex. The promoter is in the −85/+22 region and requires a functional CCATT element. Three consensus AP-1 sites are upstream[1439]. Enzyme activity has long been known to be stimulated by low phosphorus diets (in animal models)[1440], and more recently, this phenomenon has been linked to a growth hormone mechanism[1441, 1442]; its relevance in humans is not known.

Expression is also regulated by calcium, parathyroid hormone, and by the product 1α,25-dihydroxyvitamin D_3[1443, 1444]. Regulatory regions involving the responses to parathyroid hormone, calcitonin, and 1α,25-hydroxyvitamin D_3 are located in the region −0.4 to −0.5 kb[1445]. Forskolin also regulates gene expression, and an Sp1 site is involved in aspects of regulation[1446, 1447].

Complexity is seen in different models. Parathyroid hormone-related protein and Ca^{2+} have conflicting actions in a nude rat model of humoral hypercalcemia of malignancy[1448]. In differentiated Caco cells, there is upregulation of P450 27B1 expression by 1α,25-dihydroxyvitamin D_3 and epidermal growth factor, but downregulation in less differentiated Caco cell lines[1369].

Another aspect of regulation of P450 27B1 is genetic. P450 27B1 results in Type I vitamin D-dependent rickets[1449]. The genetics have been established in more than 30 patients involving at least 20 mutations[1450, 1451]. At least 13 missense mutations have been observed, none of which encode an active protein. Some of the mutants are splicing defects[1452]. Some mutations in *CYP27B1* are also involved in what is termed pseudovitamin D-deficiency rickets[1453, 1454]. Beyond these

debilitating mutations, little information is available about actual polymorphisms.

6.53.3. Substrates and Reactions

P450 27B1 can catalyze the 1α-hydroxylation of both 25-hydroxy and 24(*R*),25-dihydroxyvitamin D_3[1383, 1455] (Figure 10.16). The intrinsic activity (k_{cat}/K_m) for the recombinant human enzyme is better for 24(*R*),25-hydroxy vitamin D_3, but this does not mean that this is the favored substrate in the cell, because of the balance of vitamin D metabolites regulated by P450s 24A1 and 27A1 (ref. [29]). Apparently the 25-hydroxy group is an obligatory requirement[1378, 1455].

6.53.4. Knowledge about Active Site

Some information is available from the natural mutants of P450 27B1, even if the basis for loss of activity is not obvious. Inouye's group[1414] has provided evidence that Arg107, Gly125, and Pro497 are not simply involved in binding substrate but required for proper folding. It was also suggested that Arg389 and Arg453 are involved in heme binding and that Asp164 stabilizes the bundle of the four helices D, E, I, and J. Thr321 is suggested to be involved in O_2 activation[1414]. The natural mutants L343F and E189G show partial activity and the individuals bearing these have only marginal impairment[1456].

6.53.5. Inhibitors

Little has been done because impairment of this enzyme is a clinical problem. Some thiavitamin D analogs have been evaluated in animal models[1457].

6.53.6. Clinical Issues

The significance of the enzyme is due to the pleiotropic actions of the active form of vitamin D, 1α,25-dihydroxyvitamin D_3, which include regulation of calcium homeostasis, control of bone cell differentiation, and modification of immune responses[1458]. The 1α-hydroxylation reaction is rate limiting and hormonally controlled. The expression of the gene is usually tightly regulated (*vide supra*), but gene defects are responsible for vitamin D-dependent rickets Type I[1459]. At least 30 different mutations are known in patients[1449, 1460]. Even the "mild" phenotype of Type I rickets is due to deficiency in P450 27B1 (ref. [1461]).

CYP27B1 knockout mice have been characterized and show a typical rickets phenotype[1462]. Another mouse model in which the gene has been ablated showed skeletal, reproductive, and immune dysfunction[1463]. Rickets was also observed in a conditional knockout model[1464].

Patients with severe renal insufficiency show attenuated 1α-hydroxylation activity[1465].

Another aspect of P450 27B1 research involves cancer. Increased activity was reported in parathyroid tumors[1466]. Some splice variants of the *CYP27B1* gene (coding for truncated proteins) were amplified in human (brain) gliomas[1467]. Reports have also appeared on the relationship of P450 27B1 expression to various biological processes in human nonsmall cell lung carcinomas[1366], colon tumors[1468–1470], and prostate cancers[1471, 1472], generally with decreased expression in tumors.

Finally, 1α,25-dihydroxyvitamin D_3 is used to treat psoriasis, and patients can develop resistance. An experimental model for therapy involves enhancement of the endogenous production of 1α,25-dihydroxyvitamin D_3 by gene therapy[1473].

6.54. P450 27C1

As with some of the other P450s, the only knowledge currently available is the existence of the *CYP27C1* gene in the human genome[705]. Suggestions that this enzyme is involved in vitamin D metabolism are still only speculatory.

6.55. P450 39A1

An expression-cloning approach was utilized to isolate a cDNA from (*Cyp7b1*$^{-/-}$) mice that could, when expressed, catalyze the 7α-hydroxylation of 24-hydroxycholesterol[1474]. P450 39 has a microsomal location with a preference for the substrate 24-hydroxycholesterol and is expressed in liver. Presumably these characteristics of mouse P450 39 apply to the human ortholog but no further information is yet available. Potential relevance of this enzyme is in the inactivation of

24-hydroxycholesterol, a ligand for the LXR nuclear hormone receptor (see Section 6.56 on P450 46, *vide infra*).

6.56. P450 46A1

An expression cloning approach was utilized to clone cDNAs encoding both murine and human cholesterol 24-hydroxylase, P450 46A1 (ref. [1475]). The mouse and human sequences are 95% identical. Expression is predominantly in brain (neurons in several regions). The enzyme is in the endoplasmic reticulum; alternate substrates have not been explored but may be unlikely.

The significance of P450 46A1 rests in the fact that the brain is the most cholesterol-rich tissue in the body[1476]. LXRs, members of the nuclear hormone receptor family, are activated by 24-hydroxycholesterol. LXRβ and P450 46 have overlapping expression patterns in brain[1476]. The system may be counter-balanced by P450 39, which catalyzes the 7α-hydroxylation of 25-hydroxycholesterol (Section 6.55).

Recently, there has been considerable interest in the relationship between P450 46A1 and Alzheimer's Disease. P450 46A1 had a marked difference in distribution in the brains of normal and Alzheimer's patients, with less staining of neuronal cells but more of glial cells in diseased patients[1477]. Also, elevated levels of 25(*S*)-hydroxycholesterol were found in cerebral spinal fluid in the early stages of dementia[1478]. Associations[1479] and lacks of associations[1480] between *CYP46* SNPs and Alzheimer's Disease have been reported. One of the issues in the research with P450 46A1 is the apparently very low rate of cholesterol 24-hydroxylation (although an exact rate can be deduced from the published information). Recently, Pikuleva's laboratory has found that 24-hydroxycholesterol is a much better substrate for recombinant P450 46A1 than is cholesterol, being oxidized to as yet uncharacterized product[1480a].

6.57. P450 51A1

Lanosterol is an important intermediate in cholesterol synthesis, and 14α-demethylation has been established as a step in the pathway. Yoshida's laboratory had studied the yeast enzyme for many years and then demonstrated the reaction in rat liver microsomes in 1994[1481]. Subsequently the reaction was also demonstrated in rat brain microsomes[1482].

6.57.1. Sites of Expression and Abundance

Waterman's group identified the human *CYP51A1* gene and two pseudogenes[1483]. mRNA blot analysis showed the highest levels in testis, ovary, adrenal, prostate, liver, kidney, and lung. In mouse testis, P450 51A1 was localized in both round and elongated spermatids[1484]. The enzyme is also found in (rodent) oocytes[1485].

6.57.2. Regulation and Polymorphism

Polymorphisms in the human *CYP51A1* gene have not been reported nor have debilitating mutants been defined. However, Kelley *et al.*[1486] reported a patient with Antley–Bixler syndrome and ambiguous genitalia with lanosterol accumulation and an apparent defect in P450 51A1.

With regard to regulation of the human gene, primer extension studies indicated predominant transcription initiation sites in liver, lung, and kidney, and placenta 250 and 249 bp upstream from the translation start site and a second major site at −100 bp, with the absence of TATA and CAAT patterns and a GC-rich sequence in the promoter region[1483]. Multiple (rat) testis-specific transcripts arise from differential polyadenylation site usage[1487].

In human adrenocortical H295R cells (in culture), cholesterol deprivation led to a 2.6–3.8-fold induction of P450 51A1 mRNA, which was suppressed by the addition of 25-hydroxycholesterol[1488]. In the liver and other somatic tissues, *CYP51* is regulated by a sterol/sterol-regulatory element binding protein (SREBP)-dependent pathway[1489]. In testis, cAMP/cAMP-responsive element modulator $(CREM)_1$-dependent regulation predominates. Sp1 functions to maximize the sterol regulatory pathway of P450 51 (ref. [1490]).

Insulin is an essential factor in "basal" expression of P450 51 in rat liver, with possible involvement of SREBP-1c involvement[1491]. In a porcine vascular endothelial cell model (and in arterial wall), LDLs downregulated P450 51 through an SREBP-2 mechanism[1492].

6.57.3. Substrates and Reactions

Stimulation of human P450 51 activity by b_5 in a reconstituted system has been reported by Kelly's laboratory[1493].

The normal mammalian substrate for P450 51 is 24,25-dihydrolanosterol[1494], with the 14α-demethylation process proceeding in what are assumed to be three consecutive steps, as with some other P450s, for example, 11A1, 17A1, 19A1. Interestingly, both human and yeast (*Candida albicans*) P450 51 showed relatively little selectivity among a closely related group of analogs[1494]. It is also interesting to note that even though this P450 has a relatively defined role in a physiological process, the kinetic parameters are relatively poor among P450s (k_{cat}/K_m = 300 $M^{-1}s^{-1}$)[1494].

6.57.4. Knowledge about Active Site

A crystal structure of human P450 51 is not yet available but high resolution structure of the soluble *Mycobacterium tuberculosis* P450 51 is (ref. [1495]). Two notable features are a bent I helix and an open conformation of the BC loop. The bacterial structure has been utilized in consideration of mammalian models, and the SRS predictions do not seem to apply well[1495]. Further, the mutation hotspots for known azole-resistant *C. albicans* P450 51 mutants tend to be outside the predicted active site and suggest the contribution of long-range effects on ligand binding[1496, 1497].

Studies on Arg448 indicate that, despite sequence conservation, aspects of the role of this and other residues are different for human and yeast P450 51 (ref. [1498]).

6.57.5. Inhibitors

Most of the interest in inhibition has been with fungal P450 51, as a target for antimycotic drugs. The goal is to select candidate drugs inhibitory to fungal P450 51, but not human P450 51.

Some work on the interaction of azoles with human P450 51 has been published[1499]. Although human P450 51 has been suggested as a target for cholesterol-lowering drugs, apparently little has been done and potential toxicity due to the steroidogenic and potential germ cell side effects (*vide infra*) could be an issue.

6.57.6. Clinical Issues

Most of the work discussed here is from experimental studies on the possible role of P450 51 in reproduction, and the translation of phenomena from animal models to humans is still somewhat speculatory. However, the very high level of P450 51 expression in postmeiotic haploid spermatids is striking. The action of P450 51 is proposed to lead to the production of signaling steroids in haploid germ cells[1500]. Meiosis-activating substances (MAS) are produced by 14-reduction of products of the action of P450 51 on lanosterol[1500]. Follicular fluid MAS (FF-MAS) is formed from lanosterol in rat spermatids[1501]. Yoshida's group has reported gonadotropin-dependent expression of P450 51 in rat ovaries and the production of MAS[1502].

The reaction and possible physiological significance of the system in reproduction have been reviewed recently by Rozman[1503]. Leydig cells and acrosomes of spermatids have the highest P450 51 levels, and primary mouse oocytes and granulose cells also contain P450 51. The MAS may have a role in fertilization[1503].

7. Concluding Remarks

Some information has been presented about the known P450s. Because the genome is nearly completed, no more human P450s are likely to be added unless our views of marker sequences change.

Although much of the interest in human P450 is directed toward drug metabolism, the majority of P450s appear to be involved in the metabolism of endobiotics (Table 10.2). In one sense, the fact that about 5 or 6 of the P450s are so dominant in xenobiotic metabolism (Figure 10.3) is not surprising when we recognize that only about 15 of the 57 use xenobiotics as substrates (Table 10.1) and consider the dominant levels of expression of a few P450s that have rather broad selectivity (Figure 10.4).

Aside from the current problems already mentioned, including practical issues, what are some of the future challenges and in what areas should work be done? Some of the problems are very basic, such as the goals of deriving more experimental three-dimensional structures, answering questions about catalytic mechanisms, and understanding the complexities of regulation of these genes.

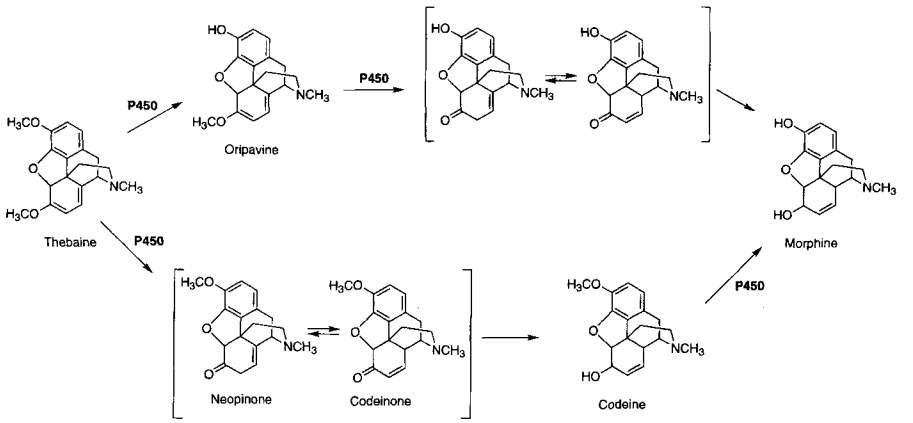

Figure 10.19. Steps in mammalian morphine synthesis[1504, 1505].

There is considerable opportunity to improve screening and predictability for use with new chemical entities in the pharmaceutical industry. Another open issue is whether large-scale SNP analysis can be practical in drug development.

Another area is understanding the relevance of variations in P450s (both SNPs and expression levels) to diseases, not only drug therapies. For instance, one can ask if some of the "minor" sterol products produced by P450 3A4 have any impact on cardiovascular disease. How important are some of the P450s that oxidize arachidonic acid to various products (in humans)? Does a P450 2D6 deficit have any relevance to long-term health?

Finally, there are two more major problems in understanding what human P450s do. One problem is the oxidation reactions, largely involving endobiotics, for which no P450s have been defined. One example is the synthesis of endogenous morphine in the body, which appears to require multiple oxidations and is subject to short-term regulation[1504, 1505] (Figure 10.19). Although P450 2D6 can oxidize codeine to morphine[1506], this enzyme does not contribute to the endogenous formation of morphine[1507]. What other oxidative reactions remain to be discovered and characterized? In the past year, the oxidative dealkylation in DNA repair was demonstrated to involve an α-ketoglutarate-dependent dioxygenase[1508, 1509]. Perhaps similar roles for P450s may be found. The other issue is identifying functions for the remaining human P450s, of which there are at least 15 (Table 10.2). Doing this on a step-by-step basis as in the past will not be very efficient, and there is an opportunity for the introduction of novel approaches.

Note added in proof. Recently Pai *et al.*[1510] have demonstrated an unusual phenomenon with the pseudogene, *CYP2D7*. A frame shifted allelic variant yields expression of an enzyme specifically in the brain, not liver, and this enzyme is more efficient than P450 2D6 in converting codeine to morphine (Figure 10.19).

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11

Cytochrome P450 and the Metabolism and Bioactivation of Arachidonic Acid and Eicosanoids

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1. Introduction

The convergence of important advances in the identification of several lipid-derived mediators as inter- and intracellular signaling molecules, and in the biochemistry of oxidative lipid metabolism, has focused interest in the functional roles of pathways responsible for their formation, and the physiological significance of their products. Among these, the studies of the enzymes of the arachidonic acid (AA) cascade, consisting of prostaglandin H_2 synthase[1, 2], lipoxygenases[3], and cytochrome P450[4, 5], constitute a premier example of the biological importance of these reactions and of their products (eicosanoids). Studies of the last two decades, have implicated the enzymes of the AA cascade in the pathophysiology of diseases such as hypertension, diabetes, and cancer, and some of these enzymes serve as molecular targets for drugs of extensive use in clinical medicine, including many nonsteroidal anti-inflammatory, antipyretic, and anti-asthmatic drugs[1–3]. The biological and signaling properties of eicosanoids are derived from the enzymatic, regio-, and stereoselective oxygenation of AA, a rather simple molecular template. While the informational content stored in the AA metabolites, is limited compared to that contained in complex informational molecules such as proteins or nucleic acids, low energy cost, versatility, and rapid turnover, makes them efficient on/off molecular switches for rapid and efficient intra- or intercellular signaling. Metabolism by prostaglandin H_2 synthase generates a cyclic endoperoxide, prostaglandin H_2 (PGH_2) that serves as the precursor for the formation of prostaglandins, prostacyclin, and thromboxanes[1, 2]. Metabolism by lipoxygenases leads to the formation of several regioisomeric hydroperoxides, the precursors of leukotrienes, regioisomeric *cis/trans* conjugated hydroxyeicosatetraenoic acids (HETEs), lipoxins, and hepoxilins[3]. Metabolism by microsomal cytochrome P450s (P450s) generates several hydroxy- and epoxy-AA derivatives[4, 5]. The reactions catalyzed by prostaglandin H_2 synthase and lipoxygenases are mechanistically similar to those of the free-radical-mediated autooxidation of polyunsaturated fatty acids in that they are initiated by hydrogen atom abstraction from a bis-allylic methylene carbon, followed by coupling of the resulting carbon radical to ground state molecular oxygen. The kinetics, regiochemistry, and chirality of these

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reactions are under strict enzymatic control. In contradistinction to the cytochrome P450-catalyzed, redox coupled, activation of molecular oxygen and delivery to ground state carbon, prostaglandin H_2 synthase and the lipoxygenases are typical dioxygenases that catalyze substrate carbon activation instead of oxygen activation.

It is apparent from the literature that the P450 gene superfamily of hemoproteins is, as a group, one of the most intensively studied enzyme systems and yet our knowledge of their endogenous metabolic or physiological roles remains limited. This is partly due to the complexity of mammalian P450 isoforms, and the wide structural diversity of substrates known to be metabolized by these proteins. This catalytic versatility pointed to, and served as the basis for, many of the documented roles for P450 in the metabolism of foreign chemicals, and has contributed to establish its toxicological and pharmacological importance. In the last few years, there has been an increasing interest in the understanding of the physiological significance of the P450 enzyme system, and its role(s) in the metabolism of endogenous substrates. In this regard, the studies of the P450 branch of the AA cascade have provided a new focus to these efforts, and far-reaching results from several laboratories are generating new paradigms in fatty acid metabolism, as well as in cell and organ physiology[4–10].

The studies of the role of P450 in the metabolism and bioactivation of AA were initiated in 1981 with the demonstration that liver and kidney microsomal fractions, as well as purified P450 isoforms[11–13] actively catalyzed the NADPH-dependent, oxidative metabolism of AA to products that were different from prostanoids and leukotrienes[11–13]. The widely documented physiological importance of AA suggested that these observations were unique and likely to be functionally significant, and led to the rapid structural characterization of most P450-eicosanoids, their chemical synthesis, and subsequent biological evaluation[4–10]. Interest in these novel P450 reactions was stimulated by: (a) the initial demonstration that some of the products displayed potent biological activities, including the inhibition of Na^+ reabsorption in the distal nephron[14], (b) the documentation of P450 participation in the *in vivo* metabolism of endogenous AA pools[15], and (c) the proposal of a role for these enzymes in the pathophysiology of genetically controlled experimental hypertension[6, 7]. These earlier studies established P450 AA monooxygenation as a formal metabolic pathway, P450 as an endogenous member of the AA metabolic cascade, and more importantly, suggested functional roles for this enzyme in the bioactivation of the fatty acid and thus, in cell and organ physiology. Many of the biological activities attributed to the P450-derived eicosanoids, as well as the potential physiological importance of these reactions, have been reviewed[6–10].

Prior to the demonstration of AA metabolism by P450, several groups demonstrated the role of microsomal P450s in the ω/ω-1 hydroxylation of prostanoids[16–20] and, more recently, leukotrienes[20]. Most of these reactions are considered to be involved in eicosanoid catabolism and excretion, but their potential relevance in eicosanoid bioactivation or inactivation, and/or in the control of organ/cell eicosanoid levels has only begun to be explored. We will first discuss the role of P450 in the metabolism of eicosanoids, and then concentrate on the studies of its role in AA metabolism and bioactivation.

2. Metabolism of Eicosanoids

During the metabolism of eicosanoids, depending on the nature of the oxygenated substrate, P450 catalyzes both NADPH-dependent and -independent reactions. This differential requirement for NADPH-mediated changes in the redox state of the heme-iron illustrates the marked differences in oxygen chemistries for these reactions, that is, the isomerization of AA peroxides, vs the more demanding activation and delivery of a reactive form of atomic oxygen to ground state carbon–hydrogen bonds.

2.1. NADPH-Independent Reactions

P450 catalyzes the isomerization of a variety of fatty acid hydroperoxides, including 15-hydroperoxyeicosatetraenoic acid (15-HPETE)[21, 22], and of the prostaglandin H_2 (PGH_2) endoperoxide[23]. A distinctive feature of some P450 fatty acid peroxide isomerases is their inability to accept electrons from NADPH and to activate molecular

oxygen[21–23]. Moreover, while all these enzymes possess a heme-thiolate prosthetic group, their overall homology to other members of the P450 gene superfamily is limited and suggests an early evolutionary functional specialization[23–26]. The mechanism by which the hemoprotein cleaves the peroxide oxygen–oxygen bond, that is, homolytic or heterolytic scission, plays a decisive role in determining the catalytic outcome of these reactions and is highly dependent on the nature of the P450 isoform, the chemical properties of the organic peroxide, and the nature of the oxygen acceptor[21–23, 27]. A homolytic pathway was proposed for the formation of 11- and 13-hydroxy-14, 15-epoxyeicosatrienoic acids (EETs) from 15-HPETE by rat liver microsomes[22]. Prostacyclin and thromboxane synthases are P450-like proteins containing a heme-thiolate prosthetic group[23–26]. The heterolytic cleavage of PGH_2 and an oxygen atom transfer or oxenoid mechanism has been proposed to account for the P450-catalyzed formation of prostacyclin (PGI_2) and thromboxane A_2 (TXA_2)[23]. The participation of P450s in the biosynthesis of these important mediators of endothelial cell and platelet function was one of the first demonstrations of a role for this enzyme system in vascular biology. However, its pharmacological and clinical implications remain to be fully explored.

2.2. NADPH-Dependent Reactions

P450 plays an important role in the NADPH-dependent metabolism of several bioactive oxygenated eicosanoids[16–20]. These reactions are of importance in that they: (a) increase eicosanoid structural diversity and, hence, modify informational content, (b) may alter the pharmacological profile of the substrate, and (c) may participate in the regulation of steady state and/or stimulated levels of physiologically relevant molecules. While these oxidations were generally viewed as catabolic, that is, yielding an attenuated biological activity, recent studies indicate that some ω-oxidized prostanoids show unique and potent biological properties[6–10]. However, in most cases the sequence of steps leading to ω/ω-1 oxidized prostanoids from endogenous AA pools remains to be clarified.

Typically, the P450-dependent metabolism of eicosanoids results in the hydroxylation of their terminal (C_{20} or ω-carbon) or penultimate carbon atoms (C_{19} or ω-1 carbon). However, the epoxidation of infused PGI_2 by a perfused kidney preparation[28], and the metabolism of 5,6- and 8,9-EET by prostaglandin H_2 synthase, were described several years ago[29, 30]. The former leads to a variety of 5,6-oxygenated prostanoids[29]. Oxidation of the latter was stereodependent, that is, 8(*S*),9(*R*)-EET formed 11(*R*)-hydroxy-8(*S*),9(*R*)-epoxyeicosatrienoic acid exclusively, whereas the 8(*R*),9(*S*)-enantiomer formed both C_{11} and C_{15} hydroxylated metabolites[30]. A detailed study of the secondary metabolism of 12(*R*)-HETE and 14,15-EET by P450 has been reported[31, 32]. The efficient ω/ω-1 oxidation of EETs by rat CYP4A isoforms to the corresponding regioisomeric epoxy-alcohols at rates comparable to those observed with lauric acid, a prototype substrate for these enzymes, was published recently[33]. One of these metabolites, the ω-alcohol of 14,15-EET, was shown to bind and activate the peroxisomal proliferator activated receptor alpha ($PPAR_\alpha$) type of nuclear receptor[33]. It is important to note that, with the exception of the ω/ω-1 hydroxylation of prostanoids and leukotrienes, none of the transformations described above has been shown to occur *in vivo* from endogenous precursors.

2.2.1. ω/ω-1 Oxidation of Prostanoids

Since the initial report of *in vivo* ω-1 hydroxylation of prostanoids in 1966[34, 35], ω- and ω-1 hydroxylation has become a recognized route of prostanoid metabolism. Early studies of prostanoid ω/ω-1 hydroxylation demonstrated these reactions were NADPH-dependent, localized to the endoplasmic reticulum[16, 36], and catalyzed by microsomal P450 (ref. [36]). Incubations with purified enzymes or recombinant P450s showed that most of these reactions were catalyzed by members of the 4 gene family of P450s[20, 36–43]. In general, while CYP4F isoforms are more active in the metabolism of eicosanoids than fatty acids, the opposite appears to be true for most CYP4A isoforms[20, 44]. Approximately 18 CYP4F isoforms have been identified in rats (4F1, 4F4, 4F5, and 4F6)[45], mice (4f13, 4f14, 4f15, 4f16, 4f17, 4f37, 4f39, and 4f40)[45], and humans (4F2, 4F3, 4F8, 4F11, 4F12,

Table 11.1. Metabolism of Fatty Acids and Prostanoids by Cytochrome P450 4A Isoforms

4A isoform	Species	Enzymatic activities[a]
4A1	Rat	ω-oxidation of laurate and arachidonate
4A2	Rat	ω/ω-1 oxidation of laurate and arachidonate
4A3	Rat	ω/ω-1 oxidation of laurate and arachidonate
4A8	Rat	ω/ω-1 oxidation of laurate and arachidonate
4A4	Rabbit	ω-oxidation of palmitate, arachidonate, and of prostaglandins A, E, D, and $F_{2\alpha}$
4A5	Rabbit	ω/ω-1 oxidation of laurate and palmitate, some ω-oxidation of PGA_1 and arachidonate
4A6	Rabbit	ω-oxidation of laurate, palmitate, and arachidonate. Low PGA_1 ω-oxidation
4A7	Rabbit	ω-oxidation of laurate, palmitate, arachidonate, and PGA_1, inactive toward PGE_2
4a10	Mouse	ω-oxidation of laurate
4a12	Mouse	ω/ω-1 oxidation of laurate and arachidonate
4a14	Mouse	ω/ω-1 oxidation of laurate
4A11	Human	ω/ω-1 oxidation of laurate and arachidonate
4a22	Human	Unknown

[a]Compiled from references [4]–[10], [17]–[20], [36]–[44], [46]–[52].

and 4F22)[45]. On the other hand, approximately 11 CYP4A isoforms have been cloned and/or isolated and purified from rats (4A1, 4A2, 4A3, and 4A8)[45], mouse (4a10, 4a12, and 4a14)[45], and rabbits (4A4, 4A5, 4A6, and 4A7)[45]. In stark contrast with the known multiplicity of rodent CYP4A and of rodent and human CYP4F isoforms, only two highly homologous CYP4A genes, *CYP4A11* and *CYP4A22*, have been identified in humans[45–48]. Most CYP4A enzymes that have been characterized enzymatically, are either inactive toward prostanoids or catalyze their ω- or ω/ω-1 hydroxylation at rates that are generally substantially lower than fatty acid hydroxylation (Table 11.1)[20, 36–44, 49–51]. A special case is that of rabbit lung CYP4A4, an isoform induced during pregnancy[20, 37, 42, 52] and active in the ω-hydroxylation of several prostanoids, including PGE_2 (Table 11.1)[20, 52]. As with AA, none of the CYP4A isoforms characterized to date is selective for the ω-1 carbon of prostanoids (Table 11.1)[20, 36–42, 49–52]. A more specific role for CYP4F isoforms as predominantly prostanoid and eicosanoid ω/ω-1 hydroxylases has emerged during the last few years[20]. The cDNA coding for CYP4F8 was cloned from human seminal vesicles, and the recombinant protein was shown to catalyze the ω-1 hydroxylation of PGH_2, the precursor of all prostanoids[43]. Based on its catalytic activity, and its high levels of expression in the seminal vesicles it was proposed that CYP4F8 is involved in the formation of 19-hydroxy-PGE_2, present at high concentrations in human seminal fluid[34, 43]. CYP4F12 is a regioselective AA ω-3 hydroxylase[53] but, it is also active in the hydroxylation of prostanoids and several prostanoid analogs[53].

2.2.2. ω/ω-1 Oxidation of Leukotrienes and Other Eicosanoids

The ω-oxidation of leukotriene B_4 (LTB_4), an important proinflammatory mediator[54, 55], has been documented in whole animals, isolated cells, and subcellular fractions[20, 56–60], and shown to be catalyzed by a unique P450 isoform, distinct from those involved in fatty acid and prostanoid metabolism[20, 56–60]. Soon after, the cDNA coding for CYP4F3 was cloned and expressed, and recombinant CYP4F3 shown to catalyze the ω-oxidation of LTB_4 with a K_m of 0.71 μM[61, 62]. A role for

CYP4F3 as an endogenous LTB_4 hydroxylase is supported by its selective expression in human polymorphonuclear leukocytes, and its lack of activity toward fatty acids such lauric, palmitic, and AAs[20, 61, 62]. CYP4F3 also supports the ω-hydroxylation of lipoxygenase metabolites such as lipoxins A and B, and of 5- and 12-HETE[62]. A splice variant of CYP4F3, CYP4F3B, is expressed in liver and kidney, and shows significant structural and functional similarities to CYP4F2[63]. Human CYP4F2 and rat 4F1 are active LTB_4 ω-hydroxylases capable of HETE ω-hydroxylation[20, 62, 64]. CYP4F2 is expressed in human liver and kidney, and responsible for most of the hepatic hydroxylation of LTB_4[65]. Four members of the rat 4F gene subfamily (CYPs 4F1, 4F4, 4F5, and 4F6) have been cloned[45]. Recombinant CYPs 4F1, 4F4, and 4F5 catalyze the ω-hydroxylation of LTB_4, and CYP4F1 also metabolizes lipoxins and HETEs[64, 66]. The ω-oxidation of 12(*S*)-HETE by polymorphonuclear leukocytes was demonstrated in 1984 by Wong *et al.*[67] and Marcus *et al.*[68]. Moreover, the latter authors further showed that endogenous AA pools are converted to 12,20-dihydroxyeicosatetraenoic acid by a co-incubated mixture of human platelets and polymorphonuclear leukocytes, thus providing one of the first examples of intercellular eicosanoid metabolism[68]. Both 5- and 15-HETE are known to undergo ω-oxidation by P450[69, 70].

3. Metabolism of Arachidonic Acid: The Arachidonic Acid Monooxygenase

As with the other enzymes of the AA metabolic cascade, P450 metabolizes only free, nonesterified forms of AA and thus, *in vivo* metabolism requires the release of the fatty acid from selected glycerophospholipid pools. CYP P450, prostaglandin H_2 synthase, and lipoxygenases are capable of metabolizing polyunsaturated fatty acids other than AA, however, it is the unique nature of the AA containing phospholipids, and the control of its release by hormonally sensitive phospholipases that makes the oxidative metabolism of AA distinctive, and functionally important. Under conditions favoring primary metabolism, the P450 AA monooxygenase oxidizes AA by one or more of the following of reactions: (a) *bis-allylic oxidation (lipoxygenase-like reaction)* to generate any of six regioisomeric HETEs containing a *cis,trans*-conjugated dienol functionality (5-, 8-, 9-, 11-, 12-, and 15-HETEs) (Figure 11.1), (b) *Hydroxylations at or near the terminal sp^3 carbon (AA ω/ω-1 hydroxylase)* affording 16-, 17-, 18-, 19-, and 20-HETEs (16-, 17-, 18-, 19-, and 20-HETE) (ω, ω-1, ω-2, ω-3, and ω-4 alcohols) (Figure 11.1), and (c) *Olefin epoxidation (AA epoxygenase)* furnishing four regioisomeric EETs

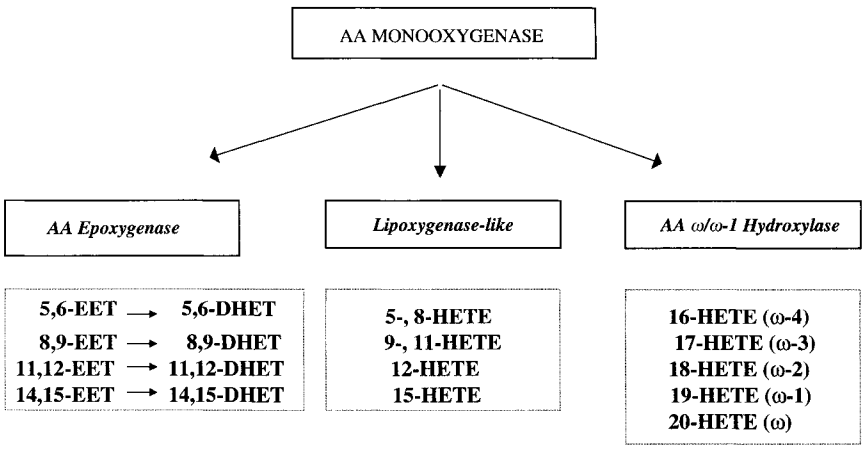

Figure 11.1. The cytochrome P450 arachidonic acid monooxygenases.

(5,6-, 8,9-, 11,12-, and 14,15-EETs) (Figure 11.1). The classification of P450-derived eicosanoids in Figure 11.1 continues to provide a rational and useful framework for most of the studies of this branch of AA metabolic cascade[4, 5].

The chemistry of the P450-derived eicosanoids is highly dependent on the tissue source of enzymes, animal species, sex, age, hormonal status, diet, and exposure to xenobiotics[4, 5]. For example, EETs are the predominant products generated by most liver microsomal fractions (≥70% of total products), while most kidney microsomal fractions generate mainly a mixture of 19- and 20-HETE (77% of total products)[5]. Studies with microsomal, purified, and/or recombinant forms of rat, rabbit, and human P450s[4–10] showed that the hemoprotein controls, in an isoform-specific fashion, oxygen insertion into the fatty acid template at three levels: (a) *type of reaction*, that is, olefin epoxidation, bis-allylic oxidation, or hydroxylations at the C_{16}–C_{20} sp^3 carbons, (b) *regioselectivity of oxygen insertion*, that is, epoxidation at either of the four olefin bonds, allylic oxidation initiated at any of the three bis-allylic methylene, or hydroxylation at C_{16}–C_{20} (ref. [37]), and (c) the *enantiofacial selectivity* of oxygenation leading to chiral products. The broad structural, functional, regulatory, and catalytic redundancy displayed by many P450 isoforms, as well as their often overlapping patterns of tissue expression, continues to complicate the task of assigning catalytic roles to a P450 or to a group of P450 isoforms. This is of current interest because many of the P450 eicosanoids are biologically active[6–10], and the identification and characterization of P450 isoforms involved in the *in vivo* metabolism of AA is needed for accurate molecular descriptions of their mechanism of action, regulatory control, and ultimately, physiological significance. For example, several CYP 2B, 2C, 2D, 2E, and 2J proteins have been shown to catalyze the *in vitro* oxidation of AA to hydroxy- and/or epoxy-acids[71–87]; however, their participation in metabolism of the endogenous fatty acid pools remains unclear[5, 86]. In a few cases, a role for individual 2 gene family isoforms in endogenous AA bioactivation has been suggested based on enzymatic and/or immunological evidence[4–7, 86, 87]. Nevertheless, the identities of the P450 isoforms responsible for organ-specific AA metabolism remains preliminary (and many cases speculative) and these unresolved issues continue to offer a major challenge for this area of research.

3.1. bis-Allylic Oxidation (Lipoxygenase-Like Reactions)

The products of these reactions are structurally similar to those of plant and mammalian lipoxygenases, and yet there is no evidence that hydroperoxide intermediates are formed during the P450-catalyzed reactions[4, 5, 88, 89]. A mechanism for P450-dependent HETE formation involving bis-allylic oxidation at either C7, C10, or C13, followed by acid-catalyzed rearrangement to the corresponding *cis-trans* dienols was proposed, and the intermediate 7-, 10-, and 13-HETEs isolated[88, 89]. Since 12(*R*)-HETE is the predominant enantiomer generated by a P450-catalyzed reaction[90], it was thought that all the mammalian 12(*R*)-HETE was a product of the P450 enzyme system[91]. However, the cloning and characterization of mammalian 12(*R*)-lipoxygenases has led to a reevaluation of the role of P450s in 12(*R*)-HETE biosynthesis[92, 93]. The formation of 12(*S*)- and 12(*R*)-HETE by P450-independent and -dependent pathways in bovine cornea epithelium has been reported[94, 95]. Importantly, *in vitro* studies showed that 12(*R*)-HETE is a powerful and enantioselective inhibitor of Na^+/K^+ ATPase[94]. Finally, the enzymatic formation of 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid (12(*R*)-HETrE), an ocular proinflammatory and vasodilatory substance in rabbits, has been described[96]. Areas in need of clarification are: (a) the role of P450 in the biosynthesis of endogenous HETE and HETrE pools, (b) the identity and molecular properties of the P450 isoforms responsible for these reactions, and (c) the contributions of P450 and 12-lipoxygenases to organ-specific 12(*R*)-HETE and 12-HETrE biosynthesis.

3.2. Hydroxylation at C_{16}–C_{20} (ω/ω-1 Hydroxylase Reactions)

3.2.1. Introduction

The hydroxylation of saturated medium-chain fatty acids at their ultimate and penultimate carbons

was one of the first enzymatic activities attributed to microsomal P450s[97]. In general, medium-chain saturated fatty acids (C_{12}–C_{16}) are far better substrates for the microsomal ω/ω-1 hydroxylases than AA[16, 17, 20, 97], and reaction rates decrease as the substrate carbon-chain length increases from C12 to C18. For example, lauric acid, a fatty acid absent from most mammalian tissues, is metabolized by the microsomal ω/ω-1 hydroxylases or by purified CYP4A isoforms at rates significantly higher than AA[33, 50, 51]. Common oxygen chemistries and reaction mechanisms for these reactions are suggested by the fact that, regardless of the carbon length of the fatty acid or its degree of saturation, the ω/ω-1 hydroxylases deliver a reactive form of oxygen to ground state, sp^3 carbons. However, the unequal chemical reactivities of the carbon atoms in the AA molecular template impose additional steric requirements on the P450 catalyst. Hydroxylation at the thermodynamically less reactive C_{16} through C_{20} rather than at the chemically comparable C_2 through C_4 indicates a rigid and highly structured binding site for the AA molecule. This binding site must position the acceptor carbon atom(s) in optimal proximity to the heme-bound active oxygen, with complete segregation of the AA-reactive olefins and bis-allylic methylene carbons. Studies with CYP102 (P450BM3), a high turnover bacterial AA hydroxylase of known atomic structure[98, 99], suggested a rigid active-site binding geometry for AA, and indicated that the regiochemistry of P450 oxygen insertion was determined by the fatty acid binding coordinates, and not by chemical properties of the acceptor carbon or the heme-bound active oxygen species[98, 99]. Thus, X-ray crystallography, molecular modeling, site-specific mutational analysis, as well as enzymatic studies indicate that the "substrate access channel" in CYP BM-3, holds the AA molecule in a rigid orientation that: (a) precludes significant rotation and/or displacement along the channel's longitudinal axis, and (b) shields the heme-bound oxidant from non-acceptor carbons[98, 99].

