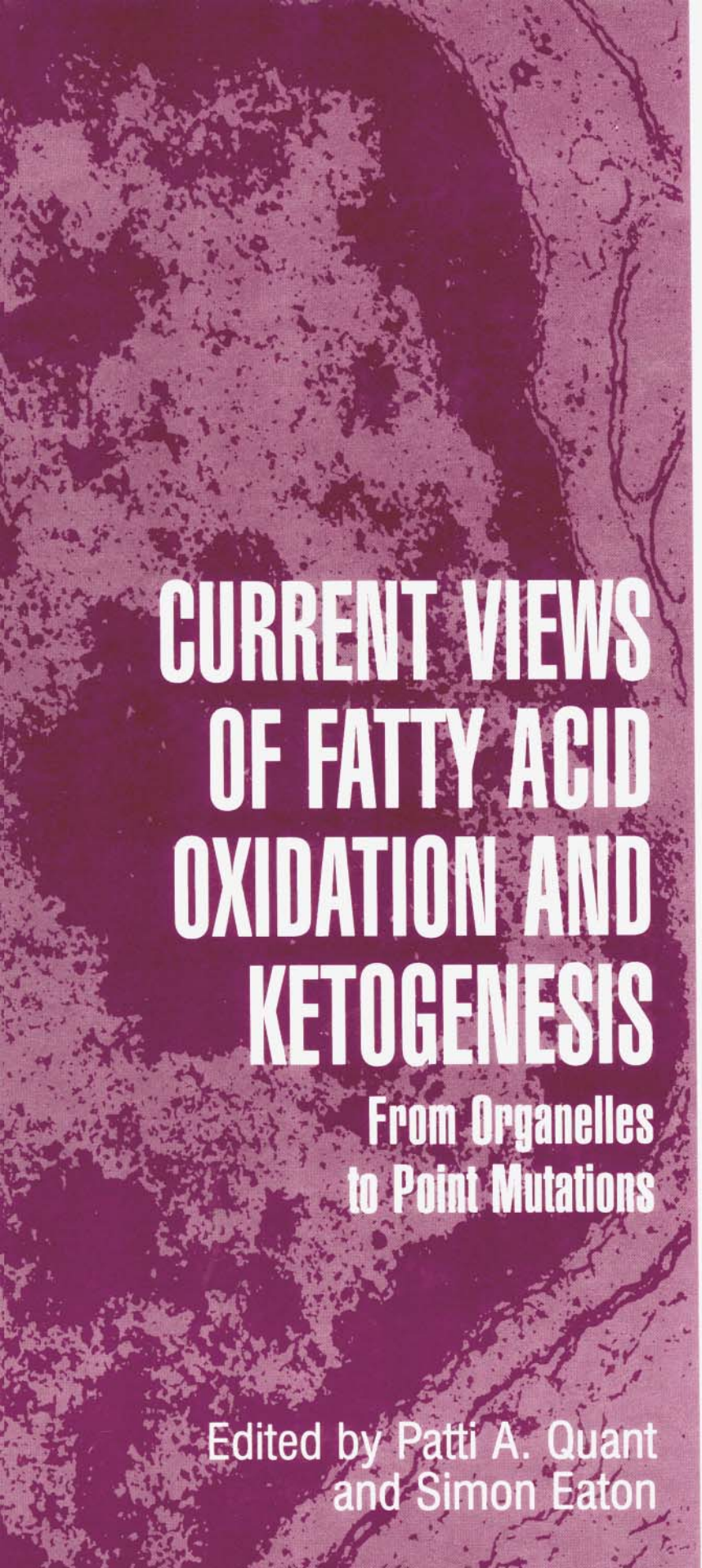

ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY

Volume 466

CURRENT VIEWS OF FATTY ACID OXIDATION AND KETOGENESIS

From Organelles to Point Mutations

Edited by Patti A. Quant and Simon Eaton

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Patti A. Quant and Simon Eaton

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Dedication

Patti Quant would like to dedicate this book to her mother, Kathleen Isobel Roe and to the memory of her father, Walter George Roe. Simon Eaton would like it to be dedicated to his grandfather, Clement Eaton.

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PREFACE

In September 1991, Victor Zammit and I were in the Department of Biochemistry, the University of Cambridge, discussing our collaborative research project when we realized the potential value and need for a conference specifically concerned with fatty acid oxidation and ketogenesis. The idea, once seeded, was indulged and flourished into the first "Fatty Acid Oxidation & Ketogenesis (FAOx&K) Conference" that was held in the Department at Eastertime, 1992. It was attended by colleagues mainly from the U.K., France and Spain. From these modest beginnings a tradition for holding a conference every second year has grown and this Book results from the 4th International FAOx&K Conference that was held in London at the Institute of Child Health & Great Ormond Street Hospital for Children NHS Trust, University College London Medical School in the new Conference Suite and was attended by colleagues from over twenty countries and five different continents. I would like to thank all my colleagues who have contributed to the conferences and, most importantly, to this Book.

The first two conferences were held in the University of Cambridge and were organized entirely by me but Simon Eaton, who came to work in London with me in February 1997, became Conference Secretary and co-organized the last two meetings. His contribution to the conferences has been invaluable and without his dedicated help and effort neither the later meetings nor this Book would have been possible. I would like to thank him personally for his help and support and most of all for his sense of humour that I must have sorely tried during the preparation of this manuscript. My heart-felt thanks to you, Simon!

There are many other colleagues to whom I am indebted. I would like to thank specifically Dr Philip K. Tubbs and Dr Martin D. Brand for taking me on as their PhD student in Cambridge and introducing me to my passions of fatty acid metabolism and biochemical research respectively. Without Philip's enthusiasm for his subject and Martin's passion for rigorous analysis my interest would not have been kindled and, hence, this Book would not have emerged. Since that time I have been encouraged and supported by many, many close colleagues in the field of fat metabolism, far too numerous to mention, whose friendship and professional advice I have needed, valued and appreciated. However, I would like to make special mention of the following colleagues: Professor Jean Girard; Dr Jean-Paul Pégorier; Danielle & Pierre Robin and Dr Victor Zammit. Drs Keith R. F. Elliott; Philip K. Tubbs and Martin D. Brand have given me continued friendship and support since my undergraduate and postgraduate student days respectively and Professors Bryan Winchester; Lewis Spitz; Peter Milla and E. David Saggerson during my time in London, for which I am extremely grateful. Everyone knows

that it is the postgraduate students and post-doctoral researchers who accrue all the results in any research group and, therefore, I would like to acknowledge and thank all the past and present members of the "Quantlab" who have contributed to this work. Finally, I would like to thank the Trustees of the Sir Halley Stewart Trust and Professor W. Jacobson in particular, for their real interest in and financial support for my fatty acid metabolism research and the development of my career. Their commitment to my research and their understanding of the clinical significance and academic importance of the research in this field has made this Book possible.

The recent explosion of interest in molecular genetics and its associated techniques has tended to overshadow the more traditional metabolic biochemistry. My hope is that this Book will re-ignite enthusiasm for rigorous research in the field of fatty acid oxidation, using the new methods and approaches available to us.

March, 1999
Patti A. Quant

I would like to thank Patti Quant for initiating this series of conferences, and allowing me to become involved in the running of the third and fourth meetings. It was attending the second conference in Cambridge that eventually led to me coming to work with her here in London, and I would also like to thank Patti for her help in writing all those fellowship proposals, for initiating me into the "dark art" of control analysis in fatty acid oxidation and for her advice and help. Speaking of initiations, I am also indebted to Professor Kim Bartlett for introducing me to fatty acid oxidation and HPLC and for his continued enthusiasm and guidance, and Dr Stan Sherratt for introducing me to the box of tricks known as the mitochondrion. I am also grateful to for support given by Professors Lewis Spitz and Bryan Winchester in London, and to the many friends and colleagues in London and Newcastle and elsewhere who have provided ideas and help over the years. I am also very thankful for the grant support given to me by the British Heart Foundation over the last five years.

I believe that this conference and book indicate that a multi-disciplinary approach involving biochemistry, physiology, molecular biology and clinical medicine can be extremely productive towards an understanding of a fundamental pathway.

March, 1999
Simon Eaton

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CAMBRIDGE, COLLEAGUES, CARNITINE AND KETOGENESIS …

Philip K. Tubbs

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Unlike everybody else at the Conference I have done no laboratory research for some 15 years, so I should not really be a participant at all. That I am here is due to three things: arm-twisting from Patti Quant, a desire to meet friends from former times ("old friends" might be politically incorrect) and a still lively, though frustrated interest in the subject. I have to say that if Patti had not come to work with me on ketogenesis in Cambridge nobody else would be here either: she would have bestowed her energy for experimental work and the forging of international contacts in another field and, no doubt, a very productive conference on something would be taking place elsewhere.

My own interest in fatty acid metabolism dates from nearly 45 years ago, my Ph.D. supervisor Guy Greville having worked with A.L. Lehninger on ketogenesis. At that time the pathway of fatty acid oxidation (though not of synthesis) had been studied in some detail, using crude and purified enzymes, but nothing was known of its control, or even why isolated mitochondria would not accept long-chain fatty acids or their Coenzyme A thioesters as substrates unless some "consommé" (boiled tissue extract) was added. Soon joined by David Pearson and James Chase, I set out in the 1960's to investigate how Coenzyme A spent its time in mitochondria and intact organs; there were after all some 27 intermediates to be expected between palmitate and acetyl-CoA. We soon extended the hunt to carnitine, because Jon Bremer of Oslo, whom I am delighted to meet here, and the late Irving Fritz had identified carnitine as being the "consommé factor". Previously, it was known only as a vitamin needed exclusively by mealworms, hardly a subject of much general relevance.

We crystallized carnitine acetyltransferase (studied in depth by Chase) and, by linking this to assays for CoA and acetyl-CoA, we found that the acylation state of both CoA and carnitine in rat liver depended on the dietary state; the perfusion of hearts with

different substrates had analogous effects. Carnitine and its esters were present in very much higher amounts than the CoA compounds, especially in heart. One interesting finding was that the acetyl-CoA/CoA and acetylcarnitine/carnitine ratios varied in parallel, so that they remained in virtual equilibrium. This led us to propose in 1967 that a major function of the carnitine acetyltransferase system is to "buffer" the acetyl-CoA content of tissues, analogous to the role of phosphocreatine/creatine in buffering the ATP/ADP ratio. This function may be very important because, although acetyl-CoA occupies a central place in metabolic maps, the amount of it in the heart is often inadequate to support the metabolic flux of the next single beat—dangerous brinkmanship!

James Chase and I tried to explore the mechanism of how carnitine permitted mitochondria to oxidize palmitate and we made some good progress in this. Using the 2-bromopalmitoyl derivatives of CoA and carnitine as potent and specific inhibitors, we found that the oxidation of palmitoyl-CoA required the sequential action of distinct "outer" and "inner" carnitine palmitoyltransferases (now known as CPT I and CPT II) separated functionally by the mitochondrial inner membrane. How the palmitoyl moieties crossed the membrane was unclear; because isolated liver mitochondria contained no carnitine we believed that the membrane was impermeable to it and that the "inner" transferase was vectorially mounted within the membrane, accepting only carnitine substrates on the outer face and CoA ones on the matrix side.

We began to investigate this theory using "inside-out" submitochondrial particles from heart, but at this point (1972) our progress on carnitine was brought to a halt by the tragic death of James Chase at the age of 30. Had he remained alive the carnitine translocase system would certainly have been discovered several years earlier; further than this, his loss deprived enzyme and metabolic biochemistry of a very gifted person.

However, work continued on liver ketogenesis. As Greville and I had pointed out in 1968 (see vol. 4 of *Essays in Biochemistry*), there seemed considerable obstacles in the way of ketogenesis at the level of HMG-CoA synthase. This enzyme has to accept acetoacetyl-CoA, one of its substrates, in the face of at least a 10^4-fold excess of the other, the extremely similar acetyl-CoA. This remarkable feat of specificity was studied by Bruce Middleton, another person I am most happy to see at the Conference; for technical reasons he initially purified the synthase from yeast but, subsequently, another colleague, Denise Lowe, obtained it in pure form from liver mitochondria. Her study of it was very careful and thorough. At one time Bruce and I were forbidden to engage in further research, of any kind whatsoever, so appalled were the Cambridge neighbours of the Biochemistry Department by the smell of reagents we designed; this prohibition was never rescinded but we ignored it. We originally believed that HMG-CoA synthase might occur in a binary complex with the thiolase which assembles acetoacetyl-CoA from two molecules of acetyl-CoA (and which Middleton has shown to be a thiolase specially adapted to this task), but no such complex has been found. The mechanism of HMG-CoA synthase involves an acetyl-enzyme intermediate, unlike the formally similar reaction catalysed by citrate synthase. Patti Quant later produced a great deal of work substantiating the earlier suggestion we had made that liver ketogenesis may be controlled, in the short term, by the synthase inactivating itself by accepting succinyl-CoA as an analogue of acetyl-CoA; the resulting succinylation mimics the acetylation step of the normal catalytic reaction. This unique form of metabolic control is supplemented by variation in the transcription rate of the HMG-CoA synthase gene and, hence, in the actual amount of the enzyme (which can be very large—it is one of the most abundant proteins in the matrix of rat liver mitochondria).

During the early 1970's Harold Stewart, Keith Stanley and I tried to detect the intermediates of β-oxidation which, as mentioned earlier, would be numerous if the process were to involve enzymes which are functionally dissociated. However, intact mitochondria oxidizing palmitoylcarnitine were found to contain appreciable quantities only of saturated acyl-CoA derivatives and even these did not show the kinetic behaviour of true intermediates. Unsaturated and 3-hydroxyacyl intermediates were formed only by disrupted mitochondria, or if respiratory inhibitors (such as rotenone) were present. We concluded that the relevant enzymes must be functionally linked in a β-oxidation "black box", as would be expected in such an iterative process; subsequent work has confirmed those results and, indeed, some multifunctional enzymes of fatty acid oxidation have been isolated from mitochondria. I am glad that Bruce Middleton is one person who has done this.

As I have mentioned already, after James Chase's death there was a hiatus in our Cambridge work on the function of carnitine. This was brought to an end by the arrival in 1974 of Rona Ramsay (now back at St Andrew's in her native Scotland). She soon found that heart mitochondria contained carnitine, as do those from liver if they are isolated very rapidly. She went on to show that mitochondria possessed a carrier, or "translocase" as it is now usually called, which catalyses a one-for-one exchange between carnitine (or acylcarnitine) on the cytosol side of the inner membrane and the same substrates in the matrix. As a result of this exchange the net influx of acyl groups into mitochondria is equal to the net efflux of free carnitine. We devised positively-charged acylcarnitine analogues for use as a "stop-inhibitor" of the translocase process and, using this, Rona carried out a very thorough study of the system (which had been independently discovered by Pande and colleagues in Montreal). Incidentally, it appears that these inhibitors of the translocase have been forgotten in recent years; the best of them is 16-trimethylamino-palmitoylcarnitine.

One property of the translocase is specially noteworthy: its affinity for long-chain acylcarnitine is very much higher than for carnitine itself. This suggests a basic rôle for carnitine, additional to the "acetylation buffer" already mentioned. Every fourth reaction of the β-oxidation "spiral" (the excision of an acetyl unit by a thiolase enzyme) requires free CoASH, so that if the CoA of the mitochondrial matrix became over-acylated the whole process would come to a halt. If acyl-CoA were formed directly from fatty acids in the same compartment (the matrix) as oxidation this might readily happen. However, the carnitine system guards against this "fail-nonsafe" situation, because if the acyl-CoA/CoASH ratio (and hence the acylcarnitine/carnitine ratio) rises the translocase will selectively export acylcarnitine from the matrix and import carnitine; this will lower the ratio and restore β-oxidation. It remains (I think!) to be seen if this effect can be conclusively demonstrated.

Enough is enough. Patti asked me to launch the Conference by briefly outlining what we had done on fatty acid oxidation in Cambridge prior to her arrival (itself 15 years ago). It was all fun to do and I miss it. Apart from that I have four feelings at this Conference: envy at the methodology and the availability of materials which now exist; admiration of so many recent findings; gladness that so many things we thought about long ago (and of course many that we didn't) remain to be done and finally one that all participants will share—gratitude that Patti continues to organize this series of conferences on fatty acid oxidation and ketogenesis.

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BIOGENESIS OF THE RAT LIVER MITOCHONDRIAL CARNITINE PALMITOYLTRANSFERASE I

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1. INTRODUCTION

Carnitine acyltransferases are involved in the transfer of acyl groups from acyl-CoAs to L-carnitine i.e. in the reversible conversion of acyl-CoA into acylcarnitine. These enzymes have a wide and overlapping chain-length specificity and a range of cellular localizations and metabolic functions. The best characterized carnitine acyltransferases are the mitochondrial carnitine palmitoyltransferases (CPT; EC 2.3.1.21): the CPT I and CPT II. The latest progress and current view of the structural, functional, regulatory and clinical aspects of the mitochondrial CPTs have been reviewed.[1–4] After a general overview of the physiological and cellular importance of CPT I, we will focus on recent insights into CPT I biogenesis in the context of protein import into mitochondria.

2. PHYSIOLOGICAL IMPORTANCE OF THE MITOCHONDRIAL CARNITINE PALMITOYLTRANSFERASE I

2.1. Role in Mitochondrial Fatty Acid β-Oxidation

Mammalian mitochondrial β-oxidation of fatty acids, the process by which fatty acids are oxidized,[2] provides the primary source of energy for the heart and skeletal muscle. In liver, when blood glucose levels are low, the capacity for fatty acid β-oxidation

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increases allowing the production of ketone bodies (acetoacetate and β-hydroxybutyrate), which serve as alternative fuels of respiration in nonhepatic tissues. Cytosolic long-chain fatty acids (LCFA) are first activated by long-chain acyl-CoA synthase on the outer mitochondrial membrane (OMM). The long-chain acyl-CoAs (LC-CoA) are substrates for the CPT system which allows them to overcome the impermeability of the inner mitochondrial membrane (IMM) and thus to be transported into the mitochondrial matrix, where the β-oxidation of LC-CoAs takes place. The CPT system requires the sequential action of CPT I (anchored at the OMM), the carnitine-acylcarnitine translocase (an integral protein of the IMM) and CPT II (located at the inner face of the IMM). In this system, CPT I catalyses the rate-limiting step of fatty acid oxidation.[1] A unique feature of CPT I is its potent inhibition by malonyl-CoA, the first intermediate of fatty acid biosynthesis.[5] This dual role of malonyl-CoA as intermediate and inhibitor provides not only a mechanism for physiological regulation of β-oxidation in liver and other tissues but also a coordinated control of fatty acid synthesis, esterification and oxidation.

By contrast to CPT II which has been found as one isoform, mammalian tissues express two isoforms of CPT I, the liver (L-CPT I) and the muscle (M-CPT I) forms, that are approximately 62% identical in amino acid sequence.[6–10] The liver isoform is present in the mitochondria of liver, pancreas, kidney, brain and most other tissues, while M-CPT I is the isoform expressed in skeletal muscle as well as in white and brown adipocytes.[3] L-CPT I is highly expressed in the fetal heart, but its expression decreases after birth with a concomitant increase in the expression of the M-CPT I.[3,4] Unlike L-CPT I which displays altered sensitivity to malonyl-CoA under different physiopathological conditions,[11–16] M-CPT I is very sensitive to malonyl-CoA inhibition but does not undergo any alteration of its sensitivity to malonyl-CoA.[3] The concentration of malonyl-CoA is much lower in heart than in rat liver,[1] but it is sufficient to inhibit fatty acid oxidation. The important question in cardiac metabolism, which is still unsolved, is how can fatty acid oxidation proceed in the presence of malonyl-CoA?

LCFA oxidation occurs mainly in mitochondria but rat liver microsomes and peroxisomes contain also both membrane-bound/malonyl-CoA-sensitive and soluble/malonyl-CoA-insensitive (luminal) CPT-like enzymes.[17–19] Thus, a similar fatty acid transport system operates in mitochondria, peroxisomes and microsomes, but it seems that the components involved in these systems are all different.[20] The physiological role of these fatty acid transport systems in microsomes and peroxisomes remains unclear. The microsomal CPTs may have a role in providing fatty acids for transport of proteins through the Golgi apparatus and for acylation of secreted proteins. Since oxidation of very long-chain fatty acids is confined to peroxisomes, a possible role for the peroxisomal CPTs may be to shuttle chain-shorted products out of peroxisomes for further oxidation in mitochondria.

2.2. Role in Cellular "Fuel Sensing" and Apoptosis

The malonyl-CoA/CPT I partnership not only plays an important role in the control of fatty acid oxidation, but it also provides the basis for "fuel sensing" which transduces to the cell information about the relative avaibility of fatty acid and glucose. CPT I participates in the control of the cytosolic LC-CoAs turnover. LC-CoA esters are involved in the regulation of metabolism and in cell signaling.[21] They are potent regulators of enzymes and ion channels,[22–24] are required for the budding of transport vesicles from Golgi cisternae[25–27] and are involved in protein acylation such as palmitoylation and myristoylation.[28–30] In pancreatic β-cell, it has been established that LCFA are acute

potentiators of glucose-stimulated insulin secretion by a still undefined mechanism.[31–35] Moreover, high levels of LC-CoA are known to interfere with glucose metabolism not only in skeletal muscle where they play an important role in insulin resistance,[36,37] but also in pancreatic β-cell where they cause insulin secretory dysfunction that characterizes non-insulin-dependent diabetes mellitus.[33,38,39] In Zucker Diabetic Fatty rats, it has been recently shown that LCFA induce β-cell apoptosis by enhancing *de novo* synthesis of ceramide, a key component of the signal transduction pathway of apoptosis.[40] Moreover, chronic lowering of β-cell CPT I activity in transgenic rats leads to the development of an insulin-deficient diabetes due to the decrease of the β-cell mass.[41] Additional studies show that LCFA are able to activate programmed-cell death in other cell types.[42,43] Furthermore, inhibition of the cellular malonyl-CoA-sensitive CPTs by etomoxir enhances LCFA induced-apoptosis.[42] A variety of key events in apoptosis focus on mitochondria[44] including the release of caspase activators, changes in electron transport, loss of mitochondrial transmembrane potential, altered cellular oxidation-reduction and participation of antiapoptotic Bcl-2 family proteins.[45] A recent study has shown that rat liver CPT I was able to interact *in vitro* with the OMM Bcl-2 protein.[46] Whether CPT I plays a direct role in the prevention of apoptosis or an indirect role by confering a protective effect on cell viability by affecting the clearance of cytosolic LCFA still remains to be elucidated.

The OMM localization of CPT I is essential for the enzyme to fulfil its metabolic and cellular function. Maintenance of its functional activity and sensitivity to malonyl-CoA implies a correct anchorage at the OMM. This raises the crucial question of how CPT I is specifically targeted to mitochondria and inserted into the OMM, and not to other organelles.

3. CHARACTERISTICS OF THE IMPORT OF L-CPT I INTO THE OMM

The past decade has significantly increased our knowledge of the mechanisms and components involved in protein targeting and import into mitochondria.[47] A general import pathway has emerged mainly from studies in yeast *S. cerevisiae* and *N. crassa.* In mammalian cells, the various steps involved in mitochondrial biogenesis are thought to be similar. Yeast mitochondrial precursors can be efficiently imported into mammalian mitochondria and vice versa. Indeed, upon heterologous expression in yeast, the rat L-CPT I is efficiently targeted to mitochondria[48–50] and is correctly inserted into the OMM.[50]

3.1. *In vitro* Import of L-CPT I into Mitochondria

The first evidence showing that L-CPT I can be post-translationally imported into freshly isolated rat liver mitochondria came from *in vitro* import assays.[51] The criterion used was the tight membrane association of the native rat L-CPT I.[52] L-CPT I is anchored at the OMM by two hydrophobic transmembrane segments (H1, residues 48–75 and H2, residues 103–122) with both N-and C-termini exposed to the cytosol whereas the linker region between H1 and H2 is located in the intermembrane space.[53] Membrane insertion of proteins can be assessed by measuring their resistance to alkaline extraction.[51,54–56] This procedure distinguishes both soluble and peripherally membrane-bound proteins from integral proteins. *In vitro* transcription-translation was performed to synthetize radiola-

belled L-CPT I. When the latter was incubated in an import mixture with freshly isolated rat liver mitochondria, L-CPT I became alkaline-resistant, and thus membrane-inserted, as was the native protein.[51] Digitonin treatment of mitochondria confirmed that the imported L-CPT I was inserted into the OMM (Cohen I., Girard J. and Prip-Buus C., unpublished results). Thus, *in vitro* import of L-CPT I leads to its insertion into the OMM and represents a dynamic process that is time- and temperature-dependent.[51] The successive steps involved in the import of L-CPT I into the OMM are schematically presented in Figure 1.

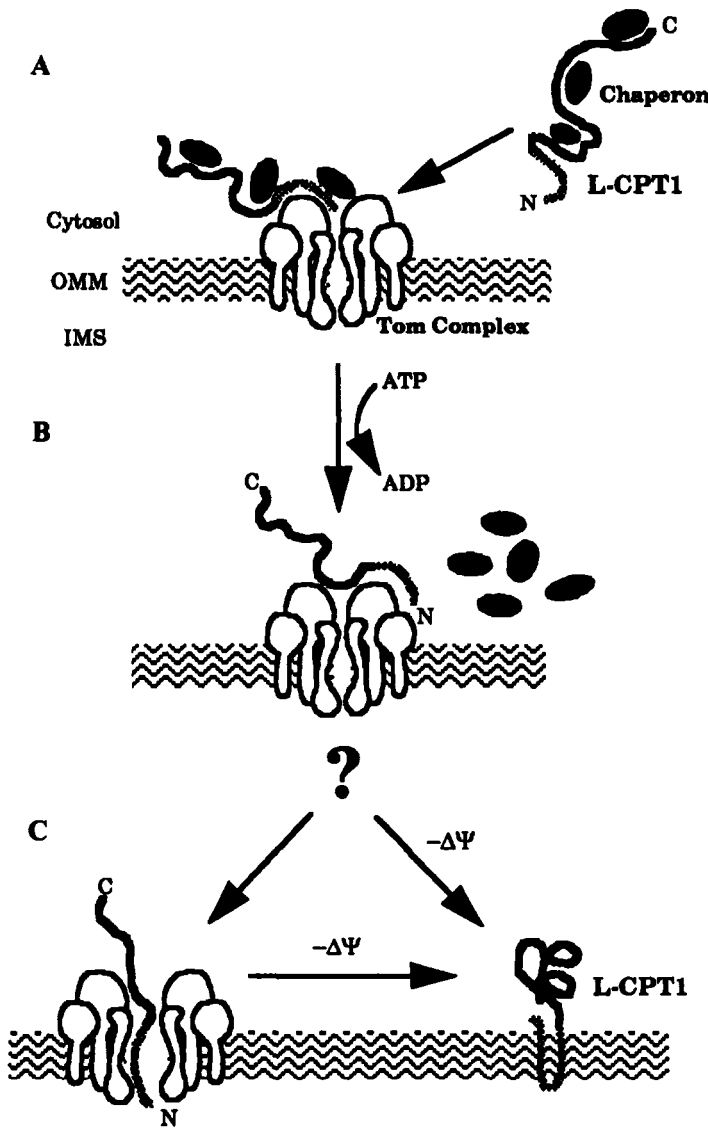


Figure 1. Import of L-CPT I into mitochondria.

3.2. Import-Competent State

It is generally assumed that a newly synthetized mitochondrial precursor must adopt an import-competent conformation in order to be efficiently imported into the mitochondria. The conformational state of precursors in the cytosol is usually different from that of the native proteins. In the case of L-CPT I, the protein present in the reticulocyte lysate is in a highly trypsin-sensitive conformation (unfoldfed state), whereas once inserted into the OMM, it becomes trypsin-resistant (folded state) (Cohen I., Girard J. and Prip-Buus C., unpublished results), similar to what was observed for the native rat L-CPT I.[50,53] Various cytosolic factors contribute to maintain the mitochondrial precursors in an import-competent state and prevent their aggregation as well as their degradation by cellular proteases.[47] The best characterized chaperones are the cytosolic Heat Shock Protein Hsp70[57] and the rat liver mitochondrial stimulating factor (MSF).[58,59] Both Hsp70 and MSF possess ATPase activities. Hsp70 is involved in intracellular protein traffic and has a general function in protein import into mitochondria as well as into endoplasmic reticulum and nuclei. Hsp70 binds to proteins enriched in hydrophobic amino acid residues, recognizes aggregated cytosolic proteins and is able to unfold them in an ATP-dependent manner. MSF recognizes more specifically mitochondrial precursors and targets them to mitochondria. After binding of MSF to a mitochondrial precursor, the MSF-precursor complex docks at the mitochondrial surface, and then, by contrast to Hsp70, ATP hydrolysis is required for the release of MSF, allowing the mitochondrial precursor to pursue its import.[60,61] *In vitro* import of L-CPT I needs the presence of ATP.[51] Upon ATP depletion, binding of L-CPT I to mitochondria is unaffected (Cohen I., Girard J. and Prip-Buus C., unpublished results), whereas its insertion into the OMM is impaired.[51] This suggests that MSF could be involved in the import of L-CPT I and that the absence of ATP may not affect the formation of the MSF/L-CPTI complex or its docking to mitochondria, but may cause the inhibition of the release of MSF, and thus may significantly impair further membrane insertion of L-CPT 1 (Fig. 1).

3.3. Recognition by Mitochondrial Receptors

The OMM translocation machinery, known as the Tom complex (for Translocase of the Outer Membrane), mediates recognition of mitochondrial precursors (receptor function) on the mitochondrial surface, their translocation across the OMM (general insertion pore, GIP), and insertion of resident OMM proteins. In fungal mitochondria, two distinct receptor systems function in parallele: the Tom70-Tom71-Tom37 subcomplex and the Tom20-Tom22 subcomplex. The first subcomplex is used by a few specific proteins, while the majority of mitochondrial precursors are directly recognized by the Tom20-Tom22 receptors. Proteins which interact first with the Tom70-Tom71-Tom37 subcomplex are then transferred to Tom20-Tom22.[47] This initial recognition step ensures both the specificity and the efficiency of the mitochondrial import process. Pretreatment of mitochondria with a low concentration of trypsin causes a marked decrease in the import efficiency of mitochondrial precursors.[62] This results in part from the removal of the large cytosolic domain of both Tom20 and Tom70.[63–66] OMM insertion of L-CPT I into trypsin-pretreated rat liver mitochondria was reduced[51] to a similar extent to what was observed for other OMM proteins.[56,67] The effect of trypsin pretreatment of mitochondria on the import effciency of L-CPT I was dose-dependent and specific for trypsin, since proteinase K pretreatment did not alter the import efficiency (Cohen I., Girard J.

and Prip-Buus C., unpublished results). Thus, the import of L-CPT I is dependent upon trypsin-sensitive mitochondrial receptors (Fig. 1). In mammals, only a small number of proteins have been identified as components of the mammalian Tom complex.[68–72] Thus, the identity of the putative receptors involved in the mitochondrial recognition of the rat L-CPT I awaits further characterization.

3.4. Post-Receptor Import Step

Functional studies have shown that mitochondrial proteins may use different receptor systems but then pass a common channel, named general import pore (GIP). This GIP guides polypeptides through the OMM and is thought to be formed by the Tom40 protein that is deeply embedded in the membrane[73,74] and by the small Tom5, Tom6, Tom7 proteins.[75,77] The import of porin into the OMM needs the presence of at least Tom40, Tom5 and Tom7.[76,77] The presence of a mitochondrial membrane potential ($\Delta\psi$) is required for the translocation of most intramitochondrial precursors across the IMM.[47] By contrast, the post-receptor import step leading to the insertion of a protein into the OMM does not require the presence of $\Delta\psi$. The import of L-CPT I into the OMM is $\Delta\psi$-independent[51] (Fig. 1). The process by which a protein is inserted into the OMM is still a matter of debate. Current models for the mechanism of membrane insertion of α-helical transmembrane segments suggest that integration into the bilayer is coupled with the vectorial movement of the polypeptide through the import machinery. It is not known whether this movement arises from the release of the transmembrane segment laterally from the Tom complex or from the disassembly of the complex when it engages the transmembrane segment. For bitopic proteins, this occurs once during its translocation, whereas the process might be repeated for polytopic (multispanning) proteins. Thus, insertion of L-CPT I may be the result of threading back and forth across the OMM or, like the mechanism proposed for β-barrel proteins, the result of simultaneous partitioning of the transmembrane segments as a bulk domain, in order to acquire a sufficient hydrophobic character to favour bilayer integration.

4. ROLE OF THE N-TERMINAL DOMAIN OF L-CPT I IN MITOCHONDRIAL IMPORT

4.1. Current Working Models for Protein Import into the OMM

The information that specifies correct mitochondrial targeting and sorting of newly synthetized mitochondrial precursors resides within their sequences.[47] For most matrix proteins, and especially for the rat CPT II,[78] the N-terminal presequences function as cleavable matrix-targeting signals. These matrix-targeting signals are characterized by a high net positive charge and by the ability to adopt an amphiphilic helical structure upon binding to a membrane surface.[79,80] By contrast, OMM proteins, such as CPT I,[51] do not contain cleavable presequences, and therefore must be targeted by means of internal signals.[81] The mitochondrial signals involved in the import and sorting of matrix and IMM proteins have been extensively studied.[47] However, the nature of the internal targeting signals present in OMM proteins as well as the mechanisms of their recognition and of their insertion into the membrane are poorly understood, especially in the case of polytopic OMM proteins.

One current model for protein import into the OMM is based on the *S. cerevisiae*

bitopic Tom70. Targeting and insertion of Tom70 into the OMM is mediated by its unique transmembrane segment (11–29 amino acids) referred to as a "signal anchor sequence" selective for the OMM.[56] 1–10 amino acids constitute a hydrophilic, positively charged segment, which on its own exhibits negligible targeting information, but seems to enhance the rate of import of Tom70.[56] The signal anchor sequence of Tom70 is structurally and functionally similar to those found in type-II and type-III integral proteins of the endoplasmic reticulum.[82–84] The position of the OMM signal anchor sequence within the polypeptide is irrelevant. In the case of Tom70, it is located at the N-terminus, whereas it lies at the C-terminal end for the mammalian Bcl-2 protein[85] and for the yeast Tom6.[75,86]

An alternative to the signal anchor sequence model is the combination of a matrix-targeting signal with a hydrophobic stop-transfer sequence.[81,87] The former signal specifies targeting of a protein to mitochondria and initiates its translocation across the OMM, whereas the hydrophobic stop-transfer sequence arrests and anchors the translocating polypeptide into the OMM. The *N. crassa* mitochondrial receptor Tom22, an OMM bitopic protein that exposes its N-terminal domain to the cytosol and its C-terminal portion in the intermembrane space, seems to follow this model. Indeed, the positively charged portion of the cytosolic domain of Tom22, proximal to the transmembrane segment, harbours information essential for its OMM import.[88] This import sequence resembles matrix-targeting sequences in that it is enriched in serine, tyrosine, and threonine residues, and is potentially amphiphilic.[89]

Integral polytopic OMM proteins fall into two classes, namely those that contain transmembrane β-sheets (porin) and those with α-helical hydrophobic transmembrane segments (CPT I). The targeting signals for polytopic OMM proteins are still not defined. For the porin, the targeting information seems to be not located at the N-terminus, but in other regions of the protein, which include its extreme C-terminus.[90] However, evidence is accumulating that a single stretch of amino acids does not suffice as a targeting signal, because point mutations in certain charged residues of porin influence its import efficiency.[91] Thus, the targeting signal of a polytopic OMM protein might not necessarily reside in a short linear sequence of the polypeptide, but could reside within a folded structure composed by several regions located in different parts of the protein.

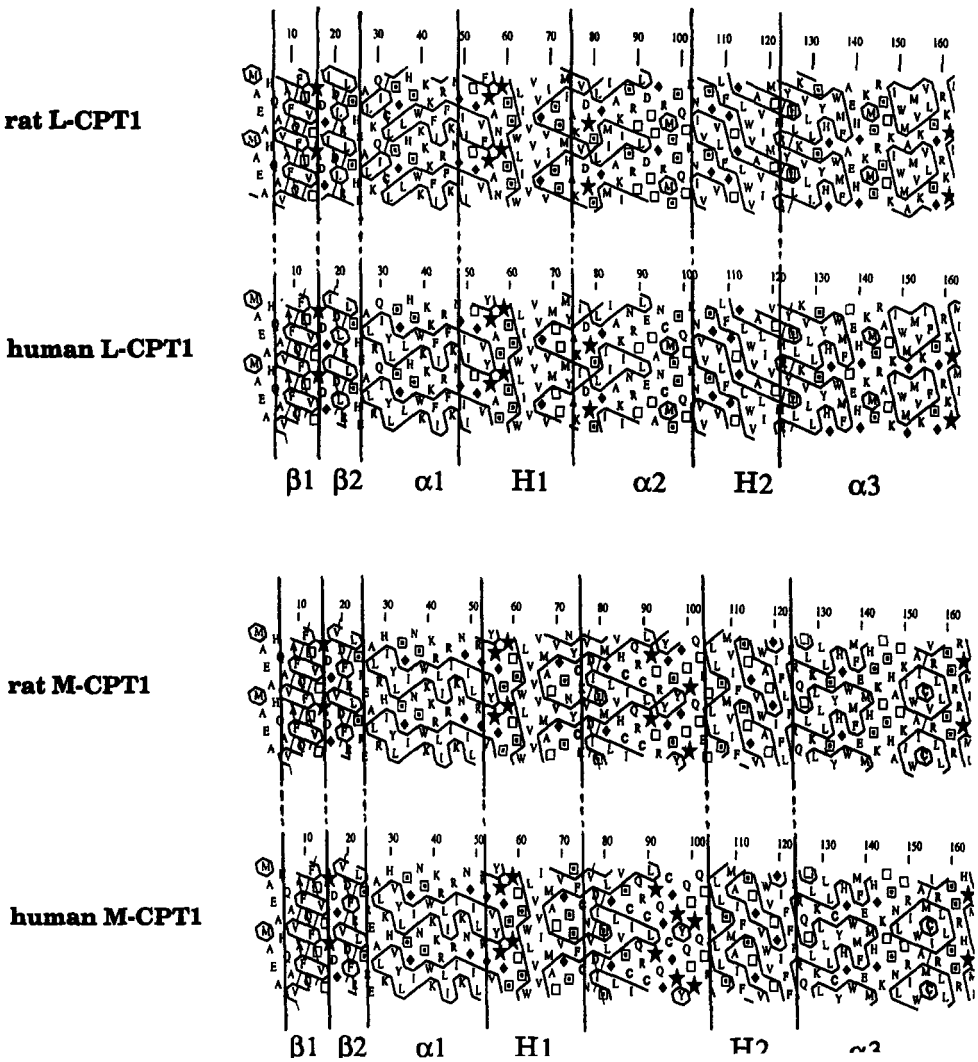
4.2. The N-Terminal Domain of L-CPT I Mediates Its Import into the OMM

Amino acid sequences alignment of rat L-CPT I and CPT II reveals in L-CPT I the presence of an extended N-terminal domain (about 150 amino acids) which bears no similarity to CPT II.[6] Deletion of the N-terminal domain of L-CPT I abrogates the ability of the protein to interact with the mitochondrial receptors, and thus to be specifically imported into rat liver mitochondria.[51] Conversely, fusion of the L-CPT I N-terminal domain to the cytosolic mouse dihydrofolate reductase (DHFR) or to the mature form of CPT II (which lacks the matrix-targeting signal and is thus incompetent for import) allows them to be targeted to mitochondria both *in vitro* and *in vivo,* and to be inserted into the OMM.[51] Thus, the OMM targeting signal of L-CPT I resides within the first 147 residues of the protein and explains the inability of **L-CPT IΔ31–148** to be associated with mitochondria.[48]

Hydrophobic cluster analysis (HCA) is a very efficient method to analyse and compare protein sequences.[92,93] Schematically, HCA uses a highly degenerated code for the sequences, where only two main states are initially considered: hydrophobic andhdrophilic.

A number of structural features of a globular protein, with or without transmembrane segments, can be derived from the simple examination of its HCA plot and concerns the structural segmentation, the identification of structural domains and some indications on the secondary structure and loops. For the identification of structural segmentation, the plot is examined to analyse the horizontal distribution and size of the various hydrophobic clusters that have been automatically drawn by the program. The HCA method is more efficient when several homologous sequences can be compared. Comparison of the HCA plots of the rat and human L-CPT I and M-CPT I is shown in Figure 2.

A segment containing mostly hydrophobic (VILFWMY) amino acids and virtually no DENQHKR residues is often indicative of completely buried secondary structure, mostly transmembrane α-helices (when the length is about 20 residues). Two of such hydrophobic clusters are recovered in the HCA plots of the four known CPT I proteins



(Fig. 2) and correspond to the two transmembrane segments (H1 and H2) of these proteins. According to the examination of the HCA plots, H1 comprises residues 47–75 (L-CPT Is) or 53–75 (M-CPT Is), whereas H2 corresponds to residues 103–123 (L-CPT Is) or 105–124 (M-CPT Is). Mosaic (zig-zag) clusters that contain highly hydrophobic residues alternating with hydrophilic ones are often associated with amphiphilic β-strands. Two putative highly conserved amphiphilic β-strands (β1, 8–14 amino acids; β2, 19–23 amino acids) are observed in the extreme N-terminus of all CPT I proteins (Fig. 2). Finally, longer horizontal clusters often denote amphiphilic α-helices. At least three amphiphilic α-helices (α1, α2, and α3) seem to be present in the N-terminal domain of the liver CPT I isoforms (Fig. 2). They are successively comprised within residues 25–47, 76–102 and 123–161. For the muscle form, α1 and α3 are also present and are included within residues 25–52 and 125–161, respectively. Residues 76–104 of the M-CPT I proteins are enriched in proline (H) and glycine (u) (Fig. 2). Proline introduces the largest constraints in a polypeptide chain and is considered to be a systematic break in the clusters. Proline often stops or distorts helices and β-strands whereas glycine has a large conformational flexibility. Thus, residues 76–104 are unlikely to adopt an α-helix but could be considered as a hinge region between H1 and H2. Whether this structural discrepancy between the liver and muscle isoforms has some repercussions on the functional properties of the enzymes remains to be established. In conclusion, the use of the HCA method reveals that the N-terminal domains of the four known CPT I proteins not only show a high degree of amino acid identity but also exhibit similar structural segmentations (Fig. 2). We propose the following prediction for the secondary structural segmentation of the N-terminal domain of the CPT I family: β1–β2–α1–H1-α2 or loop–H2–α3. As other secondary structure prediction methods, the 2-D structural informations obtained by the HCA method leads to the elaboration of a working 2-D model that could be helpfull for the comprehension of the structure-function relationships of the CPT I family.

As described above, L-CPT I contains two hydrophobic transmembrane segments. The first import mechanism hypothesis which can be formulated is that either of the transmembrane segments could function as a signal anchor sequence. Whether H1 and H2 play an equivalent role or only one of them acts as a specific signal anchor sequence still remains to be elucidated. Alternatively, H1 or H2 could function as a stop-transfer sequence in cooperation with a matrix-signal sequence which could be ensured by either α1, α2 or α3. Thus, further studies will be required to determine whether OMM insertion of L-CPT I follows the signal anchor sequence or stop-transfer model.

4.3. Membrane Orientation of OMM Proteins

The distribution of charges on either side of the membrane anchor is responsible for the orientation of proteins of the bacterial inner membrane ("positive-inside rule")[94] and the membrane of the endoplasmic reticulum ("charge-difference rule").[95,96] However, the net positive charge of OMM proteins does not seem to play such an important role in conferring protein topology in the lipid bilayer.[88,97] It has been proposed that orientation of the insertion of a transmembrane segment is determined by the absence or the presence of a retention signal on the OMM surface.[81] Amphiphilicity of the N-terminal hydrophilic region of Tom70 (amino acids 1–10) has been identified as an important determinant in conferring protein topology (Nin-Ccyto) in the lipid bilayer. Substitution of this small hydrophilic region with a strong matrix-targeting signal causes retention of the N-terminus of the protein on the cytosolic face of the OMM, resulting in the insertion of the entire protein in a reverse orientation (Ncyto-Cin).[98] Thus,

amphiphilicity rather than the number and position of basic amino acids, has been suggested to be a potential candidate for the retention signal.[97] Both the N- and C-termini of L-CPT I are exposed to the cytosol while the short loop connecting H1 and H2 is located in the intermembrane space.[53] It has been observed that the N-terminal domain of L-CPT I anchored reporter proteins at the OMM leaving them exposed to the cytosolic face of the membrane, as is the large cytosolic C-terminal region of L-CPT I.[51] This suggests that the N-terminal domain of L-CPT I could also participate in the determination of membrane topology of the protein. Additional work will be needed to establish if one of the amphiphilic regions (β1, β2, α1 or α3)present in the N-terminal domain of L-CPT I (Fig. 2) functions as a retention signal involved in membrane orientation of the enzyme.

5. FUNCTIONAL IMPORTANCE OF THE N-TERMINAL DOMAIN OF L-CPT I

Following mitochondrial targeting and OMM insertion, L-CPT I must fold correctly to attain its native functional conformation. It is now well established that L-CPT I possesses both catalytic and malonyl-CoA binding domains.[48] Both appear to be exposed on the cytosolic face of the OMM.[53] Analysis of the primary sequence of L-CPT I shows that its large cytosolic C-terminal region is the only part of the molecule that exhibits homology with CPT II and other members of the acylcarnitine transferase family.[6] This strongly suggests that the catalytic site of L-CPT I resides within its cytosolic C-terminal domain. The native functional conformation of L-CPT I is characterized by a highly folded state.[50,53] Heterologous expression in yeast has shown that when the large cytosolic C-terminal domain of L-CPT I is anchored at the OMM by the signal anchor sequence of Tom70, it exhibits a less folded structure.[51] Unfolding of this domain was accompanied by a huge decrease in functional activity and by a total loss of sensitivity to malonyl-CoA.[51] This confirms a previous study showing that the proteolytic cleavage of the N-terminal end of L-CPT I induced both the loss of activity and of malonyl-CoA sensitivity.[53] However, the functional implication of the N-terminal domain of L-CPT I seems to be more crucial for the maintenance of malonyl-CoA sensitivity than for functional activity. Heterologous expression in yeast of truncated L-CPT I lacking either the 18, 35, 52, 73 or 83 first N-terminal amino acids results in proteins that, although exhibiting about 64 to 69% of the activity of the wild type L-CPT I, were largely malonyl-CoA insensitive.[99] Loss of malonyl-CoA sensitivity was accompanied by a marked decrease in the affinity for malonyl-CoA.[99] The authors concluded that the malonyl-CoA binding site is separate from the catalytic site and that the first conserved 18 N-terminal amino acids are critical for malonyl-CoA binding and inhibition. However, fusion of the N-terminal domain of L-CPT I to the mature form of CPT II is unable to confer malonyl-CoA sensitivity to CPT II,[51] allowing two hypothesis to be formulated. The first is that if malonyl-CoA binds only to the N-terminal domain of L-CPT I, the CPT II component of the fusion protein must have had a tertiary conformation that was unable of interacting with this domain. The second is that the C-terminal region of L-CPT I is also critical for malonyl-CoA binding and that the necessary site(s) is missing in CPT II. From all these studies, it is still incertain whether the malonyl-CoA binding site resides in a linear sequence including the N-terminus of L-CPT I or in a structural domain stabilized by the interaction between the N- and C-terminal regions of the enzyme.

Far from being simple hydropohobic domains anchoring proteins at a membrane, it is now clear that the transmembrane α-helices of integral membrane proteins can participate in highly specific interactions. These interactions involve sufficient energy to drive their folding and oligomerization in some cases, and are being shown to contribute to an increasingly diverse set of functional roles.[100–102] Changes in the disposition of the hydrophobic domains within the lipid bilayer is expected to affect interactions between the N- and C-termini of a protein with subsequent effects on its kinetics characteristics.[103] Transmembrane segments of L-CPT I may also form such strong specific interactions with either each other or with the OMM that could be important for the maintenance of the folded functional conformation of the enzyme. Moreover, L-CPT I displays altered sensitivity to malonyl-CoA inhibition under different physiopathological situations.[11–16] It has been shown that increasing the fluidity of the OMM resulted in a decrease in the malonyl-CoA sensitivity of L-CPT I.[104,105] Thus, changes in the membrane microenvironment arround the two transmembrane segments of L-CPT I may affect their putative interactions inducing alterations in the functional properties of the enzyme.

6. CONCLUDING REMARKS

Because of its pivotal role in fuel metabolism, CPT I is viewed as a potent target site for pharmacological intervention, specially in pathological states characterized by excessive rates of fatty acid oxidation such as diabetes and heart diseases. The recent knowledge of the primary structure of the CPT I proteins has resulted in the emergence of new research fields. In particular, the elucidation of the structure/function/regulatory relationships of CPT I represents one of the main goals for the coming years. The first progress in this area reveals that the N-terminal domain of L-CPT I plays a crucial role in L-CPT I biogenesis and in the maintenance of an optimal conformation for both enzymatic activity and malonyl-CoA sensitivity. However, many critical questions remain still unsolved. To pose just some of them: On what residues of L-CPT I does malonyl-CoA bind and how does it exert its inhibitory effect? Is there a facilitated transport across the OMM of the acylcarnitines produced by CPT I on the cytosolic face of the OMM? Do CPT I and CPT II, which are rich in mitochondrial contact-sites,[106] interact with the carnitine-acylcarnitine translocase? Is functional CPT I under monomeric state or is it assembled into a complex? What are the features responsible for the different kinetic characteristics and inhibitor sensitivities of the liver and muscle CPT I isoforms? What is the conformational state(s) of these enzymes and how is it related to their sensitivity to malonyl-CoA inhibition? Development of new approaches and powerful tools in molecular biology will result in the emergence of more insights in the field of CPT I enzymes.

REFERENCES

1. McGarry, J.D., Woeltje, K.F., Kuwajima, M. & Foster, D.W. (1989) *Diabetes Metab. Rev.*, **5**, 271–284. Regulation of ketogenesis and the renaissance of carnitine palmitoyltransferase.
2. Eaton, S., Bartlett, K. & Pourfarzam, M. (1996) *Biochem. J.*, **320**, 345–357. Mammalian mitochondrial beta-oxidation.
3. McGarry, J.D. & Brown, N.F. (1997) *Eur. J. Biochem.*, **244**, 1–14. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis.

4. Park, E.A. & Cook, G.A. (1998) *Mol. Cell Biochem.*, **180**, 27–32. Differential regulation in the heart of mitochondrial carnitine palmitoyltransferase-I muscle and liver isoforms.
5. McGarry, J.D., Mannaerts, G.P. & Foster, D.W. (1977) *J. Clin. Invest.*, **60**, 265–270. A possible role for malonyl-CoA in the regulation of hepatic fatty acid oxidation and ketogenesis.
6. Esser, V., Britton, C.H., Weis, B.C., Foster, D.W. & McGarry, J.D. (1993) *J. Biol. Chem.*, **268**, 5817–5822. Cloning, sequencing, and expression of a cDNA encoding rat liver carnitine palmitoyltransferase I. Direct evidence that a single polypeptide is involved in inhibitor interaction and catalytic function.
7. Britton, C.H., Schultz, R.A., Zhang, B., Esser, V., Foster, D.W. & McGarry, J.D. (1995) *Proc. Natl. Acd. Sci. USA*, **92**, 1984–1988. Human liver mitochondrial carnitine palmitoyltransferase I: characterization of its cDNA and its chromosomal localization and partial analysis of the gene.
8. Yamazaki, N., Shinohara, Y., Shima, A. & Terada, H. (1995) *FEBS Lett.*, **363**, 41–45. High expression of a novel carnitine palmitoyltransferase I like protein in rat brown adipose tissue and heart: isolation and characterization of its cDNA clone.
9. Esser, V., Brown, N.F., Cowan, A.T., Foster, D.W. & McGarry, J.D. (1996) *J. Biol. Chem.*, **271**, 6972–6977. Expression of a cDNA isolated from rat brown adipose tissue and heart identifies the product as the muscle isoform of carnitine palmitoyltransferase I (M-CPT I). M-CPT I is the predominant CPT I isoform expressed in both white (epididymal) and brown adipocytes.
10. Yamazaki, N., Shinohara, Y., Shima, A., Yamanaka, Y. & Terada, H. (1996) *Biochim. Biophys. Acta*, **1307**, 157–161. Isolation and characterization of cDNA and genomic clones encoding human type carnitine palmitoyltransferase I.
11. Saggerson, E.D. & Carpenter, C.A. (1981) *FEBS Lett.*, **129**, 225–228. Effects of fasting, adrenalectomy and streptozotocin-diabetes on sensitivity of hepatic carnitine acyltransferase to malonyl-CoA.
12. Cook, G.A. (1984) *J. Biol. Chem.*, **259**, 12030–12033. Differences in the sensitivity of carnitine palmitoyltransferase to inhibition by malonyl-CoA are due to differences in Ki values.
13. Clouet, P., Henninger, C., Pascal, M. & Bezard, J. (1985) *FEBS Lett.*, **182**, 331–334. High sensitivity of carnitine acyltransferase I to malonyl-CoA inhibition in liver of obese Zucker rats.
14. Gamble, M.S. & Cook, G.A. (1985) *J. Biol. Chem.*, **260**, 9516–9519. Alteration of the apparent Ki of carnitine palmitoyltransferase for malonyl-CoA by the diabetic state and reversal by insulin.
15. Grantham, B.D. & Zammit, V.A. (1986) *Biochem. J.*, **239**, 485–488. Restoration of the properties of carnitine palmitoyltransferase I in liver mitochondria during re-feeding of starved rats.
16. Decaux, J.F., Ferré, P., Robin, D., Robin, P. & Girard, J. (1988) *J. Biol. Chem.*, **263**, 3284–3289. Decreased hepatic fatty acid oxidation at weaning in the rat is not linked to a variation of malonyl-CoA concentration.
17. Derrick, J. & Ramsay, R. (1989) *Biochem. J.*, **262**, 801–806. L-Carnitine acyltransferase in intact peroxisomes is inhibited by malonyl-CoA.
18. Broadway, N.M. & Saggerson, E.D. (1995) *Biochem. J.*, **310**, 989–995. Solubilization and separation of two distinct carnitine acyltransferase from hepatic microsomes: characterization of the malonyl-CoA-sensitive enzyme.
19. Singh, H., Beckman, K. & Poulos, A. (1996) *J. Lipid Res.*, **37**, 2616–2626. Evidence of two catalytically active carnitine medium/long chain acyltransferases in rat liver peroxisomes.
20. Pande, S.V., Bhuiyan, A.K.M.J. & Murthy, M.S.R. (1992) *Current concepts in carnitine research (Carter, A.L. ed.)*, 165–178. Carnitine palmitoyltransferase: How many and how to discriminate?
21. Faergeman, N.J. & Knudsen, J. (1997) *Biochem. J.*, **323**, 1–12. Role of long-chain fatty acyl-CoA esters in the regulation of metabolism and in cell signaling.
22. Huang, W.-H., Wang, Y. & Askari, A. (1989) *J. Biol. Chem.*, **264**, 2605–2608. Mechanism of the control of (Na+ +K+)-ATPase by long-chain acyl coenzyme A.
23. Deeney, J.T., Tornheim, K., Korchak, H.M., Prentki, M. & Corkey, B.E. (1992) *J. Biol. Chem.*, **267**, 19840–19845. Acyl-CoA esters modulate intracellular Ca^{2+} handling by permeabilized clonal pancreatic b-cells.
24. Nishizuka, Y. (1992) *Science*, **258**, 607–614. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C.
25. Pfanner, N., Orci, L., Glick, B.S., Amherdt, M., Arden, S.R., Malhotra, V. & Rothman, J.E. (1989) *Cell*, **59**, 95–102. Fatty acyl-coenzyme A is required for budding of transport vesicles from Golgi cisternae.
26. Rothman, J.E. & Orci, L. (1992) *Nature*, **355**, 409–415. Molecular dissection of the secretory pathway.
27. Ostermann, J., Orci, L., Tani, K., Amherdt, M., Ravazzola, M., Elazar, Z. & Rothman, J.E. (1993) *Cell*, **75**, 1015–1025. Stepwise assembly of functionally active transport vesicles.
28. Towler, D.A., Gordon, J.I., Adams, S.P. & Glaser, L. (1988) *Annu. Rev. Biochem.*, **57**, 69–99. The biology and enzymology of eucaryotic protein acylation.

29. Schults, A.M., Henderson, L.E. & Oroszlan, S. (1988) *Amu. Rev. Cell* Biol., **4**, 611–647. Fatty acylation of proteins.
30. Bouvier, M., Moffett, S., Loisel, T.P., Mouillac, B., Hebert, T. & Chidiac, P. (1995) *Biochem. Soc. Trans.*, **23,** 116–120. Palmitoylation of G-protein-coupled receptors: a dynamic modification with functional consequences.
31. Prentki, M. & Corkey, B.E. (1996) *Diabetes*, **45**, 273–283. Are the β-cell signaling molecules malonyl-CoA and cytosolic long-chain acyl-CoA implicated in multiple tissue defects of obesity and NIDDM?
32. Stein, D.T., Esser, V, Stevenson, B.E., Lane, K.E., Whiteside, J.H., Daniels, M.B., Chen, S. & McGarry, J.D. (1996) *J. Clin. Invest.*, **97**, 2728–2735. Essentiality of Circulating Fatty Acids for Glucose-stimulated Insulin Secretion in the Fasted Rat.
33. Lee, Y., Hirose, H., Zhou, Y.T., Esser, V., McGarry, J.D. & Unger, R.H. (1997) *Diabetes,* **46**, 408–413. Increased lipogenic capacity of the islets of obese rats: a role in the pathogenesis of NIDDM.
34. Stein, D.T., Stevenson, B.E., Chester, M.W., Basit, M., Daniels, M.B., Turley, S.D. & McGarry, J.D. (1997) *J. Clin. Invest.,* **100**, 398–403. The insulinotropic potency of fatty acids is influenced profoundly by their chain length and degree of saturation.
35. Dobbins, R.L., Chester, M.W., Stevenson, B.E., Daniels, M.B., Stein, D.T. & McGarry, J.D. (1998) *J. Clin. Invest.*, **101**, 2370–2376. A fatty acid-dependent step is critically important for both glucose- and non-glucose-stimulated insulin secretion.
36. McGarry, J.D. (1992) *Science*, **258**, 766–770. What if Minkowski had been ageusic? An alternate angle on diabetes.
37. McGarry, J.D. (1998) *Am. J. Clin. Nutr.*, **67**, 500S–504S. Glucose-fatty acid interactions in health and disease.
38. Zhou, Y.P. & Grill, V.E. (1994) *J. Clin. Invest.*, **93**, 870–876. Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle.
39. Bollheimer, L.C., Skelly, R.H., Chester, M.W., McGarry, J.D. & Rhodes, C.J. (1998) *J. Clin. Invest.*, **101**, 1094–1101. Chronic exposure to free fatty acid reduces pancreatic beta cell insulin content by increasing basal insulin secretion that is not compensated for by a corresponding increase in proinsulin biosynthesis translation.
40. Shimabukuro, M., Zhou, Y.T., Levi, M. & Unger, R.H. (1998) Proc. *Natl. Acad. Sci. USA*, **95**, 2498–2502. Fatty acid-induced β cell apoptosis: A link between obesity and diabetes.
41. Esser, V., Wyne, K.L., Comerford, S.A., Hammer, R.E. & McGarry, J.D. (1998) *European Association for the Study of Diabetes, Diabetologia*, **41,** A23.
42. Paumen, M.B., Ishida, Y., Muramatsu, M., Yamamoto, M. & Honjo, T. (1997) *J. Biol. Chem.*, **272**, 3324–3329. Inhibition of carnitine palmitoyltransferase I augments sphingolipid synthesis and palmitate-induced apoptosis.
43. de Vries, J.E., York, M.M., Poemen, T.H., de Jong, Y.F., Cleutjens, J.P., van der Vusse, G.J. & van Bilsen, M. (1997) *J. Lipid Res.*, **38**, 1384–1394. Saturated but not mono-unsaturated fatty acids induce apoptotic cell death in neonatal rat ventricular myocytes.
44. Green, D.R. & Reed, J.C. (1998) *Science*, **281**, 1309–1312. Mitochondria and Apoptosis.
45. Adams, J.M. & Cory, S. (1998) *Science*, **281**, 1322–1326. The Bcl-2 Protein Family: Arbiters of Cell Survival.
46. Paumen, M.B., Ishida, Y., Han, H., Muramatsu, M., Eguchi, Y, Tsujimoto, Y. & Honjo, T. (1997) *Biochem, Biophys. Res. Commun.*, **231**, 523–525. Direct interaction of mitochondrial membrane protein carnitine palmitoyltransferase I with Bcl-2.
47. Neupert, W. (1997) *Annu. Rev. Biochem.,* **66**, 863–917. Protein import into mitochondria.
48. Brown, N.F., Esser, V, Foster, D.W. & McGarry, J.D. (1994) *J. Biol. Chem.,* **269**, 26438–26442. Expression of a cDNA for rat liver carnitine palmitoyltransferase I in yeast establishes that catalytic activity and malonyl-CoA sensitivity reside in a single polypeptide.
49. de Vries, Y., Arvidson, D.N., Waterham, H.R., Cregg, J.M. & Woldegiorgis, G. (1997) *Biochemistry*, **36**, 5285–5292. Functional Characterization of Mitochondrial Carnitine Palmitoyltransferase I and II Expressed in the Yeast *Pichia pastoris.*
50. Prip-Buus, C., Cohen, I., Kohl, C., Esser, V., McGarry, J.D. & Girard, J. (1998) *FEBS Lett.*, **429**, 173–178. Topological and functional analysis of the rat liver carnitine palmitoyltransferase 1 expressed in Saccharomyces cerevisiae.
51. Cohen, I., Kohl, C., McGarry, J.D., Girard, J. & Prip-Buus, C. (1998) **273**, In press. The N-terminal Domain of Rat Liver Carnitine Palmitoyltransferase 1 Mediates Import into the Outer Mitochondrial Membrane and Is Essential for Activity and Malonyl-CoA Sensitivity.

52. Woeltje, K.F., Kuwajima, M., Foster, D.W. & McGarry, J.D. (1987) *J. Biol. Chem.*, **262**, 9822–9827. Characterization of the mitochondrial carnitine palmitoyltransferase enzyme system. II. Use of detergents and antibodies.
53. Fraser, F., Corstorphine, C.G. & Zammit, V.A. (1997) *Biochem. J.*, **323**, 711–718. Topology of carnitine palmitoyltransferase I in the mitochondrial outer membrane.
54. Fujiki, Y., Fowler, S., Shio, H., Hubbard, A.L. & Lazarow, P. (1982) *J. Cell Biol.*, **93**, 103–110. Polypeptide and phospholipid composition of the membrane of the rat liver peroxisomes: comparison with endoplasmic reticulum and mitochondrial membranes.
55. Ono, H. & Tuboi, S. (1987) *Eur. J. Biochem.*, **168**, 509–514. Integration of porin synthetized *in vitro* into outer mitochondrial membranes.
56. McBride, H.M., Millar, D.G., Li, J.-M. & Shore, G.C. (1992) *J. Cell Biol*, **119**, 1451–1457. A Signal-Anchor Sequence Selective for the Mitochondrial Outer Membrane.
57. Deshaies, R.J., Koch, B.D., Werner-Washburne, M., Craig, E.A. & Schekman, R. (1988) *Nature*, **332**, 800–805. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides.
58. Hachiya, N., Alam, R., Sakasegawa, Y., Sakaguchi, M., Mihara, K. & Omura, T. (1993) *EMBO J.*, **12**, 1579–1586. A mitochondrial import factor purified from rat liver cytosol is an ATP-dependent conformational modulator for precursor proteins.
59. Hachiya, N., Komiya, T., Alam, R., Iwahashi, J., Sakaguchi, M., Omura, T. & Mihara, K. (1994) *EMBO J.*, **13**, 5146–5154. MSF, a novel cytoplasmic chaperone which functions in precursor targeting to mitochondria.
60. Hachiya, N., Mihara, K., Suda, K., Horst, M., Schatz, G. & Lithgow, T. (1995) *Nature*, **376**, 705–708. Reconstitution of the initial steps of mitochondrial protein import.
61. Komiya, T., Sakaguchi, M. & Mihara, K. (1996) *EMBOJ.*, **15**, 399–407. Cytoplasmic chaperones determine the targeting pathway of precursor proteins to mitochondria.
62. Hartl, F.-U, Pfanner, N., Nicholson, D.W. & Neupert, W. (1989) *Biochim. Biophys. Acta*, **988**, 1–45. Mitochondrial protein import.
63. Schneider, H., Sollner, T., Dietmeier, K., Eckerskorn, C., Lottspeich, F., Trulzsch, B., Neupert, W. & Pfanner, N. (1991) *Science*, **254**, 1659–1662. Targeting of the master receptor MOM19 to mitochondria.
64. Ramage, L., Junne, T., Hahne, K., Lithgow, T. & Schatz, G. (1993) *EMBO J.*, **12**, 4115–4123. Functional cooperation of mitochondrial protein import receptors in yeast.
65. Schlossmann, J., Dietmeier, K., Pfanner, N. & Neupert, W. (1994) *J. Biol. Chem.*, **269**, 11893–11901. Specific Recognition of Mitochondrial Preproteins by the Cytosolic Domain of the Import Receptor MOM72.
66. Schlossmann, J. & Neupert, W. (1995) *J. Biol. Chem.*, **270**, 27116–27121. Assembly of the Preprotein Receptor MOM72/MAS70 into the Protein Import Complex of the Outer Membrane of Mitochondria.
67. Court, D.A., Nargang, F.E., Steiner, H., Hodges, R.S., Neupert, W. & Lill, R. (1996) *Mol. Cell. Biol.*, **16**, 4035–4042. Role of the Intermembrane-Space Domain of the Preprotein Receptor Tom22 in Protein Import into Mitochondria.
68. Goping, I.S., Millar, D.G. & Shore, G.C. (1995) *FEBS Lett.*, **373**, 45–50. Identification of the human mitochondrial protein import receptor, huMas20p. Complementation of delta mas20 in yeast.
69. Hanson, B., Nuttall, S. & Hoogenraad, N. (1996) *Eur. J. Biochem.*, **235**, 750–753. A receptor for the import of proteins into human mitochondria.
70. Komiya, T. & Mihara, K. (1996) *J. Biol. Chem.*, **271**, 22105–22110. Protein Import into Mammalian Mitochondria.
71. Armstrong, L.C., Komiya, T., Bergman, B.E., Mihara, K. & Bornstein, P. (1997) *J. Biol. Chem.*, **272**, 6510–6518. Metaxin is a component of a preprotein import complex in the outer membrane of the mammalian mitochondria.
72. Iwahashi, J., Yamazaki, S., Komiya, T., Nomura, N., Nishikawa, S.I., Endo, T. & Mihara, K. (1997) *J. Biol. Chem.*, **272**, 18467–18472. Analysis of the Functional Domain of the Rat Liver Mitochondrial Import Receptor Tom20.
73. Kiebler, M., Pfaller, R., Sollner, T., Griffiths, G., Horstmann, H., Pfanner, N. & Neupert, W. (1990) *Nature*, **348**, 610–616. Identification of a mitochondrial receptor complex required for recognition and membrane insertion of precursor proteins.
74. Baker, K.P., Schaniel, A., Vestweber, D. & Schatz, G. (1990) *Nature*, **348**, 605–609. A yeast mitochondrial outer membrane protein essential for protein import and cell viability.
75. Kassenbrock, C.K., Cao, W. & Douglas, M.G. (1993) *EMBOJ.*, **12**, 3023–3034. Genetic and biochemical characterization of ISP6, a small mitochondrial outer membrane protein associated with the protein translocation complex.

76. Honlinger, A., Bomer, U., Alconada, A., Eckerskorn, C., Lottspeich, F., Dietmeier, K. & Pfanner, N. (1996) *EMBO J.*, **15**, 2125–2137. Tom7 modulates the dynamics of the mitochondrial outer membrane translocase and plays a pathway-related role in protein import.
77. Dietmeier, K., Honlinger, A., Bomer, U., Dekker, P.J., Eckerskorn, C., Lottspeich, F., Kubrich, M. & Pfanner, N. (1997) *Nature*, **388**, 195–200. Tom5 functionally links mitochondrial preprotein receptors to the general import pore.
78. Brown, N.F, Esser, V., Gonzalez, A.D., Evans, C.T., Slaughter, C.A., Foster, D.W. & McGarry, J.D. (1991) *J. Biol. Chem.*, **266**, 15446–15449. Mitochondrial import and processing of rat liver carnitine palmitoyltransferase II defines the amino terminus of the mature protein. Possibility of differential modification of the rat and human isoforms.
79. Roise, D., Horvath, S.J., Tomich, J.M., J.H., R. & Schatz, G. (1986) *EMBO J.*, **5**, 1327–1334. A chemically synthesized pre-sequence of an imported mitochondrial protein can form an amphiphilic helix and perturb natural and artificial phospholipid bilayers.
80. Epand, R.M., Hui, S.W., Argan, C, Gillespie, L.L. & Shore, G.C. (1986) *J. Biol. Chem.*, **261**, 10017–10020. Structural analysis and amphiphilic properties of a chemically synthesized mitochondrial signal peptide.
81. Shore, G.C., McBride, H.M., Millar, D.G., Steenaart, N.A.E. & Nguyen, M. (1995) *Em J. Biochem.*, **227**, 9–18. Import and insertion of proteins into the mitochondrial outer membrane.
82. Wickner, W.T. & Lodish, H.F. (1985) *Science*, **230**, 400–407. Multiple mechanisms of protein insertion into and across membranes.
83. von Heijne, G. (1988) *Biochem. Biophys. Acta*, **947**, 307–333. Transcending the impenetrable: how proteins come to terms with membranes.
84. Singer, S.J. (1990) *Annu. Rev. Cell Biol.*, **6,** 247–296. The structure and insertion of integral proteins in membranes.
85. Nguyen, M., Millar, D.G., Yong, V.W., Korsmeyer, S.J. & Shore, G.C. (1993) *J. Biol. Chem.*, **268**, 25265–25268. Targeting of Bcl-2 to the Mitochondrial Outer Membrane by a COOH-terminal Signal Anchor Sequence.
86. Cao, W. & Douglas, M.G. (1995) *J. Biol. Chem.*, **270**, 56794–56798. Biogenesis of ISP6, a small carboxyl-terminal anchored protein of the receptor complex of the mitochondrial outer membrane.
87. Steenaart, N.A.E. & Shore, G.C. (1997) *J. Biol. Chem.*, **272**, 12057–12061. Alteration of a Mitochondrial Outer Membrane Signal Anchor Sequence That Permits Its Insertion into the Inner Membrane.
88. Rodriguez-Cousino, N., Nargang, F.E., Baardman, R., Neupert, W., Lill, R. & Court, D.A. (1998) *J. Biol. Chem.*, **373,** 11527–11532. An Import Signal in the Cytosolic Domain of the Neurospora Mitochondrial Outer Membrane Protein TOM22.
89. Eisenberg, D., Weiss, R.M. & Terwilliger, T.C. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 140–144. The hydrophobic moment detects periodicity in protein hydrophobicity.
90. Court, D.A., Kleene, R., Neupert, W. & Lill, R. (1996) *FEBS Lett.*, **390**, 73–77. Role of the N- and C-termini of porin in import into the outer membrane of Neurospora mitochondria.
91. Smith, M.D., Petrak, M., Boucher, P.D., Barton, K.N., Carter, L., Reddy, G., Blachly-Dyson, E., Forte, M., Price, J., Verner, K. & McCauley, R.B. (1995) *J. Biol. Chem.*, **270**, 28331–28336. Lysine Residues at Positions 234 and 236 in Yeast Porin Are Involved in Its Assembly into the Mitochondrial Outer Membrane.
92. Gaboriaud, C., Bissery, V., Benchetrit, T. & Mornon, J.P. (1987) *FEBS Lett.*, **224**, 149–155. Hydrophobic Cluster Analysis: an efficient new way to compare and analyse amino acid sequences.
93. Lemesle-Varloot, L., Henrissat, B., Gaboriaud, C., Bissery, V., Morgat, A. & Mornon, J.P. (1990) *Biochimie*, **72**, 555–574. Hydrophobic analysis: procedures to drive structural and functional information from 2-D-representation of protein sequences.
94. von Heijne, G. (1992) *J. Mol. Biol.*, **225**, 487–494. Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule.
95. Hartmann, E., Rapoport, T.A. & Lodish, H.F. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 5786–5790. Predicting the orientation of eukaryotic membrane-spanning proteins.
96. Harley, C.A., Holt, J.A., Turner, R. & Tipper, D.J. (1998) *J. Biol. Chem.*, **249**, 24963–24971. Transmembrane protein insertion orientation in yeast depends on the charge difference across transmembrane segments, their total hydrophobicity, and its distribution.
97. Steenaart, N.A.E., Silvius, J.R. & Shore, G.C. (1996) *Biochemistry*, **35**, 3764–3771. An amphiphilic lipid-binding domain influences the topology of a signal-anchor sequence in the mitochondrial outer membrane.
98. Li, J.-M. & Shore, G.C. (1992) *Science*, **256**, 1815–1817. Reversal of the Orientation of an Integral Protein of the Mitochondrial Outer Membrane.

99. Shi, J., Zhu, H., Arvidson, D.N., Cregg, J.M. & Woldegiorgis, G. (1998) *Biochemistry*, **37**, 11033–11038. Deletion of the Conserved First 18 N-Terminal Amino Acid Residues in Rat Liver Carnitine Palmitoyltransferase I Abolishes Malonyl-CoA Sensitivity and Binding.
100. Lemmon, M.A. & Engelman, D.M. (1992) *Curr. Opin. Struct. Biol*, **2**, 511–518. Helix-helix interactions inside lipid bilayers.
101. Popot, J.-L. (1993) *Curr. Opin. Struct. Biol.*, **3**, 532–540. Integral membrane protein structure transmembrane a-helices as autonomous folding domains.
102. Lemmon, M.A. & Engelman, D.M. (1994) *FEBS Lett.*, **346**, 17–20. Specifity and promiscuity in membrane helix interactions.
103. Popot, J.-L., De Vitry, C. & Attela, A. (1994) *Membrane Protein Structure: Experimental approaches (White, S.H.ed), 41–96.*
104. Kolodziej, M.P. & Zammit, V.A. (1990) *Biochem. J.*, **272**, 421–425. Sensitivity of inhibition of rat liver mitochondrial outer-membrane carnitine palmitoyltransferase by malonyl-CoA to chemical- and temperature-induced changes in membrane fluidity.
105. Zammit, V.A., Corstorphine, C.G., Kolodziej, M.P. & Fraser, F. (1998) *Lipids*, **33**, 371–376. Lipid molecular order in liver mitochondrial outer membranes, and sensitivity of carnitine palmitoyltransferase I to malonyl-CoA.
106. Fraser, F. & Zammit, V.A. (1998) *Biochem. J.*, **329**, 225–229. Enrichment of carnitine palmitoyltransferase I and II in the contact sites of rat liver mitochondria.

SUBCELLULAR DISTRIBUTION OF MITOCHONDRIAL CARNITINE PALMITOYLTRANSFERASE I IN RAT LIVER

Evidence for a Distinctive N-Terminal Structure of the Microsomal but Not the Peroxisomal Enzyme

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1. SUMMARY

Mitochondria, microsomes and peroxisomes all express overt (cytosol-facing) carnitine palmitoyltransferase activities that are inhibitable by malonyl-CoA and are collectively termed CPT_o. In order to quantify the relative contribution of the different membrane systems towards overall hepatocyte activity, all three membrane fractions and a high-speed supernatant (soluble) fraction were prepared quantitatively from rat liver homogenates. The overt (malonyl-CoA-sensitive) carnitine palmitoyltransferase activity (CPT_o) associated with the different fractions were measured. In parallel experiments, rat livers were perfused *in situ* with oxygenated medium containing dinitrophenyl (DNP) - etomoxir in order to label covalently (with DNP-etomoxiryl-CoA) and quantitatively the molecular species responsible for CPT_o activity in each of the membrane systems under near-physiological conditions. Mitochondria accounted for only 65% of total cellular overt CPT activity, with the microsomal and peroxisomal contributions accounting for the remaining 25% and 10%, respectively. A single major protein with an identical molecular size (Mr 88,000) was labelled by DNP-etomoxir perfusion in all three membrane fractions. The abundance of this 88kDa protein in each fraction was quantitatively positively related to the respective specific activities of overt CPT. The same protein was immunoreactive with three anti-peptide antibodies raised against linear epitopes within the N- and C-terminal and loop (L) domains of the mitochondrial outer membrane

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CPT I of the liver mitochondrial outer membrane (L-CPT I). However, whereas reaction with anti-L and anti-C antipeptide antibodies were proportional to the respective overt CPT activities and DNP-etomoxir labelling in all three membrane fractions, reaction with anti-N peptide antibody was much stronger for microsomal CPT. We conclude that in all three membrane systems overt CPT activity is associated with the same or highly similar molecular species to mitochondrial outer membrane CPT I, but that the protein expressed in microsomes has a modified N-terminal domain, which gives the microsomal enzyme its higher malonyl-CoA sensitivity and may target the protein to its microsomal location.

2. INTRODUCTION

The overt CPT activities (CPT_o) of mitochondria, microsomes and peroxisomes are inhibited by malonyl-CoA,[1–3] in contrast to the latent CPT isoforms. Of all the hepatic overt CPT activities, only the protein responsible for the activity in the mitochondrial outer membrane (L-CPT I) is well-characterised.[4–6] Results published in abstract form[7] have indicated that a peroxisomal protein resembling L-CPT I may be responsible for the overt CPT activity of these organelles, but the authors suggested that it is immunologically distinct from L-CPT I. A microsomal protein of Mr 47,000 has previously been postulated to represent the microsomal CPT_o on the basis that, when microsomes are incubated with [^{3}H]-etomoxir, CoA and ATP, a protein of this molecular size is labelled most prominently.[8] Similar observations were made by[9] who, however, also observed that under these *in vitro* conditions at least one other protein of Mr 87,000 was also prominently labelled. More recently, it has been confirmed that microsomes express a malonyl-CoA-sensitive overt CPT which has an apparent molecular size of 300 kDa in detergent extracts of the membranes.[10] On the basis of its different kinetic behaviour, ease of solubilisation and stability of catalytic activity when solubilised, it was concluded that the microsomal enzyme is distinct from the mitochondrial outer membrane CPT I.[10] So, current opinion is that, apart from the common sensitivity of all the overt CPT enzymes to malonyl-CoA inhibition, the proteins responsible for this activity in the mitochondrial outer membrane, microsomes and peroxisomes are distinct molecular entities.