3.2.2. Enzymology, Isoform Specificity

AA ω/ω-1 hydroxylation has been observed in microsomal fractions from several organs, including liver, kidney, brain, lung, intestine, olfactory epithelium, and anterior pituitaries[4–10]. However, it is in renal tissues that these reactions are best characterized, most prevalent, and have been assigned their most important functional roles[4–10]. Extensive biological, enzymatic, and molecular evidence shows that the CYP4A isoforms are the predominant, and functionally relevant, AA ω/ω-1 hydroxylases in the mammalian kidney[4–10, 100]. The *CYP4A* gene subfamily encodes a group of structurally and functionally conserved proteins that are specialized for fatty acid oxidation and that show little or no activity toward xenobiotics[16, 20]. The expression of the CYP4A fatty acid hydroxylases is regulated by a variety of physiological and pathophysiological effectors such as age, sex hormones, dietary lipids, fasting, starvation, mineralocorticoids, insulin, diabetes, and hypertension[4–10, 100–108]. Moreover, the sexual dimorphic, androgen sensitive, expression of rat kidney CYPs 4A2 and 4A8, and of mouse kidney Cyp4a12 have been demonstrated[100, 104, 109, 110].

In rats and rabbits, the 4A gene subfamily is composed of four highly homologous genes[45]. Amino acid sequence analysis showed that the rat 4A proteins could be divided into two groups that share ≥71% overall homology (Figure 11.2)[45]. CYPs 4A1 and 4A8 (76% sequence identity) constitute one group, and the other is composed of the highly homologous CYPs 4A2 and 4A3 (98% sequence identity) (Figure 11.2)[45]. The high level of nucleotide sequence identity shared by the CYPs 4A2 and 4A3 genes extends into their intronic areas, suggesting that they arose from a relatively recent gene duplication event[45]. The three characterized murine Cyp4a genes are localized in a ~200 kb segment of chromosome 4

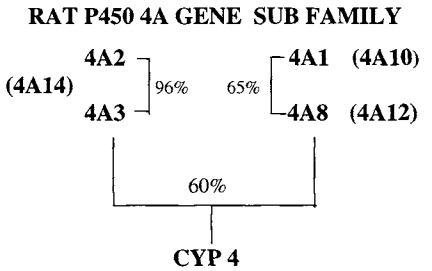

Figure 11.2. Nucleotide sequence identity between rat and murine CYP4A isoforms.

(ref. [110]). Cyps 4a10 and 4a12 are the murine homologs of rat CYPs 4A10 and 4A8, respectively (Figure 11.2)[45]. The presence of a single murine gene (CYP4a14) highly homologous to both rat CYPs 4A2 and 4A3 indicates that the 4A2/4A3 gene duplication event occurred after the evolutionary separation of rat and mouse (Figure 11.2). Southern analysis and the human genome database show that *CYPs 4A11* and *4A22* are likely to be the only members of the *CYP4A* gene subfamily in humans[46–48, 108]. These two genes share 96% nucleotide sequence identity, contain 12 exons, and have similar intron/exon distributions[48]. The cDNA coding for CYP4A11 has been cloned, expressed, and characterized as an active renal fatty acid ω-hydroxylase[44, 46, 47, 51]. On the other hand, the enzymatic activity of CYP4A22 is unknown, and its mRNA is expressed in kidney at levels that can be detected only after RT-PCR amplification[48].

Table 11.1 summarizes the published fatty acid metabolic properties for several purified and recombinant CYP4A isoforms. All enzymatically characterized CYP4A proteins (either purified or recombinant proteins) catalyze saturated fatty acid ω-oxidation and most also hydroxylate AA at either the C_{20}, or the C_{19} and C_{20} carbon atoms[16–19, 38–42, 44, 46, 47, 49 52]. To date, none of them have been shown to be selective for fatty acid ω-1 hydroxylation. Despite their high structural homology, CYP4A2 metabolizes AA while CYP4A3 is either inactive[50], or reacts at very low rates[49, 51]. Both CYP 4A2 and 4A3 are, on the other hand, active lauric acid ω/ω-1 hydroxylases[50, 51]. A microsomal form of recombinant rat CYP4A2 was shown to oxidize AA to 20-HETE and 11,12-EET[49]. In contrast, two different laboratories showed that purified recombinant CYP4A2 oxidizes AA to only 19- and 20-HETE[50, 51]. All three murine Cyp4a proteins are active lauric acid hydroxylases, but only Cyp4a12 catalyzes AA ω/ω-1 hydroxylation, providing an explanation for the low levels of 20-HETE synthase activity present in microsomes isolated from the kidneys of female *129SvJ* mice[100]. An unresolved issue is that of the relative roles played by CYPs 4A11 and 4F2 in the biosynthesis of 20-HETE by human kidney. As discussed, recombinant CYP4F2 is an active LTB_4 hydroxylase[20, 65], and it has been reported to be active toward AA ω-hydroxylation[20, 44]. On the other hand, kinetic and immunological evidence suggested that both CYPs 4A11 and 4F2 may contribute to the biosynthesis of 20-HETE by human kidney microsomes and nephron segments[44].

The liver microsomal P450 hydroxylation of AA at C_{16}, C_{17}, C_{18}, and C_{19}, but not at C_{20}, was induced after treatment of the animals with α-naphthoflavone or dioxin[111, 112]. Reconstitution experiments using purified liver CYPs 1A1 and 1A2, the major liver P450 isoforms induced by these chemicals, demonstrated that CYPs 1A1 and 1A2 were more or less regioselective for oxidations at the AA C_{16}–C_{19} carbons (87% and 44% of total products for CYP1A1 and 1A2, respectively)[111]. Furthermore, while CYP1A1 oxidized AA preferentially at C_{19}, oxygenation by CYP1A2 occurred predominantly at C_{16} (ref. [111]). It is of interest that despite a very limited sequence homology, CYPs 1A and 4A show distinct regioselectivities for the adjacent C_{19} and C_{20} carbons of AA. Purified CYP2E1, an isoform induced in rat liver by diabetes, fasting, and alcohol, converts AA stereoselectively to 19(*S*)- and 18(*R*)-HETE as its major reaction products[85]. Finally, members of the 2J gene subfamily are also active AA ω-1 hydroxylases[86, 87] and, recently, Cyp 2j9, an isoform expressed in mouse brain was cloned, expressed and shown to be a regioselective AA ω-1 hydroxylase[113].

The potent biological activities attributed to the products of the AA ω/ω-1 hydroxylases have stimulated an intense search for the physiological and/or pathophysiological roles of these reactions[4–10]. Among these, 20-HETE[4–10] has been characterized as: (a) a powerful vasoconstrictor of the renal and cerebral microcirculations, (b) an inhibitor of vascular calcium-dependent K channels, (c) a regulator of Na^+/K^+ ATPase activity, and (d) a modulator of Ca^{++} and Cl^- fluxes. Furthermore, analysis of the segmental distribution of CYP 4A isoforms along the rat nephron is consistent with many of the proposed renal actions of 19- and 20-HETE[114]. A role for 20-HETE as a powerful mitogen in cultured kidney epithelial cells, as well as in vasopressin, parathyroid hormone and norepinephrine signaling has been described[6–10]. Of importance during analyses of the functional significance of the AA ω/ω-1 hydroxylases is the recognition that, as discussed, many P450 fatty acid ω/ω-1 hydroxylases also play important roles in the metabolism of

bioactive eicosanoids such as prostanoids and leukotrienes.

The expression of several CYP4A isoforms is under transcriptional control by the nuclear $PPAR_{\alpha}$[105]. The coordinated, $PPAR_{\alpha}$-controlled, induction of peroxisomal fatty acid β-oxidation and microsomal ω/ω-1 hydroxylation, has suggested a role for CYP4A isoforms in hepatic lipolysis and fatty acid homeostasis, and a role for these isoforms in PPARα-signaling has been advanced based on gene knockout studies[115]. The potential for an involvement of CYP4A isoforms in fatty acid and lipid homeostasis is opening new opportunities for an understanding of the physiological roles of these enzymes, *vis-a-vis* their recognized functional roles as AA hydroxylases.

3.3. Olefin Epoxidation (Epoxygenase Reactions)

3.3.1. Introduction

The demonstration of NADPH-dependent metabolism of AA to 11,12- and 14,15-dihydroxyeicosatrienoic acids (DHETs) by microsomal incubates indicated a role for P450 in AA epoxidation[13]. Soon after, 5,6-, 8,9-, 11,12-, and 14,15-EET were isolated and shown to be products of the P450-dependent metabolism of AA[116]. In mammals, the epoxidation of polyunsaturated fatty acids to nonallylic, *cis*-epoxides is unique to the P450 enzyme system and, in contrast with fatty acid ω/ω-1 hydroxylation, is more or less selective for AA[4, 5]. Thus, while the enzymatic or nonenzymatic reduction and/or isomerization of polyunsaturated fatty acid hydroperoxides can yield epoxides or epoxy-alcohol derivatives, these products are structurally different from those generated by the P450 enzymes[3, 22]. The EETs are bis-allylic epoxides and as such, remarkably resistant to attack by nucleophiles such as water and glutathione (GSH). However, in most cells and organ tissues, the EETs are metabolically unstable and are rapidly esterified to glycerophospholipids, degraded by fatty acid β-oxidation pathways[117, 118], conjugated to GSH[120], and/or hydrated and excreted[119]. Cytosolic epoxide hydrolase and GSH-transferases catalyze the enzymatic conversion of EETs to the corresponding *vic*-DHETs (Figure 11.2)[119], and GSH-conjugates[120], respectively. The biological role(s) and *in vivo* significance of EET hydration or GSH conjugation remain mostly unexplored, although recently a role for cytosolic epoxide hydrolase in the regulation of antihypertensive EETs levels has been proposed[121, 122]. The catalysis of AA epoxidation, or the presence of endogenous EET pools has been demonstrated using microsomal fractions or samples obtained from numerous tissues, including liver, kidney, lung, skin, pituitary, brain, adrenal, endothelium, and ovaries[4–10].

3.3.2. Enzymology, Isoform Specificity

The isoform multiplicity of the AA epoxygenase was first suggested by changes in the regio- and stereoselectivity of the microsomal enzymes, resulting from animal treatment with known P450 inducers[71]. For example, animal treatment with phenobarbital inverted the overall enantiofacial selectivity of the rat liver microsomal AA epoxygenases (Table 11.2), and caused marked increases in the organ levels of endogenous of 8(*S*),9(*R*)-EET, an effect likely due to the induction of CYPs 2B1 and 2B2, both of which are highly stereoselective 8(*S*),9(*R*)-epoxygenases[71]. These studies showed that the regio- and stereochemical selectivity of the microsomal AA epoxygenase was under regulatory control and could be altered, *in vivo*, by animal manipulation[4, 5, 71]. Subsequently, it was demonstrated that arachidonate epoxidation was highly asymmetric and that P450s control, in an

Table 11.2. Effect of Phenobarbital Treatment on the Enantioselectivity of the Liver Microsomal Arachidonic Acid Epoxygenase

EET enantiomer (% distribution)	Liver microsomes	
	Control	Phenobarbital
8(*S*),9(*R*)-EET	32 ± 2	78 ± 2
8(*R*),9(*S*)-EET	68 ± 2	22 ± 2
11(*S*),12(*R*)-EET	19 ± 1	83 ± 2
11(*R*),12(*S*)-EET	81 ± 1	17 ± 2
14(*S*),15(*R*)-EET	67 ± 2	25 ± 3
14(*R*),9(*S*)-EET	33 ± 2	75 ± 3

Microsomes were isolated from the livers of control- and phenobarbital-treated rats (10 days; 0.05% w/v phenobarbital in the drinking water). Values are averages ± SE calculated seven different experiments. See ref. [71] for experimental details.

isoform-specific fashion, the regio- and enantioselectivities of the reaction[4, 5, 71, 73–77, 79]. These properties of the AA epoxygenase are in contrast with those of prostaglandin H_2 synthases, where the known isoforms of the enzyme oxidize AA to the same single product[1, 2].

Another distinctive feature of the AA epoxygenase pathway is the ability of a single P450 isoform to epoxidize, stereoselectively, multiple olefins of the AA template. For example, purified recombinant rat kidney CYP2C23 generates 11,12-EET as its major reaction product (58% of total) but, it is also an efficient AA 8,9-, and 14,15-epoxygenase[77]. Despite this limited regioselectivity, CYP2C23 is highly stereoselective and forms the corresponding 8(*R*),9(*S*)-, 11(*R*),12(*S*)-, and 14(*S*), 15(*R*)-EETs enantiomers with optical purities of 94%, 89%, and 75%, respectively[77]. On the other hand, the other two 2C AA epoxygenases expressed in rat kidney, CYPs 2C11 and 2C24, show moderate regioselectivity for the 11,12- and 14,15-olefins[123], and epoxidized the 8,9- and 14,15-olefins with opposing enantiofacial selectivities[123]. Extensive studies with a variety of organ purified and/or recombinant epoxygenases, including several CYP 2B and 2C isoforms, showed that: (a) with the exception of rat CYP2B12, which generates 11,12-EET as nearly the only reaction product[74], most do not catalyze the selective epoxidation of a single AA olefin to the exclusion of the other three[4, 5], and (b) most mammalian P450 isoforms preferentially epoxidize the 11,12- and 14,15-double bonds[4, 5]. The role that single amino acid residues play in the regio- and stereochemical selectivity of AA epoxidation was revealed by replacements introduced into rat and bacterial P450s 2B1 and BM3, respectively[73, 99]. Recombinant CYP2B1 metabolizes AA to predominantly 11,12- and 14,15-EET[73]. Replacement of isoleucine 114 for alanine in CYP2B1, changed its regioselectivity toward the preferential epoxidation of the AA 5,6- and 8,9-olefins[73]. On the other hand, a single active-site replacement, phenylalanine 78 for valine, changed P450 BM3 from a predominantly AA 18(*R*)-hydroxylase into a regio- and enantioselective 14(*R*),15(*S*)-epoxygenase (14(*R*), 15(*S*)-EET, ≥98% of total products)[99]. These studies indicate that the outcome of the reactions catalyzed by many of these highly homologous P450 isoforms is determined by a few amino acid residues, strategically located within the confinements of what, in most other cases, is known to be a rather promiscuous active-site cavity.

Reconstitution experiments using purified P450 isoforms and/or recombinant proteins show that most AA epoxygenases belong to the CYP 2 gene family[4–10]. The CYP2B and 2C subfamily isoforms identified so far as epoxygenases include rat 2B1, 2B2, 2B12, 2C11, 2C23, and 2C24; rabbit 2B4, 2C1, and 2C2; mouse 2b19, 2c37, 2c38, 2c39, and 2c40; and human 2C8, 2C9/2C10, 2C18, and 2C19 (refs [4]–[10], [71]–[83], [123]). On the other hand, CYPs 2J2 and 2J4 have also been identified as organ-specific epoxygenases and ω-1 hydroxylases[86, 87]. CYPs 1A1, 1A2, and 2E1 are active AA ω/ω-1 oxygenases, that also produce low and variable amounts of EETs (≤20% of total products)[71, 80, 85]. A P450 purified from the livers of dioxin-treated chick embryos has structural features typical of proteins of the 1A gene subfamily, but metabolizes AA to EETs as the major reaction products[124]. Recent studies characterized 11,12-EET as an "endothelium-derived hyperpolarizing factor" (EDHF)[125] and CYP2C34, the porcine homolog of human CYPs 2C8 and 2C9, as a coronary artery EDHF synthase[126]. While members of the CYP2C gene subfamily share extensive sequence homology, this structural homology is often accompanied by significant catalytic heterogeneity[4, 5]. For example, CYPs 2C8 and 2C9 proteins are ~90% homologous in their amino acid sequences, yet recombinant CYPs 2C8 and 2C9 epoxidize AA with distinct regio- and stereochemical selectivities[81].

Comparisons of the regio- and enantioselectivity of the microsomal epoxygenases with that of purified recombinant P450 isoforms, as well as antibody inhibition experiments indicate that CYPs 2C11 and 2C9, and 2C23 and 2C8 are the major AA epoxygenases in rat and human liver and kidney, respectively[5, 77, 81, 123]. Thus, for example, of the three major 2C epoxygenases expressed in the rat kidney, CYPs 2C11, 2C23, and 2C24 (ref. [123]), only CYP2C23 mimics the regio- and stereochemical selectivity of the microsomal enzymes[77, 123]. CYP2C23 was shown to be abundantly expressed in rat kidney, and anti-P450 2C23 antibodies were selective inhibitors of the renal microsomal epoxygenase[5, 123]. Furthermore, with the exception of CYPs 2C23 and 2C11, none of the members of the CYP2 gene family expressed in kidney, including 2A, 2B, 2C, 2E, and 2J isoforms, can account for the degree of regio- and stereoselectivity displayed by the rat

renal microsomal epoxygenase[77–123]. Sequence comparisons show that the degree of homology between CYP2C23 and the remaining 2C rat proteins is limited, indicating its early evolutionary divergence from the other CYP2C proteins. The regulation of renal CYP2C23 levels by dietary salt intake[123], and its hormonally controlled expression in the renal microcirculation[127, 128] has suggested important roles for this enzyme in kidney physiology. The application of recombinant DNA methods and heterologous protein expression should continue to facilitate unequivocal assignments of regio- and enantioselectivities as well as epoxygenase activities to individual P450 isoforms. As more of these recombinant isoforms become available, they will be useful in defining their: (a) contribution to the epoxidation of endogenous AA pools, (b) tissue and/or organ-specific distribution, and (c) regulation by physiologically meaningful stimuli.

Several powerful *in vitro* biological activities have been described for the products of the AA epoxygenases. The EETs[4–10] have been described as: (a) mediators for the release of several peptide hormones, (b) inhibitors of Na^+ reabsorption in the distal nephron, (c) vasodilators in several microvascular beds and activators of Ca^{++}-dependent vascular K^+ channels, mediators of Ca^{++} influx in several isolated cell systems, powerful mitogens, and mediators of EGF and Angiotensin II signaling.

3.3.3. P450 Arachidonic Acid Epoxygenase: A Member of the Endogenous Arachidonic Acid Metabolic Cascade

In view of their known catalytic versatility, the *in vitro* catalysis of AA epoxidation by microsomal P450s was not completely unexpected. It was therefore apparent that the uniqueness and significance of the P450 AA epoxygenase reaction was going to be defined by whether or not the enzyme system participated in the *in vivo* metabolism of the fatty acid. Since asymmetric synthesis is an accepted requirement for the biosynthetic origin of most eicosanoids, the demonstration of chiral EET pools in several rat and human organs and plasma proved their enzymatic origin and established the AA epoxygenase as a formal metabolic pathway and a member of the AA cascade[4, 5, 15]. Moreover, the analysis of the effects of known P450 inducers on the levels and stereochemistries of endogenous EETs confirmed the role of P450 in the *in vivo* epoxidation of AA[15]. These experiments documented a new metabolic function for the P450 enzyme system in the oxidation and bioactivation of endogenous fatty acids such as AA, and demonstrated that the tissue levels and chemical properties of the endogenous EETs reflect the organ biosynthetic capacity, as well the contribution of tissue-specific regio- and stereoselective EET metabolism[4, 5, 117–119]. The presence of endogenous chiral EETs has been shown in rat liver, lung, kidney, brain, plasma, and urine; in rabbit lung, kidney, and urine; and in human liver, kidney, lung, brain, plasma, and urine[4–10].

A distinctive feature of endogenous EET pools in rat liver and kidney is their presence as esters of several glycerophospholipids (~99% of the total liver EETs)[117] with 55% of the total liver EETs in phosphatidylcholine, 32% in phosphatidyl-ethanolamine, and 12% in phosphatidylinositols[117]. Chiral analysis of the fatty acids at *sn-2* revealed an enantioselective preference for 8(*S*),9(*R*)-, 11(*S*), 12(*R*)-, and 14(*R*), 15(*S*)-epoxyeicosatrienoates in all three classes of phospholipids[117]. EET-phospholipid formation involves a multistep process, initiated by the P450 enantioselective epoxidation of AA, ATP-dependent activation, and enantiomer-selective lysophospholipid acylation[117]. This EET *in vivo* esterification process is unique since most endogenously formed eicosanoids are either secreted, excreted, or further oxidized. Furthermore, these studies also show, in contrast to other classes of eicosanoids, the potential for the rapid, hormonally controlled, generation of preformed bioactive EETs via hydrolytic reactions, thus obviating the need for AA oxidative metabolism. The asymmetric nature of the esterified EETs established the existence of novel oxidized glycerolipid pools and demonstrated their enzymatic synthesis from endogenous precursors and under normal physiological conditions[117]. Greater than 90% of the circulating EETs in rat and human plasma were also found esterified to the phospholipids present in the VLDL, LDL, and HDL lipoprotein fractions[129].

The biosynthesis of endogenous phospholipids containing esterified EET moieties in several human, rat, and rabbit organs suggested a new and potentially important functional role for

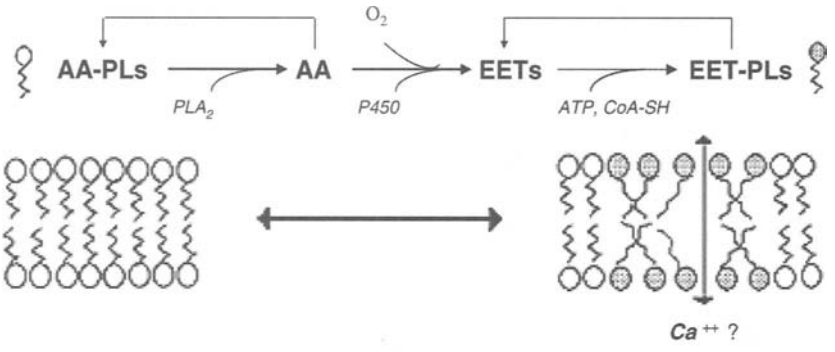

Figure 11.3. Regulation of membrane microenvironments by P450 epoxygenase metabolites.

P450 in membrane biology. A few years ago, based on studies of the capacity of synthetic epoxyeicosatrienoyl-phosphocholines to alter the Ca^{++} permeability of synthetic liposomes, we proposed that microsomal P450s could participate in the real-time control of cellular membrane microenvironments and, hence their functional properties (Figure 11.3)[5, 130]. This proposal envisioned as an initial step, the phospholipase-catalyzed release of AA from membrane phospholipids, followed by sequential P450-dependent epoxidation, EET activation to the corresponding acyl-CoA thioesters, and enzymatic lysophospholipid acylation to generate the EET-containing phospholipid pools present in many mammalian organs. The process could then be terminated by phospholipase-A_2-mediated EET release and enzymatic hydration to the corresponding DHETs. Under conditions favorable for EET acylation we were unable to show the acylation of lysolipids by DHETs[117]. Many of the enzymatic steps described above have been characterized *in vitro* using either purified proteins or microsomal membranes[5]. Inasmuch as it is well documented that oxidation of the fatty acid moieties present in membrane-bound phospholipids has profound effects on membrane structural properties, fluidity, and permeability, the formation and incorporation of EETs into cellular lipids may provide the molecular basis underlying some of the EET biological properties, many of which can be attributed to their ability to alter the physicochemical properties of cellular membranes[6–10].

3.4. Functional Roles of the P450 Arachidonic Acid Monooxygenase

Recent reviews provide excellent and detailed descriptions of the biological roles attributed to the products and enzymes of the AA monooxygenase[6–10]. We address here what we considered to be the two most relevant issues, and for which a more or less generalized consensus exists regarding their potential physiological importance.

3.4.1. Vascular Reactivity; Ion channel regulation

Because of their physiological and pathophysiological implications, the vasoactive properties of EETs and 20-HETE are under extensive scrutiny. The consensus is that EETs (5,6-and 11,12-EET in particular) and 20-HETE are powerful vasodilators and vasoconstrictors of small diameter vascular beds, respectively[6–10], and that these actions are associated with their ability to inactivate (20-HETE) or activate (EETs) Ca^{++}-activated vascular smooth muscle K^+ channels[6–10, 125, 131]. It has been proposed that an EET-mediated hyperpolarization event is required to complete the vasodilatory response of vascular smooth muscle cells to hormones such as, for example, bradykinin[8, 9, 127, 128]. The identification of 11,12-EET as an EDHF[9, 125, 126], and of 20-HETE as an anti-EDHF molecule[7, 8, 10, 131] are supported by the demonstration of hormonally controlled 20-HETE and EET biosynthesis by isolated vascular smooth

muscle and endothelial cells, respectively[7–10, 125, 131]. By facilitating the introduction of molecular and mechanistic approaches to the characterization of the renal and vascular roles of the P450 eicosanoids, these studies are generating conceptually a coherent and mechanistic understanding of their ion transport and vasoactive properties[4–10]. The accumulating evidence for a role of microsomal P450s in vascular biology is creating new and important avenues for research, and generating new concepts and experimental approaches for the study of cardiovascular and renal physiology.

3.4.2. Blood Pressure Control and Hypertension

An important contribution to the studies of the functional significance of the AA monooxygenase was the proposal of a role for renal P450s in the pathophysiology of experimental hypertension[6, 7]. In addition to its potential clinical relevance, animals models of genetically controlled hypertension afforded the opportunity to associate functional phenotypes with alterations in gene structure, function, and/or regulation. From integrations of the renal responses to P450-eicosanoids, and correlations between their biosynthesis and the development of hypertension, pro- and antihypertensive roles were identified for the AAω/ω-1 hydroxylases and epoxygenases[6, 7]. For example, the developmental phase of hypertension, in the Spontaneously Hypertensive Rat model (SHR model), was shown to be accompanied by increases in renal CYP4A2 expression and 20-HETE synthase activity[6, 7]; and chemical[132] or antisense nucleotide[133] inhibition of renal 20-HETE biosynthesis or CYP4A expression, lowered the blood pressure of hypertensive SHR rats. Importantly, 20-HETE is a powerful vasoconstrictor of the renal microcirculation[6–10], may mediate the autoregulatory responses of renal afferent arterioles[6–10], is formed *in situ* by CYP4A isoforms[6–10, 114], and its vasoactive properties are consistent with its proposed prohypertensive role[6–10]. Dahl Salt Sensitive (DS) rats fed high salt diets become hypertensive while comparable Dahl Salt Resistant (DR) animals remain normotensive. An antihypertensive role for CYP4A2 and 20-HETE was proposed based on: (a) P450 inhibitor studies, and their effects on tubular Na^+ transport[10], (b) differences between DS and DR rats in CYP4A expression and 20-HETE synthase activity[10, 134], and (c) normalization of Cl^- transport in DS rats by 20-HETE[10, 135].

The inhibition of distal nephron Na^+ reabsorption by 5,6-EET[6–8, 14], the induction of kidney CYP2C23 and EET biosynthesis by excess dietary salt[76, 123], and EET-induced dilation of microcirculatory beds[6–10] suggested antihypertensive functions for the EETs[6, 7]. In agreement with this: (a) clotrimazole inhibition of the rat kidney epoxygenases caused reductions in renal EET biosynthesis[136], and the development of clotrimazole-dependent, salt-sensitive hypertension[136], (b) high salt diets failed to induce the activity of the kidney AA epoxygenase in hypertensive DS rats[136], and (c) the EETs contribute to the prostanoid- and NO-independent dilation of renal afferent arterioles[6–10, 127, 128]. In summary, the pro- and antihypertensive roles attributed to the ω-hydroxylase and epoxygenase eicosanoids can be rationalized in terms of their biological properties and their site of action[10]. In the renal tubule, EETs and 20-HETE block Na^+ transport and function as antihypertensive molecules[6–10]. In the renal vasculature, the EETs and 20-HETE have opposing activities as either powerful vasodilators (EETs) or a vasoconstrictor (20-HETE), and can act, therefore, as anti- or prohypertensive mediators, respectively[6–10].

Conclusive evidence for a role of P450s in renal and cardiovascular physiology was provided by the demonstration that the disruption of the murine Cyp4a14 gene caused a type of hypertension that, like most human hypertension, was sexually dimorphic, and more severe in males[100]. As shown in Figure 11.4, lack of a Cyp4a14 gene product(s) raises the mean arterial blood pressure of male Cyp4a14 (−/−) mice by 38 mm of Hg, respectively[100]. Hypertensive Cyp4a14 (−/−) mice show increases in plasma androgen levels[100], and in renal 20-HETE synthase activity (Figure 11.4)[100]. Castration reduced kidney microsomal 20-HETE biosynthesis, and normalized the blood pressure of hypertensive Cyp4a14 (−/−) mice (Figure 11.4), while, on the other hand, androgen administration raised systemic blood pressures and microsomal 20-HETE biosynthesis, regardless of the animal's genotype[100]. Northern

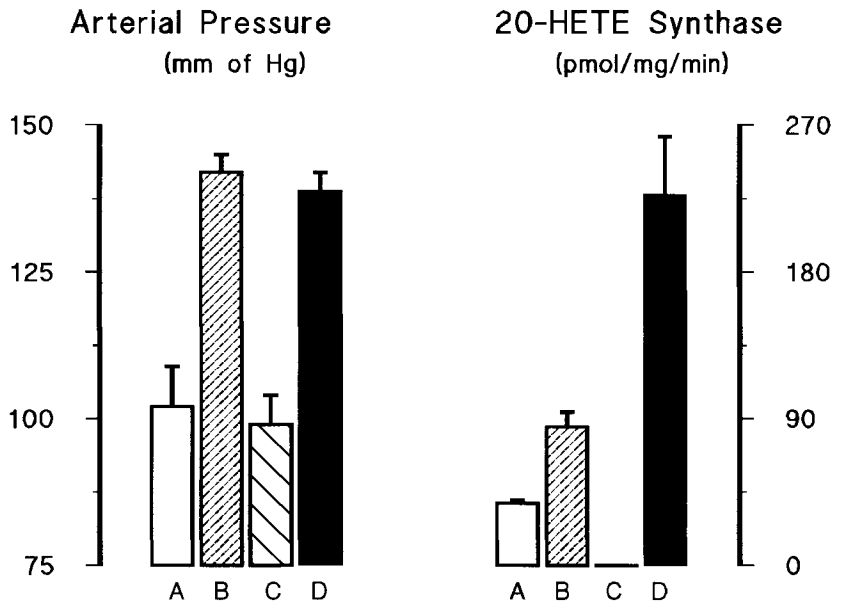

Figure 11.4. Genetically controlled or experimentally-induced alterations in the levels of plasma androgens levels are associated with changes in systemic blood pressure and renal 20-HETE synthase activity. The mean arterial blood pressure of conscious, wild-type, and Cyp4a14 (−/−) adult male mouse, was determined and described[100]. Androgens were administered by means of implanted 5α-dehydrotestosterone containing pellets (21-day release pellets; 5 mg/day; Innovative Research of America, Sarasota, FL). After 10 days of treatment, groups of animals were utilized for either pressure measurements, or the determination of kidney microsomal 20-HETE synthase activity. For more details see ref. [100]. A, wild-type; B, Cyp4a14 (−/−); C, castrated Cyp4a14 (−/−); D, castrated Cyp4a14 (−/−) mice, treated for 10 days with 5-α-dehydrotestosterone. Values show are averages ± SE, calculated from groups of at least 10 (Arterial Pressure) or 7 (20-HETE synthase) mice.

blot analyses showed that either disruption of the Cyp4a14 gene, or androgen administration caused the upregulation of Cyp4a12, an active, androgen sensitive, renal 20-HETE synthase[100], and provided a convenient explanation to paradoxical effects of Cyp4a14 disruption in renal AA ω-hydroxylase activity. Based on: (a) the known hemodynamic effects of 20-HETE[6–10], and (b) the increased renovascular resistance and impaired afferent arteriole autoregulatory capacity of Cyp4a14 (−/−) mice[100], it was proposed that androgen-mediated increases in the renal biosynthesis of vasoconstrictor 20-HETE were responsible for the hypertensive phenotype of Cyp4a14 (−/−) mice[100]. The prohypertensive effects of androgens were confirmed by administering 5-α-dehydrotestosterone to male or female Sprague–Dawley rats. In these animals, chronic treatment with 5-α-dehydrotestosterone raised the systolic blood pressure of male and female rats by 29 and 57 mm of Hg, respectively, and caused parallel increases in 20-HETE biosynthesis and in the kidney levels of CYP4A8 mRNAs[137].

The characterization of hypertensive Cyp4a14 knockout mice showed that the pressure effects of this gene were apparently independent of the intrinsic AA monooxygenase activity of its encoded protein but, rather were associated with changes in the regulation of alternate AA ω/ω-1 hydroxylases (Cyp4a12)[100]. Based on the known prohypertensive properties of 20-HETE[6–10], and the described gene-dependent, androgen-mediated, control of 20-HETE renal expression and activity, we concluded that blood pressure regulation by kidney CYP4A proteins involves a combination of transcriptional and hemodynamic mechanisms that determine the levels and site of expression of CYP 4A ω-hydroxylases and, ultimately, the level and site(s) of 20-HETE biosynthesis. Support for this conclusion was provided by the demonstration that

dissected rat renal microvessels, the target organ for most of the prohypertensive effects of 20-HETE, possess an androgen-regulated CYP4A8 20-HETE synthase[137].

The mechanism(s) by which Cyp4a14 gene product(s) control plasma androgen levels are yet to be defined; however, ample precedent supports a role for the kidney androgen receptor in regulating renal Cyp 4A and 2C expression[101–104]. Furthermore, in male and female rats, the androgen-mediated increases in systemic blood pressure, and in the levels of kidney CYP4A8 transcripts[137], are accompanied by a marked decrease in the levels of renal CYP2C23 epoxygenase protein and diminished microsomal EET biosynthesis[137]. The androgen-mediated counter-regulation of renal CYP4A ω-hydroxylases and 2C epoxygenases[101], suggests that its effects on blood pressure results from coordinated, nephron site-specific, increases in the biosynthesis of prohypertensive 20-HETE, and decreases in antihypertensive EET formation. Finally, in mice, activation of PPARα upregulates the expression of Cyps 4a10 and 4a14 (but not 4a12)[138], and the counter-regulation of rat Cyps 4A and 2C isoforms by PPARα ligands is published[139]. Nevertheless, the pressure effects of PPAR ligands in mice have yet to be fully defined, although, they have been characterized as antihypertensive in rats[10, 140, 141]. The studies summarized suggest that blood pressure regulation by renal P450s involves combinations of regulatory (transcriptional) and functional (tubular and hemodynamic) components, and that the organ balance of pro- and antihypertensive mediators and thus, its functional status, is dictated by: (a) the nephron segment-specific expression and regulation of the corresponding genes, (b) the enzymatic properties of the encoded proteins, and (c) the expression and activities of ancillary enzymes responsible for EET and/or HETE metabolism, disposition or activation.

4. Conclusion

The studies of the P450 AA monooxygenase have uncovered new and important roles for P450 in the metabolism of endogenous substrates, and added P450 to the list of enzymes that participate in the metabolism of AA, a fatty acid that serves as the precursor for the biosynthesis of several physiologically important lipid mediators. The functional relevance of this metabolic pathway is suggested by the many important biological activities attributed to its products. These studies, as well as the documented endogenous roles of P450s in cholesterol, steroid, and vitamin metabolism are contributing to establish this enzyme system as a major participant in the regulation of cell, organ, and body physiology. Among these, the phenotypic analysis of mice carrying disrupted copies of the CYP4a14 gene unveiled new and important roles for the P450 enzymes in cardiovascular physiology and the control of systemic blood pressures, and suggested the human homologs of the rodent CYP 2C and 4A AA epoxygenases and ω-hydroxylases as candidate genes for the study of their role in the pathophysiology of hypertension, and cardiovascular disease.

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12

Cytochrome P450s in Plants

Kirsten Annette Nielsen and Birger Lindberg Møller

1. Introduction

Plants are sessile organisms that cannot avoid exposure to adverse climatic conditions or attack from herbivores and pests by escaping. To survive and protect themselves, they are dependent on the ability to (a) redirect their overall metabolism to meet environmental constraints, (b) construct physical barriers that are difficult to penetrate, (c) produce chemicals that make the plant toxic to pests and herbivores, and (d) communicate with the environment, for example, to attract pollinators. Cytochrome P450 enzymes (P450s) play a key role in enabling plants to achieve these main goals.

1.1. Natural Products

Plants are the best organic chemists in nature as evidenced by their ability to synthesize all necessary carbon compounds with carbon dioxide as the sole carbon source and by their ability to synthesize a vast number of natural products. Currently, structures for more than 100,000 different natural products isolated from plants are known[1–3], and with time this number will increase into millions. Natural products are classified as phytoanticipins, phytoalexins, and/or attractants. In the last decade, the majority of the biosynthetic pathways responsible for natural product synthesis have been shown to include P450s as key enzymes. Such pathways include the biosynthetic pathways for cyanogenic glucosides, glucosinolates, isoflavonoids, and alkaloids. In addition, a number of plant P450s have been shown to catalyze detoxification of harmful agents including herbicides[4].

1.2. Chemical Warfare

The chemical warfare between plants and herbivores and pests is complex and takes place at many trophic levels. The plant *Apium graveolens* (celery) is known to combat *Helicoverpa zea* (corn earworm) by producing allelochemicals including furanocoumarins in a P450-dependent series of reactions[5]. The herbivore, however, is able to detoxify the furanocoumarins. The detoxification pathway is induced by jasmonate, a wound-induced plant signal compound. Jasmonate activates the transcription at least of four herbivore P450 genes[6]. The continuous chemical warfare between plants on one side and herbivores and pests on the other may enforce plants to constantly evolve new natural products and insects to find means to detoxify these. This requires recruitment of enzymes with altered biological functions probably mediated by modifications and duplications of existing genes. P450s are key players in securing recruitment of these new functions as exemplified by the recruitment of an allele specific P450 in Drosophila to acquire resistance to chemical insecticides[7].

1.3. Chemical Communication

Plants are dependent on intense communication with their surroundings via biochemical

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signalling, for example, to mediate pollination and seed spreading[1]. In cases with insect-mediated pollination, plants synthesize insect attractants. A special group of secondary metabolites, glucosinolates, is produced within the taxonomic order Capparales. Glucosinolates have dual roles acting both as attractants for specialized insects and as deterrents for generalist herbivores. P450s are key enzymes in the biosynthesis of glucosinolates[8, 9]. *Nicotiana tabacum* (tobacco) from the taxonomic order Solanes produces cembranoid-type terpenes as insect attractants. The cembranoid terpene gland exudates contain α- and β-epimers of cembra-2,7,11-triene-4,6-diol and these attract different pollinating insects. Unfortunately, they also enhance oviposition of the unwanted insect *Myzus nicotiana* (red aphid)[10].