Therefore, in the present study we have measured CPT_o activity in mitochondrial, microsomal, peroxisomal and high-speed supernatant fractions prepared from rat liver on a quantitativle basis. We have also quantified the relative expression of proteins that bind dinitrophenyl (DNP)-etomoxiryl-CoA (an oxirane ring-containing inhibitor of hepatic CPT I[11]) in each of these fractions in the intact liver under conditions in which all three activities were optimally inhibited. Our results indicate that the overt CPT activity of microsomes and peroxisomes is associated with a protein that is very similar, if not identical, to mitochondrial CPT I. In microsomes too, the CPT_o activity is associated with a protein of Mr 88,000, but in this membrane fraction, there is evidence that the N-terminal domain has different properties from those of mitochondrial and peroxisomal CPT I.

3. METHODS

Subcellular fractions were prepared by standard differential and density gradient centrifugation protocols. Purified fractions of mitochondria and peroxisomes were prepared by Percoll[12,13] and Nycodenz[14] centrifugation, respectively. The following marker

enzyme activities were measured as described previously:[4,13] cytochrome c oxidase (mitochondria), NADPH-Cytochrome c oxido-reductase (microsomes), uricase (peroxisomes), lactate dehydrogenase (cytosol). These activities were used to correct for cross-contamination between the different fractions. CPT_o activities were measured using an optimal concentration of palmitoyl-CoA ($135\mu M$ in the presence of 1% albumin) as described previously.[15] The various subcellular fractions were substantially pure as judged by the marker enzyme activity measurements; the corrections needed to compensate for cross-contamination of fractions were relatively minor (not shown).

Quantitative labelling of DNP-etomoxiryl-CoA-binding proteins was performed in the intact liver prior to preparation of the various subcellular fractions. Animals were anaesthetised with pentobarbitone (60mg/kg) and their livers (weighing 6–7 g) were perfused *in situ* for 30min with 250ml of oxygenated (95% O_2 : 5% CO_2) recirculating Krebs-Henseleit medium[16] containing $2.5\mu mol$ DNP-etomoxir, at 37 °C. At the end of the perfusion, the liver was excised, cooled, homogenised and subcellular fractions were prepared. Liver perfusion with DNP-etomoxir (which is converted into the effective inhibitory species DNP-etomoxiryl-CoA within the cell) resulted in >95% inhibition of overt CPT_o activity in all three membranous fractions. The component proteins in aliquots of subcellular fractions were separated on 8% polyacrylamide gels and transferred on to nitrocellulose. The resulting blots were probed with rabbit anti-DNP and sheep anti-CPT I peptide antibodies (raised against linear epitopes N, L and C of mitochondrial outer membrane CPT I as described by[4]) followed by appropriate secondary antibodies conjugated to alkaline phosphatase. Bound secondary antibody was visualised by the action of the phosphatase on nitro blue tetrazolium (NBT) and 5-bromo-l-chloro-3-indolyl phosphate (BCIP). The intensity of the resulting bands was quantified using a densitometer (Molecular Dynamics).

4. RESULTS

4.1. Overt Carnitine Palmitoyltransferase Activity (CPT_o)

The overt carnitine palmitoyltransferase activities in each fraction were measured with an optimal concentration of palmitoyl-CoA (above). A previous report suggested that palmitoyl-CoA is not a good substrate for overt CPT in microsomes.[10] However, we have not been able to confirm this, as the palmitoyl-CoA requirement for the enzymes in all three membranous fractions was very similar to that described previously for mitochondrial outer membrane CPT I.[17,18] Peroxisomes displayed the highest specific activity of overt CPT (see below) and their overall contribution, computed on a per gram liver basis was appreciable and of the same order of that in microsomes, which had a relatively low specific activity, but which occurred at much higher protein densities within the cell. Mitochondria accounted for about 65% of total CPT_o activity. These estimates are very similar to those reported by.[19] It is evident, therefore, that the peroxisomal and microsomal forms of overt CPT constitute an important component of the overall CPT activity with access to the cytosolic pools of acyl-CoA and malonyl-CoA.

4.2. DNP-Etomoxryl-CoA Labelling of Cell Proteins

In order to identify and quantify the relative amounts of malonyl-CoA-inhibitable CPT proteins expressed in each fraction we labelled them in the intact liver *in situ* prior to the quantitative preparation of subcellular fractions. This ensured that *in vitro* arte-

facts due to non-specific binding of unesterified 2-oxirane compound were obviated. In previous studies, this *in vitro* approach has resulted in the spurious labelling of several proteins.[9] In addition, labelling of the proteins prior to preparation of liver homogenates enabled quantification on the same basis as for CPT_o activity. A minimal quantity of DNP-etomoxir, sufficient to inhibit 90–95% of the activities in all three membrane fractions was used. Preliminary experiments established that recirculating perfusion of rat liver (6–8 g) with 250ml of medium containing 2.5μmol of DNP-etomoxir for 30min at 37°C was sufficient to achieve this. This indicated that this amount of DNP-etomoxir generated enough etomoxiryl-CoA to modify covalently all the malonyl-CoA-sensitive CPT expressed on the surfaces of all three membrane systems. Use of only 0.5μmol DNP-etomoxir resulted in only partial inhibition of these activities, whereas use of excess inhibitor (>10μmol) resulted in the non-specific labelling of several proteins, particularly in peroxisomes (not shown, see below). The extent of DNP-etomoxiryl-CoA binding to each fraction was quantified by SDS-gel separation of the constituent proteins, electroblotting on to nitrocellulose and quantification with anti-DNP antibody.

A representative Western blot is shown in Figure 1. In all three membrane fractions only a protein that migrates with an apparent mass of 88kDa was reproducibly labelled by DNP-etomoxiryl-CoA when quantitative CPT inhibition had been achieved. A smaller protein was sometimes labelled in peroxisomes, but its extent of labelling was very variable and not proportional to CPT activity. While for mitochondria and peroxisomes the labelling of the 88kDa protein was as expected from the studies in,[6,7,20,21] the result was surprising for microsomes. This in view of the previous suggestion[8] that CPT activity in these membranes is associated with a molecular species of Mr 47,000. Evidence that the labelled Mr 88,000 protein represents the microsomal overt CPT stems from the observations that: (i) the quantitative inhibition of CPT_o in all the fractions was achieved under conditions which also labelled this protein and (ii) that the capacity for specific

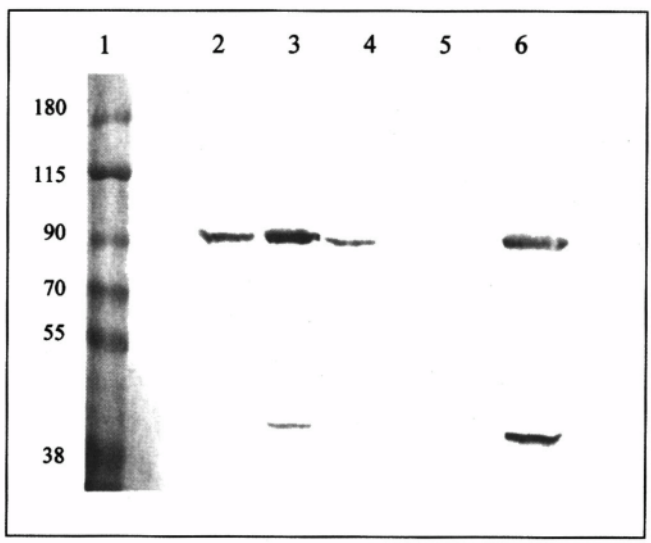

Figure 1. Anti-DNP antibody reproducibly detects only one DNP-etomoxiryl-CoA-labelled protein (88 kDa) in mitochondria, microsomes and peroxisomes prepared from rat livers previously perfused with DNP-etomoxir.

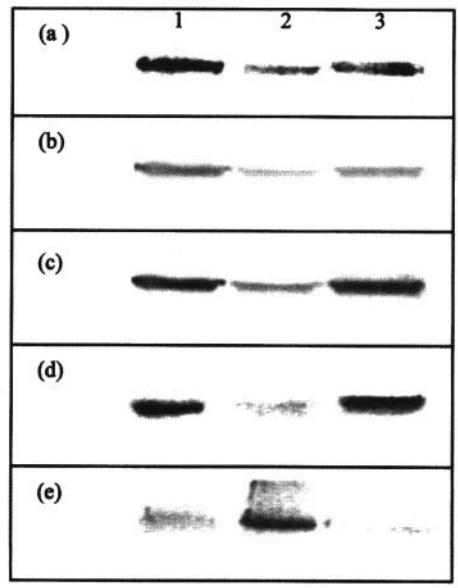

Figure 2. Microsomal CPT_o reacts disproportionately strongly with anti-N antibody raised against the Val14 ñ Lys29 epitope of liver mitochondrial CPT I. Reactions with the two other epitopes are strictly proportional to DNP-etomoxiryl-CoA labelling. Subcellular fractions were prepared as described in the text and 100 µg protein of each was subjected to SDS-PAGE, followed by probing with (a) anti-DNP, (b) anti-L, (c) anti-C, and (d) anti-N antibodies. See text for details.

binding of DNP-etomoxiryl-CoA by the different membranous fractions (expressed per mg protein) was positively related to the respective specific activities of CPT_o. Thus, peroxisomes showed the highest specific for both DNP-etomoxiryl-CoA binding and overt CPT activity (per mg membrane protein), whereas microsomes had the lowest values for both parameters (Figs. 2 and 3).

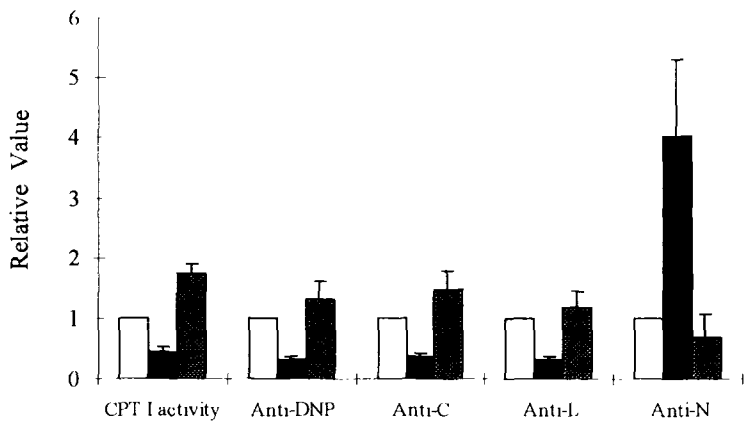

Figure 3. Quantification of immunoreactivity of 88 kDa CPT_o protein in each of the three subcellular fractions, compared with CPT_o specific activity. Reaction of microsomal CPT_o with anti-N antibody is disproportionately higher than that expected from reaction with other antibodies or CPT_o activity. Data obtained as described in Figure 2 were quantified using densitometry. They are compared with the relative values of the CPT_o activity observed in each fraction. In order to standardise the expression of the data, the values for mitochondria have been set at 100% and those for the other fractions expressed as a percentage.

4.3. Immunological Cross-Reaction of DNP-Etomoxiryl-CoA-Binding Species with Anti-Mitochondrial CPT I Antibodies

Blots of the constituent proteins of the three membrane fractions were probed with the three antibodies raised against linear epitopes of mitochondrial outer membrane L-CPT I described by.[4] All three antibodies detected the Mr 88,000 protein in mitochondria, microsomes and peroxisomes. However, important differences were observed in the relative intensities of the reaction of the antibodies with respect to CPT_o activity and DNP-etomoxir labelling (Fig. 2). Quantification of the bands (performed by densitometry) is presented in Fig. 3, together with the relative CPT_o activities in each fraction. It can be seen that, whereas the relative intensities of interaction of anti L- and anti-C peptide antibodies were strictly proportional to CPT_o activity and expression (as judged by DNP-etomoxir labelling) for all three membrane fractions, the intensity of reaction of the microsomal CPT_o with anti-N antibody was anomalous. As expected from our previous studies[4] anti-N antibody gave a very weak reaction with intact CPT I in mitochondrial extracts. This was also the case for the peroxisomal protein. However, the signal obtained with the microsomal protein was much stronger than would have been anticipated from the relatively low expression of either CPT_o activity or DNP-etomoxir labelling in the microsomal fraction. This indicated that in microsomes, the N-terminal domain of CPT I is subtly different from that in either mitochondria or peroxisomes. Its strong reaction with anti-N antibody is reminisent of that obtained for the mitochondrial protein after it is N-terminally proteolytically cleaved.[4] In that instance we suggested that loss of the extreme N-terminus unmasks the N-epitope (Vall4-Lys29) when the protein is partially refolded on nitrocellulose. However, we do not consider that this is a likely explanation for the strong detection of this epitope in the Western blots of the microsomal protein, as the band detected is of an identical molecular size as that of either mitochondrial or peroxisomal CPT I (Figs. 1 and 2).

5. DISCUSSION

CPT_o activity in microsomes and peroxisomes is accompanied by the presence of only one molecular species of Mr 88,000 that binds DNP-etomoxiryl-CoA. Several lines of evidence suggest that this protein is very similar, if not identical, to mitochondrial outer membrane CPT I. Thus: (i) their sizes appear to be identical on SDS-gels, (ii) their relative abundance in the three membrane fractions studied varies in parallel and (iii) anti-peptide antibodies raised against known linear sequences of mitochondrial CPT I cross-react with the same protein in peroxisomes and microsomes. These observations raise the strong possibility that the CPT_o activity of all three fractions is due to the expression of a protein identical, or very similar, to mitochondrial CPT I. Such a possibility was raised (in abstract form,[7]) for peroxisomal overt CPT. It is noteworthy that in[9] substantial labelling of a microsomal protein of Mr 87,000 by [^{3}H]-etomoxir in the presence of ATP and CoA was also observed. Moreover, the labelling of this protein could be attenuated by prior incubation of the microsomes with malonyl-CoA, suggesting that in spite of its lower degree of labelling under *in vitro* conditions, this 87,000kDa protein represents the actual CPT_o protein. However, the possibility that this 87,000kDa protein could represent microsomal overt CPT was discounted by those authors because of their contention that it was a regulatory subunit (a theory which has since been shown to be untenable, at least for mitochondrial CPT I, see[6,22]). Therefore, our present results suggest

that the current ideas about the molecular size of microsomal overt CPT need to be revised.

The suggestion that overt CPT in different cell membrane systems could be due to the same, or very similar, molecular species is not without parallels. Thus, long-chain acyl-CoA synthase, which catalyses the formation of the acyl-CoA esters utilised by CPT_o, exists in all three membrane systems as an identical molecular species.[23] Similarly, Bcl-2, the anti-apoptotic protein which has been shown, by yeast two-hybrid screening, to interact specifically with CPT I[24] is located not only in the mitochondrial outer membrane, but also in the endoplasmic reticular and nuclear membranes.[25,26] Targeting of such widely distributed proteins to different cytosol-facing membranes may involve the use of multiple[27] or hierarchical[28,29] targeting sequences, co-translational import directly from cytosolic polysomes[30] or the use of splice variant mRNA species that differ only in their 5′-untranslated but which are important in directing site-specific translation regions (e.g. for long-chain acyl-CoA synthetase[31]).

In the light of our observations, it is evident that work is now required to verify the degree of identity between the molecular species responsible for CPT_o activity in mitochondria, microsomes and peroxisomes and to identify the factors that determine their specific targeting to these various intracellular sites. In this context, we want to highlight the important inference we now make about the apparent distinctiveness of the secondary structure of the N-terminal domain of microsomal CPT_o compared to that of CPT_o expressed in the mitochondrial outer membrane and the peroxisomes. Our data indicate that the secondary structure of the N-terminal domain of microsomal CPT_o differs in some crucial respect. In view of our previous observation[4] that the N-terminal domain is very important in determining the malonyl-CoA sensitivity of mitochondrial CPT I, it is tempting to speculate that the 50% lower IC_{50} of the microsomal enzyme for malonyl-CoA inhibition[19] may derive from this uniquely modified N-terminus. It is also possible that any such modification may be responsible for the differential targeting of the protein to the microsomes on the one hand and the mitochondrial outer membrane and peroxisomes on the other. Putative internal sequences capable of targeting the protein to both peroxisomes and the mitochondrial outer membrane exist in the deduced primary amino acid sequence for liver CPT I.[6]

We have recently described the topology of mitochondrial CPT I within the outer membrane,[4] as well as its distribution to the specialised regions of the membrane that form contact sites with the mitochondrial inner membrane.[5] It is of interest that these contact sites are also involved in mitochondrial-endoplasmic reticular contact involving a specialised population of the microsomes.[32] In view of the (near) identity of mitochondrial and microsomal overt CPT molecular species, it would be of interest to explore whether they also occupy closely-associated microenvironments within the cell.

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REFERENCES

1. Lilly, K., Bugaisky, G., Umeda, P. & Bieber, L. (1990) *Arch Biochem Biophys* **280**, 167–174 The medium-chain carnitine acyltransferase activity associated with rat liver microsomes is malonyl-CoA sensitive.

2. McGarry, J.D., Mannaerts, G.P. & Foster, D.W. (1978) *Biochim Biophys Acta* **530**, 305–313 Characteristics of fatty acid oxidation in ral liver homogenates and the inhibitory effect of malonyl-CoA.
3. NicA'Bhaird, N. & Ramsay, R. (1992) *Biochem J* **286**, 637–640 Malonyl-CoA inhibition of peroxisomal carnitine octanoyltransferase.
4. Fraser, F., Corstorphine, C.G. & Zammit, V.A. (1997) *Biochem J* **323**, 711–718 Topology of carnitine palmitoyltransferase I in the mitochondrial outer membrane.
5. Fraser, F. & Zammit, V.A. (1998) *Biochem J* **329**, 225–229 Enrichment of carnitine palmitoyl transferases I and II in the contact sites of rat liver mitochondria.
6. Esser, V., Britton, C.H., Weis, B.C., Foster, D.W. & McGarry, J.D. (1993) *J Biol Chem* **268**, 5817–5822 Cloning, sequencing, and expression of a cDNA encoding rat liver carnitine palmiloyltransferase I. Direct evidence that a single polypeptide is involved in inhibitor interaction and catalytic function.
7. Murthy, M.S.R., Esser, V., McGarry, J.D. & Pande, S.V. (1996) *FASEB J* **10**, A506 The malonyl-CoA sensitive CPT of peroxisomes resembles the mitochondrial CPT I but is a distinct protein.
8. Murthy, M.S.R. & Pande, S.V. (1994) *J Biol Chem* **269**, 18283–18286 Malonyl-CoA-sensitive and -insensitive carnitine palmitoyltransferase activities of microsomes are due to different proteins.
9. Lilly, K., Chung, C., Kerner, J., VanRenterghem, R. & Bieber, L. (1992) *Biochem Pharmacol* **43**, 353–361 Effect of etomoxyryl-CoA on different carnitine acyltransferases.
10. Broadway, N.M. & Saggerson, E.D. (1995) *Biochem J* **310**, 989–995 Solubilization and separation of two distinct carnitine acyltransferases from hepatic microsomes: characterization of the malonyl-CoA-sensitive enzyme.
11. Esser, V. *et al.* (1993) *J Biol Chem* **268**, 5810–5816 Inhibitors of mitochondrial carnitine palmitoyltransferase I limit the action of proteases on the enzyme. Isolation and partial amino acid analysis of a truncated form of the rat liver isozyme.
12. Kolodziej, M.P. & Zammit, V.A. (1990) *Biochem J* **272**, 421–425 Sensitivity of inhibition of rat liver mitochondrial outer-membrane carnitine palmitoyltransferase by malonyl-CoA to chemical- and temperature-induced changes in membrane fluidity.
13. Kolodziej, M.P. & Zammit, V.A. (1990) *Biochem J* **267**, 85–90 Re-evaluation of the interaction of malonyl-CoA with the rat liver mitochondrial carnitine palmitoyltransferase system by using purified outer membranes.
14. Derrick, J. & Ramsay, R. (1989) *Biochem J* **262**, 801–806 L-carnitine acyltransferase in intact peroxisomes is inhibited by malonyl-CoA.
15. Drynan, L., Quant, P.A. & Zammit, V.A. (1996) *Biochem J* **317**, 791–795 The role of changes in the sensitivity of hepatic mitochondrial overt carnitine palmitoyltransferase in determining the onset of the ketosis of starvation in the rat.
16. Krebs, H. & Henseleit, K. (1932) *Hoppe-Seyler's Z Physiol Chem* **210**, 33–66 Untersuchungen über die Harnstoffbildung im Tierkörper.
17. Grantham, B.D. & Zammit, V.A. (1986) *Biochem J* **239**, 485–8 Restoration of the properties of carnitine palmitoyltransferase I in liver mitochondria during re- feeding of starved rats.
18. Grantham, B.D. & Zammit, V.A. (1988) *Biochem J* **249**, 409–414 Role of carnitine palmitoyltransferase I in the regulation of hepatic ketogenesis during the onset and reversal of chronic diabetes.
19. Ramsay, R. (1993) *Biochem. Life Sci Adv* **12**, 23–29 A comparison of the malonyl-CoA sensitive carnitine palmitoyltransfease activities on cytoplasmic substrates and their distribution in mitochondria, peroxisomes and microsomes.
20. Zammit, V.A., Corstorphine, C.G. & Kelliher, M.G. (1988) *Biochem J* **250**, 415–420 Evidence for distinct functional molecular sizes of carnitine palmitoyltransferases I and II in rat liver mitochondria.
21. Kolodziej, M.P, Crilly, P.J., Corstorphine, C.G. & Zammit, V.A. (1992) *Biochem J* **282**, 415–421 Development and characterization of a polyclonal antibody against rat liver mitochondrial overt carnitine palmitoyltransferase (CPT I). Distinction of CPT I from CPT II and of isoforms of CPT I in different tissues.
22. Esser, V., Brown, N.F., Cowan, A.T., Foster, D.W. & McGarry, J.D. (1996) *J Biol Chem* **271**, 6972–6977 Expression of a cDNA isolated from rat brown adipose tissue identifies the product as the heart/muscle isoform of carnitine palmitoyltransferase I (M-CPT I). M-CPT I is the predominant CPT I isoform expressed in both while (epidydimal) and brown adipocytes.
23. Suzuki, H. *et al.* (1990) *J Biol Chem* **265**, 8681–8685 Structure and regulation of rat long-chain acyl-CoA synthase.
24. Paumen, M., Ishida, Y., Muramatsu, M., Yamamoto, M. & Honjo, T. (1997) *J Biol Chem* **272**, 3324–3329 Inhibition of carnitine palmitoyltransferase I augments sphingolipid synthesis and palmitate-induccd apoptosis.
25. Yang, E. & Korsmeyer, S. (1996) *Blood* **88**, 386–401 Molecular thanatopsis: A discourse on the Bcl-2 family and cell death.

26. Reed, J. (1994) *J Cell Biol* **124**, 1–6 Bcl-2 and the regulation of programmed cell death.
27. McNew, J. & Goodman, J. (1996) *Trends Biochem Sci* **21**, 54–58 The targeting and assembly of peroxisomal proteins: some old rules do not apply.
28. Hurt, E. & Schatz, G. (1987) *Nature* **325**, 499–452 A cytosolic protein contains a cryptic mitochondrial targeting signal.
29. Coleman, A. & Robinson, C. (1986) *Cell* **46**, 321–322 Protein import into organelles: Hierarchical target Signals.
30. Verner, K. (1993) *Trends Biochem Sci* **18**, 366–371 Co-translational protein import into mitochondria: an alternative view.
31. Suzuki, H., Watanabe, M., Fujino, T. & Yamamoto, T. (1995) *J Biol Chem* **270**, 9676–9682 Multiple promoters in rat acyl-CoA synthetase gene mediate dillerential expression of multiple transcipts with 5'-end heterogeneity.
32. Vance, J.E. (1990) *J Biol Chem* **265**, 7248–7256 Phospholipid synthesis in a membrane fraction associated with mitochondria.

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TOPOLOGY OF HEPATIC MITOCHONDRIAL CARNITINE PALMITOYLTRANSFERASE I

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1. INTRODUCTION

Before long-chain fatty acids can enter the mitochondria and get access to the β-oxidation pathway, they must first be activated to acyl-CoA in a reaction that requires ATP and coenzyme-A. The acyl-CoA still cannot cross the mitochondrial inner membrane and must react with carnitine to form the corresponding carnitine ester. This reaction is catalyzed by the enzyme carnitine palmitoyltransferase (CPT).[1] The acylcarnitine itself is also unable to diffuse into the mitochondrial matrix so that the transport is achieved by a specific protein, the carnitine acylcarnitine translocase.[2] Following transport across the mitochondrial inner membrane, acylcarnitines are converted back to the corresponding acyl-CoA and carnitine. This reaction is catalyzed by another carnitine palmitoyltransferase which is a different enzyme than that involved in the formation of the acylcarnitine outside the mitochondria.[1] Hence, there are two CPTs, one associated with the inner aspect of the mitochondrial inner membrane, CPT-II[3–7] and one that lies

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outside the mitochondrial inner membrane barrier, CPT-I. This overt or outer CPT is of physiological importance since it is regulated through inhibition by malonyl-CoA, an intermediate in the biosynthetic pathway of fatty acids.[8–12] Also of major importance in the hepatic regulation of the overt CPT are changes in the activity and sensitivity to inhibition by malonyl-CoA in changing physiological and pathophysiological states such as starvation and diabetes.[12–16] Quantitatively, the most significant change that occurs with the onset of diabetes[15] or the feeding-starvation transition[17] is at least a 10-fold increase in the apparent K_i for malonyl-CoA.

1.1. Location of CPT-I

The exact location of CPT-I has been the subject of much debate. Until recently its location was thought to be on the outer surface of the mitochondrial inner membrane.[18] However, data presented by Murthy and Pande[7] suggested that CPT-I is associated with the mitochondrial outer membrane. Using limited proteolysis of intact mitochondria and isolated mitochondrial outer membranes, their data indicated that the protease Nagarse had little effect on CPT activity but that the inhibitory effects of malonyl-CoA were greatly reduced. These data suggested that the outer CPT, (CPT-I) has a malonyl-CoA binding site facing the cytosol and an active acyl-CoA site facing the intermembrane space, clearly suggesting two separate binding sites. They also reported that other proteases such as trypsin and papain had essentially the same effects but we reported that the effects of trypsin were somewhat different from the actions of the other two proteases,[19] and we presented an explanation for those differences.[20]

1.2. Do all Inhibitors of CPT-I Act at a Common site?

The relationship between acyl-CoA and malonyl-CoA binding sites has also been subject to debate. It is not clearly established whether they bind at the same site or even whether they bind to the same polypeptide.[21–25] Data presented by Murthy and Pande suggests two different binding sites. The suggestion that all inhibitors of CPT-I act at a common site did not seem logical to us since many of these inhibitors have very different structures and mechanisms of inhibition. For example, malonyl-CoA is a competitive inhibitor with respect to acyl-CoA and noncompetitive with respect to carnitine,[17,26] 4-hydroxyphenylglyoxylate (HPG), the active metabolite of oxfenicine, is a competitive inhibitor with respect to carnitine but noncompetitive with respect to acyl-CoA.[27] N-Benzyladriamycin-14-valerate (AD 198), an analogue of adriamycin, is a noncompetitive inhibitor with respect to carnitine but uncompetitive with respect to acyl-CoA[28] while glyburide is noncompetitive with respect to acyl-CoA but uncompetitive with respect to carnitine.[26] DL-2-Bromopalmitoyl-CoA was synthesized as a substrate analogue,[29] and is expected to bind to the active site. One of the first studies we did was to examine the effects on the inhibitory actions of these different chemical compounds that are produced by exposure of intact mitochondria to three different proteases—Nagarse, papain and trypsin—with the hope of learning whether they bind inside or outside the mitochondrial outer membrane. As indicated in Table 1, exposure of intact mitochondria to Nagarse at 37°C for 20min had relatively slight effects on activity of the outer CPT (13% and 30% reduction with 5 and 10μg/mL Nagarse, respectively), but the inhibitory effects of malonyl-CoA were reduced to a greater extent (60% or greater loss of inhibition in all experiments). Experiments conducted with papain gave almost identical results to those with Nagarse when higher concentrations of papain were used (Table 1). Effects of

Table 1. Effects of Protease Treatment of Intact Mitochondria on Carnitine Palmitoyltransferase Activity and its Inhibition by Malonyl-CoA. Intact mitochondria were incubated with proteases at the concentrations indicated and then assayed for outer CPT activity using 40 μM palmitoyl-CoA and 0.5 mM carnitine. Results are means ± S.E.M. of 3–10 different preparations. Where no S.E.M. is indicated, results are means of 2 separate experiments with different preparations of mitochondria. Percentage inhibition by malonyl-CoA is indicated in parentheses. Abbreviation: n.d., not determined. Data are reproduced with permission from ref. 20.

		Carnitine Palmitoyltransferase Activity (nmol/min/mg protein)		
Protease, μg/ml		Control	+50 μM malonyl-CoA	+100 μM malonyl-CoA
None		9.4 ± 0.9	3.8 ± 0.8 (62 ± 5)	2.0 ± 0.6 (80 ± 4)
Nagarse,	5	8.2 ± 1.4	6.4 ± 1.0 (21 ± 1†)	5.5 ± 0.9 (32 ± 1†)
	10	6.6 ± 0.7*	5.2 ± 0.8 (22 ± 3†)	4.7 ± 0.6 (29 ± 2†)
Papain,	20	8.2 ± 0.4	6.0 ± 0.4 (26 ± 1†)	5.5 ± 0.5 (34 ± 1†)
	40	5.7 ± 0.5*	4.4 ± 0.3 (23 ± 1†)	4.0 ± 0.3 (31 ± 1†)
Trypsin,	5	7.0 ± 0.3*	2.6 ± 0.5 (63 ± 6)	1.0 ± 0.2 (83 ± 2)
	10	6.3 ± 0.2†	2.0 ± 0.3 (69 ± 3)	0.9 ± 0.2 (86 ± 3)
	25	5.2 ± 0.3†	1.5 ± 0.2 (73 ± 4)	0.8 ± 0.1 (84 ± 1)
	100	3.7†	1.4 (62)	n.d.
	200	2.2 ± 0.5†	0.7 ± 0.6 (66 ± 4)	0.6 ± 0.2 (79 ± 5)

*$P < 0.1$; †$P < 0.05$; ‡$P < 0.001$ (compared to control, Student's *t*-test)

trypsin on CPT activity and inhibition by malonyl-CoA are also given in Table 1. Exposure to increasing concentrations of trypsin decreased the activity of CPT in a concentration-dependent manner. However, in contrast to the suggestions of Murthy and Pande,[7] the inhibitory effects of malonyl-CoA were not decreased as a result of exposure to trypsin, in fact, virtually identical inhibition values were obtained at all concentrations of trypsin examined for both 50 μM and 100 μM malonyl-CoA, even though up to 80% of the activity was lost.

Table 2 shows the inhibitory effects on outer CPT activity of five different inhibitors before and after exposure to Nagarse, papain and trypsin. Inhibition by malonyl-CoA was greatly reduced by protease treatment and inhibition by HPG was affected to an equivalent extent. However, trypsin had no effect on inhibition by either malonyl-CoA or HPG. These results are especially interesting in view of the fact that HPG is the only inhibitor other than malonyl-CoA that undergoes a major change in its inhibitory potency by alterations in the physiological state of the animal.[30] Inhibition of CPT by AD 198 and DL-2-bromopalmitoyl-CoA were not affected by prior treatment of the mitochondria with any concentration of Nagarse, papain or trypsin that we evaluated. Inhibition of CPT by glyburide was altered only slightly by the highest concentration of trypsin used in Table 2 and was not affected by either Nagarse or papain. These data indicate that neither AD 198, glyburide nor 2-bromopalmitoyl-CoA bind at the same site as malonyl-CoA.

There are several other natural endobiotic compounds such as CoA, acetyl-CoA, succinyl-CoA, methylmalonyl-CoA and propionyl-CoA that are inhibitors of CPT-I and are structurally similar to malonyl-CoA.[21] Therefore, it was of interest to find out whether the inhibitory effects of these compounds were also affected by proteolysis.[31] As reported by Murthy and Pande[7] and us,[19,20] the inhibitory effects of malonyl-CoA were greatly reduced as a result of exposure of the intact hepatic mitochondria to Nagarse. The

Table 2. Inhibition of Carnitine Palmitoyltransferase by Several Known Inhibitors in Control and Protease-Treated Mitochondria. Intact mitochondria were assayed for outer CPT activity in presence and absence of different inhibitors in control and protease-treated mitochondria. All inhibitors were present throughout a 5 min preincubation with palmitoyl-CoA and a 5 min assay that was initiated by adding carnitine. Results are means ± S.E.M. of 3 different preparations, where no standard error is indicated, results are means of 2 separate experiments with different mitochondrial preparations. For specific activity of CPT see Table 1. Data are reproduced with permission from ref 20.

	Percent Inhibition					
Inhibitor	No Protease	Nagarse (5 μg/ml)	Trypsin (5 μg/ml)	Trypsin (10 μg/ml)	Trypsin (25 μg/ml)	Papain (20 μg/ml)
Malonyl-CoA (100 μM)	80 ± 4	32 ± 1†	83 ± 2	86 ± 3	85 ± 1	36 ± 1†
HPG (5 mM)	86 ± 4	6 ± 4†	85 ± 2	83 ± 1	84 ± 1	23†
AD 198 (300 μM)	92 ± 1	93 ± 1	93 ± 2	91 ± 3	96 ± 2	92 ± 1
Glyburide (200 μM)	82 ± 1	80 ± 2	81 ± 3	80 ± 3	67 ± 3	83 ± 1
2-Bromopalmitoyl-CoA (1 μM)*	84 ± 2	76 ± 5	79 ± 3	83 ± 3	86 ± 1	81

*When 2-Bromopalmitoyl-CoA was preincubated with mitochondria in the presence of 0.5 mM L-carnitine, inhibition was 100% in all cases.
†$P < 0.001$ (compared to control, Student's *t*-test)

inhibitory effects of succinyl-CoA and methylmalonyl-CoA were likewise reduced as a result of Nagarse treatment. However, Nagarse had no effect on the inhibitory actions of CoA or acetyl-CoA or propionyl-CoA. There were no differences between inhibition obtained with acetyl-CoA and propionyl-CoA.[31] These data suggest that inhibitors that are CoA esters of short chain dicarboxylic acids bind identically to malonyl-CoA and the others (monocarboxylic acid esters of CoA and free CoA) act at the acyl-CoA binding site. The order of potency of these inhibitors was: malonyl-CoA >> succinyl-CoA ≥ CoA > methylmalonyl-CoA > acetyl-CoA = propionyl-CoA. Therefore, malonyl-CoA cannot be considered as the only physiological inhibitor of CPT-I, though it is clearly the most potent.

In order to further discriminate the exact orientation of the malonyl-CoA and acyl-CoA binding sites with respect to the mitochondrial outer membrane, we[32] made use of (+)-hemipalmitoylcarnitinium which is a substrate analogue for CPT[33] and Ro 25-0187 which is a malonyl-CoA analogue. Using isolated mitochondrial outer membranes we confirmed that (+)-hemipalmitoylcarnitinium is a potent inhibitor of CPT-I ($I_{50} = 20\,\mu M$). Nagarse treatment of the outer membranes caused a 20% loss of CPT-I activity (data not shown), but there was no change in the sensitivity of CPT-I to inhibition by this active site-directed inhibitor. The malonyl-CoA analog Ro 25-0187 is also a potent inhibitor of CPT-I ($I_{50} = 0.2\,\mu M$) and in contrast to results with the substrate analogue, Nagarse treatment of the outer membranes resulted in complete loss of CPT-I sensitivity to inhibition. These results are very important because: (i) they confirm that the outer CPT of isolated mitochondrial outer membranes behaves exactly as the overt CPT of intact mitochondria; (ii) CPT-I located on the mitochondrial outer membrane has its malonyl-CoA binding site facing the cytosol and its active site is protected within the mitochondrial outer membrane; (iii) these data confirm that the malonyl-CoA site and the active site are entirely different; and finally (iv) since there is no remaining inhibition by Ro 25-0187 after Nagarse treatment and since there is no difference in inhibition by (+)-hemipalmitoylcarnitinium before and after Nagarse treatment, there seems to be no interaction of either of these inhibitors with an alternate site.

1.3. Protection of CPT-I from Proteolysis and its Implications

We have reported that it is possible to protect CPT against the effects of proteolysis by preincubating the intact mitochondria with malonyl-CoA or HPG before protease treatment, and that preincubation with the other inhibitors does not have any protective effects.[19,20] We have also performed experiments in order to ascertain whether it was possible to protect against proteolytic actions of Nagarse by preincubating the mitochondria with natural endobiotic compounds such as CoA, acetyl-CoA, succinyl-CoA, methylmalonyl-CoA and propionyl-CoA inhibitors or with palmitoyl-CoA and carnitine the other natural substrates of CPT-I.[31] Data presented in Fig. 1 shows that succinyl-CoA and methylmalonyl-CoA protect against the actions of Nagarse but not to the same extent as malonyl-CoA; however, propionyl-CoA offered no protection and CoA and acetyl-CoA not only did not protect but, in fact, enhanced the proteolytic actions of Nagarse. Preincubation of CPT with carnitine gave no protection against the actions of Nagarse but preincubation with palmitoyl-CoA enhanced proteolysis in a similar manner to CoA and acetyl-CoA. These data offer additional proof that malonyl-CoA, succinyl-CoA, and methylmalonyl-CoA bind to a site that is distinct from the substrate binding site to alter proteolytic effects and that acetyl-CoA and propionyl-CoA bind, as CoA does, to the active site. Substrate of the CPT reaction cause a probable conformational change in the enzyme that results in increased proteolytic effects.

1.4. Kinetic Analysis of CPT-I Inhibition Using Pairs of Inhibitors

On the basis of competitive binding alone, it has been concluded that all analogues of malonyl-CoA bind to the same site.[21] Since our proteolysis data did not support the conclusion that all inhibitors of CPT-I act at a common site, we performed additional studies in which pairs of CPT-I inhibitors were analyzed.[32] The theory behind double inhibitor studies has been developed by Yonetani and Theorell.[34] In their studies two

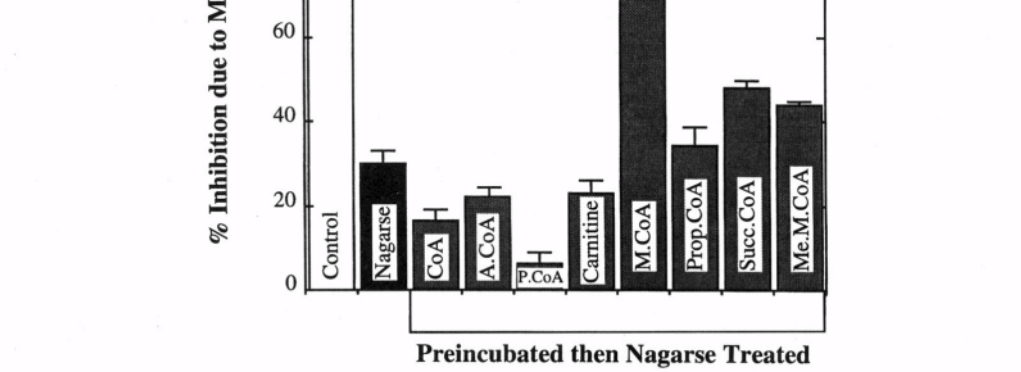

Figure 1. Protection Against Actions of Nagarse by Inhibitors and Substrates of carnitine palmitoyltransferase I.

inhibitors were present at the same time, but the concentration of one inhibitor was kept constant while the concentration of the second inhibitor was varied. From the shape of a plot of 1/activity *vs* the inhibitor concentration, it is possible to deduce whether the two inhibitors compete for the same site (the two lines are parallel), or bind at two independent sites (the two lines converge). We used this procedure to study the effects of CoA with malonyl-CoA and HPG with malonyl-CoA in control and Nagarse treated mitochondria.[32] Using a system in which the concentration of malonyl-CoA was varied but the concentration of CoA was kept fixed at 50 μM, for the control system it was observed that as the concentration of malonyl-CoA was varied at a fixed concentration of CoA, the plots converged consistent with two inhibitors binding at two different sites.[34] This suggests that malonyl-CoA binds at a specific malonyl-CoA binding site (or with a malonyl-CoA binding protein) that is quite different from the active (acyl-CoA binding) site, while CoA inhibits CPT activity by binding at the acyl-CoA binding site. However, after Nagarse treatment, this plot gave two parallel lines indicating that both inhibitors were now binding to the same site. The suggestion here is that Nagarse eliminates to a large extent the malonyl-CoA binding site (or the malonyl-CoA binding protein) so that both inhibitors must now be acting at one common site. Similar plots were obtained when the concentration of malonyl-CoA was fixed and the concentration of CoA was varied.[32]

With HPG a different picture emerged. The plots of 1/v as a function of HPG or malonyl-CoA showed parallel lines in agreement with two inhibitors competing for one site.[34] After Nagarse treatment, these plots were also in accord with two inhibitors binding at the same site.

Recent data of Fraser, Corstorphine, and Zammit[35] using trypsin and proteinase K treatment of intact mitochondria and outer-membrane-ruptured mitochondria suggest that both the active site and the malonyl-CoA binding site are exposed on the cytosolic side of the membrane and that CPT-I has two transmembrane domains. Thus, we have conducted additional studies of the topology of CPT-I using intact hepatic mitochondria and isolated mitochondrial outer membranes with Nagarse (subtilisin BPN'), papain, and trypsin using a variety of incubation conditions.

2. MATERIALS AND METHODS

Male Sprague-Dawley rats (180–240g), obtained from Harlan Industries, Inc. (Indianapolis, IN, U.S.A.), were fed Purina Rat Chow (Ralston Purina Co., Richmond, IN, U.S.A.) and water *ad libitum.* On the day of the experiment, rats were killed by decapitation and their livers were removed rapidly for preparation of mitochondria.

Intact mitochondria were isolated by the method of Johnson and Lardy[36] with the modifications previously published.[17] Mitochondrial outer membranes were isolated by the method of Parsons *et al.*[37] and their purity assessed as described previously.[16] Protease treatment of intact mitochondria and protection experiments were carried out as described previously.[19,20] Briefly, this method consisted of incubating the mitochondria (5mg/ml), and outer membranes (1 mg/mL) with Nagarse (5μg/mL) at 37°C for 10min after which the proteolytic activity was stopped by addition of 200μl of 20% (w/v) BSA/mL of incubation volume plus 40mL of ice-cold isolation medium. After centrifugation (5,600 *xg* for 10min), the mitochondria were resuspended (4mg/mL) in isolation medium and used as indicated. Protein determination was by a biuret method.[38] In some experiments intact mitochondria or the isolated outer membranes were first incubated

with malonyl-CoA at the concentrations indicated at 37°C for 5min prior to protease treatment. In those experiments the inhibitors were present during protease treatment, but were removed by the washing procedure.

Carnitine palmitoyltransferase was assayed using the method of Bremer[11] as modified and reported previously.[14] Each assay contained, in a total volume of 1 mL:82mM sucrose, 70mM KCl, 70mM imidazole, 1μg antimycin A, and 2mg bovine serum albumin. For assaying the outer CPT each assay also contained 0.5 mM L-carnitine (0.4 mCi of L-[methyl-^{3}H] carnitine) and 40μM palmitoyl-CoA. Inhibitors were added at the concentrations indicated in legends to tables and figures. Adenylate kinase was assayed using the procedure of Bergmeyer[39] as modified by Janski and Cornell.[40]

Palmitoyl-CoA, imidazole, L-carnitine hydrochloride, EDTA, acetyl-CoA, ATP, AMP, KCl, NADH, $MgCl_2$, oxaloacetate, phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, 5,5'-dithiobis-(2-nitrobenzoic acid), essentially fatty acid-free bovine serum albumin, malonyl-CoA, Nagarse (subtilisin BPN', P 4789), papain (P 4762) and trypsin (T 0134) were purchased from Sigma(St. Louis, MO, U.S.A.). Catalogue numbers are given in parentheses following each protease. L-[methyl-^{3}H]Carnitine hydrochloride was obtained from Amersham Corp. (Arlington Heights, IL, U.S.A.). Hydroxy-phenylglyoxylate was a gift from Pfizer (Sandwich, Kent, U.K.). Ro 25-0187 was a gift from Dr. Guy Heathers (Hoffman-LaRoche, Nutley, NJ, USA) and (+)-Hemipalmitoylcarnitinium was a gift from Dr. Richard D. Gandour (Virginia Polytechnic Institute and State University, Blacksburg, VA, USA).

3. RESULTS AND DISCUSSION

Figure 2 shows the effects of Nagarse on CPT-I activity and inhibition by malonyl-CoA as a function of incubation time. As shown, incubation of intact mitochondria up to 30 minutes in absence of Nagarse has no significant effect on CPT-I activity or malonyl-CoA inhibition. However, when the mitochondria is treated with 5μg/mL of Naragse, both CPT-I activity and malonyl-CoA inhibition decrease as a function of incubation time. As reported earlier,[19,20] treatment of intact mitochondria with 5μg/mL of Nagarse up to 10min at 37°C has little effect on CPT-I activity but the magnitude of malonyl-CoA inhibition is significantly reduced. These data suggest that the malonyl-CoA binding domain is exposed to Nagarse on the cytosolic side of the outer membrane, while the acyl-CoA binding domain is not, that is, it is facing the inter membrane space. However, when the incubation time is increased, CPT-I activity decreases even further and by 30min it has decreased by about 60%. Malonyl-CoA has no inhibitory effects after 30min of incubating the intact mitochondria with Nagrase at this concentration.

The fact that CPT-I activity is now decreased by such a large amount suggests that either the acyl-CoA binding domain is facing the cytosol and it needed more time for the protease to act upon it, or that the outer membrane is ruptured allowing exposure of the acyl-CoA binding domain to the protease. These observations also suggest that proteolysis of the intact mitochondria with Nagarse is affecting the apparent K_i for malonyl-CoA inhibition. To address this question, Dixon plots[41] were constructed as reported previously.[42] The data presented in Fig. 3 shows that exposure of intact mitochondria to Nagarse increases the apparent K_i values for malonyl-CoA. This is an important observation since changes in the apparent K_i values for malonyl-CoA inhibition have also been reported in such states as starvation[13] and diabetes.[14,15]

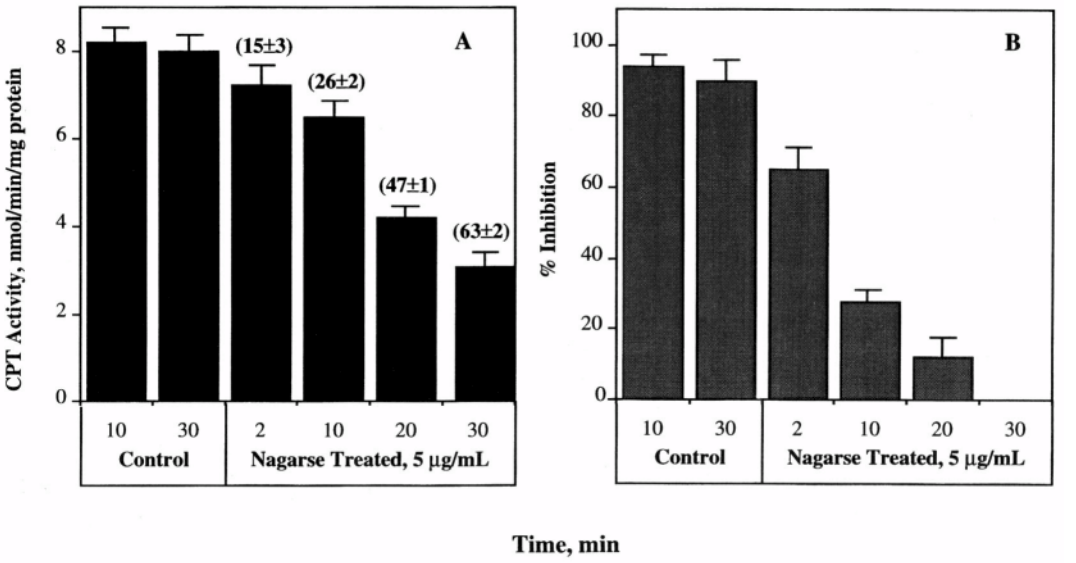

Figure 2. Effect of Nagarse on CPT Activity (A) and Malonyl-CoA inhibition (B) as a Function of Time.

Effects of trypsin (10µg/mL) on CPT-I activity time course were similar to those of Nagarse, but there was no corresponding loss of malonyl-CoA inhibition even when the enzyme activity had been destroyed by about 60%, Figure 4. This data is in contrast to a recent report by Fraser *et al.*[35] suggesting that trypsin at a concentration of 10µg/mL has no effect on CPT-I activity. Their data is presented as percent activity as a function of exposure time to trypsin with no indication of the specific activity of the enzyme before

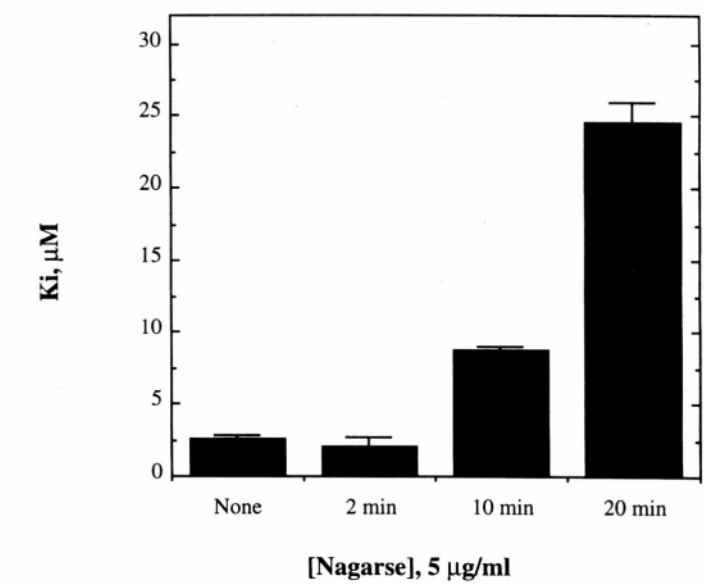

Figure 3. Effects of Nagarse on the apparent K_i of CPT-I for malonyl-CoA.

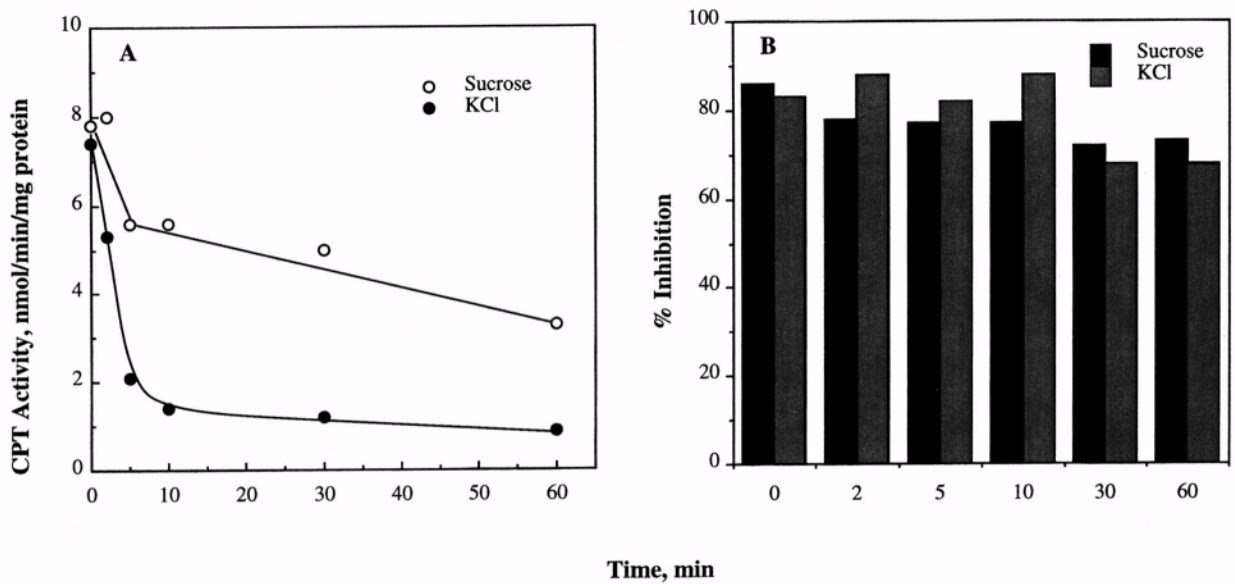

Figure 4. Effect of Trypsin on CPT Activity (**A**) and Malonyl-CoA Inhibition (**B**) as a Function of Time.

trypsin treatment. In our studies presented in Fig. 4, it is interesting to note that if the isolation medium was changed from 250mM sucrose to 150mM KCl, then loss of CPT-I activity as a function of exposure time to trypsin was much faster and also greater. This suggests that changes in salt concentration may some how expose the acyl-CoA binding domain to trypsin even further. However, the KCl medium had no effect on malonyl-CoA inhibition as was observed when sucrose was the isolating/incubation medium.

Nagarse treatment of the intact mitochondria did not alter the integrity of the mitochondrial outer membrane, since adenylate kinase which is an intermembrane space enzyme was not released, Figure 5. However, exposure of the intact mitochondria to trypsin caused release of adenylate kinase in a concentration dependent manner. These data suggest that trypsin has damaged the mitochondrial outer membrane, possibly allowing exposure of CPT active domain to trypsin within the outer membrane. Preincubation of the intact mitochondria with malonyl-CoA prior to trypsin treatment did not protect against loss of adenylate kinase (data not shown) but did protect against loss of CPT-I activity as reported before [19,20].

Effects of Nagarse and trypsin on CPT activity and malonyl-CoA inhibition using swollen mitochondria are presented in Figure 6. Swelling alone without any protease treatment slightly increased CPT activity suggesting possible rupture of the mitochondrial inner membrane and exposure of CPT-II. Malonyl-CoA inhibition is reduced from 92 ± 1% in unswollen state to 60 ± 2% when swollen, again suggesting exposure of CPT-II or a conformational change in the malonyl-CoA binding domain such that the ligand does not have full access for optimum effects. Nagarse treatment of the swollen mitochondria did not increase CPT-I activity any further, however, malonyl-CoA inhibition was reduced even more. Exposure of the swollen mitochondria to trypsin had no additional effects on CPT-I activity or malonyl-CoA inhibition. These data suggest that whilst the mitochondria is in the swollen state, the malonyl-CoA binding domain is further exposed to Nagarse on the cytosolic side of the membrane and that the protease has limited access to the acyl-CoA binding domain that is either within the membrane or facing the inter membrane. These data further suggest that it is unlikely for CPT-II to be exposed as the result of swelling since the increase in CPT activity is very small even though malonyl-CoA inhibition is much less in this state. Figure 6 also shows the results

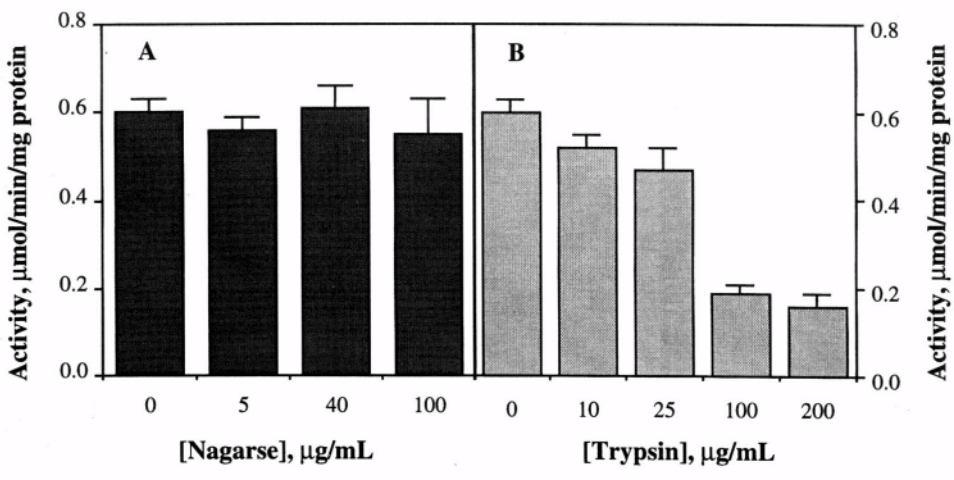

Figure 5. Effect of Protease Treatment of Intact Mitochondria on Adenylate Kinase content.

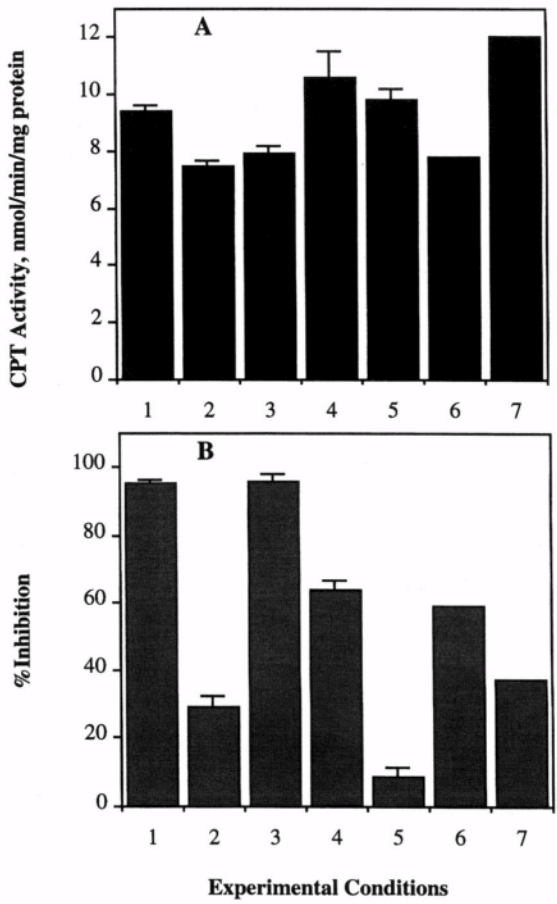

Figure 6. Effect of Proteases on CPT Activity (Panel A) and Malonyl-CoA Inhibition (Panel B) in Intact and Swollen Mitochondria.

of preincubating the swollen mitochondria with 50μM malonyl-CoA prior to Nagarse treatment. Our previous data had shown that preincubation of the intact mitochondria with malonyl-CoA protects against proteolytic actions of Nagarse.[19,20] Here we see that the protection is very limited, malonyl-CoA inhibition is now about 40% instead of being about 10% (compare conditions 5 and 7 in Figure 6). This means that under these conditions some domain of the CPT protein that is involved in malonyl-CoA inhibition is still exposed to Nagarse.

Using isolated mitochondrial outer membranes, Nagarse at increasing concentrations up to 40μg/mL had very little effect on CPT-I activity, Fig. 7 panel A, but the inhibitory effects of malonyl-CoA were reduced in a dose dependent manner still suggesting that while the malonyl-CoA domain is accesible to Nagarse, the acyl-CoA domain is not, Fig. 7 panel B. However, when the concentration of Nagarse was increased to 200 μg/mL, CPT-I activity was reduced significantly with malonyl-CoA inhibition being almost zero, Fig. 7 panels A and B. These data suggest that the malonyl-CoA binding domain is completely destroyed by this concentration of Nagarse and that the acyl-CoA binding domain is facing outwards and it needs a higher concentration of Nagarse to

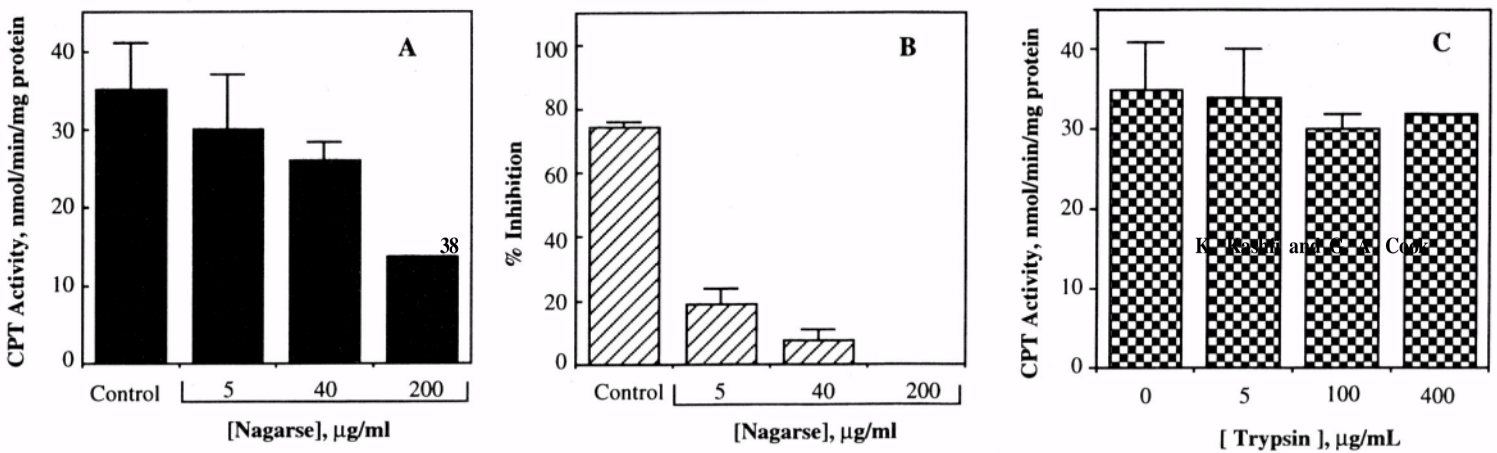

Figure 7. Effects of Proteases on CPT Activity and Inhibition by Malonyl-CoA in Isolated Mitochondrial Outer Membranes.

chew it up or that at this concentration Nagarse is able to compromise the outer membrane vesicle, get inside and have access to the acyl-CoA site. Trypsin up to concentrations of 400μg/mL had no effect on CPT-I activity of the outer membranes, Fig. 7 panel C, or the malonyl-CoA sensitivity (data not shown).

Effects of papain on CPT-I activity of the outer membranes were very similar to those of Nagarse (data not shown). Papain also caused a concentration dependant loss of malonyl-CoA inhibition with the outer membranes similar to that seen with Nagarse, Figure 8. We had previously shown that it was possible to protect against the proteolytic actions of these proteases by preincubating the mitochondria with malonyl-CoA prior to protease treatment. The same general observation is true when isolated outer membranes are used. Data shown in Fig. 8 shows protection against proteolytic actions of papain by preincubation of the outer membranes with malonyl-CoA. When the outer membranes were incubated with relatively low (5μg/mL) concentrations of any of the proteases and then co-sonicated, CPT-I activity was significantly reduced (data not shown). These observations suggest that co-sonication breaks open the outer membrane vesicles so that the protease can have access to the acyl-CoA binding domain.

4. SUMMARY

Our earlier work using intact mitochondria and isolated mitochondrial outer membranes confirms the observations of Murthy and Pande[7] that CPT-I is located on the mitochondrial outer membranes and supports the notion that this enzyme has a malonyl-CoA binding domain facing the cytosol and an acyl-CoA binding domain facing the inter

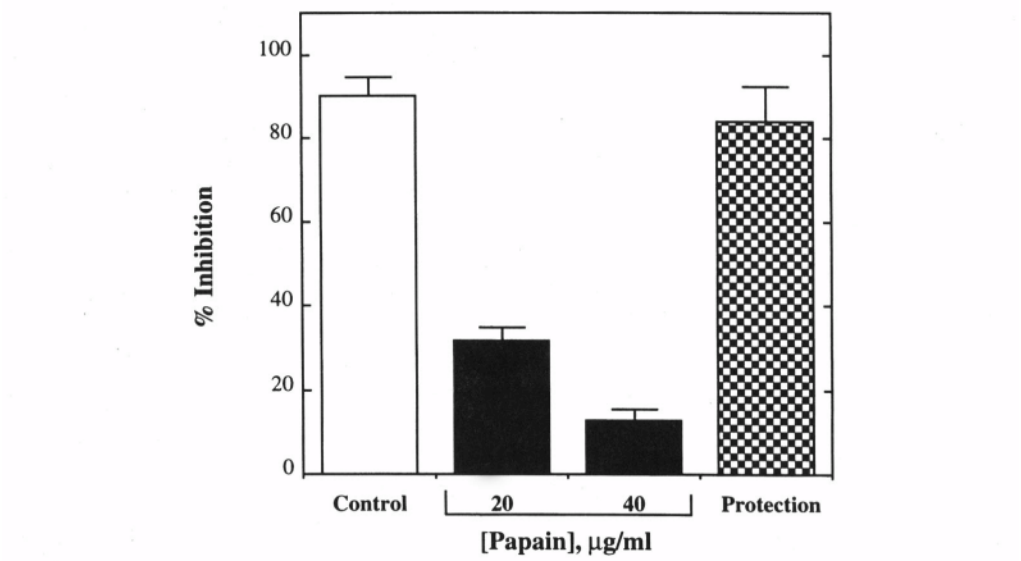

Figure 8. Effects of Papain on Malonyl-CoA Inhibition and Protection from Proteolysis by Malonyl-CoA in Isolated Mitochondrial Outer Membranes.

membrane space. Our data also suggests that coenzyme A binds at the active site of CPT-I, as does acyl-CoA, 2-bromopalmitoyl-CoA, and (+)-hemipalmitoylcarnitinium, but malonyl-CoA does not bind at that site. Inhibition of CPT-I at the malonyl-CoA binding site by HPG and Ro 25-0187, which have no CoA moiety, contributes to a resolution of this question in that the CoA itself is not essential for the binding of malonyl-CoA to its regulatory site, but the dicarbonyl function which is present in malonyl-CoA, HPG, and Ro 25-0187 is absolutely essential.

Our re-evaluation of the topology of hepatic mitochondrial CPT-I confirms the original observations that this enzyme has at least two different binding domains, one domain binding malonyl-CoA, HPG, and Ro-25-187 and the other domain binding acyl-CoA and other inhibitors of CPT-I. Furthermore, the malonyl-CoA binding domain is exposed to the cytosolic face of the membrane. Our data showing that treatment of the intact mitochondria with trypsin causes release of adenylate kinase which indicates that trypsin has damaged the mitochondrial outer membrane, possibly allowing trypsin to enter the intermembrane space and act on CPT from within the outer membrane. Since trypsin's action is limited to arginine and lysine residues, an alternative explanation could be that the portion of the protein domain responsible for malonyl-CoA inhibition may not contain these residues. The latter explanation is plausible, since malonyl-CoA was able to protect against loss of activity and sensitivity to inhibition, but did not protect against loss of adenylate kinase, suggesting that rupture of the outer membrane is not necessarily related to loss of CPT activity. These results suggest that some protein domain that is necessary for CPT activity is exposed on the outer surface of the outer membranes. Therefore, it seems likely that trypsin would have to be able to hydrolyse protein domains of CPT that are inaccessible to Nagarse and papain.

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REFERENCES

1. Fritz, I.B. & Yue, K.T.N. (1963) Long chain carnitine acyltransferase and the role of acylcarnitine derivatives in the catalytic increase of fatty acid oxidation induced by carnitine. *J. Lipid Res.* **4**, 279–288.
2. Pande, S.V. (1975) A mitochondrial carnitine acylcarnitine translocase system. *Proc. Natt. Acad. Sci. U.S.A.* **72**, 883–887.
3. Bremer, J. (1983) Carnitine-metabolism and function. *Physiol. Rev.* **63**, 1420–1480.
4. Bieber, L.L. (1988) Carnitine. *Annu. Rev. Biochem.* **57**, 261–283.
5. Kopec, B. & Fritz, I.B. (1973) Comparison of properties of carnitine palmitoylteansferase I with those of carnitine palmitoyltransferase II, and preparation of antibodies. *J. Biol. Chem.* **248**, 4069–4073.
6. Hoppel, C.L. & Tomec, R.J. (1972) Carnitine palmitoyltransferase: location of two enzymatic activities in rat liver mitochondria. *J. Biol. Chem.* **247**, 832–841.
7. Murthy, M.S.R. & Pande, S.V. (1987) Malonyl-CoA binding site and overt carnitine palmitoyltransferase activity reside on the opposite sides of the outer mitochondrial membrane. *Proc. Natl. Acad. Sci., U.S.A.* **84**, 378–382.
8. McGarry, J.D., Mannaerts, G.P. & Foster, D.W. (1977) A possible role for malonyl-CoA in the regulation of hepatic fatty acid oxidation and ketogenesis. *J. Clin. Invest.* **60**, 265–270.
9. McGarry, J.D., Leatherman, G.F. & Foster, D.W. (1978) CPTase I: The site of inhibition of fatty acid oxidation by malonyl-CoA. *J. Biol. Chem.* **253**, 4128–4136.

10. McGarry, J.D. & Foster, D.W. (1980) Regulation of hepatic tatty acid oxidation and ketone production. *Annu. Rev. Biochem.* **49**, 395–420.
11. Bremer, J. (1981) The effect of fasting on the activity of liver carnitine palmitoyltransferase and its inhibition by malonyl-CoA. *Biochim. Biophys. Acta* **665**, 628–631.
12. Saggerson, E.D. & Carpenter, C.A. (1981) Effects of fasting, adrealectomy and streptozotocin-diabetes on sensitivity of hepatic carnitine acyltransferase to malonyl-CoA. *FEBS Lett.* **129**, 225–228.
13. Cook, G.A., Otto, D.A. & Cornell, N.W. (1980) Differential inhibition of ketogenesis by malonyl-CoA in mitochondria from fed and starved rats. *Biochem. J.* **192**, 955–958.
14. Gamble, M.S. & Cook, G.A. (1985) Alteration of the apparent K_i of carnitine palmitoyltransferase for malonyl-CoA by the diabetic state and reversal by insulin. *J. Biol. Chem.* **260**, 9516–9619.
15. Cook, G.A. & Gamble, M.S. (1987) Regulation of carnitine palmitoyltransferase by insulin results in decreased activity and decreased apparent K_i values for malonyl-CoA. *J. Biol. Chem.* **262**, 2050–2055.
16. Kashfi, K., Weakley, L.J. & Cook, G.A. (1988) The Effects of Diabetes on the Carnitine Palmitoyltransferase of Isolated Hepatic Mitochondrial Outer Membrane. *Biochem. Soc. Trans.* **16**, 1010–1011.
17. Cook, G.A. (1984) Differences in the sensitivity of carnitine palmitoyltransferase to inhibition by malonyl-CoA are due to differences in K_i values. *J. Biol. Chem.* **259**, 12030–12033.
18. Norum, K.R., Farstad, M. & Bremer, J. (1966) The submitochondrial distribution of acid:CoA ligase (AMP) and palmityl-CoA:carnitine palmityltransferase in rat liver mitochondria. *Biochem. Biophys. Res. Commun.* **24**, 797–804.
19. Kashfi, K. & Cook, G.A. (1991) Malonyl-CoA Inhibits Proteolysis of Carnitine Palmitoyltransferase. *Biochem. Biophy. Res. Commun.* **176**, 600–605.
20. Kashfi, K. & Cook, G.A. (1992) Proteinase Treatment of Intact Hepatic Mitochondria has Differential Effects on Inhibition of Carnitine Palmitoyltansferase by Different Inhibitors. *Biochem. J.* **282**, 909–914.
21. Mills, S.E., Foster, D.W. & McGarry, J.D. (1983) Interaction of malonyl-CoA and related compounds with mitochondria from different rat tissues. *Biochem. J.* **214**, 83–91.
22. Kiorpes, T.G., Hoerr, D., Ho, W. Weaner, L.F., Inman, M.G. & Tutwiler, G.F. (1984) Identification of 2-tetradecylglycidyl coenzyme A as the active form of methyl 2-tetradecyglycidate (methyl paloxirate) and its characterization as an irreversible, active site-directed inhibitor of carnitine palmitoyltransferase A in isolated rat liver mitochondria. *J. Biol. Chem.* **259**, 9750–9755.
23. Bird, M.I. & Saggerson, E.D. (1984) Binding of malonyl-CoA to isolated mitochondria. Evidence for high- and low-affinity sites in liver and heart and relationship to inhibition of carnitine palmitoyltransferase activity. *Biochem. J.* **222**, 639–647.
24. Declercq P.E., Venincasa, M.D., Mills, S.E., Foster, D.W. & McGarry, J.D. (1985) interaction of malonyl-coA and 2-tetradecylglycidyl-CoA with mitochondrial carnitine palmitoyltransferase I. *J. Biol. Chem.* **260**, 12516–12522.
25. Grantham, B.D. & Zammit, V.A. (1986) Binding of [^{14}C]malonyl-CoA to rat liver mitochondria sfter blocking the active site of carnitine palmitoyltransferase I. *Biochem. J.* **233**, 589–593.
26. Cook, G.A. (1987) The hypoglycemic sulfonylureas glyburide and tolbutamide inhibit fatty acid oxidation by inhibiting carnitine palmitoyltransferase. *J. Biol. Chem.* **262**, 4968–4972.
27. Stephens, T.W., Higgins, A.J., Cook, G.A. & Harris, R.A. (1985) Two mechanisms produce tissue-specific inhibition of fatty acid oxidation by oxfenicine. *Biochem. J.* **227**, 651–660.
28. Kashfi, K., Israel, M, Sweatman, T.W., Seshadri, R. & Cook, G.A. (1990) Inhibition of Mitochondrial Carnitine Palmitoyltransferases by Adriamycin and Adriamycin Analogues. *Biochem. Pharmacol.* **40**, 1441–1448.
29. Chase, J.F.A. & Tubbs, P.K. (1969) Conditions for the self-catalysed inactivation of carnitine acetyltransferase. A novel form of enzyme inhibition. *Biochem. J.* **111**, 225–235.
30. Stephens, T.W. & Harris, R.A. (1987) Effect of starvation and diabetes on the sensitivity of carnitine palmitoyltransferase I to inhibition by 4-hydroxyphenylglyoxylate. *Biochem. J.* **243**, 405–412.
31. Kashfi, K., Mynatt, R.L. & Cook, G.A. (1994) Hepatic Carnitine Palmitoyltransferase-I Has Two Independent Inhibitory Binding Sites for Regulation of Fatty Acid Oxidation. *Biochem. Biophys. Acta* **1212**, 245–252.
32. Cook, G.A., Mynatt, R.L. & Kashfi, K. (1994) Yonetani-Theorell Analysis of Hepatic Carnitine Palmitoyltransferase-I Inhibition Indicates Two Distinct Inhibitory Binding Sites. *J. Biol. Chem.* **269**, 8803–8807.
33. Gandour, R.D., Leung, O., Greway, A.T., Ramsay, R.R., Bhaird, N.N., Fronczek, F.R., Bellard, B.M. & Kumaravel, G. (1993) (+)-Hemipalmitoylcarnitinium strongly inhibits carnitine palmitoyltransferase-I in intact mitochondria. *J. Med. Chem.* **36**, 237–242.
34. Yonetani, T. & Theorell, Y. (1982) The Yonetani-Theorell graphical method for examining overlapping subsites of enzyme active centers. *Methods Enzymol.* **87C**, 500–509.

35. Fraser, F., Corstorphine, C.G. & Zammit, V.A. (1997) Topology of carnitine palmitoyltransferase I in the mitochondrial outer membrane. *Biochem. J.* **323**, 711–718.
36. Johnson, D. & Lardy, H. (1967) Isolation of liver or kidney mitochondria. *Methods Enzymol* **10**, 94–96.
37. Parsons, D.F., Williams, G.R. & Chance, B. (1966) Characteristics of isolated and purified preparations of the outer and inner membranes of mitochondria. *Ann. New. York. Acad. Sci.* **137**, 643–666.
38. Layne, E. (1957) Spectrophotometric and turbidimetric methods for measuring proteins. *Methods Enzymol* **3**, 450–451.
39. Bergmeyer, H.U., Gawehn, K. & Grossl, M. (1974) Enzymes as biochemical reagents. In: *Methods of Enzymatic Analysis* (Eds. Bergmeyer, H.U. & Gawehn, K.), vol 1, pp. 425–556, Academic Press, London and New York.
40. Janski, A.M. & Cornell, N.W. (1980) Subcellular distribution of enzymes determined by rapid digitonin fractionation of isolated hepatocytes. *Biochem. J.* **186**, 423–429.
41. Dixon, M. (1953) The determination of enzyme inhibitor constants. *Biochem. J.* **55**, 170–171.
42. Kashfi, K., Cagen, L. & Cook, G.A. (1995) Diabetes and Proteolysis: Effects on Carnitine Palmitoyl-transferase-I and Malonyl-CoA Binding. *Lipids* **30**, 383–388.

POSSIBLE INVOLVEMENT OF CYTOSKELETAL COMPONENTS IN THE CONTROL OF HEPATIC CARNITINE PALMITOYLTRANSFERASE I ACTIVITY

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1. INTRODUCTION

Mitochondrial fatty acid oxidation in liver provides a major source of energy to this organ and supplies extra-hepatic tissues with ketone bodies as a glucose-replacing fuel.[1,2] Carnitine palmitoyltransferase I (CPT I), the carnitine palmitoyltransferase of the mitochondrial outer membrane, catalyzes the translocation of long-chain fatty acids into the mitochondrial matrix.[1–5] Moreover, recent determination of flux control coefficients of the enzymes involved in hepatic long-chain fatty acid oxidation shows that CPT I plays a pivotal role in controlling the flux through this pathway under different substrate concentrations and patho-physiological states.[6,7] CPT I is subject to long-term regulation in response to alterations in the nutritional and hormonal status of the animal.[1,2,5] Short-term control of CPT I activity involves inhibition by malonyl-CoA, the product of the reaction catalyzed by acetyl-CoA carboxylase.[8] Since the latter enzyme is a key regulatory site of fatty acid synthesis *de novo* (cf.[1–5]), malonyl-CoA inhibition of CPT I allows an elegant explanation for the coordinate control of the partition of hepatic fatty acids into esterification and oxidation. As a matter of fact, evidence has accumulated during the last two decades highlighting the physiological importance of malonyl-CoA inhibition of CPT I not only in liver but also in extra-hepatic tissues.[1,5]

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Recently, however, a novel mechanism of control of hepatic CPT I activity has been put forward. Studies using permeabilized hepatocytes have shown that various agents exert short-term changes in CPT I activity in parallel with changes in the rate of long-chain fatty acid oxidation.[3,9] These short-term changes in hepatic CPT I activity are assumed to be mediated by a malonyl-CoA-independent mechanism, since they survive cell permabilization, extensive washing of the permeabilized cells (to allow complete removal of malonyl-CoA) and subsequent preincubation of the cell ghosts at 37°C before determination of CPT I activity (to allow recovery of the original conformational state of CPT I).[10] Evidence has also been presented showing that the stimulation of hepatic CPT I by the phosphatase inhibitor okadaic acid (OA), used as a model compound to study the short-term regulation of CPT I, does not involve the direct phosphorylation of CPT I.[10] It has been recently shown that the OA-induced stimulation of CPT I is prevented by KN-62, an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II (Ca^{2+}/CM-PKII),[11] and by taxol, a stabilizer of the cytoskeleton.[12] These observations suggest that both activation of Ca^{2+}/CM-PKII and disruption of the cytoskeleton may be necessary for the OA-induced stimulation of CPT I to be demonstrated. It is conceivable that these two processes may be related, since Ca^{2+}/CM-PKII is one of the protein kinases more actively involved in the control of the integrity of the cytoskeleton by phosphorylating cytoskeletal proteins.[13] However, the events underlying this novel mechanism of control of CPT I activity are as yet unknown. The present paper summarizes the studies we have recently performed to determine the possible role of cytoskeletal components in the malonyl-CoA-independent short-term control of hepatic CPT I activity.

2. EXPERIMENTAL EVIDENCE FOR THE INVOLVEMENT OF CYTOSKELETAL COMPONENTS IN THE CONTROL OF HEPATIC CPT I ACTIVITY

A number of reports have recently described the existence of specific interactions between the mitochondrial outer membrane and cytoskeletal elements (reviewed in refs.[14,15]). In the context of CPT I regulation, OA activates hepatic CPT I[9] and disrupts the cytoskeleton of hepatocytes by causing hyperphosphorylation of cytoskeletal proteins.[16] The most important observations that strenghten the notion that extramitochondrial cell components (most likely cytokeratin intermediate filaments) are involved in the control of hepatic CPT I activity are the following:

(i) Permeabilized hepatocytes were treated with trypsin in very mild conditions and CPT I activity was subsequently determined. As shown in Fig. 1, when hepatocytes were incubated without further additions and then permeabilized with digitonin, trypsin was able to stimulate CPT I by approximately 50% in these cell ghosts. Preincubation of hepatocytes with OA led to a similar activation of CPT I in the permeabilized-cell system (Fig. 1). However, trypsin was unable to produce any further stimulation of CPT I in ghosts prepared from OA-pretreated hepatocytes (Fig. 1). The cytoskeletal stabilizer taxol has been shown to prevent the changes in hepatic CPT I activity induced by a number of cellular effectors including OA.[12] Likewise, when hepatocytes were pretreated with OA in combination with taxol, the stimulatory effect of trypsin

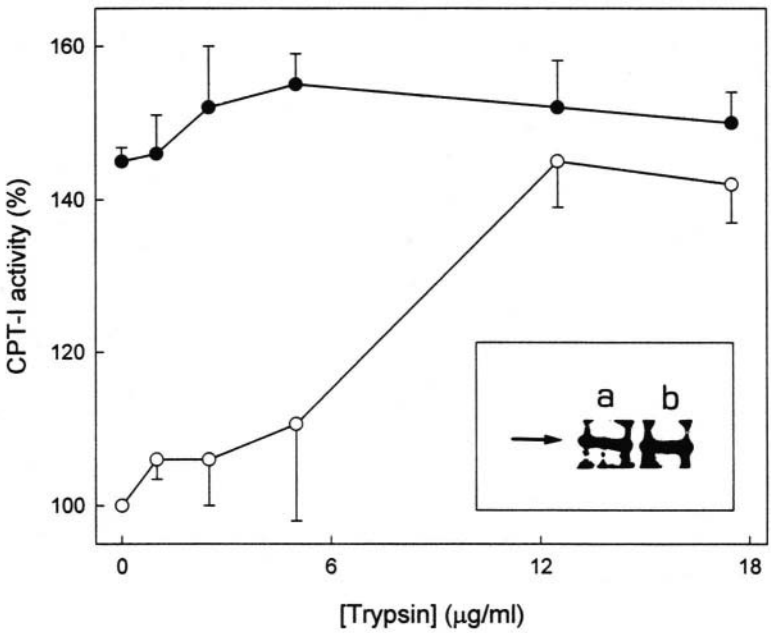


Figure 1. Effect of mild trypsin digestion on CPT I activity in permeabilized hepatocytes. Hepatocytes were preincubated for 15 min in the absence (open circles) or in the presence (filled circles) of 0.5 μM OA. Cells were then permeabilized with digitonin and thorougly washed with digitonin-free medium as described in ref.[10] Permeabilized hepatocytes were subsequently resuspended at 1.5–2.0 mg protein ml^{-1} and treated with varying concentrations of trypsin at 4 °C. Trypsin action was stopped after 2 min and CPT I activity was subsequently determined in those permeabilized hepatocytes. Results correspond to 4 different cell preparations. *Inset*: Mitochondria were isolated from permeabilized hepatocytes that had been treated without (lane a) or with (lane b) 17.5 μg ml^{-1} trypsin and CPT I was detected by Western blotting. The arrow points to the 88-kDa band.

was evident (results not shown). It was also shown that trypsin does not cleave CPT I under the experimental conditions used (Fig. 1).

(ii) Hepatocytes were incubated with colchicine (a microtubule disruptor), cytochalasin B (a microfilament disruptor), or IDPN (an intermediate-filament disruptor). As shown in Table 1, neither colchicine nor cytochalasin B affected CPT I activity. In contrast, IDPN produced a significant increase in CPT I activity (Table 1). Interestingly, the effects of IDPN and OA were basically non-additive (Table 1). Furthermore, stabilization of the cytoskeleton with taxol prevented the stimulation of CPT I induced by IDPN and OA, either alone or in combination (Table 1). It should be pointed out that neither taxol nor IDPN changed by themselves the malonyl-CoA concentration in hepatocytes (Table 1). In addition, neither of these two compounds affected the OA-induced decrease of intracellular malonyl-CoA levels (Table 1).

(iii) Two cytoskeletal fractions were isolated from rat hepatocytes to study their possible inhibitory effect on CPT I. One of them (Fraction I) was a total-cytoskeleton fraction, whereas the other (Fraction II) was a cytokeratin-enriched fraction. Isolated hepatic mitochondria were then incubated with the cytoskeletal fractions and CPT I activity was subsequently determined. As shown in Fig. 2, the two cytoskeletal fractions produced a dose-dependent inhibition of CPT I activity. In agreement with the effect of IDPN described above,

Table 1. Effect of disruptors of the cytoskeleton on CPT I activity and malonyl-CoA levels
Hepatocytes were preincubated for 45 min in the absence or in the presence of the modulators of the integrity of the cytoskeleton as indicated. Incubations were continued for 15 additional min with or without 0.5 μM OA, and then aliquots were taken to determine the level of malonyl-CoA as well as the activity of CPT I by the "one-step" assay.[10] Results correspond to the number of experiments indicated in parentheses for CPT I activity and to 3 different experiments for malonyl-CoA concentration.

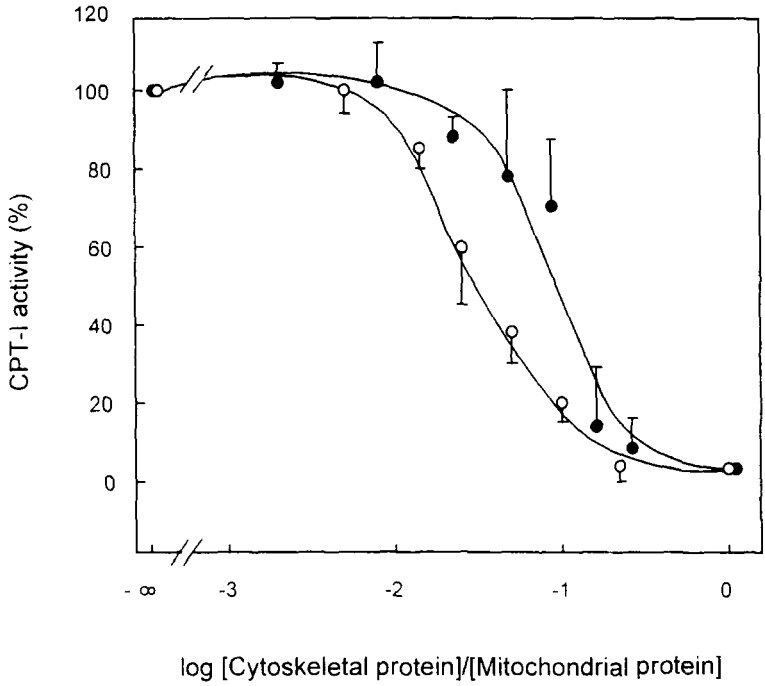
Hepatocyte preincubation	OA	CPT I activity (%)	[Malonyl-CoA] (%)
No additions	No	100 ± 17 (8)	100 ± 16
	Yes	154 ± 15* (8)	7 ± 5*
Colchicine (50 μM)	No	98 ± 12 (4)	96 ± 14
	Yes	155 ± 11* (4)	5 ± 6*
Cytochalasin B (10 μM)	No	96 ± 11 (4)	104 ± 1
	Yes	151 ± 18* (4)	7 ± 3*
IDPN (5 mM)	No	143 ± 11* (6)	103 ± 15
	Yes	166 ± 15* (6)	6 ± 6*
Taxol (10 μM)	No	102 ± 9 (4)	101 ± 12
	Yes	104 ± 8 (4)	13 ± 8*
Taxol (10 μM) + IDPN (5 mM)	No	96 ± 6 (6)	101 ± 9
	Yes	99 ± 6 (6)	7 ± 4*

*Significantly different ($P < 0.01$) from incubations with no additions

the fraction that was more enriched in intermediate-filament components (Fraction II) produced a more potent inhibition of CPT I (Fig. 2).

In short, all these data suggest that disruption of interactions between CPT I and cytoskeletal component(s) may de-inhibit CPT I and, therefore, increase enzyme activity. This is in line with the current notion that the dynamics and intracellular distribution of mitochondria in living cells may result from specific interactions of mitochondria with components of the cytoskeleton.[14,15] In the case of rat-brain mitochondria, accruing evidence indicates that specific interactions occur between mitochondrial-outer-membrane proteins and cytoskeletal proteins. A well described example being the interaction between porin, microtubule-associated protein 2, and the neurofilamental proteins NF-H and NF-M.[15,17] The existence of direct contact sites between intermediate filaments and the mitochondrial outer membrane has been reported not only in neurons, but also in smooth muscle myocytes and adrenal cortex cells.[14,15] As far as we know, although rat-liver mitochondria have been shown to interact with microtubules,[15] direct evidence for their interaction with intermediate filaments is still lacking.

It has been suggested that a function of the interactions between mitochondria and intermediate filaments may be to locate mitochondria in precise sites within the cell.[14,15] This idea is based on experiments showing a parallel redistribution of mitochondria and intermediate filaments upon cell exposure to agents that disrupt intermediate filaments or in certain stress situations.[15,18] The mitochondrial alterations observed in desmin-null mice also support this hypothesis.[19] Since the organization of intermediate filaments changes dramatically in a number of liver pathologies,[19,20] our observations predict that CPT I activity as affected by cytoskeletal components may change under pathophysiological situations in which the organization of the cytoskeleton is altered, e.g. in transformed cells. In this respect, Paumen *et al.* have observed that inhibition of CPT I with etomoxir leads to a stimulation of ceramide synthesis and to palmitate-induced cell death.[21] These authors suggested that cells that express high CPT I activity are expected



Firure 2. Effect of cytoskeletal fractions on CPT I activity. Rat liver mitochondria (1.5 2.0 mg protein ml ^{1}) were incubated for 30 min with varying amounts of cytoskeletal fractions (filled circles, Fraction I; open circles, Fraction II) and CPT I activity was subsequently determined as indicated in Experimental Procedures. Results correspond to 4 different experiments.

to withstand palmitate-induced apoptosis.[21] Thus, we have recently observed that CPT I specific activity is similar in mitochondria isolated from hepatoma cells and normal hepatocytes, but just about half in permeabilized hepatocytes than in permeabilized hepatoma cells; in addition, CPT I is not activated by OA in hepatoma cells.[22] These observations support the notion that in hepatocytes OA liberates CPT I from certain constrictions imposed by extra-mitochondrial cell components that do not operate either in isolated mitochondria or in transformed liver cells. Whether liberation of CPT I from those potential constrictions may help hepatoma cells to escape from apoptosis is currently under study in our laboratories. It is worth noting anyway that treatment of hepatocytes with OA -a well-known tumour promoter-renders a "CPT I regulatory phenotype" similar to that shown by hepatoma cells.

3. EXPERIMENTAL EVIDENCE FOR THE INVOLVEMENT OF CA^{2+}/CM-PKII IN THE CONTROL OF HEPATIC CPT I ACTIVITY

Ca^{2+}/CM-PKII is one of the protein kinases more actively involved in the control of the integrity of the cytoskeleton by phosphorylating cytoskeletal proteins.[13] Ca^{2+}/CM-PKII becomes constitutively activated when autophosphorylated in key serine or threonine residues on the autonomy site of the enzyme.[13] Autophosphorylation is sufficient to disrupt the autoinhibitory domain of Ca^{2+}/CM-PKII, leading to a permanent de-

inhibition of the kinase.[13] It is thus conceivable that permanent activation of Ca^{2+}/CM-PKII ensues upon inhibition by OA of the phosphatases involved in the dephosphorylation (= deactivation) of Ca^{2+}/CM-PKII. Ca^{2+}/CM-PKII autophosphorylation has indeed been demonstrated in hepatocytes upon challenge to OA.[11] We have recently obtained further evidence for the existence of a link between Ca^{2+}/CM-PKII and the cytoskeleton in the context of CPT I regulation. This notion is based mostly on the following observations:

(i) Purified autophosphorylated Ca^{2+}/CM-PKII was directly added to isolated mitochondria or permeabilized hepatocytes and CPT I activity was determined. Ca^{2+}/CM-PKII was able to significantly stimulate CPT I in permeabilized cells (140 ± 8% stimulation) but not in isolated mitochondria (6 ± 7% stimulation).

(ii) We next attempted to reconstitute the whole-cell experimental system in a simple manner by incubating isolated mitochondria together with cytoskeletal Fraction II and purified Ca^{2+}/CM-PKII. As shown in Fig. 3, the inhibition of CPT I produced by exposure of isolated mitochondria to cytoskeletal Fraction II was reverted by addition of exogenous Ca^{2+}/CM-PKII.

(iii) Intact hepatocytes were labelled with ^{32}Pi and cytokeratins were immunoprecipitated. As shown in Fig. 4, two major cytokeratin bands were phosphorylated upon hepatocyte challenge to OA. These two bands were assigned to cytokeratins 8 and 18 on the basis of their molecular mass (54 and 45kDa,

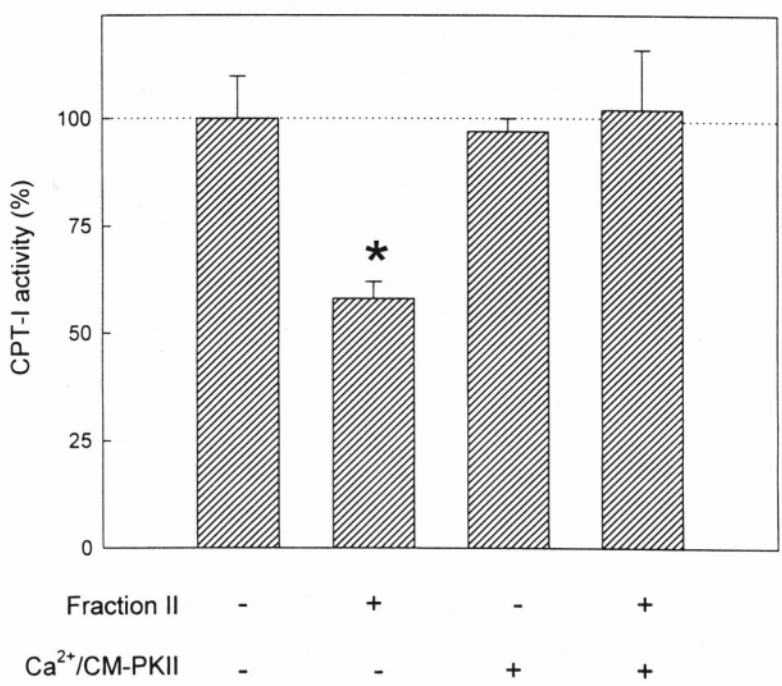


Figure 3. Effect of Ca^{2+}/CM-PKII on CPT I activity. Rat liver mitochondria (1.5–2.0 mg protein ml^{-1}) were preincubated for 30 min in the absence (–) or in the presence (+) of cytoskeletal Fraction II (0.05–0.06 mg protein ml^{-1}). Purified Ca^{2+}/CM-PKII was subsequently added (+) or not (–) to the incubations, which were run for 10 additional min. Aliquots were subsequently taken to determine CPT I activity. Results correspond to 4 different experiments. *Significantly different ($P < 0.01$) from incubations with no additions.

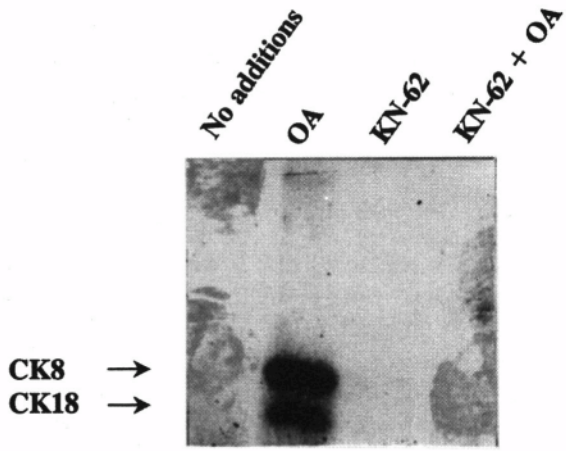


Figure 4. Phosphorylation of cytokeratins in intact hepatocytes. Hepatocytes were loaded with ^{32}Pi and further incubated for 15 min in the absence or in the presence of 30 μM KN-62. Incubations were continued for an additional 15 min period with or without 0.5 μM OA. Cells were subsequently disrupted and cytokeratins were immunoprecipitated with an anti-pan cytokeratin monoclonal antibody. Immunoprecipitates were subjected to SDS-PAGE and autoradiography. The experiment was repeated three times and similar results were obtained.

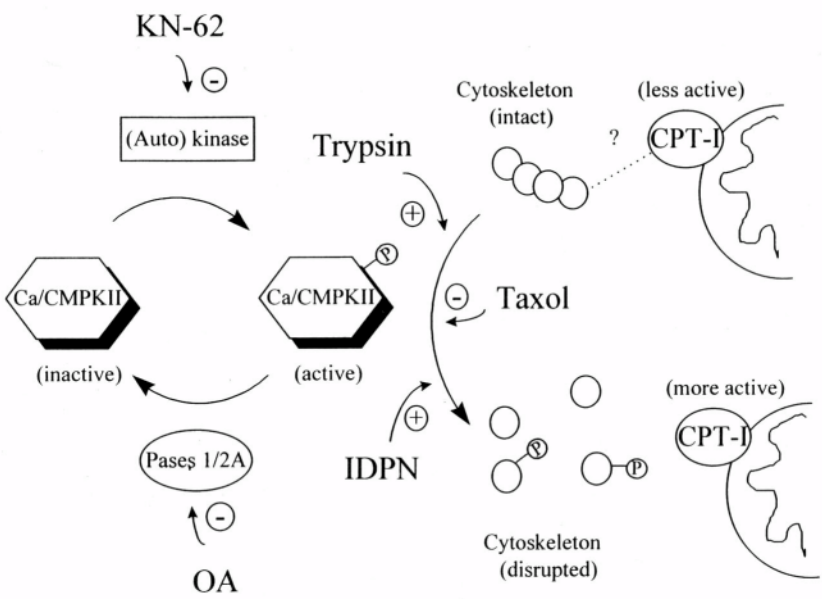
respectively) and high abundance in rat liver (e.g.[23,24]). Moreover, the OA-induced phosphorylation of these two bands was prevented by KN-62, the Ca^{2+}/CM-PKII inhibitor that antagonizes the OA-induced stimulation of CPT I.[11] We also showed by Western blotting that the ^{32}P-labelled bands do, indeed, correspond to cytokeratins 8 and 18, and not to proteins which have co-precipitated with the cytokeratins, and that cytokeratins 8 and 18 become significantly phosphorylated upon exposure of intact hepatocytes to OA (6mol of phosphate incorporated per mol of cytokeratin 8, and 3mol of phosphate incorporated per mol of cytokeratin of 18).

In short, the present data point to the existence of a link between Ca^{2+}/CM-PKII and the cytoskeleton in the context of CPT I regulation. Phosphorylation of cytokeratin intermediate filaments by Ca^{2+}/CM-PKII in whole cells has been shown to lead to the disruption of these structures,[23,24] and, therefore, this observation fits with the OA- and IDPN-induced stimulation of CPT I. It is worth noting that neither cAMP-dependent protein kinase nor protein kinase C affected CPT I activity in permeabilized hepatocytes in spite of their ability to phosphorylate cytokeratins *in vitro*.[23,25,26] However, several lines of evidence indicate that neither of these two protein kinases play an important role in the direct control of intermediate filament integrity in intact hepatocytes. Thus, it has been shown that protein kinase C may be responsible for maintaining basal levels of phosphorylation of cytokeratins 8 and 18 without altering cytokeratin filament assembly.[25] As a matter of fact, exposure of intact hepatocytes to phorbol esters induces cytokeratin phosphorylation but does not alter organization of intermediate filaments filament, which appear as fully assembled networks.[26] In addition, phosphopeptide maps of hepatic cytokeratins phosphorylated *in vivo* and *in vitro* clearly indicate that cAMP-dependent protein kinase is not much involved in cytokeratin phosphorylation in intact cells.[23] In contrast, and in line with data in the present paper, Ca^{2+}/CM-PKII has been shown to play a major role in the phosphorylation and functional integrity of hepatic cytokeratins *in vivo*.[23] It has been also suggested that activation of Ca^{2+}/CM-PKII (and not of other protein kinases) is responsible for the OA-induced disruption of hepatocyte cytoskeleton.[16] All this points to a functional involvement of this protein kinase in the control of the integrity of hepatic cytoskeleton.

4. MALONYL-COA-DEPENDENT AND MALONYL-COA-INDEPENDENT CONTROL OF CPT I ACTIVITY

Together with previous observations,[10–12] the present data allow to design a model that explains the OA-induced malonyl-CoA-independent control of hepatic CPT I. As shown in Scheme 1, OA may activate Ca^{2+}/CM-PKII by increasing its degree of phosphorylation upon inhibition of protein phosphatases 1 and 2A; this effect would be overcome by KN-62, an inhibitor of Ca^{2+}/CM-PKII autophosphorylation. Activated Ca^{2+}/CM-PKII would phosphorylate cytoskeletal components, perhaps cytokeratins 8 and 18, thereby disrupting putative inhibitory interactions between the cytoskeleton and CPT I. Stimulation of CPT I upon disruption of the cytoskeleton would be also achieved by challenge of intact hepatocytes to IDPN or by treatment of permeabilized hepatocytes with trypsin in mild conditions. Stabilization of the cytoskeleton with taxol may prevent the malonyl-CoA-independent acute stimulation of CPT I.

It is obvious that the notion that fatty acid translocation into mitochondria may be controlled by modulation of the interactions between CPT I and cytoskeletal components (i.e. by a malonyl-CoA-independent mechanism) does not diminish the importance of malonyl-CoA as a physiological modulator of CPT I activity.[5,8] On one hand, since the pioneering work of McGarry and coworkers,[8,27] changes in long-chain fatty acid oxidation under many different patho-physiological situations have been shown to be linked to changes in intracellular malonyl-CoA concentration and/or changes in the sensitivity of CPT I to malonyl-CoA.[1,2,5] On the other hand, several observations suggest that malonyl-CoA-dependent and malonyl-CoA-independent acute control of hepatic CPT I activity might operate in concert. Firstly, we have recently shown that stimulation of the AMP-activated protein kinase—a major protein kinase involved in the control of



Scheme I. Proposed model for the malonyl-CoA-independent acute control of hepatic CPT I activity. See the text for abbreviations and further details. +, activation; –, inhibitiond.

hepatic lipid metabolism—leads to an activation of hepatic CPT I by malonyl-CoA-dependent and malonyl-CoA-independent mechanisms.[28] Secondly, a fraction of hepatic acetyl-CoA carboxylase, the enzyme responsible for the synthesis of malonyl-CoA, has been recently suggested to be bound to the cytoskeleton.[29] Thirdly, it has been put forward that the 280-kDa isoform of acetyl-CoA carboxylase might interact with the outer leaflet of the mitochondrial outer membrane in order to channel malonyl-CoA for CPT I inhibition.[30] Fourthly, the recent observation that the bulk of the CPT I protein seems to face the cytoplasmic side of the mitochondrial outer membrane[31] makes more likely that interactions between CPT I and cytoskeletal components might occur. Although the physiological role of the malonyl-CoA-independent mechanism of regulation of hepatic CPT I activity is as yet unknown, it is worth noting that hormonal challenge of hepatocytes (e.g. glucagon, insulin) leads to changes in CPT I activity that parallel changes in long-chain fatty acid oxidation and that are retained after washing of the permeabilized cells.[1] In the context of the emerging role of cytoskeletal filamentous networks in intracellular signaling,[32] current research in our laboratories is focussed on the possible existence of a coordinate control of CPT I and acetyl-CoA carboxylase activities by modulation of interactions between the cytoskeleton and the mitochondrial outer membrane.

ACKNOWLEDGMENTS

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REFERENCES

1. McGarry, J.D., Woeltje, K.F., Kuwajima, M. & Foster, D.W. (1989) *Diabetes Metab. Rev.* **5**, 271–284. Regulation of ketogenesis and the renaissance of carnitine palmitoyltransferase.
2. Zammit, V.A. (1994) *Diabetes Rev.* **2**, 132–155. Regulation of ketone body metabolism. A cellular perspective.
3. Guzmán, M. & Geelen, M.J.H. (1993) *Biochim. Biophys. Acta* **1167**, 227–241. Regulation of fatty acid oxidation in mammalian liver.
4. Eaton, S., Bartlett, K. & Pourfarzam, M. (1996) *Biochem. J.* **320**, 345–357. Mammalian mitochondrial β-oxidation.
5. McGarry, J.D. & Brown, N.F. (1997) *Eur. J. Biochem.* **244**, 1–14. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis.
6. Drynan, L., Quant, P.A. & Zammit, V.A. (1996) *Biochem. J.* **317**, 791–795. Flux control exerted by mitochondrial outer membrane carnitine palmitoyltransferase over β-oxidation, ketogenesis and tricarboxylic acid cycle activity in hepatocytes isolated from rats in different metabolic states.
7. Spurway, T., Sherrat, H.S.A., Pogson, C.I. & Agius, L. (1997) *Biochem. J.* **323**, 119–122. The flux control coefficient of carnitine palmitoyltransferase I on palmitate β-oxidation in rat hepatocyte cultures.
8. McGarry, J.D. & Foster, D.W. (1980) *Annu. Rev. Biochem.* **49**, 395–420. Regulation of hepatic fatty acid oxidation and ketone body production.
9. Guzmán, M. & Geelen, M.J.H. (1992) *Biochem. J.* **287**, 487–492. Activity of carnitne palmitoyltransferase in mitochondrial outer membranes and peroxisomes in digitonin-permeabilized hepatocytes. Selective modulation of mitochondrial enzyme activity by okadaic acid.
10. Guzman, M., Kolodziej, M.P., Caldwell, A., Costorphine, C.G. & Zammit, V.A. (1994) *Biochem. J.* **300**, 693–699. Evidence against direct involvement of phosphorylation in the activation of carnitine palmitoyltransferase by okadaic acid in rat hepatocytes.

11. Velasco, G., Guzmán, M., Zammit, V.A. & Geelen, M.J.H. (1997) *Biochem. J.* **321**, 211–216. Involvement of Ca^{2+}/calmodulin-dependent protein kinase II in the activation of carnitine palmitoyltransferase I by okadaic acid in rat hepatocytes.
12. Velasco, G., Sánchez, C., Geelen, M.J.H. & Guzmán, M. (1996) *Biochem. Biophys. Res. Commun.* **224**, 754–759. Are cytoskeletal components involved in the control of hepatic carnitine palmitoyltransferase I activity?
13. Braun, A.P. & Schulman, H. (1995) *Annu. Rev. Physiol.* **57**, 417–445. The multifunctional calcium/calmodulin-dependent protein kinase: from form to function.
14. Bereiter-Hahn, J. & Vöth, M. (1994) *Microsc. Res. Tech.* **27**, 198–219. Dynamics of mitochondria in living cells: shape changes, dislocations, fusion, and fission of mitochondria.
15. Leterrier, J.F., Rusakov, D.A., Nelson, B.D. & Lindén, M. (1994) *Microsc. Res. Tech.* **27**, 233–261. Interactions between brain mitochondria and cytoskeleton: evidence for specialized outer membrane domains involved in the association of cytoskeleton-associated proteins to mitochondria *in situ* and *in vivo.*
16. Holen, I., Gordon, P.B. & Seglen, P.O. (1992) *Biochem. J.* **284**, 633–636. Protein kinase-dependent effects of okadaic acid on hepatocytic autophagy and cytoskeletal integrity.
17. Lindén, M. & Karlsson, G. (1996) *Biochem. Biophys. Res. Commun.* **218**, 833–836. Identification of porin as a binding site for MAP2.
18. Collier, N.C., Sheetz, M.P. & Schlesinger, M.J. (1993) *J. Cell Biochem.* **52**, 297–397. Concomitant changes in mitochondria and intermediate filaments during heat shock and recovery of chicken embryo fibroblasts.
19. Fuchs E. & Cleveland D.W. (1998) *Science* **279**, 514–519. A structural scaffolding of intermediate filaments in health and disease.
20. Omary, M.B. & Ku, N.O. (1997) *Hepatology* **25**, 1043–1048. Intermediate filament proteins of the liver: emerging disease association and functions.
21. Paumen, M.P., Ishida, Y., Muramatsu, M., Yamamoto, M. & Honjo, T. (1997) *J. Biol. Chem.* **272**. 3324–3329. Inhibition of carnitine palmitoyltransferase I augments sphingolipid synthesis and palmitate-induced apoptosis.
22. Velasco, G., Passilly, P., Guzmán, M. & Latruffe, N. (1998) *Biochem. Pharmacol.* (in press). Loss of response of carnitine palmitoyltransferase I to okadaic acid in transformed hepatic cells.
23. Toivola, D.M., Goldman, R.D., Garrod, D.R. & Eriksson, J.E. (1997) *J. Cell Sci.* **110**, 23–33. Protein phosphatases maintain the organization and structural interactions of hepatic keratin intermediate filaments.
24. Inagaky, M., Inagaki, N., Takahashi, T. & Takay, Y. (1997) *J. Biochem.* (*Tokyo*) **121**, 407–414. Phosphorylation-dependent control of structures of intermediate filaments: a novel approach using site- and phosphorylation state-specific antibodies.
25. Ku, N.O. & Omary, B. (1994) *J. Cell Biol.* **127**, 161–171. Identification of the major physiologic phosphorylation site of human keratin 18: potential kinases and a role in filament reorganization.
26. Cadrin, M., McFarlane-Anderson, N., Aasheim, L.H., Kawahara, H., Franks, D.J., Marceau, N. & French, S.W. (1992) *Cell. Signal.* **4**, 715–722. Differential phosphorylation of CK8 and CKI8 by 12-O-tetradecanoyl-phorbol-13-acetate in primary cultures of mouse hepatocytes.
27. McGarry, J.D., Mannaerts, G.P. & Foster, D.W. (1977) *J. Clin. Invest.* **60**, 265–270. A possible role for malonyl-CoA in the regulation of hepatic fatty acid oxidation and ketogenesis.
28. Velasco, G., Geelen, M.J.H. & Guzmán, M. (1997) *Arch. Biochem. Biophys.* **337**, 169–175. Control of hepatic fatty acid oxidation by 5′-AMP-activated protein kinase involves a malonyl-CoA-dependent and a malonyl-CoA-independent mechanism.
29. Geelen. M.J.H., Bijleveld, C., Velasco, G., Wanders, R.A.J. & Guzmán, M. (1997) *Biochem. Biophys. Res. Commun.* **233**, 253–257. Studies on the intracellular localization of acetyl-CoA carboxylase.
30. Ha, J., Lee, J.K., Kirn, K.S., Witters, L.A. & Kim, K.H. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 11466–1147031. Cloning of human acetyl-CoA carboxylase-β and its unique features.
31. Fraser, F., Costorphine, C.G. & Zammit, V.A. (1997) *Biochem. J.* **323**, 711–718. Topology of carnitine palmitoyltransferase I in the mitochondrial outer membrane.
32. Forgacs, G. (1995) *J. Cell Sci.* **108**, 2131–2143. On the possible role of cytoskeletal filamentous networks in intracellular signaling: an approach based on percolation.

EFFECTS OF 3-THIA FATTY ACIDS ON β-OXIDATION AND CARNITINE PALMITOYLATRANSFERASE I ACTIVITY IN CULTURED RAT HEPATOCYTES

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1. INTRODUCTION

3-Thia fatty acids represent non-β-oxidizable fatty acid analogues in which a S atom substitutes for the β-methylene group in the chain. They are known to reduce serum triacylglycerol and cholesterol.[1] The hypolipaemia is detectable within 3 hours of administration[2] and is explained initially by an increase in mitochondrial β-oxidation and a decrease in triacylglycerol secretion,[3,4] followed by an increase in activity of peroxisomal β-oxidation enzymes after ~12 hours.[2]

These fatty acids are taken up by hepatocytes at the same rate as normal fatty acids[5] and they are also activated to CoA esters, but at only half the rate of normal fatty acids.[6] They are substrates for carnitine palmitoyltransferase (CPT) I and make complexes with acyl-CoA dehydrogenase,[7] however, because of the sulphur atom they cannot be β-oxidized.

Addition of tetradecylthioacetic acid (TTA) to isolated rat hepatocytes leads to an increase in oxidation of palmitate[5] and a strong decrease in *de novo* synthesis of fatty acids[5] and cholesterol (Risan, K.A. & Spydevold, Ø., unpublished results). Hepatocytes isolated from rats 24 hours after administration of TTA show also an elevated activity of CPT I.[8] However, little is known about the acute effects of 3-thia fatty acids.

In the present work we have studied effects of 3-thia fatty acids on β-oxidation and CPT I activity in freshly cultured (4–6 hours) rat hepatocytes. By using digitonin-permeabilized cells[9] we were able to measure CPT I activity directly in hepatocytes which are possibly a more physiologically relevant system than isolated mitochondria.

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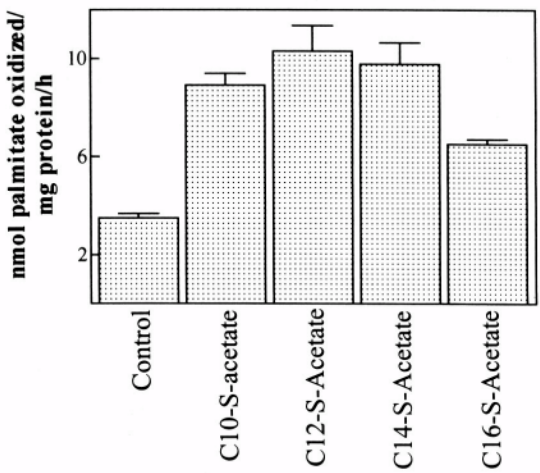

Figure 1. *Effect of chain length of 3-thia fatty acid on β-oxidation in cultured hepatocytes.* Hepatocytes were incubated with 200 µM 3-thia fatty acid with varying chain length and 200 µM [1–^{14}C] palmitate. Results are given as mean ± SD of 3 parallel incubations.

2. RESULTS

When 3-thia fatty acids are added to isolated hepatocytes they stimulate fatty acid oxidation (Fig. 1). The strength of this effect depends on the chain length of 3-thia fatty acid used. Dodecylthioacetic acid (DTA) and tetradecylthioacetic acid (TTA) are most potent, shorter and longer 3-thia fatty acids have a reduced effect.

When palmitate is used as substrate the addition of equimolar amounts of DTA stimulates the oxidation ~3 fold (Fig. 2A). With the shorter laurate as substrate (Fig. 2B) the effect decreases and is no longer detectable with octanoate (Fig. 2C). This pattern of responsiveness suggests that 3-thia fatty acids affect the mitochondrial CPT system, since oxidation of long fatty acids, in contrast to short ones, is dependent on the transport of acylcarnitines across the mitochondrial inner membrane.

Incubation of hepatocytes with DTA results in a very rapid activation of CPT I, which reaches its maximum already after 10min (Fig. 3A). Malonyl-CoA sensitivity

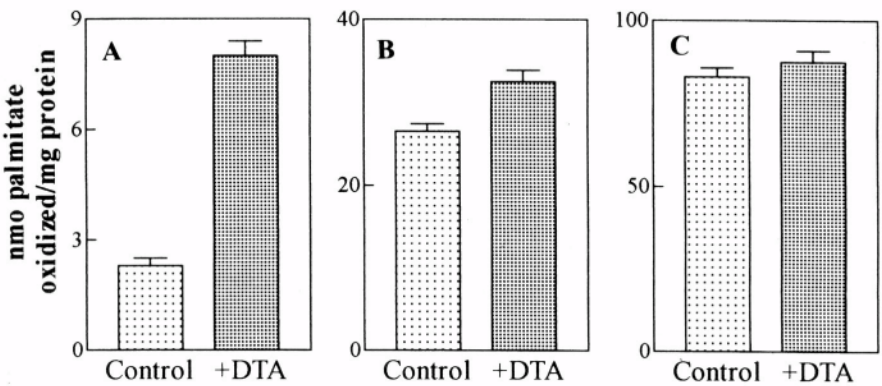

Figure 2. *Effects of DTA on β-oxidation of various fatty acids in cultured hepatocytes.* Hepatocytes were incubated with or without 200 µM DTA and with 200 µM [1–^{14}C] palmitate (A) or [1–^{14}C] laurate (B) or [1–^{14}C] octanoate (C) as substrate. Results are given as mean ± SD of 3 parallel incubations.

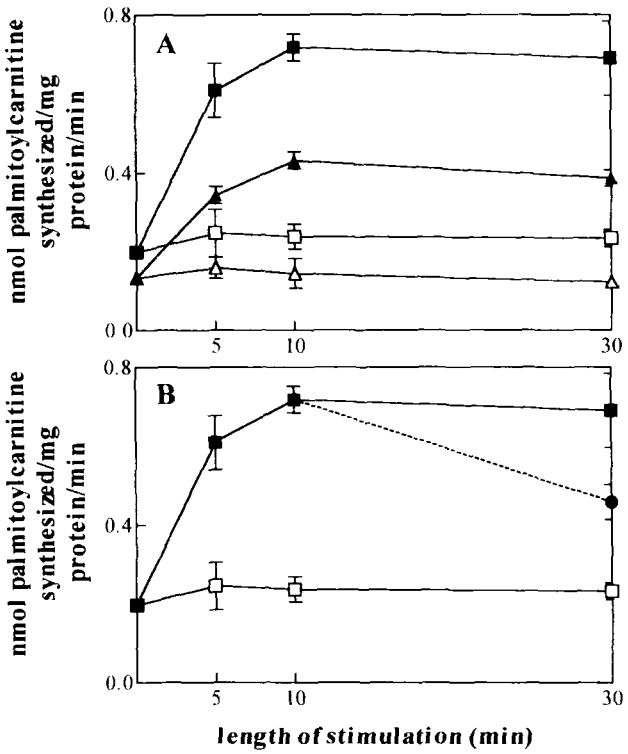

Figure 3. *Time course of DTA dependent activation (A) and inactivation (B) of CPT I in cultured hepatocytes* (A) Hepatocytes were incubated without (open symbols) or with (closed symbols) 200 μM DTA for indicated periods before permeabilization and assay of CPT I activity with (open or closed triangles) or without (open or closed squares) 50 μM malonyl-CoA. Results are given as means ± SD of 3 parallel incubations. (B) Hepatocytes were incubated without (open squares) or with (closed squares) 200 μM DTA. After 10 min DTA was removed, the cells were washed and cultured with DTA-free medium. After 20 min CPT I activity was determined (closed circles). Results are given as means ± SD of 3 parallel incubations.

remains unchanged with a 65–70% inhibition throughout the whole period of incubation. When DTA is removed from the medium there is a rapid decrease in activity of CPT I with halving of the stimulatory effect observed after ~20min (Fig. 3B).

Incubations with palmitic acid gives also some stimulation of CPT I activity, but much smaller compared to DTA (Fig. 4). Incubation with an oxo derivative with the same chain length as DTA gives stimulation comparable to DTA.

3. DISCUSSION

It has been shown in feeding experiments that TTA, only 3 hours after administration, causes a drop in blood lipids, primarily by affecting mitochondrial β-oxidation.[4]

We now have shown that the main target for the short-term effect of 3-thia fatty acids is a reversible activation of CPT I which is stimulated ~3–4 fold in freshly activated hepatocytes. This stimulation is a very fast process, which cannot be explained by gene transcription. Maximal activity is reached after only 10min, a time too short for new protein synthesis to take place. Consequently 3-thia fatty acids have to affect the already

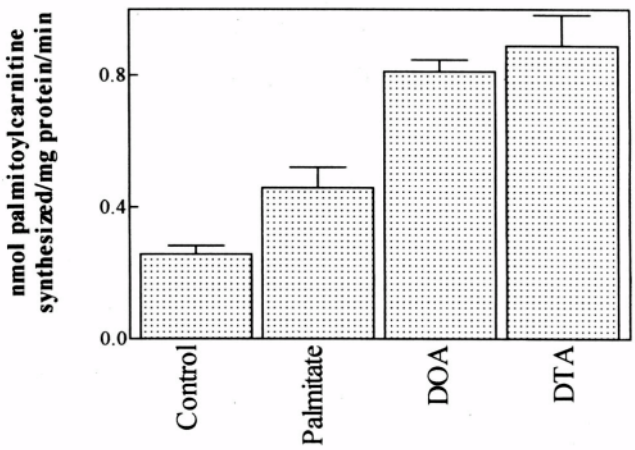

Figure 4. *Effects of palmitate and fatty acid derivatives on CPT I activity in cultured hepatocytes.* Hepatocytes were incubated with 200 µM of either palmitate or dodecyloxoacetic acid (DOA) or DTA. CPT I activity was then assayed. Results are given as mean ± SD of 2 parallel incubations.

existing enzyme. This activation does not change the malonyl-CoA sensitivity. It is also independent of level of malonyl-CoA in the cells because similar stimulation is observed in hepatocytes from both fasted and fasted-carbohydrate refed rats (results not shown), where malonyl-CoA levels are very different. This stimulation is not specific for thia fatty acids but can be reproduced also by oxa fatty acids and partially by normal fatty acids. This indicates that what we observe is probably a fatty acid effect, however, because of slower metabolism of oxo and thia derivatives there is a higher concentration of them in the cell and this may be the cause of this stronger effect.

The nature of this activation is unknown. Recently it has been suggested that cytoskeletal components take part in the regulation of CRT I[10] but it unknown whether fatty acids can interfere with this system in the cells.

REFERENCES

1. Aarsland, A., Aarsaether, N. Bremer, J. & Berge, R.K. (1989) *J. Lipid Res.*, **30**, 1711–1718. Alkylthioacetic acids (3-thia fatty acids) as non-beta-oxidizable tatty acid analogues: a new group of hypolipidemic drugs. III. Dissociation of cholesterol- and triglyceride-lowering effects and the induction of peroxisomal beta-oxidation.
2. Asiedu, D.K., Skorve, J., Willumsen, N., Demoz, A. & Berge, R.K. (1993) *Biochim. Biophys. Acta*, **1166**, 73–76. Early effects on mitochondrial and peroxisomal beta-oxidation by the hypolipidemic 3-thia-fatty acids in rat livers.
3. Skrede, S., Bremer, J., Berge, R.K. & Rustan, A.C. (1994) *J. Lipid Res.*, **35**, 1395 1404. Stimulation of fatty acid oxidation by a 3-thia fatty acid reduces triacylglycerol secretion in cultured rat hepatocytes.
4. Asiedu, D.K., Demoz, A., Skorve, J., Grav, H.J. & Berge, R.K. (1995) *Biochem. Pharmacol.*, **49**, 1013–1022. Acute modulation of rat hepatic lipid metabolism by sulphur-substituted fatty acid analogues.
5. Skrede, S., Narce, M., Bergseth, S. & Bremer, J. (1989) *Biochim. Biophys. Acta*, **1005**, 296 302. The effects of alkylthioacetic acids (3-thia fatty acids) on fatty acid metabolism in isolated hepatocytes.
6. Aarsland, A. & Berge, R.K. (1991) *Biochem. Pharmacol.*, **41**, 53-61. Peroxisome proliferating sulphur- and oxy-substituted fatty acid analogues are activated to acyl coenzyme A thioesters.
7. Lau, S.M., Brantley, R.K. & Thorpe, C. (1988) *Biochemistry*, **27**, 5089-5095. The reductive half-reaction in Acyl-CoA dehydrogenase from pig kidney; studies with thiaoctanoyl-CoA and oxaoctanoyl-CoA analogues.

8. Skrede, S. & Bremer, J. (1993) *Biochim. Biophys. Acta*, **1167**, 189–196. Acylcarnitine formation and fatty acid oxidation in hepatocytes from rats treated with tetradecylthioacetie acid (a 3-thia fatty acid).
9. Guzmán, M. & Geelen, M.J. (1988) *Biochem. Biophys. Res. Commun.*, **151**, 781–787, Short-term regulation of carnitine palmitoyltransferase activity in isolated rat hepatocytes.
10. Velasco, G., S·nchez, C., Geelen, M.J.H. & Guzmán, M. (1996) *Biochem. Biophys. Res. Commun.*, **224**, 754–759. Are cytoskeletal components involved in the control of hepatic carnitine palmitoyltransferase I activity?

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CARNITINE ACYLTRANSFERASES AND ASSOCIATED TRANSPORT PROCESSES IN THE ENDOPLASMIC RETICULUM

Missing Links in the VLDL Story?

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1. MICROSOMAL CARNITINE ACYLTRANSFERASES

1.1. Background

The presence of carnitine acyltransferase (CAT) activity in liver microsomes was first reported in 1976[1] and in 1990 it was reported that both rough and smooth endoplasmic reticulum from liver contained a CAT activity which was highly sensitive to inhibition by malonyl-CoA[2]—a property more generally associated with the carnitine palmitoyltransferase activity found in mitochondrial outer membranes (CPT_1). Subsequently, Murthy & Bieber[3] purified a CAT activity from rat liver microsomes which exhibited a M_r of 50,000 on SDS polyacrylamide gel electrophoresis and of 54,000 on gel filtration. However, this purified CAT activity was totally insensitive to malonyl-CoA and was not irreversibly inhibited by etomoxiryl-CoA (see below).[3,4] No immunological cross-reactivity with any other known CAT could be demonstrated.[4] At this stage there was no reason to suppose that microsomes contained more than one CAT activity and it appeared to us that the enzyme purified by Murthy & Bieber[3] had, in some way, lost malonyl-CoA sensitivity in the course of purification. However, this proved not to be the case. Rather, we found that liver microsomes contained two CAT activities.[5] One of these, which we now designate as microsomal CAT_A, was a malonyl-CoA-sensitive enzyme whilst the other, which we now designate as microsomal CAT_B, was a malonyl-CoA-insensitive acyltransferase. After treatment of microsomes with deoxycholate these could clearly be separated by gel filtration (Superdex 200) or by anion-exchange chromatography (Resource Q) and discriminated by differences in their fatty acyl-CoA substrate chain-lengthspecificity.[5] CAT_B activity was mainly latent in 'intact' microsomes, but was

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easily released by freezing and thawing in hypotonic buffer. By contrast, CAT_A was overt in microsomes having the 'outside-out' orientation, remained membrane-associated after freezing and thawing and was only released by detergent. It was also irreversibly inhibited by etomoxiryl-CoA. Solubilized CAT_A activity eluted from a Superdex 200 column in a peak corresponding to a M_r of approximately 300,000 and is almost certainly associated with numerous other membrane proteins at this stage. By contrast, solubilized CAT_B activity exhibited a M_r of approximately 60,000 on gel filtration—a value not greatly different from that assigned to the malonyl-CoA-insensitive enzyme purified by Murthy & Bieber.[3] With hindsight, it seems likely that CAT_A was not apparent in early purification studies[3,4] because the detergent used, octylglucoside, destroys this activity.[5] By contrast, both CAT_A and CAT_B activities survive exposure to deoxycholate.[5] A parallel study by Murthy & Pande[6] also provided evidence for distinct malonyl-CoA-sensitive and—insensitive forms of CAT in liver microsomes based upon these having different sub-microsomal localizations and different sensitivity to the inhibitor etomoxiryl-CoA. In addition Murthy & Pande[6] suggested that the malonyl-CoA-sensitive enzyme (CAT_A) had a M_r of 47–50,000 because a labelled band was found at this position on SDS polyacrylamide gel electrophoresis after treatment of microsomes with [^{3}H]etomoxiryl-CoA. However, we believe this assignment of M_r for CAT_A to be incorrect (see below). It has been suggested that CAT_B is a stress-regulated protein, a member of the thioredoxin superfamily which has been cloned and studied as the GRP58 (glucose regulated protein), ERP_{61} or HIP-70 (hormone-induced protein).[7,8] However, transient or stable expression of the cDNA corresponding to GRP58/ERP_{61}/HIP-70 in human kidney 293 cells or in HeLa cells results in very little increase in CAT activity[8] and GRP58/ERP_{61}/HIP-70 expressed in baculovirus-infected Sf9 insect cells exhibits no CAT activity.[9] At this stage therefore, the identity of microsomal CAT_B remains in question.

1.2. Recent Studies on the Possible Identity of Microsomal CAT_A

Although CAT_A can be solubilized from rat liver microsomes using mild detergents such as deoxycholate[5] and reconstituted into liposomes of known phospholipid composition[10] without loss of activity, further purification of the enzyme away from other membrane components requires harsher conditions, which inactivate the enzyme. For example, substantial further purification can be achieved by extraction into 8mM CHAPS with 4 M urea followed by anion-exchange chromatography and gel filtration (N. M. Broadway & E. D. Saggerson, unpublished work) but the protein then has to be detected by indirect means. Because of these difficulties, purification of microsomal CAT_A to homogeneity has not yet been achieved. It was during attempts to purify CAT_A, prior to sequencing, that we arrived at the notion that microsomal CAT_A and mitochondrial CPT_1 may be very similar proteins.

1.2.1. Possible Detection of CAT_A by Reaction with DNP-Etomoxiryl-CoA. Incubation of rat liver microsomes (purified by sucrose-density gradient centrifugation) with DNP-coupled etomoxiryl-CoA, followed by SDS polyacrylamide gel electrophoresis of membrane proteins and probing with an anti-DNP primary antibody, showed that DNP-etomoxiryl-CoA inhibited CAT_A activity by >95% and labelled several protein bands.[11] Of these, it was particularly noteworthy that one had a M_r of 88,000, similar to liver mitochondrial CPT_1,[12] and another had a M_r of 49–50,000, similar to that assigned to CAT_A by Murthy & Pande.[6] The abundance of the 88kDa polypeptide in both mitochondrial outer membranes and microsomes was increased 2–3 fold on fasting (24 hours)

and these changes were paralleled by similar fold increases in the activities of mitochondrial CPT_1 and microsomal CAT_A.[11] By contrast, fasting decreased the abundance of the 49-50 kDa microsomal band.[11] These findings suggested that the 49–50kDa band was unlikely to be CAT_A and raised the possibility that a protein similar to mitochondrial CPT_1 was present in the microsomal membranes.

1.2.2. Possible Detection of CAT_A with An Anti-CPT_1 Antibody. More convincing evidence for the presence of a polypeptide similar to CPT_1 in microsomes was seen in Western blotting studies. Here an antipeptide antibody against amino acids 428-441 (REEDPEASIDSYAK) of rat liver mitochondrial CPT_1[13] (a generous gift from Dr. V. A. Zammit) recognized, at roughly equal intensity, a single 88 kDa polypeptide in both mitochondrial outer membranes and microsomes.[11] On running samples of mitochondrial outer membrane and microsomal membrane in the same gel lanes, it was observed that the microsomal band had identical mobility to the mitochondrial CPT_1 band.[11] In both types of membrane the intensity of this 88 kDa band was increased by fasting.[11] Furthermore, Figure 1 shows a significant positive correlation ($r = 0.761$, $P < 0.05$) between the specific activity of CAT_A and the abundance (by Western blotting) of the 88 kDa polypeptide in liver microsomal membranes from fed and 24 hour-fasted rats and from suckling rats. Measurements of marker enzyme activities allowed us to discount the possibility that the 88 kDa band represented CPT_1 from mitochondrial outer membrane contaminating the microsomal preparation. Typically, our microsomal preparations were contaminated less than 0.6% by mitochondrial outer membranes[5] and, as calculated previously,[5] less than 8% of the malonyl-CoA-inhibitable CAT activity of liver microsomal membranes can be accounted for by CPT_1 derived from mitochondria (i.e. more than 92% of the CAT activity of microsomal membranes must be due to an enzyme that is a genuine resident therein).

At this point we began to form the hypothesis that microsomal CAT_A might actually be CPT_1 as a result of targeting of CPT_1 to the endoplasmic reticulum as well as to the mitochondrial outer membrane.

1.2.3. Liver CPT_1 Can Insert Into the Endoplasmic Reticulum in a Cell Free-System. The cDNA for rat liver CPT_1 (an integral protein in the mitochondrial outer membrane) predicts a polypeptide of 773 amino acids (88kDa).[12] Unlike CPT_2 (the latent mitochondrial CPT), no N-terminal signal peptide is cleaved from nascent CPT_1 during mitochondrial import.[12] The deduced sequences of both liver and muscle isoforms of CPT_1 (L-CPT_1 and M-CPT_1 respectively) contain two largely hydrophobic domains

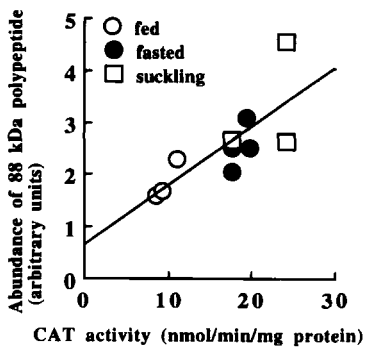

Figure 1. Correlation between CAT_A specific activity and the abundance of a 88 kDa polypeptide in rat liver microsomes. See text for further details.

encompassing amino acids 53–75 (TM_1) and 103–122 (TM_2) in the rat liver sequence.[12] A study of the topology of L-CPT_1 *in situ* in the liver mitochondrial outer membrane using antibodies, proteases and immobilized substrates and inhibitors[13] has provided evidence suggesting that these two domains could traverse the membrane and that both the N- and C-termini of the protein are oriented towards the cytosol. In addition, the first 18 N-terminal amino acids of CPT_1s are absolutely conserved between L-CPT_1 and M-CPT_1,[12] suggesting that this N-terminal region may be very important in structure/function and/or subcellular targeting.

Using reverse transcription PCR we have assembled cDNA clones encoding rat liver CPT_1.[11] A clone containing a premature stop codon was particularly useful. This encoded amino acids 1–328, i.e. it contained the conserved N-terminus as well as both putative domains TM_1 and TM_2. On translation *in vitro*, formation of the expected 36kDa polypeptide product was observed.[11] Of particular importance was the observation that this *in vitro* translation product inserted into canine pancreatic microsomes. True insertion was adjudged to have occurred because the truncated CPT_1 could not be extracted on treatment of the membranes with Na_2CO_3 (0.1 M, pH 11.5) for 60 minutes on ice.[11] Furthermore, it seems likely that the insertion was cotranslational since, if pancreatic microsomes were only added after translation had been terminated with cycloheximide, no truncated CPT_1 was recovered in the Na_2CO_3-extracted membrane pellet.[11] This is the predicted behaviour of an N-terminally anchored protein of the endoplasmic reticulum membrane and emphasizes that the integration of the translation product into import-competent microsomal membranes is a specific process. When a potential N-glycosylation site was introduced on the C-terminal side of TM_2 at residue 182, there was no change in the mobility on SDS polyacrylamide gel electrophoresis of the membrane-inserted translation product.[11] Since the canine pancreatic microsomes were N-glycosylation-competent, it was, therefore, inferred that the N-glycosylation site was oriented in a cytosolic fashion as shown in Figure 2. This would be expected if the overall orientation of the truncated CPT_1 in the microsomal membrane was similar to that deduced for L-CPT_1 in the mitochondrial outer membrane.[13] Deletion from the DNA construct of either the bases encoding the N-terminal amino acids 1–25 or the bases encoding the TM_2 domain did not abolish the ability of the translation products to insert into microsomal membranes. We conclude that the N-terminus of L-CPT_1 is not required for targeting the protein to the endoplasmic reticulum. We also suggest that the TM_1 domain may act as a signal-anchor sequence for insertion into the endoplasmic reticulum, although this conclusion would be strengthened if work in progress in our laboratory showed that deletion of this TM_1 domain abolished microsomal insertion.

We, therefore, have evidence that is not at variance with the novel notion that L-CPT_1 could target to both the endoplasmic reticulum and the mitochondrial outer membrane. In which case, it is possible that CPT_1 and microsomal CAT_A are one and the same protein. From our homology searches of expressed sequence tags it is possible to identify several clones encoding liver and muscle CPT_1, but at present there no clones reported which might encode a different (e.g. microsomal) isoform of CPT_1. However, as at present there do not appear to be any clones encoding the extreme N-terminus of L- or M-CPT_1 in the data base, we cannot formally exclude the possibility that splice variants of CPT_1 might exist with different N-termini. Further experiments are needed to resolve this issue but it is important to emphasize that the present work demonstrates that a polypeptide with an identical N-terminal region to L-CPT_1 is capable of inserting into microsomal membranes. If L-CPT_1 were to be promiscuous in its membrane targeting this would have

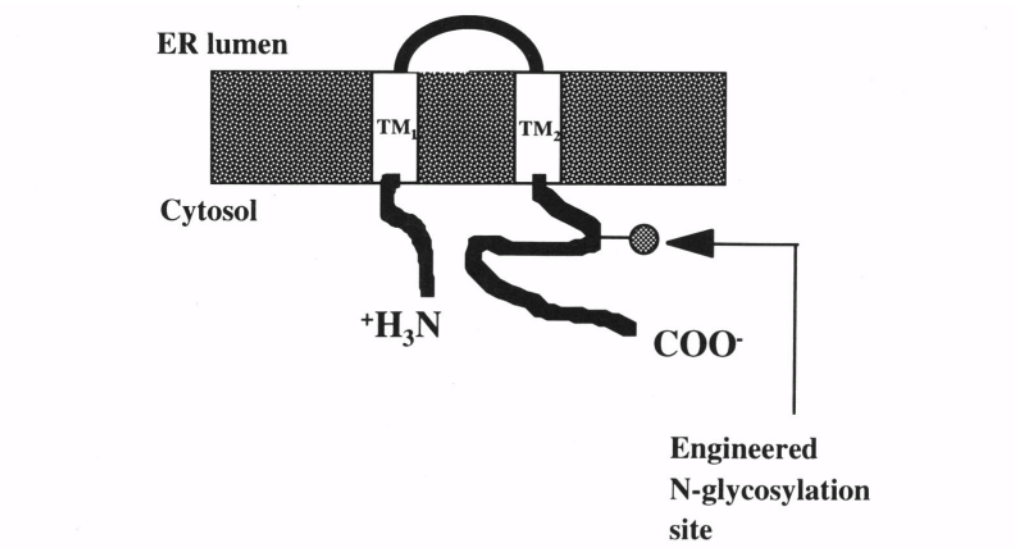

Figure 2. Proposed orientation of the truncated form of L-CPT$_1$ after co-translational insertion into canine pancreatic microsomes. See text for further details.

important implications both in the general cell biological sense and, specifically, with regard to the regulation of assembly of VLDL triacylglycerol (see Section 3).

2. TRANSPORT OF FATTY ACYLCARNITINE ACROSS THE LIVER MICROSOMAL MEMBRANE

Having CAT_A and CAT_B on either side of the microsomal membrane appears to serve no obvious purpose unless, by analogy with the well-characterized mitochondrial system,[14–18] these are linked to some form of transport system to move fatty acylcarnitines across the membrane. It is impractical to study directly the transport of radiolabelled long-chain fatty acylcarnitines into or out of sealed microsomal vesicles because these metabolites bind non-specifically to many cellular proteins. We, therefore, devised a way to do this indirectly, based on the use of the lumenal enzyme ethanol acyltransferase (EAT) as a 'reporter'. In the endoplasmic reticulum EAT[19,20] catalyzes the reaction:

palmitoyl-CoA + ethanol ethylpalmitate + CoASH.

Confirming,[19] we demonstrated[21] (Table 1) that exogenous palmitoyl-CoA was not a substrate for EAT if the microsomal vesicles were sealed (based on >90% latency of mannose 6-phosphatase). We also found that exogenous palmitoylcarnitine alone did not provide acyl substrate for EAT. However, ethylpalmitate was readily made from exogenous palmitoylcarnitine by sealed microsomes if exogenous free CoASH was simultaneously added (Table 1). These findings would be consistent with the presence of a transport process which allowed palmitoylcarnitine to access the microsomal lumen, together with a transport process for free CoASH, which is needed as co-substrate for CAT_B (Fig. 3). Similarly (Table 1), EAT activity was demonstrated when palmitoyl-CoA + carnitine were

Table 1. Use of ethanol acyltransferase as a lumen reporter enzyme to demonstrate transfer of fatty acylcarnitine across the liver microsomal membrane.
Assays were performed at 30°C in 10mM Tris/HCl buffer (pH 7.4) containing 300mM sucrose, bovine albumin (1 $mg.ml^{-1}$), 10mM$MgCl_2$, 0.8mM EDTA and 1mM dithiothreitol. Palmitoyl-CoA and palmitoylcarnitine were used at 40mM, CoASH at 100μM and carnitine at 200mM. All measurements were made with and without 123mM ethanol, with the difference between these measurements indicating ethylpalmitate formation. To unseal microsomal vesicles, alamethicin ($10\mu g.ml^{-1}$) was added. Assays were initiated by the addition of 0.7mg of liver microsomal protein. All values are means ± SEM.

Microsomal state	Substrates added	Formation of ethylpalmitate ($nmol.15min^{-1}.mg\ protein^{-1}$)
sealed	[^{14}C]palmitoyl-CoA	0.04 ± 0.22
unsealed	[^{14}C]palmitoyl-CoA	5.19 ± 0.55
sealed	[^{14}C]palmitoylcarnitine	0.21 ± 0.03
sealed	[^{14}C]palmitoylcarnitine + CoASH	2.44 ± 0.10
sealed	[^{14}C]palmitoyl-CoA + carnitine	0.12 ± 0.04
sealed	[^{14}C]palmitoyl-CoA + carnitine + CoASH	1.25 ± 0.09

added to sealed microsomes, provided free CoASH was also present. This latter route for provision of acyl substrate for lumenal EAT activity would additionally involve the CAT_A activity which is overt in sealed microsomal preparations (Fig. 3).

At present the transport processes required to move fatty acylcarnitine and free CoASH from the cytosolic side to the lumenal side of the endoplasmic reticulum are uncharacterized.

3. WHY IS THERE A NEED TO GENERATE FATTY ACYL-COA THIOESTERS IN THE LUMEN OF THE ENDOPLASMIC RETICULUM?

At present we do not know what contribution microsomal CATs or the transport processes proposed in Fig. 3 make to the phenotype of the liver cell. A general involve-

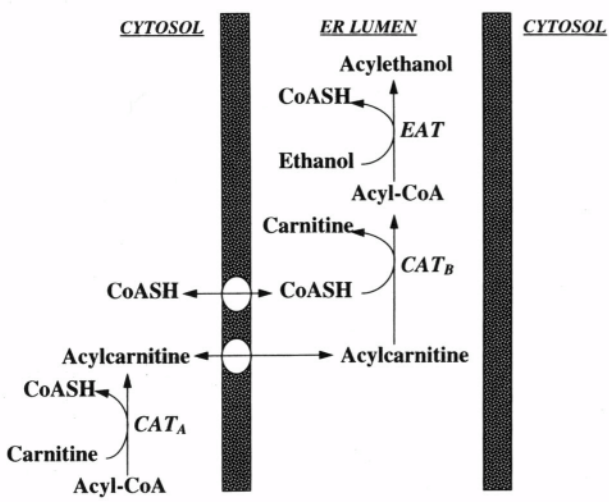

Figure 3. A scheme to show the suggested involvement of CAT_A and CAT_B in the generation of fatty acyl-CoA thioesters within the lumen of the endoplasmic reticulum. As shown, transport processes to facilitate the transmembrane movement of acylcarnitine and of free CoASH are also necessary. It is also envisaged[21] (though not shown in the figure) that there must be a transport process for free carnitine to exit the lumen.

ment of fatty acyl-CoA in promoting transport of proteins through the Golgi apparatus has been demonstrated[22-24] and important secreted proteins, such as major histocompatibility antigens, are known to be fatty acylated somewhere in the secretory pathway.[25–28] It is also possible that some lumenal fatty acyl-CoA is needed for membrane biogenesis or for remodeling of glycerolipids or sphingolipids. Certainly microsomal CAT_A is not confined to liver since we have demonstrated malonyl-CoA-inhibitable CAT activity in kidney microsomal membranes that were freed of mitochondria by sucrose density gradient fractionation (N. M. Broadway & E. D. Saggerson, unpublished work). In the context of a lipoprotein secreting tissue such as liver (or small intestine), one can readily envisage a role for fatty acyl-CoA in the endoplasmic reticulum lumen. Recent studies suggest that the assembly of apoprotein B-containing lipoprotein particles occurs in two stages.[29-36] Apoprotein B is cotranslationally threaded through the rough endoplasmic reticulum membrane and simultaneously acquires some core lipid in a process requiring the microsomal triacylglycerol transfer protein (MTP).[37,38] At this initial stage it is generally envisaged that the core triacylglycerol (TAG) may be transferred *en bloc* though the rough endoplasmic reticulum membrane. However, the bulk of the particle TAG is acquired later by a separate process, possibly in the lumen of the smooth endoplasmic reticulum.[36] In the main this bulk TAG acquisition is not by a direct, *en bloc*, transfer. Rather, a cytosolic storage pool of TAG has to undergo complete[39–41] or partial (to diacylglycerol or monoacylglycerol)[42] lipolysis before reassembly of the bulk TAG that is secreted. From such labelling studies it is clear that the reassembly of the secreted TAG must be catalyzed by enzymes that are spatially separated from those involved in the assembly of the storage pool of TAG. A recent reevaluation of the topology of liver diacylglycerol acyltransferase (DGAT) has shown that there are two microsomal pools of DGAT; one oriented to each side of the ER membrane.[43] We have recently confirmed this observation (J. M. Gooding & E. D. Saggerson—unpublished work). Since diacylglycerol can readily 'flip' across biological membranes (at least the erythrocyte membrane[44]) one can readily envisage how endoplasmic reticulum lumenal TAG could be reassembled from diacylglycerol and lumenal fatty acyl-CoA. It is well established that insulin acutely suppresses hepatic secretion of VLDL TAG [41,45–48] and it is, therefore, of note that insulin increases the hepatic level of malonyl-CoA[49]—an established inhibitor of microsomal CAT_A. We have also shown that sulphonylurea drugs, which inhibit VLDL TAG secretion at a post-lipolytic stage,[41] similarly inhibit microsomal CAT_A.[50] In view of the evidence implicating elevated plasma VLDL as a major risk factor for development of atherosclerosis, it is important to clarify the roles played by the microsomal CATs and their associated transport processes in VLDL assembly. Hitherto unrealized sites of regulation or therapeutic intervention may be revealed.

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REFERENCES

1. Markwell, M.A.K., Tolbert, N.E. & Bieber, L.L. (1976) *Arch. Biochem. Biophys.,* **176**, 479–488. Comparison of the carnitine acyltransferase activities from rat liver peroxisomes and microsomes.
2. Lilly, K., Bugaisky, G.E., Umeda, P.K. & Bieber, L.L. (1990) *Arch. Biochem. Biophys.,* **280**, 167–174. The medium-chain carnitine acyltransferase activity associated with rat liver microsomes is malonyl-CoA sensitive.
3. Murthy, M.S.R. & Bieber, L.L. (1992) *Protein Expression Purif.,* **3**, 75–79. Purification of the medium-chain/long-chain (COT/CPT) carnitine acyltransferase of rat liver microsomes.
4. Chung, C.-D. & Bieber, L.L. (1993) *J. Biol. Chem.,* **268**, 4519–4524. Properties of the medium/long chain carnitine acyltransferase purified from rat liver microsomes.

5. Broadway, N.M. & Saggerson, E.D. (1995) *Biochem. J.*, **310**, 989–995. Solubilization and separation of two distinct carnitine acyltransferases from hepatic microsomes: characterization of the malonyl-CoA-sensitive enzyme.
6. Murthy, M.S.R. & Pande, S.V (1994) *J. Biol Chem.*, **269**, 18283–18286. Malonyl-CoA-sensitive and -insensitive carnitine palmitoyltransferase activities of microsomes are due to different proteins.
7. Murthy, M.S.R. & Pande, S.V. (1993) *Mol. Cell. Biochem.*, **122**, 133–138. Carnitine medium/long chain acyltransferase of microsomes seems to be the previously cloned approximately 54kDa protein of unknown function.
8. Murthy, M.S.R. & Pande, S.V. (1994) *Biochem. J.*, **304**, 31–34. A stress-regulated protein, GRP58, a member of thioredoxin superfamily, is a carnitine palmitoyltransferase isoenzyme.
9. Bourdi, M., Demady, D., Martin, J.L., Jabbour, S.K., Martin, B.M., George, J.W. & Pohl, L.R. (1995) *Arch. Biochem. Biophys.*, **323**, 397–403. cDNA cloning and baculovirus expression of the human liver endoplasmic reticulum p58: characterization as a protein disulfide isomerase isoform, but not as a protease or a carnitine acyltransferase.
10. Broadway, N.M. & Saggerson, E.D. (1997) *Biochem. J.*, **322**, 435–440. Effect of membrane environment on the activity and inhibitability by malonyl-CoA of the carnitine acyltransferase of hepatic microsomal membranes.
11. Broadway, N.M., Pease, R.J. & Saggerson, E.D. (1998) *Biochem. J.*—submitted for publication. The liver isoform of carnitine palmitoyltransferase 1 inserts into endoplasmic reticulum membranes in a cell-free system.
12. McGarry, J.D. & Brown, N.F. (1997) *Em J. Biochem.*, **244**, 1–14. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis.
13. Fraser, F, Corstorphine, C.G. & Zammit, V.A. (1997) *Biochem. J.*, **323**, 711–718. Topology of carnitine palmitoyltransferase 1 in the mitochondrial outer membrane.
14. Indiveri, C., Tonazzi, A. & Palmieri, F. (1990) *Biochim. Biophys. Acta*, **1020**, 81–86. Identification and purification of the carnitine carrier from rat liver mitochondria.
15. Indiveri, C., Tonazzi, A., Prezioso, G. & Palmieri, F. (1991) *Biochim. Biophys. Acta*, **1065**, 231–238. Kinetic characterization of the reconstituted carnitine carrier from rat liver mitochondria.
16. Indiveri, C., Tonazzi, A. & Palmieri, F. (1994) *Biochim. Biophys. Acta*, **1189**, 65–73. The reconstituted carnitine carrier from rat liver mitochondria: evidence for a transport mechanism different from that of the other mitochondrial translocators.
17. Indiveri, C., Tonazzi, A., Giangregorio, N. & Palmieri, F. (1995) *Eur. J. Biochem.*, **228**, 271–278. Probing the active site of the reconstituted carnitine carrier from rat liver mitochondria with sulfhydryl reagents. A cysteine residue is localized in or near the substrate inding site.
18. Indiveri, C., Iacobazzi, V., Giangregorio, N. & Palmieri, F. (1997) *Biochem. J.*, **321**, 713–719. The mitochondrial carnitine carrier protein: cDNA cloning, primary structure and comparison with other mitochondrial transport proteins.
19. Polokoff, M.A. & Bell, R.M. (1978) *J. Biol. Chem.*, **235**, 7173–7178. Limited palmitoyl-CoA penetration into microsomal vesicles as evidenced by a highly latent ethanol acyltransferase activity.
20. Treloar, T., Madden, L.J., Winter, J.S., Smith, J.L. & de Jersey, J. (1996) *Biochim. Biophys. Acta*, **1299**, 160-166. Fatty acid ethyl ester synthesis by human liver microsomes.
21. Gooding, J.M. & Saggerson, E.D. (1998) *Biochem. J.*—submitted for publication. Uptake of carnitine and palmitoylcarnitine by rat liver microsomes.
22. Pfanner, N., Glick, B.S. & Rothman, J.E. (1987) *J. Cell Biol.*, **110**, 955–961. Fatty acylation promotes fusion of transport vesicles with Golgi cisternae.
23. Pfanner, N., Orci, L., Glick, B.S., Amherdt, M., Arden, S.R., Malhotra, V. & Rothman, J.E. (1989) *Cell*, **59**, 95–102. Fatty acyl-coenzyme A is required for budding of transport vesicles from Golgi cisternae.
24. Lawrence, J.B., Moreau, P., Cassagne, C. & Morre, D.J. (1994) *Biochim. Biophys. Acta*, **1210**, 146–150. Acyl transfer reactions associated with cis Golgi apparatus of rat liver.
25. Hoeg, J.M., Meng, M.S., Ronan, R., Demosky, S.J., Fairwell, T. & Brewer, H.B. (1988) *J. LipidRes.*, **29**, 1215–1220. Apolipoprotein B synthesized by Hep G2 cells undergoes fatty acid acylation.
26. Huang, G., Lee, D.M. & Singh, S. (1988) *Biochemistry*, **27**, 1395–1400. Identification of the thiol ester lipids in apolipoprotein B.
27. Kaufman, J.F., Krangel, M.S. & Strominger, J.L. (1984) *J Biol. Chem.*, **259**, 7230–7238. Cysteines in the transmembrane region of major histocompatibility complex antigens are fatty acylated via thioester bonds.
28. Magee, A.I. & Schlesinger, M.J. (1982) *Biochim. Biophys. Acta*, **649**, 279–289. Fatty acid acylation of eukaryotic cell membrane proteins.
29. Borén, J., Wettesten, M., Sjöberg, A., Thorlin, T., Bondjers, G., Wiklund, O. & Olofsson, S-O. (1990) *J.*

Biol. Chem., **265**, 10556–10564. The assembly and secretion of ApoB 100 containing lipoproteins in Hep G2 cells.

30. Spring, D.J., Chen-Liu, L.W., Chatterton, J.E., Elovson, J. and Schumaker, V.N. (1992) *J. Biol. Chem.*, **267**, 14839–14845. Lipoprotein assembly. Apolipoprotein B size determines lipoprotein core circumference.
31. Borén, J., Rustaeus, S. & Olofsson, S-O. (1994) *J. Biol. Chem.*, **269**, 25879–25888. Studies on the assembly of apoliporotein B-100- and B-48-containing very low density lipoproteins in McA-RH7777 cells.
32. Swift, L.L. (1995) *J. Lipid Res.*, **26**, 395–406. Assembly of very low density lipoproteins in rat liver: a study of nascent particles recovered from the rough endoplasmic reticulum.
33. Rustaeus, S., Lindberg, K., Borén, J. & Olofsson, S-O. (1995) *J. Biol. Chem.*, **270**, 28879–28886. Brefeldin A reversibly inhibits the assembly of ApoB containing lipoproteins in McA-RH7777 cells.
34. Cartwright, I.J. & Higgins, J.A. (1995) *Biochem. J.*, **310**, 897–907. Intracellular events in the assembly of very-low-density-lipoprotein lipids with apoliporotein B in isolated rabbit hepatocytes.
35. Gordon, D.A., Jamil, H., Gregg, R.E., Olofsson, S-O. & Borén, J. (1996) *J. Biol. Chem.*, **271**, 33047–33053. Inhibition of the microsomal triglyceride transfer protein blocks the first step of apolipoprotein B lipoprotein assembly but not the addition of bulk core lipids in the second step.
36. Cartwright, I.J., Higgins, J.A., Wilkinson, J., Bellavia, S., Kendrick, J.S. & Graham, J.M. (1997) *J. Lipid. Res.*, **38**, 531–545. Investigation of the role of lipids in the assembly of very low density lipoproteins in rabbit hepatocytes.
37. Gregg, R.E. & Wetterau, J.R. (1994) *Curr. Opin. Lipidol.*, **5**, 81–86. The molecular basis of abetalipoproteinaemia.
38. Leiper, J.M., Bayliss, J.D., Pease, R.J., Brett, D.J., Scott, J. & Shoulders, C.C. (1994) *J. Biol. Chem.*, **269**, 21951–21954. Microsomal triglyceride transfer protein, the abetolipoproteinemia gene product, mediates the secretion of apoliporotein B-containing lipoproteins from heterologous cells.
39. Gibbons, G.F., Bartlett, S.M., Sparks, C.E. & Sparks, J.D. (1992) *Biochem. J.*, **287**, 749–753. Extracellular fatty acids are not utilized directly for the synthesis of very-low-density lipoprotein in primary cultures of rat hepatocytes.
40. Gibbons, G.F. & Wiggins, D. (1994) *Adv. Enzyme Regul.*, **35**, 179–198. Intracellular triacylglycerol lipase: its role in the assembly of hepatic very-low-density lipoprotein (VLDL).
41. Gibbons, G.F. & Wiggins, D. (1992) *Biochem. J.*, **284**, 457–462. The lipolysis/esterification cycle of hepatic triacylglycerol. Its role in the secretion of ver-low-density lipoprotein and its response to hormones and sulphonylureas.
42. Yang, L-Y, Kuksis, A., Myher, J.J. & Steiner, G. (1995) *J. Lipid Res.*, **36**, 125–136. Origin of triacylglycerol moiety of plasma very low density lipoproteins in the rat: structural studies.
43. Owen, M.R., Corstorphine, C.C. & Zammit, V.A. (1997) *Biochem. J.*, **323**, 17–21. Overt and latent activities of diacylglycerol acyltransferase in rat liver microsomes: possible roles in very-low-density lipoprotein triacylglycerol secretion.
44. Allan, D., Thomas, P. & Michell, R.H. (1978) *Nature*, **276**, 289–290. Rapid transbilayer diffusion of 1,2-diacylglycerol and its relevance to control of membrane curvature.
45. Gibbons, G.F. (1990) *Biochem. J.*, **268**, 1–13. Assembly and secretion of hepatic very-low-density lipoprotein.
46. Duerden, J.M. & Gibbons, G.F. (1990) *Biochem. J.*, **272**, 583–587. Storage, mobilization and secretion of cytosolic triacylglycerol in hepatocyte cultures.
47. Bartlett, S.M. & Gibbons, G.F. (1988) *Biochem. J.*, **249**, 37–43. Short- and longer-term regulation of very-low-density lipoprotein secretion by insulin, dexamethasone and lipogenic substrates in cultured hepatocytes.
48. Sparks, J.D. & Sparks, C.E. (1994) *Biochim. Biophys. Acta*, **1215**, 9–32. Insulin regulation of triacylglycerol-rich lipoprotein synthesis and secretion.
49. Zammit, V.A. (1996) *Biochem. J.*, **314**, 1–14. Role of insulin in hepatic fatty acid partitioning: emerging concepts.
50. Broadway, N.M. & Saggerson, E.D. (1995) *FEBS Lett.*, **371**, 137–139. Inhibition of liver microsomal carnitine acyltransferases by sulphonylurea drugs.