1.4. Medicinal Agents

Humans take advantage of plant natural products as drugs or lead compounds in medicine, for example, as anesthetics and anticarcinogens. Alkaloid-containing plants have been used in human medicine for thousands of years. One very large and structurally diverse group of alkaloids are the tetrahydrobenzylisoquinoline alkaloids. *Papaver somniferum* (opium poppy) from the taxonomic order Ranunculales produces more than 100 such L-tyrosine derived alkaloids including the potent anesthetic morphine[11, 12]. The biosynthesis of tetrahydrobenzylisoquinoline alkaloids involves P450s with unique catalytic properties[13].

The nutraceuticals daidzein and genistein belong to the isoflavonoids and are phytoestrogens preventing breast and prostate cancers[14]. The dietary compounds are synthesized especially in Leguminosae belonging to the Fabales order. Isoflavonoid biosynthesis also requires a unique P450 enzyme that catalyzes aryl group migration[15]. Alkaloids and isoflavonoids play important roles in plant defense and their biosynthesis are tightly regulated and inducible processes[2, 3].

The purpose of this review is to highlight recent key findings on plant P450s. The genome sequencing programs have identified the P450s as the largest superfamily in plants. The catalytic properties of most of these P450s remain elusive. We focus on P450s involved in the synthesis of natural products belonging to the groups of cyanogenic glucosides, glucosinolates, alkaloids, and isoflavonoids. Special emphasis is on the plant model *Arabidopsis thaliana* for which the complete genome sequence is available[16] and which is easily amenable to genetical modifications[17] and provides an excellent model plant for metabolic engineering. Exploitation of P450s to reach increased production levels for desired natural compounds and transfer of entire biosynthetic pathways into other plant species will be discussed. The first part is a short presentation of different tools used to achieve gene—to function relationship, the bottleneck in P450 functional genomics[18].

2. The P450 Superfamily in Plants

So far genomic and expressed sequence tag (EST) sequencing projects have revealed a total of 1,059 plant P450 sequences[19]. Phylogenetic analyses based on translated raw DNA sequence data have spaced the P450s into 10 clans that include 59 families and an extensive number of subfamilies[18, 20]. In the *A. thaliana* genome alone, a superfamily of 272 cytochrome P450 genes including 26 pseudo genes were annotated and named[16, 21]. These genes represent members of 45 out of the 59 currently assigned plant P450 families[20, 22]. P450s constitute the largest and continuously expanding superfamily in plants. From the *Oryza sativa* cvs *japonica* and *indica* (rice cultivars)[23, 24] genome sequence projects, as many as 458 predicted P450 genes were annotated by the end of September 2002[25].

2.1. Nomenclature

The large number of P450 enzymes found in the Plant Kingdom are named and categorized based on protein sequence identity and phylogenetic relationships[26]. P450s assigned to the same family share more than 40% sequence identity at the amino acid level. Correspondingly, P450s assigned to the same subfamily share more than 55% sequence identity.

In plants, the identity rule has some exceptions due to gene duplications and shuffling as pointed out in Werck-Reichart *et al.* (2002)[22]. The plant P450s are categorized into the following

families: CYP51, CYP71-99, CYP701-727, and CYP736[20]. The CYP51 family is unique because the sequence identity of the P450s belonging to this family is well-enough conserved across phyla to contain plant, fungal, bacterial as well as animal sequences[27]. The precise structure of the sterols that serve as substrates for CYP51s varies among different eucaryotes. This variability has been suggested to represent adaptation to the availability of different sterol precursors in different Kingdoms.

Plant P450s are membrane-bound proteins. They are classified into the A-type and the non-A-type P450s[28, 29]. It has been proposed that the plant-specific A-type P450s originate from a single ancestral P450[28]. A-type P450s share a simple gene organization with a single phase 0 intron with a highly conversed position[29]. Typically, the P450 genes are found to cluster with close relatives on short stretches of all five *A. thaliana* chromosomes indicating recent duplication events[29]. P450s involved in the biosynthesis of plant natural products belong to the A-type. In this review, we focus on A-type P450s belonging to the CYP71, CYP79, CYP80, CYP83, and CYP93 families and their respective involvement in the biosynthesis of cyanogenic glucosides, glucosinolates, alkaloids, and isoflavonoids. A short description of the biological function of non-A-type plant P450s is confined to members of the CYP85 and CYP90 families that are involved in the production of polyhydroxylated steroidal molecules.

3. Tools Available to Identify Biological Functions

Only very few of the 246 predicted P450 enzymes present in *A. thaliana* have had a biological function assigned. Functional assignments of the *A. thaliana* P450s are restricted to 23 enzymes belonging to 14 of the 45 plant families represented in the *A. thaliana* genome. These identified enzymatic activities are: *CYP51*, obtusifoliol 14α-demethylase[30]; *CYP72B1*, brassinolide 26-hydroxylase[31]; *CYP73A5*, cinnamate-4-hydroxylase[32]; *CYP74A1*, allene oxide synthase[33, 34]; *CYP74B2*, hydroperoxide lyase[35]; *CYP75B1*, flavonoid 3′-hydroxylase[36]; *CYP79A2*, phenylalanine *N*-hydroxylase[37]; *CYP79B2* and *CYP79B3*, tryptophan *N*-hydroxylases[38]; *CYP79F1* and *CYP79F2*, methionine *N*-hydroxylases[39–41]; *CYP83A1* and *CYP83B1* enzymes converting indole-3-acetaldoxime, *p*-hydroxyphenylacetaldoxime, and phenylacetaldoxime into the corresponding *S*-alkyl-thiohydroximates[42–44]; *CYP84A1*, ferulic acid hydroxylase[45]; *CYP85A1*, steroid C-6-hydroxylase[46]; *CYP86A1* and *CYP86A8*, fatty acid ϖ-hydroxylases[47, 48]; *CYP88A3* and *CYP88A4* enzymes converting *ent*-kaurenoic acid to GA_{12}[49]; *CYP90A1*, steroid C-23 hydroxylase[50]; *CYP90B1*, steroid C-22 hydroxylase[51]; *CYP98A3*, 3′-hydroxylase of phenolic esters[52]; and *CYP701A3*, *ent*-kaurene oxidase[53].

3.1. Phylogenetic Relationships

The categorization of P450s from different plant species into families and subfamilies based on sequence identity and phylogenetic relationships as discussed above typically does not concomitantly lead to an assignment of biological and/or enzymatic function. In some families, the P450s all appear to catalyze the same enzymatic reaction. In other families, members of the same family clearly catalyze very different enzymatic reactions. These differences are illustrated with the following examples. The CYP73 family is composed of one subfamily, CYP73A with 37 members. CYP73As from *Helianthus tuberus* (artichoke)[54], *Phaseolus aureus* (mung bean)[55], *Medicago sativa* (alfalfa)[56], *Petroselium crispum* (parsley)[57], *Popupus tremuloides* (querken aspen)[58, 59], *A. thaliana*[32], *Triticum aestivum* (wheat)[60], *Cicer arietinum* (chickpea)[61], have all been demonstrated to be cinnamate 4-hydroxylases. The rest of the members of the CYP73A subfamily are therefore with great confidence assigned as cinnamate 4-hydroxylases solely based on their amino acid sequence identity. Members of the CYP74A subfamily have been characterized as allene oxide synthases in *A. thaliana*[33, 34], *Linum usitatissium* (flaxseed)[62], *Hordeum vulgare* (barley)[63], and *Lycopersicon esculentum* (tomato)[64]. However, members of the closely related CYP74B subfamily possess fatty acid hydroperoxide lyase activity as demonstrated in *A. thaliana* and *L. esculentum* (tomato)[35, 64]. Members of a single subfamily may also catalyze different and consecutive steps in a biosynthetic pathway as reported for members of CYP90A and CYP90B (see Section 4.1). The different steps of

entire biosynthetic pathways may be mediated by P450s belonging to the same subfamily as exemplified by the CYP71C subfamily (see Section 5.3.2). In contrast to the latter examples, members of five different subfamilies of the CYP79 family are all *N*-hydroxylases (see Section 5.2). A final example on the existence of numerous subfamilies with widely different biological functions is the 18 subfamilies CYP71A to CYP71R in the CYP71 family[20]. Enzymatic activities have solely been demonstrated for members of subfamilies CYP71C, CYP71D, and CYP71E as described in detail in Section 5.1.1. One member of a fourth subfamily, the CYP71A10 was shown to possess enhanced detoxifying properties against phenylurea-derived herbicides, an activity unlikely to be the major biological function of the enzyme[65]. Of the 110 known members of the CYP71 family, 97 belong to the subfamilies CYP71A to CYP71D. No catalytic function has been assigned to any of the 37 members of the CYP71B subfamily.

The difficulties in assigning function to a P450 solely based on its amino acid sequence will be partly alleviated as more catalytic functions become known and diagnostic sequence elements identified. The matter is particularly complicated for the A-type P450s involved in natural product synthesis. Plants are known to produce more than 100,000 different natural products with P450s involved in most pathways and sometimes being multifunctional[66–68]. To illustrate the preponderance of A-type P450s, they account for 153 out of the predicted 246 P450 genes in the *A. thaliana* genome.[20] In general, an A-type P450 is thought to possess high substrate specificity and its function to be limited to a single or a few parallel biosynthetic pathway(s).

The wide diversity in amino acid sequences found among the P450s is evident by the fact that in *A. thaliana*, P450s[16, 20] belong to 45 of the 59 plant P450 families.

3.2. Mutant Collections in *A. thaliana*

The biological function of some *A. thaliana* P450s have been elucidated *in planta* by taking advantage of the availability of knockout mutants in this model plant. Mutant collections have been generated by either T-DNA insertion[69–71], ethyl methanesulphonate (EMS) mutagenesis[72, 73], or ionizing radiation[74]. Special attention in screening programmes has been paid toward phenotypic mutants showing aberrant growth characteristics. This way, non-A-type P450s was shown to affect, for example, dwarfism[50, 51] (Section 4) and A-type P450s to affect excessive lateral root formation[43] (Section 7). Genetic analysis of phenotypes recognized by a lack of blue-green autofluorescence caused by absence of sinapoyl malate identified additional members of A-type P450s. Sinapoyl malate is a phenylpropanoid that serves as a biochemical sunscreen[75] (Section 7).

Methodologies to provide gain-of-function in mutants in existing knockout collections use activation tagging in weak-mutant-allelic backgrounds[76]. This facilitates identification of dominant suppressor genes, which will show enhanced expression after incorporation of multimeric, positive *cis*-acting elements close to suppressor genes. Using activation tagging, it was found that expression levels of a gene encoding a non-A-type P450 proved to influence regulation of light responsiveness and accumulation of steroid phytohormones[31] (Section 4.1).

3.3. Reverse Genetics

Reverse genetics provides a tool to identify the mutant genotype causing specific phenotypic characteristics. Using T-DNA tagged phenotypic mutants (Section 3.2), genomic DNA sequences flanking the T-DNA integration site are identified. Subsequently, the wild-type allele is identified and cloned and inserted into the mutant to revert its phenotype into wild type. Catalytic properties of the P450 are thereafter studied by heterologous expression of the plant cDNA in microorganisms.

3.4. Heterologous Expression in Microorganisms

Heterologous expression of individual cDNAs in *Escherichia coli* followed by enzyme assays in the presence of putative substrates have been used extensively for characterization of plant P450s[77], for example, for the CYP79s involved in

cyanogenic glucoside and glucosinolate synthesis[9, 78]. Recombinant P450 protein can be subjected to classical protein characterization including CO difference spectroscopy[79] and recording of substrate-binding spectra[80] and finally assayed for desired catalytic properties. The first plant P450 cDNA was isolated from ripening fruits of *Persea americana* (avocado)[81]. It was designated CYP71A1. Expression of the cDNA in *Saccharomyces cerevisia*[82] yielded high amounts of recombinant protein, but the predicted catalytic property of CYP71A1 was not identified[83].

Cinnamic acid 4-hydroxylase from *Helianthus tuberosus* (Jerusalem artichoke) was the first plant P450 to be functionally characterized[54]. CYP73A1 was designated as the first member of the CYP73 family. This cDNA was isolated from an expression library using antibodies raised against the isolated P450 protein (Section 3.5). Cinnamic acid 4-hydroxylase catalyzes an essential step in the phenylpropanoid pathway and it is considered to be ubiquitous in plants (see Section 3.1).

3.5. Isolation of Enzymes

Cinnamate 4-hydroxylases catalyze the hydroxylation of *trans*-cinnamic acid into *trans*-*p*-coumaric acid. The ability to monitor this enzyme activity in Jerusalem artichoke allowed isolation of the P450 enzyme CYP73A1 using conventional chromatography and generation of specific antibodies[84, 85].

A general isolation procedure based on dye affinity chromatography has been developed and has been used to isolate CYP79A1 that converts L-tyrosine into *p*-hydroxyphenylacetaldoxime[86]. This *N*-hydroxylase catalyzes the first committed step in the production of the cyanogenic glucoside dhurrin in *Sorghum bicolor*. Isolated CYP79A1 was catalytically active as demonstrated by its ability to convert tyrosine into *p*-hydroxyphenylacetaldoxime when reconstituted in artificial liposomes in the presence of NADPH-cytochrome P450 oxidoreductase, NADPH, and molecular oxygen[87]. Based on partial amino acid sequencing, the corresponding cDNA sequence was cloned from expression libraries of sorghum seedlings and subsequently used to produce recombinant protein[88, 89] (see Section 5.1.2).

3.6. Homology-Based Cloning

The CYP79A1 cDNA sequence[88] has been used to design degenerate DNA oligonucleotide primer sequences for identification of homologous genes in other cyanogenic crops like *Manihot esculenta* (cassava) using polymerase chain reactions (PCR). Cassava was found to express two P450 isoforms belonging to the CYP79 family. They showed 53% and 54% amino acid sequence identity, respectively, to CYP79A1[90]. Because the sequence identity to the CYP79A1 is below 55%, the two cassava homologues established a new subfamily and were named CYP79D1 and CYP79D2. The two isoforms exhibit 85% sequence identity and the recombinant proteins catalyze the same biochemical reaction (Sections 5.2). A similar PCR strategy served to identify additional CYP79 homologues from *Triglochin maritima* (seaside arrowgrass)[91].

Based on known cinnamate 4-hydroxylase sequences from Jerusalem artichoke and mung bean[54, 55], a homology search in an EST library identified an EST clone with 84–86% sequence identity, which was then used as a probe to isolate the *CYP73A5* from a genomic library[32].

To identify and clone cDNAs encoding inducible P450s involved in the biosynthesis of tetrahydrobenzylisoquinoline alkaloids, a PCR strategy based on the conserved sequence elements in the haem-binding domain of A-type P450s was applied[11, 92]. Based on mRNA isolated from induced, tetrahydrobenzylisoquinoline alkaloid producing plant tissue, 17 different P450 sequences were found. The sequences were compared with existing sequence data and heterologous expression assays based on predicted enzymatic activities that identified two alleles of (*S*)-*N*-methylcoclaurine 3′-hydroxylase[11] (Section 6).

4. Non-A-Type P450s Mediating Steroid Biosynthesis

Like vertebrates and fungi, plants produce polyhydroxylated steroidal hormones to regulate and control tissue morphology. In plants these types of hormones are designated brassinosteroids[93–96] and they are built on a campestanol carbon skeleton (Figure 12.1). The brassinosteroids

are nonessential phytohormones with impact on morphological characteristics, for example, leaf shape and dwarfism[50, 51]. Biological functions of brassinosteroids are controlled by specific receptors and suppressors (see Figure 12.2). These mediate signal transduction and control regulation of target genes including those for brassinosteroid biosynthesis[97, 98]. Brassinosteroids may potentiate plant fitness and defense in response to pathogen attack, since brassinosteroids induce systemic defense responses in tobacco and rice[99].

The biosynthetic pathway for brassinosteroids has not yet been fully elucidated. Models as presented in figure 12.3 for two parallel pathways assigned as "the early C-6 oxidation" and "the late C-6-oxidation" pathways have been suggested[100-102]. Non-A-type plant P450s participate in the production of the plant sterols that are brassinosteroid precursors. A key enzyme is obtusifoliol 14α-demethylase. This belongs to the CYP51 family and gene sequences encoding this ubiquitous plant enzyme that has been obtained from *S. bicolor* (sorghum)[103, 104] and *T. aestivum* (wheat)[105, 106]. Recently, an orthologue of obtusifoliol 14α-demethylase was identified in *A. thaliana* based on its ability to complement a lanosterol 14α-demethylase mutant of yeast[29]. The expression level of another gene *CYP72B1*, also assigned as *BAS1* belonging to the A-type family and encoding a brassinolide 26-hydroxylase, has been shown to regulate light perception and control accumulation of brassinosteroids[31]. Two non-A-type P450 families with known enzymatic activities, CYP90 and CYP85, participating in brassinosteroid biosynthesis are selected as representatives for detailed description (Sections 4.1 and 4.2).

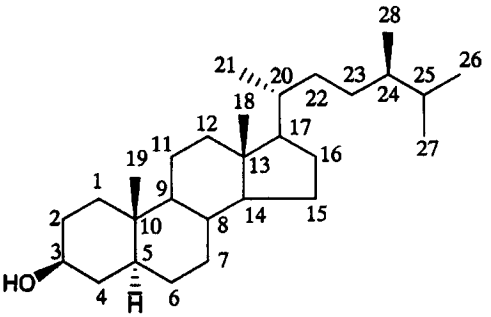

Figure 12.1. Polyhydroxylated steroids in plants indicating the carbon numbers of brassinosteroids as reprinted with permission from Bishop and Yokota (2001)[94].

4.1. CYP90s

CYP90A1, also assigned as *constitutive photomorphogenesis and dwarfism* (cpd) from *A. thaliana* encodes an enzyme in steroid biosynthesis that catalyzes hydroxylation (Figure 12.3) of cathasterone to testarone and of 6-deoxycathasterone to 6-deoxytestarone[50]. CYP90A1 was the first plant P450 identified by reverse genetics using a morphological screen for aberrant growth

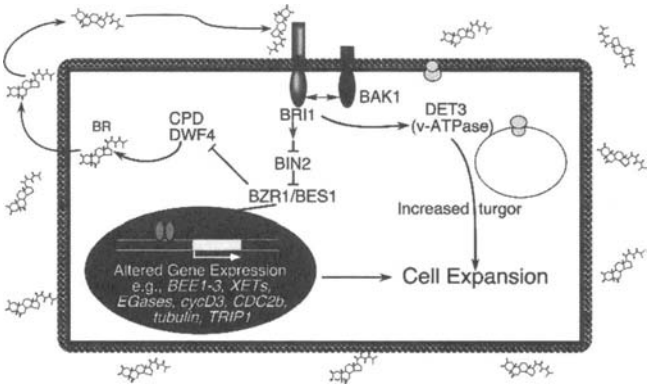

Figure 12.2. A model of the regulatory machinery for brassinosteroid sensing and biosynthesis. Upon perception of brassinosteroids, the receptor BRII signals via a phosphorylation cascade to regulate gene expression and cell expansion. Reprinted with permission from Thummel and Chory (2002)[97].

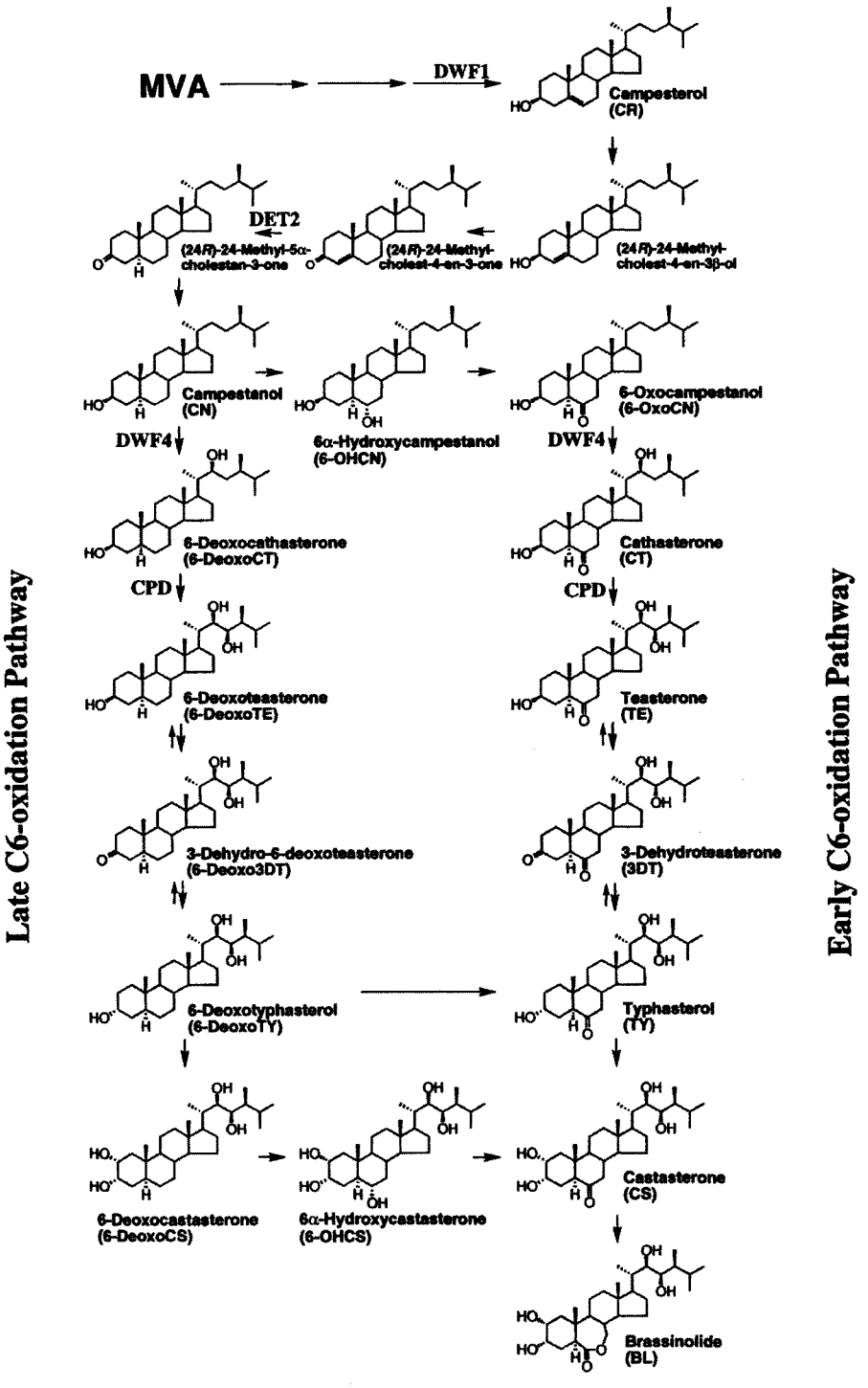

Figure 12.3. The proposed late and the early C-6 oxidation pathways for biosynthesis of brassinolides as outlined and reprinted with permission from Nogushi *et al.* (2000)[102].

characteristics among a collection of T-DNA tagged mutants[69]. CYP90A1 shared 24% amino acid sequence identity to the rat testosterone-16α-hydroxylase, CYP2B1[107]. Transcription of *CYP90A1* is negatively controlled by brassinosteroids[95, 108], most likely as part of a regulatory mechanism to ensure optimal physiological levels of endogenous brassinosteroids during growth (see Figure 12.2). The ability of CYP90A1 to hydroxylate the steroid side chain of both cathasterone and 6-deoxycathasterone illustrates that steps in "the early C-6 oxidation" and "the late C-6-oxidation" pathways may be mediated by the same enzyme. Homologues categorized into the CYP90A family have subsequently been identified in *Saccharum* sp. (sugar cane) and in *Vigna radiata* (mung bean)[20]. CYP90A encoding genes are expected to be ubiquitous in the Plant Kingdom.

A second dwarfed phenotype, *dwarf 4 (dwf4)*, in *A. thaliana* was also characterized using reverse genetics[51]. The mutation affects a 22-α-hydroxylase, assigned as CYP90B1, that hydroxylates the brassinosteroid side chain (Figure 12.3). The enzymatic activity of CYP90B1 provides the substrate of CYP90A1. CYP90B1 shares 40% amino acid sequence identity with CYP90A1 and, like CYP90A1, functions in "the early C-6 oxidation" as well and in "the late C-6-oxidation" pathways[46]. Although the *A. thaliana* CYP90B1 is currently the only member of this subfamily, it is thought to be ubiquitous in plants. Two additional subfamilies CYP90C and CYP90D have been established each containing one gene from *A. thaliana*[20]. The enzymatic activities of these P450s remain to be elucidated.

4.2. CYP85s

The CYP85 family is also involved in brassinosteroid biosynthesis. cDNA sequences encoding enzymes belonging to this non-A-type family has been obtained from *A. thaliana* and *Solanum lycopersicon* (potato). Members of the CYP85 family share approximately 35% identity to those of CYP90[95]. Recombinant versions of the two CYP85 plant genes were expressed in yeast and both enzymes were shown to catalyze multiple steps from 6-deoxoteasterone to teasterone, from 3-dehydro-6-deoxoteasterone to 3-dehydroteasterone, from 6-deoxotyphasterol to typhasterol, and from 6-deoxocastasterone to castasterone[46, 109]. The enzymatic activity of CYP85 enables crosstalk between "the early C-6 oxidation" and "the late C-6-oxidation" pathways for brassinosteroid formation and transforms the two pathways into a metabolic grid (Figure 12.3). Transcriptional activity of the gene is negatively regulated by brassinosteroids[95].

5. A-Type P450s Mediating Plant Protection

Plants need to defend and protect themselves against attack from herbivores and microorganisms. Toward this goal, plants produce a vast array of natural products some of which mediate broad resistance toward herbivores and pests and some of which are highly specific. Accordingly, the ability of plants to produce natural products enhances plant fitness by efficiently counteracting otherwise damaging biotic and abiotic stresses.

5.1. Broad Defense: Cyanogenic Glucosides

Cyanogenesis is the ability of plants to release hydrogen cyanide upon tissue damage. Cyanogenesis is an old trait widely distributed in the Plant Kingdom[78, 110–112] and currently documented in more than 2,650 plant species[113]. Cyanogenesis is mediated by cleavage of cyanogenic glucosides into the corresponding cyanohydrin and glucose by the action of β-glucosidase. Subsequent cleavage of the cyanohydrin into a ketone or aldehyde and hydrogen cyanide proceeds catalyzed by an α-hydroxy-nitrilase or non-enzymatically. Cyanogenic glucosides belong to the class of natural products known as phytoanticipins. They are also present in healthy plant tissues anticipating and ready to combat pathogen attack. Cyanogenic glucosides are present in many important crop plants like barley, sorghum, and cassava[113].

The release of poisonous hydrogen cyanide upon tissue disruption may render the presence of cyanogenic glucosides in a crop plant, a nutritional problem. This is of special concern in

cassava where use of this crop as a staple food requires careful processing to remove the cyanogenic glucosides or their degradation products before consumption[114]. In barley, major focus has been on cyanide potential in malt (5-day-old seedlings) and breeding programs have established genotypes assigned as low, medium, and high producers[115]. Despite domestication and controlled breeding, null-mutants have neither been identified in cassava nor in barley. It has been hypothesized that the ability of humans to remove cyanide by food processing explains why humans have continued to select and use cyanogenic crops as important components in the diet. In the early phases of plant breeding, selection of cyanogenic crops may have afforded protection from herbivore damage and may have helped to prevent theft of the crop[116].

Cyanogenic glucosides are derived from the amino acids L-valine, L-isoleucine, L-leucine, L-phenylalanine, and L-tyrosine and from the nonprotein amino acid cyclopentenyl glycine[78, 110]. Typically, a cyanogenic plant contains only one or two different cyanogenic glucosides. The biosynthetic pathway for cyanogenic glucosides has been elucidated using dhurrin production in *S. bicolor* as a model system. A general scheme for biosynthesis of cyanogenic glucosides involving two membrane-bound P450s and a soluble UDPG-glucosyltransferase was established as described below (see Figure 12.4).

5.1.1. Biosynthesis

Initial studies on the biosynthetic pathway for the L-tyrosine-derived cyanogenic glucoside dhurrin demonstrated that the covalent bond linking the α-and β-carbon atoms in L-tyrosine was preserved throughout dhurrin synthesis[117]. Subsequently, it was shown that the C–N bond in the parent amino acid is preserved during the biosynthetic process[118]. These studies were carried out by administration of double-labeled tyrosine to excised, biosynthetically active sorghum seedlings. Based on these observations, a biosynthetic pathway including an aldoxime, a nitrile, and an α-hydroxynitrile as intermediates was proposed, although no such compounds were detectable[119, 120]. A major breakthrough in the elucidation of the dhurrin pathway was based on the

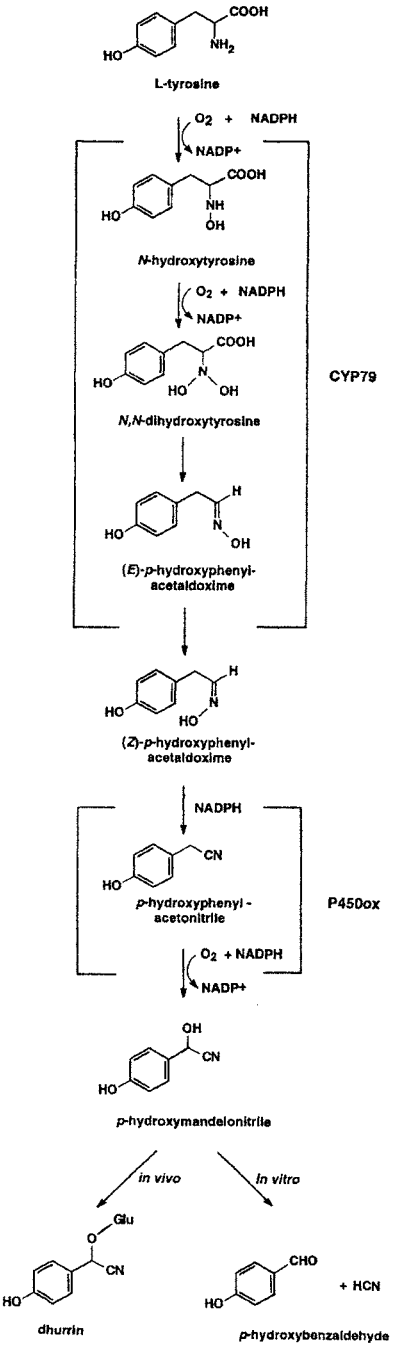

Figure 12.4. The biosynthetic pathway for the cyanogenic glucoside dhurrin is catalyzed by two multifunctional cytochrome P450s, CYP79A1, and CYP71E1 (P450ox) and by a glucosyltransferase, UGT85B1.

isolation of a biosynthetically active microsomal system. Upon administration of NADPH, molecular oxygen, and radiolabeled L-tyrosine to this experimental system in the presence of a large surplus of unlabeled putative intermediates, it was possible to trap radiolabeled *N*-hydroxytyrosine, (*E*)-*p*-hydroxyphenylacetaldoxime, (*Z*)-*p*-hydroxyphenylacetaldoxime, and *p*-hydroxymandelonitrile[121, 122].

The enzymes responsible for cyanogenic glucoside synthesis have been characterized using the sorghum microsomal system as biological starting material[86, 123]. The conversion of the parent amino acid L-tyrosine into the corresponding (*Z*)-aldoxime is catalyzed by CYP79A1 (Figure 12.4). This multifunctional P450 monooxygenase constitutes the first identified member of the CYP79 family and catalyzes two consecutive *N*-hydroxylations, a decarboxylation and a dehydration reaction. Isolated CYP79A1 was successfully reconstituted into artificial liposomes also containing isolated NADPH cytochrome P450 oxidoreductase[87]. Administration of radiolabeled tyrosine to this reconstituted enzyme system in the presence of putative intermediates as unlabeled compounds permitted identification of *N*-hydroxytyrosine, *N,N*-dihydrotyrosine, and (*E*)-*p*-hydroxyphenylacetaldoxime as intermediates in the conversion of tyrosine to the (*Z*)-aldoxime. From stoichiometric analyses, it was shown that two molecules of oxygen are consumed in this conversion. Enzyme assays carried out in an $^{18}O_2$ atmosphere using either tyrosine or *N*-hydroxytyrosine as substrates demonstrated that the two oxygen atoms introduced in the *N*-hydroxylation steps are enzymatically distinguishable as demonstrated by specific loss of the oxygen atom introduced by the first *N*-hydroxylation reaction in the subsequent conversion of *N,N*-dihydroxytyrosine into the (*Z*)-aldoxime[78, 112, 124]. This demonstrates that the intermediate *N,N*-dihydroxytyrosine is bound to the active site of CYP79A1 in a manner that prevents free rotation around the C–N single bond.

The further conversion of the (*Z*)-aldoxime into the cyanohydrin was demonstrated to also be mediated by a multifunctional P450 using the microsomal system isolated from sorghum as the biological starting material. This P450 was assigned CYP71E1 as the first member of the CYP71E subfamily. CYP71E1 catalyzes an unusual dehydration of an oxime to the corresponding nitrile, which subsequently is *C*-hydroxylated to the cyanohydrin (Figure 12.4)[123]. The nitrile intermediate in the CYP71E1 catalyzed reaction was demonstrated using trapping experiments[123, 125]. A single oxygen molecule is consumed in the CYP71E1 catalyzed reaction sequence[126, 127].

The last step in cyanogenic glucoside synthesis involves conversion of a cyanohydrin into the corresponding cyanogenic glucoside. Using dye-column affinity chromatography, a soluble UDP-glucose:*p*-hydroxymandelonitrile-*O*-glucosyltransferase, designated UGT85B1[128], was isolated from etiolated sorghum seedlings and shown to glucosylate the cyanohydrin function of *p*-hydroxymandelonitrile to produce dhurrin (Figure 12.4). Reconstitution of CYP79A1 and CYP71E1 into artificial liposomes in the presence of UGT85B1 resulted in the formation of dhurrin, that is, in reconstitution of the entire pathway for dhurrin production from its parent amino acid tyrosine[128] (Figure 12.5).

cDNA sequences encoding CYP79A1, CYP71E1, and UGT85B1 have been isolated[88, 125, 128] and functionally active proteins were obtained by heterologous expression of each of the cDNA clones in *E. coli*. The entire pathway for dhurrin synthesis has been transferred to *A. thaliana*[129], a plant species that in nature does not possess the ability to produce cyanogenic glucosides. Sequential introduction of each of the three enzymes into *A. thaliana* demonstrated that dhurrin is produced only after coordinated expression of all three sorghum genes[129]. Importantly, expression of UGT85B1 proved obligatory despite the availability in the *A. thaliana* genome of 120 family 1 glycosyl transferase genes[21, 130]. In transgenic plants co-expressing CYP79A1 and CYP71E1[131], *p*-hydroxymandelonitrile is the final product produced by the enzymes introduced. In such transgenic plants, *p*-hydroxymandelonitrile is metabolized by endogenous enzymes into a large number of different products. This is in sharp contrast to the results obtained when CYP79A1 and CYP71E1 are expressed together with UGT85B1, in which case only dhurrin formation is observed[129]. The transgenic dhurrin-producing *A. thaliana* plants showed improved resistance against the flea beetle *Phyllotreta nemorum*, which is a crucifer specialist[129].

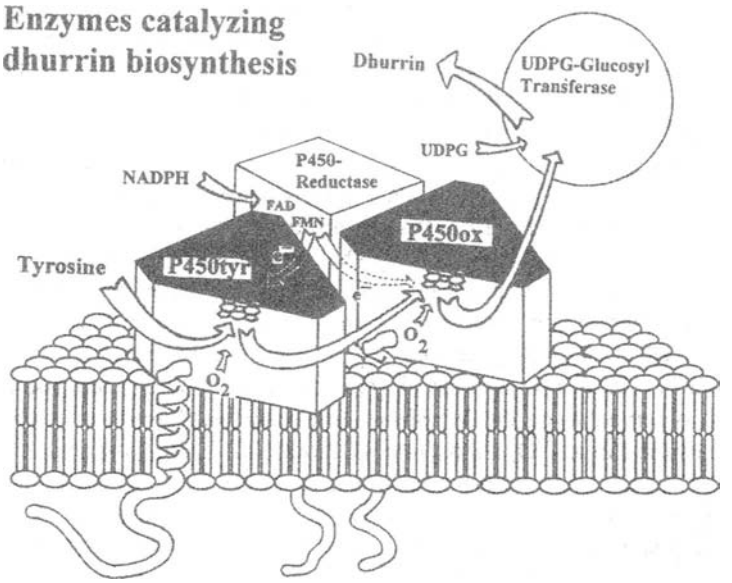

Figure 12.5. A model for metabolon formation of the three biosynthetic enzymes CYP79A1 (P450Tyr), CYP71E1 (P450ox), and UGT85B1 (glucosyltransferase) at the cytosolic surface of endoplasmic reticulum. Modified after Nelson and Strobel (1988).

5.1.2. Substrate Channeling and Metabolon Formation

Administration of radiolabeled tyrosine to etiolated sorghum seedlings resulted in a 49% incorporation into dhurrin, but surprisingly no radio labeled intermediates involved in this conversion were detectable[119, 120]. Biosynthetic studies using highly active microsomal enzyme preparations demonstrated efficient channeling of the intermediates in the pathway and provided an explanation as to why no intermediates accumulate[122] (Section 5.1.1). Likewise, biosynthetic studies with recombinant CYP79A1 and CYP71E1 reconstituted with NADPH cytochrome P450 oxidoreductase (ATR2) in artificial liposomes demonstrated efficient flux through the pathway with barely detectable levels of intermediates accumulating. Upon inclusion of cytosolic sorghum extracts or heterologously expressed UGT85B1 in the assays, almost complete stereospecific glycosylation of *p*-hydroxymandelonitrile into dhurrin was observed[123, 128]. These different sets of data suggest that the combined presence of CYP79A1, CYP71E1, and UGT85B1 results in the formation of an active metabolon (Figure 12.5).