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RECIPROCAL ENZYMATIC INTERFERENCE OF CARNITINE PALMITOYLTRANSFERASE I AND GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE IN PURIFIED LIVER MITOCHONDRIA

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ABSTRACT

(i) Highly purified mitochondrial fractions were practically devoid of microsomal contamination and of acyl-CoA ligase activity. (ii) In mitochondria, glycerol-3-phosphate acyltransferase (GPAT) activity was supported by two enzymes, the first being very active at low palmitoyl-CoA / albumin ratios and sensitive to external agents (external form), the second being detected only at higher palmitoyl-CoA / albumin ratios and insensitive to external agents (internal form). (iii) Carnitine palmitoyltransferase I (CPT I) activity was shown to inhibit external GPAT activity only. (iv) Glycerol-3-phosphate exerted an inhibitory effect on CPT I, even when GPAT was inactive. Reciprocal interaction of CPT I and GPAT was discussed with regard to the balance existing between fatty acid oxidation and esterification metabolic pathways.

1. INTRODUCTION

In 1973, McGarry *et al.*[1] inhibited carnitine acyltransferase with (+)-decanoylcarnitine in ketotic livers and showed that there was no fundamental defect in the esterification system. They concluded that inhibition of esterification in livers of fasted or

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diabetic rats should be related to the level of mitochondrial fatty acid oxidation, but no clear explanation was brought about to substantiate this idea. Carnitine palmitoyltransferase I (EC 2.3.1.21; CPT I) has been located in mitochondrial outer membranes,[2] while glycerol-3-phosphate acyltransferase (EC 2.3.1.15; GPAT) activity was detected both in mitochondrial and microsomal fractions.[3,4] The microsomal form was shown to be very sensitive to N-ethylmaleimide contrary to the mitochondrial enzyme.[5,6] Mitchell & Saggerson[7] suggested the existence in mitochondria of two pools of GPAT supported by outer and inner membranes. As CPT I and GPAT activities constitute key steps of fatty acid oxidation and esterification pathways, respectively, it was interesting to study the way they might interfere, particularly at the mitochondrial level. Besides, as the difference of acyl-CoA ligase (EC 6.2.1.3; ACL) activity in microsomal and mitochondrial fractions could affect differentially CPT I and GPAT activities, all using fatty acids activated by CoA as substrates, it was necessary to re-evaluate acyl-CoA ligase activity in the mentioned purified fractions. It was shown that (i) in liver cells, mitochondrial CPT I and GPAT are provided with acyl-CoAs by endoplasmic reticulum essentially (if not exclusively), (ii) the mitochondrial GPAT form should be of physiological significance in the balance of fatty acid fluxes towards pathways of oxidation and esterification and (iii) CPT I should exert a direct regulatory effect on mitochondrial outer GPAT.

2. MATERIALS AND METHODS

2.1. Reagents

[U-^{14}C]glycerol-3-phosphate was obtained from NEN Life Science Products (NEN, Paris, France) and L-[methyl-^{3}H]carnitine from Amersham Radiochemical Centre (Amersham, Bucks., U.K.). Percoll was from Pharmacia LKB Biotechnology (Uppsala, Sweden). Papain was from Boehringer (Meylan, France). All other biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Chemicals obtained from Prolabo (Paris, France) and Merck (Darmstadt, Germany) were of analytical grade.

2.2. Animals

Male rats of the Wistar strain were purchased from Dépré (Saint-Doulchard, France). Animals had free access to tap water and food containing 5% lipids (pellets AO3-type from U.A.R., Villemoisson/Orge, France). Rats used for preparing highly purified mitochondrial fractions were starved for 20 h before sacrifice, while rats used for GPAT measurements had constant access to food and drinking water.

2.3. Isolation of Subcellular Fractions

All the following operations were performed at 4°C. Livers were quickly removed and transferred into medium containing 0.25 M sucrose, 10mM Tris-HCl and 1 mM EGTA, pH 7.4. The tissue was minced and homogenized with 10 volumes of the above medium containing 1% fatty acid-free albumin, with three brief strokes of a loose-fitting Teflon pestle rotating at 300 rpm in a ice-cooled Potter-Elvehjem homogenizer.

2.3.1. Crude Mitochondrial Fraction (CMF). Part of the homogenate was spun down at 2,500 rpm (JA-20 rotor; J-21 Beckman centrifuge) for 10min. The supernatant was centrifuged at 13,000 rpm for 2 min and the mitochondrial pellet obtained was washed twice

after resuspension in a medium containig 0.3M sucrose, 10mM Tris-HCl and 1 mM EGTA, pH 7.4. The final pellet was suspended in 0.3 M sucrose.

2.3.2. Highly Percoll-purified Mitochondrial Fraction (HPMF). Last part of the homogenate was spun down at 4,000 rpm and, immediately after, at 2,500 rpm for 10min. The supernatant was added progressively with neutralized Percoll until 31% by volume of the final mixture. This mixture (20ml) was layered on to the top of 20ml of a medium containing 0.3 M sucrose, 10mM Tris-HCl and 1 mM EGTA, added with neutralized Percoll (31% by volume of the final medium). After centrifugation at 20,000 rpm for 20 min, the fluffy layer above a small cushion of Percoll at the bottom of the tube was withdrawn, diluted with 0.25 M sucrose and centrifuged at 3,000 rpm for 10min. The pellet was washed once more with 0.3 M sucrose, pH 7.4, and stored in the same medium.

2.3.3. Treatment of Mitochondria by Papain. Mitochondrial protein (10mg) from HPMF was exposed to various amounts of papain (0 to 300 μg) in a medium containing 0.3M sucrose, 10mM Tris-HCl and 1 mM EGTA, pH 7.4 (in a total volume: 1.8ml) for 15min in ice-cooled tubes. The treatment was stopped by the addition of 80 mg of fatty acid-free albumin in 0.4ml of 0.3 M sucrose, then by dilution with 30ml of a medium containing 0.25 M sucrose, 10mM Tris-HCl and 1 mM EGTA, pH 7.4, and centrifugation at 13,000 rpm for 5 min. The pellet was washed once more and was stored in 0.3 M sucrose.

2.3.4. Microsomal Fraction (MCS). The supernatant above the first mitochondrial pellet (see in CMF preparation) was centrifuged at 18,000 rpm for 20 min to precipitate the remaining mitochondria, peroxisomes and part of microsomes, as indicated by marker enzyme activities (data not shown). The supernatant was centrifuged at 40,000 rpm for 60 min (Ti-60 rotor; L8-55 Beckman centrifuge). The pellet was suspended with the medium containing 0.25M sucrose, 10mM Tris-HCl and 1 mM EGTA, pH 7.4, and was layered onto 4ml of 1.2M sucrose in stoppered 25 mi-tubes. The centrifugation as above of the preparation allowed to separate the microsomal pellet from the bottom translucent layer of glycogen. The pellet was stored in 0.25 M sucrose.

2.4. Biochemical Measurements

Activities of monoamine oxidase for mitochondria and aryl-ester hydrolase for microsomes were estimated as Weissbach *et al.*[8] and Beaufay *et al.*,[9] respectively. The assay used for acyl-CoA ligase activity was carried out as the procedure of Noy & Zakim,[10] in which the [^{14}C]palmitic acid substrate was bound to phosphatidylcholine. This very sensitive reaction which requires less than 20 μg protein, was stopped after 1 min. Glycerol-3-phosphate acyltransferase (GPAT) and carnitine palmitoyltransferase I (CPT I) activities were measured as Bremer[11] and Bates & Saggerson,[12] respectively. Palmitoyl-CoA (50 μM) bound to fatty acid-free albumin was the common substrate. The incubation medium for GPAT is added with 0.4 mM L-carnitine in some experiments (to trigger CPT I activity simultaneously), while the incubation medium for CPT I is added with 0.5 mM glycerol-3-phosphate in the last experiment (to trigger GPAT activity simultaneously). GPAT and CPT I assays were stopped after 7min.

Rapid protein estimations were performed by spectrophotometry[13] just before starting the incubations and were later corrected by the procedure using bicinchoninic acid.[14]

Table 1. Specific activity of marker enzymes in mitochondrial and microsomal fractions isolated from rat liver.

	Monoamine oxidase	Aryl-ester hydrolase
Crude mitochondrial fraction	8.73 ± 0.15	840 ± 22
Highly percoll-purified mitochondrial fraction	14.80 ± 0.10	92 ± 17
Microsomal fraction	0	4220 ± 65

Monoamine oxidase and aryl-ester hydrolase are marker enzymes of mitochondria (outer membranes) and microsomes, respectively. Values are expressed as nmol / min per mg protein and are means ± S.E.M (n *.004 4)

3. RESULTS

3.1. Purity of Mitochondrial Fractions and Acyl-CoA Ligase Activity

As shown in Table 1, crude mitochondrial fractions prepared by differential centrifugation (as other slightly different conventional methods) appeared to exhibit pronounced aryl-ester hydrolase (EC 3.1.1.2) activity. As this activity was found specifically in microsomes,[9] mitochondrial fractions prepared conventionally are always contaminated by microsomes. In mitochondrial fractions isolated on Percoll gradient under the conditions precisely described in the Materials and Methods section, the above microsomal activity was extremely low. The pattern was similar for acyl-CoA ligase (ACL), its specific activity was very high in microsomal fractions and almost negligible in Percoll-purified mitochondrial fractions (Fig. 1). Taking into account the specific activity of aryl-ester hydrolase in microsomal fractions, the amount of microsomal protein present in purified mitochondrial fractions could be estimated around 2% and was sufficient to account for the residual ACL activity in these fractions. It was, therefore, clear that a totally pure liver mitochondrial fraction could not esterify fatty acids with CoA.

3.2. Mitochondrial and Microsomal GPATs

Figure 2 presents glycerol-3-phosphate acyltransferase (GPAT) specific activities in purified mitochondrial and microsomal fractions for increasing palmitoyl-CoA / albumin ratios. For lowest ratios (lowest free palmitoyl-CoA concentrations), GPAT mitochondrial form was clearly more active than the microsomal form, with a notably lower apparent Km. The dramatic and specific inhibition of microsomal GPAT by N-ethylmaleimide

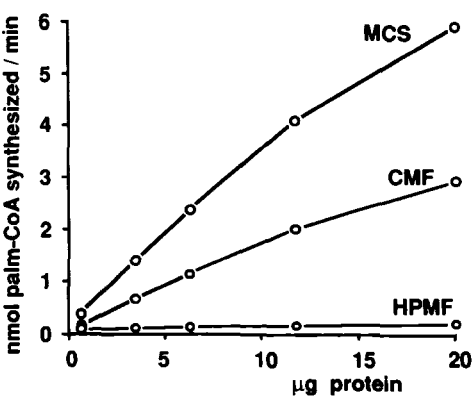

Figure 1. Acyl-CoA ligase activity in microsomal fractions (MCS), crude mitochondrial fractions (CMF) and highly purified mitochondrial fraction (HPMF). Values are expressed as nmol of palmitoyl-CoA (palm-CoA) synthesized/min for the indicated amounts of protein. Results which were obtained from subcellular fractions of the liver of one rat, are representative of fractions isolated from four rats.

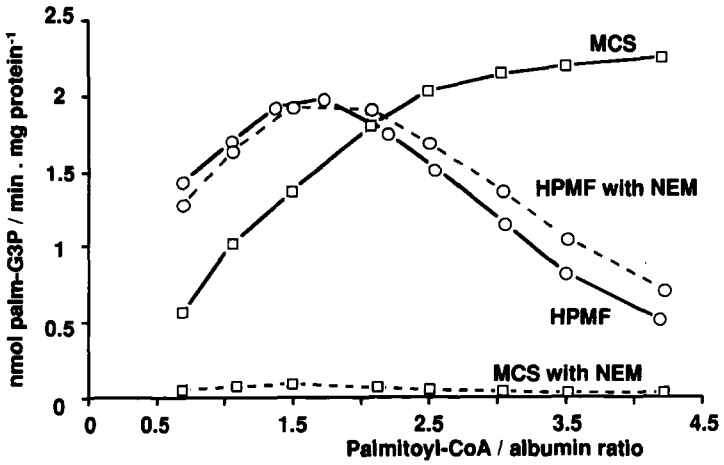

Figure 2. Glycerol-3-phosphate acyltransferase specific activity in mitochondrial and microsomal fractions as the palmitoyl-CoA / albumin ratio. Values are expressed as nmol of palmitoyl-glycerol-3-phosphate (palm-G3P) synthesized / min per mg of protein. (open circles) highly purified mitochondrial fraction (HPMF); (open squares) microsomal fraction (MCS); (–) without NEM; (—) with NEM. Results are representative of four independent experiments.

(NEM)[5,6] emphasized the absence of contamination of mitochondrial fractions with microsomal protein, while the inefficiency of NEM on mitochondrial GPAT confirmed the specificity of this enzyme in intact mitochondria and the purity of the mitochondrial fractions used. Another difference between microsomal and mitochondrial GPATs could be shown at highest palmitoyl-CoA/albumin ratios with the increasing activity of the former enzyme and the declining activity of the latter (Fig. 2). At these ratios,

palmitoyl-CoA should exhibit detergent properties, altering somehow GPAT activity in mitochondrial membranes. As the detergent sensitivity may be related to a particularly exposed location of the enzyme in membranes, mitochondria have also been previously treated with papain, to determine whether the detergent-sensitive GPAT form corresponded to a papain-suppressible form. Figure 3 shows that mitochondria exhibited, in addition, a papain-insensitive GPAT activity. This activity was of very weak amplitude at lowest palmitoyl-CoA / albumin ratios, but expressed maximum activity for about a ratio of 3. As papain suppressed the GPAT activity observed in untreated mitochondria at lowest ratios, it was possible to draw, by difference, part of the total mitochondrial GPAT activity depending on the papain-sensitive form (Fig. 3). It was then obvious that the low Km observed with total mitochondrial GPAT belonged in fact to the papain-sensitive form.

3.3. Carnitine Palmitoyltransferase I (CPT I) and GPAT Activity in Mitochondria

Addition of carnitine to the medium used for measuring GPAT activity in mitochondria initiates CPT I activity immediately. Figure 4 shows that total mitochondrial GPAT was lowered by carnitine addition all over the range of palmitoyl-CoA / albumin ratios assayed. No inhibitory effect was observed when L-carnitine was replaced by D-carnitine (data not shown) and the inhibition obtained with L-carnitine was almost com-

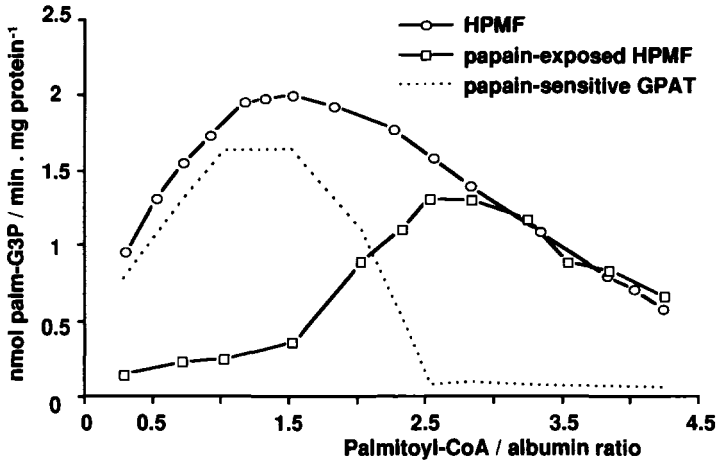

Figure 3. Glycerol-3-phosphate acyltransferase specific activity in papain-pretreated mitochondria. Values are expressed as nmol of palmitoyl-glycerol-3-phosphate (palm-G3P) synthesized/min per mg of protein. (open circles) highly purified mitochondrial fraction (HPMF); (open squares) HPMF pretreared with papain (20 µg/mg protein); (—) dotted line corresponds to the difference of activity between both curves at each palmitoyl-CoA / albumin ratio. Results are representative of four independent experiments.

pletely prevented by malonyl-CoA addition (Fig. 4). Data suggest that inhibition of GPAT activity was related to CPT I activity and not due to L-carnitine by itself. This effect may reach more than 50% for lowest palmitoyl-CoA / albumin ratios, considered to be the closest of the actual cellular values. Figure 5 shows the effect of carnitine on papain-sensitive GPAT (ratio 1.2) using mitochondria previously treated with increasing amounts of papain. This treatment appeared to reduce GPAT activity almost regularly as the proteolysis strength, while the activity of CPT I (located on the external side of the outer membrane[2]) was hardly affected. The inhibition by L-carnitine was strictly pro-

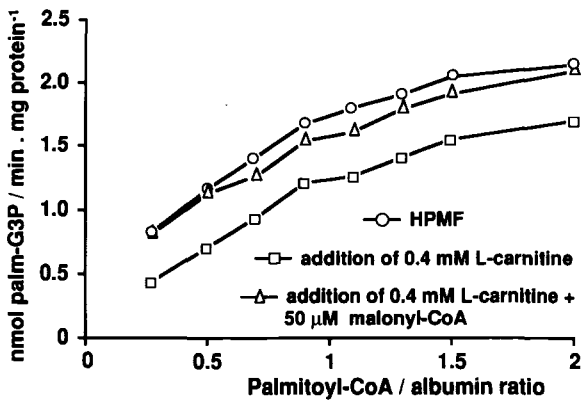

Figure 4. Effect of L-carnitine addition on glycerol-3-phosphate acyltransferase specific activity in highly purified mitochondrial fractions (HPMF). Values are expressed as nmol of palmitoyl-glycerol-3-phosphate (palm-G3P) synthesized/min per mg of protein. (open circles) HPMF alone; (open squares) addition of 0.4 mM L-carnitine or (open triangles) 0.4 mM L-carnitine + 50 µM malonyl-CoA. Results are representative of four independent experiments.

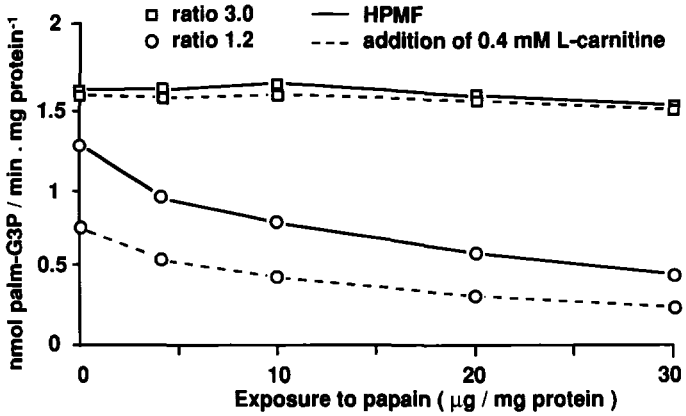

Figure 5. Effect of L-carnitine addition on glycerol-3-phosphate acyltransferase specific activity in highly purified mitochondrial fractions (HPMF) pretreated with various amounts of papain. Values are expressed as nmol of palmitoyl-glycerol-3-phosphate (palm-G3P) synthesized/min per mg of protein. (– solid line) HPMF alone; (— dotted line) addition of 0.4 mM L-carnitine; values of palmitoyl-CoA / albumin ratio: (open circles) 1.2; (open squares) 3.0. Results are representative of four independent experiments.

portional to the initial GPAT activity for all strengths of treatment, suggesting that each CPT I protein reacts negatively (directly or indirectly) on a neighbouring GPAT protein. Therefore, this GPAT form and CPT I should be located together in mitochondrial outer membranes.

When GPAT activity is studied at a relatively high palmitoyl-CoA/albumin ratio (ratio 3), pretreatment of mitochondria with various amounts of papain was almost without effect on this activity (Fig. 5), confirming the specific insensitivity of this GPAT form to papain (see Fig. 3). Under the same conditions, addition of L-carnitine did not inhibit GPAT at all, despite CPT I activity was elevated at this ratio (see Fig. 6). These data suggest that this GPAT, which appeared out of reach of papain, might be also clearly distant from CPT I protein in membranes, suggesting a location of this GPAT far from CPT I (already known to be located in outer membranes[2]), hence likely in inner membranes.

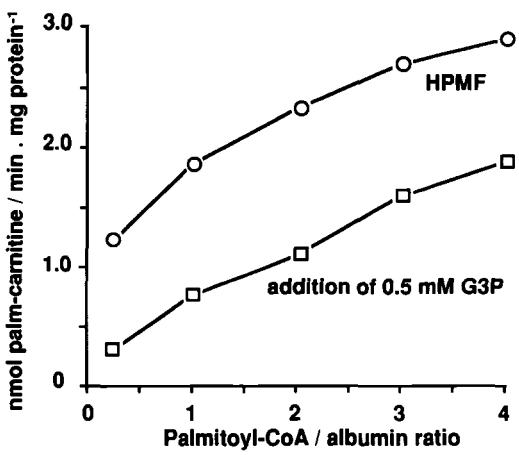

Figure 6. Effect of glycerol-3-phosphate (G3P) addition on carnitine palmitoyltransferase I specific activity in highly purified mitochondrial fractions (HPMF) as the palmitoyl-CoA/albumin ratios assayed. Values are expressed as nmol of palmitoyl-carnitine (palm-carnitine) synthesized / min per mg of protein. (open circles) HPMF, (open squares) addition of 0.5 mM G3P. Results are representative of four independent experiments.

CPT I activity appeared to be reciprocally lowered when glycerol-3-phosphate was added to the medium all over the range of palmitoyl-CoA / albumin ratios assayed (Fig. 6). This addition should initiate papain-sensitive and insensitive GPATs as the ratios applied, but the mode of inhibition of CPT I seemed to be independent of GPAT activity, since this inhibition still occured for very low GPAT activities (highest ratios, around 4.5; see Fig. 2).

4. DISCUSSION

4.1. Acyl-CoA Ligase Activity

The first interest of this work is to have shown that mitochondria isolated practically without contact with microsomes were almost devoid of acyl-CoA ligase activity. Mitochondrial fractions prepared conventionally by differential centrifugation have been shown to contain a acyl-CoA ligase activity as high as in microsomal fractions.[15] These conventional mitochondrial fractions were containing 10–20% microsomes, on a protein basis, but the microsomal contamination could not be lowered below 5% by further purification on a Percoll gradient.[16] The procedure described in this study avoided contacts which occured when mitochondria and microsomes were pelletet together and triggered almost unbreakable bonds. However, tight contacts between mitochondria and endoplasmic reticulum occur naturally[17,18] and could explain the impossibility to decrease below 2% the microsomal contamination in mitochondrial fractions. The natural and/or artificial presence of microsomal protein in mitochondrial fractions accounted for the immunochemical detection of acyl-CoA ligase in these mitochondrial fractions.[19] The practical interest of our finding is that mitochondrial reactions using acyl-CoAs as substrates are equally supplied from previous endoplasmic reticulum reactions and that activity of acyl-CoA ligase cannot interfere with other mitochondrial enzyme activities, being not located in the same membrane.

4.2. Mitochondrial GPAT Location

The second interest of the study is to have re-evaluated the mitochondrial location of GPATs by a way that differs from those used by Mitchell & Saggerson.[7] Indeed, drastic treatments to separate outer from inner membranes do not result in really pure membranes and are likely to alter enzyme activities (see also in[20]). However, the results obtained by Mitchell & Saggerson[7] are sufficiently clear to complete ours obtained with whole organelles. Our data confirm that mitochondria possess two GPATs, the first form directly sensitive to papain and palmitoyl-CoA, taken as a detergent, and being likely located within the outer membrane, the second one less accessible to external treatments, also less active than the former GPAT and being possibly anchored within the inner membrane. The respective location of either GPATs is supported additionally by the differential effect of CPT I activity. CPT I has been located within outer membranes[2] and its catalytic site is oriented on the outer side.[21] Its activity appeared to modify the activity of the GPAT form the most sensitive to external treatments. It was suggested that CPT I and the external GPAT should coexist within the same membrane, as CPT I activity interfered only with external GPAT activity and not with the GPAT activity of inner membranes (devoid of CPT I activity).

4.3. Reciprocal Interference of CPT I and GPAT

In preliminary studies (unpublished data), we defined that a correct incubation medium intended for mitochondrial fatty acid oxidation should present a acyl-CoA/albumin ratio lower than 1.5. Indeed, the radioactivity of acid-soluble products released during mitochondrial oxidation of [1-^{14}C] or [10-^{14}C]oleoyl-CoA was comparable for all ratios assayed below 1.5. Higher ratios gave rise to less labelled acid-soluble products from [10-^{14}C]oleoyl-CoA, because β-oxidation was interrupted likely after membrane permeabilization with higher free oleoyl-CoA concentrations (see in[22]). Hence, mitochondrial outer GPAT should be of physiological significance, because of its activity at a low ratio, but also because this activity can be modulated efficiently by CPT I activity. The inhibitory effect of CPT I activity, which can be relieved by malonyl-CoA (or D-carnitine used instead of L-form), did not result in definitive alteration of GPAT activity and was possibly due to effects of CPT I activity on the membrane environment of the close GPAT. This situation resembles that obtained with perfused liver, in which triglyceride formation was low in fasted rats, but was as high as in fed rats after inhibition of carnitine acyltransferase step by (+)-decanoylcarnitine.[1] The inhibition of glycerol-3-phosphate on CPT I activity appeared to be due to a different mechanism, as it occured even for ratios suppressing practically all GPAT activity. This point deserves further studies at the level of outer membranes for CPT I and inner membranes for CPT II.

The presented data illustrate some aspects of reciprocal relationships existing between two enzymes next to each other within the same membrane and belonging to opposite metabolic pathways.

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REFERENCES

1. McGarry, J.D., Meier, J.M. & Foster, D.W. (1973) *J. Biol. Chem.*, **248**, 270–278. The effect of starvation and refeeding on carbohydrate and lipid metabolism in vivo and in the perfused rat liver.
2. Murthy, M.S.R. & Pande, S.V. (1987) *Proc. Natl. Acad. Sci.* USA, **84**, 378–382. Malonyl-CoA binding site and the overt carnitine palmitoyltransferase activity reside on the opposite sides of the outer mitochondrial membrane.
3. Stoffel, W. & Schiefer, H.G. (1968) *Hoppe-Seylers Z. Physiol. Chem.,* **349**, 1017–1026. Biosynthesis and composition of phosphatides in outer and inner mitochondrial membranes.
4. Daae, L.N.W. & Bremer, J. (1976) *Biochim. Biophys. Acta,* **210**, 92–104. The acylation of glycerophosphate in rat liver. A new assay procedure for glycerophosphate acylation, studies on its subcellular and submitochondrial localization and determination of the reaction products.
5. Lands, W.E.M. & Hart, P. (1965) *J. Biol. Chem.,* **240**, 1905–1911. Metabolism of glycerolipids. VI, specificities of acyl coenzyme A:phospholipid acyltransferases.
6. Bates, E.J. & Saggerson, E.D. (1979) *Biochem. J.*, **182**, 751–762. A study of the glycerolphosphate acyltransferase and dihydroxyacetonephosphate acyltransferase activities in rat liver mitochondrial and microsomal fractions. Relative distribution in parenchymal and non-parenchymal cells, effects of N-ethylmaleimide, palmitoyl-CoA concentration, starvation, adrenalectomy and anti-insulin serum treatment.

7. Mitchell, J.R.D. & Saggerson, E.D. (1994) *Int. J. Biochem.,* **26**, 181–187. A study of glycerolphosphate acyltransferase in rat liver mitochondria-submitochondrial localization and some properties of the solubilized enzyme.
8. Weissbach, H., Smith, T.E., Daly, J.W., Witkop, B. & Udenfriend, S. (1960) *J. Biol. Chem.,* **235**, 1160–1163. A rapid spectrophometric assay of monoamine oxydase based on the rate of disappearance of kynuramine.
9. Beaufay, H., Amar-Costesec, A., Feytmans, E., Thines-Sempoux, D., Wibo, M., Robbi, M. & Berthet, J. (1974) *J. Cell. Biol.,* **61**, 188–200. Analytical study of microsomes and isolated subcellular membranes from rat liver. I. Biochemical methods.
10. Noy, N. & Zakim, D. (1985) *Biochim. Biophys. Acta,* **833**, 239–244. Substrate specificity of fatty-acyl-CoA ligase in liver microsomes.
11. Bremer, J. (1981) *Biochim. Biophys. Acta*, **665**, 628–631. The effect of fasting on the activity of liver carnitine palmitoyltransferase and its inhibition by malonyl-CoA.
12. Bates, E.J. & Saggerson, D. (1977) *FEBS Lett.,* **84**, 229–232. A selective decrease in mitochondrial glycerolphosphate acyltransferase activity in livers from streptozotocin-diabetic rats.
13. Clouet, P., Niot, I. & Bézard, J. (1989) *Biochem. J.,* **263**, 867–873. Pathway of a-linolenic acid through the mitochondrial outer membrane in the rat liver and influence on the rate of oxidation. Comparison with linoleic and oleic acids.
14. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. & Klenk, D.C. (1985) *Anal. Biochem.*, **150**, 76–85. Measurement of protein using bicinchoninic acid.
15. De Jong, J.W. & Hülsmann, W.C. (1970) *Biochim. Biophys. Acta,* **197**, 127–135. A comparative study of palmitoyl-CoA synthetase activity in rat-liver, heart and gut mitochondrial and microsomal preparations.
16. Niot, I., Pacot, F., Bouchard, P., Gresti, J., Bernard, A., Bézard, J. & Clouet, P. (1994) *Biochem. J.,* **304**, 577–584. Involvement of microsomal vesicles in part of the sensitivity of carnitine palmitoyltransferase I to malonyl-CoA inhibition in mitochondrial fractions of rat liver.
17. Shore, G.C. & Tata, J.R. (1977) *J. Cell. Biol.,* **72**, 714–725. Two fractions of rough endoplasmic reticulum from rat liver. Recovery of rapidly sedimenting endoplasmic reticulum in association with mitochondria.
18. Meier, P.J., Spycher, M.A. & Meyer, U.A. (1981) *Biochim. Biophys. Acta,* **646**, 283–297. Isolation and characterization of rough endoplasmic reticulum associated with mitochondria from normal rat liver.
19. Miyazawa, S., Hashimoto, T. & Yokota, S. (1985) *J. Biochem.,* **98**, 723–733. Identity of long-chain acyl-coenzyme A synthetase of microsomes, mitochondria, and peroxisomes in rat liver.
20. Pande, S.V. & Murthy, M.S.R. (1990) *Biochim. Biophys. Acta,* **1044**, 262–268. Freeze-thawing causes masking of membrane-bound outer carnitine palmitoyltransferase activity: implications for studies on carnitine palmitoyltransferases deficiency.
21. Fraser, F., Corstorphine, C.G. & Zammit, V.A. (1996) *Biochem. Soc. Trans.,* **24**, 184S. Evidence that both the acyl-CoA and malonyl-CoA binding sites of mitochondrial overt carnitine palmitoyltransferase (CPT I) are exposed on the cytosolic face of the outer membrane.
22. Lehninger, A.L. & Remmert, L.F. (1959) *J. Biol. Chem.,* **234**, 2465–2471. An endogenous uncoupling and swelling agent in liver mitochondria and its enzymic formation.

CHARACTERIZATION OF A RESPONSE ELEMENT FOR PEROXISOMAL PROLIFERATOR ACTIVATED RECEPTOR (PPRE) IN HUMAN MUSCLE-TYPE CARNITINE PALMITOYLTRANSFERASE I

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1. INTRODUCTION

Metabolic control of gene transcription in bacteria or yeast is a well-documented issue since the pioneer studies of gene expression. Recent observations indicate that transcriptional control of gene expression by metabolites can also be functional in mammals.[1–4] The identification of mammalian nuclear receptors for fatty acids and eicosanoids (PPAR) is of relevance because it establishes a link between the metabolism of fatty acids and transcriptional control, suggesting a molecular mechanism by which dietary fatty acids can modulate lipid homeostasis. The fact that fatty acids are ligands of PPAR,[5,6] a nuclear receptor that induces gene transcription of a suit of genes involved in lipid metabolism, indicates that fatty acids and related molecules can regulate the metabolism by short- (allostreric control) and long-term (transcriptional control) mechanisms.

We have identified the mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase gene as a PPAR target, and we have found that this receptor, which mediates the induction of the gene by fatty acids, binds as a PPAR-RXR heterodimer to a

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response element for peroxisomal proliferator activated receptor (PPRE) in the promoter region of the rat and pig genes.[7,8] Rat mitochondrial HMG-CoA synthase PPRE is in fact a nuclear receptor-responsive element (NRRE) since, in addition to **RXRα-PPAR** heterodimer, other nuclear receptors, like chicken ovalbumin upstream-promoter transcription factor (COUP-TF) or hepatocyte nuclear factor 4 (HNF-4) it bind to the element.[9,10]

Other genes involved in mitochondrial lipid metabolism, such as acyl-CoA synthetase[11] or medium-chain acyl-CoA dehydrogenase (MCAD),[12] are also targets of PPAR, indicating that different steps of fatty acid metabolism, like activation, oxidation, and utilization of fatty acids are regulated by the levels of the substrate, the fatty acids. Therefore, we speculated that the main control step in fatty acid **β-**oxidation, the outer membrane component of carnitine palmitoyltransferase enzyme system, CPT I, could also be a PPAR target.

Two isoforms of CPT I have been described, which have been designated LCPT I and MCPT I, since these isoforms are mainly expressed in liver and muscle respectively. The MCPT I gene is expressed not only in skeletal muscle but also in heart and brown and white adipose tissue.[13] This expression pattern may be of great significance since fatty acids are a major source of energy for heart, skeletal muscle and brown adipose tissue. We have amplified by polymerase chain reaction (PCR) the 5′ region of the human heart and brown adipose tissue CPT I gene[14] and demonstrated, first, the transcriptional activity of this fragment and second, the presence of a PPRE in the 5′-flanking region of this gene. In CV1 cells, the activation of the CPT I gene by PPAR was dependent on the addition of exogenous ligands.

2. RESULTS AND DISCUSSION

2.1. Fatty Acids Regulate the Mitochondrial Synthase Gene Expression Mediated by PPAR: Localization of the PPRE

There is considerable evidence that fatty acids can activate PPAR as potently as peroxisome proliferators do.[15–17] On the other hand, previous results in our laboratory revealed that fatty acids induce an increase in the mitochondrial HMG-CoA synthase mRNA levels[18] and gene transcription.[19] We were therefore interested in determining whether fatty acids could have a role in the regulation of the mitochondrial HMG-CoA synthase gene expression mediated by PPAR. Figure 1 shows that the highest stimulation of the mitochondrial synthase-CAT reporter plasmid was observed after the induction with fatty acids in the presence of PPAR. In agreement with previous results[16,17] we observed that monounsaturated oleic acid is less potent than linoleic acid in the activation of PPAR.

Experiments of 5′nucleotides deletion, electrophoretic mobility shift assays (EMSA) and site-directed mutagenesis localized a PPRE in the mitochondrial HMG-CoA synthase promoter (between positions –104 and –85),[7] which is composed, like other PPREs,[20] of an imperfect direct repeat of the consensus binding sequence for the nuclear receptor superfamily with a spacing of a single base pair (DR-1), and a conserved sequence flanking the second repeat of DR-1. Figure 2 A shows the localization of putatives PPREs in the mitochondial HMG-CoA synthase gene of three different species.

Besides the characterization of mitochondrial HMG-CoA synthase as a PPAR

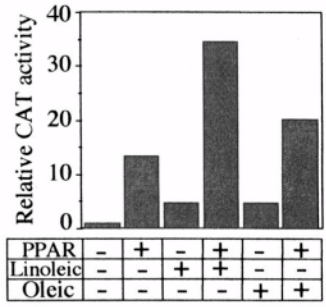

Figure 1. *Transcriptional activation of the rat mitochondrial HMG-CoA synthase gene promoter by fatty acids.* HeLa cells were cotransfected with 100 ng of the expression vector for mPPARα and reported plasmid pSMPCAT1 (−1148 to +28) in the presence or absence of 150 mM linoleic acid or 250 mM oleic acid as indicated at the bottom of the figure. Average value of β-galactosidase-normalized CAT activity are expressed as relative CAT activity, with the activity in the absence of both PPAR and fatty acids defined as 1.

target gene we had two more objectives: i) to identify other nuclear receptors able to bind to the mitochondrial HMG-CoA synthase PPRE, and ii) to extend our previous observation on mitochondrial HMG-CoA synthase gene to other genes related with fatty acids metabolism.

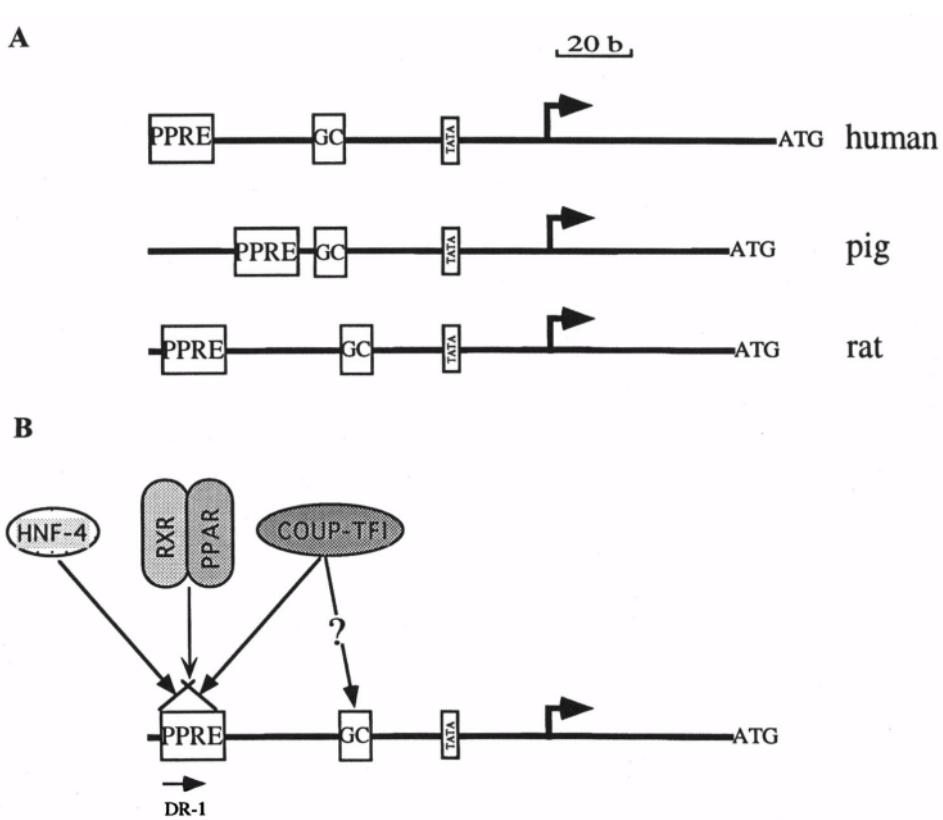

Figure 2. *Up-stream elements (UPEs) organization of human, pig and rat mitochondrial HMG-CoA synthase genes.* (A) The transcription start site, indicated by arrows, was determined by 5-RACE for the human[25] and pig[26] genes, and by primer extension and S1 experiments for the rat [27] gene. The functionality of PPRE was showed experimentally for the rat[7] and pig gene.[8] Human PPRE showed 17/19 identity with rat PPRE.[25] The scale at the top is in nucleotides. (B) Rat mitochondrial HMG-CoA synthase PPRE is a NRRE. Proposed model for the action of COUP-TF I[9] and HNF-4[10] on the rat gene.

2.2. COUP-TF I and HNF-4 Binds to a Nuclear Receptor-Responsive Element (NRRE) in the Mitochondrial HMG-CoA Synthase Gene

Rat PPRE of mitochondrial HMG-CoA synthase is a nuclear receptor responsive element (NRRE). We have shown (see Fig. 2 B) that despite the binding of PPAR/RXR heterodimers to the PPRE, which transactivates the promoter activity, COUP-TF can bind as a homodimer to rat PPRE, competing with the PPAR-RXR binding and therefore abolishing the PPAR transactivation.[9] We also showed that COUP-TF can form non-functional heterodimers with PPAR or RXR yielding the same effect: the lack of transactivation in the presence of PPAR.[9] HNF-4 is another nuclear receptor that can bind to rat PPRE. This binding does not require the complete element, only the DR-1 of the element, and it produces a similar effect in transcription activity to COUP-TF.[10] Besides the binding to a PPRE just described, COUP-TF can trans-activate promoter constructs that carry only the GC and TATA box. Moreover, the effect of COUP-TF on this small piece of DNA is tissue-[9] and species-specific (Judith Mallolas, and Pedro F. Marrero, unpublished). These results suggest that the mitochondrial HMG-CoA synthase gene is subjet to different regulation by the interplay of multiple members of the nuclear hormone receptor superfamily.

2.3. Human MCPT I gene 5′ Flanking Region Contains a Consensus PPRE and It is Activated by PPAR

In addition to HMG-CoA synthase, involved in ketogenesis, genes involved in fatty acid activation, like acyl-CoA synthetase,[11] or in β-oxidation, like medium-chain acyl-CoA dehydrogenase[12] are also target of PPAR. This transactivation pathway is reminiscent of a prokaryotic operon organization, in which fatty acids induce the expression of the genes responsible for their metabolism. We speculated that the main control step in fatty acid β-oxidation, the outer membrane component of carnitine palmitoyltransferase enzyme system, CPT I, could also be a PPAR target.

The 5′ flanking regions of CPT genes is now known.[14,21–23] Human Muscle type CPT I had been cloned,[14] and the complete sequence of the human gene from a BAC clone containing the end of the q arm of chromosome 22 is in GenBankTM (U62317). The analysis of the 5′-flanking region of this gene by TFSEARCH routine, performed using the Kyoto center's GenomeNet WWW Server, shows the presence of a putative PPAR binding sequence upstream of exon 1A (see Fig. 3A). The comparison of this sequence with the consensus sequence required for the binding of the PPAR-RXR heterodimer, as proposed by Palmer *et al.*,[20] shows the coincidence of 17 out of 20 bases (see Fig. 3B).

We performed gel mobility shift assays to analyze whether PPAR-RXR heterodimers bind to the putative PPAR binding sequence of the human muscle type CPT I gene. As can be seen in Fig. 4A neither PPARs nor RXR alone binds significantly to this sequence. However, incubation of this probe with a mixture of PPAR (α, β or γ) and RXRα resulted in a prominent complex. An oligonucleotide containing a mutated PPRE was not able to compete with the wild type probe for the formation of the complex (see Fig. 4B). The binding of the three subtypes of PPAR to the MCPT I PPRE is as strong as the binding to the mitochondrial HMG-CoA synthase PPRE, which allows the formation of the strongest complexes for all PPAR subtypes[24] (data not shown).

To investigate the effect of the observed binding of PPAR to the human MCPT I gene promoter on its transcriptional activity, we made constructs in which

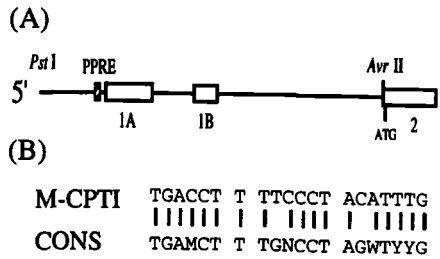

Figure 3. *Scheme of the 5'-flanking region of the human MCPT I.* (A) The positions of exons 1A, 1B and 2 containing the translation initiation codon (open rectangles) and the presence of the PPRE (dashed box) are indicated. (B) Comparison of the sequence of the proposed PPRE with the consensus. (C) Electrophoretic mobility shift assay of the muscle CPT I PPRE with PPAR-RXR heterodimers. PPAR α, γ and δ and RXRa were translated in vitro, incubated with the proposed CPT I PPRE labelled probe, and analyzed by electrophoretic mobility shift assay. Additions were as indicated on the top of the figure.

the 5′ flanking region of this gene was linked to a promoter-less bacterial chloramphenicol acetyltrasferase (CAT) gene. These plasmids, pMCPT I-CAT, were introduced into cultured CV1 cells by the calcium phosphate method, with or without an expression vector for PPARs, together with a plasmid that contains the β-galactosidase coding region driven by the SV40 promoter as a control of the efficiency of the transfection. Following transfection, cells were incubated in the presence or absence of a

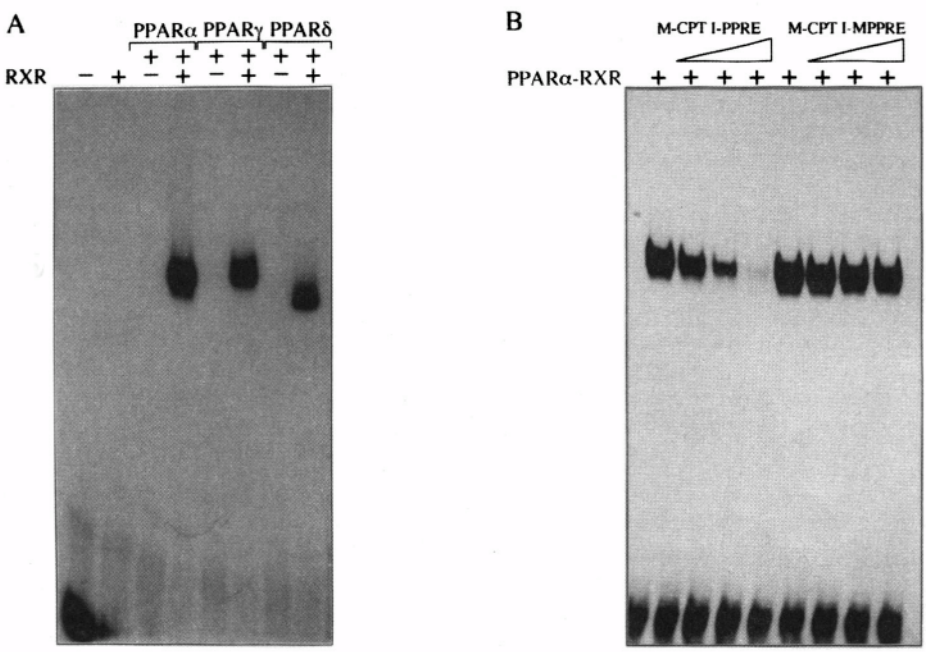

Figure 4. *PPAR-RXR heterodimers bind to human MCPT I promoter.* (A) PPAR α, γ and δ and RXRα were translated in vitro, incubated with the proposed CPT I PPRE labeled probe, and analyzed by electrophoretic mobility shift assay. Additions were as indicated on the top of the figure. Shown in (B) is a competition of the complex PPARα-RXR-PPRE with a 25 to 100-fold molar excess of two different unlabeled oligonucleotides: MCPT I PPRE, containing the proposed PPRE or MCPT I MPPRE with the proposed PPRE mutated.

PPAR activator and after 48 h the cells were harvested and CAT activity was measured.

As can be seen in Table I cotransfection of MCPT I-CAT and PPARs expression vectors led to a marked increase in CAT activity in the presence of the PPAR activators. Surprisingly, even though PPARδ is able to bind the MCPT I PPRE in vitro it does not activate the expression of the chimeric gene even in the presence of linoleic acid as activator.

2.4. Human MCPT I PPRE Confer PPAR Sensitivity in Its Natural Contexts and PPAR Responsiveness to Normally Unresponsive Promoter

The functionality of human MCPT I PPRE was studied in two ways. First, a pair of oligonucleotides containing the human MCPT I PPRE were inserted into pBLCAT2, a plasmid containing the CAT gene under the control of the thymidine kinase gene promoter, generating pTKCAT-MCPT I. As can be seen in Table I this sequence conferred PPAR responsiveness to the otherwise unresponsive thymidine kinase gene promoter. Second, scrambling of the DR-1 (pMCPTI-CAT-M), by site directed mutagenesis obliterates the response to PPAR (see Table I) of human MCPT I promoter. The results demonstrate that this human MCPT I element is able to confer PPARα and γ responsiveness both on its natural context and on a normally unresponsive promoter.

2.5. PPAR Could Mediate Fatty Acid Regulation of Lipid-Metabolism Related Genes in Hepatic and Extra-Hepatic Tissues

Our data indicate that mitochondrial HMG-CoA synthase and muscle CPT I isotype genes are both targets of PPAR. Other genes involved in mitochondrial lipid metabolism, such as acyl-CoA synthetase[11] or medium-chain acyl-CoA dehydrogenase (MCAD),[12] are also target of PPAR. Therefore PPAR is a pivotal factor for lipid home-

Table 1. *PPARα and γ-dependent activation of the human MCPT I.* pMCPT I-CAT (−909 to +126,[14]), pMCPT I-CAT-M (−909 to +126, containing a 5′-flanking mutated-region by changing nucleotides −769 to −758,[14] or pTKCAT-MCPT I containing the CAT gene under the control of TK gene promoter and a fragment from the 5′ region of the MCPT I containing the proposed PPRE (−782 to −748,[14]), were cotransfected with expression vectors for PPARα, γ and δ into CV1 cells in the presence of LY (30μM), PGJ2 (10μM) or linoleic acid (30μM) respectively as activators of the different isoforms of PPAR. Values of β-galactosidase-normalized CAT activity are expressed relative to the activity in the absence of both PPARs and activators.

	Co-Transfection			
Transfection	none	PPARα + LY	PPARδ + C18:2	PPARγ + PGJ2
MCPT I-CAT	1	4,10	1,17	3,80
MCPT I-CAT-M	1	0,65	0,90	0,86
TKCAT-MCPT I	1	5,1	1,11	4,40

The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; CAT, chloramphenicol acetyltransferase; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-responsive element; NRRE, nuclear receptor responsive element; RXR, retinoid X receptor; hRXRa, human 9-cis-retinoic acid receptor α; mPPARα, mouse peroxisome proliferator-activated receptor a; COUP-TF, chicken ovalbumin upstream-promoter transcription factor; HNF-4, hepatocyte nuclear factor 4; EMSA, electrophoretic mobility shift analysis; tk, thymidine kinase; NEFA, nonesterified fatty acids

ostasis control in which free fatty acids, or their derivatives could activate their own metabolism by regulating gene expression. In this model, a cis-acting element, the PPRE, is present in house-keeping and tissue specific genes, indicating that fatty acid control of gene expression could be active in hepatic and extra-hepatic tissues.

BIBLIOGRAPHY

1. Forman, B.M., Goode, E., Chen, J., Oro, A.E., Bradley, D.J., Perlmann, T, Noonan, D.J., Burka, L.T., McMorris, T., Lamph, W.W., Evans, R.M. & Weinberg, C. (1995) Cell. 81, 687–693.
2. O'Malley, B.W. (1989) Endocrinology. 125, 1119–1120.
3. Green, S. & Wahli, W. (1994) Mol. Cell. Endocrinol. 100, 149–153.
4. Wang, X., Sato, R., Brown, M.S., Hua, X. & Goldstein, J.L. (1994) Cell. 77, 53–62.
5. Forman, B.M., Chen, J. & Evans, R.M. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 4312–4317.
6. Kliewer, S.A., Sundseth, S.S., Jones, S.A., Brown, P.J., Wisely, G.B., Koble, C.S., Devchand, P., Wahli, W, Willson, T.M., Lenhard, J.M. & Lehmann, J.M. (1997) Proc. Natl. Acad. Sci. U.S.A. 94,4318–4323.
7. Rodríguez, J.C., Gil-Gómez, G., Hegardt, F.G. & Haro, D. (1994) J. Biol. Chem. 269, 18767–18772.
8. Ortiz, J.A., Mallolas, J., Nicot, C., Bofarull, J., Rodíguez, J.C., Hegardt, F.G., Haro, D., and Marrero, P.F. (1998) Biochem. J. Submitted.
9. Rodríguez, J.C., Ortiz, J.A., Hegardt, F.G. & Haro, D. (1997) Biochem. J. 326, 587–592.
10. Rodríguez, J.C., Ortiz, J.A., Hegardt, F.G. & Haro, D. (1998) Biochem. Biophys. Res. Commun. 242, 692–696.
11. Schoonjans, K., Watanabe, M., Suzuki, H., Mahfoudi, A., Krey, G, Wahli, W, Grimaldi, P, Staels, B. Yamamoto, T. & Auwerx, J. (1995) J. Biol. Chem. 270, 19269–19276.
12. Gulick, T., Cresci, S., Caira, T., Moore, D.D. & Kelly, D.P. (1994) Pro. Natl. Acad. Sci. U.S.A. 91, 11012-11016.
13. McGarry, J.D. & Brown, N.F. (1997) J. Biochem. 244, 1–14.
14. Yamazaki, N.Y., Y. Hashimoto, Y. Shinohara, Y. Shima, A. & Terada, H. (1997) FEBS-Lett. 409, 401–106.
15. Issemann, I., Prince, R., Tugwood, J. & Green, S. (1992) Biochem. Soc.Trans. 20, 824–827.
16. Keller, H., Dreyer, C., Medin, J., Mahfoudi, A., Ozato, K. & Wahli, W. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 2160–2164.
17. Gottlicher, M., Widmark, E., Li, Q. & Gustafsson, J.A. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 4653–4657.
18. Casals, N, Roca, N., Guerrero, M., Gil-Gómez, G., Ayté, J., Ciudad, C.J. & Hegardt, F.G. (1992) Biochem. J. 283, 261–264.
19. Gil-Gómez, G., Ayté, J. & Hegardt, F.G. (1993) Eur. J. Biochem. 213, 773–779.
20. Palmer, C.N., Hsu, M.H., Griffin, K.J. & Johnson, E.F. (1995) J. Biol. Chem. 270, 16114–16121.
21. Montermini, L., Wang, H., Verderio, E., Taroni, F., DiDonato, S. & Finocchiaro, G. (1994) Biochim. Biophys. Acta. 1219, 237–240.
22. Park, E.A., Steflen, M.L., Song, S.L., Park, V.M. & Cook, G.A. (1998) Biochem. J. 330, 217–224.
23. Gelb, B.D. (1993) Genomics 18, 651–655.
24. Juge-Aubry, C., Pernin, A., Favez, T., Burger, A.G., Wahli, W., Meier, C.A. & Desvergne, B. (1997) J-Biol-Chem. 272, 25252–25259.
25. Boukaftane, Y. & Mitchell, G.A. (1997) Gene. 195, 121–6.
26. Adams, S.H., Alho, C.S., Asins, G, Hegardt, F.G. & Marrero, P.P. (1997) Biochem. J. 324, 65–73.
27. Ayté, J., Gil-Gómez, G., Haro, D., Marrero, P.P. & Hegardt, F.G. (1990) Proc. Natl. Acad. Sci. 87, 5682–5684.

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KINETIC INVESTIGATION OF CARNITINE PALMITOYLTRANSFERASES IN HOMOGENATES OF HUMAN SKELETAL MUSCLE USING L-AMINO-CARNITINE AND MALONYL-COA

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1. INTRODUCTION

Carnitine palmitoyltransferase I and II together with the acylcarnitine translocase form a shuttle system responsible for the transfer of acylgroups into the mitochondria. For a review see.[1] Diseases caused by defects in the three enzymes involved are known[2–4] but the most frequently affected enzyme is the CPT II.[1] Deficiencies of CPT I are not known for muscle isoenzyme[1] but for liver type CPT I. In patients with CPT II deficiency several mutations have been detected.[5,6] Until now the kinetic consequences of such mutations on metabolism of muscle mitochondria have not been well characterized. Increased sensitivity for inhibition by palmitoylcarnitine and malonyl-CoA of the CPT II have been detected in muscle homogenates of patients with CPT II deficiency.[7] It has been shown that CPT I has a higher molecular size than CPT II due to an additional N-terminal domain consisting of 150 aminoacids.[1] In addition, CPT I contains hydrophobic domains thought to be responsible for the membrane spanning section of the enzyme. Therefore, CPT I is a integral protein of mitochondrial outer membrane whereas CPT II is bound at the inner surface of mitochondrial inner membrane.[1] The sensitive inhibition of CPT I by malonyl-CoA is an important factor in the regulation of β-oxidation.[8,9] In contrast, CPT II is insensitive to malonyl-CoA[8,10] but this enzyme can be inhibited very sensitively by L-aminocarnitine (L-AC).[11] CPT I on the other hand is only poorly inhibited by L-AC.[11] The different sensitivities of both CPTs to the two inhibitors should allow an experimental approach for the kinetic investigation of both enzymes in homogenates of human skeletal muscle. The selective solubilization of CPT II by mild detergents such as Tween 20[12] and the separation from the particulate CPT I by centrifugation offers a further possibility for kinetic investigation of this enzymes. Mostly, the CPT activity is measured in the forward direction in homogenates prepared from frozen muscle.[7] Under these conditions the total activity is the sum of the activities for both CPT I and CPT II.

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The aim of the present study was to investigate kinetically CPT I and CPT II in homogenates of skeletal muscles obtained from orthopaedic patients in the course of hip surgery. Homogenates from frozen muscle were investigated with and without malonyl-CoA and with increasing additions of L-AC. Results were compared with corresponding investigations of CPT I and CPT II in the pellet and supernatant fractions after Tween separation.

2. MATERIAL AND METHODS

Human muscle specimens (*M. vastus lateralis*) without metabolic myopathies were obtained from patients who were undergoing hip surgery.

2.1. Muscle Homogenates

Frozen muscle tissue was homogenized in 9 volumes Chappel-Perry-buffer containing 50mM Tris buffer (pH 7.5), 100 mM KCl, 5mM $MgCl_2$ and ImM EDTA. Homogenates were used in a final dilution of 1:30 or 1:10 (w/v).

For Tween separation of both CPTs, 1% Tween was added to the homogenate. After 15min of incubation the homogenates were centrifuged for 30 min at 20,000 rpm using a Beckman centrifuge (J2-HC) equipped with the JA-20 rotor. CPT activity was investigated in the supernatant and in the pellet redissolved with the same concentration of Tween 20.

2.2. Chemicals

All chemicals were obtained from Sigma Chemie (Deisenhofen/Germany). L-aminocarnitine was a gift from Sigma Tau (Rom/Italy). **[^{14}C]-**carnitine was obtained from NEN Life Science Products (Köln/Germany). All chemicals were reagent grade purity.

2.3. Enzyme Assay

CPT was determined using a radiochemical isotope forward assay as described previously.[7] In brief, the assay system contained 10mM Tris-HCl-buffer (pH 7.6), 0,1% fatty acid-free bovine serum albumin, 1 mM dithiothreitol, 0.08 mM palmitoyl-CoA and **5 mM [^{14}C]-carnitine** in a final volume of 1 ml and the assay temperature was 30 °C. Experiments were performed either in the presence or in the absence of 0.4 mM malonyl-CoA to inhibit CPT I. Concentration of L-AC was varied between 0 and 30mM. Incubations were started by the addition of homogenate and stopped after 10min by addition of ammonium sulfate and isobutanol. Labelled palmitoylcarnitine was measured after separation of the organic phase using a scintillation counter (Beckmann LS 6500).

3. RESULTS

The kinetic properties of carnitine palmitoyltransferases were investigated in homogenates of *M. vastus lateralis* from 8 orthopaedic patients. As shown in Table 1 the total activity of CPT was **1.6 ± 0.4 I.U./g** NCP with a range between 0.8 and 2.2I.U./g NCP.

Table 1. Activities of CPT I and CPT II in homogenates of human *M. vastus lateralis* Total activity of CPT was measured radiochemically in muscle homogenates as described in Methods. Further measurements were performed in the additional presence of 0.4 mM malonyl-CoA. The malonyl-CoA-insensitive activity was attributed to CPT II. Acitivies were related either at non collagen protein (NCP) or the weight of frozen sample (s.w.). Data as mean of at least two separate measurements of each muscle.

Biopsy	CPT I + CPT II [U/g NCP]	CPT II [U/g NCP]	CPT II [U/g w.w.]	Residual activity after malonyl-CoA inhibition [%]
1	1.62	0.55	4.54	34
2	1.57	0.63	6.24	40
3	1.32	0.64	8.74	48
4	0.80	0.22	2.28	27
5	1.46	0.70	9.06	48
6	2.07	0.62	7.20	30
7	1.73	0.46	4.57	27
8	2.15	0.85	12.24	39
mean ± S.D.	1.59 ± 0.43	0.58 ± 0.18	6.86 ± 3.15	36.6 ± 8.6

Since muscle mitochondria were completely destroyed during homogenization of the tissue the total CPT activity should consist of CPT I and CPT II. After inhibition of CPT I, by addition of 0.4 mM malonyl-CoA, a residual activity remained (37 ± 9%) which can attributed to CPT II. The activity of CPT II was 0.6 ± 0.2 I.U./g NCP or 6.9 ± 3.2 I.U./g frozen tissue. Parallel CPT measurements in muscle homogenates in the absence and in the presence of malonyl-CoA should yield information about both the CPT isoenzymes without the necessity to separate them from each other. Fig. 1 illustrates the inhibition of carnitine palmitoyltransferases by L-AC. Enzymatic activity of CPT was plotted versus the inhibitor concentration. Normalised data of three different experiments are shown as means ± S.D. In the absence of L-AC 37% of the total CPT activity was insensitive to malonyl-CoA. Increasing additions of the L-AC decreased the total CPT activity with a large range of concentration. Whereas the inhibition of about 30% of the total activity needs the addition of 100 µM L-AC only, the full inhibition occurs at 30 mM inhibitor.

In contrast, the malonyl-CoA-insensitive CPT activity (open circles) was very sensitive to inhibition by L-AC. After addition of 500 µM this CPT activity was completely inhibited. As marked by arrow 1 in the presence of about 20 µM L-AC 50% of the malonyl-CoA-insensitive CPT-activity was inhibited. This concentration can be taken as I_{50}. Subtracting the malonyl-CoA-insensitive activity from the total CPT activity the malonyl-CoA-sensitive inhibitor curve (triangles) was obtained: At concentrations higher than 500 µM L-AC this curve is identical with the inhibitor curve of the total CPT I + CPT II activity. At inhibitor concentrations lower than 500 µM L-AC, however, practically no inhibition of CPT I occurs. The I_{50} concentration was at about 5mM (Arrow 2). In additional studies it was found that L-AC inhibits the CPT II competitively to carnitine with a K_i of 3.5 µM L-AC (data not shown).

If the malonyl-CoA addition to muscle homogenates really causes a specific inhibition of CPT I, then the double inhibitor titration protocol should allow detection of changed relationships between CPT I and CPT II. Therefore, the double inhibitor titration was applied for CPT measurements in samples where the distribution between both enzymes was changed by Tween separation. For that purpose muscle homogenate was

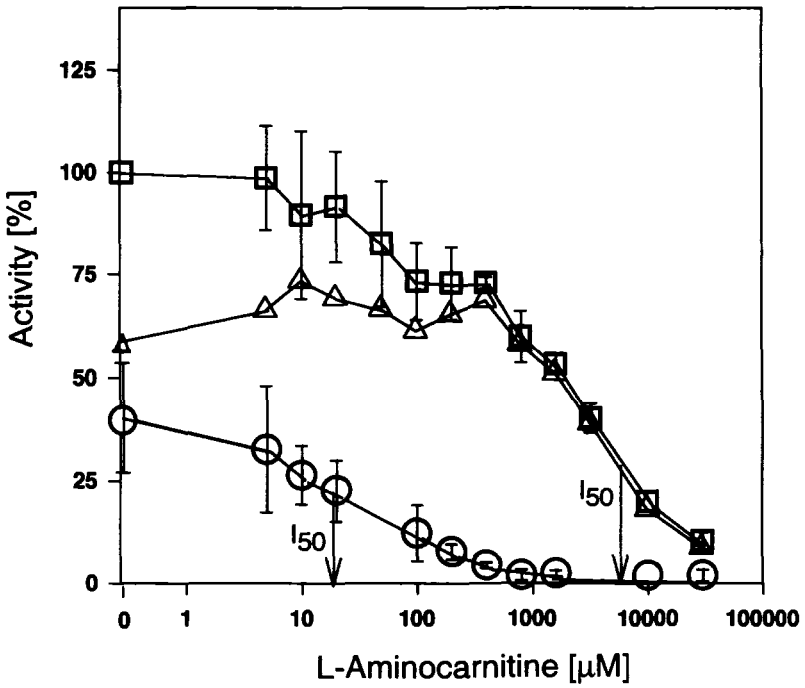

Figure 1. Inhibition of the activities of total CPT (squares), CPT I (circles) and CPT II (triangles) by L-aminocarnitine Activity of the total CPT was measured radiochemically as described in Methods using palmitoyl-CoA and [^{14}C]-L-carnitine (forward assay). CPT II-activity was assayed in the additional presence of 0.4 mM malonyl-CoA. CPT I activity was calculated by subtracting CPT II from the sum of CPT I and CPT II activities. L-aminocarnitine was varied between 0–30 mM as indicated. Values are means of three typical experiments ± S.D. Activities are expressed as percentage of the activity seen without the addition of aminocarnitine and malonyl-CoA. The error bars indicate the standard deviation.

incubated with 1% Tween 20. After incubation time of 10min the homogenate was centrifugated to sediment the cell structures including the particulate CPT I-activity which remaines under these conditions. The supernatant should contain the solubilized CPT II. As shown in Fig. 2, in the pellet fraction the restidual activity in the presence of malonyl-CoA was reduced to 20% indicating that part of CPT II activity was released from the mitochondria but a substantial part remained in this fraction. This remaining activity was sensitively inhibited by L-AC (I_{50} approximately 20 μM).

In comparison to the pellet fraction, the CPT in the supernatant fraction was much less sensitive to inhibition by 0.4 mM malonyl-CoA as it can be seen from the residual activity of about 88% but the sensitivity to L-AC was increased. At 100 μM L-AC the residual activity of CPT was about 20% indicating that a part of CPT I remained still in the supernatant probably due to incomplete sedimentation of mitochondrial membranes during the centrifugation.

4. DISCUSSION

Studies of CPT deficiencies require exact assays of the two mitochondrial CPT activities in biopsy specimens from human skeletal muscle. Attempts to investigate CPT

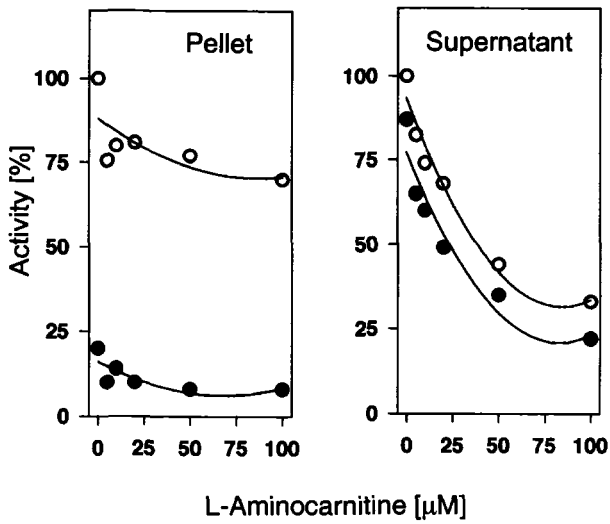

Figure 2. Inhibition of the activities of CPT I and CPT II by malonyl-CoA and L-aminocarnitine in Tween solubilized CPT II and particulate CPT I activity CPT II was released from muscle mitochondria by incubation of muscle homogenate with 0.5% Tween and separated by centrifugation (supernatant) from particulate CPT I (pellet). Activity of CPT was measured radiochemically without (open circles) and with (filled circles) 0.4 mM Malonyl-CoA as well as in the presence of L-aminocarnitine as indicated.

II in isolated muscle mitochondria revealed several difficulties. Whereas CPT I is normally active in isolated intact mitochondria, activity of CPT II is latent under these conditions. Under some conditions, such as freeze-thawing of mitochondria, the releasing of the CPT II activity is accompanied by a masking of CPT I activity. Therefore, the apparent total CPT activity decreases after such procedures.[10,13] Similar studies with liver mitochondria, however, revealed clearly increased activities of total CPT after membrane disruptive procedures.[13]

In this paper we present our results from studies in homogenates of frozen skeletal muscle. These homogenates were obtained by using glass/glass homogenizers under conditions were the mitochondrial matrix enzymes are completely released as shown by the release of the marker enzyme citrate synthase.[13]

From the total CPT activity measured in such homogenates 74% was inhibitable by 0.4 mM malonyl-CoA. This malonyl-CoA-sensitive activity was attributed to CPT I. There are several reports showing that only CPT I but not CPT II is inhibitable by malonyl-CoA.[8,14–16] The malonyl-CoA-insensitive activity, therefore, represents the activity of CPT II. Our estimate of 37% of the total activity was similar to that in earlier reports[17] and to the level reported by others.[10] This means that under the conditions used no significant masking of CPT I seems to occur.

The Tween separation experiments add further evidence tsupporting this view. The mild detergent Tween 20 solubilizes selectively CPT II from mitochondrial inner membranes but keeps the CPT I bound at the mitochondrial outer membranes.[18] By centrifugation it should be possible then to change significantly the ratio between both CPT-enzymes in the supernatant and the pellet-fraction. As shown in Fig. 2 this is obviously the case. In the supernatant the malonyl-CoA-insensitive fraction increased to 90% of total and decreased to 20% in the pellet fraction.

The remaining 10% activity of malonyl-CoA-sensitive CPT activity in the super-

natant is probably caused by incomplete sedimentation of mitochondrial membranes. Whereas the remaining 20% malonyl-CoA-insensitive activity in the pellet fraction is probably caused either by an incomplete releasing of CPT 1 activity and/or an incomplete separation of released CPT I from the mitochondrial membranes.

Recent progress in understanding the relationship between the structure and function of CPT, in relation to the inhibiton by malonyl-CoA, support our approach. At present there are no reports of malonyl-CoA inhibition of CPT II. If CPT I were to be released during the Tween separation, the enzyme should loose its activity since the malonyl-CoA-sensitivity of the CPT I is an intrinsinc property and not caused by its binding to mitochondrial outer membrane. Furthermore, CPT II seems not to be inhibited by malonyl-CoA. It can, however, not be ruled out that mutated CPT II can be inhibitable by malonyl-CoA as was proposed earlier.[19]

DL-aminocarnitine and its naturally in fungis occuring L-isomer have been identified as potent inhibitors of mitochondrial CPT.[20] As shown in Figs. 1 and 2, it is clear that CPT I and CPT II have very different sensitivities to L-AC with differences in the K_i of more than two orders of magnitude. The I_{50} for CPT II was found to be about 20 μM whereas more detailed studies revealed a K_i of 3.5 μM. The type of inhibition was competitive in respect to carnitine (Hertel, K., Gellerich, F. N. & Zierz, S., unpublished results). The sensititivity of the inhibition of CPT II by L-AC is one order of magnitude higher than was found by Chiodi *et al.* for CPT II in rat liver mitochondria.[11] L-AC concentrations of about 500 μM which even completely inhibit CPT II have no effect on the CPT I (I_{50} = 4 mM). To date a preferential inhibition of CPT II by low concentrations of L-AC has been demonstrated in rat hearts and rat liver mitochondria.[11,21,22]

In conclusion, the different behaviour of CPT I and CPT II with respect to mild detergents such as Tween and to inhibitors such as malonyl-CoA or L-AC allows the investigation of kinetic properties of the enzymes in homogenates of human skeletal muscle. We, therefore, propose this technique as an approach for investigating the kinetic consequences of mutations of CPT II in human muscle.

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REFERENCES

1. McGarry, J.D. & Brown, N.F. (1997) The mitochondrial carnitine palmitoyl-transferase system. From concept to molecular analysis. *Eur. J. Biochem.* **244**:1–14.
2. DiMauro, S. & DiMauro, P.M. (1973) Muscle carnitine palmitoyltransferase deficiency and myoglobinuria. *Science* **182**:929–930.
3. Huizing, M., Iacobazzi, V, Ijlst, L., Savelkoul, P., Ruitenbeek, W., van den Heuvel, L.P., Indiveri, C., Smeitink, J.A.M., Trijbels, J.M.F., Wanders, R.J.A. & Palmieri, F. (1997) Cloning of te human carnitine-acylcarnitine carrier cDNA and indentification of the molecular defect in a patient. *Am. J. Hum. Gen.* **61**:1239–1245.
4. Tein, I., Demaugre, F, Bonnefont, J.P. & Sauddubray, J.M. (1989) Normal muscle CPT 1 and CPT 2 activities in hepatic presentation patients with CPT 1 deficiency in fibroblastst. Tissue specific isoforms od CPT 1? *J. Neural. Sci.* **92**:229–245.

5. Wieser, T, Dechauer, M. & Zierz, S. (1998) Carnitine palmitoyltransferase II deficiency: three novel mutations. *Ann. Neurol.* **42**:414–415.
6. Taroni, F, Verderio, E., Dworzak, F., Willems, P.J., Cavadini, P. & DiDonato, S. (1993) Identification of a common mutation in the carnitine palmitoyltransferase II gene in familial recurrent myoglobinuria patients. *Nat. Genet.* **4**:314–320.
7. Zierz, S. & Engel, A.G. (1985) Regulatory properties of a mutant carnitine palmitoyltransferase in human skeletal muscle. *Eur. J. Biochem.* **149**:207–214.
8. McGarry, J.D., Leatherman, G.F. & Foster, D.W. (1978) Carnitine palmitoyl-transferase I. The site of inhibition of hepatic fatty acid oxidation by malonyl-CoA. *J. Biol. Chem.* **253**:4128–4136.
9. Fraser, F., Corstorphine, C.G. & Zammit, V.A. (1997) Topology of carnitine palmitoyltransferase I in the mitochondrial outer membrane. *Biochem. J.* **323**:711–718.
10. Pande, S.V., Lee, T.S. & Murthy, M.S.R. (1990) Freeze-thawing causes masking of membrane-bound outer carnitine palmitoyltransferase activity: implications for studies on carnitine palmitoyltransferases deficiency. *Bioch. Biophys. Acta* **1044**:262–268.
11. Chiodi, P., Maccari, F. & Ramacci, M.T. (1992) Tissue lipid accumulation by L-aminocarnitine, an inhibitor of carnitine-palmitoyltransferase-2. Studies in intact rats and isolated mitochondria. *Biochim. Biophys. Acta* **1127**:81–86.
12. Woeltje, K.F., Kuwajima, M., Foster, D.W. & McGarry, J.D. (1987) Chracterization of the mitochondrial carnitine palmitoyltrasnferase enzyme system. II Use of dteregents aand antobodies. *J. Biol. Chem.* **262**:9822–9827.
13. Zierz, S. & Engel, A.G. (1987) Are there two forms of carnitine palmitoyltransferase in muscle ? *Neurology* **37**:1785–1790.
14. Cook, G.A. (1984) Differences in sensitivity of carnitine palmitoyltransferase to inhibition by malonyl-CoA are due to differences in K_i values. *J. Biol. Chem.* **259**:12030–12033.
15. Murthy, M.S.R. & Pande, S.V. (1987) Malonyl-CoA binding site and the overt carnitine palmitoyltransferase activity reside on the opposite sides of the outer mitochondrial membrane. *Proc. Natl. Acad. Sci.* **84**:378–382.
16. Declercq, P. (1987) Characterization of the mitochondrial carnitine palmitoyltransferase enzyme system. *J.Biol.Chem.* **262**:9812–9821.
17. Zierz, S. & Engel, A.G. (1987) Different sites of inhibition of carnitine palmitoyltransferase by malonyl-CoA, and by acetyl-CoA and CoA, in human skeletal muscle. *Biochem. J.* **245**:205–207.
18. Murthy, M.S.R. (1987) Some differences in the properties of carnitine palmitoyltransferase activities of the mitochondrial outer and inner membranes. *J. Biol. Chem.* **248:** 727–733.
19. Zierz, S. (1994) Limited trypsin proteolysis renders carnitine palmitoyltransferase insensitive to inhibition by malonyl-CoA in patients with muscle carnitine palmitoyltransferase deficiency. *Clin Invest* **72**:957–960.
20. Jenkins, D.L. & Griffith, O.W. (1985) DL-aminocarnitine and Acetyl-DL-aminocarnitine. Potent inhibitors of carnitine acyltransferases and hepatic triglyceride catabolism. *J. Biol. Chem.* **260**: 14748–14755.
21. Hülsmann, W.C., Schneijdenberg, C.T.W.M. & Verkleij, A.J. (1991) Accumulation and excretion of long-chain acylcarnitine by rat hearts; studies with aminocarnitine. *Biochim. Biophys. Acta* **1097**:263–269.
22. Murthy, M.S.R., Ramsay, R.R. & Pande, S.V. (1990) Acyl-CoA chain length affects the specifity of various carnitine palmitoyltransferases with respect to carnitine analogues. *Biochem. J.* **267**:273–276.

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PROCESSING OF CARNITINE OCTANOYLTRANSFERASE PRE-mRNAs BY *CIS* AND *TRANS*-SPLICING

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1. ABSTRACT

Trans-splicing is a mechanism by which two pre-mRNAs are processed to produce a mature transcript that contains exons from both precursors. This process has been described mostly in trypanosoma, nematodes, plant/algal chloroplasts and plant mitochondria [Bonen et al. (1993) FASEB J. 7, 40–46]. Our studies clearly demonstrate that a trans-splicing reaction occurs in the processing of the carnitine octanoyltransferase (COT) gene in rat liver. Three different mature transcripts of COT have been found in vivo, the canonical cis-spliced mRNA and two trans-spliced transcripts, in which either exon 2 or exons 2 and 3 are repeated. Splicing experiments in vitro also indicate the capacity of exon 2 to act either as a donor or as an acceptor of splicing, allowing the trans-splicing reactions to occur.

1. INTRODUCTION

Trans-splicing has been described mostly in trypanosoma, nematodes, plant/algal chloroplasts and plant mitochondria. Several cases of *in vitro t*rans-splicing of artificial

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pre-mRNAs in mammalian cells have also been reported. [1–6] Functional conservation in the splicing machinery between lower eukaryotes and mammals has also been demonstrated and *trans*-splicing may occur in mammalian cells.[7] Some examples of possible natural *trans*-splicing in mammalian cells have been reported[8–11] but none has been demonstrated *in vitro.*

Carnitine acyltransferases (CATs) are a family of enzymes that transport fatty acyl-CoAs across membranes in a range of organelles so that the CoA-esters can be metabolized further. The CAT enzyme involved depends on the length of the fatty acid moeity to be transported. Carnitine acetyltransferase acts with acetyl-CoA as substrate while mitochondrial carnitine palmitoyltransferase (CPT) I and CPT II,[12] transport long-chain acyl groups into the mitochondrial matrix, where they undergo **β-oxidation.** Peroxisomal carnitine octanoyltransferase (COT) transports medium-chain fatty acids across the membrane in peroxisomes in a similar way to CPT I in mitochondria. Peroxisomal **β-oxidation** may account for a significant proportion of all liver fatty acid oxidation and COT is involved in this process.[13]

Recently, we isolated a clone from a rat liver library with identical exonic organization to that previously reported,[14] but with exon 2 repeated. The possibility of this exonic repetition in the COT gene was ruled out by genomic Southern blot. RT-PCR amplifications of total RNAs from rat liver and sequencing of the fragments revealed that in addition to the canonical organization there were two other forms, in which exon 2 or exons 2 and 3 were repeated. RNase H digestion assays with total rat liver RNA showed three different transcripts. The occurence of this process can be explained by the presence of three exon-enhancer sequences in exon 2. Analysis by Western blot of the COT proteins using specific antibodies showed that two proteins corresponding to the expected Mr are present in rat peroxisomes.

2. RESULTS

2.1. Isolation and Characterization of Liver COT cDNAs

A cDNA clone from a rat liver cDNA library was isolated using the *Xba*I-*Eco*RI fragment of the reported cDNA as a probe.[14] The exonic organization of the isolated cDNA was not the same as that reported;[15] instead, exon 2 was repeated. This abnormal exon2-exon2-exon3 organization of the isolated clone could be due to three different possibilities: i) a product of an artefactual rearrangement, ii) a duplication of exon 2 in the COT gene or iii) a *trans*-splicing product occurring naturally in this gene.

To rule out the occurrence of an artefactual organization, RT-PCR with different primers was carried out using as templates the cDNAs produced after the rat liver mRNAs had been reacted with reverse transcriptase using either oligo dTs or random hexamers as primers. When primers located in exon 1 and exon 3 were used, a three-band pattern was obtained (Fig. 1A). One of these was of the expected size corresponding to exon1-exon2-exon3 (254 bp) but the other two were larger. Sequencing of these bands unequivocally showed that they were formed by exon1-exon2-exon2-exon3 (390 bp) and exon1-exon2-exon3-exon2-exon3 (515 bp) in addition to the canonical band (254 bp). Figure 1B. corresponds to the sequence of a fragment in which exon 2 and 3 were repeated with an exact junction between them. To confirm these data, PCR amplifications were carried out with a forward primer for exon 1 and reverse primers complementary to exons 2, 4, 5, and 7. The same three-band pattern was obtained in all cases (Fig. 1A). Sequencing of all these bands showed that the repetition affected only exons 2 and 3.

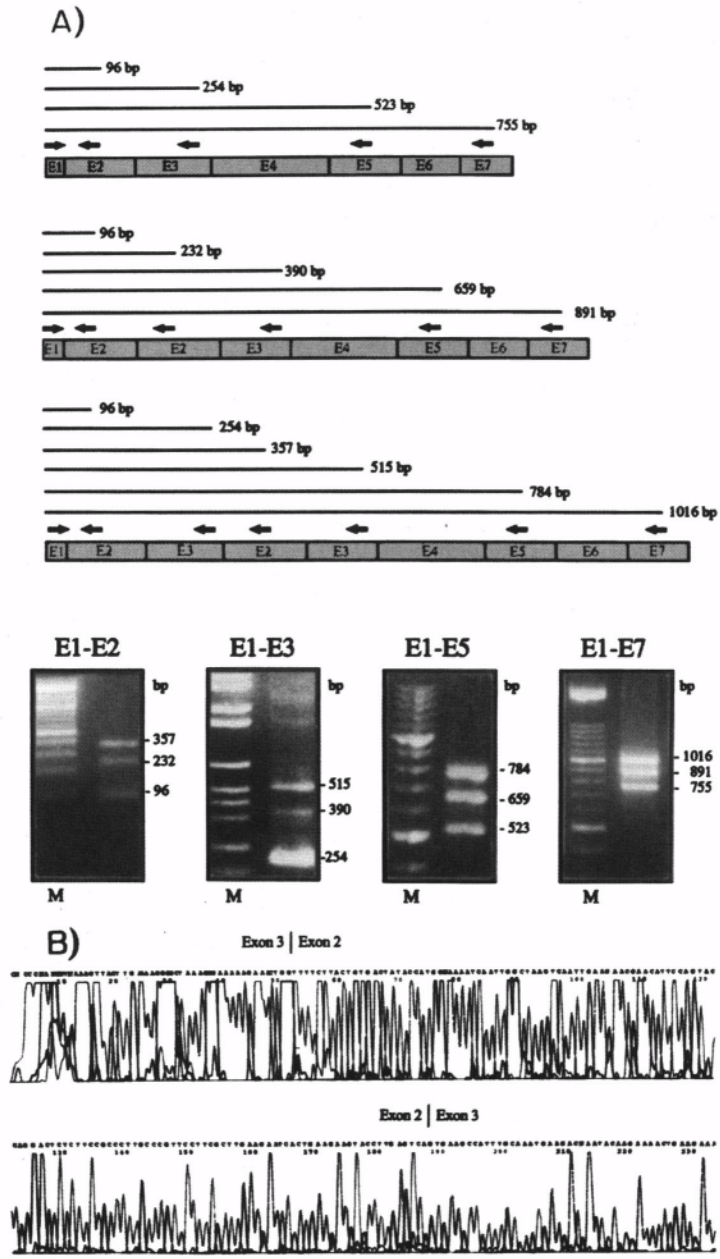

Figure 1. Three-band pattern obtained by RT-PCR amplification of the mRNAs of COT rat liver.
RT reaction was performed as described in.[16] RT-PCR products were obtained using four sets of primers, E1f-E2r, E1f-E3r, E1f-E5r and E1f-E7r. E1f (5′GAGT GCAGAGAGCCAAGCCGGG3′), E2r (5′GGAAT-GTTCGTTCTT CAATTGA3′), E3r (5′TGATGCAATGTCTTGCCAAC3′), E5r (5′GGTTCATGTCTA-GAGGAG3′) and E7r (5′GGCCCAACAGGTTCATTCCAG3′). (A) Schematic representation of the possible origin of PCR products is shown at the top. Exons are indicated by boxes and capital letters, arrows indicate the annealing regions of the PCR primers, lines above denote expected size for the three possible exonic organizations. Representative agarose gels of PCR products obtained with the four sets of primers are shown at the foot of the figure. M indicates the molecular DNA markers. The size and the sequence analysis of PCR products correspond to the exonic representation. PCR products were sequenced with an ABI prism automatic sequencer using nested primers. The chromatogram shows one of the sequences and the accurate junction exon 2-exon 2 and exon 3-exon 2 is marked (Fig. 1 B).

Genomic Southern blot analysis showed that COT is a single-copy gene and that exons 2 and 3 (Data not shown) are present only once in genomic DNA. Moreover, Southern blot and restriction mapping of three different clones obtained from a rat genomic library confirmed these data.

2.2. Evidence of Three Different Transcripts in Rat Liver COT

The difference in size between the three transcripts was so small that they could not been detected by Northern blot analysis. To improve the sensitivity of the method and to determine whether three different transcripts of COT were present as independent molecules, total RNA was hybridized with a specific oligonucleotide within exon 5 of the COT mRNA and digested with RNase H, which digests double-stranded regions of RNA-DNA hybrids. If our hypothesis was right, the treatment would result in the appearance of four different bands (Fig. 2A): the band of highest molecular mass would be the most 3′ and the other three, of lower molecular mass, would be at the most 5′ regions of the transcripts. Figure 2B shows a Northern blot using the whole cDNA COT as a probe. The results confirm these predictions. The band corresponding to the 3′ fragment after RNase H digestion (lanes 2–5) ran with a slightly faster mobility (2,434 bp) than the uncut transcript (lane 1) (3,000bp). Moreover, three bands with faster mobility corresponding to the 5′ fragment after RNase H digestion were also seen (807, 682 and 546 bp). The results of these analyses are consistent with the presence of 3 transcripts of COT which differ only in the most 5′ region.

2.3. The Exon 2 of the COT Gene Contains Natural Sequences that Trans-Splice with Human Nuclear Extracts *in vitro*

In order to study the possible role of the sequences around exon 2 of the COT gene, two truncated pre-mRNAs were prepared (Fig. 3); a donor pre-mRNA, containing only exon 2 and the 5′ splice site of intron 2 (A pre-mRNA), and an acceptor pre-mRNA, containing the branch point region and the 3′ splice site of intron 1 and exon 2 (B pre-mRNA).

Figure 3 shows a *trans*-splicing assay in which the donor, A, was ^{32}P-labelled during transcription and the acceptor, B, was added unlabelled to the *trans*-splicing reaction in HeLa nuclear extracts. The combination of both pre-mRNAs (Fig. 3, lanes 2–5) showed a band of high molecular mass that was dependent on ATP. This band was cut, assayed by RT-PCR and sequenced and it turned out to be the *trans*-splicing product. Another band also appeared (exon2) which was assumed to be one of the two intermediates of the first-step *trans*-splicing reaction because of its size (191 nt) and because there is a correlation between the amount of this band and the amount of the *trans*-splicing product. From this experiment, we can conclude that exon 2 and the splice site regions around it are strong *trans*-splicing signals.

2.4. Western Blot Analysis of the COT Proteins in Rat Liver Peroxisomes

To test whether the three mature mRNAs were translated *in vivo,* Western blots of peroxisomal proteins using specific antibodies against a selected hydrophilic peptide of COT were carried out (Fig. 4). Two proteins were immunolocalized as expected: one corresponding to the *cis*-spliced exon l-exon2-exon3...etc. mRNA, which is indistinguishable from the protein translated from the *trans*-spliced form exonl-exon2-

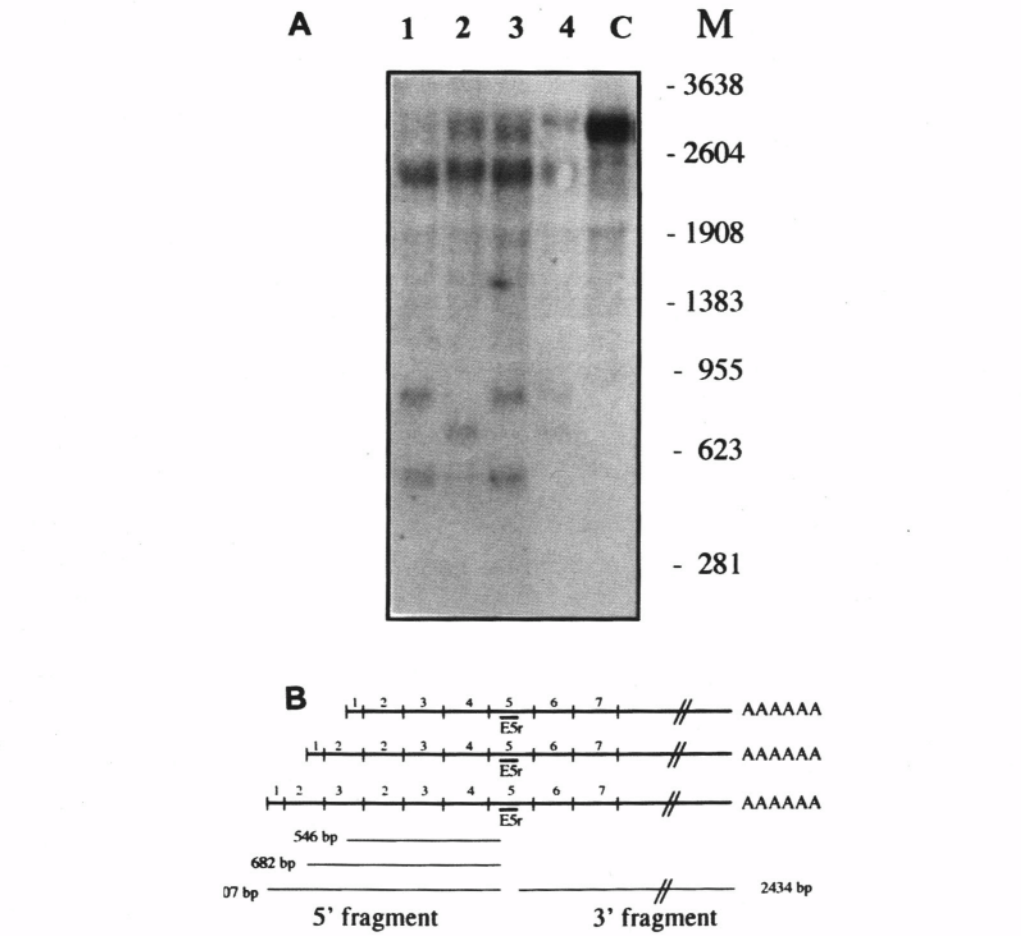

Figure 2. RNase H Digestion of RNA. Samples of 20 µg of total RNA were incubated separately with an oligonucleotide that anneals to the exon 5 (E5r) for 30 min at 37 °C and then 1h at 30 °C after addition of 0.8 U of RNase H (Boehringer Mannheim). The digested RNA was analysed by Northern Blot. Lane C denotes control non-digested RNA. The ^{32}P-labelled cDNA probe contained the fragment corresponding to positions 31–1,700 bp in the cDNA sequence from COT.[14] RNA molecular size markers (M) are indicated to the right. B. The diagram shows the products formed after binding the primer E5r to RNA and digesting with RNAse H.

exon2-exon3... etc. mRNA, since the first in-frame ATG is located in the second exon 2. The second protein, translated from the *trans*-spliced mRNA composed by exons 1-2-3-2-3-4 etc. should also appear. The theoretical Mr of these translated proteins are 70,302 Da and 80,572 Da, respectively. The occurrence of the two forms of the COT enzyme in peroxisomes may be a direct consequence of this *trans*-splicing mechanism in the COT gene.

3. DISCUSSION

Dandekar *et al.* suggested that *trans*-splicing is a regular event in mammalian cells.[17] Later, the capability of mammalian cells to perform the *trans*-splicing reaction with appropriate foreign RNAs was also demonstrated.[18]

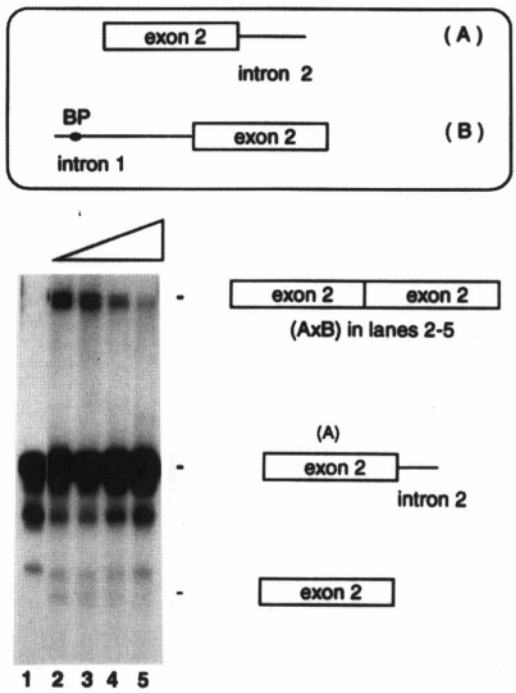

Figure 3. *Trans*-splicing of COT pre-mRNAs *in vitro*. The A and B constructs were obtained as described in.[16] A pre-mRNA was 220 nucleotides (nt) long: 55 nt from the plasmid polylinker region (PL) + exon 2 (136 nt) + 25 nt of the 5′ end of intron 2 + 4 nt PL; B pre-mRNA was 278 long: 23 nt PL + 115 nt of the 3′ end of intron 1 + exon 2 (136 nt) + 4 nt PL. BP indicates branch-point. The ^{32}P-labelled A pre-mRNA was mixed with increasing concentrations of unlabelled B pre-mRNA and incubated in *trans*-splicing conditions in nuclear HeLa extracts. Lane 1 shows the incubation reaction of labelled A pre-mRNA with 50 ng of B pre-mRNA without ATP. A pre-mRNA was incubated with increasing amounts of B pre-mRNA (10 ng, 25 ng, 50 ng, and 100 ng). The *trans*-splicing product (A × B), (lanes 2–5) was confirmed by RT-PCR and sequencing. The exon 2 band might correspond to the intermediate exon 2 without the attached intron.

The mechanism responsible for *trans*-splicing in lower eukaryotic cells or mammalians cells is not well known. Solnick[1,3] had reported that *trans*-splicing in mammals *in vitro* can occur only if the 5′ and 3′ substrate RNAs form base pairs via a segment of complementarity in their introns. However, other authors[2,5,22,23] clearly showed that *trans*-splicing did not need complementarity in the introns. Bruzik and Maniatis[6] showed that the occurrence of an exonic splicing enhancer (ESE) in the downstream exon is necessary for *trans*-splicing to occur. In addition, SR proteins have been shown to mediate alternative splicing[21–24] and to commit pre-mRNAs to the splicing pathway.[25,26] Splicing enhancers facilitate the assembly of protein complexes on mRNAs containing a 3′ splice site, and these complexes are sufficiently stable to interact with 5′ splice sites located on

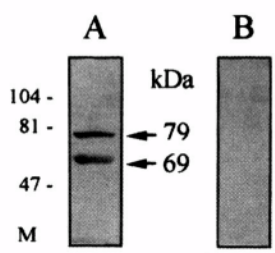

Figure 4. Immunolocalization of peroxisomal COT. The peptide ANEDEYKKTEEI corresponding to the N-terminus of COT protein (sequence 43–54,) was used to obtain the antibodies as described elsewhere.[16] Rat liver peroxisomes were isolated in a Nycodenz cushion as described elsewhere.[18] 10 mg of rat liver peroxisomal extracts were separated by SDS/PAGE and subjected to immuno-blotting using specific A43 antibodies (A) for carnitine octanoyltransferase or preimmune sera (B). Two bands corresponding to Mr of approximately 69 and 79 kDa are observed. The markers (M) were used to determine the approximate molecular weights of the species indicated in the figure.

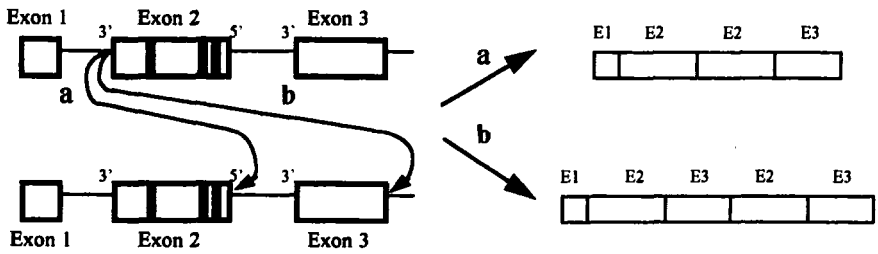

Figure 5. Model for *trans*-splicing in COT pre-mRNAs. The exons are represented by boxes, the introns by lines. The exonic splicing enhancers (ESE) are represented in exon 2 in thicker lines. The 3′ acceptor site of intron 1 reacts with the 5′ donor sites of introns 2 or 3 of other molecules of pre-mRNA (*trans*-splicing). The organization of the mRNAs is shown at the right.

separate mRNAs. Exon 2 of the COT contains three purine-rich ESEs at positions 49 (GAAGAACGAA), 106 (GAAGAA) and 117 (GAAGAAG), which are similar to those previously described.[24] Whether these ESEs are necessary to produce *trans*-splicing in the COT gene remains to be demonstrated.

Conrad *et al.*[27,28] demonstrated that a *cis*-splice acceptor can act as a *trans*-splice acceptor when the 5′ donor splice site is not well defined. The *trans*-splicing was dominant over the *cis*-splicing with a poorly-defined, short, untranslated exon. This is apparently the case shown here. The short, untranslated, poorly-defined exon 1 with 30 nt[29] may not be long enough to define intron 1, and it becomes outron-like, which allows *trans*-splicing and *cis*-splicing to take place simultaneously.

From this study we can conclude that the two forms of the COT enzyme in peroxisomes may be a result of the *trans*-splicing mechanism in the COT pre-mRNAs. The strength of the 3′ acceptor splice site, the presence of several ESEs in exon 2 and the fact that exon 1 is untranslated with only 30 nt, may be responsible of this process (Fig. 5).

4. ACKNOWLEDGMENTS

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4.1. Abbreviations

COT, Carnitine Octanoyltransferase. RT-PCR, reverse transcriptase-polymerase chain reaction. ESE, exonic splicing enhancer. PL, plasmid polylinker region.

¶ The sequences reported in this paper have been deposited in the GenBank data base (accession nos. AF056298 and AF056299)

REFERENCES

1. Solnick, D. (1985) *Cell* **42**, 157–164. *Trans* Splicing of mRNA Precursors.
2. Konarska, M.M., Padgett, R.A. & Sharp, PA. (1985) *Cell* **42**, 165–171. *Trans* Splicing of mRNA Precursors *in vitro*.

3. Solnick, D. (1986) *Cell* **44**, 211–211. Does *Trans* Splicing *in vitro* require base pairing between RNAs?
4. Sharp, P.A. & Konarska, M.M. (1986) *Cell* **44**, 211–211. Does *Trans* Splicing *in vitro* require base pairing between RNAs? .
5. Chiara, M.D. & Reed, R, (1995) *Nature* (London) **375**, 510–513. A two-step mechanism for **5′** and **3′** splice-site pairing .
6. Bruzik, J.P. & Maniatis, T. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7056–7059. Enhancer-dependent interaction between **5′** and **3′** splice sites in *trans.*
7. Bruzik, J.P. & Maniatis, T. (1992) *Nature* (London) **360**, 692–695. Spliced leader RNAs from lower eukaryotes are *trans*-spliced in mammalian cells.
8. Sullivan, P.M., Petrusz, P., Szpirer, C. & Joseph, D.R. (1991) *J. Biol. Chem.* **266**, 143–154. Alternative processing of androgen-binding protein RNA transcripts in fetal rat liver.
9. Shimizu, A. & Honjo, T. (1993) *FASEB J.* **7**, 149–154. Synthesis and regulation of trans-mRNA encoding the immunoglobulin ε heavy chain.
10. Vellard, M., Sureau, A., Soret, J., Martinerie, C. & Perbal, B. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 2511–2515. A potencial splicing factor is encoded by the oposite strand of the *trans*-spliced **c-*myb* exon.**
11. Breen, M.A. & Ashcroft, S.J.H. (1997) *FEBS Lett.* **409**, 375–379. A truncated isoform of Ca^{2+}/calmodulin-dependent protein kinase II expressed in human islets of Langerhand may result from *trans*-splicing.
12. Mc Garry, J.D. and Brown, N.F. (1997) *Eur. J. Biochem.* **244**, 1–14. The mitochondria! carnitine palmitoyltransferase system. From concept to molecular analysis.
13. Ramsay, R.R., Derrick, J.P., Friend, A.S. and Tubbs, P.K. (1987) *Biochem. J.* **244**, 271–278. L-Carnitine acyltransferase in intact peroxisomes is inhibited by malonyl-CoA.
14. Chatterjee, B., Song, C.S., Kim, J.M. & Roy, A.K.. (1988) *Biochemistry* **27**, 9000–9006. Cloning, sequencing, and regulation of rat liver carnitine octanoyltransferase transcription stimulation of the enzyme during peroxisome proliferation.
15. Choi, S.J., Oh, D.H., Song, C.S., Roy, A.K. & Chatterjee, B. (1995) *Biochim. Biophys. Acta* **1264**, 215–222. Molecular cloning and sequence analysis of the rat liver carnitine octanoyl-transferase cDNA, its natural gene and the gene promoter.
16. Caudevilla, C, Serra, D., Miliar, A., Codony, C., Asins, G, Bach, M. & Hegardt, F.G. (1998) *Proc. Nat. Acad. Sci.* **95** (in press).
17. Dandekar, T. & Sibbald, P. (1990) *Nucleic Acids Res.* **18**, 4719–725. *Trans*-splicing of pre-mRNA is predicted to occur in a wide range of organisms including vertebrates.
18. Eul, J., Graessmann, M. & Graessmann, A. (1995) *EMBO J.* **13**, 3226–3235. Experimental evidence for RNA trans-splicing in mammalina cells.
19. Konforti, B.B. & Konarska, M.M. (1995) *RNA* **1**, 815–827. A short **5′** splice site RNA oligo can participate in both steps of splicing in mammalian extracts.
20. Ghetti, A. & Abelson, J.N. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 11461–11464. *In vitro trans*-splicing in *Saccharomyces cerevisiae.*
21. Horowitz, D.S. & Krainer, A.R. (1993) *Trends Genet.* **10**, 100–106. Mechanisms for selecting **5′** splice sites in mammalian pre-mRNA splicing.
22. Watakabe, A., Tanaka, K. & Shimura, Y. (1993) *Genes Dev.* **7**, 407–418. The role of exon sequences in splice site selection.
23. Lavigueur, A., La Branche, H., Kornblihtt, A.R. & Chabot, B. (1993), *Genes Dev.* **7**, 2405–2417. A splicing enchancer in the human fibronectin alternate DE1 exon interacts with SR proteins and stimulates U2 snRNP binding.
24. Sun, Q., Mayeda, A., Hampson, R.K., Krainer, A.R. & Rottman, F.M. (1993) *Genes Dev.* **7**, 2598–2608. General splicing factor SF2/ASF promotes alternative splicing by binding to an exonic splicing enhancer.
25. Fu, X.D. (1993) *Nature* (London) **365**, 82-85. Specific commitment of different pre-mRNAs to splicing by single SR proteins.
26. Bruzik, J.P. (1996) *Microb. Pathog.* **21**, 149–155. Splicing glue: a role for SR proteins in *trans* splicing?
27. Conrad, R., Thomas, J., Spieth, J. & Blumenthal, T. (1991) *Mol. Cell. Biol.* **11**, 1921–1926. Insertion of part of an intron into the **5′** untranslated region of a *Caenorhabditis elegans* gene converts it into a *trans*-spliced gene.
28. Conrad, R., Liou, R.F. & Blumenthal, T. (1993) *EMBO J.* **12**, 1249–1255. Conversion of a *trans*-spliced *C. elegans* gene into a conventional gene by introduction of a splice donor site.
29. Berget, S.M. (1995) *J. Biol. Chem.* **270**, 2411–2414. Exon recognition in vertebrate splicing.

SELECTIVE MODULATION OF CARNITINE LONG-CHAIN ACYLTRANSFERASE ACTIVITIES

Kinetics, Inhibitors, and Active Sites of COT and CPT-II

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1. ABSTRACT

Carnitine acyltransferases in mitochondria, peroxisomes and the endoplasmic reticulum are different gene products and serve different metabolic functions in the cell. Here we summarize briefly evidence that carnitine octanoyltransferase (COT) from the peroxisomes and carnitine palmitoyltransferase II (CPT-II) from the mitochondria (both matrix facing enzymes) differ kinetically and demonstrate that they differ in their sensitivity to conformationally constrained inhibitors that mimic the reaction intermediate. Medium chain inhibitors are 15 times more effective on COT than on CPT-II and long chain inhibitors, such as hemipalmitoylcarnitinium, 80 times more effective on the mitochondrial enzyme. Thus, it may be possible to develop inhibitors to inhibit mitochondrial β-oxidation with minimal effects on peroxisomal β-oxidation and other acyl-CoA dependent reactions.

1. INTRODUCTION

The carnitine acyltransferases (CPT) catalyze the reversible acyl group transfer between the CoA and carnitine pools (reviewed in).[1,2] Only carnitine acetyltransferase (CAT), the soluble peroxisomal COT and the mitochondrial inner carnitine palmitoyltransferase (CPT-II) are not regulated allosterically. The mitochondrial outer carnitine

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palmitoyltransferase (CPT-I), the peroxisomal overt carnitine octanoyltransferase (COT), and the enzyme in the endoplasmic reticulum (CPT_{ER}) are all inhibited by physiological concentrations of malonyl-CoA, the precursor for fatty acid synthesis.[3–5] CPT levels and the IC_{50} levels for malonyl-CoA inhibition change with metabolic and hormonal status. For example, fasting increases CPT levels and decreases sensitivity to malonyl-CoA.[5,6] Insulin inhibits CPT activity but glucagon increases it.[7] Hormonally induced changes in CPT-I correlate with changes in fatty acid oxidation, in accord with the role of CPT-I in the regulation of the entry of fatty acyl groups into mitochondria. Hypolipodemic drugs, such as 2-tetradecylglycidic acid (TDGA) and etomoxir, inhibit CPT-I and, hence, decrease fatty acid oxidation and ketogenesis.[8,9]

However, not just CPT-I, but all CPT enzymes that use cytoplasmic substrates are regulated by malonyl-CoA and inhibited by TDGA and etomoxir.[10–12] Thus, these drugs will also interfere with peroxisomal oxidation and influence microsomal acyl-CoA utilizing pathways such as lipid synthesis and lipid modification of proteins for export. In view of recent indications that non-mitochondrial acyl-CoA pools may influence membrane turnover,[13] this may not be desirable, so inhibitors specific to CPT-I would be better. We have studied the kinetics and inhibition of two of the CPT family of enzymes as the first step towards finding differences that could be exploited to inhibit **β-oxidation** without affecting peripheral function.

2. RESULTS

2.1. Kinetic Studies on COT and CPT-II

Purified preparations of COT from beef liver peroxisomes[14] and CPT-II[15] from the inner membrane of beef liver mitochondria, the two enzymes that can be isolated in stable form, were used for the kinetic studies. Based on the kinetic patterns obtained when both substrates were varied and on product inhibition studies, we concluded that the mechanism of COT was a rapid equilibrium random one.[16] In contrast, that of CPT-II was ordered with the CoA substrate binding first (acyl-CoA in the forward direction, CoA in the reverse) to prepare the carnitine binding site. The kinetic constants are given in Table 1.

Table 1. Steady-state kinetic parameters for COT and CPT-II. The data, taken from Nic a'Bhaird *et al.*,[15] was obtained using purified enzymes in the absence of bovine serum albumin.

	Kinetic Constants	
Parameters	COT	CPT-II
Forward		
K_m DecCoA (mM)	0.34	3.5
PalCoa (mM)	0.6	0.5
K_m L-carnitine (mM)	108	1,500
V_{max} (mmol.min^{-1}.mg^{-1})	36	14.6
Reverse		
K_m Dec-L-carn (mM)	8.4	46
Pal-L-carn (mM)	7.4	n.d.
K_m CoA (mM)	16	112
V_{max} (mmol.min^{-1}.mg^{-1})	34	32

Based on early work on CAT and our own and other kinetic studies on the CPT enzymes, an active site with 3 distinct regions can be visualised for these proteins ([16] and Fig. 1). The CoA site is common to all the enzymes. The acyl group site determines the specificity and, as seen in Table 1, the discrimination by this site is greater in CPT-II than in COT. The variation in k_{cat}/K_M for the acyl-CoA substrate with chain length is relatively small for COT, but is larger for CPT-II by 3-fold. The effect is to make CPT-II more specific for the longer chain substrates, whereas the specificity of COT is more general. The carnitine site is less well characterized although both charges are important.[17,18] It is clear from the kinetic data that the carnitine is different in COT and CPT-II, in that, in the absence of the CoA substrate, carnitine does not bind to CPT-II. Specificity studies using carnitine analogues also suggest that there are differences between CPT-I, CPT-II and COT in the carnitine site.[19,20]

2.2. Inhibitors

The design of the inhibitors used in this study was based on the proposed tetrahedral intermediates containing CoA, carnitine and the acyl group[21] (see Fig. 1). Conformationally constrained analogues that mimic the carnitine and acyl parts of the tetrahedral intermediate were synthesized.[21–23] The value of K_i depends on how well the structure of the inhibitor fits the shape of the catalytic centre. In addition, inhibitors that mimic reaction intermediates should bind better than substrates. Two series of effective inhibitors of the acyltransferases have been synthesized and characterized, the morpholiniums and the hemiacylcarnitiniums (2-hydroxymorpholiniums).[21] Figure 2 shows the structures of these inhibitors and Table 2 the IC_{50} or K_i values obtained for the inhibition of the reverse reaction catalyzed by COT and by CPT-II (acylcarnitine + CoA to acyl-CoA + carnitine).

A previous study showed that the substrate analogue, palmitoylcholine, and the product, palmitoylcarnitine gave similar K_i values against the forward reaction of CPT-II, suggesting that the COO^- group of the carnitine moeity contributes very little to the binding of a substrate analogue. However, comparing the cholinium and carnitinium results in Table 2, it is apparent that the COO^- contributes strongly to the binding of the tetrahedral intermediate analogue. Indeed, the COO^- moeity present in the carnitiniums improves the binding by 20-fold in both enzymes. We have recently demonstrated by site directed mutagenesis that Arg505 in COT, which is normally an asparagine in the aligned choline acetyltransferase sequences but is conserved in all the carnitine-dependent enzymes, is essential for the affinity for carnitine. When Arg505 is changed to asparagine in COT, the affinity for choline (143mM) is the same as the wild-type, but the affinity for carnitine (measured as the K_M) increases by more than three orders of magnitude from 0.1 mM to 160mM.[24]

Differences in the acyl site of the COT and CPT-II are apparent from the data in Table 2. For both enzymes, lengthening the acyl chain on the conformationally constrained analogue gives better binding, but the advantage conferred by the longer chain is 3 to 5-fold more in CPT-II than it is in COT. This agrees with the K_M differences noted in Table 1. In other words, CPT-II discriminates for longer chain length more than does COT, which means that the environment in the acyl binding site must be slightly different for the two enzymes.

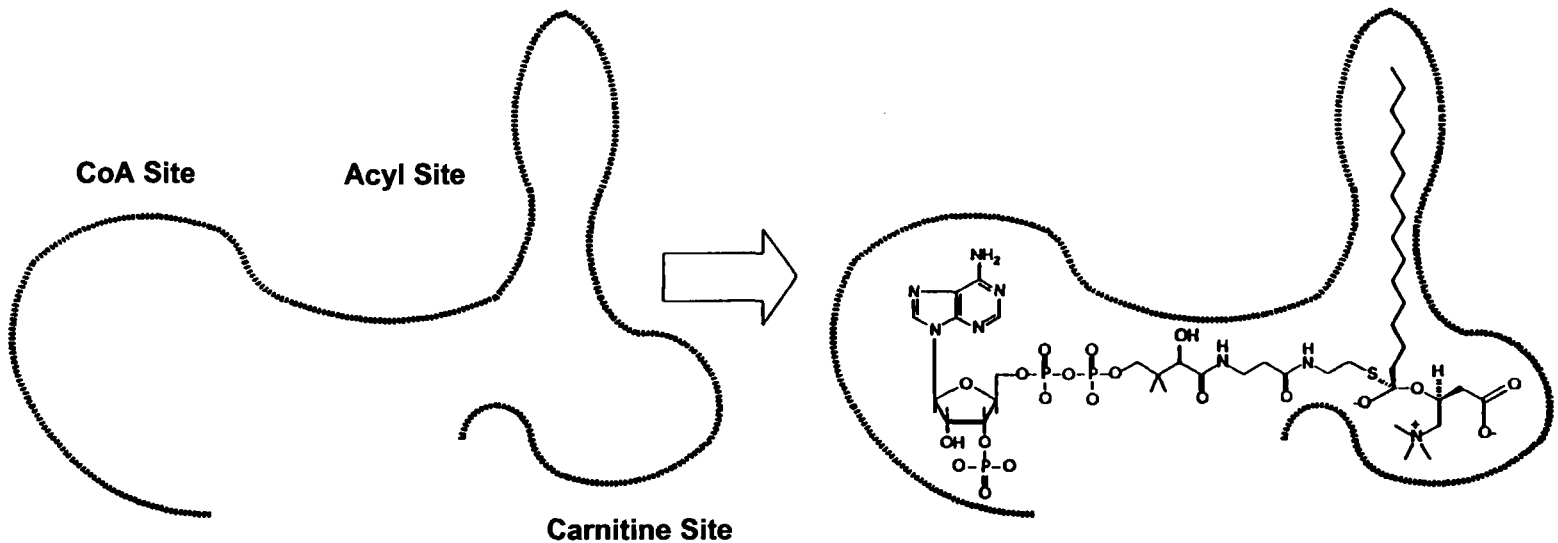

Figure 1. Schematic representation of the active site of COT and the structure of the tetrahedral intermediate in the site.

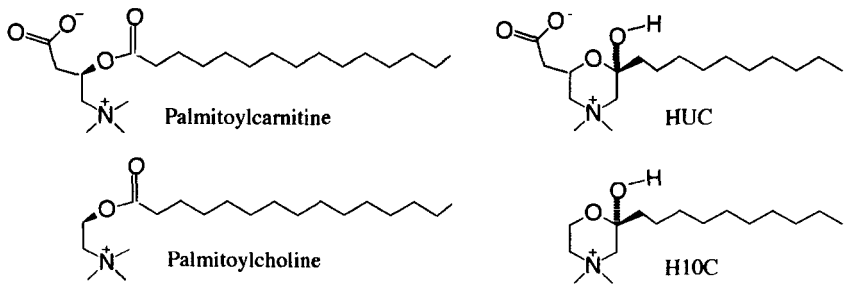

Figure 2. Structures of the substrate, palmitoylcarnitine, and the inhibitors, hemiundecanoylcarnitinium (HUC), palmitoylcholine (PmCh), and hemidecanoylcholinium (H10C).

3. CONCLUSIONS

3.1. Inhibitors

Hemicholiniums are good inhibitors of CPT-II, less so of COT. Preliminary data suggest that CPT-I, like CPT-II, follows an ordered mechanism,[25] so that the active site of these two enzymes may be similar. If these inhibitors work as well on CPT-I as they do on CPT-II, then the hemiacylcarnitiniums could be the first inhibitors to inhibit fatty acid oxidation with less effect on the peripheral processes.

3.2. Enzymes

The random mechanism followed by COT indicates that the substrate sites are well-formed in the resting enzyme. In contrast, in the resting state of CPT-II, the carnitine site is either closed or unformed so that binding of carnitine alone is poor. The conformationally constrained inhibitors do bind to CPT-II because mixed inhibition is observed when either the acyl-CoA or the carnitine substrate is varied. (If the inhibitor could bind only to the acyl-CoA-enzyme complex as is the case for carnitine, competitive inhibition would be observed when carnitine was varied). Thus, the structure of the tetrahedral intermediate found in these inhibitors induces the change in the carnitine site that is normally induced by CoA. Clearly, the affinity at the acyl site contributes strongly to the

Table 2. Inhibition constants for hemicholiniums (H10C-H15C), hemiacylcarnitiniums (HUC, HPC), and palmitoylcholine (PmCh) on the reverse reaction (acyl-carnitine + CoA to acyl-CoA + carnitine).

	IC_{50} (mM)*			K_i (mM)*	
Inhibitor	CPT-II	COT	Inhibitor	CPT-II	COT
H10C	235 ± 115	>4,000	HUC	8.0 ± 0.25	113 ± 19
H12C	116 ± 24	2,100 ± 270			
H13C	44 ± 6.5	1,300 ± 320			
H14C	32 ± 9	1,700 ± 300			
H15C	micelles	micelles	HPC	0.16 ± 0.05	13 ± 0.3
			PmCh	28 ± 6	454 ± 10

*The values given are means ± sd (for n = 2- 4)

initial binding of the inhibitor to the site. Further, the acetyl analogue does not inhibit in CPT-II. As described above, increased acyl chain length contributes strongly to the affinity of both inhibitors and substrates for CPT-II.

These kinetic studies suggest that it may indeed be possible to inhibit β-oxidation with minimal effect on the lipid recycling reactions in the rest of the cell.

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REFERENCES

1. Bieber, L.L. (1988) *Annu. Rev. Biochem.* **57**, 261–283. Carnitine.
2. Ramsay, R.R. & Arduini, A. (1993) *Arch. Biochem. Biophys.* **302**, 307–314. The carnitine acyltransferases and their role in modulating acyl-CoA pools.
3. McGarry, J.D., Leatherman, G.F. & Foster, D.W. (1978) *J. Biol. Chem.* **253**, 4128–4136. CPTase I: The site of inhibition of fatty acid oxidation by malonyl-CoA.
4. Robinson, I.N. & Zammit, V.A. (1982) *Biochem. J.* **206**, 177–179. Sensitivity of carnitine acyltransferase I to malonyl-CoA inhibition in isolated rat liver mitochondria quantitatively related to hepatic malonyl-CoA concentration *in vivo.*
5. Ramsay, R.R. (1993) *Life Sci. Adv. (Biochem.)* **12**, 23–29. A comparison of the malonyl-CoA sensitive carnitine palmitoyltransferase activities acting on cytoplasmic substrates and their distribution in mitochondria, peroxisomes and microsomes.
6. Saggerson, E.D. & Carpenter, C.A. (1982) *Biochem. J.* **208**, 673–678. Response to starvation of hepatic carnitine activity and its regulation by malonyl-CoA—sex differences and effects of pregnancy.
7. Guzman, M. & Geelan, M.J.H. (1988) *Biochem. Biophys. Res. Commun.* **151**, 781–787. Short-term regulation of carnitine palmitoyltransferase activity in isolated rat hepatocytes.
8. Tutwiler, G.F., Brentzel, H.J. & Kiorpes, T.C. (1985) *Proc. Soc. Exp. Biol. Med.* **178**, 288–296. Inhibition of mitochondrial carnitine palmitoyltransferase A *in vivo* with methyl 2-tetradecylglycidate (methyl palmoxirate) and its relationship to ketonemia and glycemia.
9. Wolf, H.P.O. & Engel, D.W. (1985) *Eur. J. Biochem.* **146**, 359–363. Decrease of fatty acid oxidation, ketogenesis, and gluconeogenesis in isolated perfused rat liver by phenylalkyl oxirane carboxylate (B 807–27) due to inhibition of CPT-I (EC 2.3.1.21).
10. Declercq, P.E., Falck, J.R., Kuwajima, M., Tyminiski, H., Foster, D.W. & McGarry, J.D. (1987) *J. Biol. Chem.* **262**, 9812–9821. Characterization of the mitochondrial carnitine palmitoyltransferase enzyme system. I. Use of inhibitors.
11. Lilly, K., Bugaisky, G.E., Umeda, P.K. & Bieber, L.L. (1990) *Arch. Biochem. Biophys.* **280**, 167–174. The medium chain carnitine acyltransferase activity associated with rat liver microsomes is malonyl-CoA sensitive.
12. Ramsay, R.R., unpublished.
13. Arduini, A., Mancinelli, G., Radatti, G.L., Dottori, G, Molajoni, F. & Ramsay, R.R. (1992) *J. Biol. Chem.* **268**, 12673–12681. Role of carnitine & carnitine palmitoyltransferase as integral components of the pathway for membrane phospholipid fatty acid turnover in intact human erythrocytes.
14. Ramsay, R.R., Derrick, J., Friend, A. & Tubbs, P.K. (1987) *Biochem. J.* **244**, 271–278. Carnitine palmitoyltransferase: Purification and properties of the easily solubilized enzyme (CPT-A) from the outer side of the mitochondrial inner membrane.
15. Clarke, P.R.H. & Bieber, L.L. (1981) *J. Biol. Chem.* **256**, 9861–9868. Isolation and purification of mitochondrial carnitine octanoyltransferase activities from beef heart.
16. Nic a'Bhaird, N., Kumaravel, R.D., Gandour, R.D. & Ramsay, R.R. (1993) *Biochem. J.* **294**, 645–651. Comparison of the active sites of the purified carnitine acyltransferases from peroxisomes and mitochondria using a reaction intermediate analogue.

17. Colucci, W.J. & Gandour, R.D. (1988) *Bioorg. Chem.* **16,** 307–334. Carnitine acetyltransferase: a review of its biology, enzymology, and bioorganic chemistry.
18. Saeed, A., McMillin, J.B., Wolkowicz, P.E. & Brouillette, W.J. (1993) *Arch. Biochem. Biophys.* **305**, 307–312. Carnitine acyltransferase enzymic catalysis requires a positive charge on the carnitine cofactor.
19. Jenkins, D.L. & Griffith, O.W. (1985) *J. Biol. Chem.* **260**, 14748–14755. DL-Aminocarnitine and acetyl-DL-aminocarnitine. Potent inhibitors of carnitine acyltransferases and hepatic triglyceride metabolism.
20. Murthy, M.S.R., Ramsay, R.R. & Pande, S.V. (1990) *Biochem. J.* **267**, 273–276. Acyl-CoA chain-length affects the specificity of various carnitine palmitoyltransferases with respect to carnitine analogues.
21. Gandour, R.D. (1992) In *Current Concepts in Carnitine Research* (Carter, A.L., ed.) CRC Press, Boca Raton, Florida, pp. 93–105. Designing active site probes of carnitine acyltransferases: potentially potent regulators of lipid metabolism.
22. Gandour, R.D., Blackwell, N.L., Colucci, W.J., Chung, C, Bieber, L.L., Ramsay, R.R., Brass, E.T. & Fronczek, F.R. (1992) *J. Org. Chem.* **57**, 3426–3431. Syntheses, structures, and enzymatic evaluations of hemiacylcarnitiniums, a new class of carnitine acyltransferase inhibitors.
23. Colucci, W.J., Gandour, R.D., Fronczek, F.R., Brady, P.S. & Brady, L.J. (1987) *J. Am. Chem. Soc.* **109**, 7915–7916. A "siameso" inhibitor: chiral recognition of a prochiral bilaterally symmetric molecule by carnitine acetyltransferase.
24. Cronin, C.N. (1997) *Eur. J. Biochem.* **247**, 1029–1037. cDNA cloning, recombinant expression, and site-directed mutagenesis of bovine liver carnitine octanoyltransferase.
25. Greway, A.T., Heathers, G.P., McDonald, M.E., Kozak, M.Z., Tilley, J.W., Kaplan, G.L., Kierstead, R.D., Millin, J.G., Guthrie, R.W., Kachensky, D.F., Gandour, R.D. & Higgins, A.J., *unpublished.*

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CONFOCAL LASER SCANNING MICROSCOPY OF HUMAN SKIN FIBROBLASTS SHOWING TRANSIENT EXPRESSION OF A GREEN FLUORESCENT CARNITINE PALMITOYLTRANSFERASE 1 FUSION PROTEIN

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The mitochondrial outer membrane enzyme carnitine palmitoyltransferase 1 (CPT1) is a main site of regulation of intracellular long-chain fatty acid transport. At least two isoforms of CPT1 are expressed in the body: L-CPT1 (the "liver-type" isoform) and M-CPT1 (the "muscle-type" isoform).[1] Skin fibroblasts from healthy humans are known to contain only one isoform of CPT1: the liver-type, which is encoded by the gene *CPT1A*. Skin fibroblasts from patients with a liver-type CPT1 deficiency do not express either of the two known CPT1 isoforms (neither liver- nor muscle-type), and therefore could provide an excellent background to study CPT1 by means of molecular complementation.

In this chapter, we describe the first experiments we carried out with a gene fusion of a complementary DNA of the human gene for muscle-type carnitine palmitoyltransferase (*CPT1B*)[2] and a gene encoding an "enhanced" green fluorescent protein (*GFP*).[3]

We wished to express the human *CPT1B* gene in human skin fibroblasts, taking the following facts into consideration:

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(1) *CPT1B* is normally not expressed in skin fibroblasts, therefore the *CPT1B* promoter itself would not be suitable for efficient expression of a *CPT1B* transgene in these cells. A constitutive or skin fibroblast-specific promoter is needed to express *CPT1B,* for that reason we use the human cytomegalovirus immediate early (CMV-IE) promoter, a broadly used strong promoter for transgene expression in mammalian cells.
(2) Human skin fibroblasts are generally regarded as recalcitrant to transfection. Various transformation procedures have been applied by different groups,[4,5,6,7] each claiming different levels of relative and absolute success. We wished to transiently express CPT1-GFP in order to get an impression of what would be possible with regard to transfection efficiencies, in our hands. If promising efficiencies could be obtained, an optimised protocol may allow functional studies.
(3) As human skin fibroblasts are recalcitrant to transfection, we wished to include an easily traceable marker, which can either be used as an in-frame fusion protein, or as a separately expressed protein. The widely applicable green fluorescent protein (GFP) reporter system, originally isolated from the jellyfish *Aequorea victoria,* and adapted for efficient expression in human cells and for, elevated levels of fluorescence,[3,8] provides these properties. The GFP system has been used for example, to study protein trafficking to the mitochondrial matrix[9] and the mitochondrial outer membrane.[10] Therefore, by using confocal laser scanning imaging we may get an impression of the intracellular localization of a CPT1-GFP fusion protein after transient or stable transfection.
(4) Since we aim to study CPT1 deficiencies in a conditional knockout mouse model (see elsewhere in this volume), the application of a fused marker protein in gene alteration approaches would serve a long-term goal. Applications like the monitoring of gene expression before (knock-in) and after a conditionally mediated knockout event would provide important tools in gene function analyses. Using the human gene for M-CPT1, experiments with fusions to GFP may provide important data to be applied in gene-targeting of the murine *CPT1* genes.
(5) Once the localisation of mitochondrial enzymes in a two- or three-dimensional perspective is studied, it is important to realise what may be expected. In textbooks, schematic representations of the three-dimensional shape of mitochondria are often globular structures which resemble flattened balls rather than flexible tubes (see e.g. reference,[11] page 487). It should be stressed however, that in real life mitochondria may "line up" or may be tubular shaped and have a thread-like, sometimes branched, appearance, as shown in reference,[11] on page 483 and 485.

A nearly full length coding region for human muscle-type CPT1, based on a complementary DNA sequence of the *CPT1B* gene[2] was fused to enhanced *GFP* in the mammalian expression vector pEGFP-N2 (Clontech, Palo Alto, CA, USA). Since the CPT1 C-terminal part is expected to be a much larger domain than its N-terminal counterpart relative to the transmembrane domains,[1,12] we chose to fuse GFP to the C-terminus of CPT1 as we aimed to achieve optimal freedom of the GFP domain to reach its fluorescent state. In the resulting construct the CMV-IE promoter drives the expression of the fused genes, and the transcript encodes CPT1 as the N-terminal domain, with GFP at the C-terminus. The plasmid provides a *neo* gene, driven by two promoters for selection in bacteria and mammalian cells.

We used two protocols for skin fibroblast transfection: one commercially available preparation (Lipofectamine, Life Technologies, Paisley, Scotland) was applied according to the manufacturers instructions, and the other protocol was according to the calcium precipitation method,[13] with slight adaptations.[14] The former protocol is used in our laboratories for transient expression in hamster lung fibroblasts, and the latter for stable transfection of hamster lung fibroblasts and both transient- and stable transfection of the human cervix epithelial carcinoma cell line HeLa. The selective dose of G418 (Geneticin, Life Technologies) for human skin fibroblasts was determined in a pilot experiment. Geneticin selection was applied at 250 μg/ml after calcium phosphate transfection. In the Lipofectamine transfection experiments the cells were split into one half of the population at which G418 selection was applied, and one half which was allowed to attach to coverslips for transient expression assessment without G418 selection.

Images were taken with a confocal laser scanning microscope (True Confocal Scanner 4D, Leica, Heidelberg, Germany) equipped with an argon-krypton laser and coupled to a Leitz DM IRB inverted microscope (Leica).[15] Excitation was at 488 nm, and emission was band-pass filtered at 515nm.

An assessment of the transfection efficiency of human skin fibroblasts, compared to transfection of hamster lung fibroblasts and Human HeLa cells, indicated that the protocols we used are not suitable to obtain stable transfected skin fibroblasts. We concluded this from the fact that no stable transfected colonies of human skin fibroblasts were obtained, regardless of being CPT1-defective or being from a patient with a disorder unrelated to β-oxidation. Only transient expression with the Lipofectamine method was successful. We found an efficiency of 0.0-0.2 % of the cells which survived transfection without selection. However, compared to the efficiencies of transfection in hamster lung fibroblasts (5-10% in a parallel experiment), this still was low. Therefore, for long-term applications it is clear that different procedures should be used than the ones we applied here.

The low transfection efficiencies are, however, compensated for by the confocal laser scanning images we obtained of the few cells transiently expressing CPT1-GFP. As illustrated, these cells showed a promising result with respect to intracellular localisation of green fluorescent signal (Fig. 1). Punctuated and thread-like structures are clearly more fluorescent than the background level in the cytosol, and the nucleus remains clear of green fluorescent signal. These images are comparable to the microscopical data known from a variety of cells with mitochondrion specific stains like Rhodamine 123,[11] or GFP fusions targeted to the mitochondrial matrix.[9] Moreover, in human skin fibroblasts, the compound 2-(4-(dimethylamino)styryl)-l-methylpyridinium iodide has been successfully used to image mitochondria, showing comparable signal distribution patterns,[16] albeit that we sometimes could observe more distally localized fluorescent signals, putatively from mitochondria situated near the sites where the cells were attached to the coverslip glass. The low number of cells we have observed does not allow firm conclusions with respect to intracellular distribution of CPT1-GFP in relation to cytoskeletal compounds near these attachment sites.[17]

Control experiments with non-fused enhanced GFP resulted in homogeneously distributed green fluorescent signal, not only in the cytosol, but also in the nucleus. This uniform distribution of non-fused GFP was expected from previous reports.[3,8] Taken these facts together, our results with transient expression of CPT1-GFP in human skin fibroblasts are highly suggestive for targeting of the fusion protein to mitochondria. To investigate the targeting and topology of the CPT1-GFP fusion in more detail

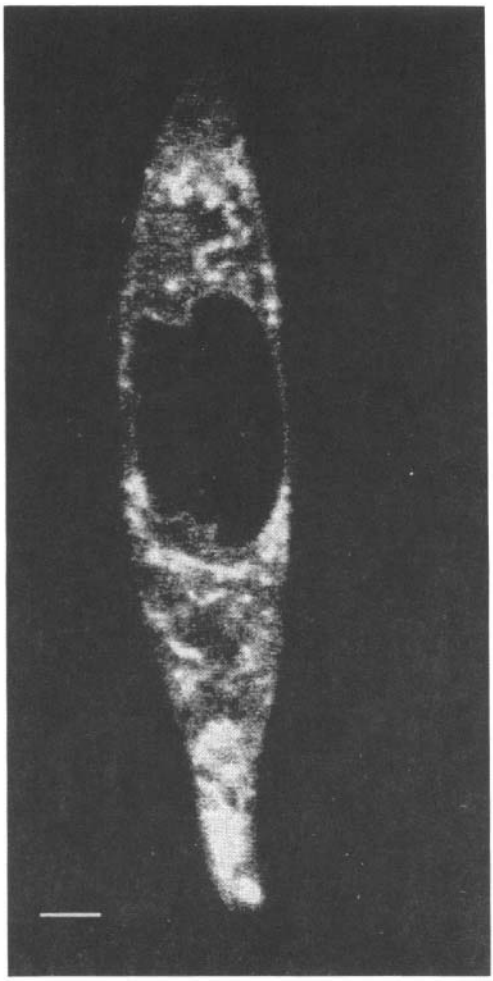

Figure 1. Transient expression of a green fluorescent carnitine palmitoyltransferase 1 fusion protein in a transfected human skin fibroblast. The intracellular distribution of the fusion protein is predominantly restricted to structures, putatively representing mitochondria. Bar represents 5 μm.

we recently have obtained stable transfected HeLa cells. These cells are currently subjected to more detailed microscopical and functional analyses (van der Leij *et al.,* in preparation).

We conclude that for long-term expression studies in human skin fibroblasts, the transfection methods we applied in this study are not appropriate. This may be improved when other transfection methods are applied. In that respect, a combination of a receptor-targeted transfection method and an immortalization strategy[7] seems attractive, since this would overcome the limited number of cell cycles of skin fibroblasts. High resolution imaging of the few cells we obtained made this study a valuable exercise, however. Much could be learned already from the few transfected cells we observed. The intracellular heterogenous distribution of CPT1-GFP points to mitochondrial targeting of this fusion protein, and these results are an encouraging basis for ongoing and future experiments.

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REFERENCES

1. McGarry, J.D. & Brown, N.F. (1997). *Eur J Biochem* **244**:1–14. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis.
2. van der Leij, F.R., Takens, J., van der Veen, A.Y., Terpstra, P. & Kuipers, J.R.G. (1997). *Biochim Biophys Acta* **1352**:123–128. Localization and intron usage analysis of the human CPT1B gene for muscle-type carnitine palmitoyltransferase I.
3. Zhang, G., Gurtu, V. & Kain, S.R. (1996). *Biochem Biophys Res Commun* **227**:707–711. An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells.
4. Fountain, J.W., Lockwood, W.K. & Collins, F.S. (1988). *Gene* **68**:167–172. Transfection of primary human skin fibroblasts by electroporation.
5. Ray, J. & Gage, F.H. (1992). *Biotechniques* **13**:598–603. Gene transfer into established and primary flbroblast cell lines: comparison of transfection methods and promoters.
6. Mayne, L.V., Priestley, A., James, M.R. & Burke, J.F. (1986). *Exp Cell Res* **162**:530–538. Efficient immortalization and morphological transformation of human fibroblasts by transfection with SV40 DNA linked to a dominant marker.
7. Veelken, H., Jesuiter, H., Mackensen, A., Kulmburg, P., Schultze, J., Rosenthal, F., Mertelsmann, R. & Lindemann, A. (1994). *Hum Gene Ther* **5**:1203–1210. Primary fibroblasts from human adults as target cells for ex vivo transfection and gene therapy.
8. Zolotukhin, S., Potter, M., Hauswirth, W.W., Guy, J. & Muzyczka, N. (1996). *J Virol* **70**:4646–4654. A "humanized" green fluorescent protein cDNA adapted for high-level expression in mammalian cells.
9. Rizzuto, R., Brini, M., De Giorgi, F., Rossi, R., Heim, R., Tsien, R.Y. & Pozzan, T. (1996). *Curr Biol* 6:183–188. Double labelling of subcellular structures with organelle-targeted GFP mutants in vivo.
10. Wolter, K.G., Hsu, Y.T., Smith, C.L., Nechushtan, A., Xi, X.G. & Youle, R.J. (1997). *J Cell Biol* 139:1281–1292. Movement of Bax from the cytosol to mitochondria during apoptosis.
11. Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. & Watson, J.D. (1983). *Molecular biology of the cell.* Garland Publishing, Inc., New York and London.
12. Fraser, F., Corstorphine, C.G. & Zammit, V.A. (1997). *Biochem J* **323**:711–718. Topology of carnitine palmitoyltransferase I in the mitochondrial outer membrane.
13. Graham, F.L. & Eb, A.J.v.d. (1973). *Virology* **52**:456–467. A new technique for the assay of infectivity of human adenovirus 5 DNA.
14. Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G. & Chasin, L. (1979). *Proc Natl Acad Sci USA* **76**:1373–1376. DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells.
15. Roelofsen, H., Vos, T.A., Schippers, I.J., Kuipers, F., Koning, H., Moshage, H., Jansen, PL. & Muller, M. (1997). *Gastroenterology* **112**:511–521. Increased levels of the multidrug resistance protein in lateral membranes of proliferating hepatocyte-derived cells.
16. Ruiters, M.H., van Spronsen, E.A., Skjeldal, O.H., Stromme, P., Scholte, H.R., Pzyrembel, H., Smit, G.P., Ruitenbeek, W. & Agsteribbe, E. (1991). *J Inherit Metab Dis* **14**:45–48. Confocal scanning laser microscopy of mitochondria: a possible tool in the diagnosis of mitochondrial disorders.
17. Bereiter-Hahn, J., Luck, M., Miebach, T., Stelzer, H.K. & Voth, M. (1990). *J Cell Sci* **96**:171–188. Spreading of trypsinized cells: cytoskeletal dynamics and energy requirements.

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CARNITINE BIOSYNTHESIS

Purification of γ-butyrobetaine Hydroxylase from rat liver

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1. SUMMARY

γ-Butyrobetaine hydroxylase catalyses the last step in carnitine biosynthesis, the formation of L-carnitine from γ-butyrobetaine, a reaction dependent on Fe^{2+}, α-ketoglutarate, ascorbate and oxygen. Initial attempts to purify the protein from rat liver showed that γ-butyrobetaine hydroxylase is unstable. We, therefore, determined the influence of various compounds on the stability of γ-butyrobetaine hydroxylase at different storage temperatures. The enzyme activity was best conserved by storing the protein at 4°C in the presence of 200 g/l glycerol and 10mM DTT. We subsequently purified the enzyme from rat liver to apparent homogeneity by liquid chromatography.

2. INTRODUCTION

Carnitine was discovered in the beginning of this century in muscle tissue extracts.[1] For a long time, the physiological role of carnitine remained obscure. An important observation was made by Fraenkel and Friedmann when they identified an essential factor required for growth of the meal worm *Tenebrio molitor*.[1,2] The identity of this essential component which they named Vitamin B_T, was later resolved by Carter *et al.*[2] as 3-hydroxy-4-trimethylaminobutyric acid (carnitine). These studies were soon followed

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by reports which shed new light on the physiological function of carnitine. Indeed, studies by Fritz[3] revealed that oxidation of palmitic acid in liver slices was greatly stimulated by adding muscle extract which turned out to be due to the carnitine present in these extracts. In the same year, Friedman and Fraenkel[4] discovered the reversible enzymatic acetylation of carnitine. This pioneering work led to the recognition that carnitine plays an indispensable role in mitochondrial fatty acid oxidation as essential element of the carnitine cycle. This cycle mediates the transport of fatty acids across the mitochondrial membrane via the concerted action of carnitine palmitoyltransferase 1 (CPT1), the mitochondrial carnitine/acylcarnitine carrier (CAC) and carnitine palmitoyltransferase 2 (CPT2).

In humans, carnitine is either obtained from the diet or synthesised *de novo* (Fig. 1). Carnitine biosynthesis in higher eukaryotes starts when protein-bound L-lysine is trimethylated by a protein-dependent methyltransferase to form ε-N-trimethyllysine.[5] Upon degradation of these proteins, free ε-N-trimethyllysine becomes available and is hydroxylated at the 3-position by ε-N-trimethyllysine hydroxylase.[5,6] Subsequently, β-hydroxy-ε-N-trimethyllysine is cleaved into γ-trimethylaminobutyraldehyde and glycine by β-hydroxy-ε-N-trimethyllysine aldolase,[5,6] after which the aldehyde is oxidized by γ-trimethylaminobutyraldehyde dehydrogenase to yield γ-butyrobetaine.[5,6,7] Finally, γ-butyrobetaine is hydroxylated at the 3-position by γ-butyrobetaine hydroxylase (γ-BBH) to produce L-carnitine[5,6,8] (see Fig. 1).

Of all the enzymes of the carnitine biosynthetic pathway, γ-BBH is the best-studied enzyme. Like ε-N-trimethyllysine hydroxylase, γ-BBH is a non-heme ferrous-iron dioxygenase that requires α-ketoglutarate, Fe^{2+} and molecular oxygen as cofactors.[9] In this class of enzymes, the hydroxylation of the substrate is linked to the oxidative decarboxylation of α-ketoglutarate. Ascorbate is needed to maintain iron in the reduced state. γ-BBH has been isolated from various sources including human kidney,[10,11] calf[12] and rat liver[13,14] and the bacterium Pseudomonas AK1.[15] A common problem in the purification of this enzyme from mammalian tissues is the poor stability of the protein.[16] We, therefore, determined the optimal storage conditions and subsequently purified γ-butyrobetaine hydroxylase from rat liver.

3. EXPERIMENTAL PROCEDURES

3.1. Materials

Q-sepharose, chromatofocusing PBE 94, phenyl sepharose HP, G25 sephadex (fine), were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden), and hydroxylapatite CHT-II, from Biorad (Hercules, USA). All other reagents were of analytical grade.

3.2. Determination of Storage Conditions

Rat liver was homogenised in 50 mM sodium phosphate buffer, pH 7.0, containing 20 mM KC1 by five passes of a Teflon pestle in a Potter-Elvehjem glass homogenizer at 500rpm. The crude homogenate was centrifuged for 60min at 20,000 x g at 4°C and the supernatant desalted on a Sephadex G25 column. Various compounds were added to the supernatant, which was stored at 4°C, –20 °C and –80 °C. After one and two weeks, γ-BBH activity was determined and compared with the activity in the fresh supernatant.

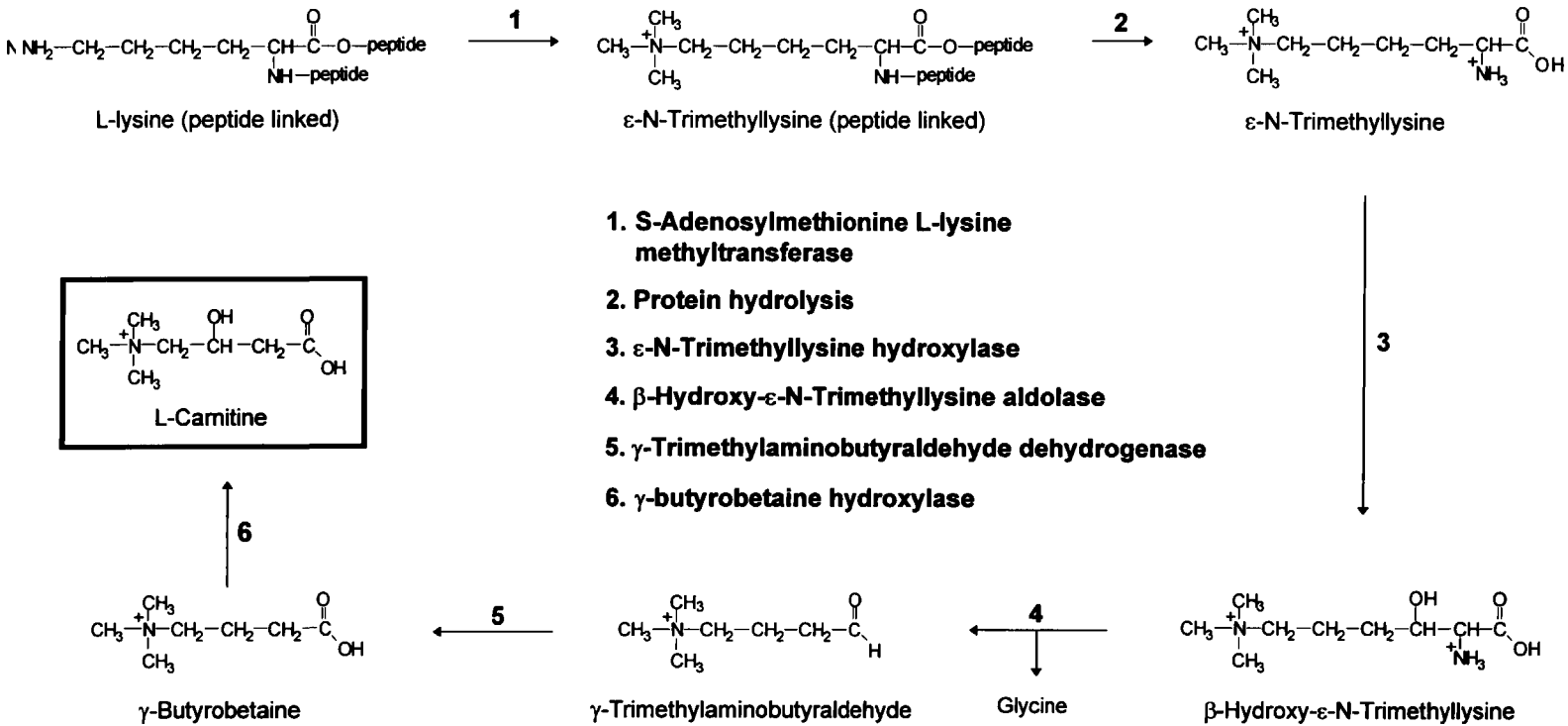

Figure 1. Schematic overview of carnitine biosynthesis.

3.3. γ-BBH Activity Measurement

γ-BBH activity was determined according to Lindstedt[15] followed by a radioisotopic determination of the produced carnitine[17] Protein concentrations were determined by the method of Bradford,[18] using bovine serum albumin as standard.

4. RESULTS

Table I shows the recovery of specific activity in cytosolic fraction of rat liver containing several additives after 1 and 2 weeks of storage. Recoveries are expressed as the percentage of the activity in the fresh cytosolic fraction homogenised in 50 mM sodium phosphate buffer, pH 7.4, containing 20mM KCl. γ-BBH activity was best conserved at 4°C in the presence of 10mM DTT and 200 g/1 glycerol. Addition of co-factors like sodium ascorbate, α-ketoglutarate and $(NH_4)_2Fe(SO_4)_2$ or combinations of the former did not appear to stabilise the enzyme. Addition of EDTA resulted in total loss of enzyme activity.

After determining the optimal storage condition, γ-BBH was purified from rat liver by liquid chromatography. The purification procedure was adapted from Lindstedt *et al.*[15] All protein isolation steps were carried out at 4°C. The livers of five Wistar rats were homogenised in a 50mM potassium phosphate buffer, pH 6.8, containing 20mM KCl and 2mM dithiothreitol (DTT), by five passes of a Teflon pestle in a Potter-Elvehjem glass homogenizer at 500 rpm. The crude homogenate was centrifuged for 60min at 20,000 x g. The supernatant was filtered through a column of glass pearls to remove floating fat particles. This filtrate was applied onto a Q-sepharose column (140ml), which was pre-equilibrated with a 20mM sodium phosphate buffer, pH 6.8, containing 75 mM KCl. The column was eluted with a linear gradient from 75 to 350mM KC1 in the same buffer. γ-BBH activity eluted as a single peak between 200 and 240 mM KCl (Fig. 2). Fractions 43-55 were pooled and concentrated with an Amicon Ultrafiltration unit using a YM10 membrane. The concentrate was dialysed against a 25 mM sodium phosphate buffer, pH 7.0, containing 100g/l glycerol, 2mM DTT and loaded on to an Econo-Pac CHT-1II hydroxylapatite column (5ml) equilibrated with the same buffer. Bound proteins were eluted with a linear gradient from 25 to 150 mM sodium phosphate. γ-BBH activity eluted as a single peak between 75mM and 100mM sodium phosphate (Fig. 3). Fractions 10 to 14 were pooled and dialyzed against a 10mM sodium phosphate buffer, pH 7.0, containing 100 g/1 glycerol and 2mM DTT. This dialysate was applied on to a PBE 94 chromatofocusing column (15ml) pre-equilibrated with a 25 mM imidazole/HCl buffer, pH 6.5, and eluted with 125ml/1 Polybuffer 74, pH 4.6. The pH of the fractions was immediately adjusted to 7.4 by the addition of 1/10 volume of 1M sodium phosphate buffer, pH 7.4. γ-BBH activity was determined in the fractions and found to elute in a single peak at pH of 5.2 (Fig. 4). Fractions 32–40 were pooled and dialysed against a 20mM sodium phosphate buffer, pH 7.4, containing 100 g/1 glycerol and 2mM DTT. An equal volume of the dialysis buffer supplemented with 1M ammonium sulphate was slowly added to the dialysate. Precipitated proteins were removed by centrifugation and the supernatant applied onto a phenyl sepharose HP column (2ml) pre-equilibrated with a 20mM sodium phosphate buffer, pH 7.4, containing 100g/l glycerol, 2mM DTT and 0.5 M ammonium sulphate. Proteins were eluted with a linear gradient from 0.5 to 0.15 M ammonium sulphate in the same buffer. Fractions were tested for γ-BBH activity and

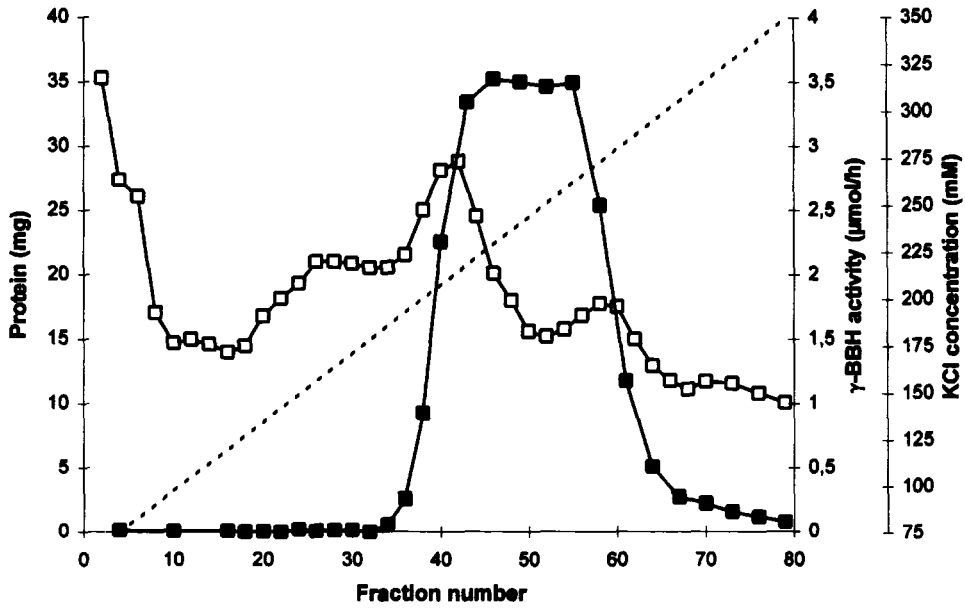

Figure 2. Fractionation of rat liver cytosol on Q-sepharose column. (□—□) protein (mg), (■—■) γ-BBH activity (µmol/min) and (----) the KCl concentration (mM).

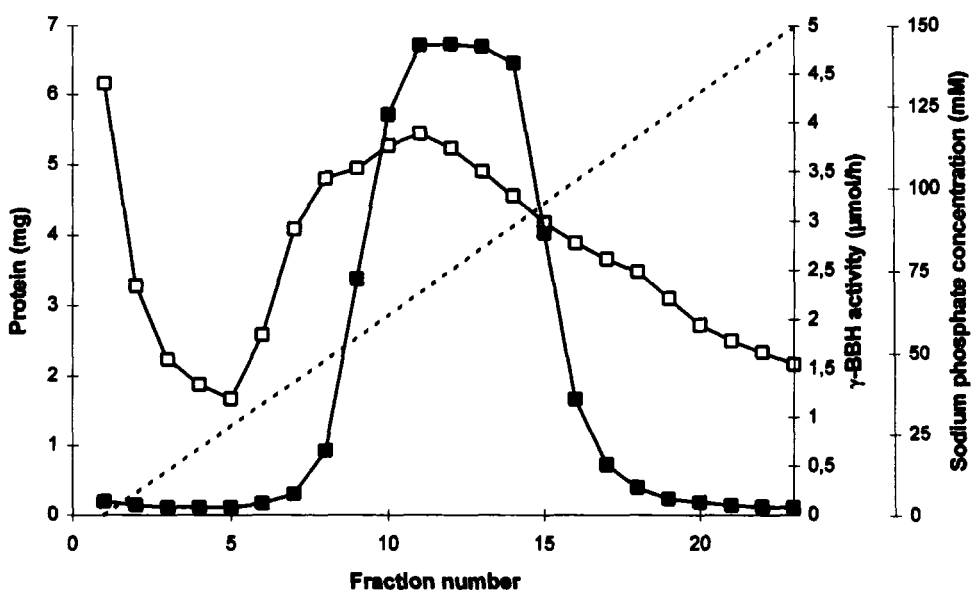

Figure 3. Fractionation of Q-sepharose pool by hydroxylapatite chromatography. (□—□) protein (mg), (■—■) γ-BBH activity (µmol/min) and (----) the sodium phosphate concentration (mM).

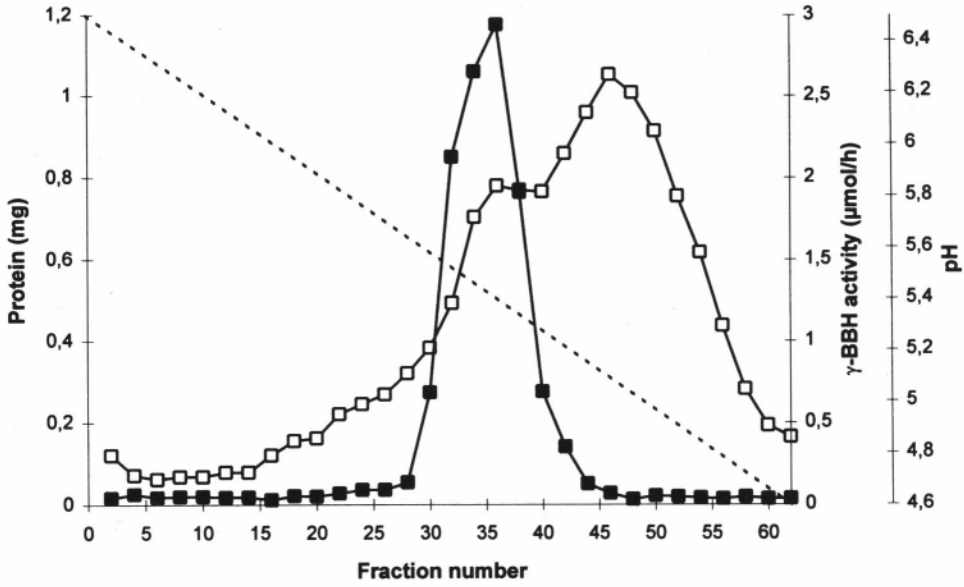

Figure 4. Fractionation of hydroxylapatite pool on PBE 94 chromatofocusing column. (□—□) protein (mg), (■—■) γ-BBH activity (μmol/min) and (----) the pH.