The possible organization of the enzymes catalyzing a specific biosynthetic pathway into multi-enzyme complexes, also denoted metabolons, has for many years been a point of discussion in plant biology. The existence of metabolons in plants becomes increasingly apparent[132], for example, in the biosynthesis of cyanogenic glucosides[122], phenylpropanoid, and flavonoid pathways[133–135]. Metabolon formation may serve to overcome kinetic constraints, for example, by mediating a considerable local increase in substrate availability and concentration and secure that labile and/or toxic intermediates are swiftly converted into more stable and less toxic constituents. Evolution of a metabolon for dhurrin synthesis would appear essential to ensure rapid conversion of the toxic *p*-hydroxymandelonitrile intermediate by UGT85B1 to prevent its dissociation into hydrogen cyanide and aldehyde at the same time as gaining efficacy in dhurrin production. To demonstrate metabolon formation and to identify the subcellular compartment into which the metabolon accumulates, expression plasmids harboring DNA sequences encoding fusion proteins between the biosynthetic enzymes and spectral variants of green fluorescent protein (GFP)[136, 137]

were designed. Fusion proteins in which each of the three enzymes, CYP79A1, CYP71E1, and UGT85B1, were *C*-terminally linked to either cyano fluorescent protein (CFP) or yellow fluorescent protein (YFP) were functionally active when heterologously expressed in *E. coli* or *A. thaliana*. Dhurrin-producing *A. thaliana* plants were obtained by simultaneous expression of CYP79A1, CYP71E1-CFP, and UGT85B1-YFP, but not by simultaneous expression of CYP79A1-YFP, CYP71E1-CFP, and UGT85B1. This indicates prevention of proper interaction between CYP79A1 and CYP71E1 when both are fused to fluorescent protein in spite of a retained functionality of each separate P450 fusion. Examination of the transgenic plants by confocal laser scanning microscopy (CLSM) demonstrated that a metabolon visualized by UGT85B1-YFP is indeed formed after coordinated expression of the three biosynthetic genes. The metabolon located in distinct domains at the cytosolic surface of the endoplasmic reticulum appressed against the plasma membrane at the periphery of biosynthetically active cells (Figure 12.6A, B, see color insert). When UGT85B1-YFP was expressed alone, it showed an even cytosolic distribution (Figure 12.6C, see color insert).

5.1.3. Substrate Specificities

The type of cyanogenic glucoside present in a given plant species is defined by the substrate specificity of the enzyme catalyzing the first committed step in the pathway. This conclusion was reached from investigations of the amino acid specificity of active microsomal systems from sorghum that is specific to L-tyrosine, the precursor of dhurrin[86], seaside arrowgrass showing specificity to L-tyrosine, the precursor of taxiphyllin[138, 139], cassava, flax, and white clover, which are all specific to L-valine and L-isoleucine, the precursors of linamarin and lotaustralin[140–145], and barley with specificity to L-leucine, the precursor of epiheterodendrin[146]. These same specificities are also observed in *in vitro* assays using recombinant protein from sorghum, cassava, and seaside arrowgrass[90, 91, 123].

The enzymes catalyzing the subsequent steps in cyanogenic glucoside synthesis, that is, the conversion of oximes into cyanohydrins are not nearly as substrate specific. Again this knowledge was obtained from studies of microsomal preparations. The broadest substrate specificity is observed with the cassava microsomal preparation that is able to metabolize oximes derived from L-valine, L-isoleucine, L-phenylalalnine, L-tyrosine as well as from cyclopentenylglycine[142]. Sorghum microsomal preparations are able to metabolize oximes derived from L-tyrosine and L-phenylalanine[127]. Barley contains five different L-leucine-derived cyanoglucosides of which only one is cyanogenic. These are thought to be formed by the action of a single P450 that is able to hydroxylate all individual carbon atoms of the nitrile intermediate and to facilitate multiple hydroxylations as well as dehydrations (Figure 12.7)[146]. So far, the only P450 known to catalyze this set of reactions is CYP71E1 isolated from sorghum.

5.2. Functional Uniformity within the CYP79 Family

To date the CYP79 family consists of six subfamilies denoted CYP79A, -B, -C, -D, -E, and -F[20]. Currently, the CYP79A subfamily has eight members covering four plant species of which sorghum, *T. aestivum* (wheat) and *H. vulgare* (barley) belong to the *Poacea*[20]. The fourth plant species is Arabidopsis that does not contain cyanogenic glucosides. Instead, Arabidopsis is able to synthesize glucosinolates, a closely related group of natural products[9, 147]. The amino acid sequence identity between CYP79A1 from sorghum and CYP79A2 from Arabidopsis is 53%, slightly below the 55%[18, 20, 22, 26] criterion usually required to assign P450s to the same subfamily. Whereas the precise catalytic properties of the CYP79C subfamily remain to be established, all other members of the CYP79 family have been shown to catalyze the conversion of an amino acid to the corresponding oxime. Subfamilies CYP79A, -D, and -Es are involved in cyanogenic glucoside synthesis whereas the subfamilies CYP79A, -B, and -F are involved in glucosinolate synthesis[9]. Introduction of the sorghum CYP79A1 gene into *A. thaliana* by genetic engineering resulted in the production of large amounts of the tyrosine-derived glucosinolate *p*-hydroxyglucosinolate[148]. This illustrates that the oxime produced by the "cyanogenic" CYP79A1 serves as an efficient substrate for the endogenous *A. thaliana* downstream biosynthetic enzymes mediating

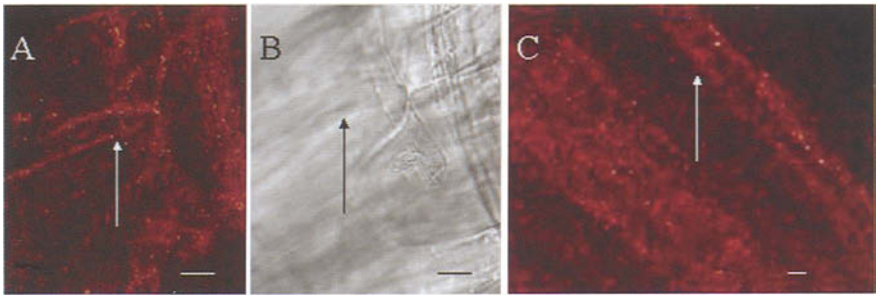

Figure 12.6. Confocal laser scanning microscopy of *A. thaliana* roots. (A) YFP fluorescence monitored using a color code gradient ranging from black over red to orange to illustrate increased fluorescence intensities. The arrow indicates the confined fluorescence at the periphery of cells co-expressing CYP79, CYP71, UGT85B1-YFP. (B) Transmitted light image to visualize the cell shape. Arrow as in (A). (C) YFP fluorescence in cells expressing UGT85B1-YFP shows even cytosolic distribution and high accumulation in and around the nucleus (arrow). Bar = 5 μm. According to Tattersall *et al.*, unpublished.

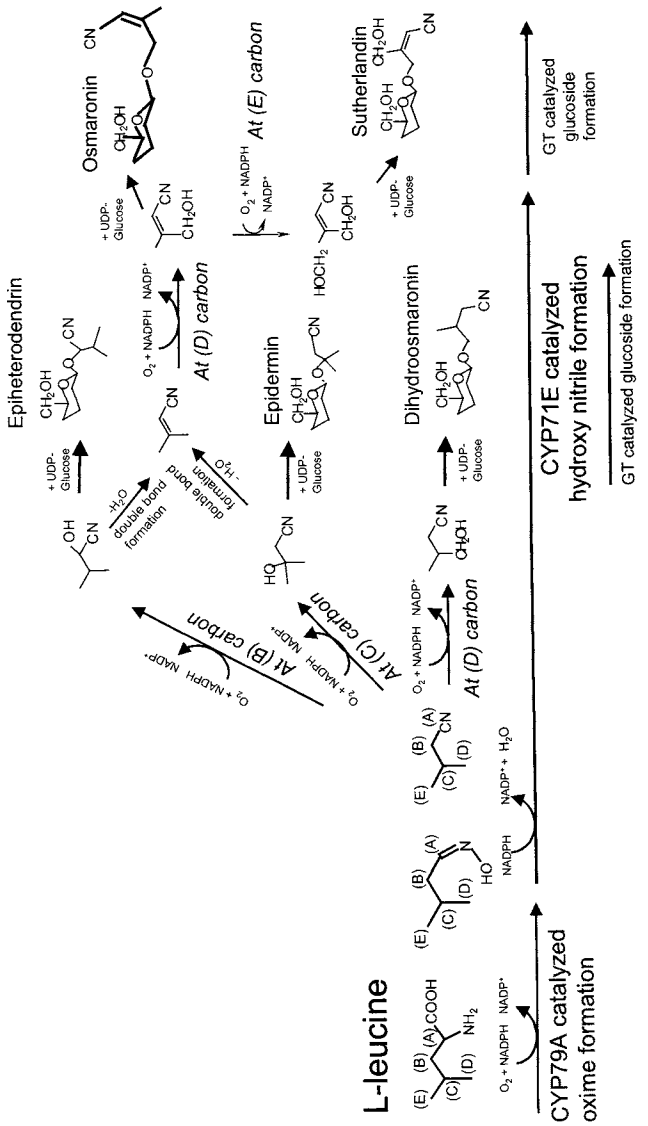

Figure 12.7. Proposed biosynthetic pathway for the different Leu-derived cyanoglucosides in barley. Reprinted with permission from Nielsen *et al.* (2002)[146].

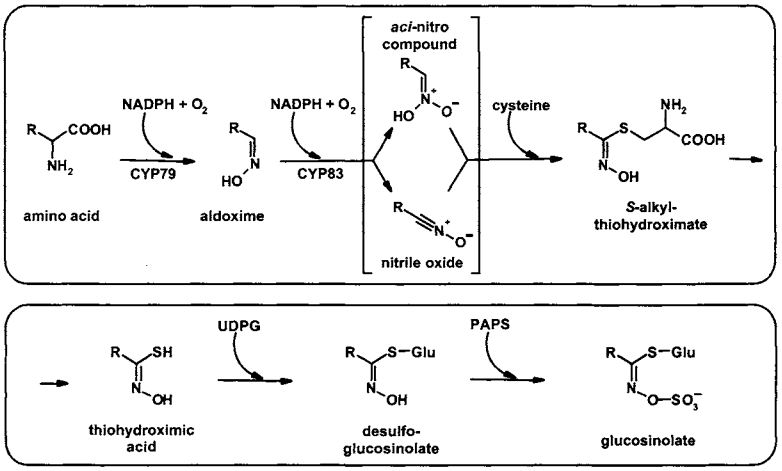

Figure 12.8. The biosynthetic pathway for glucosinolate production. Reprinted with permission from Wittstock and Halkier (2002)[9].

glucosinolate formation. Most likely, this involves formation of a metabolon as demonstrated in sorghum (Section 5.1.2). An Arabidopsis double mutant knocked out in both CY79B2 and CYP79B3 completely lack indole-derived glucosinolates, but show subtle morphological mutant phenotype. The subsequent conversion of oximes to glucosinolates is catalyzed by members of the CYP83 family (Section 7; Figure 12.8).

5.3. Functional Diversity among CYP71s

In contrast to the CYP79 family, the CYP71 family is functionally diverse and constitutes the largest A-type plant P450 family with a total of 110 members divided into 18 subfamilies.

5.3.1. CYP71A and CYP71B Subfamilies

The CYP71A subfamily contains 28 members including 17 annotations from the *A. thaliana* genome. The first member of this subfamily was derived from avocado[81]. No specific enzymatic activity has been demonstrated for the members of the CYP71A subfamily. CYP71A10 from *Glycine max* (soybean) catalyzes conversion of the phenylurea herbicides, fluometuron, linuron, chlortoluron, and diuron into more polar compounds[65]. This is unlikely to be the *in planta* biological function of the enzyme and surely does not explain the apparent evolutionary need for maintenance of 17 isoforms in the *A. thaliana* genome[21]. The CYP71B family is very large and composed of 36 members all annotated from the *A. thaliana* genome. The subfamily was first established based on a sequence with unknown biological function from *Thlaspi arvense* (field penny-cress), which like *A. thaliana* belongs to the *Brassicacea*[92].

5.3.2. CYP71C Subfamily: Grass-Specific Defense Compounds

The CYP71C subfamily is comprised of a total of 23 members with 11 from *Zea mays* (corn), 11 from *Trititum aestivum* (wheat), and a single member from *H. vulgare* (barley), all belonging to *Poacea*. The CYP71C subfamily possesses some very special enzymatic features related to the fact that together different members of this subfamily are able to mediate the synthesis of the grass-specific phytoalexin 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA)[149]. Each of the 23 members catalyzes one of four consecutive enzyme reactions

in the DIBOA pathway (Figure 12.9). Thus, coordinated enzymatic activities of CYP71C1, CYP71C2, CYP71C3v1, and CYP71C4 from maize mediate the production of DIBOA that is further metabolized to yield the cyclic hydroxamic acid 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA)[150].

Biosynthetic experiments using maize seedling and radiolabeled [3-^{13}C]-indole as precursor demonstrated that CYP71C4, CYP71C2, CYP71C1, and CYP71C3 catalyze the consecutive conversions into [3-^{13}C]-indolin-2-one, [3-^{13}C]-hydroxyindonin-2-one, 2-hydroxy-1, 4-benzoxazin-3-one (HBOA), and DIBOA, respectively (Figure 12.9). An additional hydroxylation at the C-7 position followed by C-7 specific methylation gave rise to the formation of DIMBOA. The C-7 hydroxylating enzyme was obtained by screening a maize EST collection in combination with a reverse genetics approach that revealed C-7 hydroxylation of DIBOA forming 2,4,7-trihydroxy-2H-1,4-benzoxazin-3(4H)-one (TRIBOA) by a 2-oxoglutarate-dependent dioxygenase[151] (Figure 12.10). The high sequence identity among CYP71C4, CYP71C2, CYP71C1, and CYP71C3 does not compromise substrate specificity as demonstrated by determining the catalytic activities of the recombinant proteins expressed in yeast[152].

From an evolutionary perspective, it is interesting that the phylogenetically closely related genes Bx2 (encoding CYP71C4), Bx3 (encoding CYP71C2), Bx4 (encoding CYP71C1), and Bx5 (encoding CYP71C3) co-locate to the short arm on chromosome 4 in the maize genome and to chromosome 5 on wheat genomes[153]. A fifth gene,

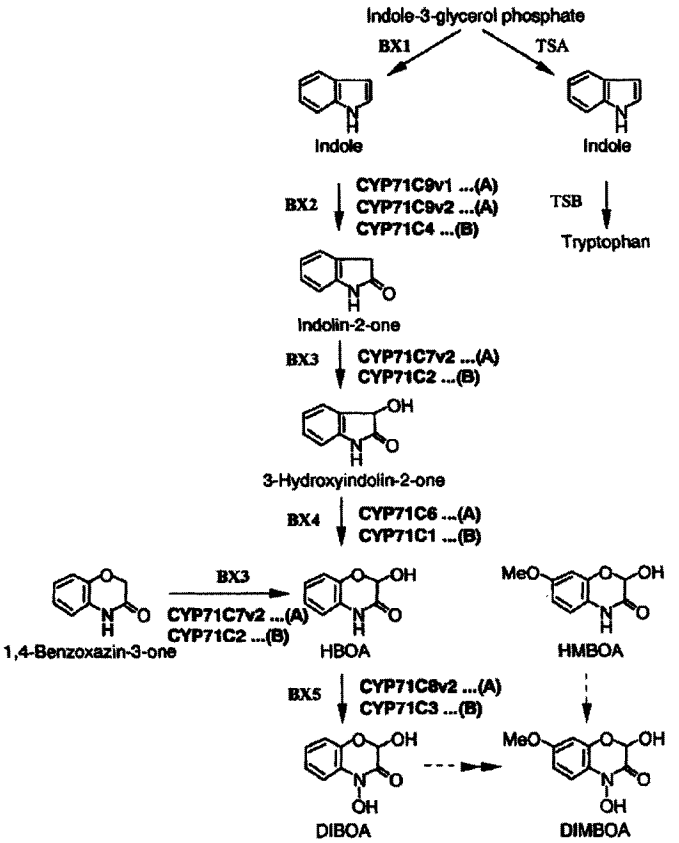

Figure 12.9. The biosynthetic pathway for DIMBOA. Bx1–Bx5 are gene names encoding the corresponding CYP71Cs as indicated in (A) wheat and (B) maize. Reprinted with permission from Nomura *et al.* (2002)[153].

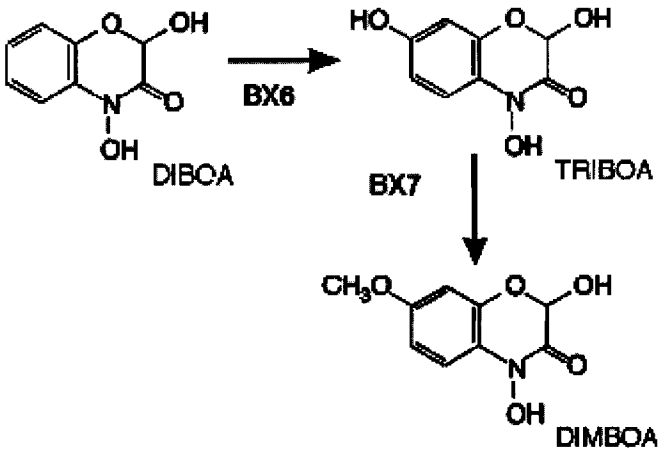

Figure 12.10. The 2-oxoglutarate-dependent dioxygenase Bx6 catalyzes hydroxylation of DIMBOA to produce TRIBOA. Reprinted with permission from Frey *et al.* (2003)[151].

Bx6, encoding the oxoglutarate-dependent dioxygenase clusters with the CYP71Cs at the short arm of chromosome 4. In maize, DIMBOA confers resistance to herbivores like *Ostrinia nubilalis* (European corn borer) and *Rhophalosiphum maydis* (maize plant aphid) and to the fungal pathogen *Helminthosporium turcicum* (Northern corn blight). The DIMBOA pathway may exemplify an evolutionary recent recruitment of new biological activities of P450s. The substrate for DIMBOA synthesis, indole or indole-3-glycerol phosphate is suggested to derive from a branch point in L-tryptophan synthesis. A sixth gene *Bx1* encoding a tryptophan synthase homologue is situated together with the cluster of DIMBOA genes on chromosome 4 in maize and was shown to be essential for DIMBOA production[149]. A homologue of this gene was activated by a herbivore elicitor, thus strengthening the suggestion of an introduction of a branch point in L-tryptophan biosynthesis for DIMBOA production in response to herbivore attack[154]. Transcription of the maize genes encoding CYP71C1 (Bx4) and CYP71C3 (Bx5) are induced in response to the maize bacterial pathogen *Acidovorax avenae* and in response to wounding[155]. No CYP71C homologues are identified in the Arabidopsis genome. However, the structure of DIMBOA is sufficiently close to the indole-derived phytoalexin camalexin that is produced by *A. thaliana* to allow speculations on a tight functional relationship between CYP71Cs and Arabidopsis P450 candidates[154]. In support of this working hypothesis, Zhou *et al.* (1999)[156] have published that a *pad3 A. thaliana* mutant unable to accumulate camalexin is defective in a putative P450 monooxygenase gene, annotated as CYP71B15[18, 20].

5.3.3. CYP71D, -F, and -R Subfamilies

CYP71D subfamily is also large and currently comprises a total of 22 members from 10 different plant species. At present, the catalytic properties of five CYP71D enzymes have been determined and the enzymes assigned to specific steps in indole alkaloid, sequiterpenoid, cyclic terpenoid, and flavonoid synthesis. Accordingly, enzymes belonging to the CYP71D subfamily do not necessarily share similar functional characteristics.

The first member to be functionally characterized was CYP71D12 from *Catharanthus rosea* (Madagascar periwinkle). CYP71D12 was identified as the tabersonine 16-hydoxylase enzyme involved in the biosynthetic pathway for the two medically important bisindole alkaloids vinblastine and vincristine[157] (Figure 12.11). Microsomal preparations from etiolated seedlings of Madagascar periwinkle were shown to be low in tabersonine 16-hydroxylase activity in comparison to light grown seedling. Interestingly, the light regulation was retained in suspension cultures of Madagascar periwinkle. A cDNA clone encoding tabersonine

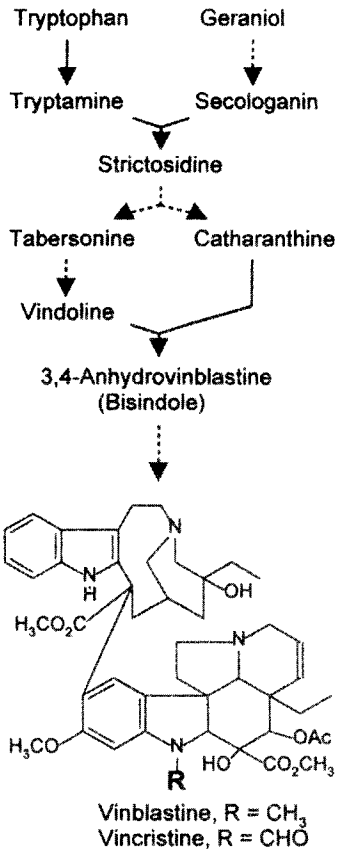

Figure 12.11. The biosynthetic pathway for the bisindole alkaloids vinblastine and vincristine. Reprinted with permission from Schroeder *et al.* (1999)[157].

16-hydroxylase was isolated from a cDNA library prepared from light-induced cells using degenerate oligodeoxynucleotide primers and verified by heterologous expression in *E. coli*. The other enzymes involved in the conversion of tabersonine to vindoline may also be light induced and this may provide a route for their isolation and cloning and for the production of vinblastine and vincristine by expression of the entire pathway from the precursors tryptamine and secologanin in cell cultures. A transcriptional regulator *O*ctadecanoid-derivative *r*esponsive *Catharanthus A*P2-domain protein (ORCA3) activates the expression of genes mediating L-tryptophan and tryptamine production as well of several genes in the synthesis of vindoline from tryptamine and secologanin[158]. A cytochrome P450, CYP72A1, was shown to convert loganin into serologanin (Figure 12.11)[159]. Activation of the ORCA3 gene is regulated by methyl jasmonate. This plant hormone is produced in response to stress and wounding[160] thereby enabling synthesis of the bisindole alkaloids as response to herbivore attack.

A second functionally identified member of the CYP71D subfamily is CYP71D20[161]. This enzyme from tobacco mediates production of the sesquiterpene capsidiol, an antimicrobial compound. The enzyme catalyzes hydroxylations of 5-epi-aristone as well as of 1-deoxy-capsidiol to capsidiol[161]. The functional and mechanistic features of CYP71D20 were determined in a coupled assay using substrate production by sesquiterpene synthases and a microsomal system[162]. CYP71D20 was found to catalyze unique stereo- and regiospecific hydroxylations first at carbon atom-1 followed by rotation of the molecule in the active site and a second hydroxylation at carbon atom-3 of the bicyclic sesquiterpene hydrocarbon skeleton. The CYP71D20 gene is induced in response to fungal elicitors like paraciticein[163].

The third functionally characterized member of the CYP71D subfamily is CYP71D9. This enzyme has been identified in soybean as a flavonoid 6-hydroxylase. It was demonstrated that hydroxylation of carbon atom-6 of the A-ring precedes 1,2-aryl migration to produce isoflavonoids as described in Section 5.4[164].

Regiospecific hydroxylation of the monoterpene (−)-4*S*-limonene at the C-3 or C-6-allylic positions to yield (−)-menthol (peppermint) or (−)-carvone (spearmint), respectively, is accomplished by the last two functionally characterized CYP71Ds, the CYP71D13 and CYP79D18 found in commercial mint species (*Mentha* sp.)[165–167].

5.4. Specialized Defense—Isoflavonoids in Legumes

Plant isoflavonoids possess a wide range of biological activities. They are efficient antimicrobial agents, inducers of the nodulation genes of symbiotic *Rhizobium* bacteria and phytoestrogens that work through the human estrogen receptor causing alterations in serum lipids and bone metabolism[2, 168]. Isoflavonoids are produced almost exclusively in the Leguminosae in the order Fabales. Isoflavonoids are produced from L-phenylalanine that condenses with 4-coumaroyl

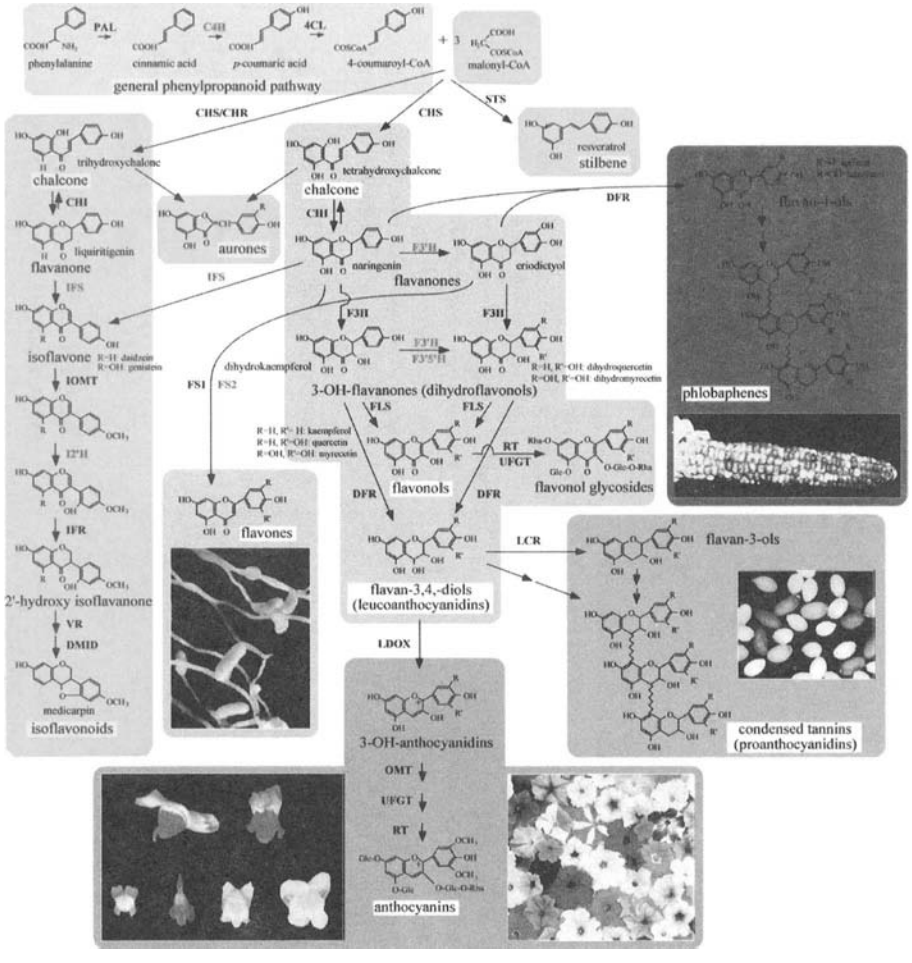

Figure 12.12. Biosynthetic pathways of flavonoid compounds from phenylalanine into isoflavonoids, flavones, flavonols, anthocyanidins, leucoanthocyanidins, proanthocyanidins, and phlobaphenes. The figure was kindly provided by Winkel-Shirley, B. (2003) as modified from Winkel-Shirley (2001). Reprinted with permission from Winkel-Shirley B., Grotewold E., Martins C., Hirsch A. M., and Quattrocchio F.

CoA and three molecules of malonyl CoA to produce chalcone and subsequently the flavanones naringenin and liquiritigenin (Figure 12.12). The synthesis of isoflavonoids from these flavanones is mediated by a CYP93C that catalyzes the migration of the B-ring to the 3-position followed by hydroxylation at the 2-position. The CYP93Cs are therefore termed 2-hydroxy-isoflavone synthases[15, 169]. CYP93C genes have been cloned from *G. max* (soybean; CYP93C1v2)[15], *Glycyrrhiza echinata* (licorice; CYP93C2)[170], and several other legumes: *Trifolium pratense* (red clover), *Trifolium repens* (white clover), mung bean, *M. sativa* (alfalfa), *Lens culinaris* Medik. (lentil), *Pisum sativum* L (snow pea), *Vicia villosa* (hairy vetch), and *Lupinus* spp. Lupin[171]. CYP93C enzymes catalyze the first committed step in the isoflavonoid pathway. Insertion of CYP93Cs into *A. thaliana* by genetic engineering enabled production of low levels of genistein in this non-leguminous plant[169, 171, 172]. Increased expression of CYP93Cv2 did not add to production[169]. When CYP93Cv2 was expressed in the tt3, tt6 double-mutant[173–175] that is blocked with respect to flavonol synthesis (see Figure 12.12), the genistein content was increased 3-fold[169]. Accordingly, competition for common substrates is an important parameter to consider in optimizing the production of desired natural products[169].

The production of the two isoflavonoids daidzein and genistein is highly induced by pathogen attack. Elicitation by crude polysaccharide preparations from yeast cell wall was used to facilitate biosynthetic studies in alfalfa cell suspension cultures[176]. A signal pathway dependent on endogenously generated nitric oxide is also responsible for the induction of daidzein and genistein synthesis[177]. Nitric oxide is generated from L-arginine by nitric oxide synthases (NOS). Although NOS belongs to the class of heme-thiolate proteins, the crystal structure of NOS clearly demonstrates that they belong to a different class of heme proteins as the P450 superfamily[178] enzymes. In alfalfa, improved protection against fungal pathogens is achieved by 4′-*O*-methylation of daidzein into formononetin followed by a number of unidentified hydroxylation steps to yield the highly antifungal phytoalexin, medicarpin (Figure 12.12)[176]. The 4′-*O*-methylation reaction has been studied in detail. Intricate physical interaction between the CYP93C isoflavonoid synthase and an isoflavone-*O*-methyltransferase, designated IOMT8 was suggested to guide 4′-*O*-methylation and to prevent 7′-*O*-methylation in spite of the fact that *in vitro* IOMT8 was found to catalyze 7′-*O*-methylation[176]. However, this intricate reaction mechanism has recently been challenged by the cloning and functional characterization of 2,7,4′-trihydroxyisoflavanone 4′-*O*-methyltransferases from *G. echinata* (licorice) and *Lotus japonicus* (Bird's foot trefoil) that exhibit high affinity for 4-*O*-methylation of daidzein[179].

6. P450 Mediated Production of Alkaloids with Medicinal Importance

In previous parts of this review, natural products with interesting medicinal uses have been mentioned like the bisindoles and isoflavonoids[14, 157]. In this context, a number of other alkaloids are important. The tetrahydrobenzylisoquinoline alkaloid berberine constitutes the first complex alkaloid for which the enzymes catalyzing the entire biosynthetic pathway from the primary precursor L-tyrosine have been identified. Biosynthesis of tetrahydrobenzylisoquinoline alkaloids involves a number of P450s with high substrate specificity and catalysing stereo- and regiospecific oxidations[180]. (*S*)-*N*-methylcoclaurine 3′-hydroxylase assigned as CYP80B1[11] catalyzes the conversion of (*S*)-*N*-methylcoclaurine to (*S*)-3′-hydroxy-*N*-methylcoclaurine, which by methylation is transformed into (*S*)-reticuline, which represents the branch point for formation of a vast number of different tetrahydroisoquinoline alkaloids including the berberine-, phenan-threne- and benzo[*c*]phenanthridine-type alkaloids (Figure 12.13).

The conversion of (*S*)-reticuline into berberine includes two remarkable P450 enzymes. The first is the unique berberine bridge enzyme (BBE) that catalyzes the introduction of a new C–C bond in its product, (*S*)-scoulerine[13, 180] (Figure 12.13). The second enzyme has been designated as canadine synthase and introduces a methylene dioxy-bridge[181]. The enzymatic mechanism for methy-lene dioxy-bridge formation[180] is outlined in Figure 12.14. Synthesis of the phenanthrine-type alkaloid morphine from (*S*)-reticuline via (*R*)-reticuline demands the involvement of three NADPH-dependent reductases

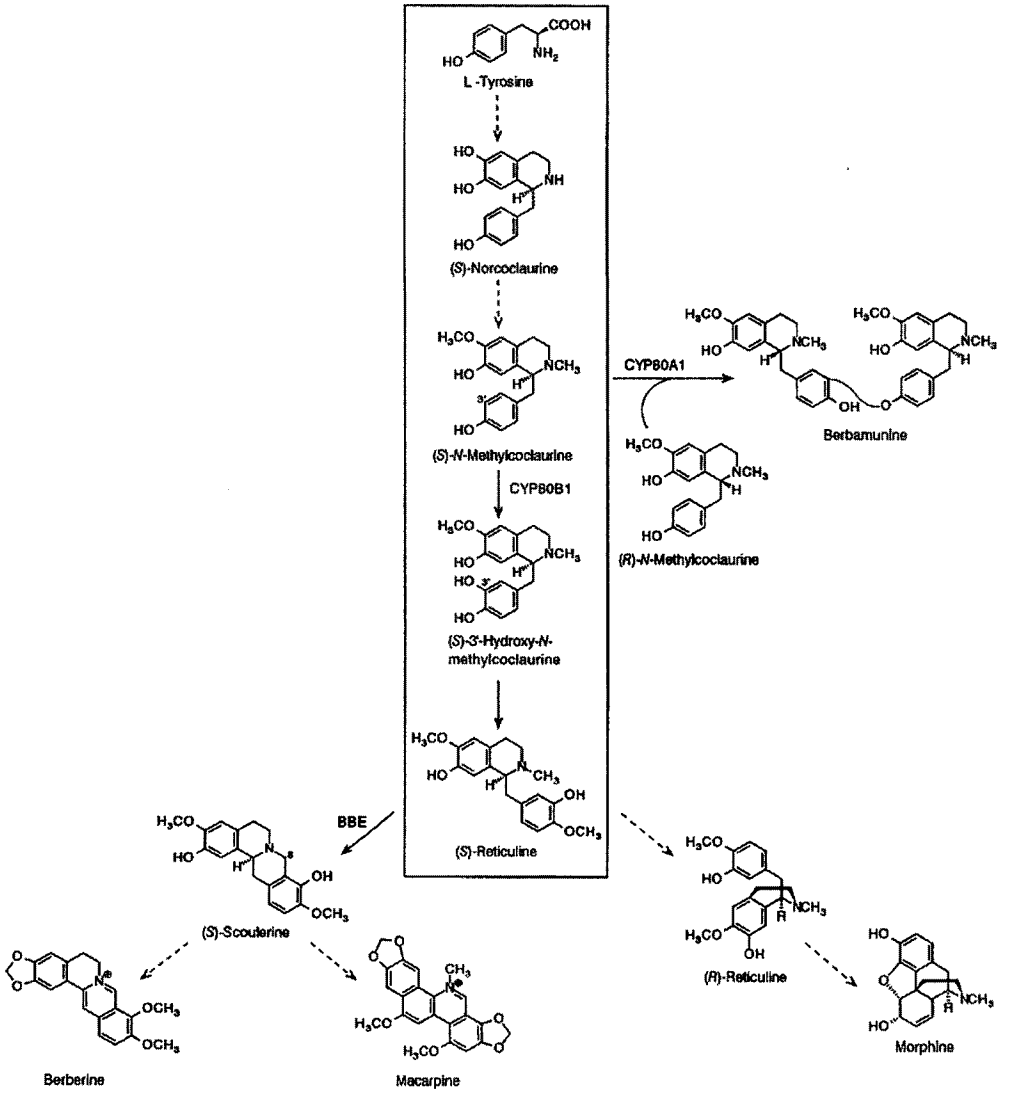

Figure 12.13. The biosynthetic pathway for L-tyrosine derived tetrahydrobenzylisoquinoline alkaloids. The core production of (*S*)-reticuline and the branch points for berberine, macarpine, and morphine production. Reprinted with permission from Chou and Kutchan (1998)[180].

and the P450 enzyme salutaridine synthase[180] that like the BBE introduces a new C–C bond in the product (Figure 12.15). So far the corresponding gene of only one of the reductases has been cloned[12]. Synthesis of the antimicrobial benzo[*c*]phenanthridine-type alkaloid macarpine (Figure 12.13) from (*S*)-reticuline involves the action of six P450s that have been studied in plant cell cultures[182–184]. New C–C bond formation and methylene dioxy-bridge formations are key catalytic features of the conversion[180].

The CYP80 family involved in the production of (*S*)-reticuline in the opium poppy is not at all represented in the Arabidopsis genome. This reflects the fact that the production of a specific alkaloid typically is restricted to a particular plant species or to a limited number of species within a family. Accordingly, it is normally not possible to

study such pathways in genetically well-characterized model plants. This greatly complicates elucidation of the biosynthetic pathways involved in alkaloid formation. Furthermore, alkaloids may accumulate very slowly over a period of months to years and in a highly tissue-specific manner. The establishment of cell cultures have helped to overcome some of these experimental difficulties[185]. Availability of native alkaloid producing plants as sources for isolation of important medicinal drugs remain of high importance because controlled production in, for example, transgenic *A. thaliana* is dependent on the availability of the genes encoding the entire pathway and hampered by technical problems in the co-expression of a multitude of heterologous genes.[186]

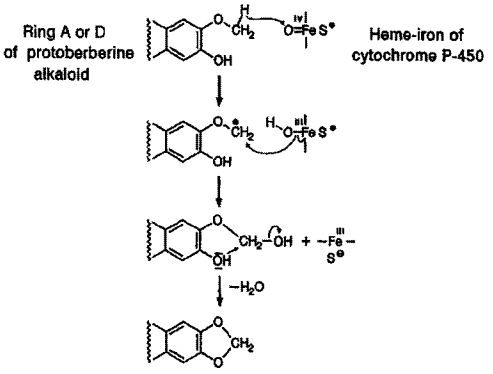

Figure 12.14. A proposed mechanism of methylene dioxy-bridge formation. Reprinted with permission from Chou and Kutchan (1998)[180].

7. Future Prospects: Crosstalk and Metabolic Engineering

The multigene family of plant P450s represents a very rich source for metabolic engineering. The A-type P450s involved in the synthesis of low molecular mass natural products is a key target because many of these compounds are of high value either as fine chemicals or as plant constituents that provide desired agronomical traits such as insect or fungal resistance. In all cases, the P450 enzymes catalyzing the first committed step in the different pathways leading to the production of natural products appear to exert a very high degree of substrate specificity. The successful transfer of the entire pathway for dhurrin formation from sorghum to *A. thaliana*[129] demonstrates that metabolon formation may be achieved also after heterologous expression of a biosynthetic pathway in a plant species that would not in nature produce the same type of natural products. Insertion of an incomplete pathway was shown to favor crosstalk with other metabolic pathways and the formation of side products[129]. When separately introduced into *A. thaliana*, sorghum CYP79A1 was able to establish highly efficient

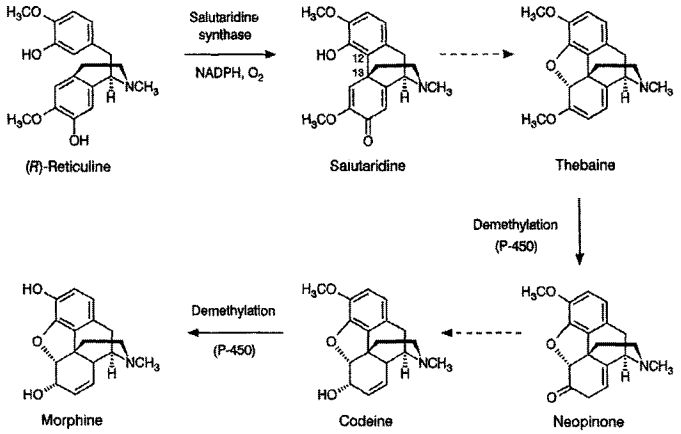

Figure 12.15. Biosynthesis of morphine from (*R*)-reticuline. Reprinted with permission from Chou and Kutchan (1998)[180].

interaction with downstream glucosinolate-producing enzymes to create a new metabolon that resulted in the accumulation of large amounts of *p*-hydroxybenzylglucosinolate in *A. thaliana*[148] and thereby changing the overall glucosinolate profile of *A. thaliana*[187].