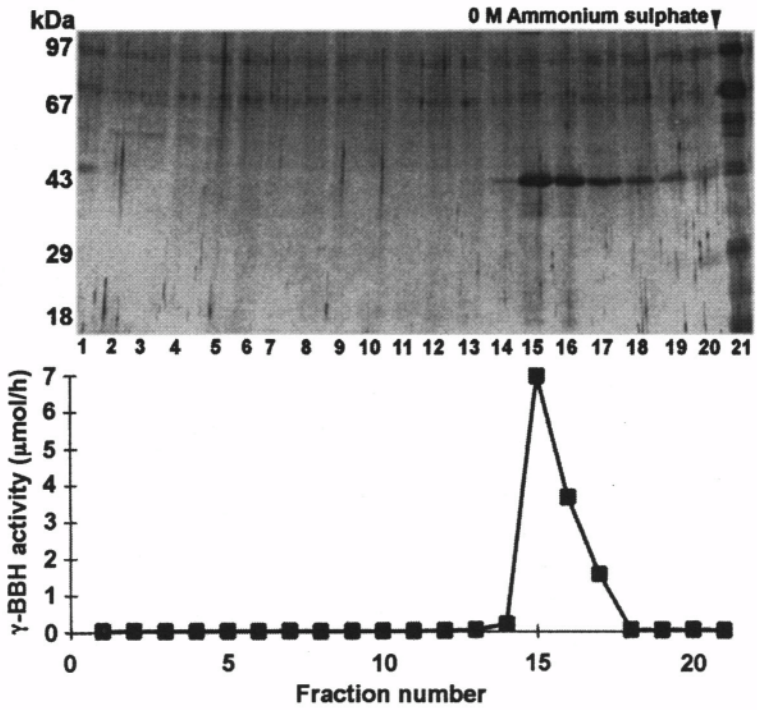

Figure 5. Purification of γ-BBH by phenyl sepharose HP chromatography. Analysis of column fractions by silver stained SDS-PAGE with the corresponding γ-BBH activity profile.

analysed by SDS-PAGE followed by silver staining. The γ-BBH activity was found to co-elute with a 44kDa protein (Fig. 5).

5. DISCUSSION

γ-BBH was purified to homogeneity by a four-step column chromatographic method. First, the influence of various compounds on γ-BBH activity at different storage temperatures was determined. Storing the homogenate at 4°C in the presence of 200g/l glycerol and 10mM DTT was found optimal for storage during the purification. For long term storage, -20 °C with the same additives is recommended An important observation was that addition of EDTA results in total loss of γ-BBH activity, which is probably caused by the chelation of γ-BBH bound Fe^{2+}. Addition of Fe^{2+} in the presence or absence of ascorbate had no effect on the stability of the enzyme.

γ-BBH was subsequently purified 280-fold from rat liver cytosol and identified as a 44 kDa protein by SDS-PAGE analysis, which agrees with the previously purified rat liver γ-BBH.[13,14] The purified protein will be used to raise an antiserum and the N-terminal amino acid sequence will be determined with the ultimate goal to identify the cDNA encoding rat γ-BBH.

REFERENCES

1. Fraenkel, G. & Friedman, S. (1957) Carnitine. *In:* Vitamins & Hormones, edited by R.S. Harris, G.F. Marian & K.V. Thimann. New York: Academic, **15,** 73–118.
2. Carter, H.E., Bhattacháryya, P.K., Weidman, K..R. & Fraenkel, G. (1952) *Arch. Biochem. Biophys.,* **38**, 405-416, Chemical studies on vitamin B_T isolation & characterization as carnitine.
3. Fritz, I.B. (1955) *Acta Physiol. Scand.*, **34**, 367–385, The effect of muscle extracts on the oxidation of palmitic acid by liver slices & homogenates.
4. Friedman, S. & Fraenkel, G. (1955) *Arch. Biochem. Biophys.,* **59**, 491–501. Reversible enzymatic acetylation of carnitine.
5. Bremer, J. (1983) *Physiol. Rev*., **63,** 1420–1480, Carnitine-Metabolism and Functions.
6. Rebouche, C.J. & Engel, A.G. (1980) *Biochim. Biophys. Acta*, **630**, 22–29, Tissue distribution of carnitine biosynthetic enzymes in man.
7. Hulse, J.D. & Henderson, L.M. (1980) *J. Biol. Chem*., **255**, 1146–1151, Carnitine Biosynthesis: Purification of 4-N-trimethylaminobutyraldehyde dehydrogenase from beef liver.
8. Englard, S. (1979) *FEBS Letters*, **102**, 297–300, Hydroxylation of γ-butyrobetaine to carnitine in human and monkey tissues.
9. Lindstedt, G. & Linstedt, S. (1970) *J. Biol. Chem.,* **245**, 4178–4186, Cofactor requirements of γ-butyrobetaine hydroxylase from rat liver.
10. Lindstedt, S. & Nordin, I. (1984) *Biochem. J*., **223**, 119–127, Multiple forms of γ-butyrobetaine hydroxylase (EC 1.14.11.1).
11. Lindstedt, G., Lindstedt, S. & Nordin, I. (1982) *Scand. J. Clin. Lab. Invest*., **42**, 477–485, γ-Butyrobetaine hydroxylase in human kidney.
12. Kondo, A., Blanchard, J.S. & Englard, S. (1981) *Arch. Biochem. Biophys.,* **212**, 338–346, Purification and properties of calf liver γ-butyrobetaine hydroxylase.
13. Lindstedt, G. (1967) *Biochemistry,* **6**, 1271–1282, Hydroxylation of γ-butyrobetaine to carnitine in rat liver.
14. Galland, S., Leborgne, F., Guyonnet, D., Clouet, P. & Demarquoy, J. (1998) *Mol. Cell. Biochem.,* **178**, 163–168, Purification and characterization of the rat liver γ-butyrobetaine hydroxylase.
15. Lindstedt, G, Lindstedt, S. & Nordin, I. (1977) *Biochemistry,* **16**, 2181–2188, Purification and properties of γ-butyrobetaine hydroxylase from Pseudomonas sp AK 1.
16. Carter, A.L., Abney, T.O. & Lapp, D.F. (1995) *J. Child Neurol,* **10(Suppl)**, 2S3–2S7, Biosynthesis and metabolism of carnitine.

17. Barth, P.G., Scholte, H.R., Berden, J.A. Van der Klei-Van Moorsel, J.M., Luyt-Houwen, I.E., Van't Veer-Korthof, E.T., Van der Marten, J.J. & Sobotka-Plojhar, M.A. (1983) *J. Neurol. Sci.,* **62**, 327–355, An X-linked mitochondrial disease affecting cardiac muscle, skeletal muscle and neutrophil leucocytes.
18. Bradford, M.M. (1976) *Anal. Biochem.*, **72**, 248–254, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.

HYPOLIPIDEMIC 3-THIA FATTY ACIDS

Fatty Acid Oxidation and Ketogenesis in Rat Liver under Proliferation of Mitochondria and Peroxisomes

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High serum levels of triglyceride (TG)-rich lipoproteins, i.e. very low density lipoproteins (VLDL) and its remnants are important risk factors for coronary artery disease.[1] Serum TGs can be lowered either by dietary treatment with fish oils or by pharmacological treatment with drugs of the fibrate class. Classically, the decrease in plasma TG concentrations upon fibrate treatment (Table 1) are thought to be the result of a decreased hepatic secretion of VLDL accompanied by an enhanced plasma TG clearance, possibly due to the induction of lipoprotein lipase (LPL) activity in peripheral tissues.[2]

Several lines of evidence have implicated apolipoprotein (apo) C-III in plasma TG metabolism. Reports have indicated that apo C-III inhibits TG hydrolysis by LPL and hepatic lipase *in vitro* and impairs the uptake of TG-rich lipoproteins by the liver. Moreover, transgenic animal studies, in which the plasma TG levels are proportional to plasma apo C-III concentrations and liver apo C-III gene expression, provided more direct evidence for the causal involvement of apo C-III in hypertriglyceridemia. Recently, it has been shown that fibrate downregulates apo C-III expression (Table 1) and this may contribute for the hypotriglyceridemic action of these drugs.[2]

In addition to lowering plasma TG, n-3 fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) are reported to have a number of additional beneficial effects on the cardiovascular system which include antihypertensive and antithrombotic actions.[3–5] EPA and DHA are major fatty acid constituents of fish oil and it has been assumed that both EPA and DHA are responsible for its hypotriglyceridemic activity. However, growing evidence indicates that EPA and DHA may possess different hypolipidemic properties. We have reported that EPA is the fatty acid primarily responsible for the TG-lowering effect of fish oil (Table 1), but the mechanism underlying this hypotriglyceridemic effect has not yet been fully elucidated. Interestingly, however, we

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Table 1. Effect of EPA, DHA, fibrate and TTA on plasma TGs and liver ApoC-III mRNA levels in rats.

Group	Plasma TG	Plasma VLDL-TG	ApoC-III mRNA
Fenofibrate	↓	↓	↓
DHA	→	→	→
EPA	↓	↓	→
TTA	↓	↓	↓

↓ the values are decreased to values for control group which are set as 100

→ the values are unchanged compared to control group

have reported that EPA, but not DHA, increased mitochondrial fatty acid oxidation in liver of rats (Table 2).

In the liver, mitochondrial fatty acid oxidation and TG-biosynthesis are the major competitors for the utilization of fatty acids as substrate. The regulatory interrelationship between β-oxidation and TG-biosynthesis has not yet been elucidated in detail by fibrates and n-3 fatty acids. Growing evidence indicates that availability of TG is a major driving force in the secretion of TG-lipoproteins by the liver. It is, therefore, conceivable that factors influencing TG-biosynthesis of fatty acid oxidation may ultimately influence plasma lipoprotein levels and metabolism.

Sulfur-substituted fatty acids, especially the 3-thia fatty acids (TTA, CH_3-$(CH_2)_{13}$-S-CH_2-COOH), that profoundly affect β-oxidation, have facilitated studies on the concerted regulation of fatty acid oxidation and TG-biosynthesis. We have demonstrated that stimulation of β-oxidation (Table 2) may affect both TG-formation (Table 1) and plasma lipoprotein homeostasis (Table 1) under both normolipidemic and hyperlipidemic conditions.

TTA is a fatty acid analogue in which a sulfur atom replaces the β-mehylene groups in the alkyl-chain (a 3-thia fatty acid). TTA, therefore, cannot be β-oxidized. Paradoxically, TTA is both mitochondrial and peroxisomal proliferator and the hepatic mitochondrial and peroxisomal fatty acid oxidation capacities are increased (Table 2). In addition to its biochemical and morphological effects, TTA decrease serum TG (Table 1) very low density lipoprotein (VLDL)-TG, cholesterol and free fatty acid (NEFA) levels in rats. Thus, the observed reduction in plasma TG levels during TTA administration could be accomplished by retarded synthesis, reduced hepatic output, enhanced clearance or a combination of these factors. 3-Thia fatty acid resulted in a slight inhibition in the activities of ATP-citrate lyase and fatty acid synthase.[6] However, the impact of

Table 2. Effect of EPA, DHA, fibrates and TTA on mitochondrial and peroxisomal β-oxidation (measured as fatty acyl-CoA oxidase) and proliferation in rat liver.

Group	Mitochondrial β-oxidation	Volume fraction of mitochondria	Peroxisomal β-oxidation	Volume fraction of peroxisomes
Fenofibrate	↑	ND	↑	ND
EPA	↑	↑	↑	↑
DHA	→	→	↑	↑
TTA	↑	↑	↑	↑

↑ increased compared to controls

→ unchanged compared to controls

↓ decreased compared to controls

ND not determined

these effects on lipogenesis, and consequently TG-biosynthesis, is not self-evident. On one hand, an inhibition of two of the enzymes involved in fatty acid synthesis is consistent with a retarded lipogenesis. On the other hand, the finding that the activity of the enzyme considered to be rate-limiting in fatty acid synthesis i.e. acetyl-CoA carboxylase, was unaffected by TTA treatment, argues against the contention that decreased fatty acid synthesis is of major importance for the TG-lowering effect observed. As NEFA levels decreased, TTA acid treatment might interfere with both the exogenous and the endogenous supply of hepatic fatty acids, affecting their availability for esterification and TG-biosynthesis. In addition, the observed inhibition of phosphatidate phosphohydrolase activity during 3-thia fatty acid[7] would further contribute to a lower TG-synthetic rate. The parallel decrease in phosphatidate phosphohydrolase activity and the hepatic and plasma TG concentrations suggests that the hypotriglyceridemic effect of TTA level may be largely due to its effect on the synthetic level. Also, this underscores the regulatory importance of phosphatidate phosphohydrolase in TG-biosynthesis.[8] In contrast, both glycerol 3-phosphate and diacylglycerol acyltransferase activities[7] increased when hepatic TG-synthesis and secretion were retarded. This argues against the initial and the last esterification steps[9] in TG-synthesis being potential sites at which TTA might modulate TG-synthesis.

A finding in the present study was the effect of TTA treatment on the lipoprotein fractions, with a decrease in both VLDL-TG (Table 1) and low density lipoprotein (LDL)-cholesterol levels. The decrease in VLDL-TG was associated with a reduction (data not shown) in the secretion of newly synthesized TG (Table 1 and Fig. 1). Since VLDL is a precursor to LDL, it is conceivable that the observed reduction in VLDL secretion and plasma VLDL levels will affect LDL formation, which might contribute to the lowering of LDL cholesterol levels. It is of interest that the reduction in plasma TG levels was slightly more pronounced (38%) than the decrease in the VLDL-TG secretion (25%), implying that alterations in the clearance of VLDL-TG might contribute to the decrease in plasma TG. In agreement with this, the activities of plasma LPL and hepatic

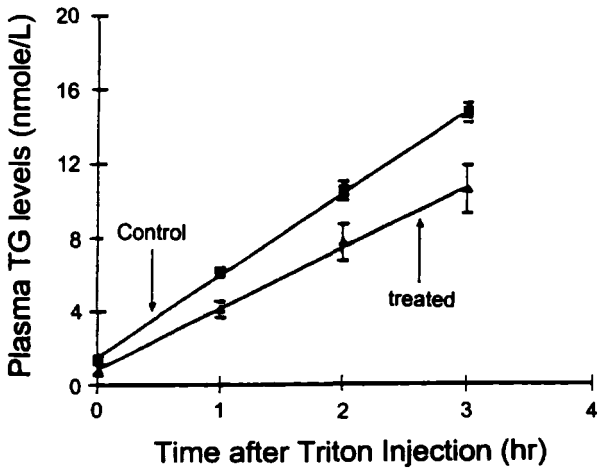

Figure 1. Effect of 3-thia fatty acid on plasma triglyceride entry rate after a single intravenous injection of Triton WR 1339. The values are expressed as means ± SD for 6 rats in each experimental group. To convert mmol/L to mg/dL, multiply by 88.5. Data from.[18]

lipase were somewhat higher (10-20%) in TTA treated animals, indicating a possible increase in the clearance potential of TG-rich lipoproteins. Moreover, the down-regulation of liver apo C-III gene expression of TTA may contribute to the hypotriglyceridemic action of this class of drugs. However, no difference was found in either the chemical composition of the apolipoprotein pattern in isolated VLDL from control or 3-thia fatty acid-treated rats, arguing against any larger conformational changes that could affect their catabolism (data not shown). At present, we cannot, therefore, exclude that minor conformational changes in VLDL could be induced by TTA administration, and further studies are needed to establish whether treatment with this drug affects the metabolic properties of VLDL. Most plausible TTA will lower the number of VLDL-particles.

In hepatocytes cultured in the presence of oleic acid, incorporation of [^{3}H]-water into secreted lipids and TG was lower than incorporation into synthesized lipids with the 3-thia fatty acids.[10] A similar phenomenon was observed with [^{3}H]-glycerol as the radioactive precursor. This suggests that some of the hypotriglyceridemic effects of 3-thia fatty acids may arise from a reduction in biosynthesis and/or secretion of TG.

The degree of inhibition of [^{3}H]-water incorporation into TG and phospholipids (PL) was at approximately the same level, indicating that the 3-thia fatty acids may affect a common step in the biosynthesis of these lipids.

The incorporation of [^{3}H]-water into synthesized diacylglycerols was reduced even more than the incorporation into the other two lipid classes. As the sulfur-substituted fatty acids had only small effects on incorporation of [^{3}H]-water into cell monoacylglycerols (data not shown), these data suggest that 3-thia fatty acids decreased TG-synthesis by affecting the step before formation of diacylglycerols. The reduced incorporation into diacylglycerols, thus, may indicate that activity of the enzyme phosphatidate phosphohydrolase could be affected.

When the synthesis of lipids is reduced due to the presence of fatty acid analogs, the NEFAs will be diverted from the esterification pathway. The level of NEFAs in the hepatocytes treated with 3-thia fatty acids tended to decrease. This indicates that the mitochondrial β-oxidation was increased, as the peroxisomal β-oxidation was unchanged in these hepatocytes. Thus, it is likely that the non-β-oxidizable fatty acid analogs reduced the availability of fatty acids for TG-synthesis due to increased mitochondrial fatty oxidation. The lack of effect on the peroxisomal β-oxidation confirms the *in vivo* data that the hypotriglyceridemic effect of the analogs can be dissociated from the proliferation of peroxisomes.[11,12]

Malonyl-CoA, the product of the acetyl-CoA carboxylase reaction, is substrate for the biosynthesis of fatty acids. It is also an inhibitor of carnitine palmitoyltransferase (CPT), the enzyme regulating mitochondrial fatty acid oxidation. Long-chain acyl-CoA inhibits malonyl-CoA synthesis by inhibiting acetyl-CoA carboxylase.[13] An acute decrease in malonyl-CoA at 4–6 hr has been found in animals treated with TTA.[14,15] At that time the levels of long-chain acyl-CoA was increased. A rise in cellular acyl-CoA after administration of 3-thia fatty acids could result in inhibition of the enzyme acetyl-CoA carboxylase. Reduced levels of malonyl-CoA could then relieve inhibition of CPT and the mitochondrial fatty acid oxidation would be stimulated.[16]

Thus, it is likely that rate of TG-synthesis is controlled by coordinate regulation of the activities of mitochondrial β-oxidation and phosphatidate phosphohydrolase. The instant hypotriglyceridemic effect observed in rats given 3-thia fatty acids can be explained by a sudden increase in mitochondrial fatty acid oxidation and a decrease in phosphatidate phosphohydrolase.[7] This alteration is accompanied by a reduction in the

availability of substrates, i.e. fatty acids for glycerolipid synthesis, even before the 3-thia fatty acids induce peroxisomal fatty acid oxidation and microsomal ω-oxidation.

Mitochondrial CPT-I is rate-limiting for mitochondrial β-oxidation through different mechanism—1) as changes in activity and the transcription rate, 2) changes in the concentration of its physiological inhibitor malonyl-CoA, and 3) changes in its sensitivity to malonyl CoA.

Recently we have performed experiments in order to study the effects of a single dose (acute effects) and repeated administration of TTA (long-term effects) on the expression of rat hepatic genes related to peroxisomal and mitochondrial β-oxidation. In the acute study we have shown that the expression of several mitochondrial and peroxisomal fatty acyl-CoA ester metabolizing enzymes were rapidly increased in rats fed TTA, but the increase seemed to follow different time-courses. Interestingly, CPT-II mRNA and, especially, CPT-I mRNA were induced early, that is at 4 and 2h, respectively, after TTA administration (Fig. 2).

The gene expression of several enzymes involved in the peroxisomal and mitochondrial β-oxidation are regulated through peroxisome proliferator activated receptor (PPAR). To our knowledge, no response element for PPAR in the genes for CPT-I or CPT-II has yet been identified.

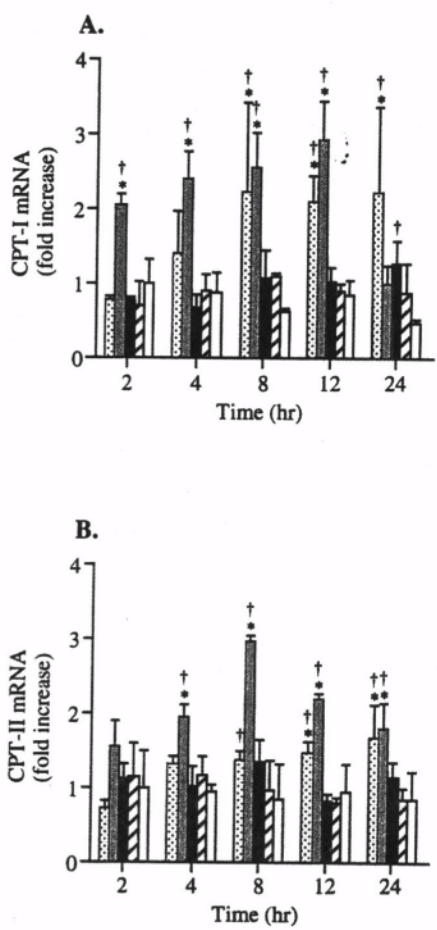

Figure 2. Time-courses on the effect of TD and TTA on relative mRNA levels of A) CPT-I and B) CPT-II. □ TTA 500 mg/kg body mass; ▩ TD 150 mg/kg body mass; ■ palmitic acid 150 mg/kg body mass.; ▨ palmitic acid 500 mg/kg body mass; □ CMC. Data are given as means ± S.D. and are from 3 different rats. *denotes significantly different from palmitic acid at equal dose, and †denotes significantly different from CMC. Data from H. Vaagenes *et al.* Biochem. Pharmacol. 1998 In press.

The major increase in the mRNA levels of fatty acyl-CoA oxidase did not appear until 8-12h after administration of TTA (Vaagenes H. *et. al.*, Biochem. Pharmacol. *in press).* The gene for this enzyme contains a PPAR-responsive element. It is, therefore, interesting to speculate whether PPAR is responsible for mediating the effects of TTA on CPT-I and/or CPT II which are induced earlier than fatty acyl-CoA oxidase.

In the long-term experiments i.e. repeated administration of TTA for days, we have shown that TTA accumulated in the hepatic tissues. Unexpectedly, under these conditions the CPT-I mRNA was not increased and the mitochondrial CPT-I activity was rather decreased. TTA can be activated to its CoA ester, and we have observed that TTA-CoA inhibits CPT-I activity. Taking the data together from the short-term versus long-term administration, it seems as if TTA has both an inhibitory and a stimulatory effect on CPT-I and there might be a balance between these two opposing effects of TTA. Thus, in the long-term study the result is an inhibitory effect of CPT-I possible due to accumulation of TTA-CoA. How the amount of TTA and/or TTA-CoA is correlated to the CPT-I activity will be studied further.

In contrast to CPT-I, the mRNA levels of CPT-II were increased in the long-term as well as in short-term experiments (Fig. 2). These results suggest that the regulation of fatty acid oxidation appears to be shifted to steps(s) beyond CPT-I and CPT-II may be an important locus in the regulation of hepatic fatty acid oxidation. The proposed sequence of oxidation of fatty acids involving peroxisomal and mitochondrial β-oxidation under peroxisomal and mitochondrial proliferation is depicted in Fig. 3. A possible explanation would be that fatty acids are partially oxidized in the peroxisomes and enter the mitochondria as medium chain fatty acids via peroxisomal CPT, like the phy-

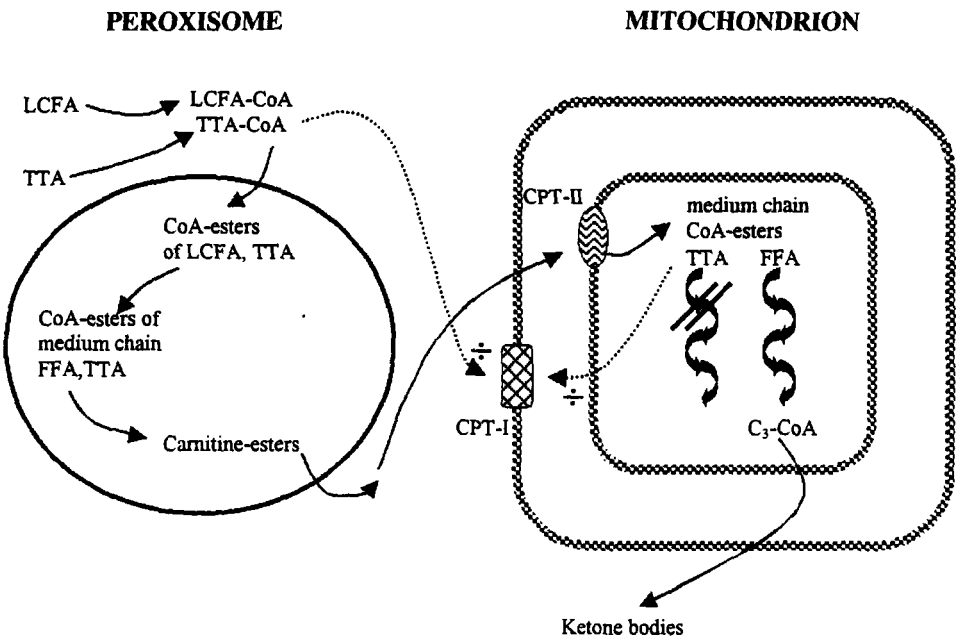

Figure 3. Model of the functional organization of oxidation of long-chain fatty acids (LCFA) and TTA in the liver of rats. +, inhibition.

tanic and pristanic acid.[17] This model implies formation of carnitine derivates of fatty acids which are good substrates for CPT-II. Indeed, increased carnitine-derivates of different fatty acids have been observed after TTA administration (data to be published).

Hepatic ketogenesis is mainly controlled by two systems: 1) entry of fatty acyl-CoA into mitochondria catalyzed by the CPT system and 2) enzymatic activity of mitochondrial 3-hydroxy-3-methyl glutaryl-coenzyme A (HMG-CoA) synthase. We have recently studied the regulation of ketogenesis at the level of gene expression of this protein after repeated administration of TTA. In these long-term experiments the plasma ketone bodies and hepatic mRNA levels of HMG-CoA synthase were increased. Moreover, immunodetectable proteins and activities of both HMG-CoA synthase and CPT-II were increased in the liver of TTA treated rats. This suggests the possibility that mitochondrial HMG-CoA synthase and CPT-II retain some control of fatty acid oxidation and ketone body formation under conditions when CPT-I is inhibited. Whether these enzymes retain some control over the relevant fluxes of fatty acid oxidation to ketone bodies will be investigated in more detail in the future.

REFERENCES

1. Hodis, H.N., Mack, W.J., Azen, S.P., Alaupovic, P., Pogoda, J.M., LaBree, L., Hemphill, L.C., Kramsch, D.M. & Blankenhorn, D.H. 1994. *Circulation* **90**:42–49. Triglyceride- and cholesterol-rich lipoproteins have a differential effect on mild/moderate and severe lesion progression as assessed by quantitative coronary angiography in a controlled trial of lovastatin.
2. Staels, B., Vu-Dac, N., Kosykh, V.A., Saladin, R., Fruchart, J.C., Dallongeville, J. & Auwerx, J. 1995. *J. Clin. Invest.* **95**:705–712. Fibrates downregulate apolipoprotein C-III expression independent of induction of peroxisomal acyl coenzyme A oxidase. A potential mechanism for the hypolipidemic induction action of fibrates.
3. Kinsella, J.E., Lokesh, B. & Stone, R.A. 1990. *Am. J. Clin. Nutr.* **52**:1–28. Dietary n-3 polyunsaturated fatty acids and amelioration of cardiovascular disease: possible mechanisms.
4. Bonaa, K.H., Bjerve, K.S., Straume, B., Gram, I.T. & Thelle, D. 1990. *N Engl. J. Med.* **322**:795–801. Effect of eicosapentaenoic and docosahexaenoic acids on blood pressure in hypertension. A population-based intervention trial from the Tromso study.
5. Leaf, A. & Weber, P.C. 1988. AC *Engl. J. Med.* **318**:549–557. Cardiovascular effects of n-3 fatty acids.
6. Skorve, J. & Berge, R.K. 1993. *Biochim. Biophys. Acta* **1167**:175–181. The hypocholesteremic effect of sulfur-substituted fatty acid analogues in rats fed a high carbohydrate diet.
7. Skorve, J., Asiedu, D., Rustan, A.C., Drevon, C.A., Al-Shurbaji, A., Berge, R.K. 1990. *J. Lipid. Res.* **31**:1627–1635. Regulation of fatty acid oxidation and triglyceride and phospholipid metabolism by hypolipidemic sulfur-substituted fatty acid analogues.
8. Bell, R.M., Coleman, R.M. 1980. *Ann. Rev. Biochem.* **49**:459–487. Enzymes of glycerolipid synthesis in eucaryotes.
9. Rustan, A.C., Nossen, J.O., Christiansen, E.N. & Drevon, C.A. 1988. *J. Lipid Res.* **29**:1417–1426. Eicosapentaenoic acid reduces hepatic synthesis and secretion of triacylglycerol by decreasing the activity of acyl-coenzyme A: 1,2-diacylglycerol acyltransferase.
10. Skorve, J., Rustan, A.C. & Berge, R.K. 1995. *Lipids* **30**:987–994. Effects of non-beta-oxidizable sulfur-substituted fatty acid analogues on synthesis and secretion of triacylglycerol and cholesterol in cultured rat hepatocytes.
11. Aarsland, A., Berge, R.K., Bremer, J. & Aarsæther, N. 1990. *Biochim. Biophys. Acta* **1033**:176–183. The hypolipidemic peroxisome-proliferating drug, bis(carboxymethylthio)-1.10 decane, a dicarboxylic metabolite of tiadenol, is activated to an acylenzyme A thioester.
12. Arsland, A., Lundquist, M, Børretsen, B. & Berge, R.K. 1990. *Lipids* **25**:546–548. On the effect of peroxisomal β-oxidation and carnitine palmitoyltransferase activity by eicosapentaenoic acid in liver and heart from rats.
13. Wong, S.H., Nestel, P.J., Trimble, R.P., Stores, G.B., Illman, R.J. & Topping, D.L 1984. *Biochim. Biophys. Acta* **792**:103–109. The adaptive effects of dietary fish and safflower oil on lipid and lipoprotein metabolism in perfused rat liver.

14. Skrede, S. & Bremer, J. 1993. *Biochim. Biophys. Acta* **1167**:189–196. Acylcarnitine formation and fatty acid oxidation in hepatocytes from rats treated with tetradecylthioacetic acid (a 3-thia fatty acid).
15. Asiedu, D.K., Demoz, A., Skorve, J., Grav, H.J., Berge, R.K. 1994. *Biochem. Pharmacol.* **49**:1013–1022. Acute modulation of rat hepatic lipid metabolism by sulphur-substituted fatty acids analogues.
16. Skorve, J., Ruyter, B., Rustan, A.C., Christiansen, E.N., Drevon, C.A. & Berge, R.K. 1990. *Biochem. Pharmacol.* **40**:2005–2012. Effect of 3- and 4-thia-substituted fatty acids on glycerolipid metabolism and mitochondrial β-oxidation in rat liver.
17. Verhoeven, N.M., Roe, D.S., Kok, R.M., Wanders, R.J., Jakobs, C. & Roe, C.R. 1998. *J. Lipid. Res.* **39**:66–74. Phytanic acid and pristanic acid are oxidized by sequential peroxisomal and mitochondrial reactions in cultured fibroblasts.
18. Skorve, J., Al-Shurbaji, A., Asiedu, D., Björkhem, I., Berglund, L., Berge, R.K. 1993. *J. Lipid Res.* **34**:1177–1185. On the mechanism of the hypolipidemic effect of sulfur-substituted hexadecanedoic acid (3-thiadicarboxylic acid) in normolipidemic rats.

15

MOLECULAR MECHANISMS OF FATTY ACID β-OXIDATION ENZYME CATALYSIS

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The β-oxidation of fatty acids is an important metabolic process that takes place in various organisms ranging from *E. coli* to *Homo sapiens.* In animal cells there are different β-oxidation systems in mitochondria and peroxisomes. Mitochondrial β-oxidation of fatty acids provides a significant part of the energy in some organs. For example, free fatty acids are the preferred substrate for energy production in heart. On the other hand, the peroxisomal β-oxidation system degrades very long-chain fatty acids and other uncommon carboxylic acids but does not provide useful energy.[1] More complexity was found in plant cells where glyoxysomes—which correspond to peroxisomes—have a unique β-oxidation system.[2] Fungi possess peroxisomal bifunctional β-oxidation enzymes employing D-specific substrates but not L-isomers.[3] The stereochemical specificity of the latter enzymes is the opposite of that for the animal peroxisomal trifunctional enzyme.[4]

During prolonged fasting or long-lasting exercise, mitochondrial β-oxidation supplies energy to various tissues, producing acetyl-CoA for liver ketogenesis to supply circulating ketone bodies as an important alternative fuel in extrahepatic tissues. In addition to the mitochondrial matrix β-oxidation enzymes, a long-chain-specific β-oxidation enzyme system with a very long-chain acyl-CoA dehydrogenase and a trifunctional β-oxidation complex has been discovered on the mitochondrial inner membrane.[5]

Although the fatty acid β-oxidation spiral comprises only four reactions, understanding of the complexity of fatty acid degradation has dramatically increased in recent years due to the discovery of a variety of new β-oxidation enzymes. This article will discuss the enzymes that catalyze the second and third steps of the β-oxidation pathway, an area of recent and substantial progress.

Current Views of Fatty Acid Oxidation and Ketogenesis: From Organelles to Point Mutations edited by Quant and Eaton, Kluwer Academic / Plenum Publishers, New York, 1999.

1. HYDRATION OF 2-*TRANS*-ENOYL-CoA

The second step of the β-oxidation spiral is the reversible hydration of 2-*trans*-enoyl-CoA to yield L-3-hydroxyacyl-CoA, catalyzed by enoyl-CoA hydratase.[1] However, in fungi 2-*trans*-enoyl-CoA is hydrated by peroxisomal D-3-hydroxyacyl-CoA dehydratase to form D-3-hydroxyacyl-CoA.[3] Enoyl-CoA hydratases are usually associated with the N-terminal region of multifunctional proteins except for the mitochondrial matrix enoyl-CoA hydratase and the *E. coli* long-chain enoyl-CoA hydratase[10] (see Table 1). D-3-hydroxyacyl-CoA dehydratases are located on the C-terminal domain of the peroxisomal D-specific bifunctional β-oxidation enzyme[3] or the central domain of 17β-hydroxysteroid dehydrogenase type IV.[8,9]

An investigation of the stereochemistry of the reaction catalyzed by enoyl-CoA hydratase[11] revealed that the β-addition/elimination reaction follows a *syn* course and the pro-R (but not pro-S) α-proton of L-3-hydroxyacyl-CoA is derived from water. On the basis of a double isotope effect study, Bahnson and Anderson[12] have suggested that crotonase-catalyzed β-elimination is concerted. However, Gerlt and Gassman[13,14] have insisted that crotonase operates by a two-step process just as all the other enzymes that catalyze β-elimination reactions. They have argued that the crotonase active site provides an electrophile at the carbonyl oxygen of thioester group to form a short, strong hydrogen bond for enhancing the acidity of the α-proton of the substrate, so that in the first step a deprotonated catalytic residue abstracts the α-proton and then this protonated catalytic residue facilitates the departure of the β-leaving group in the second step. Despite lively controversy about the lifetime of the enolized intermediate, the advocates of the concerted mechanism[12,15,16] and those of the stepwise mechanism[13,14] all support the notion that a single general acid-base functional group is required for the catalysis of a β-addition/elimination reaction because of the reaction's *syn* stereochemical course.

Site-directed mutagenesis studies in our laboratory[17] revealed that substitution of glutamine for glutamate-139 on the large subunit of the *E. coli* fatty acid oxidation complex caused a greater than 3,000-fold decrease in the k_{cat} of enoyl-CoA hydratase without a significant change in the K_m value. We identified the γ-carboxyl group of glutamate-139 as the primary catalytic acid-base functional group of the *E. coli* crotonase in 1994. Meanwhile, Muller-Newen *et al.*[18] found that a conserved glutamate-164 is the catalytic residue of rat liver mitochondrial enoyl-CoA hydratase. This glutamate residue is at position 135 of the mature enoyl-CoA hydratase, however, because a 29 residue leader sequence is cleaved when the precursor is transported into mitochondria; the catalytic residue of rat liver hydratase apparently corresponds to glutamate-139 of the *E. coli* crotonase (Figure 1).

Our studies on the pH dependence of k_{cat}/K_m of the *E. coli* crotonase have revealed two pK_a values (5.9 and 9.2).[19] A pK_a of 9.2 was assigned to glutamate-139, since a protonated catalytic residue is necessary for transferring a proton to the α-carbon of α,β-unsaturated fatty acyl-CoA thioesters. The results also suggested that crotonase possesses another catalytic residue involved in the hydration of 2-*trans*-enoyl-CoA. In order to identify the second catalytic residue, we mutated additional protic amino acid residues that are conserved in members of the enoyl-CoA hydratase family and investigated the catalytic properties of these mutant hydratases. We found that the substitution of glutamine for glutamate-119 on the large subunit of the *E. coli* fatty acid oxidation complex caused an 88-fold decrease in the catalytic rate with disappearance of a pK_a of 5.9 of the wild type enoyl-CoA hydratase.[19] The experimental data indicated that the γ-carboxyl group of glutamate-119 serves as the second general acid-base functional group in cat-

alyzing the hydration of 2-*trans*-enoyl-CoA. According to the crystal structure of rat liver enoyl-CoA hydratase reported by Engel *et al.*,[20] the γ-carboxyl group of glutamate-164 is close to oxygens of the 1-keto and 3-keto groups of acetoacetyl-CoA, an inhibitor bound to the hydratase active site, and the γ-carboxyl group of glutamate-144 is hydrogen-bonded to the oxygen of the 3-keto group while the imino group of glycine-141 forms a short, strong hydrogen bond to the oxygen of the 1-keto group. It is noteworthy that glutamate-144 and glycine-141 are at positions 115 and 112, respectively, of the mitochondrial enoyl-CoA hydratase (see Fig. 1). The results of our site-directed mutagenesis studies prompted us to propose the catalytic mechanism of the *E. coli* enoyl-CoA hydratase. An outline of the hydratase reaction is shown in Fig. 2, and the same principles are applicable to the hydratases of other multifunctional β-oxidation enzymes and to monofunctional enoyl-CoA hydratases regardless of their substrate chain length specificities.

The molecular mechanism of the *syn* β-elimination reaction catalyzed by enoyl-CoA hydratase we proposed[19] has a single concerted transition state where the general base catalyzes abstraction of the α-proton and the general acid catalyzes loss of the β-leaving group (Fig. 2). The harmonious cooperation of two general acid-base functional groups of hydratases could account for the observed high catalytic rate for the hydratase reaction, e. g., $k_{cat} \approx 775\,s^{-1}$ reported for the hydration of crotonyl-CoA.[17] It is not known why a single pK_a of 8.5 was previously reported for rat liver enoyl-CoA hydratase.[16] Nevertheless, the hypothesis that a single active-site base can mediate both proton transfers in a cyclic transition state[12,16] and the view that the *syn* stereochemical course enables the hydration reaction to be catalyzed by a single group in a stepwise manner[13,14] are no longer tenable. Other factors that could stabilize the transition state are included in the present model of hydratase catalysis (see Fig. 2). For example, hydrogen bonding of the imino group of glycine-116—which corresponds to glycine-141 of rat liver crotonase—with the carbonyl oxygen of the substrate could reduce the pK_a of the α-proton,[13,14] and its coordination with a nucleophile near the β-carbon could cause an electronic rearrangement in the acryloyl portion to enhance the electrophilicity of the β-carbon for substrate activation.[15] The replacement of glycine-116 by phenylalanine would interfere the formation of such a hydrogen bonding, and did in fact result in inactivation of the crotonase.[21]

The *syn* β-addition/elimination of water proved not to be superior to an *anti* process in chemical efficiency.[22] The reason why the reaction catalyzed by enoyl-CoA hydratases follows the *syn* stereochemical course is because the two catalytic residues of the hydratase are located on the same side of the substrate (see Fig. 2). The reaction mechanism of D-3-hydroxyacyl-CoA dehydratase catalysis is not known. If it follows the *anti* stereochemical course, the D-specific dehydratases of the peroxisomal multifunctional

```
                  *  *                    *
MP  108   AVNGYALGGGCECVLATDYRLATPDLR--IGLPETKLGIMPG
TE   92   AIQGVALGGGLELALGCHYRIANAKAR--VGLPEVTLGILPG
PT  108   AIDGLALGGGLEVAMACHARISTPTAQ--LGLPELQLGIIPG
LT  104   AINGTCLGGGLELAISCQYRIATKDKKTVLGAPEVLLGILPG
EH  104   AVNGYALGGGCELAMMCDIIYAGEKAQ--FGQPEILLGTIPG
```

Figure 1. Comparison of the amino acid sequence in the N-terminal domain of the *E. coli* multifunctional protein (MP)[42] with those of homologous regions of rat peroxisomal trifunctional enzyme (TE),[43] plant glyoxysomal tetrafunctional protein (PT),[2] pig mitochondrial long-chain-specific bifunctional enzyme (LT),[44] and rat mitochondrial enoyl-CoA hydratase (EH).[45] Gly^{116}, Glu^{119}, and Glu^{139} of MP, which are conserved in all known enoyl-CoA hydratases, are indicated by asterisks.

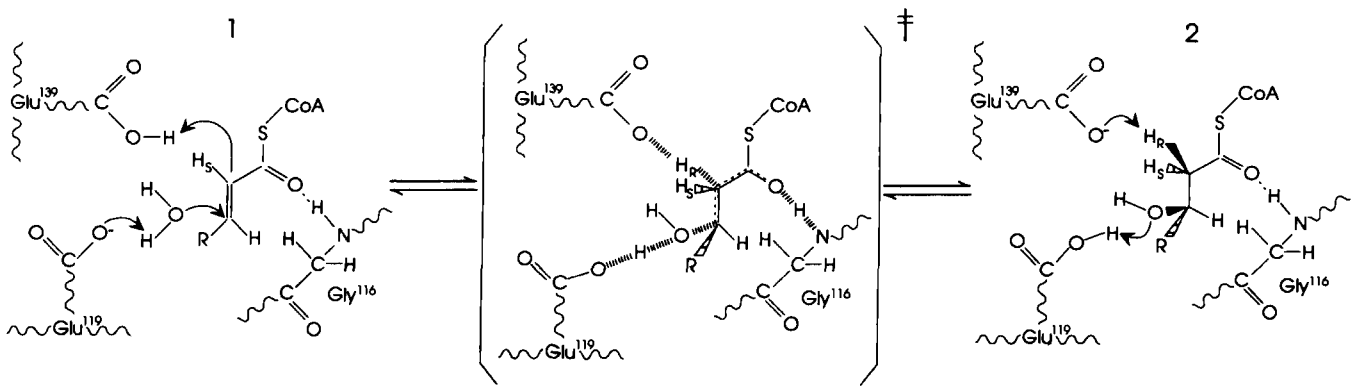

Figure 2. Schematic diagram of the second reaction of the fatty acid β-oxidation pathway catalyzed by *E. coli* enoyl-CoA hydratase. Compounds 1 and 2 are 2-*trans*-enoyl-CoA and L-3-hydroxyacyl-CoA, respectively. The protonated Glu^{139} transfers a proton to the α-carbon of the substrate on the *re* face, whereas the deprotonated Glu^{119} attracts a proton from water whose oxygen makes a nucleophilic attack on the β-carbon of the substrate. The amino group of Gly^{116} in the peptide backbone acts as a hydrogen donor to form a hydrogen bond with the carbonyl oxygen so that an electronic rearrangement occurs in the acryloyl portion of the substrate. The transition state is shown in the square brackets. The product, L-3-hydroxyacyl-CoA, can then leave the active site. Two general acid-base functional groups, the γ-carboxyl groups of Glu^{139} and Glu^{119}, play a major part in the hydratase catalysis.

proteins from mammals[8,9] and fungi[3] are expected to have catalytic residues on both sides of the substrate.

2. DEHYDROGENATION OF L- AND D-3-HYDROXYACYL-CoA

The dehydrogenation of L- and D-3-hydroxyacyl-CoAs is catalyzed by L- and D-3-hydroxyacyl-CoA dehydrogenases, respectively. These enzymes display strict substrate stereochemical specificity, but they both produce 3-ketoacyl-CoAs. The L-3-hydroxyacyl-CoA dehydrogenase binds its coenzyme NAD^+ and its substrate to a cleft between its N- and C-terminal domains.[23,24] The crystal structure of pig heart L-3-hydroxyacyl-CoA dehydrogenase was published a decade ago,[23] but the catalytic mechanism of this type of dehydrogenase was not known until we identified a conserved histidine as the catalytic residue.[25] L-3-Hydroxyacyl-CoA dehydrogenases are associated with the C-terminal region of multifunctional proteins except for those in the mitochondrial matrix[6,7,26] (see Table 1). In contrast, D-3-hydroxyacyl-CoA dehydrogenases are associated with the N-terminal region of peroxisomal multifunctional proteins.[3,8,9]

The replacement of glycine-322 with alanine in the large subunit of the *E. coli* fatty acid oxidation complex significantly increased the K_m value for NADH, so the importance of a glycine-rich sequence (GXGXXG) to the coenzyme binding was confirmed in a catalytic context.[25] Since the pH dependence of the k_{cat}/K_m of the *E. coli* dehydrogenase suggested the catalytic involvement of an amino acid residue with a neutral pK_a, we replaced histidine-450, which is the only histidine conserved in all known L-3-hydroxyacyl-CoA dehydrogenases, with either glutamine or alanine by site-directed mutagenesis. Both mutant dehydrogenases showed a more than 1,500-fold decrease in the k_{cat}, but their K_m values and the catalytic properties of other component enzymes of the fatty acid oxidation complex exhibited only a very mild change. As a result, the imidazole of histidine-450, which is located in a loop between β-strands 6 and 7, was found to be the functional group.[25] Thereafter, a conserved glutamate-462 was identified by "alanine-scanning" mutagenesis as another important element involved in dehydrogenase catalysis. The electrostatic interaction between this acidic residue and the catalytic residue forms a catalytic His^{450}-Glu^{462} pair (Figure 3). Such His-Glu couples play a pivotal role in the catalytic process of L-3-hydroxyacyl-CoA dehydrogenases by maintaining the electroneutrality in the active site and reducing the activation energy of the reaction.[27] The amino acid sequences of all known L-3-hydroxyacyl-CoA dehydrogenases are highly conserved in this key region of the active center, and the consensus sequence has been designated as the signature pattern of the L-3-hydroxyacyl-CoA dehydrogenase family[6] (Fig. 4). The catalytic mechanism delineated in Fig. 3 is applicable to other members of the dehydrogenase family, including long-chain L-3-hydroxyacyl-CoA dehydrogenase of the mitochondrial inner membrane-bound trifunctional β-oxidation complex, which is more closely related evolutionarily to the *E. coli* fatty acid oxidation complex than to the corresponding matrix β-oxidation enzymes, in spite of a functional cooperation of the two mitochondrial β-oxidation systems in the same organelle.[28]

The negative charge of glutamate-462 was found to be necessary for increasing the thermostability of the multienzyme complex, and amidation of the γ-carboxyl group of glutamate-462 is known to have an adverse effect on the 3-ketoacyl-CoA thiolase activity associated with the small subunit of the fatty acid oxidation complex.[27] These findings provided evidence that a $Glu^{510} \rightarrow Gln$ mutation of mitochondrial trifunctional β-oxidation complex, which corresponds to the $Glu^{462} \rightarrow Gln$ mutation described above,

Table 1. Different fatty acid β-oxidation enzyme systems[a]

Reaction	Bacteria (*E. coli*)	Mitochondrial inner membrane	Mitochondrial matrix	Animal peroxisome	Plant glyoxysome	Fungi (*S. cerevisial*)
1. Dehydrogenation	acyl-CoA dehydrogenase	very long-chain acyl-CoA dehydrogenase	various acyl-CoA dehydrogenase[b]	acyl-CoA oxidase	acyl-CoA oxidase	acyl-CoA oxidase
	long-chain enoyl-CoA hydratase[c]					
2. Hydration	*fatty acid oxidation complex*	*trifunctional β-oxidation complex*	enoyl-CoA hydratase			
	large subunit	**large subunit**				
3. Dehydrogenation	multifunctional fatty acid oxidation Protein	long chain enoyl-CoA hydratase :3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme	L-3-hydroxyacyl-CoA dehydrogenase[d]	trifunctional β-oxidation enzyme[f]	tetrafunctional β-oxidation enzyme	D-specific bifunctional β-oxidation enzyme
			short chain L-3-hydroxyacyl-CoA dehydrogenase[e]			
4. Thiolytic cleavage	**small subunit** 3-ketoacyl-CoA thiolase	**small subunit** long chain 3-ketoacyl-CoA thiolase	3-ketoacyl-CoA thiolase[g]	3-ketoacyl-CoA thiolase	3-ketoacyl-CoA thiolase	3-ketoacyl-CoA thiolase

a. Some bacteria and fungi have unusual β-oxidation systems different from those presented here.
b. The existence of branched-chain acyl-CoA dehydrogenases as well as medium-chain acyl-CoA dehydrogenase and its "isoenzymes" differing in their preferences to the chain-lengths of the substrates brings additional complexity to the β-oxidation system.[1]
c. This enzyme has not yet been purified.
d. The sequence difference between pig liver and heart dehydrogenases suggest that there may be tissue-specific isoenzymes.[6]
e. This enzyme is a new member of the short chain dehydrogenase family. It can catalyze the dehydrogenation of L-3-hydroxyacyl-CoAs with different chain-lengths.[7]
f. Another multifunctional protein 2 is identical to 17 β-hydroxysteroid dehydrogenase type IV, which is presumably involved in steroid metabolism even though it displays D-3-hydroxyacyl-CoA dehydratase and dehydrogenase activities.[8,9]
g. Acetoacetyl-CoA thiolase serves mainly for the metabolism of ketobodies.

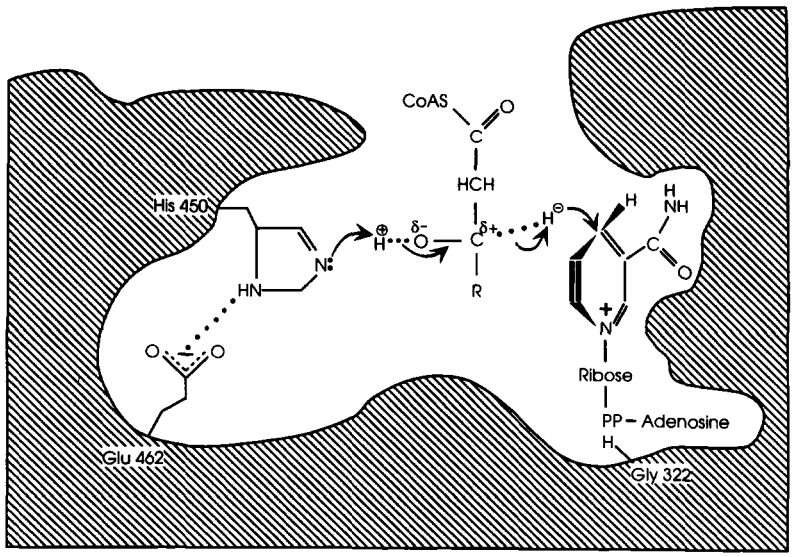

Figure 3. Schematic diagram of a model of the active site of *E. coli* L-3-hydroxyacyl-CoA dehydrogenase. Glu^{462} is not directly involved in the binding of substrate or coenzyme. The negatively charged γ-carboxyl group of Glu^{462} significantly reduces the free energy of the transition state due to an electrostatic interaction with the catalytic residue, His^{450}, in the ternary complex, thus maintaining electroneutrality in the desolvated active center during catalysis. In the dehydrogenase • coenzyme • substrate ternary complex, His^{450} serves as a general catalytic base/acid to catalyze the carbonyl/alcohol interconversion of the substrates and the coupled redox reaction of NAD^+/NADH. The substrate takes such an orientation that a large part of the CoA moiety extends into the solution, and the nicotinamide ring of the coenzyme has "B-side" specificity.

is responsible for loss of long-chain dehydrogenase activity[29] and rapid degradation of the β-oxidation complex.[30] The discovery of a catalytic His-Glu pair has contributed enormously to the understanding of the pathogenesis of long-chain L-3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency at the molecular level. In addition, patients with so-called isolated LCHAD deficiency[29] were found to be deficient in long-chain 3-ketoacyl-CoA thiolase activity as well, and this was attributed to the same mutation at the large subunit.[27]

		* *
MP	450	HFFNPVHRMPLVE
LT	462	HYFSPVDKMQLLE
PT	449	HFFSPAHIMPLLE
TE	431	HFFSPAHVMRLLE
LD	158	HFFNPVPLMKLVE

Figure 4. Alignment of the amino acid sequence between His^{450} and Glu^{462} of the *E. coli* multifunctional protein (MP)[42] with those of homologous regions of pig mitochondrial long-chain-specific bifunctional enzyme (LT),[44] plant glyoxysomal tetrafunctional protein (PT),[2] rat peroxisomal trifunctional enzyme (TE),[43] and pig liver L-3-hydroxyacyl-CoA dehydrogenase (LD).[6] The conserved histidine and glutamate residues are indicated by asterisks. The large subunit of human mitochondrial trifunctional β-oxidation complex[39] has the same amino acid sequence as that of LT in this region. Glu^{462} of MP corresponds to Glu^{474} of the large subunits of these mammalian β-oxidation complexes and to Glu^{510} of their precursors.

The short chain L-3-hydroxyacyl-CoA dehydrogenases, which are new members of the short chain dehydrogenase family,[31] have recently been found in human brain[7] and bovine liver.[32] All the aforementioned L-3-hydroxyacyl-CoA dehydrogenases display the two-domain structure,[6,23,25] whereas the short chain dehydrogenase is homotetramer[7,32] and has a one-domain subunit.[7,31] Since the comformational patterns of various short chain dehydrogenases are highly similar,[31] the three-dimentional structure of the short chain L-3-hydroxyacyl-CoA dehydrogenase is probably similar to that of **3α, 20β**-hydroxysteroid dehydrogenase.[33] For example, human brain short chain L-3-hydroxyacyl-CoA dehydrogenase (SCHAD) has a glycine-rich sequence (GXXXGXG) as the marker of the N-terminal NAD-binding region.[7] In its C-terminal region there are well-conserved tyrosine-168, lysine-172, and serine-155,[7] which may play essential roles in catalysis similar to a catalytically important "triad" involved in the short chain dehydrogenase reaction.[34] Since human brain SCHAD can bind amyloid β-peptide and is located in mitochondria and endoplasmic reticulum[7,35] (He and Yang, unpublished results), it is of great interest to determine whether this dehydrogenase has other important functions in cells and to investigate its involvement in Alzheimer's disease. It was reported that *scully*, an essential gene of *Drosophila*, encodes a homologous enzyme,[36] but the gene product has not yet been purified and characterized. Short chain L-3-hydroxyacyl-CoA dehydrogenases share some structural features with peroxisomal D-3-hydroxyacyl-CoA dehydrogenases,[3,8,9] but differ totally from members of the L-3-hydroxyacyl-CoA dehydrogenase family.[6,23,25,28] With respect to the molecular mechanism of this variety of enzyme, the most interesting question is why their stereochemical specificity is opposite to that of the D-3-hydroxyacyl-CoA dehydrogenases. If more effort is expended in the study of these vital enzymes, valuable discoveries in this new area are to be expected.

3. SUBSTRATE CHAIN-LENGTH SPECIFICITY

Substrate chain length has a profound impact on the k_{cat} and K_m values of the β-oxidation enzymes. Effects of this important factor on catalysis have been investigated by kinetic studies.[37,38] However, reasons for phenomena such as the fact that mitochondrial inner membrane-bound trifunctional β-oxidation complex cannot use short-chain substrates,[5,39] have not yet been offered at the molecular level.

Rat liver enoyl-CoA hydratase is a homohexamer, which is composed of two trimers.[20] The crystal structure of this enzyme revealed that acetoacetyl-CoA fits into the binding pocket well whereas the longer fatty acyl tail of octanoyl-CoA extends into the intertrimer space via a narrow opening.[20,40] The following were cited as strong support that the binding of short-chain substrate with the crotonase was superior to longer chain substrates: the K_m increased so dramatically with substrate chain-length that the K_m values for crotonyl-CoA and hexadecenoyl-CoA are 20μM and 500μM, respectively,[41] and the binding affinity of octanoyl-CoA was lower than that of acetoacetyl-CoA.[20,40] However, the data are not reliable. When an updated assay method was used, it was found that the K_m values for longer chain enoyl-CoAs were, in fact, significantly lower than for crotonyl-CoA.[38] Moreover, the conformation pattern of octanoyl-CoA in the enzyme active site is quite distinct from 2-*trans*-octenoyl-CoA, because the pro-R α-proton of the **α, β**-unsaturated fatty acyl-CoA is abstracted by the same catalytic residue that catalyzes the stereospecific exchange of the pro-S α-proton of saturated fatty acyl-CoA.[16] Obviously, a more sophisticated binding model that can explain these new experimental results[16,38] remains to be worked out.

The majority of the CoA-moiety of L-3-hydroxyacyl-CoA is in contact with the bulk medium while the fatty acid tail is inserted into the cleft and buried by the enzyme.[24] This orientation of the substrate is consistent with the fact that the length of the acyl chain has a significant effect on the turnover number of pig heart L-3-hydroxyacyl-CoA dehydrogenase.[37] Since detailed structural information about the liganded active site is not available, it is not known why the dehydrogenase displays the top catalytic efficiency with substrates of medium acyl chain length. A more intensive study of the substrate chain length specificity, a characteristic which contributes enormously to the complexity of fatty acid β-oxidation systems, shall be made in the future.

4. CONCLUDING REMARKS

In recent years, the identification of two catalytic glutamate residues cooperatively functioning in members of the enoyl-CoA hydratase family and the discovery of a catalytically important His-Glu couple in L-3-hydroxyacyl-CoA dehydrogenases filled a gap in the knowledge of the catalytic mechanisms of the fatty acid β-oxidation enzymes. The information derived from our studies is essential not only for the understanding of the pathogenesis of inherited β-oxidation disorders at the molecular level, but also for designing potent inhibitors to β-oxidation enzymes and granting new features to these enzymes by protein engineering. The remarkable progress in elucidating the molecular mechanisms of the enzymes of fatty acid β-oxidation is to a great extent due to exploration by scholars with expertise in site-directed mutagenesis, in stereochemistry, in clinical investigation, and in x-ray crystallography. It is expected that this multidisciplinary molecular dissection will continue to reveal the structure-function relationships of the novel β-oxidation enzymes and lead to insight into the complicated interactions of the β-oxidation enzymes with their substrates.

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REFERENCES

1. Kunau, W.-H., Dommes, V. & Schulz, H. (1995) *Prog. Lipid Res.* **34**, 267–342. β-Oxidation of fatty acids in mitochondria, peroxisomes, and bacteria: a century of continued progress.
2. Preisig-Müller, R., Gühnemann-Schäfer, K. & Kindl, H. (1994) *J. Biol. Chem.* **269**, 20475-20481. Domains of the tetrafunctional protein acting in glyoxysomal fatty acid β-oxidation. Demonstration of epimerase and isomerase activities on peptide lacking hydratase activity.
3. Hiltunen, J.K., Wenzel, B., Beyer, A., Erdmann, R., Fossa, A. & Kunau, W.-H. (1992) *J. Biol. Chem.* **267**, 6646–6653. Peroxisomal multifunctional β-oxidation protein of *Saccharomyces cerevisiae*. Molecular analysis of the FOX2 gene and gene product.
4. Filppula, S.A., Sormunen, R.T., Hartig, A., Kunau, W.-H. & Hiltunen, J.K. (1995) *J. Biol. Chem.* **270**, 27453–27457. Changing stereochemistry for a metabolic pathway *in vivo*. Experiments with the peroxisomal β-oxidation in yeast.
5. Hashimoto, T. (1992) In *New Developments in Fatty Acid Oxidation* (Coates, P.M. & Tanaka, K., eds.) pp. 19–32. Wiley-Liss, New York. Peroxisomal and mitochondrial enzymes.

6. He, X.-Y. & Yang, S.-Y. (1998) *Biochim. Biophys. Acta.* **1392**, 119–126. Molecular cloning, expression in *Escherichia coli,* and characterization of a novel L-3-hydroxyacyl coenzyme A dehydrogenase from pig liver.
7. He, X-Y, Schulz, H. & Yang, S.-Y. (1998) *J. Biol Chem.* **273**, 10741–10746. A human brain L-3-hydroxyacyl coenzyme A dehydrogenase is identical to an amyloid β-peptide binding protein involved in Alzheimer's disease.
8. Leenders, F., Tesdorpf, J.G., Markus, M., Engel, T., Seedorf, U. & Adamski, J. (1996) *J. Biol. Chem.* **271,** 5438–5442. Porcine 80-kDa protein reveals intrinsic 17 β-hydroxysteroid dehydrogenase, fatty acyl-CoA-hydratase/dehydrogenase, and sterol transfer acitivties.
9. Dieuaide-Noubhani, M., Novikov, D., Baumgart, E., Vanhooren, J.C.T., Fransen, M., Goethals, M., Vandekerckhove, J., Van Veldhoven, P.P. & Mannaerts, G.P. (1996) *Eur. J. Biochem.* **240**, 660–666. Further characterization of the peroxisomal 3-hydroxyl-CoA dehydrogenases from rat liver. Relationship between the different dehydrogenases and evidence that fatty acids and the C_{27} bile acids di- and tri-hydroxycoprostanic acids are metabolized by separate multifunctional proteins.
10. Pawar, S. & Schulz, H. (1981) *J. Biol. Chem.* **256**, 3894–3899. The structure of the multienzyme complex of fatty acid oxidation from *Escherichia coli.*
11. Willadsen, P. & Eggerer, H. (1975) *Eur. J. Biochem.* **54**, 247–252. Substrate stereochemistry of the enoyl-CoA hydratase reaction.
12. Bahnson, B.J. & Anderson, V.E. (1991) *Biochemistry* **30**, 5894–5906. Crotonase-catalyzed β-elimination is concerted: A double isotope effect study.
13. Gerlt, J.A. & Gassman, P.G. (1992) *J. Am. Chem. Soc.* **114**, 5928–5934. Understanding enzyme-catalyzed proton abstraction from carbonic acids: details of stepwise mechanisms for β-eimination reactions.
14. Gerlt, J.A. & Gassman, P.G. (1993) *Biochemistry* **32**, 11943–11952. Understanding the rates of certain enzyme-catalyzed reactions: Proton abstraction from carbon acids, acyl-transfer reactions, and displacement reactions of phosphodiesters.
15. D'Ordine, R.L., Tonge, P.J., Carey, P.R. & Anderson, V.E. (1994) *Biochemistry* **33**, 12635–12643. Electronic rearrangement induced by substrate analog binding to the enoyl-CoA hydratase active site: evidence for substrate activation.
16. D'Ordine, R.L., Bahnson, B.J., Tonge, P.J. & Anderson, V.E. (1994) *Biochemistry* **33**, 14733–14742. Enoyl-coenzyme A hydratase-catalyzed exchange of the α-proton of coenzyme A thiol esters: a model for an enolized intermediate in the enzyme-catalyzed elimination?
17. Yang, S.-Y, He, X.-Y. & Schulz, H. (1995) *Biochemistry* **34**, 6441–6447. Glutamate 139 of the large α-subunit is the catalytic base in the dehydration of both D- and L-3-hydroxyacyl-coenzyme A but not in the isomerization of Δ^3, Δ^2-enoyl coenzyme A catalyzed by the multienzyme complex of fatty acid oxidation from *Escherichia coli.*
18. Muller-Newen, G, Janssen, U. & Stoffel, W. (1995) *Eur. J. Biochem.* **228**, 68–73. Enoyl-CoA hydratase and isomerase form a superfamily with a common active-site glutamate residue.
19. He, X.-Y. & Yang, S.-Y. (1997) *Biochemistry* **36**, 11044–11049. Glutamate-119 of the large α-subunit is the catalytic base in the hydration of 2-*trans*-enoyl-coenzyme A catalyzed by the multienzyme complex of fatty acid oxidation from *Escherichia coli.*
20. Engel, C.K., Mathieu, M., Zeelen, J.P., Hiltunen, J.K. & Wierenga, R.K. (1996) *EMBO J.* **15**, 5135–5145. Crystal structure of enoyl-coenzyme A (CoA) hydratase at 2.5 angstrom resolution: a spiral fold defines the CoA-binding pocket.
21. Yang, S.-Y. & Elzinga, M. (1993) *J. Biol. Chem.* **268**, 6588–6592. Association of both enoyl coenzyme A hydratase and 3-hydroxyacyl coenzyme A epimerase with an active site on the amino-terminal domain of the multifunctional fatty acid oxidation protein from *Escherichia coli.*
22. Mohrig, J.R., Moerke, K.A., Cloutier, D.L., Lane, B.D., Person, E.C. & Onasch, T.B. (1995) *Science* **269**, 527–529. Importance of historical contigency in the stereochemistry of hydratase-dehydratase enzymes.
23. Birktoft, J.J., Holden, H.M., Hamlin, R., Xuong, N.C. & Banaszak, L.J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8262–8266. Structure of L-3-hydroxyacyl-coenzyme A dehydrogenase: preliminary chain tracing at 2.8 Å resolution.
24. Hartmann, D., Philipp, R., Schmadel, K., Birktoft, J.J., Banaszak, L.J. & Trommer, W.E. (1991) *Biochenistry* **30**, 2782–2790. Spatial arrangement of coenzyme and substrates bound to L-3-hydroxyacyl-CoA dehydrogenase as studied by spin-labeled analogues of NAD^+ and CoA.
25. He, X.-Y. & Yang, S.-Y. (1996) *Biochemistry* **35**, 9625–9630. Histidine-450 is the catalytic residue of L-3-hydroxyacyl-CoA dehydrogenase associated with the large α-subunit of the multienzyme complex of fatty acid oxidation from *Escherichia coli.*

26. Yang, S.-Y. (1992) In *New Developments in Fatty Acid Oxidation* (Coates, P.M. & Tanaka, K., eds.) pp. 183–188. Wiley-Liss, New York. The *fadBA* operon of *Escherichia coli* and evidence for the endosymbiont origin of peroxisomes.
27. He, X.-Y., Deng, H. & Yang, S.-Y. (1997) *Biochemistry* **36**, 261–268. Importance of the γ-carboxyl group of glutamate-462 of the large α-subunit for the catalytic function and the stability of the multienzyme complex of fatty acid oxidation from *Escherichia coli.*
28. Yang, S.-Y. (1994) *Comp. Biochem. Physiol.* **109B**, 557–566. The large subunit of the pig heart mitochondrial membrane-bound β-oxidation complex is a long-chain enoyl-CoA hydratase: 3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme.
29. Ijlst, L., Wanders, R.J.A., Ushikubo, S., Kamijo, T. & Hashimoto, T. (1994) *Biochim. Biophys. Acta* **1215**, 347–350. Molecular basis of long chain 3-hydroxyacyl-CoA dehydrogenase deficiency: identification of the major disease-mutation in the α-subunit of the mitochondrial trifunctional protein.
30. Kamijo, T., Wanders, J.A., Saudubray, J.-M., Aoyama, T., Komiyama, A. & Hashimoto, T. (1994) *J. Clin. Invest.* **93**, 1740–1747. Mitochondrial trifunctional protein deficiency. Catalytic heterogeneity of the mutant enzyme in two patients.
31. Jornvall, H., Persson, B., Krook, M., Atrian, S., Gonzalez-Duarte, R., Jeffery, J. & Ghosh, D. (1995) *Biochemistry* **34**, 6003–6013. Short-chain dehydrogenases/reductases (SDR).
32. Furuta, S., Kobayashi, A., Miyazawa, S. & Hashimoto, T. (1997) *Biochim. Biophys. Acta* **1350**, 317–324. Cloning and expression of cDNA for a newly identified isozyme of bovine liver 3-hydroxyacyl-CoA dehydrogenase and its import into mitochondria.
33. Ghosh, D., Weeks, CM., Grochulski, P., Duax, W.L., Erman, M., Rimsay, R.L. & Orr, J.C. (1991) *Proc. Natl. Acad. Sci. USA.* **88**, 10064–10068. Three-dimensional structure of holo 3 alpha, 20 beta-hydroxysteroid dehydrogenase: a member of a short-chain dehydrogenase family.
34. Oppermann, U.C., Filling, C., Berndt, K.D., Persson, B., Benach, J., Ladenstein, R. & Jornvall, H. (1997) *Biochmistry* **36**, 34–40. Active site directed mutagenesis of 3 beta/17 beta-hydroxy-steroid dehydrogenase establishes differential effects on short-chain dehydrogenase/reductase reactions.
35. Yan, S.D., Fu, J., Soto, C., Chen, X. Zhu, H., Al-Mohanna, F., Collison, K., Zhu, A., Stern, E., Saido, T., Tohyama, M., Ogawa, S., Roher, A. & Stern, D. (1997) *Nature* **389**, 689–695. An intracellular protein that binds amyloid-β peptide and mediates neurotoxicity in Alzheimer's disease.
36. Torroja, L., Ortuno-Sahagun, D., Ferrus, A., Hammerle, B. & Barbas, J.A. (1998) *J. Cell Biol.* **141**, 1009–1017. *scully*, an essential gene of *Drosophila,* is homologous to mammalian mitochondrial type II L-3-hydroxyacyl-CoA dehydrogenase/amyloid-β-peptide-binding protein.
37. He, X.-Y, Yang, S.-Y. & Schulz, H. (1989) *Anal. Biochem.* **180**, 105–109. Assay of L-3-hydroxy-acyl-coenzyme A dehydrogenase with substrates of different chain lengths.
38. He, X.-Y., Yang, S.-Y. & Schulz, H. (1992) *Arch. Biochem. Biophys.* **298**, 527–531. Inhibition of enoyl-CoA hydratase by long-chain L-3-hydroxyacyl-CoA and its possible effect on fatty acid oxidation.
39. Kamijo, T, Aoyama, T., Komiyama, A. & Hashimoto, T. (1994) *Biochem. Biophys. Res. Commun.* **199**, 818–825. Structureal analysis of cDNA for subunits of human mitochondrial fatty acid β-oxidation trifunctional protein.
40. Engel, C.K., Kiema, T.R., Hiltunen, J.K. & Wierenga, R.K. (1998) *J. Mol Biol.* **275**, 847–859. The crystal structure of enoyl-CoA hydratase complexed with octanoyl-CoA reveals the structural adaptations required for binding of a long chain fatty acid-CoA molecule.
41. Waterson, R.M. & Hill, R.L. (1972) *J. Biol. Chem.* **247**, 5258–5265. Enoyl coenzyme A hydratase (crotonase). Catalytic properties of crotonase and its possible regulatory role in fatty acid oxidation.
42. Yang, X.-Y.H., Schulz, H., Elzinga, M. & Yang, S.-Y. (1991) *Biochemistry* **30**, 6788–6795. Nucleotide sequence of the promoter and *fadB* gene of the *fadBA* operon and primary structure of the multifunctional fatty acid oxidation protein from *Escherichia coli.*
43. Osumi, T., Ishii, N., Hijikata, M., Kamijo, K., Ozasa, H., Furuta, S., Miyazawa, S., Kondo, K., Inoue, K., Kagamiyama, H. & Hashimoto, T. (1985) *J. Biol. Chem.* **260**, 8905–8910. Molecular cloning and nucleotide sequence of the cDNA for rat peroxisomal enoyl-CoA: hydratase-3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme.
44. Yang, S.-Y., He, X.-Y, Styles, J., Luo, M.J., Schulz, H. & Elzinga, M. (1994) *Biochem. Biophys. Res. Commun.* **198**, 431–437. Primary structure of the large subunit of trifunctional β-oxidation complex from pig heart mitochondria.
45. Minami-Ishii, N., Taketani, S., Osumi, T. & Hashimoto, T. (1989) *Eur. J. Biochem.* **185**, 73–78. Molecular cloning and sequence analysis of the cDNA for rat mitochondrial enoyl-CoA hydratase. Structural and evolutionary relationships linked to the bifunctional enzyme of the peroxisomal β-oxidation system.

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CONTROL OF MITOCHONDRIAL β-OXIDATION AT THE LEVELS OF [NAD$^+$]/[NADH] AND CoA ACYLATION

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1. INTRODUCTION

Mitochondrial β-oxidation is subject to several possible intramitochondrial controls (reviewed[1,2]). Here, we will consider control exerted at the levels of the [NAD$^+$]/[NADH] and [acyl-CoA]/[CoASH] ratios. In cells, the [ATP]/[ADP]+[Pi] ratio is fairly constant since the rate of ATP synthesis may not be directly controlled by this ratio.[3,4] The [NAD$^+$]/[NADH] and the [acyl-CoA]/[CoASH] sensitivities are consequences of the co-factor requirement of the 3-hydroxyacyl-CoA dehydrogenases or the CoASH requirement of the carnitine palmitoyl transferase II or other carnitine acyl transferases, for the thiolytic cleavage of 3-ketoacyl-CoA and for the transfer of acyl groups from car-

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nitine to CoASH, respectively. Total NAD, NADP and CoA are relatively fixed within mitochondria, at approximately 3–5 mM.

2. FEEDBACK INHIBITION

Inhibition of the 3-ketoacyl-CoA thiolase step (with the likely accumulation of 3-ketoacyl-CoA esters) or the 3-hydroxyacyl-CoA dehydrogenase, with accumulation of 3-hydroxyacyl-CoA esters, could lead to feedback inhibition of β-oxidation (see Fig. 1). The 3-hydroxyacyl-CoA dehydrogenases,[5,6] enoyl-CoA hydratases[7] and short-medium- and long- chain acyl-CoA dehydrogenases can be all be inhibited by 3-ketoacyl-CoA esters.[8] The enoyl-CoA hydratases catalyse an equilibrium but can be inhibited by their 3-hydroxyacyl-CoA products.[9] Finally, the acyl-CoA dehydrogenases are subject to inhibition by their 2-enoyl-CoA products[8,10] and, hence, feedback inhibition has been viewed as potentially very important in the regulation and control of β-oxidation flux.

3. CONTROL AT THE LEVEL OF INTRAMITOCHONDRIAL [NAD$^+$]/[NADH]

Early experiments demonstrated that when the rate of oxidation of NADH in isolated mitochondria is limited, for example by state IV conditions, rotenone inhibition of

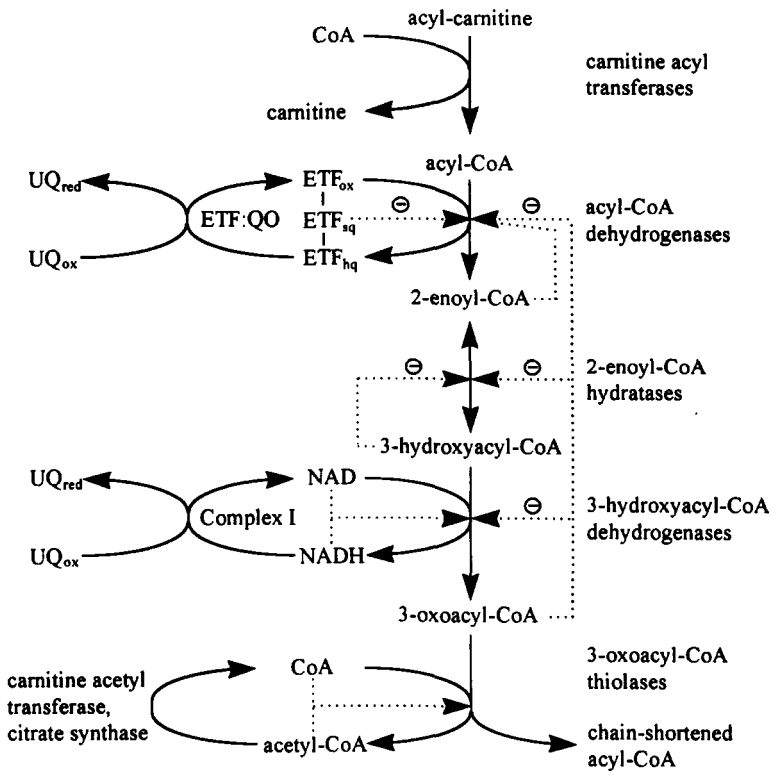

Figure 1. Feedback control of mitochondrial β-oxidation. Reprinted from[1] by permission of Portland Press.

complex I activity or by succinate-induced reversed electron flow, 3-hydroxyacyl- and 2-enoyl- CoA intermediates accumulated,[11–14] whereas they did not in state III. However, when we examined the intact CoA ester intermediates of β-oxidation of palmitate or palmitoyl-carnitine in rat skeletal muscle or liver mitochondria, we detected 3-hydroxyacyl- and 2-enoyl- CoA esters even under state III conditions.[15,16] This was not due to gross changes in intramitochondrial [NAD^+]/[NADH] as measured by a sensitive fluorescence HPLC assay. The accumulation of 3-hydroxyacyl- and 2-enoyl- CoA esters could have three possible explanations:

(i) 3-hydroxyacyl-CoA dehydrogenase activity is low. However, the flavoprotein acyl-CoA dehydrogenases appear to have a very much lower activity than any of the other of the enzymes of β-oxidation in both rat and human tissues, although comparison of isolated enzyme activities can clearly be misleading.[17–19]

(ii) NAD^+ and NADH are channelled between 3-hydroxyacyl-CoA dehydrogenase and complex I and it is turnover of this pool rather than gross [NAD^+]/[NADH] that is responsible for the accumulation of 3-hydroxyacyl- and 2-enoyl- CoA esters. Some support for this idea is from studies showing direct interaction of complex I with a variety of mitochondrial dehydrogenases.[20–22]

(iii) The 3-hydroxyacyl-CoA dehydrogenase activity of the trifunctional protein is very sensitive to [NAD^+]/[NADH] so that even the small changes in [NAD^+]/[NADH] observed in rat and skeletal muscle mitochondria could cause accumulation of 3-hydroxyacyl- and 2-enoyl- CoA esters.

We have recently investigated the third possibiility. The isolated human trifunctional protein was incubated with 2-hexadecenoyl-CoA. The [NAD^+]/[NADH] was varied at a fixed total [NAD^+ + NADH] incubating in the presence of lactate dehydrogenase and a range of [lactate] and [pyruvate]. The rate of β-oxidation was only decreased at low [NAD^+]/[NADH] ratios (Fig. 2). This indicates that the presence of 3-hydroxyacyl- and 2-enoyl-CoA esters was not due to sensitivity of the trifunctional protein to [NAD^+]/[NADH].

4. CONTROL BY ACETYL-COA

The medium-chain 3-ketoacyl-CoA thiolase and the 3-ketoacyl-CoA thiolase activity of the trifunctional protein are inhibited by acetyl-CoA.[23,24] We have also demonstrated the sensitivity of the trifunctional protein to [acetyl-CoA]/[CoA] but at a fixed [acetyl-CoA + CoASH]. This was carried out by incubating isolated trifunctional protein with 2-hexadecenoyl-CoA, NAD^+, CoASH, carnitine acetyltransferase and a range of concentrations of carnitine and acetyl-carnitine to keep the total [CoA] constant. Results from a typical titration are shown in Fig. 3.

Hence, were disposal of acetyl-CoA to ketogenesis, the tricarboxylic acid cycle or to acetyl-carnitine inhibited, feedback inhibition of β-oxidation would result and it has been suggested that this may control β-oxidation in cardiac mitochondria.[25] However, although 3-ketoacyl-CoA esters are readily observed as intermediates of peroxisomal β-oxidation,[26] we have never observed accumulation of 3-ketoacyl-CoA esters in mitochondrial incubations even at maximal β-oxidation flux and under conditions in which acetyl-CoA accumulates.[15,16,27] Therefore, the accumulation of 3-ketoacyl-CoA esters may be strongly prevented and an excess of thiolase activity "pulls" β-oxidation as the

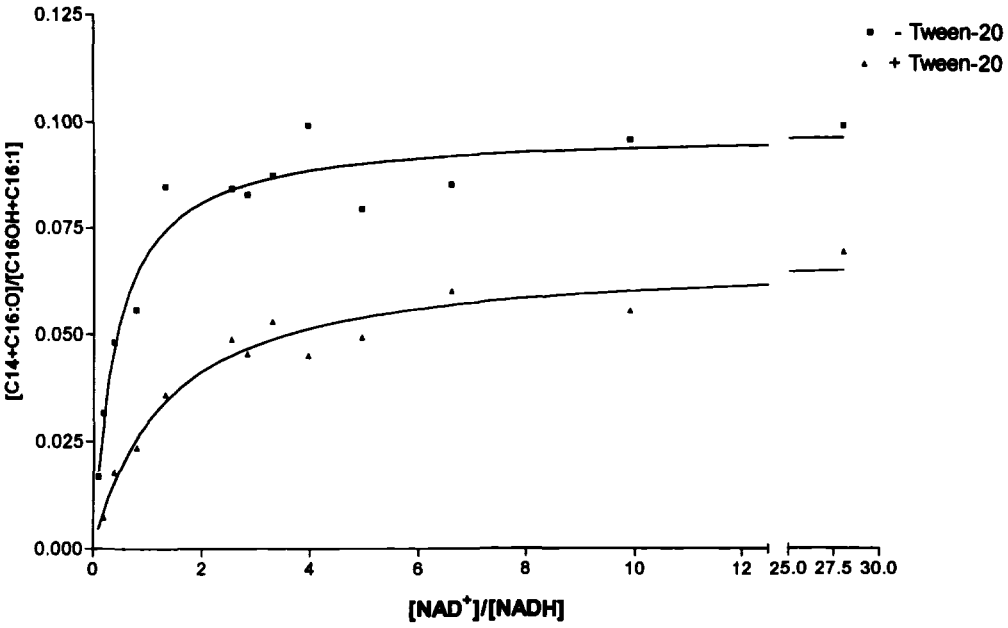

Figure 2. Dependence of trifunctional protein activity on [NAD^+]/[NADH]. Isolated human trifunctional protein was incubated with 2-hexadecenoyl-CoA, NAD^+, lactate dehydrogenase and CoASH. [NAD^+]/[NADH] was varied by altering [pyruvate]/[lactate]. CoA ester intermediates were analysed by HPLC. The ratio [tetradecanoyl-CoA + 3-ketohexadecanoyl-CoA]/[3-hydroxyhexadecanoyl-CoA + 2-hexadecenoyl-CoA] represents forward flux.

3-ketoacyl-CoA thiolases are not inhibited by their acyl-CoA products. However, it should be noted that Schulz and co-workers have demonstrated channelling of 3-ketoacyl-CoA in the isolated trifunctional protein so that the failure to detect 3-ketoacyl-CoA intermediates of β-oxidation may reflect the fact that they are channelled.[24] Further work is necessary to determine the control of 3-ketoacyl-CoA thiolase over β-oxidation.

5. CONTROL BY THE LEVEL OF COASH

As the mitochondrial CoA pool is limited, depletion of free CoASH will inhibit both CPT II and the 3-ketoacyl-CoA thiolase. Garland *et al.* found that 90–95% of intramitochondrial CoA was acylated during maximal β-oxidation flux, so that only a small amount of CoASH can sustain β-oxidation.[28] As intramitochondrial total CoA is about 3 mM, depending on intramitochondrial volume, 90–95% acylation corresponds to 150–300mM CoASH. However, results from other workers suggest that β-oxidation flux may be at least partially controlled by either lack of intramitochondrial CoASH or an elevated [acetyl-CoA]/[CoA] ratio.[25,29,30] Control via lack of intramitochondrial CoASH with accumulation of 3-ketoacyl-CoA esters and subsequent feedback inhibition of the previous steps (Fig. 1) would most likely lead to accumulation of further acyl-CoA esters, thus lowering intramitochondrial CoASH even further. Other intramitochondrial enzymes dependent on CoASH (including pyruvate dehydrogenase, branched-chain oxo-acid dehydrogenase and 2-oxoglutarate dehydrogenase) would also be inhibited. Hence

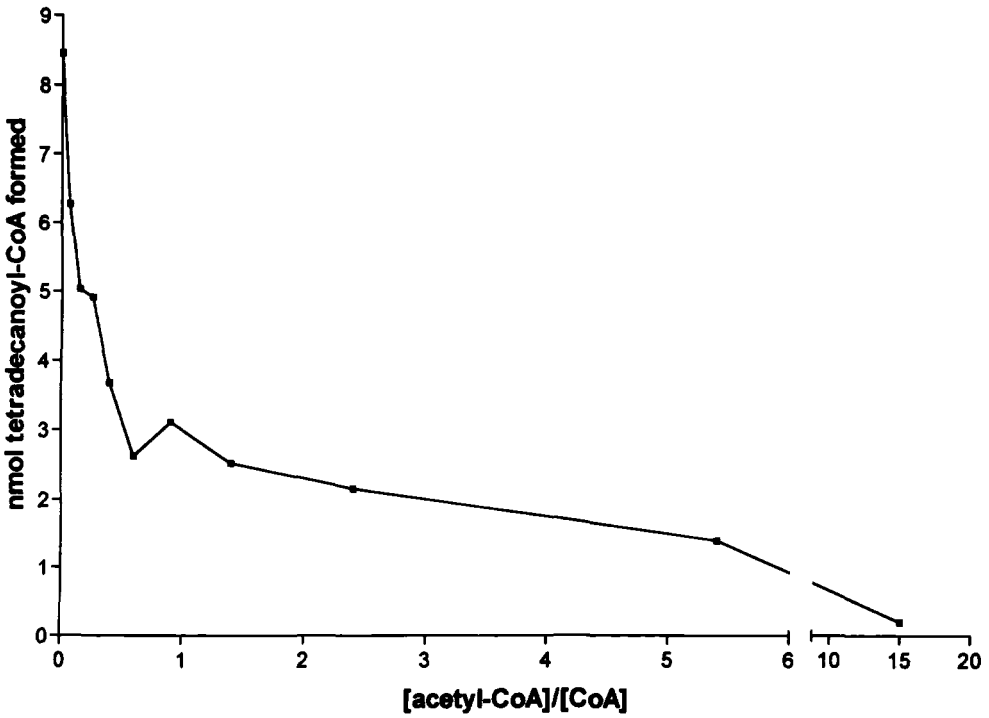

Figure 3. Dependence of trifunctional protein activity on [acetyl-CoA]/[CoA]. Incubations were carried out as for Fig. 2, except that lactate dehydrogenase was omitted, carnitine acetyl-transferase was included and [acetyl-CoA]/[CoA] was varied by altering [carnitine]/[acetyl-carnitine].

complete acylation of the mitochondrial CoA pool would result in the breakdown of mitochondrial oxidative metabolism; surely a very risky control method. An alternative control on β-oxidation in the event of lack of CoASH is to prevent entry of further acyl groups. As CPT II is also CoASH dependent, and the carnitine acyl-carnitine translocase favours export of acyl-carnitine esters,[31] this would provide an excellent way of preventing further acylation, freeing some intramitochondrial CoASH (via the reversal of CPT II activity and the export of acyl-carnitine esters).

The apparent Michaelis Constants for various intramitochondrial CoASH dependent enzymes are shown in Table 1. The measurements are clearly made under very different assay conditions in a variety of tissues so should be compared with caution. This is particularly true for the 3-ketoacyl-CoA thiolases, which exhibit ping-pong kinetics so that the K_m for CoASH is directly related to the concentration of the 3-ketoacyl-CoA substrate. However, it does appear striking that the K_m for CoASH of those enzymes causing acylation of the intramitochondrial CoA pool (CPT II, medium chain acyl-CoA synthase) are orders of magnitude higher than those of the 3-ketoacyl-CoA thiolases and the CoASH-dependent steps related to the tricarboxylic acid cycle. Indeed, the K_m of CPT II for CoASH appears to be of the order of 120 μM,[32] much higher than the K_{mapp} of the general 3-ketoacyl-CoA thiolase, 18 μM[33] and the 3-ketoacyl-CoA thiolase activity of the trifunctional protein.[34] In addition, the K_m of CPT II for acyl-CoA in the reverse direction is low. Hence control of entry of acyl groups to the mitochondria by CoASH levels appears to be a possibility on kinetic grounds.

Table 1. Michaelis constants of intra-mitochondrial CoASH and acyl-CoA dependent enzymes.

Enzyme	K_m for CoASH (μM)	Reference
CPT II	121	[32]
Trifunctional protein 3-ketoacyl-CoA thiolase	5	[34]
General 3-ketoacyl-CoA thiolase	18	[33]
Acetoacetyl-CoA thiolase	21	[33]
Pyruvate dehydrogenase	20	[46]
2-Oxoglutarate dehydrogenase	4.5	[47]
Branched chain amino-acid dehydrogenase	10.5	[48]
Acetyl-CoA synthetase	400	[49]
Medium chain acyl-CoA synthase	850	[50]
Succinic thiokinase	20	[51]
	K_m for acyl-CoA (μM)	
CPT II (reverse direction)	3.5 (decanoyl-CoA)	[32]
Carnitine acetyl-transferase	21 (acetyl-CoA)	[52]
Short-chain acyl-CoA hydrolase	1000 (acetyl-CoA)	[53]
Medium-chain acyl-CoA hydrolase	500 (hexanoyl-CoA)	[35]
Long-chain acyl-CoA hydrolase	17 (palmitoyl-CoA)	[54]
Acyl-CoA: glycine-N-acyltransferase	210 (acetyl-CoA) 9 (benzoyl-CoA)	[45]

6. INHIBITORS OF β-OXIDATION AND SEQUESTRATION OF COASH

Several inhibitors of β-oxidation have been proposed to act by sequestration of intramitochondrial CoASH. High concentrations of short chain fatty acids, such as cyclopropanecarboxylic acid which are not oxidized, cause a lowering of CoASH and inhibition of β-oxidation and of oxidation of 2-oxoglutarate and pyruvate. However, other fatty acids such as hypoglycin and pent-4-enoic acid, which inhibit mitochondrial β-oxidation and have been described as acting by sequestration of CoASH, are still inhibitory to β-oxidation at much lower concentrations than are required to sequester CoASH. Metabolites of hypoglycin and pent-4-enoic acid have subsequently been shown to have specific inhibitory effects.[35–39] Acyl-CoAs can act as competitive inhibitors of β-oxidation, for example we have shown that palmitoyl-CoA impedes flux of 2-hexadecenoyl-CoA through the isolated trifunctional protein (Eaton, Middleton & Bartlett, unpublished observations). Maleate is a potent inhibitor of β-oxidation which sequesters 2moles CoA per mole maleate, one mole as thioester and one as thioether.[40–42] We have investigated the effect of this inhibitor of β-oxidation on the oxidation of palmitoyl-CoA by rat heart mitochondria, to determine whether 3-ketoacyl-CoA esters accumulated due to an inhibition of 3-ketoacyl-CoA thiolase. In the absence of inhibitor, 2-enoyl-, 3-hydroxyacyl- and saturated acyl-CoAs accumulated (Fig. 4a). In the presence of 2mM maleate (β-oxidation flux inhibited by 75%), no 3-ketoacyl-CoA esters accumulated and the only CoA ester present was hexadecanoyl-coA, which is largely extramitochondrial (Fig. 4b). Hence, we regard this as evidence that CPT II is inhibited rather than 3-ketoacyl-CoA thiolase by lack of intramitochondrial CoASH.

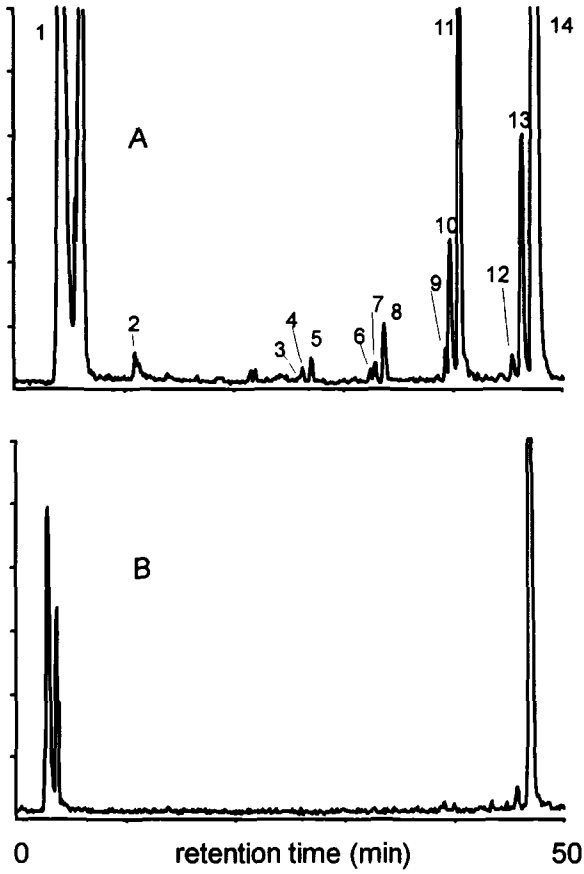

Figure 4. Radio-HPLC chromatograms showing accumulation of CoA esters from [U-^{14}C]hexadecanoyl-CoA by rat heart mitochondria in the presence of: (A) 0,(C) 2mM potassium maleate. Rat heart mitochondria were incubated with 90μM [U-^{14}C]hexadecanoyl-CoA and CoA esters analysed by radio HPLC. The chromatograms are representative of 3–5 incubations. Peak identification: 1, TCA cycle intermediates, 2, acetyl-; 3, dec-2-enoyl-; 4, 3-hydroxydodecanoyl-; 5, decanoyl-; 6, dodec-2-enoyl-; 7, 3-hydroxytetradecanoyl-; 8, dodecanoyl-; 8, tetradec-3-enoyl-; 9, tetradec-2-enoyl-; 10, 3-hydroxyhexadecanoyl-; 11, tetradecanoyl-; 12, hexadec-3-enoyl-; 13, hexadec-2-enoyl-; 14, hexadecanoyl-.

7. RECYCLING OF INTRAMITOCHONDRIAL COASH

As well as recycling of intramitochondrial CoASH by citrate synthase, there are other routes via which CoASH can be recycled. These are indicated in Table 1. Firstly, the carnitine acyltransferases, coupled with carnitine-acylcarnitine translocase, can export acyl groups, thus recycling intramitochondrial CoASH. Secondly, acyl-CoA hydrolases can hydrolyse the thioester link directly. They have been shown to be active in recycling CoASH from medium and short chain acyl-CoAs during inhibition by hypoglycin metabolites.[35,38,39] As well as having a high K_{mapp} for acyl-CoA esters, medium acyl-CoA hydrolase is inhibited by CoASH, thus further limiting their action. Another important route both of detoxification and recycling of CoASH, is glycine conjugation by the matrix enzyme acyl-CoA: glycine-N-acyltransferase, which has maximal activity towards aromatic acyl-CoA esters and is the route of appearance of hippuric acid.[43–45] Finally, intramitochondrial acyl-CoA synthases are reversible so that acylation of the CoA pool can be reversed in the presence of pyrophosphate and AMP.[40]

8. CONCLUSIONS

We conclude that β-oxidation flux can be controlled by the [NAD^+]/[NADH] and [acyl-CoA]/[CoA] ratios in intact mitochondrion. The gross intramitochondrial [NAD^+]/[NADH] ratio may not exert control directly over β-oxidation because of the channelling of NAD(H) between 3-hydroxyacyl-CoA dehydrogenases and complex I. Although control of β-oxidation, by CoA acylation or acetylation and feedback inhibition via the 3-ketoacyl-CoA thiolases, is possible it appears to be unlikely to have much impact in intact mitochondrion because: (i) 3-ketoacyl-CoA esters are not observed as intermediates of mitochondrial β-oxidation (ii) the K_m for CoASH of the 3-ketoacyl-CoA thiolases is comparable to that of mitochondrial CoASH-dependent dehydrogenases and much lower than that of CPT II. However, further work characterising the dependence of mitochondrial β-oxidation on these conserved-moiety cycles is necessary.

We hypothesise that because a range of different intermediates is observed at different chain lengths and there are several possible intramitochondrial controls over β-oxidation flux, that the control of β-oxidation flux is shared between different enzymes rather than in a single, "rate-limiting" step. This is not unexpected as it has been shown that in multi-step pathways, control is likely to be shared and traditional concepts of rate-limiting steps and feedback inhibition appear to be erroneous.[55,56]

REFERENCES

1. Eaton, S., Bartlett, K. & Pourfarzam, M. (1996) *Biochem. J.* **320**, 345–357. Mammalian mitochondrial β-oxidation.
2. Osmundsen, H., Bartlett, K., Pourfarzam, M., Eaton, S. & Sleboda, J. (1996) *in Channelling in Intermediary Metabolism* (Agius, L. & Sherratt, H.S.A., Eds.) Portland Press, pp. 293–313. Substrate Channelling in β-oxidation—myth or reality?
3. Jeffrey, F. & Malloy, C. (1992) *Biochem. J.* **287**, 117–123. Respiratory control and substrate effects in the working heart.
4. Harris, D. & Das, A. (1991) *Biochem. J.* **280**, 561–573. Control of mitochondrial ATP synthesis in heart.
5. He, X., Yang, S. & Schulz, H. (1989) *Anal. Biochem.* **180**, 105–109. Assay of L-3-hydroxyacyl-CoA dehydrogenase with substrates of different chain lengths.
6. Schifferdecker, J. & Schulz, H. (1974) *Life Sci.* **14**, 1487–1492. The inhibition of L-3-hydroxyacyl-CoA dehydrogenase by acetoacetyl-CoA and the possible effect of this inhibitor on fatty acid oxidation.
7. Waterson, R. & Hill, R. (1972) *J. Biol. Chem.* **247**, 5258–5265. Enoyl coenzyme A hydratase (crotonase). Catalytic properties of crotonase and its possible regulatory role in fatty acid oxidation.
8. Davidson, B. & Schulz, H. (1982) *Arch. Biochem. Biophys.* **213**, 155–162. Separation, properties, and regulation of acyl coenzyme A dehydrogenases from bovine heart and liver.
9. He, X.-Y., Yang, S.-Y. & Schulz, H. (1992) *Arch. Biochem. Biophys.* **298**, 527–531. Inhibition of enoyl-CoA hydratase by long-chain L-3-hydroxyacyl-CoA and its possible effect on fatty acid oxidation.
10. Powell, P.J., Lau, S.M., Killian, D. & Thorpe, C. (1987) *Biochem.* **26**, 3704–3710. Interaction of acyl coenzyme A substrates and analogues with pig kidney medium-chain acyl-CoA dehydrogenase.
11. Stanley, K. & Tubbs, P. (1975) *Biochem. J.* **150**, 77–88. The role of intermediates in mitochondrial fatty acid oxidation.
12. Stanley, K.K. & Tubbs, P.K. (1974) *FEES Lett.* **39**, 325–328. The occurrence of intermediates in mitochondrial fatty acid oxidation.
13. Lopez-Cardozo, M., Klazinga, W. & van den Bergh, S.G. (1978) *Eur. J. Biochem.* **83**, 629–634. Accumulation of carnitine esters of beta-oxidation intermediates during palmitate oxidation in rat liver mitochondria.
14. Bremer, J. & Wojtczak, A.B. (1972) *Biochim. Biophys. Acta.* **280**, 515–530. Factors controlling the rate of fatty acid oxidation in rat liver mitochondria.

15. Eaton, S., Turnbull, D. & Bartlett, K. (1994) *Eur. J. Biochem.* **220**, 671–681. Redox control of beta-oxidation in rat liver mitochondria.
16. Eaton, S., Bhuiyan, A., Kler, R., Turnbull, D. & Bartlett, K. (1993) *Biochem. J.* **289**, 161–168. Intramitochondrial control of the oxidation of hexadecanoate in skeletal muscle. A study of the acyl-CoA esters which accumulate during rat skeletal-muscle mitochondrial beta-oxidation of [U-^{14}C]hexadecanoate and [U-^{14}C]hexadecanoyl-carnitine.
17. Jackson, S., Kler, R.S., Bartlett, K., Briggs, H., Bindoff, L.A., Pourfarzam, M., Gardner-Medwin, D. & Turnbull, D.M. (1992) *J. Clin. Invest.* **90**, 1219–1225. Combined enzyme defect of mitochondrial fatty acid oxidation.
18. Reichmann, H. & DeVivo, D.C. (1991) *Comp. Biochem. Physiol.* **98B**, 327–331. Coordinate enzymatic activity of beta-oxidation and purine nucleotide cycle in a diversity of muscle and other organs of rat.
19. Melde, K., Jackson, S., Bartlett, K., Sherratt, H.S.A. & Ghisla, S. (1991) *Biochem. J.* **274**, 395–400. Metabolic consequences of methylenecyclopropylglycine poisoning in rats.
20. Sumegi, B. & Srere, P.A. (1984) *J. Biol. Chem.* **259**, 15040–15045. Complex I binds several mitochondrial NAD-coupled dehydrogenases.
21. Ushiroyama, T., Fukushima, T., Styre, J.D. & Spivey, H.O. (1992) *Curr. Top. Cell. Regul.* **33**, 291–207. Substrate channeling of NADH in mitochondrial redox processes.
22. Fukushima, T., Decker, R.V., Anderson, W.M. & Spivey, H.O. (1989) *J. Biol. Chem.* **264**, 16483–16488. Substrate channeling of NADH and binding of dehydrogenases to complex I.
23. Olowe, Y. & Schulz, H. (1980) *Eur. J. Biochem.* **109**, 425–429. Regulation of thiolases from pig heart. Control of fatty acid oxidation in heart.
24. Yao, K. & Schulz, H. (1996) *J. Biol. Chem.* **271**, 17816–17820. Intermediate channeling on the trifunctional beta-oxidation complex from pig-heart mitochondria.
25. Wang, H.Y., Baxter, C.F., Jr. & Schulz, H. (1991) *Arch. Biochem. Biophys.* **289**, 274–280. Regulation of fatty acid beta-oxidation in rat heart mitochondria.
26. Sleboda, J., Pourfarzam, M., Bartlett, K. & Osmundsen, H. (1995) *Biochim. Biophys. Acta* **1258**, 309–318. Effects of added l-carnitine, acetyl-coa and coa on peroxisomal beta- oxidation of [U-^{14}C]hexadecanoate by isolated peroxisomal fractions.
27. Eaton, S., Pourfarzam, M. & Bartlett, K. (1996) *Biochem. J.* **319**, 633–640. The effect of respiratory chain impairment on beta-oxidation in rat heart mitochondria.
28. Garland, P., Shepherd, D. & Yates, D. (1965) *Biochem. J.* **97**, 587–594. Steady-state concentrations of CoA acetyl-CoA and long-chain fatty acyl-CoA in rat liver mitochondria.
29. Hansford, R.G. & Johnson, R.N. (1975) *J. Biol. Chem.* **250**, 8361–8375. The steady state concentrations of coenzyme-ASH and coenzyme A thioester, citrate, and isocitrate during tricarboxylate cycle oxidations in rabbit heart mitochondria.
30. Oram, J.F., Bennetch, S.L. & Neely, J.R. (1973) *J. Biol. Chem.* **248**, 5299–5309. Regulation of fatty acid utilization in isolated perfused rat hearts.
31. Ramsay, R. & Arduini, A. (1993) *Arch. Biochem. Biophys.* **302**, 307–314. The carnitine acyltransferases and their role in modulating acyl-CoA pools.
32. Bhaird, N., Kumaravel, G., Gandour, R., Krueger, M. & Ramsay, R. (1993) *Biochem. J.* **294**, 645–651. Comparison of the active-sites of the purified carnitine acyltransferases from peroxisomes and mitochondria by using a reaction-intermediate analog.
33. Middleton, B. (1973) *Biochem. J.* **132**, 717–730. The oxoacyl-coenzyme A thiolases of animal tissues.
34. Hashimoto, T., Shindo, Y, Souri, M. & Baldwin, G. (1996) *Biochem. J.* **119**. A new inhibitor of mitochondrial fatty acid oxidation.
35. Osmundsen, H. & Sherratt, H. (1975) *FEBS Lett.* **55**, 38–41. A novel mechanism for inhibition of beta-oxidation by methylenecyclopropylacetyl-CoA, a metabolite of hypoglycin.
36. Holland, P. & Sherratt, H. (1973) *Biochem. J.* **173**, 157–171. Biochemical effects of the hypoglycaemic compound pent-4-enoic acid and related non-hypoglycaemic fatty acids. Effects of the free acids and their carnitine esters on coenzyme A-dependent oxidations in rat liver mitochondria.
37. Holland, P., Senior, A. & Sherratt, H. (1973) *Biochem. J.* **136**, 173–184. Biochemical effects of the hypoglycaemic compound pent-4-enoic acid and related non-hypoglycaemic fatty acids. Effects of their coenzyme A esters on enzymes of fatty acid oxidation.
38. Billington, D., Kean, E., Osmundsen, H. & Sherratt, H. (1978) *Biochem. Pharmacol.* **27**, 2879–2980. Mechanisms of the metabolic disturbances caused by hypoglycin and by pent-4-enoic acid *In vitro* studies.
39. Billington, D., Osmundsen, H. & Sherratt, H.S.A. (1978) Biochem. Pharmacol. **27**, 2891–2900. Mechanisms of the metabolic disturbances caused by hypoglycin and by pent-4-enoic acid *In vivo* studies.

40. Rossi, C., Alexandre, A., Carignani, G. & Siliprandi, N. (1972) *Adv. Enzyme Regul.* **10**, 171–186. The role of mitochondrial adenine nucleotide pool on the regulation of fatty acid and -ketoglutarate oxidation.
41. Pacanis, A. & Rogulski, J. (1981) *J. Biol. Chem.* **256**, 13030–13034. Studies on chemical and enzymatic-synthesis of maleyl-CoA.
42. Pacanis, A., Strzelecki, T. & Rogulski, J. (1981) *J. Biol. Chem.* **256**, 13035–13038. Effects of maleate on the content of CoA and its derivatives in rat- kidney mitochondria.
43. Al-Bassam, S. & Sherratt, H.S.A. (1981) *Biochem. Phamacol.* **30**, 2817–2824. The antagonism of the toxicity of hypoglycin by glycine.
44. Gatley, S. & Sherratt, H.S.A. (1977) *Biochem. J.* **165**, 39–47. The synthesis of hippurate from benzoate and glycine by rat liver mitochondria. Submitochondrial localisation and kinetics.
45. Bartlett, K. & Gompertz, D. (1974) *Biochem. Med.* **10**, 15–23. The specificity of glycine-N-acylase and acylglycine excretion in the organicacidaemias.
46. Pettit, F. & Reed, L. (1982) *Meth. Enzymol.* **89**, 376–391. Pyruvate dehydrogenase complex from bovine kidney and heart.
47. Ishikawa, E., Oliver, R. & Reed, L. (1966) *Proc. Natl. Acad. Sci.* **56**, 534–541. Alpha-Keto acid dehydrogenase complexes, V. Macromolecular organization of pyruvate and alpha-ketoglutarate dehydrogenase complexes isolated from beef kidney mitochondria.
48. Boyer, B. & Odessey, R. (1991) *Arch. Bioch. Biop.* **285**, 1–7. Kinetic characterization of branched chain ketoacid dehydrogenase.
49. Frenkel, E. & Kitchens, R. (1981) *Meth. Enzymol.* **71**, 317–324. Acetyl-CoA synthetase from bakers' yeast (*Saccharomyces cerevisiae*),
50. Webster, L.J., Gerowin, L. & Rakita, L. (1965) *J. Biol. Chem.* **240**, 29–33. Purification and characteristics of a butyryl-CoA synthetase from bovine heart mitochondria.
51. Cha, S., Cha, C. & Parks, R.J. (1967) *J. Biol. Chem.* 2**42**, 2577–2581. Succinic thiokinase. IV. Improved method of purification, arsenolysis of guanosine triphosphate, succinate-dependent guanosine triphosphatase activity, and some other properties of the enzyme.
52. Bloisi, W., Colombo, I., Garavaglia, B., Giardini, R., Finocchiaro, G. & Didonato, S. (1990) *Eur. J. Biochem.* **189**, 539–546. Purification and properties of carnitine acetyltransferase from human liver.
53. Snoswell, A. & Tubbs, P.K. (1978) *Biochem. J.* **171**, 299–303. Deacylation of acetyl-coenzyme A and acetylcarnitine by liver preparations.
54. Berge, R.K., and Farstad, M. (1979) *Eur. J. Biochem.* **96**, 393–401. Purification and characterization of long-chain acyl-CoA hydrolase from rat liver mitochondria.
55. Fell, D. and Thomas, S. (1995) *Biochem. J.* **311**, 35–39. Physiological control of metabolic flux—the requirement for multisite modulation.
56. Thomas, S. & Fell, D.A. (1998) *Adv. Enzyme Reg.* **38**, 65–85. The role of multiple enzyme activation in metabolic flux control.

PRODUCTION AND EXPORT OF ACYL-CARNITINE ESTERS BY NEONATAL RAT HEPATOCYTES

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1. INTRODUCTION

One of the functions of hepatic β-oxidation is to provide ketone bodies, acetoacetate and β-hydroxybutyrate, to the peripheral circulation. These can then be utilized by peripheral tissues such as brain and heart. Beta-oxidation itself produces acetyl-CoA which then has three possible fates: entry to the Krebs cycle via citrate synthase; ketogenesis or transesterification to acetyl-carnitine by the action of carnitine acetyltransferase (CAT).[1] Intramitochondrial acetyl-carnitine then equilibrates with plasma via the carnitine acyl-carnitine translocase and presumably via the plasma membrane carnitine transporter. Human studies have shown that acetyl-carnitine may provide up to 5% of the circulating carbon product from fatty acids[1] and can be taken[2] and utilized by muscle[3] and possibly brain.[4] In addition, acyl-carnitines are of important with regard to the diagnosis of inborn errors of β-oxidation.[5] For these reasons, we wished to examine the production of acetyl-carnitine and other acyl-carnitine esters by neonatal rat hepatocytes.

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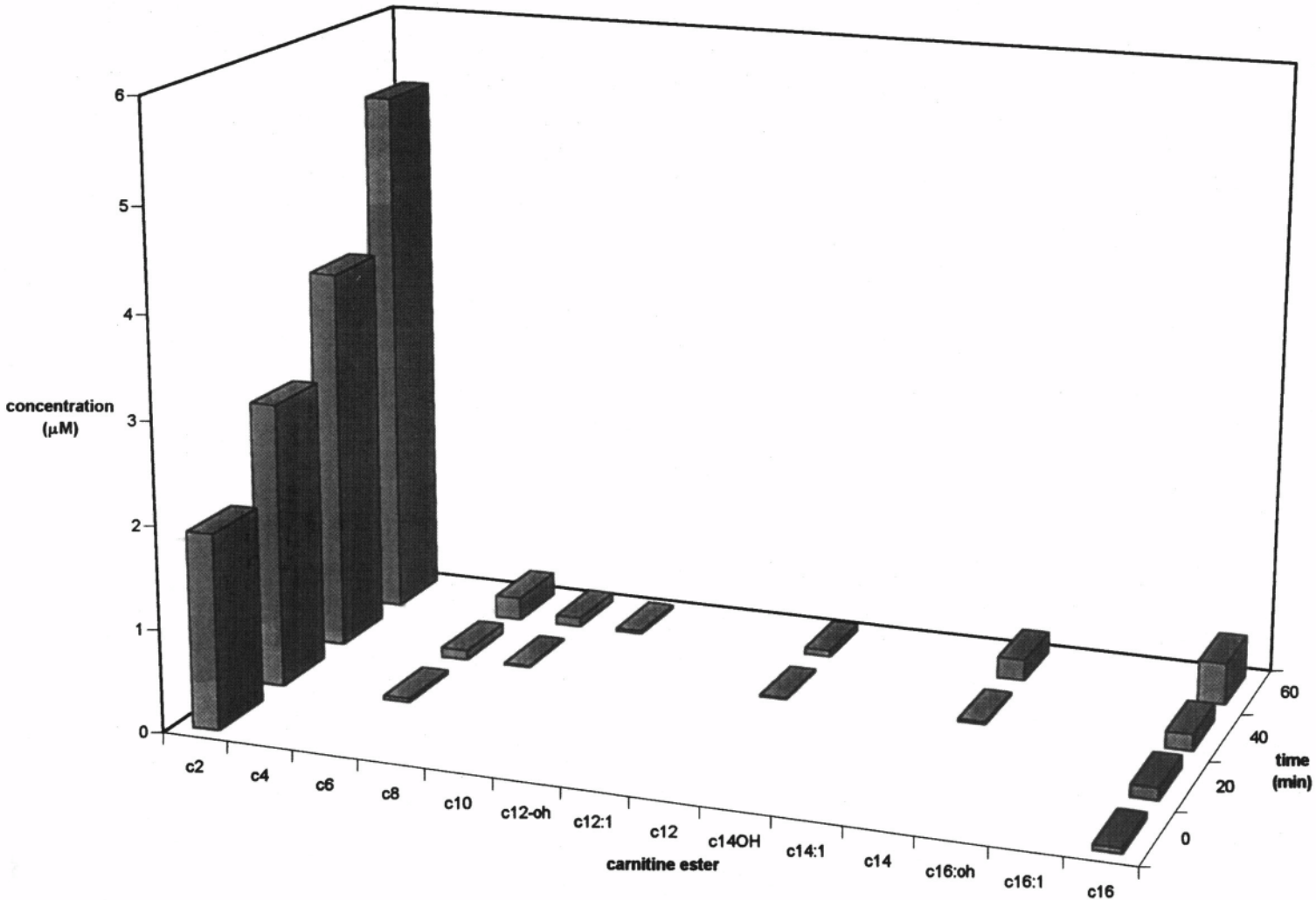

Figure 1. Extracellular accumulation of acyl-carnitines in hepatocytes isolated from neonatal rats.

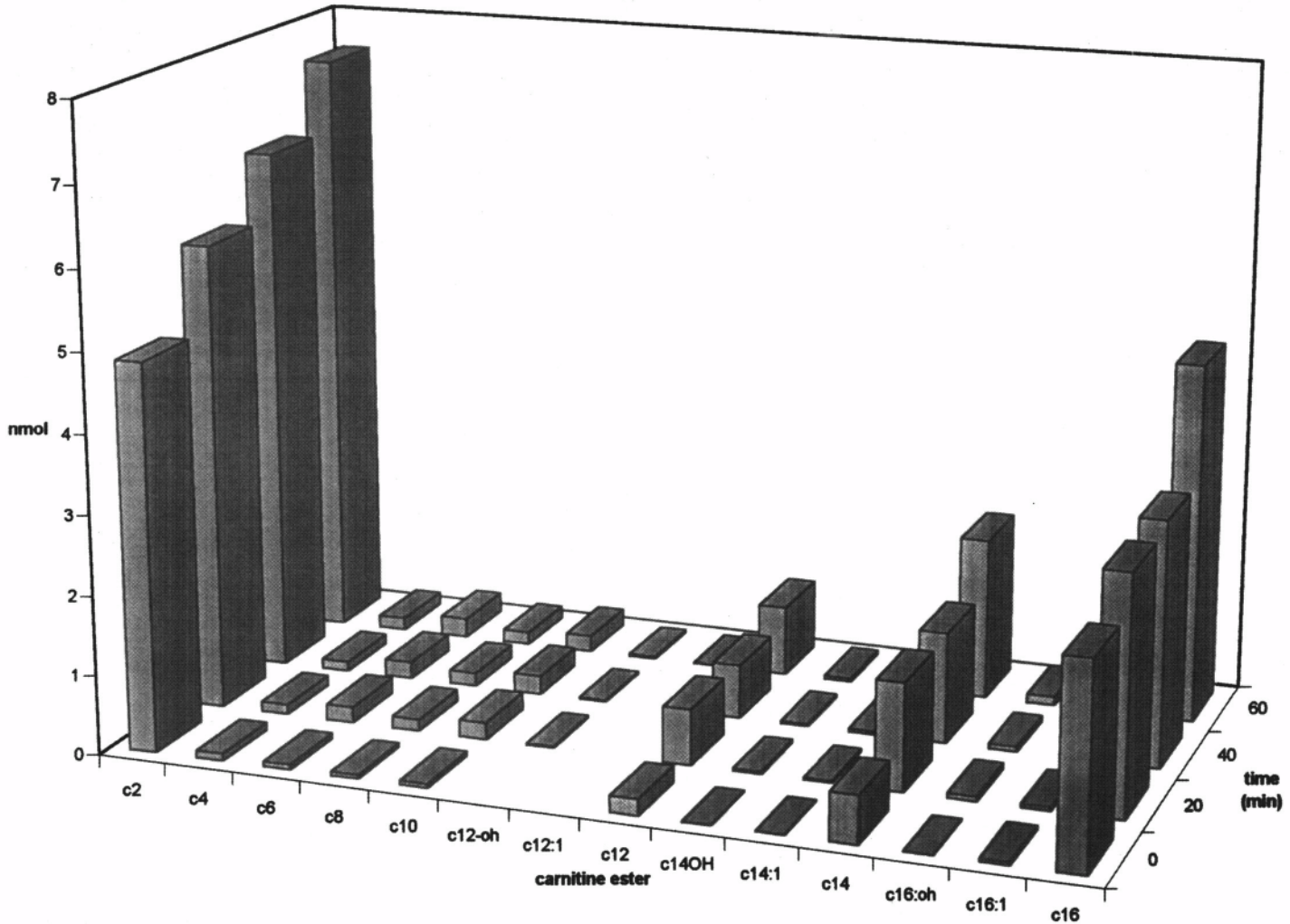

Figure 2. Intracellular accumulation of acyl-carnitines in hepatocytes isolated from neonatal rats.

2. METHODS

Hepatocytes (12×10^6), isolated from 11–13 day-old rats (peak suckling), were gassed with 95% O_2/5% CO_2 and pre-incubated for 10min in 3ml Krebs-Ringer-Bicarbonate buffer (KRB), which contained 2mM carnitine (carnitine is lost from cells during isolation), before addition of 0.5mM palmitate in KRB, regassing and further incubation for 60min. Aliquots of 0.7ml were removed and centrifuged (11,500*g*, 2min). The supernatants were collected and the pellets resuspended in 100 μl 10% (v/v) acetic acid and both pellets and supernatants were analysed for the presence of acyl-carnitines by tandem mass-spectrometry as described previously.[6] Parallel incubations carried out with [l-^{14}C]palmitate (0.1 μCi/μmol) were used for measurement of flux as acid-soluble radioactivity.[6] Cell volumes were measured using differential [^{14}C]methoxyinulin exclusion and ^3H_2O partition as described by Brand.[7]

3. RESULTS AND DISCUSSION

Small amounts of long-chain acyl-carnitine esters (primarily hexadecanoyl-carnitine) were accumulated in the medium (Fig. 1). Acetyl-carnitine increased linearly with respect to time reaching 5.5 μM at 60min, a figure comparable to circulating levels in the rat.[8]

Intracellularly, a wide range of carnitine esters was accumulated (Fig. 2). Again hexadecanoyl-carnitine and acetyl-carnitine predominated. However, significant amounts of chain-shortened acyl-carnitine esters and 2-enoyl and 3-hydroxyacyl carnitine esters were found, in keeping with the presence of the corresponding CoA esters as intermediates of β-oxidation in rat liver mitochondria.[9] Total acid soluble product over 60min was 0.12 μmol acetate units $(10^6 \text{ cells})^{-1}$, whereas, the total acetyl-carnitine formed was 1.38 nmol $(10^6 \text{ cells})^{-1}$. Hence, acetyl-carnitine formed only 1.3% of the acid soluble product at 60min. As the volume of the cells was 0.2 μl $(10^6 \text{ cells})^{-1}$, the intracellular concentrations of acetyl-carnitine and hexadecanoyl-carnitine were 0.297 and 0.178mM respectively at 60min. Export of acyl-carnitines from the cell was clearly low (comparing the intracellular and extracellular concentrations) which may be due to the high K_m with respect to carnitine of the liver plasma membrane carnitine transporter,[10,11] by which acetyl-carnitine probably exits the cell. The enzyme responsible for export of long-chain acyl-carnitines is unknown. These data suggest that in neonatal rats, acetyl-carnitine may not be important as a circulating product of hepatic β-oxidation. In humans, however, acetyl-carnitine export may be much more important as human hepatic CAT activity is 10–14 times higher than that in rat liver and, furthermore, the concentration of acetyl-carnitine in blood is higher in man.[8,12–14]

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REFERENCES

1. Eaton, S., Bartlett, K. & Pourfarzam, M. (1996) *Biochem. J.* **320**, 345–357. Mammalian mitochondrial β-oxidation.

2. Brass, E.P. & Hoppel, C.L. (1980) *Biochem. J.* **190**, 495–304. Relationship between acid-soluble carnitine and coenzyme A pools *in vivo.*
3. Bartlett, K., Bhuiyan, A.K., Aynsley Green, A., Butler, P.C. & Alberti, K.G. (1989) *Clin. Sci.* **77**, 413–416. Human forearm arteriovenous differences of carnitine, short-chain acylcarnitine and long-chain acylcarnitine.
4. Kuratsune, H., Watanabe, Y., Yamaguti, K., Jacobsson, G., Takahashi, M., Machii, T., Onoe, K., Matsumara, K., Valind, S., Kitani, T. & Langstom, B. (1997) *Biochem. Biophys. Res. Commun.* **231**, 488–493. High uptake of [2–11C]acetyl-1-carnitine into the brain: A Pet study.
5. Bartlett, K., Eaton, S. & Pourfarzam, M. (1997) *Arch. Dis. Child.* **77,** F151–F154. New developments in neonatal screening.
6. Eaton, S., Pourfarzam, M. & Bartlett, K. (1996) *Biochem. J.* **319**, 633–640. The effect of respiratory chain impairment on β-oxidation in rat heart mitochondria.
7. Brand, M.D. (1995) *in Bioenergetics: a practical approach* Brown, G. & Cooper, C. Eds. Oxford University Press, Oxford, pp. 39–62. Measurement of mitochondrial protonmotive force.
8. Bhuiyan, A.K.M.J., Bartlett, K., Sherratt, H.S.A. & Agius, L. (1988) *Biochem. J.* **253**, 337–343. Effects of ciprofibrate and 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate (POCA) on the distribution of carnitine and CoA and their acyl-esters and on enzyme activities in rats.
9. Eaton, S., Turnbull, D. & Bartlett, K. (1994) *Eur. J. Biochem.* **220**, 671–681. Redox control of β-oxidation in rat liver mitochondria.
10. Bieber, L. (1988) *Ann. Rev. Biochem.* **57**, 261–283. Carnitine.
11. Christiansen, R. & Bremer, J. (1976) *Biochim. Biophys. Acta.* **448**, 562–577. Active transport of butyrobetaine and carnitine into isolated liver cells.
12. Bronfman, M. & Leighton, F. (1984) *Biochem. J.* **224**, 721–730. Carnitine acyltransferase and acyl-coenzyme A hydrolase activities in human liver Quantitative analysis of their subcellular localization.
13. Agius, L., Wright, P.D. & Alberti, K.G. (1987) *Clin. Sci.* **73**, 3–10. Carnitine acyltransferases and acyl-CoA hydrolases in human and rat liver.
14. Bhuiyan, A.K.M.J., Jackson, S., Turnbull, D.M., Aynsley-Green, A., Leonard, J.V. & Bartlett, K. (1992) *Clin. Chim. Acta.* **207**, 185–204. The measurement of carnitine and acyl-carnitines—application to the investigation of children with suspected inherited disorders of mitochondrial fatty acid oxidation.

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TISSUE SPECIFIC DIFFERENCES IN INTRAMITOCHONDRIAL CONTROL OF β-OXIDATION

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1. INTRODUCTION

It has become clear that there are important tissue specific differences in the control and regulation of β-oxidation and that these differences may largely reside with the entry of acyl moieties into the mitochondrion by the carnitine palmitoyltransferase (CPT) /translocase system. CPT I has wide tissue-specific variations in sensitivity to its physiological inhibitor, malonyl-CoA,[1] which is probably due to the presence of different isozymes.[2,3] However, it is possible that there are additional intramitochondrial controls which vary amongst tissues. The individual reactions of the β-oxidation of long-chain fatty acids within the mitochondrion have been known for many years although their functional and topological relationship is less well known. Recently, it has been found that the long-chain activities of three of the enzymes reside on a single membrane-bound protein (the trifunctional protein,[4,5]) and, in addition, there exists a fourth acyl-CoA dehydrogenase which is similarly membrane bound.[6] Hence, the possibility of functional organization of β-oxidation enzyme activities, associated with the inner mitochondrial membrane, must be considered. The organization of β-oxidation enzymes within the mitochondrion has previously been postulated as a result of the classic studies of Stanley and Tubbs, amongst others, leading to the "leaky hosepipe" model of β-oxidation.[7,8] Their work also led to the view that the acyl-CoA dehydrogenases were the "rate-limiting step" for β-oxidation, as only saturated acyl groups accumulated under well oxygenated conditions. These workers measured acyl groups resulting from hydrolysis of CoA and car-

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nitine esters and other groups have measured carnitine esters.[9,10] To examine further the question of the control of β-oxidation, we have developed radio-HPLC methods for the direct analysis of CoA and carnitine esters resulting from β-oxidation. We have applied these methods to the diagnosis of inborn errors of β-oxidation as well as the study of the pattern of β-oxidation in various tissues.[11–13] Mitochondria isolated from control subjects (muscle, liver, fibroblasts or leucocytes) produce a characteristic pattern of CoA and carnitine esters and variations in these esters infer the probable site of a defect which can then be confirmed by direct enzyme assay.[14,15]

In the following sections we describe experiments in which we characterise the relationship of β-oxidation and the respiratory chain in three tissues: skeletal muscle, heart and liver.

2. RAT SKELETAL MUSCLE MITOCHONDRIA

Well-coupled rat skeletal muscle mitochondria were incubated with [U-^{14}C]hexadecanoyl-carnitine or [U-^{14}C]hexadecanoate under state 3 conditions and the resulting CoA and carnitine esters measured.[12] As expected, acetyl-CoA and acetyl-carnitine levels depended on the presence of carnitine or Krebs cycle acceptors for acetyl-CoA.[11] However, we were surprised to observe 3-hydroxacyl- and 2-enoyl- CoA (Figure la) and carnitine esters: previously we and others had only observed 3-hydroxyacyl- and 2-enoyl- CoA and carnitine esters in incubation made in the presence of rotenone.[9,16] Clearly some control over β-oxidation resides with the properties of the 3-hydroxyacyl-CoA dehydrogenase activity of the trifunctional protein. The most obvious reason for this would be the lowered NAD^+/NADH ratio resulting from state 4 conditions or lack of adequate oxygenation. We, therefore, developed a sensitive HPLC method to measure intramitochondrial NAD^+ and NADH during a pulse of β-oxidation. As can be seen in Fig. 1b, despite the production of significant amounts of 3-hydroxyacyl-CoA and carnitine esters between 2 and 8 min, there is no significant rise in intramitochondrial gross NADH (only NADH levels are shown for clarity, but NAD^+ levels altered as expected). This suggests that if there is a single homogenous pool of NAD^+/NADH it could not be responsible for the 3-hydroxyacyl- and 2-enoyl CoA ester accumulation. One simple explanation for this observation could be that the activity of 3-hydroxyacyl-CoA dehydrogenase is lower than the other enzyme activities of β-oxidation. However, all the experimental evidence suggests that the acyl-CoA dehydrogenases have by far the lowest activity[14,17,18] although this requires extrapolation from optimised assay conditions to the situation in the intact mitochondrion. We have, therefore, suggested: (i) that the control at the level of 3-hydroxyacyl-CoA dehydrogenase is exerted via a pool of NAD^+ in direct contact with both the trifunctional protein and with complex I of the respiratory chain and (ii) that it is the rate of turnover of this pool rather than that of bulk NAD^+/NADH that governs the rate of 3-hydroxyacyl-CoA dehydrogenase.[12] Although there is no direct evidence for this, other groups have provided evidence for an interaction between short-chain 3-hydroxyacyl-CoA dehydrogenase and complex I[19,20] and have found evidence for an interaction between 3-hydroxyacyl-CoA dehydrogenase and the respiratory chain in intact mitochondria.[21]

3. RAT HEART MITOCHONDRIA

The CoA esters and NADH measurements observed using rat heart mitochondria are shown in Figs, le and 1f respectively. Both the CoA esters observed and the NADH

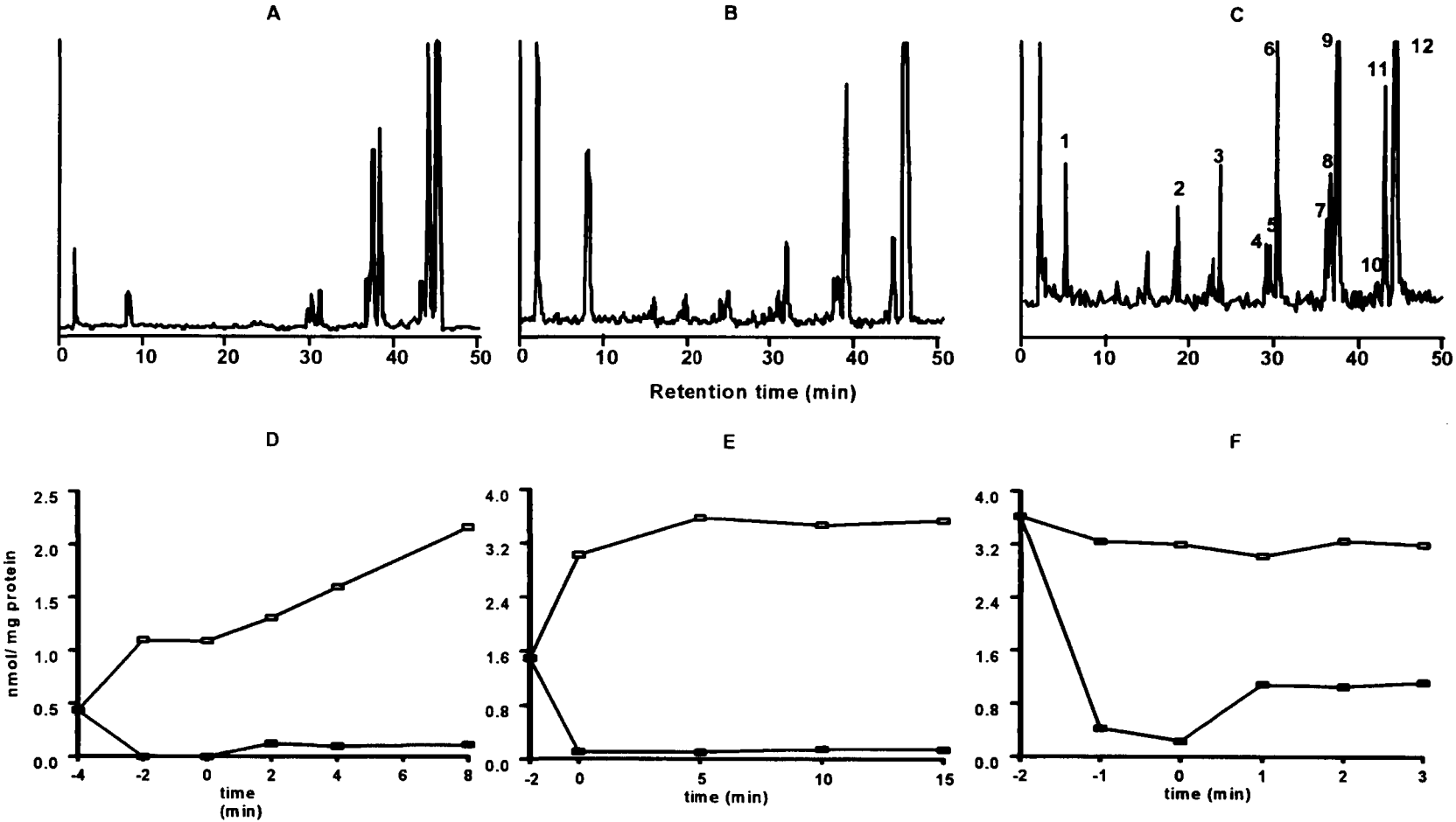

Figure 1. The generation of CoA esters (panels a, b & c) and NADH levels (panels d, e & f in the presence [open squares] and absence [filled squares] of rotenone) during β-oxidation of hexadecanoate in rat mitochondria from: a and d: skeletal muscle, b and e: heart, c and f: liver. The identity of the peaks in panels a, b & c is as follows: (1) acetyl-, (2) octanoyl-, (3) decanoyl-, (4) dodec-2-enoyl-, (5) 3-hydroxytetradecanoyl-, (6) dodecanoyl-, (7) tetradec-2-enoyl-, (8) 3-hydroxyhexadecanoyl-, (9) tetradecanoyl-, (10) hexadec-3-enoyl-, (11) hexadec-2-enoyl-, (12) hexadecanoyl-CoA esters.

levels during a pulse of β-oxidation are similar to those observed in rat skeletal muscle mitochondria. This is to be expected as β-oxidation in both muscle and heart provide up to 70% of the energy requirement of the tissue[22] and there is a similar function of carnitine acetyl transferase in these tissues in providing a buffer for acetyl units.[11,23] Schulz and co-workers have suggested that the inhibition of 3-ketoacyl-CoA thiolase by acetyl-CoA may be an important mechanism of control in heart and skeletal muscle mitochondria.[24] However, we have never observed 3-ketoacyl-CoA esters in our incubations of heart or of skeletal mitochondria, therefore, it does not appear that the observed inhibition of the purified (general) 3-ketoacyl-CoA thiolase extends to the situation in the intact mitochondrion or to the 3-ketoacyl-CoA thiolase activity of the trifunctional protein.

It thus appears that the relation of the NAD^+/NADH pool to β-oxidation is similar to that seen in skeletal muscle mitochondria i.e. that there is a sub-compartment of NAD^+/NADH in proximity to both the trifunctional protein and to complex I.

4. RAT LIVER MITOCHONDRIA

Similar experiments were carried out using rat liver mitochondria and [U-^{14}C]hexadecanoate and a CoA ester chromatogram is shown is Fig. lc.[13] The presence of acetyl-CoA and acetyl-carnitine points to a possible control at the level of disposal of acetyl-CoA to either ketogenesis or complete oxidation via the Krebs cycle.[25] As in rat skeletal muscle mitochondria, we were surprised to observe an accumulation of 3-hydroxyacyl- and 2-enoyl-CoA esters (Fig. lc) and their corresponding carnitine esters; previous measurements of CoA esters in liver mitochondria had observed only saturated esters.[16] However, these measurements had been made with uncoupled mitochondria in which the Krebs cycle was inhibited by fluorocitrate and with a substrate of much lower specific activity (so that the amounts of radioactive CoA esters detected were close to their detection limit). Again, we measured NAD^+ and NADH during a pulse of β-oxidation under identical conditions and found a steady 30% reduction of the pool during oxidation of hexadecanoate (Fig. 1d). This indicates that it is likely that the respiratory chain is responsible for the accumulation of these CoA esters. Uncoupling of mitochondria with 40 μM 2,4-dintrophenol plus 1 μg/ml oligomycin led to a stimulation in the rate of β-oxidation as assessed by total acid soluble radioactivity, as expected (DNP stimulates respiratory chain activity). Hence, although rat liver, muscle and heart mitochondria all produce 3-hydroxacyl- and 2-enoyl- CoA esters, in liver their presence can be explained by a steady-state reduction of NAD^+/NADH, whereas, in muscle and heart there is no great reduction of the NAD^+/NADH pool and we suggest sub-compartmentation of NAD^+ to account for this. It should be noted that in liver the rate of β-oxidation is probably in excess of physiological rates because of the high substrate concentration used and because other physiological controls, such as that exerted at the level of CPT I, are not operative (as malonyl-CoA is absent). The subcompartmentaton of intramitochondrial NAD^+/NADH cannot be ruled out here although an alteration in overall redox state was observed.

5. HUMAN MITOCHONDRIA

Where possible, we have investigated the accumulation of CoA and carnitine esters in mitochondria from human subjects. The accumulation of CoA esters from oxidation

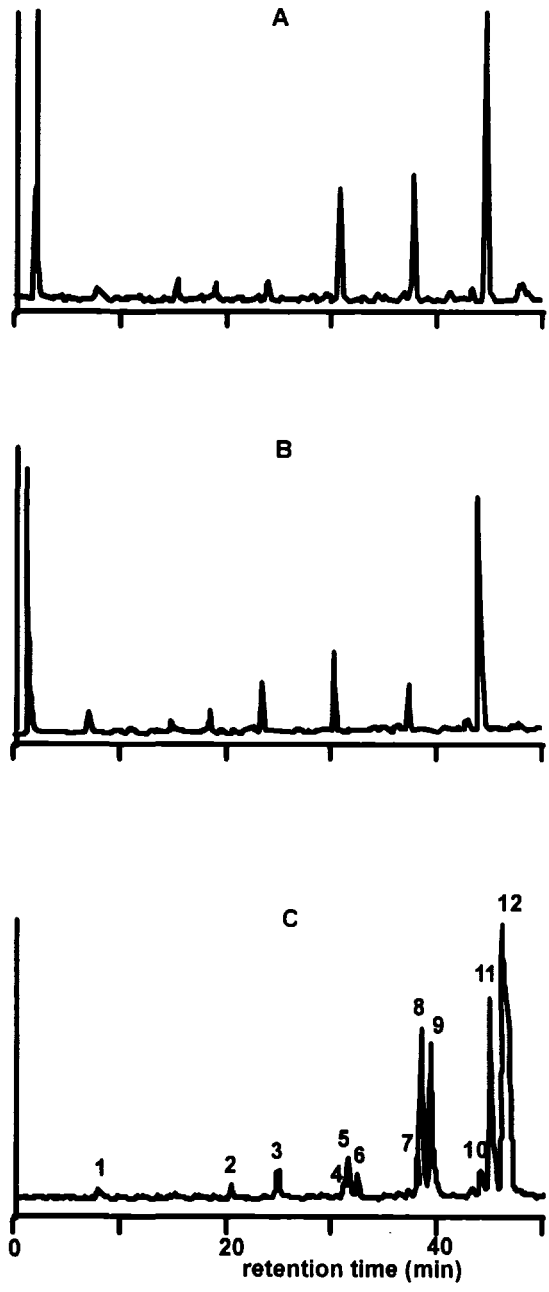

Figure 2. The generation of CoA esters during β-oxidation of hexadecanoate in human mitochondria from: a: skeletal muscle, b: heart, c: liver. Identity of peaks is as in Figure 1.

of [U-^{14}C]hexadecanoate in human muscle and heart (*post-mortem* from a patient with no evidence of myopathy or cardiomyopathy) and human liver (surgical specimen) is shown in Figs. 2a,b and c respectively. Our radio-HPLC methods have been very useful in diagnosis of inborn errors of β-oxidation, for example in cases of 3-hydroxyacyl-CoA dehydrogenase[14] and very long-chain acyl-CoA dehydrogenase deficiencies in skeletal muscle[15] as well as deficiencies in fibroblasts[26,27] and leucocytes.[28] We have also been able

to measure intermediates of β-oxidation in needle biopsies from human liver[29] which we have used for investigation of the pathogenesis of alcoholic liver disease.[36]

As can be seen from Fig. 2a, there is a striking difference between control human skeletal muscle and rat skeletal muscle mitochondria in that 3-hydroxyacyl- and 2-enoyl-CoA esters do not accumulate to any great extent in the human. This pattern has been observed in all the control subjects we have examined.[14,15,30] It is possible that this difference represents a closer association between the trifunctional protein and complex I so that the turnover of the postulated sub-compartment of NAD^+/NADH is faster than in rat skeletal muscle mitochondria, with the consequence that 3-hydroxyacyl- and 2-enoyl-CoA esters do not accumulate. Similarly, in human heart muscle mitochondria (Fig. 2b) there was no accumulation of 3-hydroxyacyl- or 2-enoyl- CoA esters in the single post-mortem heart that we studied[30] and a similar explanation to human skeletal muscle is likely. Human liver mitochondria, however, showed a striking accumulation of 3-hydroxyacyl and 2-enoyl CoA esters, similar to rat liver mitochondria. We have obtained this pattern of intermediates from incubation of mitochondria isolated both from needle biopsies (≈20 mg) and from surgical biopsies from subjects with no histological evidence for liver disease. We have not been able to investigate the accumulation of NAD^+/NADH in any human tissue although it is expected that human liver mitochondria accumulate 3-hydroxyacyl- and 2-enoyl- CoA esters for a similar reason to rat liver mitochondria, i.e. rate limitation by the respiratory chain.

Renewable tissue sources are clearly of great usefulness diagnostically and we have examined the CoA and carnitine esters resulting from β-oxidation of hexadecanoate in fibroblasts[26,27] and leucocytes.[28] In both these cell types, control cells produce only saturated esters although it is interesting to note that in platelet mitochondria, 3-hydroxyacyl and 2-enoyl- CoA esters are found, possibly due to low respiratory chain activity in this cell type.[31,32]

6. DISCUSSION

By examining the patterns of CoA and carnitine esters accumulating in mitochondria incubated with [U-^{14}C]hexadecanoate together with concomitant NADH/NAD^+ measurements, we have been able to infer tissue specific differences in intra-mitochondrial control. It appears that the re-oxidation rate of NADH produced by the 3-hydroxyacyl-CoA dehydrogenase activity of the trifunctional protein is an important factor and suggest that a sub-compartment of NAD^+ exists in close proximity between the complex I and the trifunctional protein. It is not unusual for dehydrogenases to transfer reduced coenzyme directly; indeed short chain 3-hydroxyacyl-CoA dehydrogenase and complex I have been found to interact.[19,20] NAD-dependent dehydrogenases have two different forms of chiral specificity, A and B, differing in the chirality of the transferred hydrogen on the nicotinamide ring. Dehydrogenases of opposite chiral specificity are able to transfer NADH directly between one another, whereas dehydrogenases of the same specificity can only transfer NADH between one another via the aqueous solvent.[33,34] However, complex I appears to accept NADH directly from both A and B type donors, probably reflecting its biological role in accepting NADH from a variety of mitochondrial dehydrogenases and possibly a consequence of its elaborate structure including transhydrogenase activities.[35] The possible interaction between the trifunctional protein and complex I and the chiral specificity of such an interaction remain to be established.

Although we have concentrated our work on the 3-hydroxyacyl-CoA dehydroge-

nases and complex I, it appears that much control strength lies at the acyl-CoA dehydrogenase stage of mitochondrial β-oxidation. However, as outlined in our other paper in this volume, it is not certain whether this is due to the control by the acyl CoA dehydrogenases themselves or due to the combined rate of the ETF-ETF:QO reductase-ubiquinone segment. We intend to extend further our studies on the redox control of β-oxidation to address these questions.

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REFERENCES

1. Saggerson, E.D. (1982) *Biochem. J.* **202**, 397–405. Carnitine acyltransferase activities in rat liver and heart measured with palmitoyl-CoA and octanoyl-CoA Latency, effects of K^+, bivalent metal ions and malonyl-CoA.
2. Kolodziej, M.P., P.J. Crilly, C.G. Corstorphine & V.A. Zammit (1992) *Biochem. J.* **282**, 415–421. Development and characterization of a polyclonal antibody against rat liver mitochondrial overt carnitine palmitoyltransferase (CPT I) Distinction of CPT I from CPT II and of isoforms of CPT I in different tissues.
3. Weis, B.C., V. Esser, D.W. Foster & J.D. McGarry (1994) *J. Biol. Chem.* **269**, 18712–18715. Rat heart expresses two forms of mitochondrial CPT I- the minor component is identical to the liver enzyme.
4. Uchida, Y., K. Izai, T. Orii & T. Hashimoto (1992) *J. Biol. Chem.* **267**, 1034–1041. Novel fatty acid β-oxidation enzymes in rat liver mitochondria II. Purification and properties of enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein.
5. Carpenter, K., R.J. Pollitt & B. Middleton (1992) *Biochem. Biophys. Res. Commun.* **183**, 443–448. Human liver long-chain 3-hydroxyacyl-CoA dehydrogenase is a multifunctional membrane bound β-oxidation enzyme of mitochondria.
6. Izai, K., Y. Uchida, T. Orii, S. Yamamoto & T. Hashimoto (1992) *J. Biol. Chem.* **267**, 1027–1033. Novel fatty acid β-oxidation enzymes in rat liver mitochondria. I Purification and properties of very-long-chain acyl-CoA dehydrogenase.
7. Stanley, K.K. & P.K. Tubbs (1974) *FEBS Lett.* **39**, 325–328. The occurrence of intermediates in mitochondrial fatty acid oxidation.
8. Stanley, K.K. & P.K. Tubbs (1975) *Biochem. J.* **150**, 77–88. The role of intermediates in mitochondrial fatty acid oxidation.
9. Bremer, J. & A.B. Wojtczak (1972) *Biochim. Biophys. Acta* **280**, 515–530. Factors controlling the rate of fatty acid oxidation in rat liver mitochondria
10. Lopes-Cardozo, M., W. Klazinga & S.G. Bergh *Eur. J. Biochem.* (1978) **83**, 629–634. Accumulation of carnitine esters of β-oxidation intermediates during palmitate oxidation by rat-liver mitochondria.
11. Watmough, N.J., A.K.M.J. Bhuiyan, K. Bartlett, H.S.A. Sherratt & D.M. Turnbull (1988) *Biochem. J.* **253**, 541–547. Skeletal muscle mitochondrial β-oxidation. A study of the products of oxidation of [U-^{14}C]hexadecanoate by h.p.l.c. using continuous on-line radiochemical detection.
12. Eaton, S., A.K.M.J. Bhuiyan, R.S. Kler, D.M. Turnbull & K. Bartlett (1993) *Biochem. J.* **289**, 161–168. Intramitochondrial control of the oxidation of hexadecanoate in skeletal muscle.
13. Eaton, S., D.M. Turnbull & K. Bartlett (1994) *Eur. J. Biochem.* **220**, 671–681. Redox control of β-oxidation in rat liver mitochondria.
14. Jackson, S., *et al.* (1992) *J. Clin. Invest.* **90**, 1219–1225. Combined enzyme defect of mitochondrial fatty acid oxidation.
15. Ogilvie, I., *et al. Neurology* **44**, 467–473. Very long-chain acyl coenzyme-A dehydrogenase-deficiency presenting with exercise-induced myoglobinuria.

16. Watmough, N.J., D.M. Turnbull, H.S.A. Sherratt & K. Bartlett (1989) *Biochem. J.* **262**, 261–269. Measurement of the acyl-CoA intermediates of β-oxidation by h.p.l.c. with photodiode array detection.
17. Reichmann, H. & D.C. DeVivo (1991) *Comp. Biochem. Physiol.* **98B**, 327–331. Coordinate enzymatic activity of beta-oxidation and purine nucleotide cycle in a diversity of muscle and other organs of rat.
18. Yang, S.Y., X.Y. He & H. Schulz *J. Biol. Chem.* **262**, 13027–13032. Fatty acid oxidation in rat brain is limited by the low activity of 3-ketoacyl-CoA thiolase.
19. Sumegi, B. & P.A. Srere (1984) *J. Biol. Chem.* 2**59**, 15040–15045. Complex I binds several mitochondrial NAD-coupled dehydrogenases.
20. Fukushima, T., R.V. Decker, W.M. Anderson & H.O. Spivey (1989) *J. Biol. Chem.* **264**, 16483–16488. Substrate channeling of NADH and binding of dehydrogenases to complex I.
21. Sumegi, B., Z. Porpaczy & I. Alkonyi (1991) *Biochim. Biophys. Acta.* **1081**, 121–128. Kinetic advantage of the interaction between the fatty acid beta-oxidation enzymes and the complexes of the respiratory chain.
22. Felig, P. & J. Wahren (1975) *N. Engl. J. Med.* 293, 1078–1084. Fuel homeostasis in exercise.
23. Oram, J.F., S.L. Bennetch & J.R. Neely (1973) *J. Biol. Chem.* **248**, 5299–5309. Regulation of fatty acid utilization in isolated perfused rat hearts.
24. Wang, H.Y., C.F. Baxter Jr. & H. Schulz (1991) *Arch. Biochem. Biophys.* **289**, 274–280. Regulation of fatty acid beta-oxidation in rat heart mitochondria.
25. Quant, P.A., D. Robin, P. Robin, J. Girard & M.D. Brand (1993) *Biochim. Biophys. Acta* **1156**, 135–143. A top-down control analysis in isolated rat-liver mitochondria—Can the 3-hydroxy-3-methylglutaryl-CoA pathway be rate-controlling for ketogenesis?
26. Singh Kler, R., *et al.* (1991) *J. Biol. Chem.* **266**, 22932–22938. Quantitiation of acyl-CoA and acylcarnitine esters accumulated during abnormal fatty acid oxidation.
27. Pourfarzam, M., J. Schaefer, D.M. Turnbull & K. Bartlett (1994) *Clin. Chem.* **40**, 2267–2275. Analysis of fatty acid oxidation intermediates in cultured fibroblasts: a new approach for detection of mitochondrial oxidation disorders.
28. Schaefer, J., M. Pourfarzam, K. Bartlett, S. Jackson & D.M. Turnbull (1994) *Pediatr. Res.* **37**, 354–360. Fatty acid oxidation in peripheral blood cells: characterisation and use for the diagnosis of defects of fatty acid oxidation.
29. Eaton, S., C.O. Record & K. Bartlett (1993) Clin. Chim. Acta **220**, 219–224. Investigation of beta-oxidation in human liver biopsies.
30. Eaton, S.J. (1992) PhD thesis, University of Newcastle-upon-Tyne. Regulation of fatty acid beta-oxidation.
31. Kler, R.S. (1991) PhD thesis, Universty of Newcastle-upon-Tyne. Biochemical studies of mitochondrial fatty acid oxidation.
32. Howell, N., *et al.* (1991) *Am. J. Hum. Genet.* **49**, 939–950. Leber hereditary optic neuropathy: identification of the same mitochondrial ND1 mutation in six pedigrees.
33. Srivastava, D.K. & S.A. Bernhard (1985) *Biochemistry* **24**, 623–628. Mechanism of transfer of reduced nicotinamide adenine dinucleotide among dehydrogenases.
34. Srivastava, D.K., S.A. Bernhard, R. Langridge & J.A. McClarin (1985) *Biochemistry* **24**, 629–635. Molecular basis for the transfer of nicotinamide adenine dinucleotide among dehydrogenases.
35. Ushiroyama, T., T. Fukushima, J.D. Styre & H.O. Spivey (1992) *Curt Top. Cell. Regul.* Substrate channeling of NADH in mitochondrial redox processes. **33**, 291–307.
36. Eaton, S., A. Zaitoun, C. Record, & K. Bartlett, (1996) *Clin. Sci.* **90**, 307–313. Beta-oxidation in human alcoholic and non-alcoholic steatosis.

ENDOTOXIN-INDUCED CHANGES IN VERY-LOW-DENSITY LIPOPROTEIN AND MYOCARDIAL UTILISATION OF TRIACYLGLYCEROL FROM ABNORMAL VLDL IN THE RAT

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1. INTRODUCTION

Lipids are major energetic substrates in oxidative tissues; they are calorifically efficient but poorly water soluble and transport in plasma requires hydrophilic packaging. Non-esterified fatty acids (NEFA) are carried complexed to albumin but toxicity limits their concentration and hence quantitative availability; triacylglycerols (TAG) are the favoured storage form of lipid and can be transported in large amounts as lipid-protein assemblies ("lipoproteins")—chylomicrons, synthesised in the gastrointestinal tract and very-low-density lipoprotein (VLDL), synthesised in liver from endogenous lipid and *de novo* lipogenesis. The protein components of both these lipoproteins ("apoproteins") serve a variety of functions including structural support (apoB) and receptor recognition (apoE). Apoprotein-CII (apo-CII) is an activator of the enzyme responsible for TAG hydrolysis, lipoprotein lipase (EC 3.1.1.34; LPL). LPL activity is considered rate-limiting for circulating TAG utilisation and is tissue-specific depending on tissue activity.

During systemic sepsis, plasma [TAG] is increased; this is due to increased plasma VLDL, a result of both increased hepatic lipogenesis and VLDL secretion, and of decreased adipose tissue LPL activity limiting peripheral uptake. These effects are mediated by pathogen-derived endotoxin stimulating host immune cells to release inflammatory mediators (cytokines).[3] The function of the excess VLDL-TAG produced during

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systemic inflammation is unknown, but it is abnormal in composition.[4] The heart has increased workload during early sepsis due to increased tissue demand for cardiac output. The VLDL produced in sepsis may therefore be destined for the heart. To test this radiolabelled VLDL was prepared by perfusing livers from control and endotoxic rats; the VLDL was then analysed and used to perfuse isolated working hearts from control and endotoxic rats, and the efficacy of the VLDL-TAG to act as substrate for myocardial oxidation and tissue lipid synthesis was measured.

2. MATERIALS AND METHODS

2.1. Preparation of Lipid Substrates

(i) ^{3}H-Labelled sodium oleate was prebound to fatty acid-free bovine serum albumin and added to the heart perfusate to give a final concentration of 1.1 mM (NEFA group). (ii) ^{3}H-Labelled triolein in the form of rat VLDL was prepared by rat liver perfusion. Fasted rats were pre-treated 15 h prior to experiment with endotoxin (from E. coli serotype 055:B5) 100 µg/kg body wt. or saline (control) intraperitoneally; they were anaesthetised, the portal vein and right atrium were cannulated without heparin and the inferior vena cava was ligated. The liver was perfused *in situ* with a recirculating solution comprising Waymouth's medium supplemented with glucose, amino acids, washed red cells and [^{3}H]oleate prebound to fatty acid-free albumin, gassed with O_2:CO_2 (95:5) at 37 °C. The perfusate was ultracentrifuged at 144,500 g to separate d < 1.006 layer. Thin-layer chromatography (TLC) of the ^{3}H-VLDL showed that ≥ 95% of the label was in the triacylglycerol band. VLDL were suspended in fatty-acid free bovine serum albumin, TAG content was assayed (Boehringer test kit) and added to the heart perfusate to give a final concentration of 0.4 mM TAG.

2.2. Isolated Perfused Working Heart Preparation

Hearts from fed rats were perfused in "working" mode by the Taegtmeyer *et al.*[7] modification of the method of Neely and Morgan. The heart was excised, the aorta cannulated (<2min from excision) and perfused retrogradely through the coronary arteries in "Langendorff" mode whilst lung, mediastinal, and peri-cardiac brown adipose tissue were excised, pulmonary arteriotomy performed, and the left atrium cannulated. The apparatus was switched to "working" mode and cardiac perfusion maintained through the left atrium (anterograde). A recirculating Krebs-Henseleit bicarbonate buffer solution containing $CaCl_2$ 1.3mM, glucose 10mM and fatty-acid free bovine serum albumin 2% (w/v) was filtered and gassed with O_2:CO_2(95:5) at 37°C. Afterload was 100 cmH_2O and preload (atrial filling pressure) 15 cmH_2O. After 15 min stabilisation lipid substrate (*v.s.*) was added (2min) (Time "0"). Peak systolic pressure (PSP) and heart rate (HR) were measured by pressure transducer. Aortic flow rate (AFR) and coronary flow rate (CFR) were measured by timed collections of perfusate. Measurements were made at time 0 and at 10 min intervals for 60 min. Cardiac output (CO) was calculated as (CFR + AFR). Hydraulic work (HW) was calculated as (CO x mean aortic pressure ÷ heart wt.). Rate-pressure product = PSP × HR. After t = 60min heparin (5U/ml) was added and at t = 62 min a perfusate sample was taken for LPL assay, the heart was rapidly excised, freeze-clamped in light alloy tongs cooled in liquid nitrogen, and weighed.

2.3. Measurement of Lipid Oxidation Rate

Timed perfusate samples were extracted (Folch) with chloroform/methanol/water; the aqueous supernatant phase was counted for ^3H_2O radioactivity as described[2] (TAG oxidation rate), the organic infranatant phase was dried, resolubilised in ethanol and assayed for TAG (TAG uptake rate).

2.4. Incorporation of Exogenous Lipid into Myocardial Lipid

Frozen myocardium was ground to powder and ^{3}H-myocardial lipids were extracted with chloroform :methanol (Folch). After washing, the lipids were re-solubilised in chloroform and separated by TLC using a hexane-diethylether-acetic acid system. Lipid classes were counted for ^{3}H-radioactivity.

2.5. Lipoprotein Lipase Activity

Myocardial LPL activity was estimated in post-heparin perfusate ("heparin-releasable") and in acetone/ether-dried ground tissue powders ("residual-tissue") by using a ^{3}H-labelled triolein substrate emulsion and counting radioactivity in evolved fatty acids extracted in methanol/chloroform/heptane.[5]

2.6. Analysis of VLDL Composition

Apoprotein composition was analysed by denaturing SDS-polyacrylamide gel electrophoresis/Coomassie Brilliant Blue staining and densitometry; lipid composition (NEFA, TAG, cholesterol and cholesterol ester, and phospholipid) was determined with commercial kits (Sigma).