The possibility to redirect L-tyrosine into the glucosinolate or cyanogenic glucoside pathways without loss of plant fitness[8, 129] demonstrates the existence of immanent routes for transport and storage of new classes of natural products introduced into plants by genetic engineering, and an inherent ability to redirect and optimize the flux of intermediates to counteract inbalances in primary and secondary metabolism[43]. The availability of a metabolic grid with numerous metabolic crosspoints to accommodate the synthesis of natural products upon demand is well documented. To enable the production of physiologically active amounts of DIMBOA in grasses without depleting the indole-3-glycerol phosphate pool for tryptophan synthesis, gene duplication has provided two modified genes each encoding enzymes that catalyze the same reaction but are directed toward different biochemical routes[154]. In periwinkle, a transcription factor ORCA3 upregulates the synthesis of L-tryptophan to provide efficient synthesis of the inducible bisindole alkaloids. Bisindole alkaloid synthesis is also dependent on the availability of secologanin and the rate-limiting step in its synthesis appears unaffected by ORCA3. The opposite situation where L-tryptophan accumulates due to blockage of natural product synthesis is also possible as observed in the double knockout mutant in Arabidopsis lacking the tryptophan metabolizing CYP79B2 and CYP79B3 enzymes[188]. Such plants completely lack indole-derived glucosinolates but only exhibit temperature-dependent phenotypic difference. So accumulation of free L-tryptophan does not appear to severely compromise wild-type growth characteristics, for example, by the formation of excess amounts of the tryptophan-derived indole acetic acid.

The ability to accommodate altered levels of intermediates depends on the type of compounds involved. In *A. thaliana*, tryptophan-derived oximes are key intermediates in the formation of the phytohormone indole acetic acid as well as in the synthesis of glucosinolates. CYP83A1 and CYP83B1 are the enzymes responsible for converting oximes into glucosinolates. Overexpression and knockout of these two enzyme activities result in altered phenotypes and pleiotrophic effects. Increased formation of lateral roots was associated with altered levels of indole acetic acid and provided evidence that fluxes of intermediates directed toward natural product formation may serve an important function to balance primary metabolism[43, 44, 189]. Surprisingly, disturbance of oxime metabolism affects phenylpropanoid metabolism and the monomer composition of lignin[75]. The link between these different phenomena is not yet understood.

In the synthesis of natural products, increased diversity is often achieved by a final set of modifications including hydroxylations, glucosylations, methylations, and acylations. As a result, the flavonol quercitin may be transformed into 300 different glucosides[190]. Berries of *Vitis vinifera* (grape wine) accumulate over 200 different aglycones that each may be decorated differently[191, 192]. Most likely, the synthesis of the basic structures of natural products is facilitated by metabolon formation. Dependent on cell type, developmental stage and elicitation as a result of abiotic or biotic stresses, additional enzyme activities may be bound to the basic metabolons to secure that desired specific modifications are obtained. The broad *in vitro* substrate specificity observed for *O*-methyltransferases[176, 193] and UDPG-glucosyltransferases[128, 194] may reflect that *in vivo* these will be associated to metabolons that prevent general access to their active sites. In this manner, the cell is able to maintain the potential to specifically decorate a large array of natural products without having to produce a separate enzyme for each reaction. As an added benefit, metabolon formation may prevent undesired reactions, for example, random glucolylation of plant hormones.

Based on the understanding of the basic principles for metabolon formation, in a foreseeable future it may be possible to transfer the entire pathways for synthesis of desired alkaloids into more convenient production plants from which these compounds can be isolated in high amounts. A main obstacle to reach these goals is knowledge of the proper P450, UDPG-glucosyltransferases, methyltransferases, and acyltransferases. Typically, these genes are not present in genetically well-defined model plants like *A. thaliana* and rice. They have to be traced often from exotic

plants for which no genome program and not even cDNA libraries are available. System biology technologies like metabolite profiling, proteomics, and transcriptomics may help to identify the proper enzymes and genes by unraveling coincidences of enhanced expression, protein appearance, and accumulation of specific metabolites. A genomics approach to elucidate the biosynthesis of the triterpene saponin in *Medicago truncatula* based on data mining of EST resources[195] and saponin metabolite profiles[196] resulted in the identification of three putative pathway enzymes[197]. In such approaches, metabolon formation may render it difficult to detect the true intermediates of a pathway. Reconstitution of a biosynthetic pathway by heterologous expression in a model plant is important to avoid wrong conclusions. In spite of the experimental limitations described above, progress on P450s and natural product synthesis moves quickly ahead thanks to hard work and the original approaches taken by many scientists involved in this research area.

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13

The Diversity and Importance of Microbial Cytochromes P450

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The cytochromes P450 (CYPs) of microbes are enormously diverse as revealed in discoveries from the era of molecular biology and as subsequently found in genomic investigations. One percent of the genes of a microbe can encode CYPs, but in stark contrast most bacteria studied so far can survive without CYPs. Microbial eukaryotes usually have at least one CYP, due to the essential requirement of most to synthesize sterol involving CYP51, sterol 14α-demethylase. The roles of the vast majority of microbial CYPs remain to be elucidated, but many already have important fundamental roles in nature, and others are important for biotechnological purposes. Some others have, of course, provided facile models for understanding CYP structure and activity, such as CYP101 ($P450_{CAM}$) of *Pseudomonas putida* and CYP102A1 ($P450_{BM-3}$) of *Bacillus megaterium*. The purpose of this chapter is to provide an outline of the important biomedical and environmental roles of the microbial CYPs, including many which were unsuspected when the respective microorganisms were originally studied. This includes involvement of CYP in some of the earliest metabolic alterations in the production of penicillin, some of the early biosynthetic steps allowing the production of corticosteroids, and the first application of therapeutic CYP inhibitors, the azole antifungal agents. Current and future applications involving microbial CYPs are manifestly clear, ranging from new therapeutics to biotransformations and bioremediation.

1. Introduction to Microbial CYPs

The discovery of cytochromes P450 in mammalian tissues rich with these proteins, such as liver and the adrenal gland, resulted in intense scrutiny of their roles in xenobiotic metabolism and endogenous functions[1–3]. Following their discovery came the realization that mammalian proteins required electron donor systems for activity, either NADPH-cytochrome P450 reductase (CPR) or adrenodoxin and adrenodoxin reductase in the endoplasmic reticulum or the mitochondria respectively[4, 5]. Protein biochemistry and molecular biology revealed the multiplicity of CYP forms and mammalian genomes exhibited CYP diversity.

The microbial CYP systems were studied at the same time, with yeast CYP being reported by Lindenmeyer and Smith (1964) and bacterial CYP by Appleby (1967)[6, 7]. The systems were viewed as models and this was true especially for a CYP from *P. putida* called $P450_{CAM}$ (CYP101) that allowed this bacterium to grow on camphor as a carbon source[8–12]. In pioneering work from the

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Gunsalus laboratory, the $P450_{CAM}$ system allowed biochemical and biophysical investigation of the CYP catalytic cycle as well as of the genetics of a bacterial catabolic plasmid. This typical CYP system was found to require a ferredoxin and ferredoxin reductase for catalytic activity, unlike the model for eukaryote CYPs, CYP102A1 or $P450_{BM-3}$, which was discovered in the Fulco laboratory and consisted of a fusion polypeptide containing CYP and reductase domains[13].

Studies in yeast have revealed deep insights into eukaryotic processes, and this is also true in studies on CYPs, where the first microbial CYP cloned was found to undertake an ancestral role in the superfamily. That is, CYP51 is needed for sterol biosynthesis and is found in plants, fungi, protists, animals, and some bacteria[14].

The nomenclature for CYPs is based on amino acid identity with 40% identity and above needed to place CYPs in the same family and more than 55% to place them in the same subfamily[15]. These rules can be relaxed, as is the case for CYP51s that can fall below 40% identity, if the CYPs undertake the same function. For microbial eukaryotes, the family numbers 51–69 and 501–699 are available and at the time of writing, numbers up to CYP553 are listed, but each genome reveals many more and many are not yet assigned. Bacterial CYP family numbers are initiated at CYP101 and a similar expanding scenario can be envisaged with more and more genomes.

In this chapter, we will outline historical perspectives on the discovery and importance of CYPs in biotechnology before going on to describe the diversity of functions and activities associated with microbial CYPs. The coverage is relatively extensive and is illustrative of the field, but with so many CYPs now revealed it is impossible to discuss each one individually. Obviously many of the CYPs that remain orphan in function today will emerge as being important in future studies.

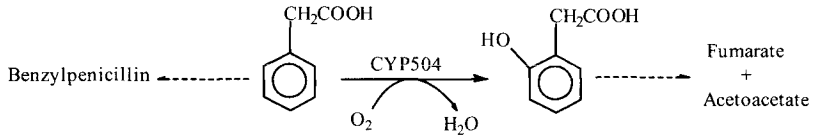
Microbial science is generally reported to begin with the fermentation of yeast observed by Pasteur, and although yeast CYP is not a cytochrome involved in respiration, it does contribute to the osmotic robustness of the microorganism, including ethanol tolerance, through the synthesis of ergosterol. This product requires CYP51 to remove the C14-methyl group of the precursor as well as a second CYP, CYP61, to undertake C22-desaturation[16]. Ergosterol in yeast has been used to produce vitamin D_2, although this has been uneconomical recently, but manipulation of this pathway has allowed production in whole-cells of hydrocortisone[17].

The modern era of biotechnology began with the discovery of antibiotics and the steps taken to improve yield. We now realize that streptomycetes contain many CYPs for drug (secondary metabolism) synthesis and this is discussed later in more detail, but *Penicillium chrysogenum* was the first utilized in antibiotic production. In early work following the pioneering studies, phenylacetate (precursor) feeding was found to elevate yields from fermentations and, of course, mutation and screening strategies increased the titer. Recently, the basis of genetic change in the Wisconsin strains revealed that a CYP mutation produced increased penicillin yield at the beginning of the genesis of improved fungal strains[18]. The gene concerned, *PahA* encodes CYP504, a CYP also identified in *Aspergillus nidulans* that allows growth on phenylacetate[19]. A *CYP504* mutant containing the substitution L181F resulted in the reduced 2-hydroxylation of phenylacetate, and this mutation channeled the carbon flux away from the side-pathway and through into increased penicillin titer.

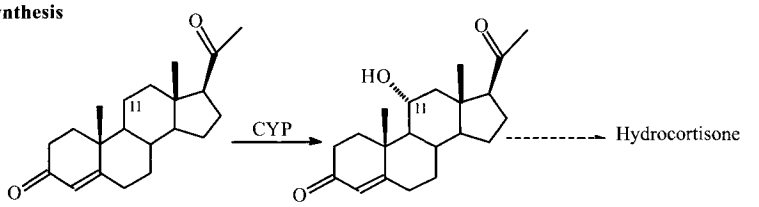
Parallel with developments after World War II in antibiotic production, the therapeutic value of corticosteroids was discovered. Interest during the early 1940s was based on rumors of experiments in performance enhancement of pilots in the Luftwaffe by corticosteroids[20]. The chemical synthesis route was inefficient and microbial hydroxylations by fungi were some of the first successful biotransformations for pharmaceutical production. 11α-Hydroxylation of a steroid was achieved by *Aspergillus ochraceous* and *Rhizopus niger*, and the 11β-hydroxylation achieved with other fungi such as *Cochliobolous lunatus*, allowing the production of cortisol. We now realize these conversions were achieved by fungal CYPs, although the genes concerned are not yet known.

A last example to note before moving onto describing the diversity of microbial CYPs and their importance, is provided by the azole antifungal compounds[21]. First developed for agriculture, where they are known as DMI compounds (demethylase inhibitors), these compounds have become central to antifungal therapy in the clinic

A. Mutation in CYP504 leads to overproduction of penicillin in *Penicillium chrysogenum*



B. Commercial application of P450 catalysed 11α-hydroxylation of progesterone in hydrocortisone biosynthesis



C. CYP51 and sterol biosynthesis, application to azole antifungal development

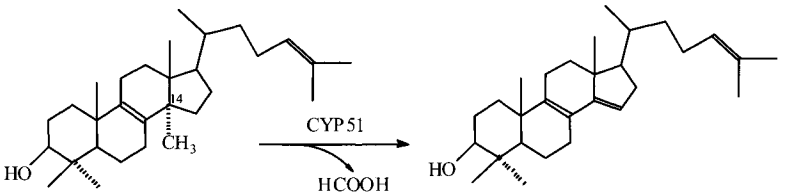


Figure 13.1. (A) The metabolism of phenylacetate by *Penicillium chrysogenum* by CYP504 is impeded during early strain improvement by mutation and selection increasing flux through to penicillin production; (B) The 11-hydroxylation of steroid by some filamentous fungi were among the first commercial biotransformations in the pharmaceutical industry allowing the production of corticosteroids, first 11α-hydroxylation by *Aspergillus* sp. and *Rhizopus* sp. and subsequently 11β-hydroxylation by *Curvularia* sp.; (C) Sterol C14-demethylation in fungi is the target of the antifungal azole compounds.

and new compounds continue to be evaluated. The mode of action in relation to CYP51 and the repercussions of that for the fungus only became clear after their development, but they were the first commercial CYP inhibitors. Some of the microbial CYP activities described here are shown in Figure 13.1.

2. Classes of Microbial CYPs

CYPs in bacteria are generally soluble proteins requiring ferredoxin and ferredoxin reductase for the two electrons needed in the CYP catalytic cycle, while CYPs in eukaryotic microbes are typically located in the endoplasmic reticulum with an associated NADPH-CPR providing the necessary reducing equivalents. As such, these are often called class I and class II, respectively (see also Chapter 4). Over the last 20 years other novel forms have been identified including the fusion protein CYP102A1 ($P450_{BM-3}$) identified in *Bacillus megaterium* by the Fulco laboratory[13, 22]. This CYP resembles the class II system with a flavoprotein reductase domain while CYP55 ($P450_{nor}$) is a stand-alone catalytic entity with an NADPH-binding site and a third class[23]. Over the last years, several new types have been identified which, in some cases, have been placed into classes. One response to this diversity is to have new classes for each new form and to not disrupt the assignments made already, and we have previously adopted that approach[24]. However, with the emergence of further forms of CYP fusion proteins, and the anticipation that more will arise, it is a suggestion here that classes should reflect novelty only in the method CYP reduction. In this way, CYP fusion proteins involving ferredoxin

and ferredoxin reductase would be subclasses of class I, while CYP fusions involving only flavoproteins would be subclasses of class II. Catalytically self-sufficient CYPs represent class III. Different subclasses can occur for the CYPs involved, but class I and class II would give the immediate impression of the type of electron transfer system concerned.

A number of new CYP fusion forms and a novel CYP operon have been cloned at the time of writing that represent new forms. The gene encoding CYP176A1 ($P450_{cin}$) was found in an operon with genes encoding a flavodoxin and flavodoxin reductase and so could represent an ancestor of the class II system. These would be placed as a class IIc after CYP102A1, class IIb[25]. The flavodoxin and flavodoxin reductase could have become fused in other class II systems. Also a novel CYP was identified from a *Rhodococcus* sp. containing a reductase domain at the C-terminus similar to dioxygenase reductase protein (containing flavin mononucleotide and NADH-binding domains) and a C-terminal ferredoxin center (2Fe2S) and the end of the polypeptide[26]. This catalytically self-sufficient enzyme will be of interest for biotechnological modifications and directed evolution studies, but as it contains a ferredoxin system, it could be placed within class Ib. A CYP51 has been observed in *Methylococcus capsulatus* fused to a C-terminal ferredoxin domain and unless this has a novel reductase partner, it can be considered a Class Ic form as it conforms to the ferredoxin/ferredoxin reductase model (Figure13.2)[27].

A further *CYP* gene has also been cloned that represents a new form. It confers a capability to metabolize the high explosive hexahydro-1,3,5-trinitro-1,3,5-triazine and has been identified in *Rhodococcus rhodochrous*, with a flavodoxin domain at its N-terminus, but appears also to need a ferredoxin reductase for activity, which may be encoded adjacent to the *CYP* gene[28]. As this gene contains a flavodoxin domain, this can be placed

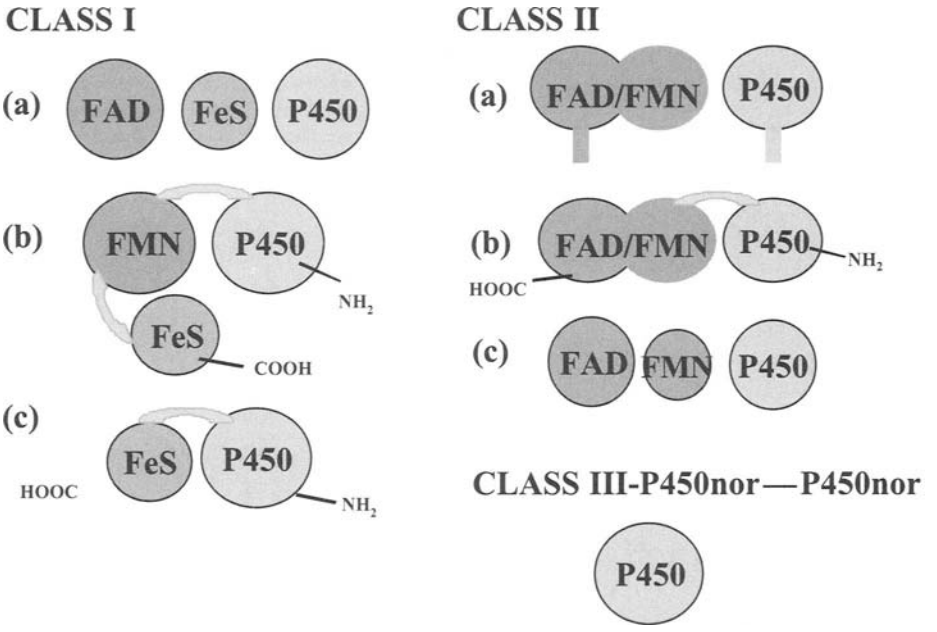


Figure 13.2. A proposal for a simplified classification of CYPs with reference to either use of a ferredoxin or alternative electron transport mechanism. Class Ia—typical bacterial system supported by ferredoxin and ferredoxin reductase, for example, CYP101; Class Ib—*Rhodococcus* sp. CYP fusion protein containing a ferredoxin domain[26]; Class Ic—*Methylococcus capsulatus* CYP51–ferredoxin fusion[27]. Class IIa—typical eukaryotic CYP/NADPH-cytochrome P450 reductase system; Class IIb—a fusion protein of a CYP and flavoprotein reductase, for example, $P450_{BM-3}$; Class IIc—$P450_{cin}$ containing separate flavodoxin and flavodoxin reductase partners[25]. Class III—stand-alone functional CYPs, for example, $P450_{nor}$[23].

with the Class II CYP systems if substantiated by protein studies. Another CYP has been detected in *Ralston metallidurans* and has a C-terminal fusion to a phthalate family oxygenase reductase module, but appears to be similar to another fusion described above[26, 29].

3. Considering the Origins and Relatedness of Microbial CYPs

Clues to the basic functions and the origins of CYPs can be found among the microorganisms. Among eighteen archaebacterial genomes probed, five contained *CYP* genes. Similarly, about two thirds of proteobacterial genomes (28 among 90 genomes probed), such as *Escherichia coli*, contain no CYPs, indicating how nonessential CYPs are to basic metabolism. This correlates with the involvement of CYPs in mechanisms of deterrence and attraction through the production of secondary metabolites, and in detoxification, and it is the general view that these selective pressures have produced much of the observed CYP diversity[14, 30–32]. However, some bacteria contain many CYPs, including *Mycobacterium smegmatis* that contains approximately 39 *CYP* genes, that is, about 1% of all genes in this microorganism, together with additional *CYP* pseudogenes[33].

CYPs have a role in bacteria to enable growth on carbon sources in the environment, such as on camphor by *P. putida* containing CYP101, or to undertake secondary metabolism as part of the biochemical warfare between organisms. Many CYPs are found in the pathways synthesizing important therapeutic compounds and this is discussed later in this chapter. The number of vital endogenous steps that have evolved for bacterial CYP function is still very small; for instance, BioI in *Bacillus subtilis* is needed to produce biotin by a pathway distinct from other bacteria[34]. Some CYPs, among the numerous forms uncovered, must be anticipated to be involved in key areas of endogenous metabolism especially in the

Table 13.1. The Numbers of CYPs in Various Sequenced Microbial Genomes

Microorganism	CYP complement (CYPome)
Prokaryotes (most have no CYPs)	
Campylobacter jejuni	1
Bacillus halodurans	1
Methanosarcinia barkeri	1
Mycobacterium leprae	1
Halobacterium species *NRC1*	1
Sulfolobus tokodaii	1
Sinorhizobium meliloti	2
Agrobacterium tumafaciens	2
Pseudomonas aeruginosa	3
Deinococcus radiodurans	3
Bacillus subtilis	8
Mycobacterium bovis	18
Streptomyces coelicolor	18
Mycobacterium tuberculosis	20
Streptomyces avermitilis	33
Mycobacterium smegmatis	39
Eukaryotes (usually have CYPs)	
Schizosaccharomyces pombe	2
Saccharomyces cerevisiae	3
Candida albicans	12
Neurospora crassa	38
Phanerochaete chrysosporium	>100

morphologically and developmentally complex organisms. This is an important area of investigation, as therapeutic CYP inhibitors need targets that are implicated in viability or pathogenicity. No such general CYP target exists across the bacteria. Table 13.1 shows some examples of the numbers of CYPs present in bacterial genomes. It is clear that the actinomycetes can be especially rich in CYPs and that, unlike prokaryotes, eukaryotic microorganisms usually contain at least a few CYPs, including CYP51 that is used for making sterols.

3.1. CYP51 and Evolution of the Superfamily

Work on yeast resulted in the cloning of the first microbial *CYP*, *CYP51*, encoding sterol 14-α-demethylase, in the Loper laboratory[35]. Orthologues of this CYP were later revealed to be present in animals[36, 37], and plants[38]. Many different CYP51s have now been identified and Figure 13.3 shows a phylogram of CYP51 sequences. Substrates differ slightly between the different kingdoms[39], especially in plants where the enzymes studied so far utilize the C4-methyl sterol obtusifoliol as the substrate and not the C4-dimethyl substrate (e.g., lanosterol) used in fungi and animals. CYP51 is found throughout eukaryotes, although some, for example, nematodes and insects obtain sterol from their diet. The primitive eukaryote *Giardia lamblia* also lacks *CYP* genes as judged by probing of its genome using conserved heme-binding motifs for CYP, but this remains unusual among eukaryote genomes that may require sterols for stabilizing membranes. In general, most eukaryote genomes searched to date have at least a few *CYP*s. The question arises: If sterol biosynthesis represents an early CYP function, when did it arise? Previous thoughts on what early function CYPs might have evolved to undertake have centered on their potential role in detoxifying oxygen[40]. Within sterol biosynthesis, squalene epoxidase is a non-P450 monooxygenase preceding CYP51 and it is probable that CYP51 conferred on primitive microorganisms a more robust membrane by producing sterols. An alternative CYP ancestor could easily have preceded CYP51, of course. Following squalene epoxidase, the next enzyme needed to make sterols is 2,3-oxidosqualene sterol cyclase (or lanosterol synthase). This is again a point of divergence in metabolism, with many plants and some protists synthesizing cycloartenol using the related cycloartenol synthase rather than lanosterol synthase, prior to producing obtusifoliol for CYP51 metabolism[41].

The original route to sterol biosynthesis is unclear as some protists synthesize lanosterol and many, like fungi, produce ergosterol as an end-product[42]. Recently, *Mycobacterium tuberculosis* was revealed to possess a CYP51 with sterol 14-demethylase activity[43], and this protein has been crystallized[44]. The true function of this protein remains to be clarified[33], as the earlier detection of mycobacterial sterols was probably the result of sequestration from medium. Other bacteria have been shown to contain sterols at relatively high concentration, as confirmed by purification and nuclear magnetic resonance (NMR) studies. The earliest of these is the methane utilizing proteobacterium *M. capsulatus* that synthesizes sterols via a lanosterol route[45], but not as far as ergosterol, sitosterol, or cholesterol. This organism contains a CYP51 and as such was the first proven bacterial sterol biosynthesis gene and protein studied[27]. The closest homologues to this CYP51 are among the mycobacteria, and then CYP170 of *Streptomyces coelicolor* and plant and protist CYP51 (Figure 13.3). More recently, another myxobacterium has been revealed to produce sterols and a cycloartenol synthase has been identified, although a CYP51 awaits identification from this bacterium[46]. We have also identified a lanosterol synthase and squalene epoxidase homologues at a locus in *M. capsulatus* (unpublished observation).

It seems reasonable to assume that sterol biosynthesis arose in gram-negative bacteria at least, and possibly in gram-positive bacteria, although the homologues here could have been the result of horizontal transfer. The need for several genes that are unlinked in order to make bacterial sterols seems to suggest that sequential horizontal transfer or independent evolution is unlikely, and that a sterol biosynthetic pathway may have evolved as a feature of prokaryotic ancestors of eukaryotes. There is a cycloartenol-type (plant-like) pathway, as well as a lanosterol-type (fungal/animal-like) pathway, in different bacteria that provides an extra level of complexity to these considerations, as it might be expected that, if ancestral, a single

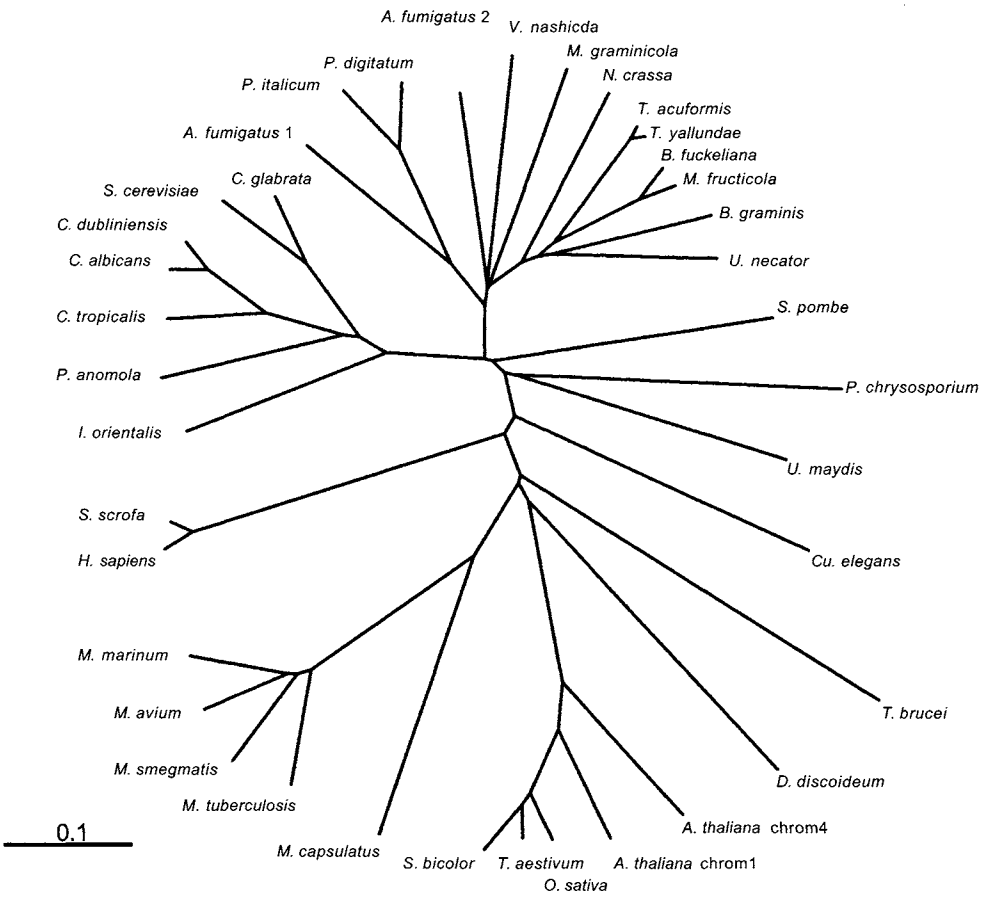


Figure 13.3. A phylogenetic tree containing sequences from bacterial, fungal, protist, plant, and animal CYP51s constructed using clustal X and Treeview 6. The sequences are *Aspergillus fumigatus* CYP51 1 (AF222068), *Aspergillus fumigatus* CYP51 2 (AF338660), *Botryotinia fuckeliana* (AF346594), *Blumeria graminis* (AF052515), *Candida albicans* (AB006856), *Candida dubliniensis* (AY034867), *Candida glabrata* (S75389), *Candida tropicalis* (M23673), *Cunninghammela elegans* (AF046863), *Issatchenkia orientalis* (S75391), *Mycosphearella graminicola* (AF263470), *Neurospora crassa* (http://drnelson.utmem.edu), *Penicillium italicum* (Z49750), *Phanerochaete chrysosporium* (http://drnelson.utmem.edu), *Pichia anomola* (AF019903), *Saccharomyces cerevisiae* (M18109), *Schizosaccharomyces pombe* (NC003424), *Tapesia acuformis* (AF208657), *Tapesia yallundae* (AF276662), *U. maydis* (Z48164), *Venturia nashicola* (AJ314649), *Dictyostelium discoideum* (http://drnelson.utmem.edu), *Trypanosoma brucei* (AF363026), *Arabidopsis thaliana* (chromosome 1 CYP51, AY05860), *Arabidopsis thaliana* (chromosome 4 CYP51, NC003071), *Oryza sativa* (AB25047), *Sorghum bicolor* (U74319), *Triticum aestivum* (AJ251798), *Homo sapiens* (U23942), *Sus scrofa* (AF198112), *Methylococcus capsulatus* (TIGR 414), *Mycobacterium avium* (TIGR 1764/3294), *Mycobacterium marinum* (Sanger mar388e06), *Mycobacterium smegmatis* (TIGR 1772/3269), and *Mycobacterium tuberculosis* (AL123456).

type of prokaryotic pathway would exist. Some clarification will emerge with more post-genomic studies.

The further evolution of CYPs must in some cases have included gene duplication events, including involving *CYP51* individually, besides genome duplication. Generally, it is accepted that pathways can evolve sequentially. The progressively more complex tailoring of sterols was selected for in the microbial era based on increased fitness of the organism, probably when exposed to physical parameters such as osmotic

stress. In fungi a further *CYP*, *CYP61*, evolved to perform the C22-desaturation of sterols and this may have evolved after a gene duplication of *CYP51*. The two gene families today have very little sequence identity (approx. 30%). The fungal sterol, ergosterol, is also found in protists such as trypanosomes and algae such as *Chlamydomonas reinhardtii*[47, 48], and so a CYP61 may be encountered elsewhere as proposed earlier[49]. Stigmasterol production in plants requires a C22-desaturation and yet no CYP61 was observed in *Arabidopsis thaliana*. The closest amino acid identity of a plant CYP to CYP61 was for CYP710, and no CYP61 has yet been seen in the green alga *C. reinhardtii*, which also makes ergosterol. It may be that other CYP families undertaking sterol C22-desaturation will need to be reclassified as CYP61 due to common function. Obviously, higher organisms and those that have homeostasis exist in a different evolutionary scenario from single-celled microbes. This presumably resulted in selection for other sterols such as cholesterol in animals and the array of phytosterols in plants. In some cases, such as in nematodes and insects that obtain sterol from their diet, sterol biosynthesis has been lost, while most bacteria have never had this requirement.

3.2. Bacterial CYP51

The identification of sterols in bacteria was first achieved in 1971 for the gram-negative bacterium *M. capsulatus*, where the end-products reported are various C4-methyl and desmethyl products[45]. A *CYP51* was identified in the genome of this bacterium with homology to other *CYP51*s and represented a new form with a (3Fe4S) ferredoxin domain fused at the C-terminus of the CYP domain via an alanine rich linker[27]. This 62-kDa protein was expressed and purified from *E. coli* and metabolized lanosterol (0.24 nmol/min/nmol protein) on addition of a ferredoxin reductase and NADPH. Other homologues of eukaryotic sterol biosynthesis, for example, lanosterol synthase and squalene epoxidase, are evident in this genome, although no genes are detected so far for the formation of end-product of 4-methyl and 4-desmethyl sterols. With the presence of many prokaryotic genomes it has become obvious that while some contain open-reading frames with considerable identity to sterol biosynthesis proteins, they are not all part of a sterol biosynthetic apparatus.

Another *CYP51*-like gene was identified in the emerging genome of *S. coelicolor* A3(2) and the protein had sterol 14-demethylase activity, but was not essential when the gene was knocked out and no sterols are made by this microbe[50]. This gene has been assigned to a different family (*CYP170A1*) and the protein shares 23 of the conserved amino acids across CYP51s, whereas the other CYP51s share approximately 40 amino acids.

One of the first important bacterial pathogens sequenced, *M. tuberculosis*, was found to contain 20 CYPs, one of which was identified as a CYP51. The hypothesis of azole inhibition of this CYP51, and parallels to antifungal activity with inhibition of fungal CYP51, stimulated the examination of the sensitivity among mycobacteria to known antifungal drugs. It was shown in *M. smegmatis* that potent activity of azole antifungals existed, except for fluconazole, but particularly for the topical agents[51], and this was also found in another study[52]. The latter report and a separate assessment of *Mycobacterium bovis* BCG sensitivity[14], showed less sensitivity in this slow-growing bacterium than in the fast-growing species, where the minimum inhibitory and bactericidal concentrations of miconazole were less than 2 μg/ml. Our further unpublished work indicates that the sensitivity of *M. tuberculosis* resembles that of *M. bovis* BCG, but other pathogenic species causing skin infections, *Mycobacterium chelonei* and *Mycobacterium fortuitum*, are sensitive and may be treatable with current topical antifungals (Table 13.2). To develop potent azole-type inhibitors as antimycobacterial agents will require screening other compounds, and it may well be that CYP51 is not the target, although both *M. tuberculosis* and *M. smegmatis* CYP51 bind azoles with high affinity[33, 52]. Indeed another *M. tuberculosis* CYP, CYP121, also shows high affinity binding for azole compounds[53]. Other CYPs from *M. tuberculosis*, such as CYP125, bind antifungal azoles poorly (unpublished observation). Further work in this area is needed to establish the mode of action, but while *M. smegmatis* is sensitive to azole compounds it contains no CYP121. It does, however, contain a strong homologue of the sole *Mycobacterium leprae* CYP, CYP164A1, with which the *M. smegmatis* CYP164A2 has 60% identity. No CYP family is

Table 13.2. Sensitivity of Mycobacteria to Azole Compounds Plus Amphotericin B Compared to Against *C. albicans*

	Minimum Inhibitory Concentration (μg/ml)							
Antifungal	*C. albicans* 505	*M. smegmatis* 700084	*M. smegmatis* 13116	*M. fortuitum* 01/2341	*M. chelonei* *LRT* 6680	*M. bovis* BCG	*M. tuberculosis* 1	*M. tuberculosis* 2
Clotrimazole	<2	<2	<2	4	<2	16	64	64
Econazole	<2	<2	<2	8	<2	8	32	32
Fluconazole	8	>256	>256	>256	>256	>256	>64	>64
Ketoconazole	<2	16	16	>256	8	32	—	—
Itraconazole	—	—	—	—	—	—	>64	>64
Imidazole	>256	>256	>256	ND	ND	ND	—	—
Miconazole	4	<22	<2	8	<2	8	32	32
Tebuconazole	4	32	32	64	ND	ND	—	—
Amphotericin B	<2	>256	>256	ND	ND	ND	—	—

Notes: Clotrimazole, econazole, and miconazole, showed most efficacy against *M. smegmatis*[51], and also against pathogenic mycobacteria such as *M. chelonei* and *M. fortuitum* that cause skin infections resistant to treatment with standard antimycobacterial agents. Less activity was seen with these compounds against the slow growing species *M. bovis* BCG and against two strains of *Mycobacterium tuberculosis*. Other azoles show lower activity and fluconazole was not active. The ND denote no assay information was available.

present in all mycobacteria to present a common antimycobacterial target, although many are present in all so far known except *M. leprae* (Table 13.3).

The genome sequences of various mycobacteria have been completed and it became apparent that *CYP51* was part of a putative hexacistronic operon that was conserved across mycobacteria except for *M. leprae*, in which no gene or pseudogene exists for *CYP51* due to divergent evolution and massive gene decay[54]. One striking similarity is the close homology between the CYP51 of mycobacteria and that from *M. capsulatus*. This is also true for the ferredoxin gene that lies downstream of *CYP51* in mycobacteria, but is fused to the CYP51 domain in *M. capsulatus*. Possibly the *CYP51* in mycobacteria was transferred by horizontal transfer from an ancestor of *M. capsulatus*, where it has possibly been recruited to a new function linked to the other genes in that putative operon/gene cluster. Also included among the genes is another *CYP*, *CYP123*. If the CYP51 performs a diffferent endogenous role from other CYP51s, this would require reclassification, possibly as a CYP170 as with the *CYP51*-like gene of *S. coelicolor*.

4. Archetypal Bacterial CYPs

Table 13.1 shows a list of some bacteria with the number of CYPs associated with their genomes to date. Most striking are the number and diversity seen among actinomycetes such as streptomycetes and mycobacteria, although some actinomycetes, such as *Corynebacterium diptheriae*, have no CYPs. Much of the CYP diversity is likely to be due to their role in secondary metabolism, as is true in the filamentous gram-negative

Table 13.3. The CYPome of *M. tuberculosis* Compared to Another Strain with Genomic Information and the Presence or Absence of these Forms in *M. bovis*, *M. avium*, *M. smegmatis*.

CYP number	Gene	H37Rv CDC1441,210	*M. bovis*	*M. avium*	*M. smegmatis*	*M. leprae*
CYP51	*Rv0754c*	+	+	+	+	
CYP121	*Rv2276*	+	+			
CYP123	*Rv0766c*	+	+	+	+	
CYP124	*Rv2266*	+	+	+	+	*ML1787*
CYP125	*Rv3545c*	+	+	+	+	*ML2024*
CYP126	*Rv0778*	+	+	+	+	*ML2229*
CYP128	*Rv2268c*	+	+			
CYP130	*Rv1256c*	+		+	+	*ML1102*
CYP132	*Rv1394c*	+	+			
CYP135A1	*Rv0357c*	+	+			
CYP135B1	*Rv0568*	+	+		+	
CYP136	*Rv3059*	+	+	+	+	*ML1742*
CYP137	*Rv3685c*	+	+			
CYP138	*Rv0136*	+	+	+	+	*ML2684*
CYP139	*Rv1666c*	+	+	+		*ML1237*
CYP140	*Rv1880c*	+	+	+	+	*ML2033*
CYP141	*Rv3121*	+				
CYP142	*Rv3518c*	+	+	+	+	
CYP143	*Rv1785c*	+	+	+		*ML1542*
CYP144	*Rv1777*	+	+	+	+	*ML1185*
CYP102						*ML0447*
CYP102						*ML2159*
CYP164				+	+	*ML2088*

Notes: Also shown is whether a pseudogene for the *M. tuberculosis CYP* exists in *M. leprae*. *ML0447* and *ML2159* (*M. leprae* ORF identifier) are identical pseudogenes in *M. leprae* that resemble *S. coelicolor A3(2) CYP102B1* and *ML 2088* is the only putative functional CYP of *M. leprae* where another member of this family is only found in *M. smegmatis*. The TIGR databases were utilized here.

myxobacterium that produces the anticancer drug epothilone[55], and the numerous forms present in the rifamycin gene cluster of *Amycolatopsis mediterranei*[56]. Some examples of bacterial CYP reactions are shown in Figure 13.4.

Many of the bacterial forms are described by David Nelson in his central website for CYPs (http://drnelson.utmem.edu/nelsonhomepage.html), but others are not yet assigned CYP numbers. The archetypal bacterial CYP is of course $P450_{CAM}$, assigned as CYP101, obtained from the bacterium *P. putida* ATCC17453[8–11]. This enzyme catalyzes the 5-*exo* hydroxylation of camphor, part of the breakdown of this carbon source for growth. The *CYP101* operon contains the *CYP* (*camC*) together with class I electron donor partners, a putidaredoxin reductase (*camA*), and putidaredoxin (*camB*). A fourth gene *camD* encodes a 5-*exo* hydroxy camphor dehydrogenase and the operon *camDCAB* is controlled by a *camR* repressor. CYP101 still provides an archetypal system for investigations of the CYP catalytic cycle and was the first CYP structure that revealed the triangular prism shape of the proteins and the heme with its cysteinyl thiolate ligand (see Chapter 3)[57, 58].

Other bacterial CYPs also undertake the breakdown of carbon sources for microbial growth. For instance, CYP108A1 ($P450_{TERP}$) metabolizes terpineol[59], and CYP176A1 ($P450_{cin}$) can metabolize cineol[25], while others can metabolize pollutants such as thiocarbamate herbicides and atrazine, as illustrated by CYP116 from a *Rhodococcus* sp.[60]. The CYP105 family of streptomycetes especially is associated with a wide variety of xenobiotic

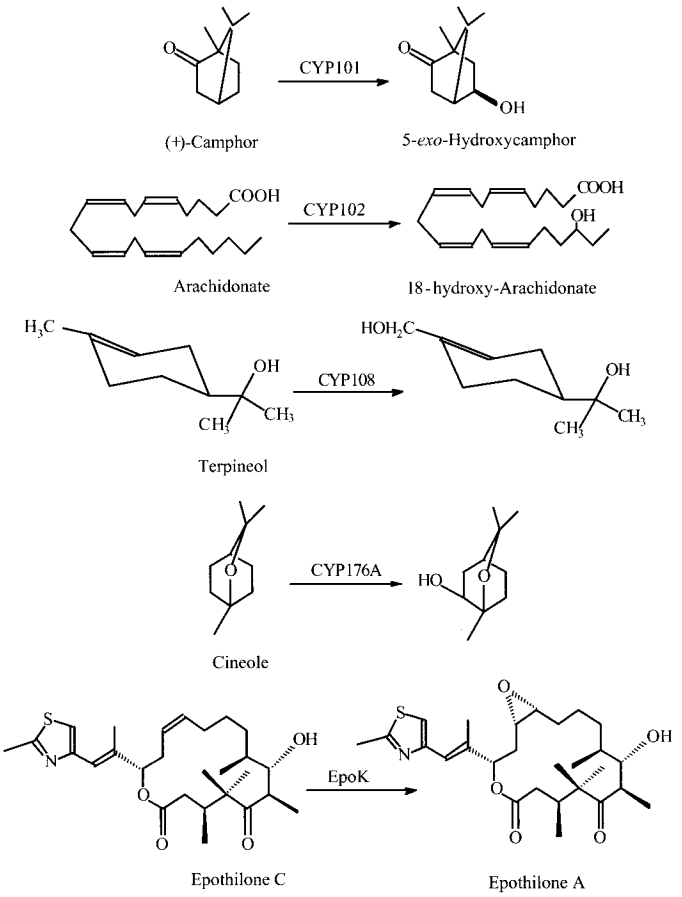


Figure 13.4. Examples of bacterial CYP reactions.

metabolism, although roles in pathways of secondary metabolism are also emerging[61].

As with all CYP activities, the substrates are largely lipophilic, and this is true of the other bacterial archetypal CYP, CYP102A1 ($P450_{BM-3}$), which as a class II system utilizing an FAD and FMN containing reductase domain, has provided a model for eukaryotic CYPs. This protein, containing both CYP and reductase domains in a soluble fusion protein that metabolizes various fatty acids, mostly at ω-2, was characterized in a series of publications from Fulco and colleagues during the 1980s[13, 62–64]. The rates of various reactions have been studied and this protein turns over substrate more efficiently than other CYPs utilizing separate redox proteins, with a rate of 17,000 min^{-1} observed for arachidonate metabolism. Structural considerations for this protein are not the object of this chapter, but resolution of the structure of the CYP domain was a landmark in the field[65], and allowed numerous subsequent investigations using site-directed mutagenesis to probe the structure as well as the reductase domain[66–68].

Besides being present in proteobacteria, CYPs are also present in archaebacteria and some of these, CYP119 from *Sulfobolus solfataricus* and CYP175 from *Thermus thermophilus*, have had their structure solved[69, 70]. While these are interesting, the absence of known endogenous function precludes their discussion here. Thermophilic CYPs have been studied and we await functions, as well as CYP stucture/function for CYPs with activity at low temperatures that may have industrial uses.

5. Biodiversity of Bacterial CYPs and the Actinomycetes

The actinomycete bacteria encompass a wide range of species, including *Rhodococcus* spp., *Corynebacterium* spp., *Mycobacterium* spp., and *Streptomyces* spp., and represent important organisms for biotechnology in terms of enzymes, natural products, biotransformations, and bioremediation. Many are saprophytic, soil-inhabiting, gram-positive bacteria with a high G+C content, and some are also life-threatening human pathogens.

As mentioned earlier, many bacteria, including within the actinomycetes *C. diptheriae*, possess no CYPs, but the actinomycetes have revealed many genomes containing numerous CYPs. Mostly these are orphans with no known function, but included are new classes of CYPs from *Rhodococcus* described above, and from genome projects the diversity is surprising, with 20 *CYPs* in *M. tuberculosis*[71], 18 in *S. coelicolor*[72, 73], 33 in *S. avermitilis*[74], and 39 in *M. smegmatis*[33]. The genomes of streptomycetes are larger than the mycobacterial genomes with almost twice as many genes arranged on a linear chromosome. Thus, *S. coelicolor* contains 18 *CYPs* among approximately 7,825 open reading frames, that is, 0.2% of genes, while *M. smegmatis* has 39 out of approximately 3,800 genes (1% of genes). This latter proportion is similar to that observed in plants. Figure 13.5 shows a phylogenetic tree of the mycobacterial CYPomes of both *M. tuberculosis* and *M. smegmatis*.

The genomes of the mycobacteria and streptomycetes do not contain many CYP families in common. Both contain a *CYP51*-like *CYP*, and in *S. coelicolor* and *S. avermitilis* this *CYP* is called *CYP170A* and lies adjacent to a sesquiterpene cyclase that may well be of related function[50, 74]. One *CYP*, *CYP125*, was originally observed in *M. tuberculosis*, and recently a *CYP125* was also found in *S. avermitilis*.[74] *CYP105s* originally found in streptomycetes and associated with xenobiotic metabolism have also now been identified in *M. smegmatis* (Figure 13.5), as well as a *CYP107*[75–78]. The only other CYP family seen in both genera is a *CYP102* identified in *S. coelicolor* and *S. avermitilis*[73, 74], and as a pseudogene seen in the dramatic gene decay observed in *M. leprae* (Table 13.3).

5.1. Mycobacterial CYPs

The diversity of mycobacterial CYPs has been mentioned and trees relating the different CYP families of *M. tuberculosis* and *S. coelicolor* indicate they are quite different, reflecting the many hundreds of millions years since divergence from a common ancestor.[14, 73] A list of CYPs of *M. tuberculosis* is shown in Table 13.2, where the presence of these families in other mycobacteria is also shown. All *M. tuberculosis* CYPs conform to the expected conserved amino acids within the sequence of a CYP, including a conserved T

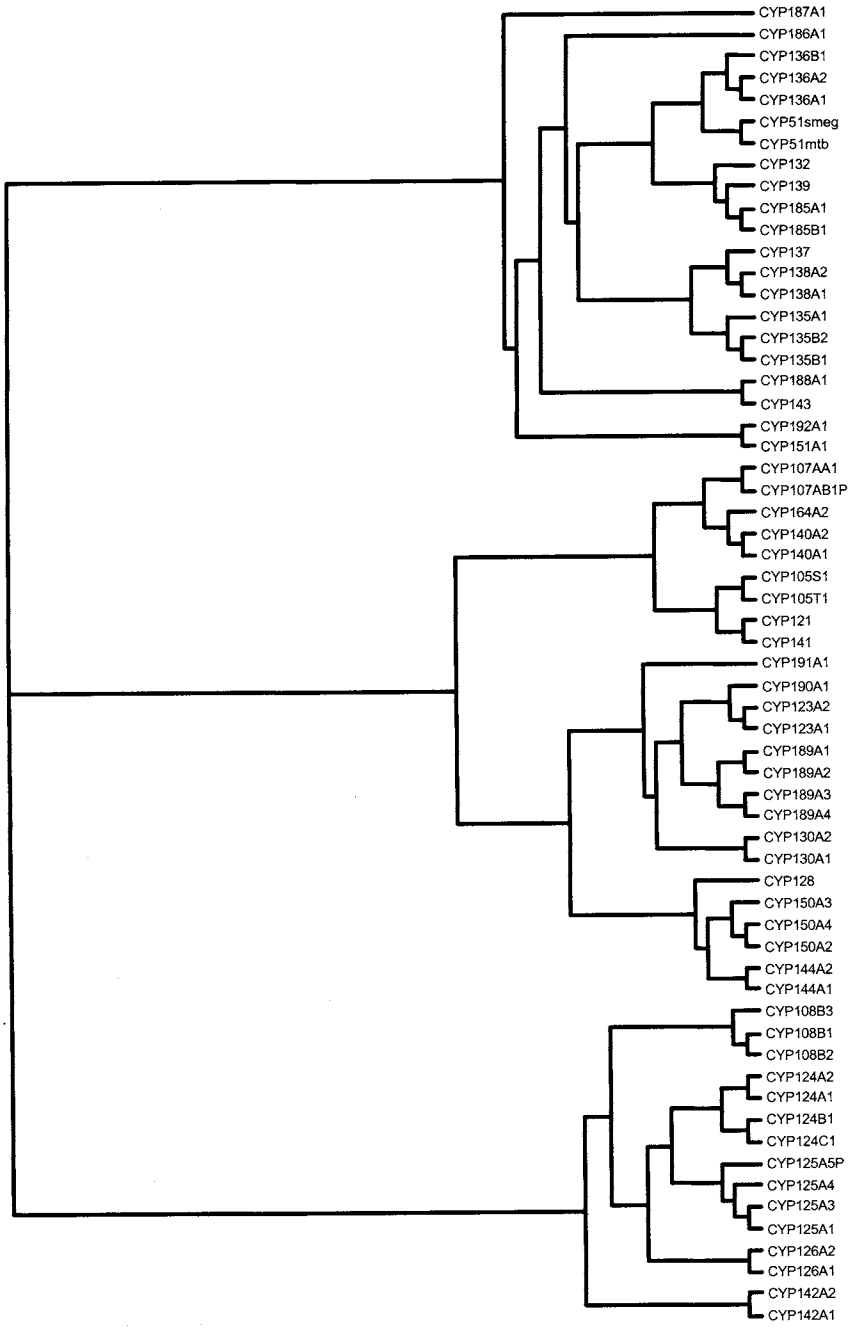


Figure 13.5. A phylogenetic tree of the CYPomes of *M. tuberculosis* and *M. smegmatis* using Clustal X and Treeview. Where present the *M. tuberculosis* CYP family member is designated A1. CYP families 121, 128, 132, 135A1, 137, 139, 141, and 143 of *M. tuberculosis* are missing in *M. smegmatis*, but a homologue of *M. leprae* CYP164A1 (CYP164A2) is included. New families of CYPs 186–192 were identified, new members of the CYP108 family and CYP105 and CYP107 family members are also found in streptomycetes. CYP125 showed two genes and a pseudogene (CYP125A3, A4, and A5P).

within the I-helix involved in oxygen-binding/electron transport, an EXXR motif in the K-helix, and a C-heme ligand in the C-terminal region.

Compared to the published sequence of the *M. tuberculosis* genome, *M. bovis*, although very closely related to *M. tuberculosis*, has only 18 *CYP*s, with *CYP130* being absent and *CYP141* being present as a pseudogene. The sequence of *M. leprae* showed massive gene decay as this organism moved toward parasitism, with only approximately 1,800 genes retained vs 3,800 in *M. tuberculosis*. Only 12 close homologues of the *M. tuberculosis CYP* complement were detected in *M. smegmatis* (Table 13.2), and this has the largest complement so far with 39 *CYP*s. When completed, the genomes of the related species *Mycobacterium avium* and *M. avium* ssp. *paratuberculosis* will have a similar number of *CYP*s, based on preliminary analysis of the data deposited at TIGR.

CYP164A2, the *M. smegmatis* homologue of *M. leprae ML2088* (*CYP164A1*), is 1,245 bp in length, encoding a predicted protein of 414 aa with a molecular weight of 44.9 kDa. By comparison, *CYP164A1* is 1,305 bp long and encodes a predicted protein of 434 aa with a molecular weight of 47.6 kDa. *M. smegmatis CYP162A2* is 60% identical (249/415) and 75% similar (313/415) in a 415 aa overlap with *CYP164A1* (BLASTP score e-130). Homology extends across all regions of the proteins, with only two gaps. In contrast, the closest *M. tuberculosis* homologue to CYP164A1, CYP140, shows only 38% identity (145/379) and 515 similarity (196/397), with 30 introduced gaps (BLASTP score 3e-58). The leprosy genome also contains a separate pseudogene of *M. tuberculosis CYP140* at locus *ML2033*, so these *CYP*s are likely to be functionally distinct.

The analysis of the CYP families present in *M. smegmatis*, reveal that, as expected, many new families covering *CYP186–192*, have been identified. The CYP family members in *M. tuberculosis CYP121*, *128*, *132*, *135A*, *137*, *139*, *141*, and *143* are not found in *M. smegmatis*. One CYP had already been identified before the *M. smegmatis* genome sequence, and this CYP was involved in morpholine utilization (*CYP151*)[79]. Interestingly, this soil microorganism has been associated with useful bioremediation properties and this may in some instances be associated with the CYP complement. Included in this CYPome are the first other members of the CYP108 family similar to $P450_{TERP}$ (CYP108A1)[59]. This may well reflect utilization of similar carbon sources to terpineol for growth. The study of *M. smegmatis* CYPs in bioremediation will be an important area of future research, as for the fungus *Phanerochaete chrysosporium* discussed later, for which 1% of the genes also encode CYPs. Common with the findings of many other genomic projects, the function of the mycobacterial CYPs remains unknown; however, as these are elucidated by gene knockouts, transcriptomics, proteomics, and metabolomics, there will be benefits for medical science and biotechnology.

5.2. Biodiversity in Streptomycetes

Streptomycetes are organisms with a complex life cycle, which involves the formation of a filamentous mycelium giving rise to aerial hyphae that produce spores. This, in part, explains the requirement for a larger genome in these bacteria that are also important producers of bioactive molecules (secondary metabolites). These metabolites represent about two thirds of the microbially derived compounds that include antibacterial (erythromycin, tetracycline), antifungal (amphotericin, nystatin), antiparasitic (avermectin), immunosuppressor (FK506), anti cancer (adriamycin), and herbicidal (bialaphos) compounds. Structural diversity is observed within all of the compounds and CYPs participate in oxidative tailoring of many of these, and thus play a key role in many of these pathways. The smell of wet earth on a spring day, resulting from geosmin, is also a product of actinomycetes/streptomycetes. Geosmin requires CYP for its biosynthesis and in *S. avermitilis* this is probably undertaken by CYP180A1[74]. With all these important biosynthetic pathways in which CYPs are known to participate (Table 13.4, Figure 13.6), there is of course interest in the cryptic pathways associated with the many orphan CYPs of streptomycete genomes for which function has yet to be detected. Apart from natural product biosynthesis, streptomycete CYPs have been identified as good biocatalysts with particular attention to xenobiotic metabolism by CYP105D1, identified

in *Streptomyces griseus*[77], and CYP105A1 from *S. griseolus*, which has been used to manipulate herbicide tolerance in plants[61]. Indeed, streptomycetes have been used as a source of drug metabolites by fermentation[80], and have also been used for stereo- and regio-specific biotransformations[81, 82].

Among the streptomycetes, two genomes have already been released into the public domain, for *S. coelicolor* A3(2) (ref. [72]) and for *S. avermitilis*[83]. The *S. coelicolor* genome of 7,825 open-reading frames contained 18 putative CYPs that were cataloged and expressed in a systematic study[73]. This laboratory strain is the model for streptomycetes and produces a number of secondary metabolites, although none are currently commercially important. The CYP roles are unclear, as the CYP51-like protein now called CYP170A1, is not involved in sterol biosynthesis and a gene deletion event was found not to be lethal[50]. Surprisingly, six of the eighteen CYPs are associated with operons with a conserved structure that have been called conservons[72]. In these, the first open-reading frame encodes a sensor kinase, then two open-reading frames of unknown function followed by a gene encoding an ATP-binding domain and finally, in some cases, encoding one or two *CYPs*. In conservon 10, the downstream *CYPs* are *CYP157A1* and *CYP154C1*; in conservon 11, the downstream *CYP* is *CYP157B1*; in conservon 12, the downstream *CYPs* are *CYP156A1* and *CYP154A1*; and in conservon 13, the downstream *CYP* is *CYP157C1*. The functions of these CYPs is intriguing, and as CYP154C1 can metabolize antibiotics, it could be related to a chemical defense and detoxification system[84]. Recently, a conservon from *S. griseus* was isolated containing a CYP157 homologue. Gene inactivation of the first gene of the conservon resulted in precocious formation of mycelium and secondary metabolism, suggesting this operon regulates the onset of differentiation[85].

Table 13.4. Streptomycete Cytochromes P450 Including CYP Assignments Where Available (www.drnelson.utmem.edu/P450.family.list.html).

Streptomyces sp.	CYP identification	Bioactive molecule produced	Function
S. griseolus	CYP105A1, 105B1, 105C1		
S. carbophilus	CYP105A3		
S. griseus	CYP105D1, 105D2, 107F1		
S. scerotialus	CYP105D3		
S. lividans	CYP105D4		
S. lavendulae	CYP105F1, 107N1, **160A1**	Complestatin	Anti-HIV
S. noursei	CYP105H1, **161A1**	Nystatin	Antifungal
S. tendae	CYP105K1, **162A1**	Nikkomycin	Insecticidal
S. fradiae	CYP105L1, 113B1, **154B1**	Tylosin	Promotant
S. clavuligerus	CYP105M1		
S. thermotolerans	CYP107C1		
S. erythraea	**CYP107A1, 107B1**	Erythromycin	Antibacterial
S. antibioticus	**CYP107D1**	Oleandomycin	Antibacterial
S. hygroscopius	CYP107G1, **122A2, 122A3**	Rapamycin	Antibacterial
S. venezuelae	**PikC (PicK)**	Pikromycin	Antibacterial
S. maritimus	CYP107R1		
S. peucetius	CYP129A2, **131A1, 131A2**	Daunorubicin	Antitumor
S. spheroides	**CYP163A1**	Novobiocin	Antibacterial
S. avermitilis	**CYP171A1**	Avermectin	Antiparasitic
S. acidscabies	**TxtC**	Thaxtomin	Phytotoxin
S. nodosus	**Orf1, Orf2**	Amphotericin	Antifungal

Notes: Many are involved in biosynthetic gene clusters of commercially important bioactive natural products and those implicated in this biosynthesis are in bold. The list does not include those found in the genomes of *Streptomyces coelicolor* and *Streptomyces avermitilis*.

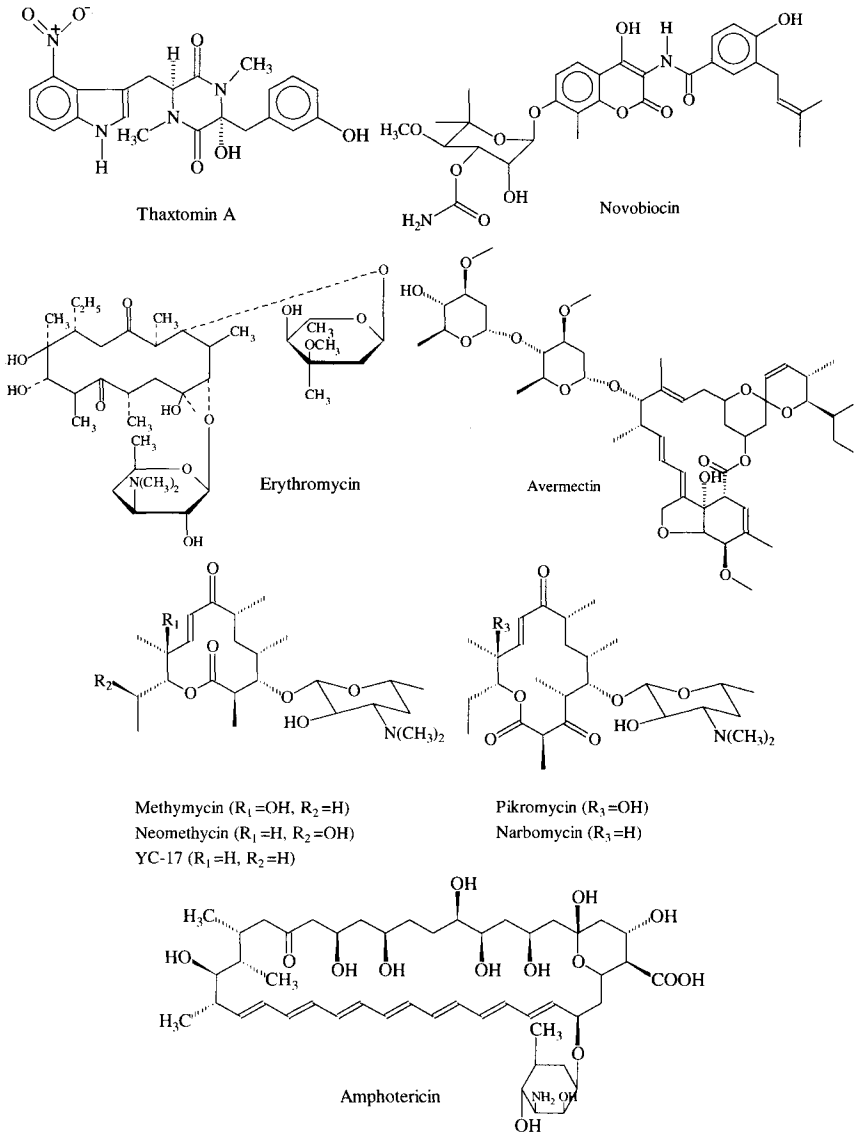


Figure 13.6. Some of the natural products used as drugs and produced by various streptomycetes.

Some of the functions of the *S. coelicolor* CYPs are in secondary metabolism operons, for example, *CYP158A2* located downstream of a Type III polyketide synthase and *CYP105N1* located downstream of a nonribosomal peptide synthase. These are currently subject to functional studies using the tools of gene disruption and metabolite/metabolome profiling, although not all the secondary metabolite genes identified have previously detected known end-products. The structural genomics of these CYPs is also in progress, with the first structure reported already for CYP154C1[84].

The second streptomycete genome completed has revealed 33 CYPs comprising 0.4% of genes. This industrial microorganism is important for the production of avermectin, an anti-helminthic agent. We have cataloged the *S. avermitilis*

CYPome and of note is the discovery of seven new families and two conservons containing CYPs[74]. The CYP157s within the conservons, as in *S. coelicolor*, deviate from the prior conservation in CYPs of an EXXR motif within the K-helix. CYP157A2 and CYP157C2 exhibited a ^{235}EVLW and ^{257}EQSLW motif, respectively. Together with the *S. coelicolor* CYP156A1 motif of ^{272}STVR, the need for E and R is not essential, leaving only the heme cysteinyl ligand as the amino acid essential in all CYPs. These motifs are the subject of current experimental examination.

There were no clusters of *S. avermitilis* CYPs in a single subfamily except for *CYP105D6* and *CYP105D7*. These *CYP105D* forms seem associated with filipin biosynthesis, so that the roles of CYP105s in xenobiotic rather than secondary metabolism may well need reconsideration. CYP171A1 was involved in C8a oxidation in avermectin biosynthesis, CYP107W1 in oligomycin biosynthesis through oxidation at C12, and CYP180A1 is predicted to be involved in geosmin biosynthesis[74]. The closest homologue of CYP180A1 in *S. coelicolor* is CYP107U1, but geosmin is made by *S. coelicolor* and so must be synthesized using a CYP from a different family. When identified, it will require a modification of the nomenclature to place CYPs with common function in the same family, an illustration of some of the problems relating homology and CYP families to function.

Other CYPs identified in this genome include *CYP102s*, one of which encodes a fusion protein with a CYP and a reductase domain unlike the single *CYP102B1* found in *S. coelicolor A3(2)*. The *CYP102*s are also seen in bacillus species that are sporulating bacteria, but the endogenous function remains unclear. The *S. avermitilis* genome also contained a *CYP125*[74]. This had only previously been seen in mycobacteria, but was not present in *S. coelicolor* and was the first *CYP* family detected in two diverse actinomycete species.

Eleven of the *CYP*s from *S. avermitilis* were located in known gene clusters involved in secondary metabolism, including that of geosmin, avermectin, filipin, and pentalenolactone biosynthesis, and again, as in *S. coelicolor*, others appear to be involved in uncharacterized pathways of secondary metabolism. *CYP178A1* is in a cluster with a nonribosomal peptide synthase, *CYP107Y1* and *CYP181A1* are associated with a Type II polyketide synthase, *CYP154D1* is adjacent to an indole dioxygenase (and maybe involved in xenobiotic breakdown), while *CYP158A3* is adjacent to a Type III polyketide synthase, as is *CYP158A2* in *S. coelicolor*, which is likely to have a common function.

The emergence of further streptomycete genomes will add to our understanding of *CYP* evolution, and the numbers of *CYP*s in unknown pathways of secondary metabolism will reveal new natural products. Given the estimate that only 1% of microorganisms are culturable, the depth of the biocatalytic reservoir of CYPs becomes evident. The reason why the streptomycetes and mycobacteria have many CYPs is not clear, as other soil bacteria contain either small numbers of CYPs (Table 13.1) or none.

5.3. CYP biodiversity in Archaebacteria

CYPs also are found among the archaea in under a third of the genomes so far sequenced. Functional information about them is totally absent. Whether they arose later in evolution, after the appearance of oxygen, is also unclear. Their ability to maintain integrity in extreme conditions of temperature, and so on, could be useful in biotechnology as is the ability to function at low temperatures. The structures of two CYPs have been obtained. First, the CYP119 structure from *S. solfataricus* has been obtained and the heat stability of the protein has been ascribed to clusters of aromatic residues[69]. The second structure of CYP175A1 from the thermophilic *T. thermophilus* HB27 has also recently been solved[70].

6. Fungal CYPs

The yeast *Saccharomyces cerevisiae* has three *CYP* genes. *CYP51* was identified in 1987[35], *CYP57* was found in 1994 to be responsible for the synthesis of dityrosine, which is needed for the yeast spore wall[86], and *CYP61* was identified in 1995 as a sterol C22-desaturase in a proteomic study linking protein information to the emergence of this genome[87]. Fungal CYPs are generally class II associated with the endoplasmic reticulum and a single reductase drives all the CYPs, as in humans.

This limited diversity of CYPs was also seen in the fission yeast *Schizosaccharomyces pombe* that had *CYP51* and *CYP61* only, the minimal CYP requirement for ergosterol biosynthesis. The number of completed fungal genomes in the public domain is limited, but will expand in the coming years with sequences for major human and plant pathogens. This will rectify the current imbalance, given the economic, biomedical, biotechnological, and scientific reasons for obtaining fungal genome data and the large scientific community that will use this information. A shotgun analysis of the genome of *Candida albicans*, a major human pathogen, has been completed, but is not yet published at the time of writing. Our analysis of this genome reveals approximately 12 putative CYPs, including *CYP51* and *CYP61*, but also a novel orphan form *CYP501* and many members of the *CYP52* family. This was surprising, as these alkane utilization proteins are found in soil/environmental yeasts like *Candida tropicalis* and *Candida maltosa*. Perhaps these CYPs are involved in utilizing the host lipid in animals, or an otherwise unrecognized environmental niche for *C. albicans* exists. Another interesting observation is the presence of a *CYP56* homologue, that in *S. cerevisiae* is involved in dityrosine formation for the spore wall after yeast meiosis to produce tetrads[86]. However, dityrosine has been detected in the mitotic cell wall of *C. albicans*[88].

It was surprising that the *Neurospora crassa* genome contained 38 CYPs in a 40 MB genome. The genome of this filamentous fungus contained many CYPs from existing families, but as usual for a eukaryotic species of a previously unvisited biological type, it contained many new families of orphan function. The families observed were *CYP51*, *61A5*, *53A4*, *54*, *55A6*, *65B1*, *65C1*, *68D1*, *505A2*, and *507A1*, besides *CYP527A1* to *CYP553A1* which represent new families. No doubt similar numbers of CYPs will be identified in other fungi, but so far *P. chrysoporium* has many more for a fungal species (http://drnelson.utmem.edu/nelsonhomepage.html).

There is interest in using fungi in bioremediation and one of those that has been used commercially is the white-rot fungus *P. chrysosporium*. This is a basidiomycete, higher fungus with about 10,000 genes. Probing the unannotated genome revealed approximately 123 heme-binding motifs, so that approximately 1% of the genes of this microorganism encode for CYPs. This organism can degrade recalcitrant pollutants and the CYP system has been implicated in this activity[89–91]. The fungus is also commonly seen as a bracket fungus that can break down wood. As plants utilize only a small number (four) of CYPs to synthesize lignin it seems unlikely that all the CYPome of *P. chrysosporium* is involved in this aspect of metabolism, so much remains to be discovered about function of the orphan CYPs. Only one NADPH-reductase for the CYPs was present and this has been expressed and characterized[91, 92].

Fungal comparative genomics is in an early stage and, as with streptomycetes, much information about secondary metabolism is anticipated. Fifteen further fungi are to be sequenced, including the pathogen *Aspergillus fumigatus*. Also of interest will be the pathogenic basidiomycete *Cryptococcus neoformans* that, as with *P. chrysosporium*, may have a large CYPome as it is also associated with life in hollow eucalyptus trees and may therefore have evolved in a similar niche. Information on these projects is available on the web at the Sanger Center and TIGR sites.

Purification of fungal CYPs from cell extracts is a difficult task due to the usual low specific content, instability, and the presence of multiple forms. The fungal steroid hydroxylase CYPs have been studied and a polycyclic aromatic hydrocarbon hydroxylase[93, 94]. The emergence of genomes and the ability to express the CYPs present in *E. coli* or yeast has greatly facilitated their study, as will the application of transcription profiling.

In an early study by British Petroleum, the use of a *Candida* sp. producing single-cell protein from oil was envisaged. Although this became economically unviable during the 1970s with the rise in oil prices, it became apparent that CYP was responsible for the initial oxidation and that the CYPs responsible were from a new family, *CYP52*[95, 96]. Other *CYP52*s have been found and studied in many yeasts, including *C. maltosa* and *Yarrowia lipolytica*[97, 98]. Typically, many *CYP52*s are present in these yeast. Eight were found in *C. maltosa*, and knocking out four of these genes (also called *ALK* genes in the yeast nomenclature) prevented growth on n-alkane[97].

Other fungal CYP families identified include a benzoate *para*-hydroxylase from *Aspergillus niger* and a cycloheximide inducible *CYP54* from *N. crassa*[99, 100]. CYP55 ($P450_{nor}$) from *Fusarium oxysporum* represented a new class of CYP, as it is soluble, and carries out nitric oxide reduction without the need for a CYP-reductase (CPR) or a requirement for molecular oxygen. It was the first eukaryotic CYP to have its structure resolved and was a member of a new class of CYP[23].

CYP56 was found to be needed for dityrosine production for the spore walls of *S. cerevisiae*[86], while *CYP57* was identified among a group of six pea pathogenicity genes as a gene on a supernumary chromosome of *Nectria haematococca*. It plays a role in detoxifying the phytoalexin pisatin produced by the plant host[101]. CYP61, as mentioned above, is responsible for sterol C22-desaturation during ergosterol biosynthesis[87, 102–104]. Interestingly, some rice planthoppers and anobiid beetles use symbiotic yeast-like symbionts as a sterol source[105]. However, unlike in the beetles, the planthopper symbiont has a defective *CYP61* containing nonsense mutations and therefore accumulates ergosta-5,7,24(28)-trienol. The selection of this change is interesting in terms of the benefits in the relationship.

Some CYPs (CYP58, 59, 64) have been found to play a role in aflatoxin and mycotoxin biosynthesis[106–108], while *CYP68* is in a gene cluster involved in giberellin biosynthesis in *Giberella fujikuroi*[109], and another CYP is in the biosynthetic pathway of paxilline synthesis by *Penicillium paxilli*[110]. Fungal genome analysis will reveal many more *CYP*s involved in biosynthetic pathways of known and unknown natural products. Further novel CYP forms can also be anticipated, such as CYP505 from *F. oxysporum* that metabolizes fatty acids and is a membrane-bound CYP with a C-terminal CPR fusion[111].

7. Azole Antifungals and the Evolution of New Resistant Genes

Antifungal treatments have become increasingly important as fungal infections have become one of the top five most frequently encountered in the clinic. This increase is associated with the opportunistic nature of these fungal microorganisms that prey on the old and young, but also increasingly on patients in intensive care as well as with HIV, during cancer chemotherapy, and after organ transplantation[112]. The infections are by a variety of fungal species that also vary geographically, as well as demographically, but among the most important are *C. albicans*, increasingly other *Candida* spp. (such as *Candida glabrata* and *Candida krusei*), *C. neoformans, A. fumigatus, Histoplasma capsulatus, Pneumocystis carinii, Coccidiodes immitis, Penicillium italicum, Fusarium* (normally associated with diseases in plants), and even man's best friend, *S. cerevisiae*. Equally frequent are the skin and nail infections produced by dermatophytic fungi (*Trichophyton rubrum, Epidermophyton* spp., *Microsporum* spp.), which represent a significant market for drugs, albeit not because of life-threatening conditions. However, of the hundreds of thousands of fungal species, only about a hundred are reported as pathogens[113].

7.1. The Fungal CYP51 System

During the 1960s and 1970s a series of agrochemical fungicides and clinical antimycotics became available that were found, in studies with the plant pathogen *Ustilago maydis*, to be inhibiting sterol C14-demethylation[114]. This was also observed for azole compounds when treating *C. albicans* infections[115]. This step of sterol biosynthesis had been postulated to be a cytochrome P450-mediated activity[116, 117]. In pioneering work by Yoshida, Aoyama, and colleagues, CYP was purified and characterized from *S. cerevisiae* and, in a series of studies, they looked at the demethylation event occurring via three sequential monooxygenase reactions, and also at the enzymatic and electron transport requirements of the system that involved a typical eukaryotic NADPH-CPR[118, 119]. The reaction sequence was tested using recombinant *C. albicans* CYP51 protein and it was shown that the acyl-carbon bond cleavage occurred by a mechanism similar to those proposed by Akhtar and colleagues for the reactions involving CYP17 and CYP19[120, 121]. In other studies, CYP was purified from *S. cerevisiae* and shown to metabolize benzo(*a*)pyrene[122]. This was presumably the form that was responsible for activating pro-carcinogens in yeast genotoxicity

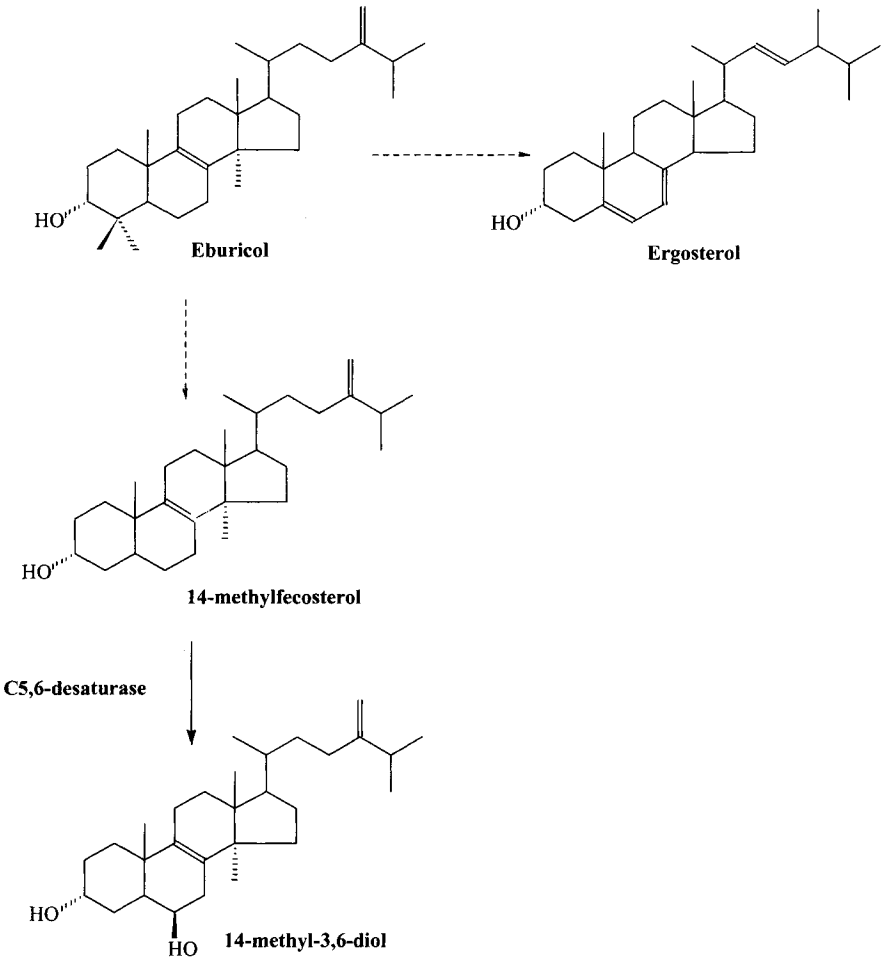


Figure 13.7. The sterols accumulating under azole treatment of *C. albicans* including the end-product, 14α-methylergosta-8,24(28)-dien-3β,6α-diol, that does not support growth and requires sterol C5-desaturase for biosynthesis. In most fungi, the substrate for CYP51 is more likely eburicol under normal conditions, and not lanosterol, although both are metabolized.

assays[123, 124]. The CYP purified was most likely CYP61, which has a low-level activity in benzo(*a*)pyrene metabolism as well as an endogenous activity in sterol C22-desaturation[49, 104, 125].

Genetic analysis of *CYP51* by gene disruption, the first for a CYP in any organism, demonstrated the essential nature of the gene product[35]. Chemical inhibition of CYP activity was also studied and genetic suppression of the effect of azole inhibition (by ketoconazole and fluconazole) was found to be mediated by sterol C5-desaturase mutants. These mutants changed the sterol accumulating under treatment from a fungistatic end-product, 14α-methylergosta-8,24(28)-dien-3β,6α-diol, to a functional end-product, 14α-methylfecosterol. This conversion required sterol C5-desaturase and therefore revealed sterol C5-desaturase to be implicated in the mode of action of azoles through introduction of a 6-OH group into 14-methyl sterol in a presumed attempted desaturation[126]. Subsequently, this was also found to suppress the genetic disruption of the *CYP51* locus[127, 128]. The discovery in the clinic of similar azole-resistant sterol

C5-desaturase mutants of *C. albicans* confirmed the central role of this biotransformation in the mode of action in a clinical setting[129, 130]. Sterol profiles of untreated and treated *C. albicans* illustrate the major sterols accumulating in wild-type and sterol C5-desaturase defective mutants (Figure 13.7).

It has also been found that the lethal effect of a sterol C4-methyl oxidase gene knockout can be suppressed by a mutation in *CYP51*[131]. These mutants accumulate lanosterol without further sterol metabolism and this indicates that lanosterol itself can support yeast growth despite previous sterol-feeding studies to the contrary[132]. Consequently, retention of a C14 methyl group on a sterol does not in itself make the sterol nonfunctional, as might be expected from the original events resulting in the evolution of the sterol pathway.

One additional point of interest here is the use of azole antifungals and CYP51 in proof of principal experiments within functional genomics, as pioneered using the yeast genome. Transcriptome studies with *S. cerevisiae* following treatment with fluconazole revealed increased CYP51 expression, showing the effect of the inhibitor and the usefulness of transcriptomics for determining drug mode of action[133, 134].

For activity, eukaryotic CYPs located in the endoplasmic reticulum require NADPH-CPR to provide the first and/or the second electron needed for the catalytic cycle (see Chapter 4)[135]. Gene disruption of yeast CPR was surprisingly not lethal[136], although the genome subsequently revealed no further *CPR* genes[137], and cells still synthesized ergosterol[138]. The source of electrons to support the requirement of CYP51and CYP61 for ergosterol biosynthesis has been studied and genetic evidence and reconstitution studies have shown that cytochrome b5 and cytochrome b5-reductase are responsible (Figure 13.7)[139]. This represents a difference from animals, where a *CPR* gene knockout in *Caenorhabditis elegans* and mouse is lethal[140,141]. Further differences exist in the yeast CPR, for which soluble forms have been produced that can support CYP activity, as shown in reconstitution assays and in genetic complementation studies using suitable yeast strains[138, 142]. Thus assumptions based on studies with mammalian systems may not be applicable across other Kingdoms or in other areas of CYP biology.

7.2. Azole Activity and Resistance in Fungi

In the early 1980s, it was first reported that resistance to the antifungal agent ketoconazole occurred in patients suffering chronic mucocutaneous candidiasis, but a general problem with antifungal resistance had never been encountered clinically. Resistance, had however, been seen in agriculture in the 1980s with the use of related fungicides generically termed demethylase inhibitors (DMIs).

Azoles bind to the heme of fungal CYP as a sixth ligand, as evidenced by the generation of Type II spectra using fungal microsomes, typically with a maximum at approximately 430 nm and a minimum at approximately 410 nm[143]. This interaction involves the N-3 of an imidazole ring or the N-4 of a triazole ring as a ligand to the heme, resulting in the formation of a low-spin, azole-bound complex. It had been known from 1972 that imidazoles could be CYP inhibitors[144]. A similar interaction was seen for the antifungal pyridyl compound buthiobate on binding to purified *S. cerevisiae* CYP51[145]. The studies on buthiobate showed saturation of the Type II spectra with a one-to-one ratio of the antifungal and CYP51, reflecting the high affinity of binding, although buthiobate and other azole antifungals can be displaced by carbon monoxide.

The orally administered antifungal drug ketoconazole was followed into clinical use by fluconazole and itraconazole, and more recently voriconazole, with further compounds still in clinical evaluation trials, including posaconazole and ravuconazole (Figure 13.8)[112]. In contrast, the range of agrochemicals that are in use is more diverse, and although newer compounds have been developed with alternate modes of action, resistance problems will emerge, necessitating a requirement for azoles. Resistance has emerged as a serious problem for agricultural and clinical use of azoles, but there is seemingly no potential causal link as with bacteria and concerns over the use of growth promotant antibiotics on the farm. The compounds have a selective effect on the pathogen over the effect on the human/plant CYP51, although direct comparisons at the level of the enzyme suggest that the sensitivities are closer to each other than might be anticipated (>10-fold)[146, 147]. Possibly CYP pools give a

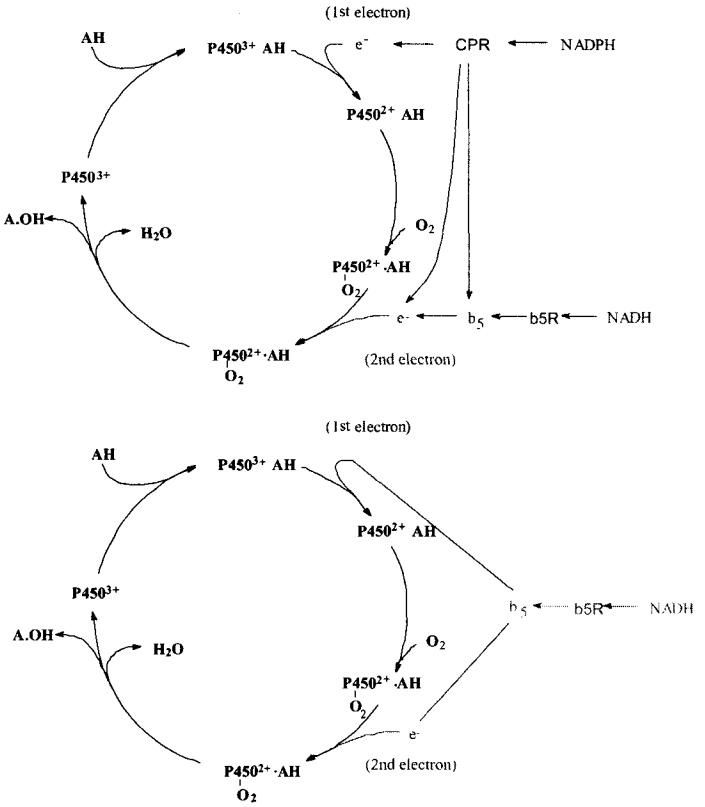


Figure 13.8. The typical CYP catalytic cycle with the potential for the second electron to be provided via cytochrome b5 rather than NADPH-cytochrome P450 reductase and the alternative observed in yeast where NADPH-cytochrome P450 reductase is not essential.

protective effect against CYP51 inhibitors in plants and humans.

With the emergence of the AIDS epidemic oropharengeal candidiasis became a serious problem and is often the presenting symptom indicating HIV infection, while in Southeast Asia it is often cryptococcosis. Aspergillosis is a serious health risk in organ-transplant patients and treatment success is much less than for common bacterial infections: here it appears that the latest azole drug voriconazole is superior to amphotericin, the previous drug of choice for this infection[112, 113]. With prolonged and often prophylactic use of azole drugs it is not surprising that resistance has emerged, requiring increasing drug treatment to control disease, especially for candidiasis. Molecular investigation of the mechanisms giving rise to resistance is ongoing the relevance of these for new azole drugs is being investigated. Some of these are shown in Figure 13.9.

Resistance to ketoconazole was studied in the 1980s in isolates obtained from patients suffering chronic mucocutaneous candidiasis. One of these, from the Darlington isolate(s), has been found to contain defective sterol C5-desaturase and altered CYP51 proteins, with the latter containing the substitutions Y132H and I471T[148]. Multiple mechanisms of resistance, between and within individual strains, is a common finding since the mid-1990s, when fluconazole resistance, primarily in HIV positive patients, became a clinical problem. First, some resistant strains of *C. albicans* from HIV patients were found to contain reduced concentrations of fluconazole[149, 150], and this was correlated with overexpression of transporters of the ABC superfamily, notably Cdr1p and Cdr2p

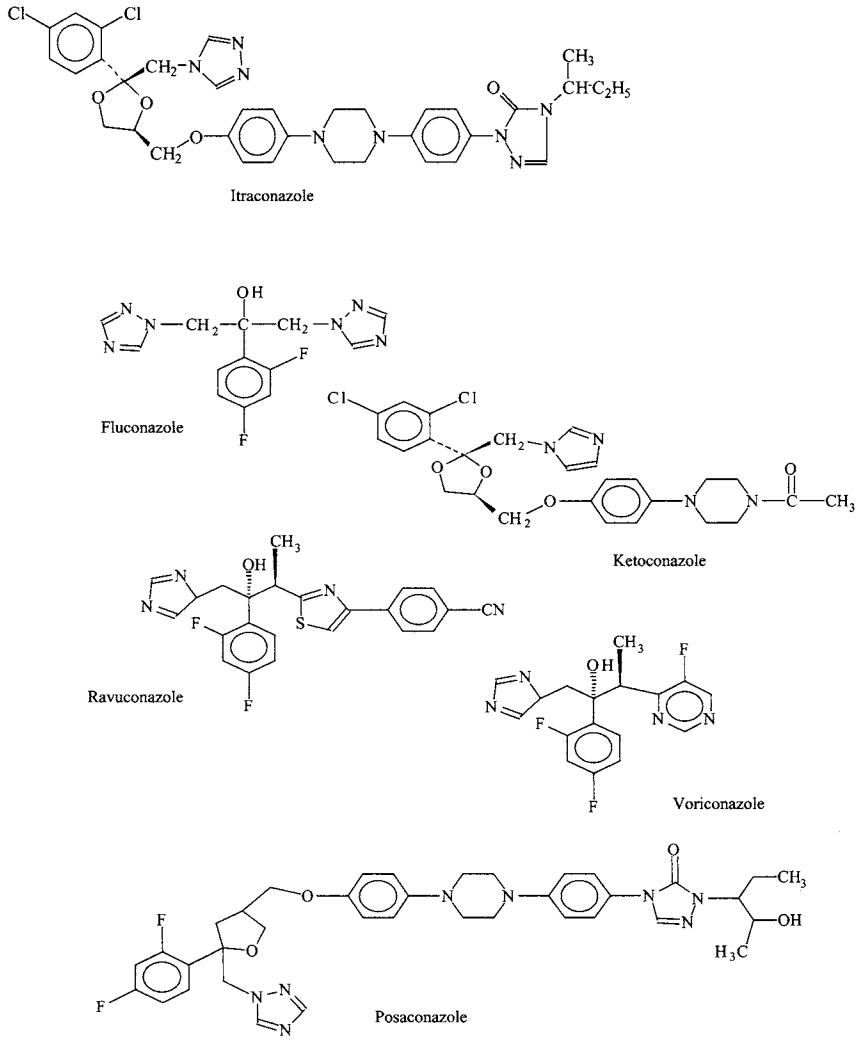


Figure 13.9. Some azole antifungals in current clinical use and under trial.

(Candida drug resistance proteins)[151, 152]. In addition, other studies implicated a major facilitator transporter of *C. albicans* in drug resistance (*MDR1*), as well as showing that *CDR1* and *CDR2* played a role in transporting the drug[151, 153, 154]. Other *C. albicans* resistant trains have shown defective sterol C5-desaturase activities, including some from AIDS patients and from patients suffering infections as a result of leukemia[129, 130].

Changes in CYP51 expression have been detected in resistant strains, but the role in causing resistance is unclear. For instance, in two studies on different series of isolates from two patients during the emergence of resistance, increased mRNA levels for CYP51 were observed in addition to increased transcription of drug transporters (CDR and MDR1) and substitutions in the amino acid sequence of CYP51 (R467K and G464S, respectively) [151, 154]. Overexpression of CYP51 on plasmids causes only a small increase in resistance, so this is not definitively identified as a cause of resistance[127]. In a different setting, a *Penicillium digitanum* resistant strain exhibited five repeats of a 126 bp sequence in the *CYP51*

promoter of a resistant strain, in comparison to one in a sensitive strain, and this may represent a resistance mechanism that results in enhanced *CYP51* expression[155]. *A. fumigatus* has two *CYP51* genes, as in other aspergilli[156], and this could also be the mechanism that makes it insensitive to fluconazole, although itraconazole resistance in *A. fumigatus* may be the result of mechanisms similar to those described in more detail here for *C. albicans*[157].

An isolate of *S. cerevisiae*, SG1, defective in sterol C14-demethylation, contained the substitution G310H, had an inactive protein, and was azole resistant[158]. The resistance was due to the second sterol C5-desaturase defect of the strain that suppressed the effect of the block in 14-demethylation. 14-Methyl-ergosta-24,(28)-dien-3,6-diol normally accumulates when 14-demethylation is blocked, but with a second defect in 5-desaturation, the functional sterol 14-methylfecosterol accumulates instead[127]. Other circumstantial evidence for mutant CYP51 resulting in azole resistance was observed[159], but it was not until 1997 that the first published evidence of changes in *C. albicans* CYP51 as a cause of resistance in practical settings appeared. These were reported for laboratory studies that had indicated that the change T315A in *C. albicans* CYP51 could alter fluconazole resistance when expressed in *S. cerevisiae*[160]. However, in a study of 18 resistant and 18 fluconazole resistant *C. albicans* isolates, a number of amino acid substitutions were observed in the resistant cohort suggestive of a causal link (Table 13.5). These included the change G464S observed in many subsequent investigations[161]. One particular problem was the absence of a parental isolate to compare resistant strains to, but a number of studies allowed sequential sets to be investigated that were obtained during the treatment of a patient and the emergence of resistance. In one set of 17 isolates, a number of molecular changes were observed, including increased transcription of Cdr1p ABC transporter that can confer cross-resistance to other azoles, and of Mdr1p, a major facilitator transporter, that confer fluconazole resistance[151]. During the study, increased transcript levels of CYP51 were also observed in later isolates. Furthermore, the substitution R467K in CYP51 produced an active protein with reduced affinity for fluconazole, as demonstrated by *in vitro* sterol biosynthesis in cell-free extracts and later in studies on the protein after expression in *S. cerevisiae*[162]. Another matched-set of *C. albicans* detected the appearance of G464S during the emergence of resistance[154]. As noted by White[153] and Loeffler *et al.*[161] the presence of homozygosity in this

Table 13.5. A List of Amino acid Substitutions Observed in CYP51 Among Clinical Fluconazole Resistant *Candida albicans*

CYP51 substitution	References
F72L	Favre *et al.*, 1999
F105L	Loeffler *et al.*, 1997
D116E	Favre *et al.*, 1999
F126L	Favre *et al.*, 1999
K128T	Sanglard *et al.*, 1998
G129A	Sanglard *et al.*, 1998
Y132H	Sanglard *et al.*, 1998
K143E	Favre *et al.*, 1999
A149V	Marichal *et al.*, 1999
D153E	Marichal *et al.*, 1999
E165Y	Marichal *et al.*, 1999
T229A	Favre *et al.*, 1999
E266Q	Sanglard *et al.*, 1998
E266D	Loeffler *et al.*, 1997
	Favre *et al.*, 1999
S279Y	Marichal *et al.*, 1999
K287R	Loeffler *et al.*, 1997
G307S	Perea *et al.*, 2001
S405Y	Favre *et al.*, 1999
S405F	Sanglard *et al.*, 1998
	Favre *et al.*, 1999
V437I	Sanglard *et al.*, 1998
	Favre *et al.*, 1999
G448E	Loeffler *et al.*, 1997
F449L	Favre *et al.*, 1999
G450E	Loeffler *et al.*, 1997
V452A	Marichal *et al.*, 1999
G464S	Loeffler *et al.*, 1997
	Sanglard *et al.*, 1998
	Marichal *et al.*, 1999
G465S	Loeffler *et al.*, 1997
	Marichal *et al.*, 1999
R467K	White, 1997
	Sanglard *et al.*, 1998
I471T	Kakeya *et al.*, 2000
V488I	Loeffler *et al.*, 1997

Notes: Not all these changes are known to cause resistance and only Y132H, G464S, and R467K have been subject to investigation at the level of protein.

diploid yeast implied two molecular events in the selection of resistant *CYP51*, an initial forward mutation followed by gene conversion of the second allele or (less likely on frequency grounds) a second identical mutational event in the second allele. This might be expected to produce higher level resistance than the heterozygous condition. Further point mutations in *CYP51* from fluconazole-resistant *C. albicans* were reported and yeast expression used to assess the resistance phenotype associated with the altered proteins and the altered sensitivity[152]. In many cases, more than one change from "wild-type" sequence existed and this allowed additive resistance changes and neutral *CYP51* polymorphisms to be detected. It is possible, as in reverse transcriptase substitutions in drug-resistant HIV, that selection could also involve changes that alter activity of the resistant protein and assist fitness.

Since these studies, a number of others have identified point mutations implicated in resistance using either *in vitro* sterol biosynthesis on cell-free extracts of the *C. albicans* strains, or more specifically, expression of the respective CYP51s and sensitivity testing using a heterologous host, *S. cerevisiae*. These studies have revealed a hugely diverse series of changes scattered across the protein and a list of these is included in Table 13.4[148, 154, 163–165]. Early molecular modeling of the CYP51 protein based on the CYP101 structure did not predict these residues as being important in azole interaction[166], although later models took into account a number of prokaryotic structures and existing information on fluconazole resistance mutations[167]. Interestingly, the latter model predicted a kink in the *C. albicans* CYP51 I-helix, and in the structure of *M. tuberculosis* CYP51 a break in the helix was found[44].

Of the mutations observed to-date, the helices B and B′, F126L, K128T, G129A, Y132H, K143R, F145L, and K147R would be close to the access channel. The Y132H mutation has been detected in a number of studies as well as in combination with different substitutions[148, 152, 163], and upon expression in yeast confers a 4-fold increase in fluconazole resistance and cross-resistance to itraconazole (2-fold) and ketoconazole (16-fold)[152]. E266D, R276H, D278E, and S279F are located at the end of the G-helix and G307S is the first substitution observed in the clinic to be related to resistance and located in the I-helix. Another cluster of residues associated with fluconazole resistance in *C. albicans* CYP51 are located before the heme-binding region consisting of G448E, F449L, G450E, and V452A, while G464S, G465S, R467K, and I471T are adjacent to the heme-cysteinyl ligand C470. While all these changes, together with further numerous unpublished alterations detected recently, need further characterization to define their effects on fluconazole binding, only F126, G464, and R467 are conserved across CYP51s. The numbers of mutants found with G464S and R467K were a surprise, as fluconazole binds above the heme as a sixth ligand with interaction of the N-1 substituent group within the active site. These substitutions below the plane of the heme are presumed to result in a change in the plane or orientation of the heme and to have a knockon effect on the location of the fluconazole bound above the heme[161, 162]. Surprisingly, in plant pathogens only a change in the equivalent residue to Y132 in *C. albicans* has so far been detected in grape powdery mildew among 19 resistant isolates[168]. This could make a simple diagnostic test for resistance feasible, as was also explored by Loeffler *et al.*[161] but the diversity of changes found in *C. albicans* CYP51 make this more complicated for clinical use.

To complete an understanding of the structural basis of resistance, biophysical studies will be required. A structure for a mammalian endoplasmic reticulum associated CYP has been achieved through expression of a soluble derivative and its crystallization[169, 170]. So far no reports of using *E. coli* and CYP51 for this purpose have occurred, but a soluble derivative that is active has been produced by expression of CYP51 containing an engineered protease site beyond the N-terminal membrane anchor. This may provide the route to resolving the structure if crystals can be obtained[142].

The nature of the diverse mutations giving rise to fluconazole resistance in *C. albicans* is remarkable, but mutations arose mainly in AIDS patients where resistance occurred in >10% during extended and prophylactic treatment[21]. With effective HIV treatment, this source of isolates has diminished as the immunocompetence of patients has improved. There will remain a problem, however, with continuation of azole use and development, and many facets of the resistance mechanisms remain to be unraveled, including

precise roles and effects of the diverse mutations in *CYP51*.

8. Conclusions

Microbial CYP biodiversity is presenting an unexpected challenge as the numbers of CYPs uncovered makes functional investigation through high-throughput genomics mandatory in addition to the more traditional, but essential approaches. The most facile organisms to study are the model organisms with well-established genetic systems, but many of the most interesting in terms of their CYP complements and environmental relevance, such as *P. chrysosporium,* will be important to tackle even if more recalcitrant. With many CYPs involved in metabolic pathways, an emphasis on changing CYPs to alter end-products will emerge. One of the most ambitious metabolic engineering projects to date involved the diversion of yeast ergosterol biosynthesis toward corticosteroid production, which involved deleting and adding CYPs to transgenic yeast[17]. Coupled to the ability to improve enzymes through processes of directed evolution, much will be possible in pathway improvement and invention as well as in biocatalysis[171].

Comparative genomics between close species and within strains will add to our information on the biological roles and evolution of CYPs. Contributions from currently unrepresented organisms from the fungi, protista, and algae will be of great interest in understanding CYP evolution. With 42 CYPs already detected in the slime-mould *Dictyostelium discoidium,* other protists are likely to have large numbers of CYPs. Where they are pathogenic, such as *Leischmeinia* and *Trypanosoma,* there will be the added interest of developing drugs to target these diseases via CYP inhibition, and azoles are already being used. The huge wave of CYP information from these projects will provide a major impetus to finding ways of establishing function and biotechnological uses in a systematic manner.

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Appendix

Human and Rat Liver Cytochromes P450: Functional Markers, Diagnostic Inhibitor Probes, and Parameters Frequently Used in P450 Studies

Maria Almira Correia

The tables in this appendix summarize the relative functional selectivities of substrates and inhibitors for the major human and rat liver cytochrome P450 isoforms (P450s). These hepatic isoforms are well recognized to catalytically participate in the metabolism of chemically diverse endo- and xenobiotics including drugs, and in the case of human liver P450s to thus contribute to clinically adverse drug–drug interactions. Consequently, these P450s are the targets of intense scrutiny in the pharmaceutical screening of existing or novel chemical agents of potential clinical relevance for drug development. At a more basic level, these tables provide information on established and/or potential diagnostic tools for the identification and/or characterization of the metabolic role of each individual P450 in the disposition of an as yet uncharacterized xeno- or endobiotic. Three critical issues are however worth consideration before use of these experimental probes: First, as discussed in Chapter 7 (Inhibition of Cytochrome P450 Enzymes), given the vast diversity of P450 isoforms and their differential active-site affinities for a given compound, the *"relative"* selectivity of a substrate or inhibitor probe for a given P450 isoform is entirely defined by the number of different P450 isoforms included in its evaluation as well as the range of substrate/inhibitor concentrations tested. Second, substrates and inhibitors determined to be "relatively selective" for a human liver isoform, may not necessarily be so for its rat liver ortholog, and vice versa. Third, the relative metabolic contribution of a P450 isoform to the *in vivo* hepatic metabolism of a given drug is directly proportional to the relative hepatic microsomal abundance of that isoform and its affinity for that compound, irrespective of its *in vitro* high metabolic profile assessed under "optimized" conditions. This issue arises because recent advances in recombinant P450 technology have made unprecedented amounts of purified human liver enzymes readily available for comparative *in vitro* characterization of drug metabolism, at relative P450 concentrations that may be irrelevant *in vivo*. Furthermore, this abundance albeit highly desirable, has nevertheless also sidelined the comparative characterization of rat liver orthologs of newly discovered human P450 isoforms. Thus, in some cases (i.e., CYP1B1), better characterized functional probes are available for the human liver enzyme than for its rat counterpart. In addition to the functional probe information, a table with

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some signature P450 spectral characteristics and parameters commonly used in P450 studies has been included as a quick reference guide.

It is to be noted that the tables in this appendix have been compiled with practical utility rather than comprehensiveness as the overall objective. Much more comprehensive coverage is available in several excellent books and reviews on human and rat liver P450s that are gratefully acknowledged as the sources of some of the information presented herein[1–27]. In particular, the reader is referred to the series *Methods in Enzymology* (Volumes 10, 52, 206, 272, and 357)[3–7], *Methods in Molecular Biology* (Volume 107, *Cytochrome P450 Protocols*)[8], Chapter 7 (Inhibition of Cytochromes P450), and Chapter 10 (Human Cytochrome P450 Enzymes). Last, but not the least, I wish to particularly acknowledge Prof. S. Rendic for having provided access to his regularly updated human P450 metabolism database through the Gentest website during the preparation of this Appendix. Wherever feasible, the literature citations in this appendix have favored those providing methodological details, assay modifications, and/or controversial information, sometimes at the expense of accurate chronological reporting of the literature.

Table A.1. Human Liver P450s: Functional Markers, Common Inducers, Diagnostic Inhibitors, and Noninvasive Probes[a]

P450s	Functional markers	Inducers	Inhibitors	Noninvasive probe
1A2	Caffeine *N*3-demethylation[b] Phenacetin *O*-deethylation[d] 7-Ethoxyresorufin *O*-deethylation[f]	Smoking Charcoal-broiled foods Cruciferous vegetables Omeprazole	Furafylline[c] Galangin[e]	Caffeine
2A6	Coumarin 7-hydroxylation[g]	Rifampin Phenobarbital	Tranylcypromine[h] R(+)-Menthofuran[i] 8-Methoxypsoralen[j]	Coumarin
2B6	(*S*)Mephenytoin *N*-demethylation[k] 7-Ethoxy-4-trifluoromethylcoumarin O-deethylation[n] Bupropion hydroxylation[p]	Phenobarbital	2-Isopropenyl-2-methyladamantane[l] 3-Isopropenyl-3-methyldiadamantane[l] Triethylenethiophosphoramide[o] Ticlopidine[q] Clopidogrel[q]	*S*-Mephenytoin?[m]
2C8	Paclitaxel 6α-hydroxylation[r]	Phenobarbital Rifampin	Trimethoprim[s]	
2C9	Diclofenac 4′-hydroxylation[t] Tolbutamide methylhydroxylation[v] Phenytoin 4-hydroxylation[x] (*S*)Warfarin 7-hydroxylation[y]	Phenobarbital Rifampin Dexamethasone	Sulfaphenazole[u] Tienilic acid[w]	Tolbutamide Warfarin Diclofenac
2C18	3-[2,3-Dichloro-4-(2-thenoyl) phenoxy]-propan-1-ol 5-hydroxylation[z]	Phenobarbital	Sulfaphenazole derivative[aa]	
2C19	(*S*)Mephenytoin 4′-hydroxylation[bb]	Phenobarbital Rifampin	(+)-*N*-3-Benzyl-nirvanol[cc] (+)-*N*-3-Benzyl-phenobarbital[cc] Ticlopidine[ee]	*S*-Mephenytoin[dd]
2D6	Debrisoquine 4-hydroxylation[ff] Bufuralol 1′-hydroxylation[hh] Dextromethorphan *O*-demethylation[jj]		Quinidine[gg] Paroxetine[ii] SCH66712[kk]	Debrisoquine Dextromethorphan
2E1	Chlorzoxazone 6-hydroxylation[ll] *p*-Nitrophenol hydroxylation[nn] *N*-Nitrosodimethylamine *N*-demethylation[pp]	Ethanol Isoniazid	4-Methylpyrazole[mm] Diethyldithiocarbamate[oo]	Chlorzoxazone
3A4	Testosterone 6β-hydroxylase[qq] Nifedipine oxidation[ss] Erythromycin *N*-demethylation[uu]	Dexamethasone Rifampin	Gestodene[rr] Ketoconazole[tt] Troleandomycin[vv]	Erythromycin 6β-Hydroxycortisol

Table A.1. (continued)

P450s	Functional markers	Inducers	Inhibitors	Noninvasive probe
	Midazolam 1′-hydroxylation[ww]		Azamulin[xx] Mifepristone[yy] Diltiazam[zz]	
3A5	Testosterone 6β-hydroxylase[aaa] Midazolam 1′-/4-hydroxylation[bbb] Alprazolam 4-/1-hydroxylation[ccc]			
3A7	DHEA 3-sulfate 16α-hydroxylation[ddd] Testosterone 6β-hydroxylation[eee]			
4A11	Lauric acid ω-hydroxylation[fff]	WY14,643[ggg]	10-(Imidazolyl)decanoic acid[hhh] HET0016[iii] 17-ODYA[jjj]	
4F2	LTB4 ω-hydroxylation[kkk]	Tretinoin[lll]	10-UDYA[mmm]	
4F12	Arachidonic acid ω-hydroxylation[nnn] Arachidonic acid ω-2-hydroxylation[nnn]			
7A1	Cholesterol 7α-hydroxylation[ooo]	Cholesterol		
8B1	Sterol 12α-hydroxylation[ppp]			

[a]For more comprehensive coverage see Chapter 10 (ref. [21]); Chemical structures of some of the agents are depicted in Table A.2.
[b]Refs [28–30].
[c]Refs [31–34].
[d]Refs [35–37].
[e]Refs [38], [39].
[f]Refs [40], [41].
[g]Refs [43–45].
[h]Refs [46–48].
[i]Ref. [49].
[j]Refs [48], [50].
[k]Ref. [51].
[l]Ref. [52].
[m]Plausible urinary excretion of the *S*-mephenytoin metabolite, nirvanol[51].
[n]Has been used but not very selective[53, 54]. Relative selectivity may be achieved at low (~5 μM) substrate concentrations.
[o]Ref. [55].
[p]Ref. [56].
[q]Inhibits CYP2B6 at 1 μM but CYP2C19 and CYP2C9 at higher (10 μM) concentration[57].
[r]Refs [58–60]. A specific monoclonal antibody that can inhibit CYP2C8 function is commercially (Gentest) available.
[s]An antibacterial diaminopyrimidine[61, 62].
[t]Refs [63], [64].
[u]Refs [18], [65], [66].
[v]Refs [67–71].

[w]Refs [72], [73].
[x]Refs [22], [23], [74].
[y]Refs [65], [75–77].
[z]Ref. [78].
[aa]4-Amino-*N*-ethyl-(2-phenyl-2H-pyrazol-3-yl)-benzenesulfonamide[79]. Also potently inhibits CYP2C8 whose contributions should be excluded.
[bb]Refs [73], [74], [80], [81].
[cc]Refs [82], [83].
[dd]Urinary excretion of the 4′-hydroxy (*S*)-Mephenytoin metabolite.
[ee]Refs [57], [84], [85]. Can distinguish CYP2C19 from other CYP2C isoforms, only after CYP2B6 contribution has been conclusively excluded by other means[57].
[ff]Refs [86–88].
[gg]Refs [89], [90].
[hh]Refs [87], [88].
[ii]Refs [90–93].
[jj]Refs [94], [95].
[kk]5-fluoro-2-[4-(2-phenyl-1H-imidazoyl-5-yl)methyl]-1-piperazinylpyrimidine[96].
[ll]Refs [97–99].
[mm]Refs [99], [100].
[nn]Ref. [101].
[oo]Refs [18], [102], [103]. Use as probe is controversial[104].
[pp]Refs [105–108].
[qq]Refs [109], [110].
[rr]Ref. [111].
[ss]Refs [112–114].
[tt]Can inhibit other P450s at concentrations >5 μM[18, 48, 115].
[uu]Refs [116, 117].
[vv]Refs [118, 119].
[ww]Refs [120, 121].
[xx]A diterpene azole antibiotic[122].
[yy]Refs [123–125].
[zz]Metabolic intermediate complex with CYP3A4 (ref. [126]).
[aaa]CYP3A5 has identical but considerably lower activities than CYP3A4. The CYP3A5 isoform may be distinguished by its relatively higher functional ratios of aflatoxin B_1 8,9-epoxidation/3α-hydroxylation, midazolam 1′-hydroxylation/midazolam 4-hydroxylation at 1–50 μM (but not higher), and alprazolam 4-hydroxylation/1′-hydroxylation at concentrations up to 2 mM; as well as its refractoriness to mechanism-based inactivation by mifepristone and diltiazam[126–131]. Specific monoclonal antibodies that distinguish the two proteins have also been reported[132].
[bbb]Refs [128], [129], [131], [132].
[ccc]Refs [130], [131].
[ddd]Dehydroepiandrosterone 3-sulfate16α-hydroxylation[133, 134].
[eee]Very low albeit detectable[135].
[fff]Refs [136], [137]. Liver CYP2E1 is also known to catalyze substantial lauric acid 12-hydroxylase activity and needs to be excluded[138, 139].
[ggg]A peroxisome proliferator[140].
[hhh]Ref. [141].
[iii]*N*-Hydroxy-*N*′(4-butyl-2-methylphenyl)-formamidine[142].
[jjj]17-Octadecynoic acid (Ortiz de Montellano, personal communication).
[kkk]Refs [143], [144].
[lll]Retinoic acid *(all trans)*[145].
[mmm]10-Undecynoic acid[146], (Ortiz de Montellano, personal communication).
[nnn]Ref. [147].
[ooo]Ref. [148].
[ppp]Ref. [149].

Table A.2. Human Liver P450s: Chemical Structures of Diagnostic Substrate and Inhibitor Probes

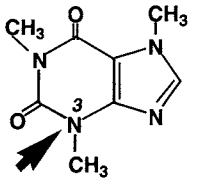
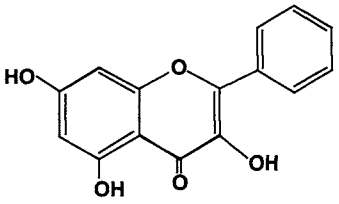
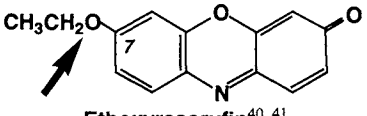
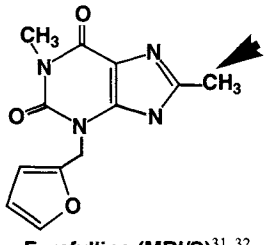
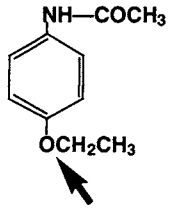
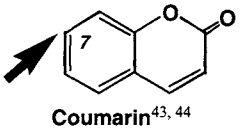
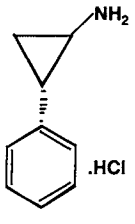
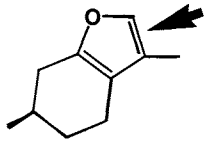
P450s	Substrate[a]	Inhibitor[b]
CYP1A2	 Caffeine[29]	 Galangin (3, 5, 7-trihydroxyflavone)[c, 38]
	 Ethoxyresorufin[40, 41]	 Furafylline (MBI/S)[31, 32]
	 Phenacetin[35]	
CYP2A6	 Coumarin[43, 44]	 Tranylcypromine[c, 46–48]
		 (R)-(+)Menthofuran (MBI/S)[49]

Table A.2. (continued)

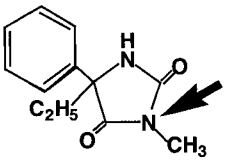
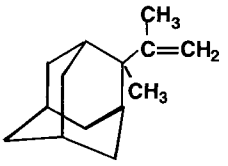
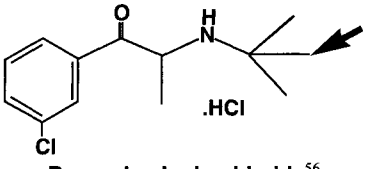
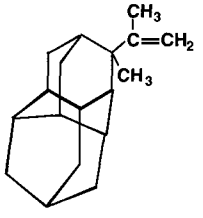
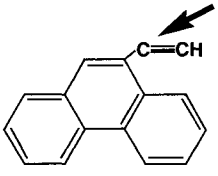
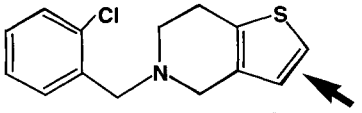
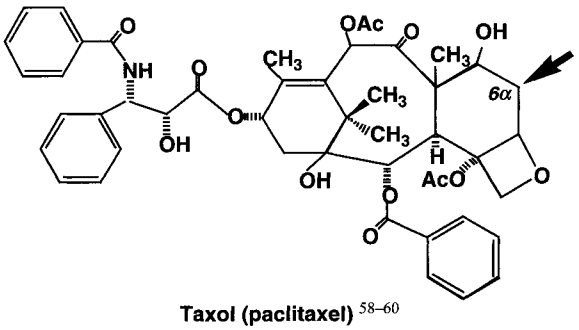
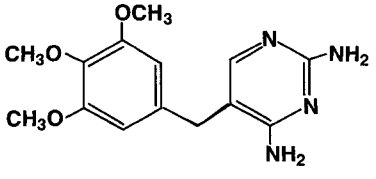
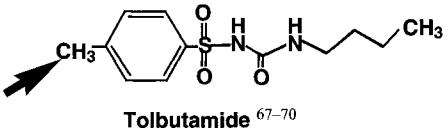
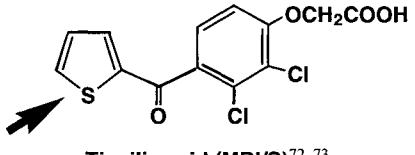
P450s	Substrate[a]	Inhibitor[b]
CYP2B6	 (S)-Mephenytoin[51]	 2-PMADA[c, d, 52]
	 Bupropion hydrochloride[56]	 3-PMDIA[c, e, 52]
		 9-Ethynylphenanthrene (MBI/S)[170]
		 Ticlopidine (MBI/S)[57]
CYP2C8	 Taxol (paclitaxel) [58–60]	 Trimethoprim[c, 61, 62]
CYP2C9	 Tolbutamide [67–70]	 Tienilic acid (MBI/S)[72, 73]

Table A.2. (continued)

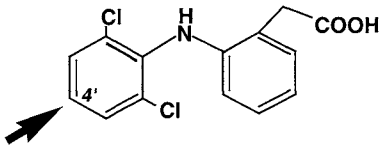
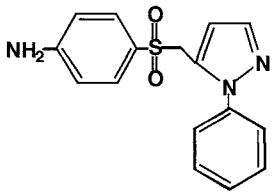
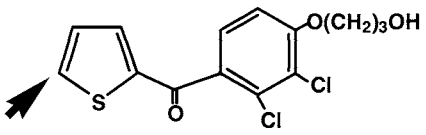
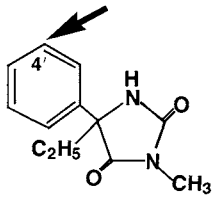
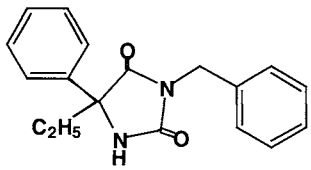
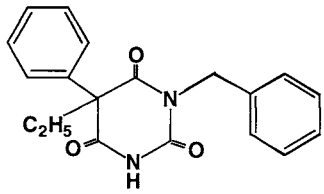
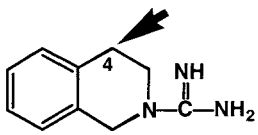
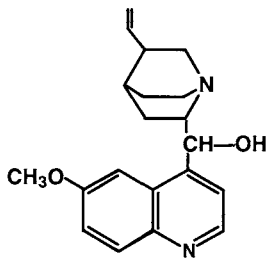
P450s	Substrate[a]	Inhibitor[b]
	 Diclofenac [63, 64]	 Sulfaphenazole[c, 65, 66]
CYP2C18	 3-[2, 3-Dichloro-4-(2-thenoyl)phenoxy]-propan-1-ol[78]	
CYP2C19	 (*S*)-Mephenytoin[71, 74, 80, 81]	 (+)-*N*-3-Benzyl-nirvanol[c, 82, 83]
		 (+)-*N*-3-Benzyl-phenobarbital[c, 82, 83]
CYP2D6	 Debrisoquine[86–88]	 Quinidine[c, 89, 90]

Table A.2. (continued)

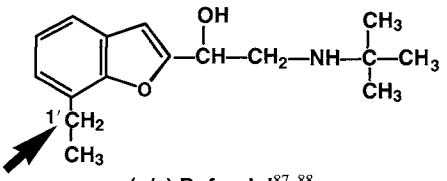
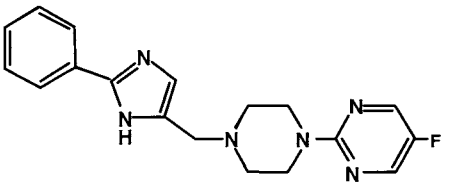
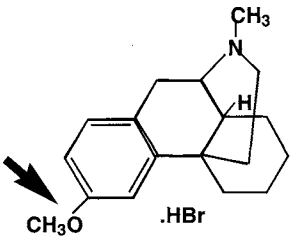
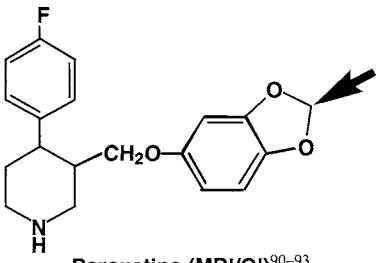
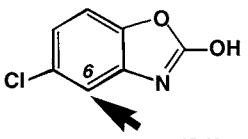
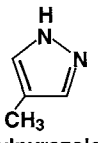
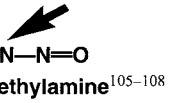
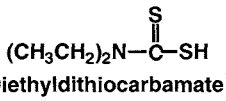
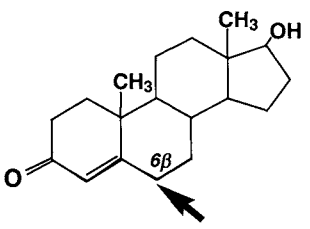
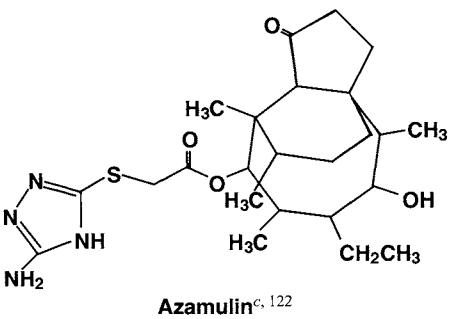
P450s	Substrate[a]	Inhibitor[b]
	 (+/–) Bufuralol[87, 88]	 SCH66712[c, f, 96]
	 Dextromethorphan Hydrobromide[94, 95]	 Paroxetine (MBI/QI)[90–93]
CYP2E1	 Chlorzoxazone[97–99]	 4-Methylpyrazole[c, 99, 100]
	 *N*-Nitrosodimethylamine[105–108]	 Diethyldithiocarbamate[18, 103, 104]
CYP3A4	 Testosterone[109–110]	 Azamulin[c, 122]

Table A.2. (continued)

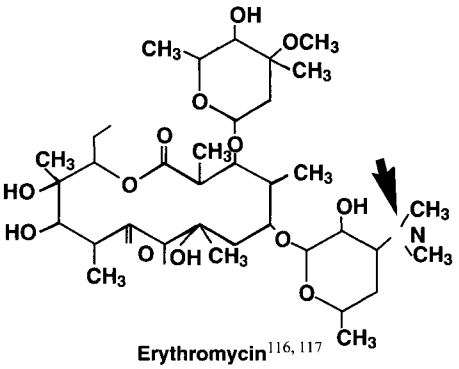
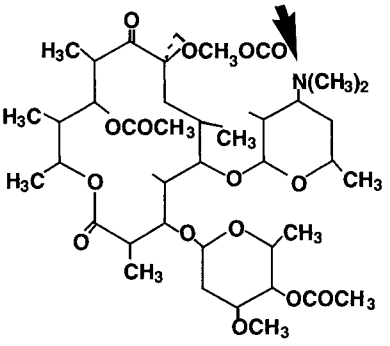
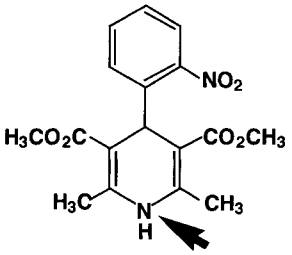
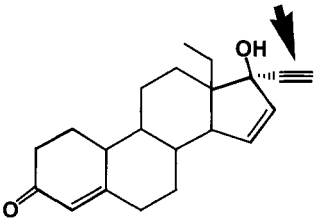
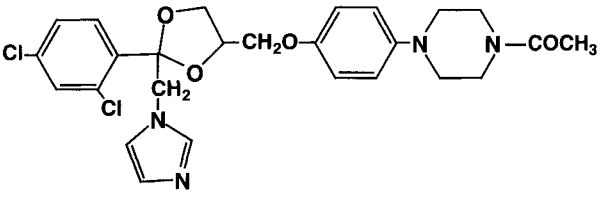
P450s	Substrate[a]	Inhibitor[b]
	 Erythromycin[116, 117]	 Troleandomycin (MBI/QI)[118, 119]
	 Nifedipine[112–114]	 Gestodene (MBI/S)[111]
	 Ketoconazole[c, 18, 48, 115, 122]	

Table A.2. (continued)

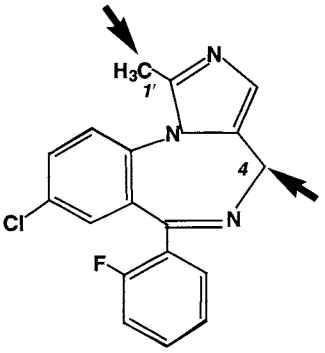
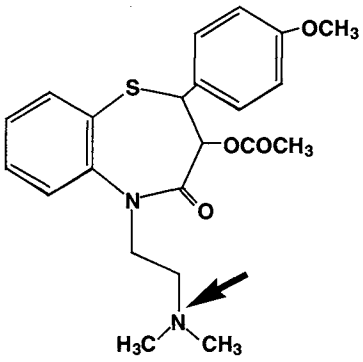
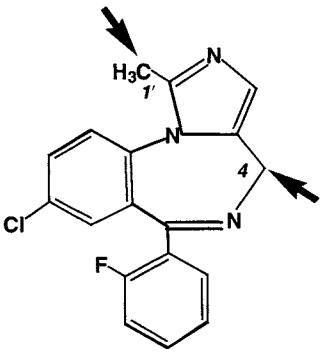
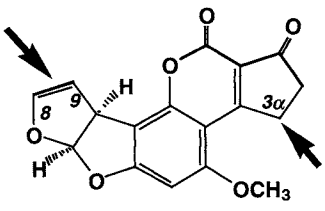
P450s	Substrate[a]	Inhibitor[b]
	 Midazolam[120, 121]	 Diltiazam (MBI/QI)[g, 126]
CYP3A5	 Midazolam[h, 128, 129, 131]	
	 Aflatoxin B1[h, 128, 248]	

Table A.2. (continued)

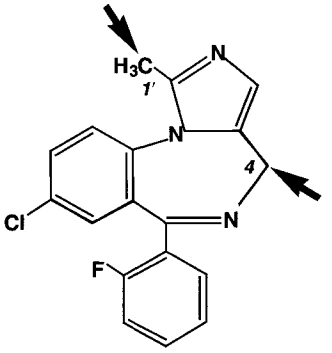
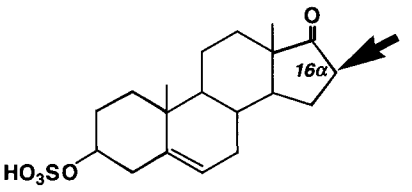
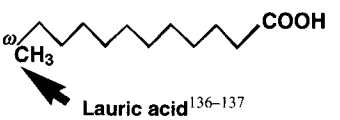
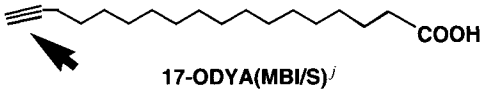
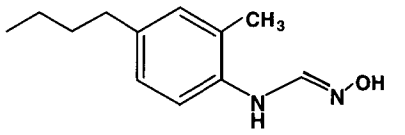
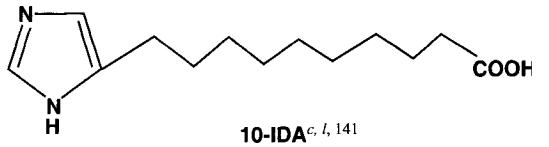
P450s	Substrate[a]	Inhibitor[b]
	 Alprazolam[h, 130, 131]	
CYP3A7	 DHEA-Sulfate[i, 133]	
CYP4A11	 Lauric acid[136–137]	 17-ODYA(MBI/S)[j]
		 HET0016[c, k, 142]
		 10-IDA[c, l, 141]

Table A.2. (continued)

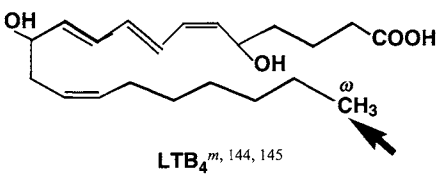
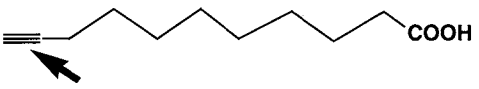
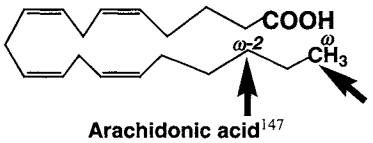
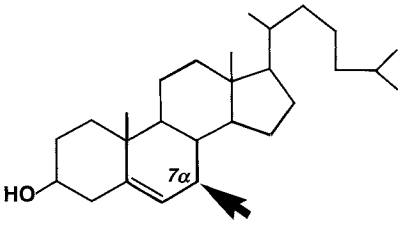
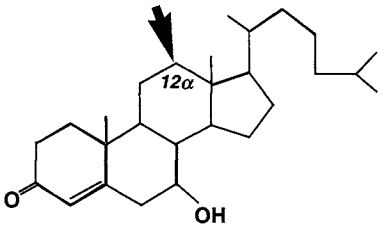
P450s	Substrate[a]	Inhibitor[b]
CYP4F2	 LTB_4[m, 144, 145]	 10-UDYA(MBI/S)[n, 146]
CYP4F12	 Arachidonic acid[147]	
CYP7A1	 Cholesterol[148]	
CYP8B1	 3-One-4-ene-7α-hydroxycholesterol[149]	

Table A.2. (continued)

Additional literature references are listed in Table A.2.

[a]Arrow(s) indicate(s) the substrate position(s) oxidized by that particular P450 isoform, enabling the assay of the corresponding oxidized metabolite(s) as its relatively selective functional probe(s).
[b]The arrow indicates the inhibitor site that is metabolically activated by that P450 isoform resulting in mechanism-based inactivation (MBI) of the enzyme that is either irreversible (suicide, S) or quasi-irreversible (QI).
[c]Inhibitor acts competitively by coordinating to the P450 heme-iron and/or ligation to the protein at the active site.
[d]2-PMADA, 2-isopropenyl-2-methyladamantane.
[e]3-PMDIA, 3-isopropenyl-3-methyldiadamantane.
[f]SCH66712, 5-fluoro-2-[4-[(2-phenyl-1H-imidazol-5-yl)methyl]-1-piperazinyl]pyrimidine.
[g]A metabolic intermediate complex (MIC) observed only with CYP3A4 but not CYPs 3A5 and 3A7.
[h]Given their ~89% sequence similarity, CYP3A4 and CYP3A5 have similar functional and inhibitory profiles. However, CYP3A5 may be distinguished from CYP3A4 by its higher metabolic ratio of midazolam 1′-/4-hydroxylation, aflatoxin B1 8,9-epoxidation to 3α-hydroxylation, and alprazolam 4-/1′-hydroxylation, as well as by its inability to form a diltiazam-MIC. Mifepristone has also been found to distinguish between the two CYP3A isoforms.
[i]DHEA-sulfate, dehydroepiandrosterone sulfate.
[j]17-ODYA, 17-octadecynoic acid. (Ortiz de Montellano, personal communication)
[k]HET0016, *N*-Hydroxy-*N*′(4-butyl-2-methylphenyl)-formamidine.
[l]10-IDA, 10-imidazolyldecanoic acid.
[m]LTB_4, leukotriene B_4.
[n]10-UDYA, 10-undecynoic acid. (Ortiz de Montellano, personal communication)

Table A.3. Rat Liver P450s: Chemical Structures of Diagnostic Substrate and Inhibitor Probes

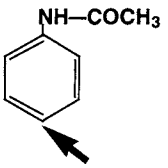
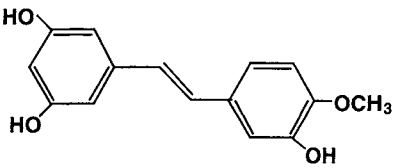
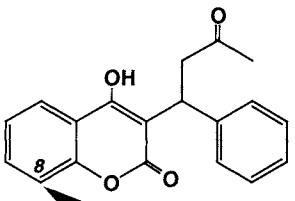
P450s	Substrate[a]	Inhibitor[b]
CYP1A1	 Acetanilide[151]	 Rhapontigenin(MBI/S)[150]
	 (*R*)-Warfarin[75, 76, 151]	
	7-Ethoxyresorufin[c, 40, 41] *(O-Deethylation)*	

Table A.3. (continued)

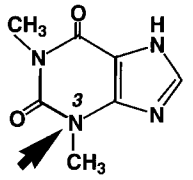
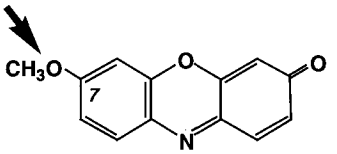
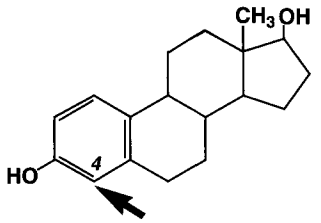
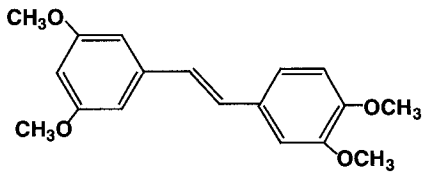
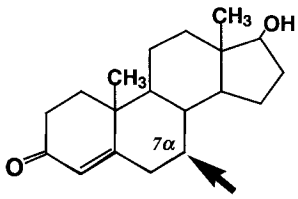
P450s	Substrate[a]	Inhibitor[b]
CYP1A2	Caffeine[c, 28] *(N-Demethylation)*	Galangin (3,5,7-trihydroxyflavone)[c–e, 38]
	 Theophylline[153]	Furafylline (MBI/S)[c, 27, 31–34]
	 7-Methoxyresorufin[40, 41]	
	Phenacetin[c, 152] *(O-Deethylation)*	
CYP1B1	 17β-Estradiol[157]	 TMS (2, 4, 3′, 5′-tetramethoxystilbene)[154]
	7-Ethoxyresorufin[c, 8, 154–156] *(O-Deethylation)*	
CYP2A1	 Testosterone[159–162]	

Table A.3. (continued)

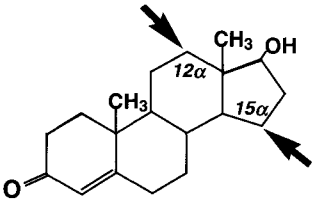
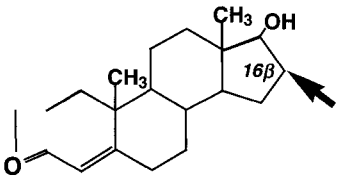
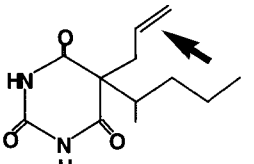
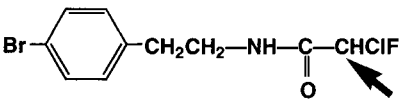
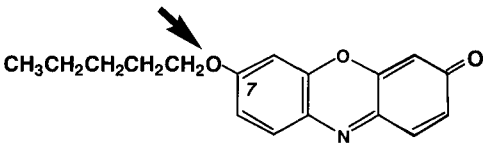
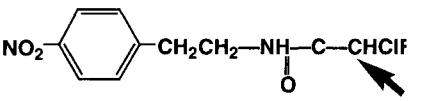
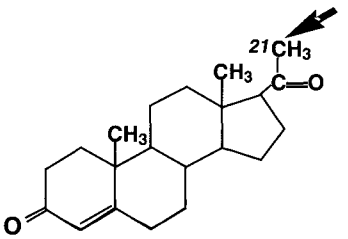
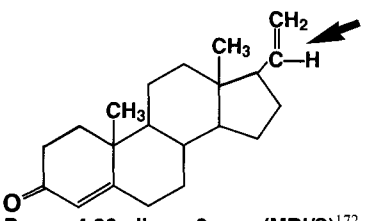
P450s	Substrate[a]	Inhibitor[b]
CYP2A2	 Testosterone[163, 164]	
CYP2B1	 Testosterone[161, 162]	 Secobarbital (MBI/S)[165, 166]
		 *N*-2-*p*-BPCFA (MBI/S)[168]
	 7-Pentoxyresorufin[167]	 *N*-2-*p*-NPCFA (MBI/S)[168]
		9-Ethynylphenanthrene (MBI/S)[c, 169]
CYP2C6	 Progesterone[171, 172]	 Pregn-4,20-diene-3-one (MBI/S)[172]

Table A.3. (continued)

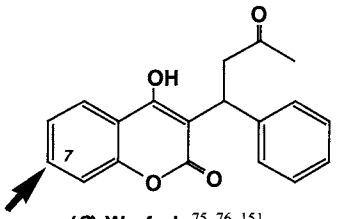
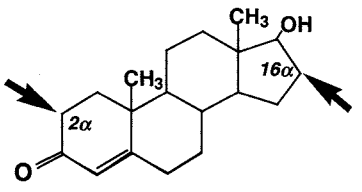
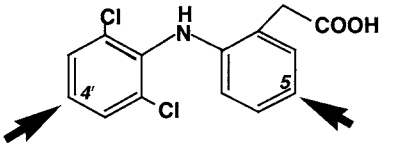
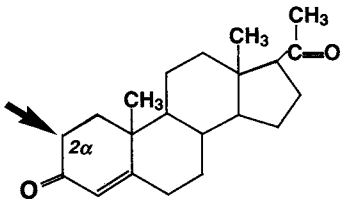
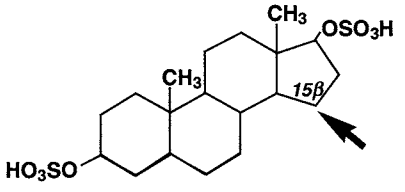
P450s	Substrate[a]	Inhibitor[b]
	 (*S*)-Warfarin[75, 76, 151]	Sulfaphenazole[c, 173]
CYP2C7	Testosterone[f, 162, 163] *(16α-Hydroxylation)* Progesterone[c, 171] *(16α-Hydroxylation)*	
CYP2C11	 Testosterone[162, 163]	 Diclofenac (MBI/S)[174, 175]
CYP2C12	 Progesterone[171]	
	 5α-androstane-3α,17βdiol, 3,17-disulfate[176]	
CYP2C13	Testosterone[c, 160–163] *(6β-Hydroxylation)*	

Table A.3. (continued)

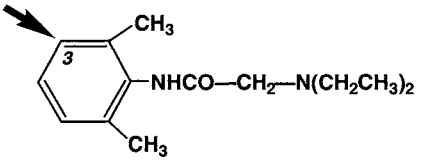
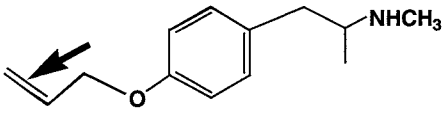
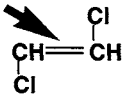
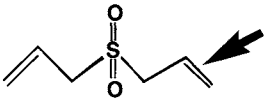
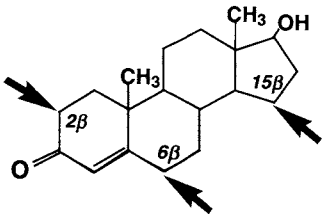
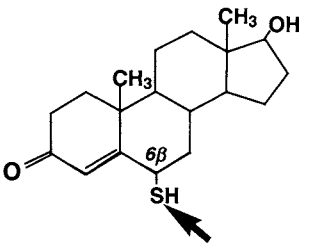
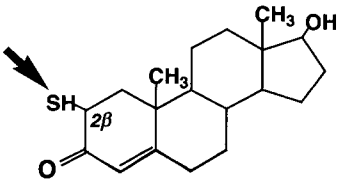
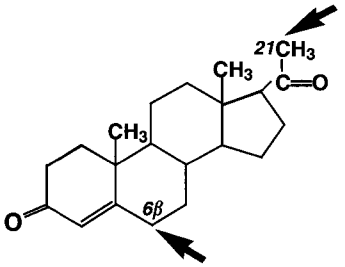
P450s	Substrate[a]	Inhibitor[b]
CYP2D2	 Lidocaine[177, 178] Debrisoquine *(4-Hydroxylation)*[c, 180] (+/–) Bufuralol *(1′–Hydroxylation)*[c, 179, 180]	 4-Allyloxymethamphetamine (MBI/S)[179]
CYP2E1	Chlorzoxazone[c, 181, 182] *(6-Hydroxylation)* *N*-Nitrosodimethylamine[c, 100, 184, 189] *(N-Demethylation)*	 *trans*-1,2-dichloroethylene (MBI/S)[183]  Diallylsulfone (MBI/S)[g, 186–189]
CYP3A2/3A23	 Testosterone[h, 162, 163, 190]	 6β-Thiotestosterone (MBI/S)[191]

Table A.3. (continued)

P450s	Substrate[a]	Inhibitor[b]
		
	Erythromycin[c, 192] *(N-Demethylation)*	2β-Thiotestosterone (MBI/S)[191]
	Nifedipine[c, 112] *(Oxidation)*	Troleandomycin (MBI/QI)[c, 193]
CYP3A9		
	Progesterone[194, 195] *(6β, 21-Dihydroxylation)*	
CYP4A1	Lauric acid[c, 196] *(ω-Hydroxylation)*	17-ODYA[c, i] (MBI/S)
CYP7A1	Cholesterol[c, 199, 200] *(7α-Hydroxylation)*	10-UDYA[c, i] (MBI/S)[c, 197, 198]

[a]The arrow(s) indicate(s) the substrate position(s) oxidized by that particular P450 isoform, enabling the assay of the corresponding oxidized metabolite(s) as its relatively selective functional probe(s).
[b]The arrow indicates the inhibitor site that is metabolically activated by that P450 isoform resulting in mechanism-based inactivation (MBI) of the enzyme that is either irreversible (suicide, S) or quasi-irreversible (QI).
[c]The chemical structures is depicted in Table A.2. The reaction used as a functional probe is shown in parentheses.
[d]A polyclonal anti-rat CYP1A1 antibody is available that recognizes CYP1A2 in immunoblotting, but that selectively immunoinhibits CYP1A1 but not CYP1A2 function.
[e]Inhibitor acts competitively by coordinating to the P450 heme-iron and/or protein binding at the active site.
[f]Steroid structure and site of 16α-hydroxylation shown under CYP2C11. Lower activity than that of CYP2C11.
[g]Phenethylisocyanate is also reported to selectively inhibit this enzyme[189].
[h]Progesterone or androstenedione 6β-hydroxylase may also be used as functional probe[162, 171].
[i]17-ODYA, 17-octadecynoic acid. (Ortiz de Montellano, P.R., unpublished observations).
[j]10-UDYA, 10-undecynoic acid, (Ortiz de Montellano, P.R., unpublished observations).

Table A.4. Commonly Used P450 Substrates and Inhibitors

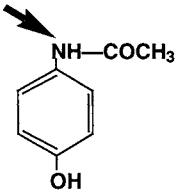
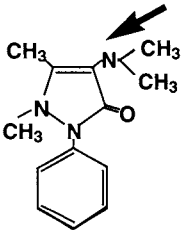
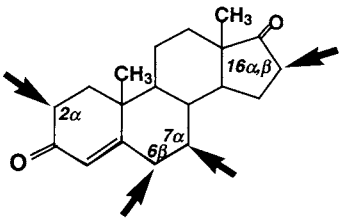
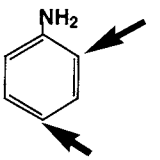
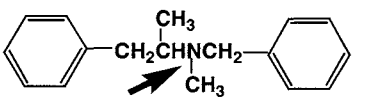
Trivial name	Chemical structure	Type of oxidation	P450 catalyst(s)
A. Substrates			
Acetaminophen[118, 200, 201]		*N*-Oxidation	CYPs 2E1, 3A, 1A2
Aminopyrine[151, 202]		*N*-Demethylation	Multiple
Androstenedione[162, 163]		*C*-Hydroxylation	Multiple
Aniline[151]		*C*-Hydroxylation	Multiple
Benzphetamine[151, 203]		*N*-Demethylation	Multiple

Table A.4. (continued)

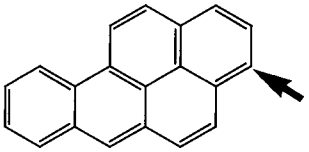
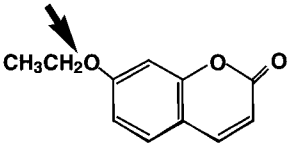
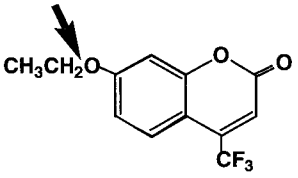
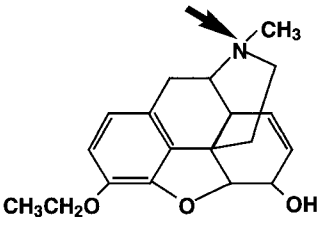
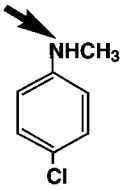
Trivial name	Chemical structure	Type of oxidation	P450 catalyst(s)
Benzo(a)pyrene[151, 204]		*C*-Hydroxylation	CYPs 1A, 1B
7-Ethoxycoumarin[151, 205]		*O*-Deethylation	Multiple
7-Ethoxy-4-trifluoromethyl-coumarin[26, 53]		*O*-Deethylation	Multiple
Ethylmorphine[151, 203]		*N*-Demethylation	Multiple
p-Chloro-*N*-methylaniline (PCNMA)[206]		*N*-Demethylation	CYPs 1A

Table A.4. (continued)

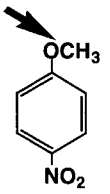
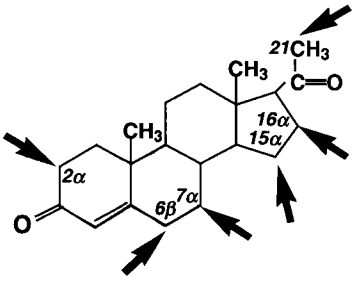
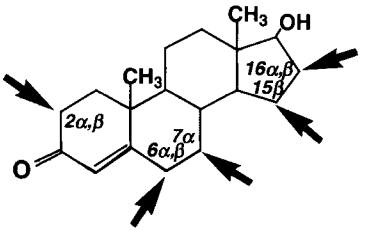
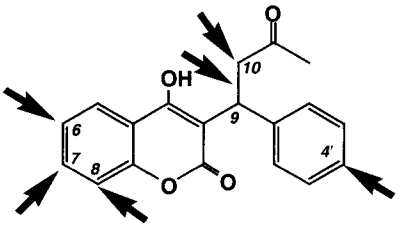
Trivial name	Chemical structure	Type of oxidation	P450 catalyst(s)
p-Nitroanisole[151]		*O*-Demethylation	Multiple
Progesterone[171]		*C*-Hydroxylation	Multiple
Testosterone[160–163, 190]		*C*-Hydroxylation	Multiple
(*R*/*S*)-Warfarin[75–77]		*C*-Hydroxylation	Multiple

Table A.4. (continued)

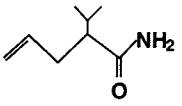
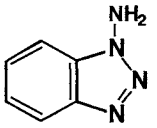
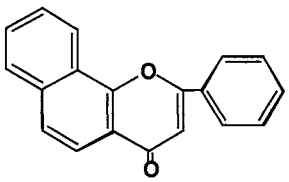
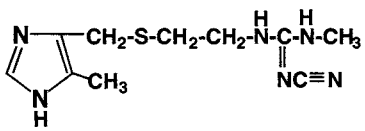
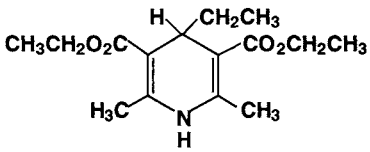
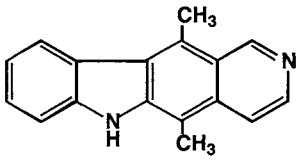
Trivial name	Chemical structure	Mode of inactivation	P450s inactivated
B. Inhibitors			
Allylisopropylacetamide[203]		MBI/S	Multiple
1-ABT[b] [207–209]		MBI/S	Multiple
7,8-Benzoflavone[18, 104]		C	CYPs 1A, 1B
Carbon monoxide[1, 2]	C=O	C	Multiple
Cimetidine[210–213]		C MBI/QI	Multiple CYPs 2C6 and 2C11
DDEP[c, 214]		MBI/S	Multiple
Ellipticine[48, 215, 216]		C	CYPs 1A, 1B, 2A6

Table A.4. (continued)

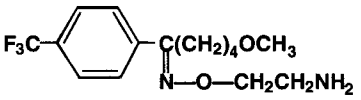
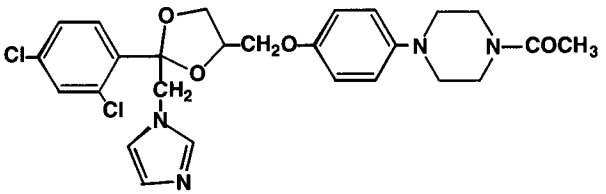
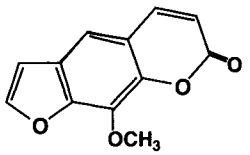
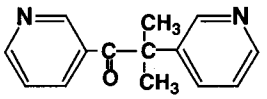
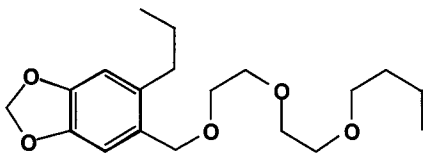
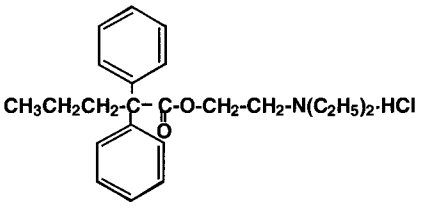
Trivial name	Chemical structure	Mode of inactivation	P450s inactivated
Fluvoxamine[217, 218]		C	CYPs 1A2, 2C19, 2C9
Ketoconazole[24, 27]		C	Multiple[d]
8-Methoxypsoralen[24, 219, 220]		MBI/S	Multiple[e]
Metyrapone[221, 222]		C	Multiple
Piperonyl butoxide[223, 224]		MBI/QI	Multiple

Table A.4. (continued)

Trivial name	Chemical structure	Mode of inactivation	P450s inactivated
SKF525A[f,225]		**MBI/S**	**Multiple**

[a]C, competitive inhibition through P450 Fe^{+2}/Fe^{+3}-heme complexation; MBI, Mechanism-based inactivation that is either quasi-irreversible (QI) via metabolic intermediate complexation or irreversible (suicidal, S).
[b]1-Aminobenzotriazole
[c]DDEP, 3,5-Dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine.
[d]Potent inhibitor of CYP3A4, but can inhibit other P450s at higher concentrations. Nonselective for rat liver P450s.
[e]Potent inhibitor of CYP2A6, but can inactivate other P450s at higher concentrations.
[f]SKF525A, Proadifen hydrochloride

Table A.5. Some Useful Parameters and Frequently Used Values in Cytochrome P450 Studies

Parameter	Type of spectrum	Wavelength (nm)		ε $mM^{-1}cm^{-1}$	Comments
		λ_{max}(nm)	$\lambda_{isobestic}$(nm)		
P450 concentration					
	$Fe^{+2}CO$–Fe^{+2}/ Difference	446–452	490	91	Dithionite-reduced sample split into two cuvettes and spectrally scanned with baseline correction between 400–500 nm. CO is bubbled into the sample cuvette and the cuvettes are similarly rescanned[226].
	$Fe^{+2}CO$–CO/ Difference	446–452	490	100	CO-bubbled microsomal sample split into two cuvettes and spectrally scanned with baseline correction between 400–500 nm. Dithionite added to the sample cuvette and the cuvettes are rescanned. Useful when substantial heme/hemoglobin contamination exists[227].
	$Fe^{+2}CO$–CO/ Difference	446–452	490	106	CO-bubbled tissue or microsomal sample split into two cuvettes and spectrally scanned with baseline correction between 400–500 nm. Dithionite added to the sample cuvette and the cuvettes are rescanned. Useful with tissue homogenates with low P450 content and substantial hemoglobin contamination[228].
	Fe^{+3}/Absolute	412–417(S)		125–162	Most P450s (low-spin) with the exception of those (CYP1A2, CYP2C7) present as high-spin species[128, 151, 229–231].
		530–536(β)		~14–18	
		564–574(α)		12–16	
	Fe^{+2}/Absolute	409–424(S)		91–116	
		540–554		23–29	
	Fe^{+3}/High-spin/ Absolute	392–395		81	CYP1A2, CYP2C7[229–231].
		540			
		645		4.7	Spin-associated peak.
	$Fe^{+2}CO$/ Absolute	446–452		114–117	Refs [229–232].
		536–549		24–33	
	EthylisoCN/ Difference	430(S)			Relative 455/430 nm peak heights are pH, high salt and P450 isoform dependent[230].
		452–455(S)			

RZ Values	0.7–1.3			Ratios of Soret peak absorbance maxima ($\sim A_{417\ nm}$ to $A_{280\ nm}$) of purified rat liver P450s. The RZ value can serve as an index of the purification extent, if non-UV absorbing detergents and buffers are used. Range from 0.7 (CYP2C11) to 1.3 (CYP2B1)[151].
P420 concentration				
$Fe^{+2}CO$–Fe^{+2}/ Difference	420	490	110	Dithionite-reduced microsomal sample split into two cuvettes and spectrally scanned with baseline correction between 400–500 nm. CO is bubbled into the sample cuvette and the cuvettes are similarly rescanned[233].
Fe^{+3}/Absolute	414(S) 535		124 11	Ref. [233]
Fe^{+2}/Absolute	427(S) 530(β) 559(α)		149 13 24	Ref. [233]
$Fe^{+2}CO$/ Absolute	421(S) 538(β) 565(α)		213 17 16	Ref. [233]
Metabolic intermediate P450 complexes (MIC)				
Methylenedioxyphenyl Fe^{+2} MIC	427, 455	490	75	The two species are in pH-dependent equilibrium and at a given pH, the ratio of the 427 and 455 peak absorbances is substrate-dependent. MIC can be displaced by irradiation at the wavelength absorption maxima or certain lipophilic ligands[235].
Methylenedioxyphenyl-Fe^{+3} MIC	437	490	—	Isosafrole-complexed CYP1A2 (Fe^{+2}) apparently exhibits spectral peaks at 413, 453, and 546 nm[221, 223, 224, 235, 236].
Alkyl/Aryl amine-Fe^{+2} MIC	456	490	64	Can be disrupted by oxidizing the P450 heme iron with $K_3Fe(CN)_6$. Arylamine complexes unstable to reduction by dithionite. Other $\varepsilon mM^{-1}cm^{-1}$ ranging from 68–80 also have been reported[191, 225, 236–239].
Heme concentration				
$Fe^{+2}Pyr - Fe^{+3}Pyr$/ Difference	557(α) 557(α) 557(α)	575 541 600	32.4 20.7 34.4	Samples dissolved in 20% pyridine/0.1 M NaOH, split in two cuvettes and spectrally scanned with baseline correction between 500–600 nm. Dithionite is added to the sample cuvette before rescanning[240, 241].

Table A.5. (continued)

Parameter	Type of spectrum	Wavelength (nm)		$\varepsilon mM^{-1}cm^{-1}$	Comments
		λ_{max}(nm)	$\lambda_{isobestic}$(nm)		
Cytochrome b_5 concentration					
	$Fe^{+2}-Fe^{+3}$/ Difference	424	409	185	Assayed after NADH reduction of microsomal samples or dithionite reduction of purified preparations[2, 226].
	$Fe^{+2}-Fe^{+3}$/Difference	557	575	20	Ref. [242].
	Fe^{+3}/Absolute	413(S) 530(β) 565(α)	438		Purified b_5 spectra[227, 243, 244]. For typical spectra see ref. [227].
	Fe^{+2}/Absolute	424(S) 526(β) 556(α)	438		For typical spectra see ref. [227].
Substrate-induced P450 difference spectra					
		λ_{max}(nm)	λ_{min}(nm)		**Typical substrates:**
	Type I	385–390	420		Hexobarbital, ethylmorphine, camphor.
	Reverse Type I	420	385–390		*n*-Butanol, phenacetin.
	Type II	425–435	390–410		Aniline, *n*-octylamine, and other primary amines. For typical spectra see refs. [227] and [232].

1. Estimation of Cytochrome P420 in Cytochrome P450 Preparations

Using the spectral baseline correction mode, determine the peak spectral absorption differences (ΔA) between 420–490 nm (P420) and 450–490 nm (P450) of CO-reduced–reduced ($Fe^{+2}CO$–Fe^{+2}) samples[226, 227, 233, 245].

[P450]: $\Delta A_{450-490\ nm}/0.091 = X$ nmol/ml (with $\varepsilon mM^{-1}cm^{-1} = 91$)

[P420]: $\Delta A_{420-490\ nm}/0.110 = Y$ nmol/ml (with $\varepsilon mM^{-1}cm^{-1} = 110$)

$(-0.041)X$ nmol/ml = correction for negative contribution of P450 to P420 peak absorption at ~420–424 nm ($\varepsilon mM^{-1}cm^{-1} = -41$)

Actual [P420] in P450 preparations: $Y-[(-0.041X)/0.110] = Z$ nmol/ml

2. Determination of Cytochrome P450 Reductase Activity

Carried out with NADPH as the electron donor and cytochrome c as the artificial electron acceptor by the method of Philips & Langdon[246] as detailed[245–247].

Using the $\Delta A_{550\ nm}$/min and an $\varepsilon mM^{-1}cm^{-1} = 21$ determine $[\Delta A_{550\ nm}/min]/0.021 = X$ nmol cytochrome c reduced/min[245–247].

Purified liver P450 reductase preparations range from 40–70 μmol cytochrome c reduced/mg protein/min. Molar concentrations of purified P450 reductase are estimated using either a molecular weight of 74 kDa (based on cDNA sequence as well as flavin and protein content) or an apparent molecular weight of 79.5 kDa (SDS-PAGE).

Acknowledgments

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