2.7. Statistics

Results are expressed as mean values ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) for repeated measurements, or by Student's *t* test with Bonferroni correction for multiple comparisons, where appropriate. Statistical significance was set at $P < 0.05$.

3. RESULTS

Working hearts were able to assimilate and oxidise VLDL-TAG (Fig. 1); pre-treatment of animals with endotoxin (LPS) before isolation of hearts had no effect on the rate of TAG uptake or oxidation, but VLDL-TAG prepared from livers of endotoxic rats was taken up and oxidised by hearts at a significantly greater rate than control VLDL prepared from non-endotoxic livers, regardless of heart endotoxic status (Fig. 1). "Endotoxic" VLDL had a greater TAG content than control VLDL when corrected for apo-B content (data not shown). Under conditions of moderate workload, cardiac output and hydraulic work were significantly increased in endotoxic VLDL-perfused hearts compared to control VLDL-perfused hearts, again regardless of endotoxic status of the heart (Fig. 2) although rate-pressure product was unchanged (data not shown). Accumulation of [^{3}H]-labelled lipid by cardiac tissue was unchanged by pre-treatment with endotoxin

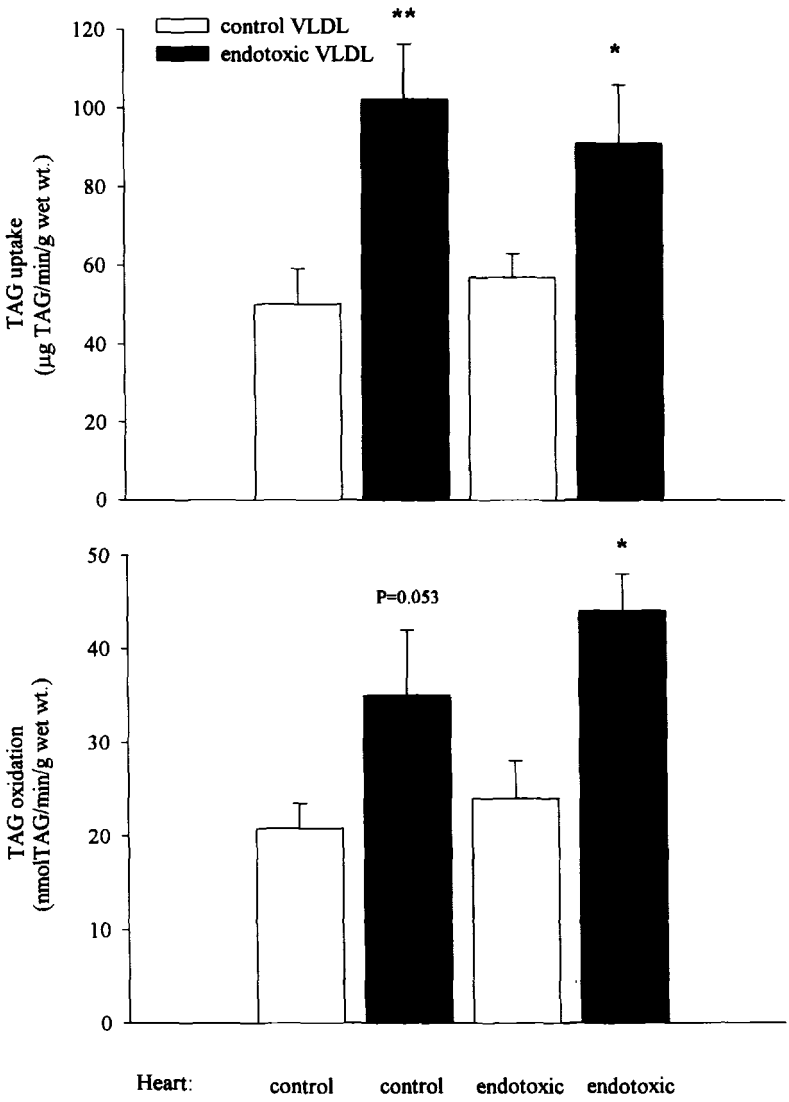

Figure 1. Utilisation of VLDL-TAG by working hearts prepared from control and endotoxic rats. Rats were pretreated with endotoxin prior to liver perfusion (VLDL preparation) or heart perfusion. Results are means ± SEM for n = 6–10 experiments, *P < 0.05; **P < 0.01 compared to control hearts perfused with control VLDL.

(Table 1). However, endotoxic VLDL was also prepared from rats treated with a lower dose of endotoxin (20 μg/kg body wt.) prior to liver perfusion; myocardial accumulation of this "low dose" endotoxic VLDL was significantly less in perfused hearts (238 ± 33 nmol/g wet wt. (n = 6)) than in control VLDL-perfused hearts (525 ± 97 nmol/g wet wt. (n = 10) P < 0.05), This was due to decreased accumulation of [^{3}H]TAG and [^{3}H] diacylglycerol but with no change in [^{3}H]cholesterol ester, [^{3}H]NEFA and [^{3}H]phospholipid accumulation. Lipoprotein lipase activity was examined in heparin-releasable (endothe-

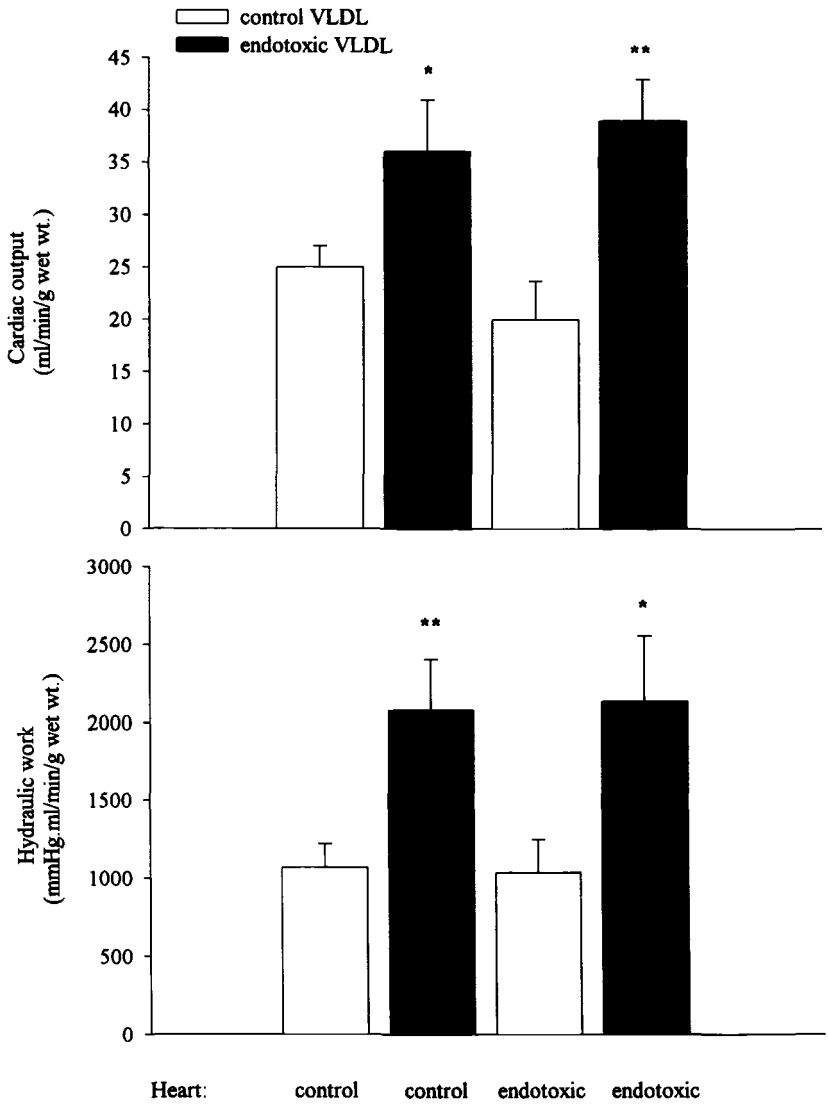

Figure 2. Mechanical function in rat hearts perfused with VLDL. Hearts from control and endotoxic rats were perfused with VLDL prepared from control and endotoxic rat livers. Results are means ± SEM for n = 6–10 experiments. *P < 0.05; **P < 0.01 compared to control hearts with control VLDL.

lial, physiologically active) and tissue-residual (nascent) components and compared to NEFA-perfused hearts (Fig. 3). Total (heparin-releasable + tissue-residual) LPL activity was significantly increased in VLDL-perfused hearts compared to hearts perfused with NEFA (1.1 mM oleate) under identical conditions; perfusion with endotoxic VLDL (VLDL-LPS) increased the proportion of the enzyme present at the endothelium (i.e. heparin-releasable) whilst decreasing the amount of residual enzyme in the tissue (i.e. causing translocation of the enzyme from intracellular to endothelial site) (Fig. 3).

Table 1. Accumulation of tissue [^{3}H]lipids by VLDL-perfused rat heart.

		Myocardial tissue [^{3}H]lipids (nmol/g wet wt.)					
VLDL:	Heart:	NEFA	CE	PL	DAG	TAG	Total
control	control	31 ± 16	7.2 ± 1.7	44 ± 2	19 ± 5	425 ± 92	525 ± 97
endotoxin	control	31 ± 7	6.9 ± 0.8	41 ± 7	17 ± 4	248 ± 28	344 ± 39
control	endotoxin	33 ± 6	6.4 ± 1.2	45 ± 17	14 ± 2	486 ± 184	584 ± 175
endotoxin	endotoxin	38 ± 10	6.5 ± 1.0	47 ± 10	17 ± 2	459 ± 169	585 ± 175

NEFA: non-esterified fatty acid; CE: cholesterol ester; PL: phospholipid; DAG: diacylglycerol; TAG: triacylglycerol. For details see text. n = 6–10; no significant differences.

4. DISCUSSION

These results indicate that whilst VLDL can act as a substrate for heart at physiological concentrations, its efficacy as a cardiac substrate is significantly improved if the VLDL is synthesised by a liver previously subjected to endotoxic stimulation—a greater proportion of the TAG is assimilated and then oxidised, and under certain dosage conditions less is esterified into myocardial tissue lipid whilst hydraulic work and cardiac output are doubled. This effect may be partly due to increased LPL activity although the mechanism for the endotoxic VLDL-induced translocation of myocardial LPL to its active endothelial site is uncertain. One report[4] suggests that the composition of VLDL may change during systemic *E.coli* sepsis with proportionately increased NEFA and TAG compared to apoprotein content in VLDL particles from septic rats. Such a modification of VLDL particle composition may explain the change in LPL activity since TAG and

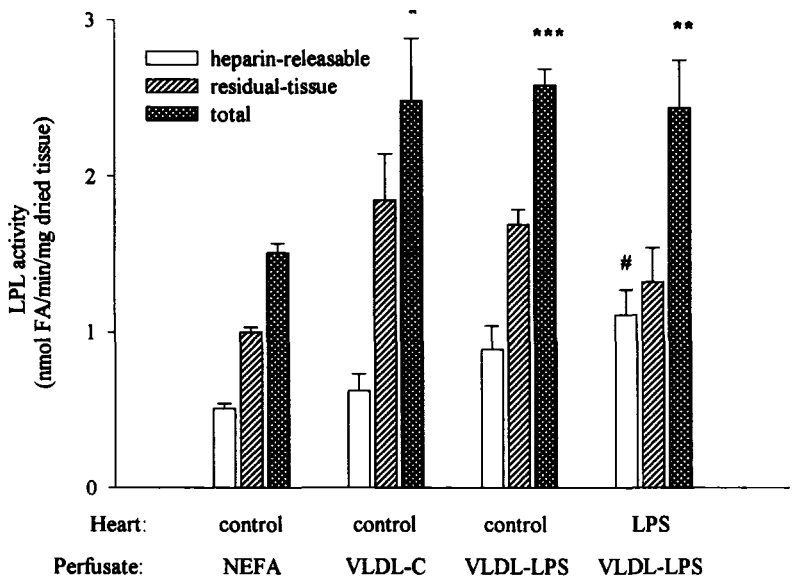

Figure 3. Effect of VLDL on LPL activity in perfused working rat heart. Hearts from endotoxic or control rats were perfused with VLDL from control (VLDL-C) or endotoxic (VLDL-LPS) rat liver perfusions; some control hearts were perfused with oleate (NEFA). Results are means ± SEM for n = 6–10 experiments. *P < 0.05, **P < 0.01, ***P < 0.001 VLDL compared to NEFA, #P < 0.05 control VLDL compared to endotoxic VLDL.

NEFA are known to modulate cardiac LPL activity,[1,6] and the increased TAG content of VLDL may render it a better substrate for LPL. Glucose was present as a co-substrate but its rate of utilisation was not measured in these experiments. The mechanism whereby endotoxaemia induces increased TAG content of VLDL is unknown but may be related to the increased hepatic lipogenesis.[3] An alternative pathway for VLDL access into the cardiomyocyte is through the VLDL-receptor[8] which binds apo-E as well as LPL; this may account for quantitatively important amounts of VLDL-TAG uptake under certain conditions but Tripp *et al.*[9] noted decreased hepatic VLDL-apo-E synthesis during systemic sepsis suggesting that this pathway is unlikely to be increased during endotoxinaemia. These results support the hypothesis that excessive, abnormal VLDL produced by the liver during systemic sepsis/endotoxinaemia may be destined for the heart.

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REFERENCES

1. Anderson, L.G., Carroll, R., Ewart, H.S., Acharya, A. & Severson, D.L. (1997) *Am-J-Physiol,* **273**, E759–E767. Fatty acids reduce heparin-releasable LPL activity in cultured cardiomyocytes from rat heart.
2. Evans, R.D. & Wang, X. (1997) *Biotechnology Techniques*, **11**, 127–132. Measurement of fatty acid oxidation in isolated perfused rat heart. A simple and accurate method.
3. Evans, R.D. & Williamson, D.H. (1991) *Biochem-Cell-Biol*, **69**, 320–321. Signals, mechanisms, and function of the acute lipid response to sepsis.
4. Lanza Jacoby, S., Wong, S.H., Tabares, A., Baer, D. & Schneider, T. (1992) *Biochim-Biophys-Acta*, **1124**, 233–240. Disturbances in the composition of plasma lipoproteins during gram-negative sepsis in the rat.
5. Nilsson Ehle, P. & Ekman, R. (1997) *Artery,* **3**, 94–209. Rapid, simple and specific assays for lipoprotein lipase and hepatic lipase.
6. Rodrigues, B., Braun, J.E., Spooner, M. & Severson, D.L. (1992) *Can-J-Physiol-Pharmacol,* **70**, 1271–1279. Regulation of lipoprotein lipase activity in cardiac myocytes from control and diabetic rat hearts by plasma lipids.
7. Taegtmeyer, H., Hems, R. & Krebs, H.A. *Biochem-J,* **186**, 701–711. Utilization of energy-providing substrates in the isolated working rat heart.
8. Takahashi, S., Suzuki, J., Kohno, M., Oida, K., Tamai, T., Miyabo, S., Yamamoto, T. & Nakai, T. (1995) *J-Biol-Chem,* **270**, 15747–15754. Enhancement of the binding of triglyceride-rich lipoproteins to the very low density lipoprotein receptor by apolipoprotein E and lipoprotein lipase.
9. Tripp, R.J., Tabares, A., Wang, H. & Lanza Jacoby, S. (1993) *J-Surg-Res,* **55**, 465–472. Altered hepatic production of apolipoproteins B and E in the fasted septic rat: factors in the development of hypertriglyceridemia.

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EFFECT OF VALPROIC ACID ON THE EXPRESSION OF ACYL-COA DEHYDROGENASES IN VARIOUS TISSUES

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1. INTRODUCTION

Valproic acid (2-propyl-n-pentanoic acid, VPA) is an anticonvulsant widely used in the treatment of various epileptic disorders.[1] It has been known that VPA administration caused severe hepatic dysfunction similar to Reye's syndrome in a small number of patients.[2,3] Deaths from hepatotoxicity were also reported. VPA affects carnitine and ammonia levels and other metabolic parameters related to fatty acid oxidation. The potential hepatotoxicity by VPA is caused by its unsaturated metabolites, such as 4-en-VPA.[4,5] Histologically, microvesicular steatosis induced by 4-en-VPA is accompanied by ultrastructural changes characterized by myeloid bodies, lipid vacuoles and mitochondrial abnormalities. An enhanced excretion of C_6 to C_{10} dicarboxylic acids by patients and rats indicates an interference with mitochondrial β-oxidation as an important pathogenesis.[5,6] If the normal pathway of fatty acid oxidation is disrupted by VPA, it results in reduced ketone body formation and decrease of free coenzyme-A (CoA) in the liver. Especially, decreased CoA would limit the activities of one or more enzymes in the pathway of fatty acid oxidation.

VPA is metabolized via four different routes; β-oxidation, glucuronide formation,

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ω-oxidation and ω-1-oxidation. Among them, β-oxidation has the most important role. However, neither the enzyme that is responsible for the dehydrogenation of valproyl-CoA in β-oxidation, nor the enzymatic basis for the inhibition of fatty acid oxidation by VPA had been known for a long period. With the progress of enzymatic and molecular studies of fatty acid β-oxidation, five mitochondrial acyl-CoA dehydrogenases have been well characterized.[7] Three of them, short chain (SCAD), medium chain (MCAD), and long chain acyl-CoA dehydrogenases (LCAD), catalyze the first step of β-oxidation of fatty acids with varying chain length, whereas two others, isovaleryl-CoA dehydrogenase (IVD) and 2-methyl-branched chain acyl-CoA dehydrogenase, catalyze the third step in the leucine and valine/isoleucine oxidation pathways, respectively.[8–10] Comparison of the amino acid sequence of four acyl-CoA dehydrogenases (SCAD, MCAD, LCAD, and IVD) revealed a distinct homology, indicating that these enzymes belong to a gene family, the acyl-CoA dehydrogenase family.[11] Ito *et al.*[12] first demonstrated that valproyl-CoA is dehydrogenated by 2-methyl-branched-chain acyl-CoA dehydrogenase, one of the enzymes that belong to the acyl-CoA dehydrogenase family. Also, VPA moderately inhibited human acyl-CoA dehydrogenases except the long chain enzyme *in vitro*.[12] However, the biochemical basis of the inhibition of fatty acid β-oxidation could not be fully explained by these observations. Decreased synthesis or an enhanced degradation of acyl-CoA dehydrogenases could be a possible mechanism. It is currently unknown whether VPA affects each step of the biosynthesis of acyl-CoA dehydrogenases or not.

In this report, we studied the effects of VPA on the amount of each ACD mRNA and protein in rat tissues, and also the post-translational processing *in vitro*.

2. MATERIALS AND METHODS

2.1. Materials, Source of Antibodies and cDNAs, and Preparation of Probes

VPA was obtained from Sigma (St. Louis, MO). Antibodies and cDNAs for various acyl-CoA dehydrogenases (SCAD, MCAD, LCAD and IVD) were gifts from Prof. Kay Tanaka, Yale university school of medicine.[7–11] cDNAs for rat ornithine transcarbamylase (OTC) and β-actin were previously synthesized in our laboratory using cDNA synthesis kit (Clontech, CA). For use as probes, cDNAs were radiolabeled with [α ^{32}P]dCTP (3,000 mCi/mmol, Amersham Corp.) using a random primer DNA labeling method.[13]

2.2 Animals and Assay of Clinical Chemistry Parameters

Male Wistar rats, weighing 100–120 g, were obtained from Charles River and were randomly divided into two groups (ten rats each). They were fed with standard commercial diet and drank water *ad libitum* during the study period. The control group of rats received normal saline (0.5ml twice a day), and the experimental group received VPA (500mg/kg, divided into two doses/d) by intraperitoneal injections for 7 days. Clinical chemistry parameters in rat blood (glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase, ammonia, glucose, β-hydroxybutyrate and aceto-acetate) were measured using diagnostic kits from Sigma (St. Louis, MO). Free and total carnitine levels in plasma and tissue were determined by the method of McGarry and Foster.[14]

2.3 RNA Preparation, Isolation of Mitochondria and Assay of Acyl-CoA Dehydrogenases

The rats were anesthetized with pentobarbital and blood samples were collected from the carotid artery. After decapitation, tissues were rapidly removed, weighed, and used for further experiments. Total RNA was prepared from each tissue by the guanidine isothiocyanate method.[15] Mitochondria was isolated from rat liver and heart by the method of Schnaitman and Greenwalt.[16] Acyl-CoA dehydrogenase activities were measured spectrophotometrically in the presence of 0.1 mM FAD as previously described.[7]

2.4 Immunoblot Analysis

Mitochondria were solubilized by boiling for 3min with a 10-fold volume of buffer containing 4% SDS, 125 mM Tris-HCl, (pH 6.8), 20% glycerol, 0.01% bromophenol blue, and 10% 2-mercaptoethanol. Samples were electro-phoresed on 12.5% SDS-polyacrylamide gel with a 4% stacking gel. After electrophoresis, the gel was electroblotted on to a Immobilon-P membrane (Millipore), according to the method of Towbin *et al.*[17] Immunoreactive protein was detected using the Protoblot Western Blot AP system (Promega). The relative amount of protein bands in the blot was determined densitometrically.

2.5 Slot Blot Hybridization

Total RNA was denatured with 1M glyoxal in 10mM sodium phosphate buffer (pH 6.8) at 50°C for 1 h. Slot-blots were prepared by applying the glyoxylated RNAs to a Nylon membrane using a microsample filtration manifold (Bio-Rad). The nylon membrane was then baked at 80 °C and boiled in 20 mM Tris-HCl (pH 8) for 10min. The membrane was prehybridized with sonicated and denatured salmon sperm DNA (250mg/ml) for 24 h at 42 °C in prehybridization buffer consisting of 50% formamide, 5 × SSC (1 × SSC, 150mM sodium chloride, and 15mM sodium citrate) 50 mM sodium phosphate buffer (pH 6.5), and 1 × Denhart's solution. The nylon membrane thus prepared was hybridized with an appropriate radiolabelled cDNA. After hybridization, the membrane was washed at room temperature with four changes of 2 × SSC, 0.1% SDS, for 15min each and then at 64°C with two changes of 0.2 × SSC, 0.1% SDS for 30min each, and was exposed to an X-ray film at –70 °C for 4–6 h.

2.6 *In Vitro* Transcription/Translation of Individual Proteins

cDNA inserts, each containing the entire coding region of precursor(p)SCAD, pMCAD, pIVD and pOTC (ornithine transcaramylase) were subcloned into pGEM blue or pBluescript transcription vector. *In vitro* transcription of cDNAs was carried out using T7 or SP6 RNA polymerase (Promega Biotec). The synthesized mRNA was translated *in vitro* using the rabbit reticulocyte lysate translation kit (Promega Biotec). The reaction mixture, containing [^{35}S]methionine, a mixture of unlabelled amino acids (minus methionine), *in vitro* transcribed mRNA, and appropriately prepared reticulocyte lysate, was incubated for 1 hr at 30 °C. The translated polypeptides were analyzed, either directly or after immunoprecipitation, by SDS-PAGE on 8% gel fluorography.

2.7 Post-Translational Processing and Assay of Stability of Mature Proteins

The freshly isolated mitochondria were suspended at a final concentration of 20 mg protein/ml in HMS (2mM Hepes, pH 7.4, 220mM mannitol, 70mM sucrose) buffer. The translation mixture (6ml) was mixed with 4ml of mitochondrial suspension and 2ml of HMS buffer and was incubated at 27 °C for 30min. The processed products were analyzed with SDS-PAGE and fluorography. For analyzing the stability of the mature enzymes, the reaction mixture was centrifuged for 5min at 8,000 × g. The isolated mitochondrial pellet was resuspended in 10 µl of HMS buffer and further incubated at 37 °C for varying periods of time, and then the samples were treated with 5 µg of trypsin/mg protein for 10min at 4°C After trypsin treatment, soybean trypsin inhibitor (8mg/ml) was added, and the mitochondrial extracts were analyzed with SDS-PAGE and fluorography.[18]

3. RESULTS

3.1. Metabolic Effects of VPA in Rats

All rats survived the experiments. There was no significant differences in body weight gain between the control and experimental group. Intraperitoneal administration of VPA (500mg/kg body weight, divided into two doses/d) to rats caused some specific changes in clinical chemistry parameters, which have also been reported in humans during valproate therapy. The glutamic-oxalacetic transaminase (GOT) and ammonia values for VPA-treated rats were higher than the controls. The serum glucose and β-hydroxybutyrate (BHB) concentrations were significantly decreased by VPA administration. The values for glutamic-pyruvic transaminase (GPT) and acetoacetate did not show statistically significant differences between two groups (Table 1). Increased excretion of urinary dicarboxylic acids were also observed in experimental animals (data not shown).

3.2 Carnitine Concentrations in Various Tissue

Reduction in the free carnitine levels in blood and tissue is another effect of VPA administration.[19] We, therefore, evaluated changes of carnitine levels in the control and VPA-treated groups. Liver, heart, muscle and plasma carnitine concentrations in control and VPA-treated rats are shown in Table 2. In liver, both free and acyl-carnitine were unaffected by VPA administration. However, in heart and plasma, VPA-treated rats

Table 1. Effect of administration of sodium valproate on various clinical chemistry parameters in rats.

	VPA (n = 10)	Control (n = 10)
GOT (IU/l)	29.2 ± 2.3*	15.4 ± 2.6
GPT (IU/l)	72.3 ± 27.1	60.6 ± 18.5
Ammonia (µg/ml)	2.9 ± 0.3*	2.1 ± 0.2
Glucose (mg/dl)	57.6 ± 14.2*	125.0 ± 30.6
BHB (mmoles/l)	0.26 ± 0.05*	1.55 ± 0.34
Acetoacetate (mmoles/l)	0.85 ± 0.31	0.75 ± 0.30

GOT: glutamic-oxalacetic transaminase, GPT: glutamic-pyruvic transaminase, BHB: β-hydroxybutyrate, *$p < 0.01$

Table 2. Carnitine concentrations in liver, heart, muscle and plasma.

		Total	Free	Acyl
Liver	Control	1.5 ± 0.1	1.1 ± 0.2	0.4 ± 0.1
	VPA	1.6 ± 0.2	1.1 ± 0.2	0.5 ± 0.2
Heart	Control	5.3 ± 0.4	3.8 ± 0.5	1.5 ± 0.1
	VPA	5.0 ± 0.6	2.8 ± 0.3*	2.3 ± 0.2**
Muscle	Control	4.1 ± 0.6	2.7 ± 0.5	1.3 ± 0.5
	VPA	4.0 ± 0.4	2.0 ± 0.3	1.9 ± 0.3
Plasma	Control	62.1 ± 8.4	45.3 ± 6.1	16.0 ± 2.3
	VPA	50.3 ± 5.1	30.8 ± 3.3**	19.7 ± 2.1

liver, heart & muscle: nmole/mg non-collagen protein
plasma: μmole/l
Results are means ± S.D. for four experiments.
*$p < 0.05$, **$p < 0.01$

clearly showed reduced concentrations of free carnitine and increased levels of acyl-carnitine.

3.3 Enzyme Activities and Immunoblot Analysis of Various Acyl-CoA Dehydrogenases in Control and VPA-treated Rats

Liver and heart mitochondria from ten normal and ten VPA-treated rats were separately pooled and used for enzyme assay and irnmunoblot analysis. Enzyme assays by a dye reduction method revealed that fatty acyl-CoA dehydrogenase (SCAD, MCAD and LCAD) activities in VPA-treated rat heart mitochondria were moderately decreased (57–79% of control), however, those in VPA-treated liver mitochondria were slightly reduced (78–95% of control). IVD activities in liver and heart were almost unchanged by VPA. We then estimated each enzyme protein by immunoblot analysis. The intensity of signals of fatty acyl-CoA dehydrogenases in VPA-treated heart decreased significantly compared to controls (Fig. 1). There were no reductions of any of the ACD proteins in liver (data not shown).

Table 3. Acyl-CoA dehydrogenase activities in liver and heart mitochondria from untreated and VPA-administered rats.

	Activities (nmol/mg protein per min)	
Subject	VPA (% of control)	Control
Liver		
SCAD	1.2 ± 0.1 (86)	1.4 ± 0.2
MCAD	3.2 ± 0.3 (78)	4.1 ± 0.7
LCAD	11.5 ± 0.6 (95)	12.1 ± 0.8
IVD	0.3 ± 0.06 (100)	0.3 ± 0.08
Heart		
SCAD	4.0 ± 0.5 (65)	6.1 ± 1.1
MCAD	5.3 ± 0.9 (57)	9.3 ± 1.8
LCAD	17.1 ± 2.0 (79)	21.9 ± 1.5
IVD	1.4 ± 0.1 (104)	1.3 ± 0.1

Results are means ± S.D. for 10 rats.

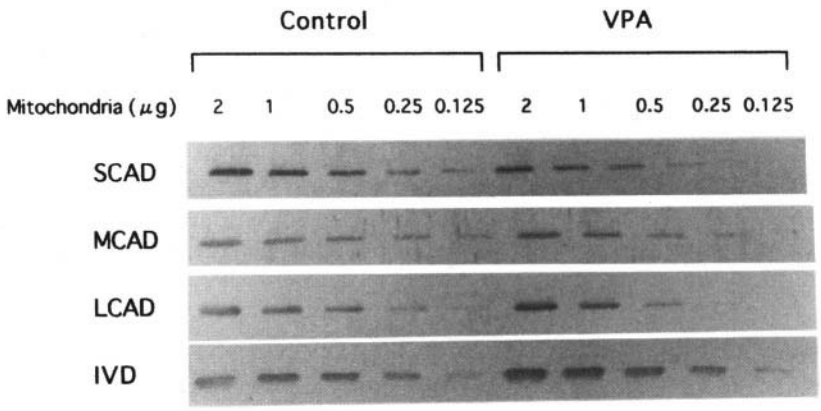

Figure 1. Immunoblot analysis of various acyl-CoA dehydrogenases in rat heart mitochondria. Heart mitochondria were isolated from control and VPA-treated rats. Serial 2-fold dilutions of mitochondrial suspension (2.0, 1.0, 0.5, 0.25 and 0.125 μg protein) were subjected to immunoblot analysis.

3.4 Slot-Blot Hybridization Analysis of mRNAs for Various Acyl-CoA Dehydrogenases

RNA preparations from ten control and ten VPA-treated rats were separately combined. Three serially diluted amounts (4, 2, and 1 μg) of each total RNA preparation were applied to nylon membranes using a microsample filtration manifold and were hybridized with a radiolabelled cDNA probe. Quantitation of each slot was performed by densitometric scanning of X-ray film. We first carried out preliminary slot-blot hybridization experiment to confirm that each species of mRNA is specifically detected by its cDNA probe. The accuracy of quantitation of mRNA amounts was also tested. The result for SCAD mRNA is shown in Figure 2. The slot blots of SCAD mRNA in the heart were more intensely labeled than those of the liver. Hybridization signals of each slot were comparable to the amount of total RNA applied. There was no hybridization to yeast tRNA at all. The same experiments using other cDNA probes (MCAD, LCAD, and IVD) also demonstrated the specificity and accuracy of quantitation (data not shown).

Figure 2. Slot-blot analysis of SCAD mRNA in rat liver and heart. Total RNA was isolated from liver and heart of adult Wistar rats. For each tissue, samples that contained 4,2,1,0.5 μg of total RNA were hybridized with SCAD cDNA probe as described under Materials and Methods. Yeast tRNA was used as a negative control.

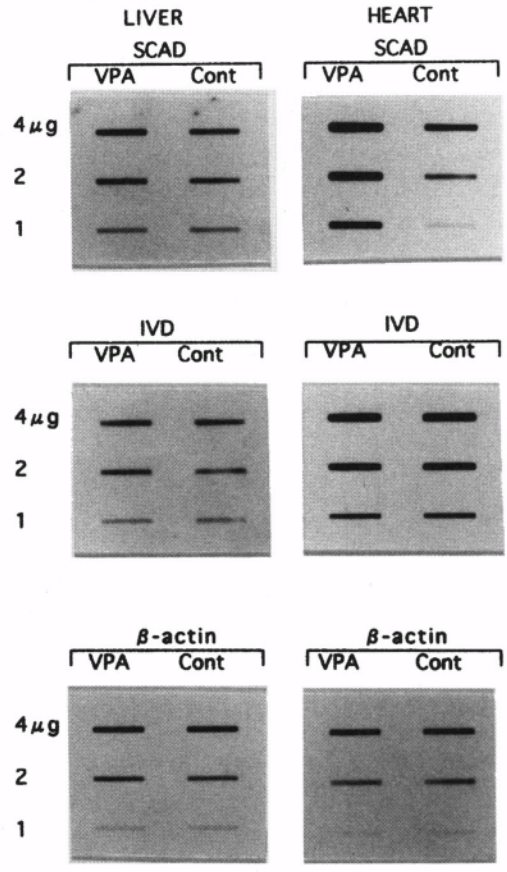

Figure 3. Slot-blot analysis of mRNAs for SCAD, IVD and β-actin from VPA-treated rat liver and heart. RNA was prepared from the livers and hearts of 10 control and 10 VPA-treated rats, respectively. Serial 2-fold dilution of total RNA (4,2,1 μg) were hybridized to the individual cDNA probes.

We then determined the amounts of mRNAs of four acyl-CoA dehydrogenases in various tissues of control and VPA-treated rats. The slot blots of SCAD, IVD and β-actin mRNAs in the liver and heart are shown in Figure 3. Densitometric measurements of mRNA of four acyl-CoA dehydrogenases are summarized in Figure 4. In the liver, kidney, and skeletal muscle, the levels of mRNAs involved in fatty oxidation (SCAD, MCAD, and LCAD) increased 1.2–1.6 fold over control. In small intestine, the mRNA levels of all four ACDs in VPA-treated rats did not show significant differences from controls. The increases of SCAD and MCAD mRNAs in the heart were particularly large, reaching the levels of approximately 2.2–2.5 fold over the control. In contrast, the amounts of the IVD mRNA and β-actin mRNA were almost the same as control in five organs.

3.5. Effects of VPA on *in Vitro* Translation

The decreased amounts of ACD proteins in the presence of increased levels of each mRNA could be caused by impaired translation or post-translational processing, or by increased degradation of enzyme protein. First, we evaluated the effect of VPA on *in vitro* translation of ACDs. The reticulocyte lysate was preincubated with four different concentrations of VPA (0, 0.5, 1.0, and 10mM) for 1h at 30 °C. Immediately after this preincubation, *in vitro* translation was started by adding *in vitro* transcribed mRNA,

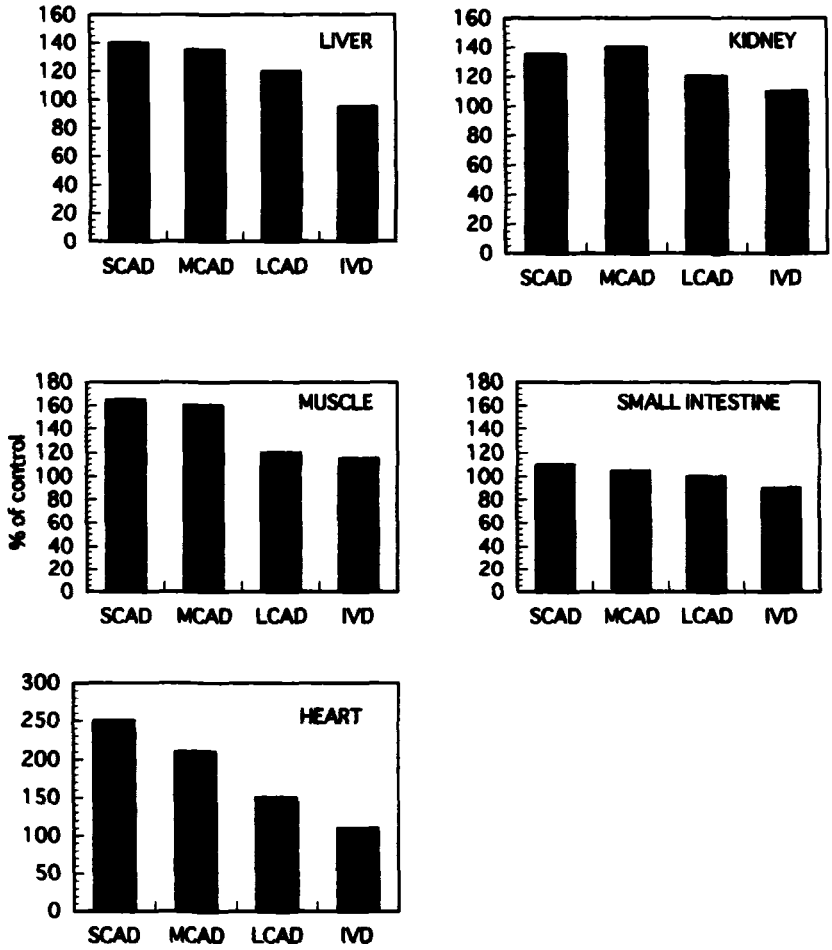

Figure 4. Tissue specific changes in the amount of mRNAs for various acyl-CoA dehydrogenases caused by VPA administration. Adult Wistar rats were divided into two groups of ten rats (Control and VPA-treated). Total RNA was prepared from liver, psoas muscle, heart, kidney and small intestine. Serial 2-fold dilutions of total RNA (4,2,1 μg) were blotted onto a nylon membrane and hybridized with the radiolabeled cDNA probes for various acyl-CoA dehydrogenases. The results on the amount of each mRNA were quantitatively analyzed using a densitometer, and expressed as a shift from the control level in percentage. Each value represents the mean of three experiments.

[^{35}S]methionine, and the minus-methionine amino acid mixture. Autoradiographs of SCAD and IVD are shown in Figure 5. The amount of synthesized protein was measured by liquid scintillation. Translational activity was not altered in the range of VPA concentrations tested. We repeated the experiments using a different batch of lysate, and the same result was obtained.

3.6. Effects of VPA on Mitochondrial Import and Processing of ACD

We prepared *in vitro* translated pSCAD using reticulocyte lysate. Mitochondria were freshly isolated from control and experimental rats. After *in vitro* translated pSCAD

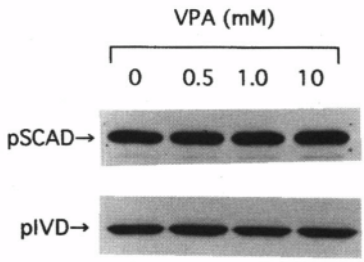

Figure 5. Effects of VPA on *in vitro* translation. pSCAD and pIVD mRNAs were translated in rabbit reticulocyte lysate, which was preincubated with VPA (0–10 mM). The translation products were analyzed by SDS-PAGE and fluorography.

was incubated with VPA-treated or control mitochondria for 30min, the reaction mixture was centrifuged, and the amount of mature (m) SCAD in the mitochondrial pellet was determined as described. In both VPA-treated and control mitochondria, the rate of import/processing of the precursor SCAD was similar in repeated experiments (Fig. 6). Thereafter, the amount of mSCAD decreased markedly faster in VPA-mitochondria than

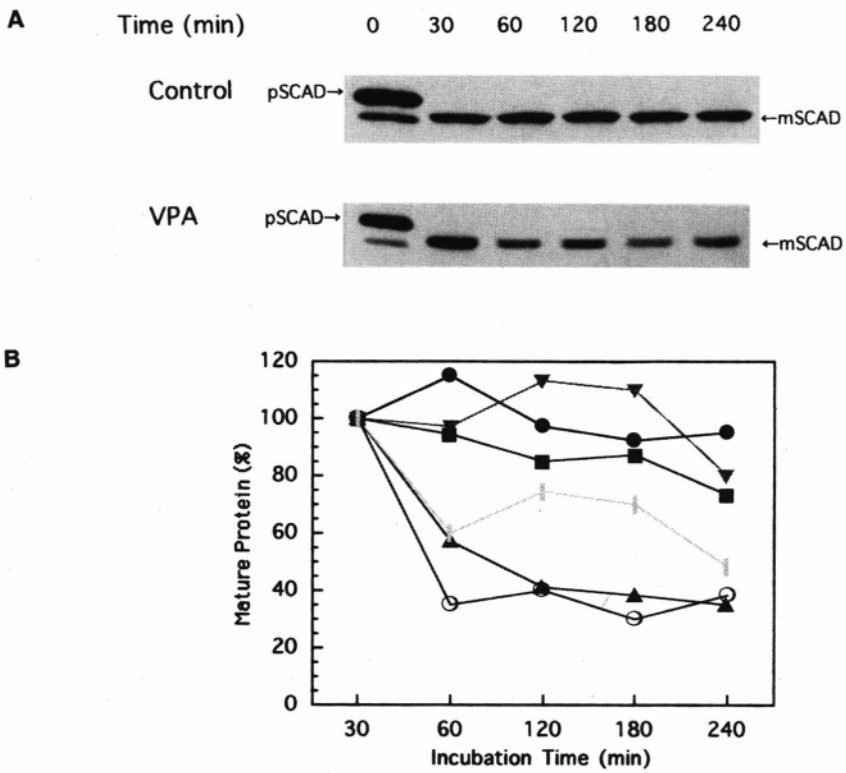

Figure 6. Time course of mitochondrial import/processing using control and VPA-treated mitochondria. [^{35}S]Methionine-labeled pSCAD, produced via *in vitro* transcription/translation, were incubated with freshly prepared mitochondria from control and VPA-treated rats at 27 °C for 30 min. The mitochondrial pellet was isolated and resuspended in HMS-buffer, and further incubated at 37 °C for varying periods of time. After trypsin treatment, the product was analyzed by SDS-PAGE and autoradiography (A). The results were quantitatively analyzed using a densitometer(B). The amount of mature protein was expressed as percentages of the amount of the respective protein after 30 min of incubation. Symbols are: (filled circles), mSCAD(control); (open circles), mSCAD(+VPA); filled squares), mIVD(control); (filled triangles/up), mIVD(+VPA); (filled triangles/down), mOTC(control); broken lines, mOTC(+VPA).

in control mitochondria. After 240 min of incubation, only 40% remained as mSCAD in the VPA-mitochondria, however, almost 100% still remained in control mitochondria. The same experiment was performed using *in vitro* translated pIVD and pOTC. The mature form of these proteins also showed increased degradation after import/processing in the VPA-treated mitochondria.

4. DISCUSSION

It has been suggested that β-oxidation inhibition by VPA or its unsaturated metabolites occurred at least by two mechanisms.[4] One is a transient sequestering of CoA. The other is inhibition of β-oxidation enzymes, including the acyl-CoA dehydrogenases. In this study, we have shown that the expression of various acyl-CoA dehydrogenases is subject to VPA-related effects at the level of their respective mRNAs and also at the level of stability of mature proteins. Our investigation provides the first comprehensive analysis at the molecular level of the regulation of acyl-CoA dehydrogenases by VPA.

First, the expression of the fatty ACD mRNAs, especially SCAD and MCAD, appears to be commonly regulated, producing an increased amount of the respective mRNAs in the VPA-treated rats. The effect of VPA was prominent in the heart where fatty oxidation is very active, reflecting tissue specific expression of ACDs. It should be noted that the degree of inhibition of ACD activities was also large in the heart. It is of particular interest that ACD mRNAs are increased several fold in riboflavin-deficient rat, in which the mitochondrial oxidation of fatty acids is severely impaired.[18] Thus, the present data and previous observations in riboflavin deficiency, together suggest that the expression of various ACD genes appear to be coordinately regulated by a feedback mechanism when fatty acid oxidation is inhibited.

The decrease in the amounts and activities of various ACDs in the presence of increased mRNA levels suggests that VPA affects the expression of various ACD genes at the stage of translation, or post-translational processing, or stability of the mature proteins. The present results show that translation of mRNAs and import/processing of precursor proteins by mitochondria was not affected by VPA, but that the stability of the mature protein itself was decreased. The loss of stability in the VPA-treated mitochondria was observed not only in the experiment of SCAD, but also IVD and OTC. Therefore, this is not a specific finding of fatty ACD. It may be possible that some mitochondrial proteins lose their stability in the VPA-treated mitochondria *in vivo*. Histopathologic examination of the liver from VPA-dosed rats (600mg/kg/day) showed various changes of mitochondrial morphology.[3] It is possible that normal structure of enzyme proteins could not be retained in the pleomorphic mitochondria, and accelerated breakdown may occur. Activation of proteinase inside the mitochondria might be associated with the phenomenon. However, the exact mechanism of increased degradation of mature ACDs in VPA-treated mitochondria is unknown at present.

From clinical findings of human VPA therapy, it was suggested that VPA inhibits the β-oxidation at the level of SCAD and MCAD. However, as suggested from *in vitro* enzymatic study by Ito *et al.*,[12] VPA and valproyl-CoA did not significantly inhibit the activities of various ACDs at various concentrations tested. They speculated that the inhibition of ACDs is not the major cause of the inhibition of fatty acid oxidation by VPA. The *in vivo* experiment in the present study showed that the degree of inhibition of ACDs were different between liver and heart. It is also important that the ACD mRNAs are more greatly changed in the heart. Similar tissue specific responses of the ACD genes were also observed in the studies of development and starvation.[20] Therefore, we should

note that ACD expression in the heart was differently regulated from that in the liver during VPA therapy. Although VPA is mainly metabolized in the liver, some potent toxic intermediates, which inhibit fatty acid oxidation, can exert their effects in other organs.[21] Since the utilization of fatty acids as an energy source is very active in the heart, the degree of inhibition could be greater in the heart than other organs. Unlike fatty ACDs, the IVD gene may not be coordinately regulated by a feedback mechanism caused by β-oxidation inhibition. In the condition of riboflavin deficiency or starvation, the expression of IVD is regulated separately from that of the enzymes related to fatty acid oxidation (SCAD, MCAD, and LCAD).[18] These findings are supported by the analysis of regulatory component of the IVD gene, which are different from those of other acyl-CoA dehydrogenase genes.[22]

Carnitine plays an important role in fatty acid oxidation by facilitating both the transport of fatty acids and excretion of toxic intermediates in the form of acyl-carnitine esters. Impairment of β-oxidation by VPA causes decreased level of plasma and muscle free carnitine and increased acyl-carnitine.[23] The results in this study show that free carnitine was decreased in the heart, where β-oxidation is most active. It has been suggested that the inhibition of β-oxidation due to VPA medication may be relieved with L-carnitine supplementation.[19] Therefore, L-carnitine supplementation might prevent not only VPA-induced hepatic dysfunction, but also might be effective in compensating β-oxidation in the heart. In addition, VPA administration reduced the *in vivo* concentrations of liver free coenzyme A.[24] Most of the metabolic disturbances caused by VPA can be explained by an increase in the acyl-CoA/CoASH ratio in the mitochondrial matrix, which may be associated with a lower acetyl-CoA concentration. The decreased availability of coenzyme A may inhibit β-oxidation, and other important metabolic pathways (urea synthesis, gluconeogenesis, etc.). Carnitine and coenzyme A are the substances that control the whole activity of fatty acid oxidation. We are going to investigate the correlation between availability of carnitine and coenzyme A and the expression of ACD genes in various metabolic disturbances.

Metabolic disturbances caused by the therapeutic range of VPA are partial and are normally well compensated by the mechanism described above. They could nevertheless significantly impair the whole activity of fatty acid oxidation when there is an additional insult, such as may occur with concomitant use of other epileptic drugs, viral infection or underlying inborn errors of metabolism.[25,26] In these conditions, if the biosynthesis of ACDs is not be regulated normally, the inhibition of fatty acid oxidation may result in serious clinical problems.

ACKNOWLEDGMENTS

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REFERENCES

1. Koch-Weser, J. & Browne, T.R. (1980) *New Engl. J. Med.* **302**, 661–666. Valproic acid.
2. Gerber, N., Dickinson, R.G., Harland R.C., Lynn, R.K., Houghton, D., Antonias, J.I. & Schimschock, J.C. (1979) *J. Pediatr.* **95**, 142-144. Reye-like syndrome associated with valproic acid therapy.

3. Dreifess, F.E., Laner, D.H., Moline, K.A. & Maxwell, J.E. (1989) *Neurology* **39**, 201–207. Valproic acid hepatic fatalities. II. US experience since 1984.
4. Kesterson, J.W., Graneman, G.R. & Machinist, J.M. (1984) *Hepatology* **4**, 1143–1152. The hepatotoxicity of valproic acid and its metabolites in rats. I. Toxicologic, biochemical and histopathologic studies.
5. Graneman, G.R., Wang, S., Kesterson, J.W. & Machinist, J.M. (1984) *Hepatology* **4**, 1153–1158. The hepatotoxicity of valproic acid and its metabolites in rats. II. Intermediary and valproic acid metabolism.
6. Mortensen, P.B., Gregersen, N., Kolvraa, S. & Christensen, E. (1980) *Biochem. Med.* **24**, 1531–1561. The occurrence of C6-C10-dicarboxylic acids in urine from patients and rats treated with dipropylacetate.
7. Ikeda, Y, Dabrowsly, C. & Tanaka, K. (1983) *J. Biol. Chem.* **258**, 1066–1076. Separation and properties of five distinct acyl-CoA dehydrogenases from rat liver mitochondria. Identification of a new 2-methyl branched chain acyl-CoA dehydrogenase.
8. Ikeda, Y, Okamura-Ikeda, K. & Tanaka, K. (1985) *J. Biol. Chem.* **260**, 1311–1325. Purification and characterization of short-chain, mediumchain, and long-chain acyl-CoA dehydrogenases from rat liver mitochondria. Isolation of the holo- and apoenzymes and conversion of the apoenzyme to the holoenzyme.
9. Ikeda, Y. & Tanaka, K.. (1983) *J. Biol. Chem.* **258**, 1077–1085. Purification and characterization of isovareryl coenzyme A dehydrogenase from rat liver mitochondria.
10. Ikeda, Y. & Tanaka, K. (1983) *J. Biol. Chem.* **258**, 9477–9487. Purification and characterization of 2-methyl-branched chain acylcoenzyme A dehydrogenase, an enzyme involved in the isoleucine and valine metabolism, from rat liver mitochondria.
11. Matsubara, Y, Indo, Y, Naito, E., Ozasa, H., Glassberg, R., Vockley, J., Ikeda, Y, Kraus, J. & Tanaka, K. (1989) *J. Biol. Chem.* **264**, 16321–16331. Molecular cloning and nucleotide sequence of cDNAs encoding the precursors of rat long chain acyl-coenzymeA, short chain acyl-coenzyme A, and isovaleryl-coenzyme A dehydrogenases. Sequence homology of four enzymes of the acyl-CoA dehydrogenase family.
12. Ito, M., Ikeda, Y, Arnez, J.G., Finocchiaro, G. & Tanaka, K. (1990) *Biochim. Biophys. Acta* **1034**, 213–218. The enzymatic basis for the metabolism and inhibitory effects of valproic acid: dehydrogenation of valproyl-CoA by 2-methyl-branched-chain acyl-CoA dehydrogenase.
13. Feinberg, A.P. & Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6–13. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity.
14. McGarry, J.D. & Foster, D.W. (1976) *J. Lipid Res.* **17**, 277–281. An improved and simplified radioisotopic assay for the determination of free and esterified carnitine.
15. Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. & Rutter, W.J. (1979) *Biochemistry* **18**, 5294–5299. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease.
16. Schnaitman, C. & Greenwalt, J.W. (1968) *J. Cell Biol.* **38**, 158–175. Enzymatic properties of the inner and outer membranes of rat liver mitochondria.
17. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4530–4534. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications.
18. Nagao, M. & Tanaka, K. (1992) *J. Biol. Chem.* **267**, 17925–17932. FAD-dependent regulation of transcription.translation, post-translational processing, and post-processing stability of various mitochondrial acyl-CoA dehydrogenases and of electron transfer flavoprotien and the site of holoenzyme formation.
19. Nishida, N., Sugimoto, T., Araki, A., Woo, M., Sakane, Y. & Kobayashi, Y. (1981) *Pediatr. Res.* **22**, 500–503. Carnitine metabolism in valproate-treated rats: The effect of L-Carnitine supplementation.
20. Nagao, M., Parimoo, B. & Tanaka, K. (1993) *J. Biol. Chem.* **268**, 24114–24124. Developmental, nutritional, and hormonal regulation of tissue-specific expression of the genes encoding various acyl-CoA dehydrogenases and α-subunit of electron transfer flavoprotein in rat.
21. Bjorge, S.M. & Baillie, T.A. (1985) *Biochem. Biophys. Res. Comm.* **132**, 245–252. Inhibition of medium-chain fatty acid β-oxidation *in vitro* by valproic acid and its unsaturated metabolite, 2-n-propyl-4-pentanoic acid.
22. Parimoo, B. & Tanaka, K. (1993) *Genomics* **15**, 582–590. Structual organization of the human isovaleryl-CoA dehydrogenase gene.
23. Stumpf, D.A., Parker, W.D. Jr. & Angelini, C. (1982) *Neurology* **35**, 1041–1045. Carnitine deficiency, organic acidemias and Reye's syndrome.
24. Thurston, J.H., Caroll, J.E., Hauhart, R.E. & Schiro, J.A. (1985) *Life Sci* **36**, 1643–1651. A single therapeutic dose of valproate affects liver carbohydrate, fat, adenylate, amino acid, coenzyme A, and carnitine metabolism in infant mice: possible clinical signficance.

25. Papadimitriou, A. & Servidei, S. (1991) *Neuromuscul. Disord.* **1**, 247–252. Late onset lipid storage myopathy due to multiple acyl CoA dehydrogenase deficiency triggered by valproate.
26. Van Hove, J.L., Zhang, W., Kahler, S.G., Roe, C.R., Chen, Y.T., Terada, N., Chace, D.H., Iafolla, A.K., Ding, J.H. & Millington, D.S. (1993) *Am. J. Hum. Genet.* **52**, 958–966. Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency: diagnosis by acylcarnitine analysis in blood.

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FORMATION OF A HUMAN "ELECTRON TRANSFERRING FLAVOPROTEIN"

Medium Chain Acyl Coenzyme A Dehydrogenase Complex, Preliminary Evidence from Crosslinking Studies

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1. INTRODUCTION

The "electron transferring flavoprotein" (ETF) is the physiological electron acceptor for the straight-chain acyl-CoA dehydrogenases (short, medium and long), involved in the β-oxidation pathway.[1] ETF is heterodimeric, containing an α subunit (32kDa), a β subunit (28 kDa) and one molecule of FAD. In contrast the associated dehydrogenases are homotetrameric, with a subunit molecular mass of 45 kDa, and contain one FAD molecule per monomer. In the mammalian system the reducing equivalents from the dehydrogenases are transferred via ETF to ETF: ubiquinone oxidoreductase (ETF: QO).[2]

A bacterial equivalent of the mammalian short chain acyl-CoA dehydrogenase, butyryl-CoA dehydrogenase, has recently been detected in a complex with ETF.[3] An ETF: trimethylamine dehydrogenase complex from *Methylotrophus methylophilus* has also been characterised using ultracentrifugation.[4]

Porcine ETF and MCAD have been shown to form a specific association, with 1: 1 stoichiometry,[5] and cross-linking studies showed preferential binding of porcine MCAD to the β subunit of ETF.[6] More recently, mammalian ETF has been detected complexed to MCAD and sarcosine dehydrogenase in the crude extract of porcine liver [Parker, A and Engel, P.C.-submitted] and these complexes have also been partially purified.

The phenomenon whereby one protein can recognise and form a protein-protein

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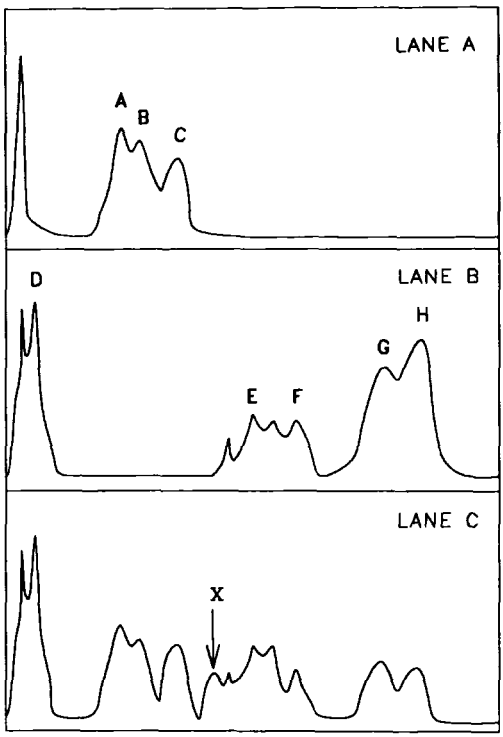

Figure 1. Formation of a cross-linked ETF: MCAD complex. ETF (9.5μM), MCAD (38μM) and octanoyl-CoA (1.5mM) were preincubated in 20mM potassium buffer, pH 8.0 (lane C). ETF (18μM) and MCAD (18μM) were also incubated separately with octanoyl-CoA in buffer (lanes A and B). After 3mins DMS (9.5 mM) was added and the mixture incubated for 30mins at 30°C. After boiling, aliquots were subjected to electrophoresis on an 8% SDS polyacrylamide gel. Densitometric scans of the Coomassie Blue-stained gels are shown.

complex with a number of different proteins is of wider occurrence. The ability of thioredoxin to interact with several reductases, for instance, is well documented.[7] In the present study the homobifunctional cross-linking reagent dimethyl suberimidate (DMS) has been employed to seek evidence for a stoichiometric ETF:MCAD complex.

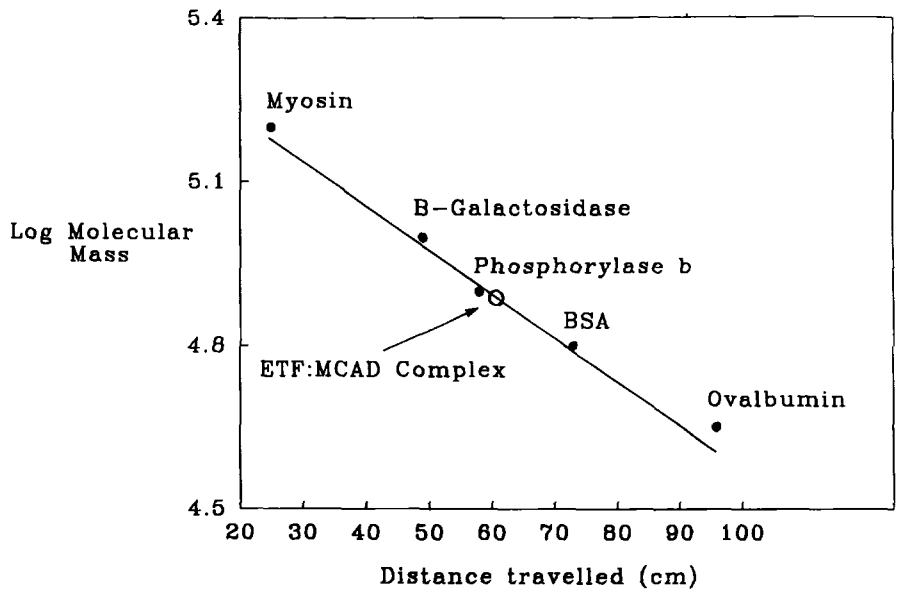

Figure 2. Molecular mass of ETF: MCAD complex determined by SDS polyacrylamide gels.

2. RESULTS

Figure 1, a densitometric scan of a Coomassie Blue stained gel, shows the results of cross-linking ETF and MCAD both separately and together. Lane A, ETF control, shows three protein bands A, B and C. Band B is assumed to be a cross-linked αβ molecule of ETF (60kDa). Bands A and C are probably the result of inter-subunit cross-linking between two β subunits (56kDa: band A) and two α subunits (64kDa: band C).

In the MCAD control, lane B, bands D, E, F, G and H probably represent mono- (45kDa), di- (90kDa), tri- (135kDa), tetra- (ISOkDa) and penta-meric (225kDa) cross-linked forms of MCAD. The two unlabelled bands in lane B are probably slight cross-linked impurities and are not present in the absence of DMS. Lane C shows the

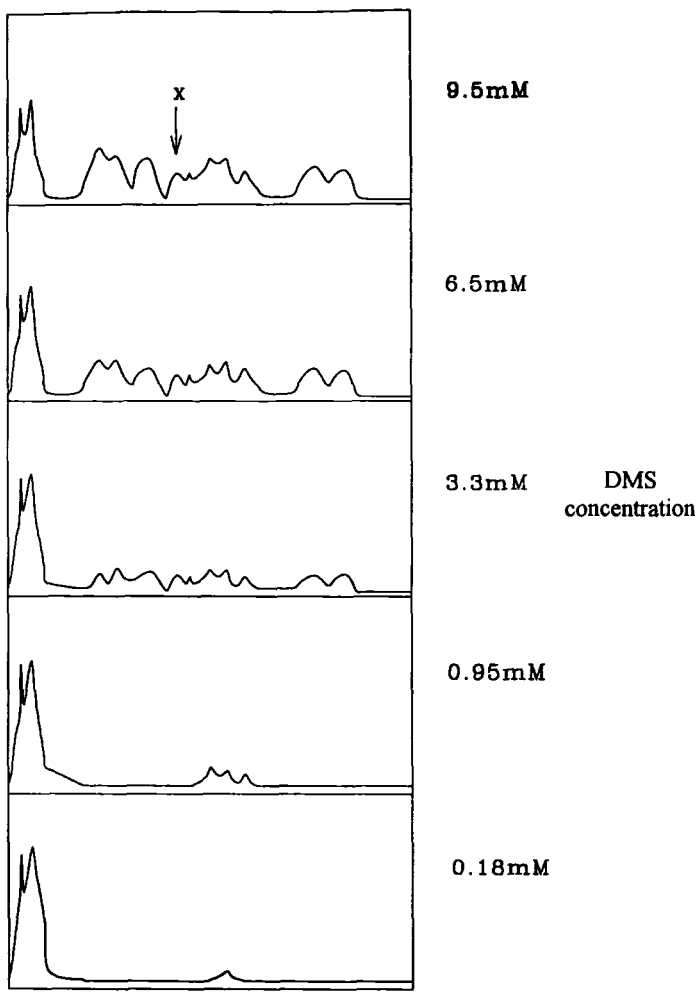

Figure 3. Formation of a cross-linked ETF:MCAD complex as a function of DMS concentration. ETF (9.5 μM), MCAD (38 μM) and octanoyl-CoA (1.5 mM) were preincubated in 20 mM potassium buffer, pH 8.0 (lane C). After 3 mins DMS (0.18–9.5 mM) was added and the mixture incubated for 30 mins at 30 °C. Controls of ETF and MCAD cross-linking separately were run concurrently. After boiling, aliquots were subjected to electrophoresis on an 8% SDS polyacrylamide gel. Densitometric scans of the Coomassie Blue-stained gels are shown.

result of mixing ETF and MCAD, in the presence of octanoyl-CoA, in the ratio of 1:4 in terms of FAD content. A new band of protein is detected, band X, with an approximate mass of 75–80 kDa (Fig. 2).

This band was found to be present whatever the ratio of MCAD to ETF or ratio of excess ETF to MCAD used (up to 4-fold excess of each protein was used). The production of the extra band is mirrored by the decrease in the formation of the postulated tetrameric and pentameric cross-linked species of MCAD (bands G and H). In the absence of octanoyl-CoA, band X was not detected. Figure 3 shows the formation of the band X as a function of DMS concentration. The formation of only the probable dimeric and trimeric forms of MCAD at 0.95 mM DMS suggest that only intra-subunit cross-linking occurs. This corresponds with the absence of band X using this DMS concentration.

3. DISCUSSION

These results provide direct evidence that a specific ETF: MCAD complex is formed in the presence of octanoyl-CoA. The molecular mass of 75–80 kDa, under denaturing conditions, implies that 1 ETF molecule interacts with 1 MCAD monomer. This work does not offer any evidence for the molecular size of such a complex in the native state although consideration must be given to the possibility that the MCAD tetramer may not be able to accommodate four ETF molecules simultaneously because of steric hindrance. Although previous work using N-succimidyl 3-(2-pyridyldithio) propionate (SPP)[6] showed that the β subunit of ETF interacted with the MCAD monomer, a clear protein band representing an ETF: MCAD complex was not produced. This may be due to the difference in the length of cross-linker used for SPP is only 6.8 Å long whereas DMS is 11 Å long.[8]

The interaction of the two flavoproteins involves electron transfer from one FAD molecule to another. The FAD binding site in ETF is located in the α-subunit.[9] Using the estimated molecular mass of band X on the gel, the present work suggests that MCAD may in fact bind to the α subunit of the ETF.

REFERENCES

1. Crane, F.L. & Beinert, H. (1956) *J. Biol. Chem.* **218**, 717–731. On the mechanism of dehydrogenation of fatty acid acyl coenzyme A: the electron transferring flavoprotein.
2. Ruzicka, F.J. & Beinert, H. (1975) *Biochem. Biophys. Res. Comm.* **66**, 622–631. A new membrane iron-sulphur flavoprotein of the mitochondrial electron transfer system.
3. Parker, A. & Engel, PC. (1998) *Biochem. Soc. Trans.* **26**, 73S. Experimental evidence for formation of complex between butyryl CoA dehydrogenase and electron transferring flavoprotein.
4. Wilson, E.K., Scrutton, N.S., Cölfen, H., Harding, S.E., Jacobson, M.P. & Winzor, D.J. (1997) *Eur. J. Biochem.* **243**, 393–399. An ultracentrifugal approach to quantitative characterisation of the molecular assembly of a physiological electron transfer complex.
5. Hall, C.L., Lambeth, J.D. & Kamin, H. (1979) *J. Biol. Chem.* **254**, 2023–2031. Acyl-CoA complexes of general acyl-CoA dehydrogenase and electron transferring flavoprotein.
6. Steenkamp, D.J. (1987) *Biochem. J.* **243**, 519–524. Preferential cross- linking of the small subunit of the electron transfer flavoprotein to general acyl-CoA dehydrogenase.
7. Holmgren, A. (1985) *Ann. Rev. Biochem.* **54**, 237–272. Thioredoxin.
8. Pierce Products Catalogue, 1997.
9. Roberts, D.L., Frerman, F.E. & Kim, J.J.P. (1996) *Proc. Natl. Acad. Sci. USA.* **93**, 14355–14360. Three dimensional structure of human electron transfer flavoprotein to 2.1-Å resolution.

CLONING AND REGULATION OF PEROXISOME PROLIFERATOR-INDUCED ACYL-CoA THIOESTERASES FROM MOUSE LIVER

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1. ABSTRACT

1.1. Acyl-CoA thioesterases hydrolyze acyl-CoAs to the corresponding free fatty acid plus CoASH. The activity is strongly induced in rat and mouse liver after feeding the animals peroxisome proliferators. To elucidate the role of these enzymes in lipid metabolism, we have cloned the cDNAs corresponding to the inducible cytosolic and mitochondrial type I enzymes (CTE-I and MTE-I) and studied tissue expression and nutritional regulation of expression of the mRNAs in mice. The constitutive expression of both mRNAs was low in liver, with CTE-I being expressed mainly in kidney and brown adipose tissue and MTE-I expressed in brown adipose tissue and heart. As expected, the expression in liver of both the CTE-I and MTE-I mRNAs was strongly induced (>50-fold) by treatment with clofibrate. A similar level of induction was observed by fasting and a time-course study showed that both mRNAs were increased already at 6 hours after removal of the diet. Refeeding normal chow diet to mice fasted for 24 hours normalized the mRNA levels with a $T_{1/2}$ of about 3–4 hours. Feeding mice a fat-free diet further decreased the expression, possibly indicating repression of expression. The strong expression of MTE-I and CTE-I in the heart was increased about 10-fold by fasting. To further characterize these highly regulated enzymes, we have cloned the corresponding genes and promoter regions. The structures of the two genes were found to be very similar, consisting of three exons and two introns. Exon-intron borders conform to general consensus sequences and especially the first exon appears to be highly conserved. The pro-

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moter regions of both the CTE-I and MTE-I genes contain putative peroxisome proliferator response elements (PPREs), suggesting an involvement of peroxisome proliferator-activated receptors in the regulation of these genes.

2. INTRODUCTION

2.1 A broad class of compounds, collectively known as peroxisome proliferators, lower plasma lipids, cause proliferation of peroxisomes and induction of several lipid metabolizing enzymes (for review, see[1]). Several studies have shown that acyl-CoA thioesterase activity is strongly induced in rat and mouse liver by treatment with peroxisome proliferators. However, the induction was evident mainly in cytosol and mitochondria[2–1] but was much weaker in peroxisomes. Characterization of the induced activity by size-exclusion chromatography showed that two types of acyl-CoA thioesterases were induced, with molecular masses of approximately 40 and 110–150kDa.[4,5] The 40kDa enzyme was tentatively named CTE-I and the 110-150kDa enzyme tentatively named CTE-II.[4] The induced activity in mitochondria was shown to be due to induction of two activities, MTE-I and MTE-II, which showed identical elution properties on size exclusion chromatography to the corresponding cytosolic type I and type II enzymes.[4] The cDNAs corresponding to these enzymes have recently been cloned by us and others.[6–10] The physiological roles of these thioesterases are still unknown, therefore further characterization of the regulation of expression and at the gene level is of importance for the understanding of the functions of these enzymes.

3. MATERIALS AND METHODS

3.1. Materials

The standard chow diet was obtained from Lactamin (R36, Vadstena, Sweden), the fat-free diet (containing 64% carbohydrate, 17.6% protein, 4.2% ash, 4% fibre and 10% water) from AnalyCen (Linköping, Sweden). Clofibrate (Atromidin) was from Zeneca (Cheshire, United Kingdom) and restriction enzymes were obtained from Pharmacia Biotech (Sweden). All chemicals used were standard commercial products of analytical grade.

3.2. Animals and Treatments

Adult male C57 BL/6 mice, obtained from B & K, Sollentuna, Sweden, were used throughout this study. Mice were maintained on a standard chow diet before commencement of the experiments. Mice were fed a fat-free diet (FF), a 0.5% (w/w) clofibrate-containing diet (Clo) or fasted as indicated. All mice had access to water ad libitum. The animals were sacrificed by CO_2 treatment followed by cervical dislocation, and weighed immediately. The various tissues were then excised, weighed and frozen in liquid nitrogen.

3.3. Northern Blot Analysis

Total RNA was isolated from mouse tissue samples using QuickPrep^R Total RNA Extraction Kit (Pharmacia Biotech, Uppsala, Sweden) or Ultraspec RNA Kit (Biotecx

Laboratories Inc., Houston, TX). Twenty micrograms of total RNA was denatured in formaldehyde/formamide and electrophoresed on 1% agarose gels containing ethidium bromide. In the case of heart, kidney and brown adipose tissue, 10 mg of RNA was used. The RNA was transferred to Hybond-N nylon membranes (Amersham International plc, Buckinghamshire, England) by capillary action using the Northern blotting technique. Specific cDNA probes for full-length mouse CTE-I and β-actin were labelled with α-^{32}P by random priming (Oligolabelling Kit, Pharmacia) and hybridised overnight at 65 °C to the RNA filters. The filters were then washed at 65 °C in decreasing concentrations of SSC/SDS solutions and exposed to X-ray film. Filters were stripped in boiling 0.5% SDS solution and re-probed as above.

3.4. Gel Electrophoresis and Western Blotting

Total mouse liver proteins (50 μg) were separated on a 10% sodium dodecylsulfate polyacrylamide gel (SDS-PAGE) and Western blotting was performed by electrophoretic transfer of the separated proteins onto a nitrocellulose filter (Nitropure, Micron Separations Inc., Westborough, MA, USA) using a Mini-Protean II device (Bio-Rad). The blots were probed with an anti-MTE-I antibody and subsequently with horseradish peroxidase-conjugated secondary antibodies and visualised by enhanced chemiluminescence (ECL™, Amersham) using X-ray film.

3.5. Gene Structure and Promoter Region Analysis for MTE-I and CTE-I

About 2×10^6 plaques of a mouse genomic λ Zap library (Stratagene) were screened with a cDNA probe containing about 2/3 (3′) of the CTE-I cDNA sequence. About two hundred positive clones were isolated of which thirty were rescreened twice. One clone was partially sequenced in the first exon and intron and used to generate primers for PCR-based screening of P1 clones by Genome Systems Inc. (U.S.A.). The gene structure for CTE-I was elucidated using primers designed from the cDNA sequence. Genomic clones corresponding to MTE-I were obtained by screening of a "mini-library" of the positive clones obtained in the first screening described above, using an MTE-I specific cDNA probe corresponding to the sequence of mitochondrial targeting signal of MTE-I. The intron/exon boundaries were sequenced using the ABI Prism Dye Terminator Ready-Reaction Kit (Perkin-Elmer) at Cybergene AB (Novum) in an ABI 373A-stretch DNA Sequencer (Perkin-Elmer). Sequence analysis was performed using the LaserGene software package (DNAStar).

The 5′-flanking regions of both CTE-I and MTE-I genomic clones were sequenced in both sense and antisense directions. Sequence analysis was carried out using the ABI Prism Dye Terminator Ready-Reaction Kit (Perkin-Elmer) at Cybergene AB (Novum) as stated previously, and the data analysed using LaserGene software package (DNAStar).

4. RESULTS

4.1. Tissue Expression and Regulation of mRNA Levels

The tissue specific expression of CTE-I and MTE-I mRNA was carried out by Northern blot analysis. CTE-I was shown to be mainly expressed in kidney, brown adipose tissue and heart, but was also detectable in control liver, white adipose tissue,

brain and testis. MTE-I was strongly expressed in heart and brown adipose tissue, with strong signals also detected in kidney and white adipose tissue. Weak expression of MTE-I was evident in liver and muscle but no detectable expression was seen in brain or testis.

4.2. Regulation of Acyl-CoA Thioesterases by Dietary Manipulation-

Previous studies by our group indicated a very strong induction of both CTE-I and MTE-I expression in mouse liver after fasting for 48 hours as compared to expression in untreated animals, where levels are barely detectable.[7] We found that this induction was already maximal after 24 hours, and carried out a time course of fasting mice for 0, 6, 12, 18, 24, 36 and 48 hours to pinpoint more closely when induction took place. After 6 hours of fasting the expression of the cytosolic acyl-CoA thioesterase was already slightly increased, with maximum induction after only 12 hours fasting. mRNA levels of the MTE-I transcript were induced in line with its cytosolic counterpart. Due to the rapid induction of both CTE-I and MTE-I by fasting, it was of interest to examine how a refeeding effect would alter the induced expression of these enzymes. An experiment was carried out where mice were fasted for 24 hours and refed a normal chow diet for 0, 3, 6, 9, 12, 24 and 30 hours. The decrease in transcription of mCTE-I was again very rapid, being strongly reduced after only 6 hours of refeeding the normal chow diet with mRNA returning to control levels within 9 hours of this treatment. The apparent half-lives of the mRNAs were estimated to be about 3–4 hours.

In view of this different tissue specificity and the rapid regulation of expression of these enzymes in liver by fasting, we investigated the effects of fasting on CTE-I and MTE-I mRNA in tissues where the enzymes are constitutively expressed, such as heart, kidney and brown adipose tissue. Interestingly, fasting for 24 hours caused a very large upregulation of both CTE-I and MTE-I in mouse heart, which was not further increased at 48 hours. In kidney, mCTE-I transcription was much more strongly upregulated by fasting than the mitochondrial transcript. The mRNA levels of both these enzymes in brown adipose tissue were unaffected by fasting conditions.

Previous experiments at this laboratory had indicated that both mCTE-I and mMTE-I were downregulated at mRNA level by feeding a fat-free diet. We carried out a long-term time course of feeding mice a fat-free diet for 1,2,4 and 7 days. After two days of this treatment, both enzymes were suppressed at mRNA level.

4.3. Regulation of Acyl-CoA Thioesterases by the Peroxisome Proliferator Clofibrate

Previous results have indicated that both CTE-I and MTE-I are inducible at mRNA level in both rat and mouse liver by peroxisome proliferators such as clofibrate and di-(2-ethylhexyl)phthalate (DEHP).[6,7] We examined the expression of CTE-I and MTE-I mRNA after various treatments with clofibrate-containing diets. In a time course carried out by feeding mice a 0.5% clofibrate-containing diet for 1, 2, 4 and 7 days, both CTE-I and MTE-I were already maximally induced after the first day of treatment and levels remained high for the duration of the experiment.

It was of interest to verify if the induction at the mRNA level was also reflected by an increase at the protein level. Western blot analysis of mouse total liver protein using an anti-MTE-I antibody, which labelled a strong band at the expected size of 45 kDa, indicated that there was a good correlation in the changes at the protein and mRNA levels. Fasting for 24 hours, treatment with a clofibrate-containing diet for 1 day or fasting

and refeeding a clofibrate-containing diet all caused an increase of MTE-I and CTE-I proteins. Fasting and refeeding a chow diet for 24 hours normalised levels of the thioesterase protein. However, treatment with a fat-free diet for 4 days caused a suppression of both CTE-I and MTE-I at mRNA level, but this suppression was not evident at protein level.

4.4. Gene Structures of MTE-I and CTE-I

The screening of a mouse genomic library, using a cDNA probe corresponding to about 2/3 of the cDNA sequence (lacking the 5′-end) resulted in positive clones. After 3 more rounds of screening about 90% remained positive, suggesting that the probe recognized several genes of this apparent multi-gene family. Screening for MTE-I was carried out by generation of a specific cDNA probe containing the 5′-end of the sequence corresponding to the mitochondrial leader peptide, which was used to screen a "mini-library" consisting of a pool of the positive clones obtained in the first screening. This resulted in the isolation of two clones, one of which was completely sequenced. About 2.8kb of the 5′-flanking region was also sequenced in both directions. This clone was found to contain the entire gene for MTE-I, and found to consist of 3 exons and 2 introns. The first exon contains 680 bp, the second exon contains 202 bp and the third exon about 695bp.

One of the clones obtained from the first screening, corresponding to CTE-I, was partially sequenced and the sequence information was used to design oligo oligonucleotides for P1-clone screening. A P1 clone containing the entire CTE-I gene was obtained and completely sequenced and based on the sequence the gene structure was determined. Similarly to the MTE-I gene, it contains three exons and two introns, with the consensus sequences for the intron borders commencing with the sequence GTG and ending in the sequence CAG. The first exon, of 465 base pairs, is highly conserved between CTE-I and MTE-I (showing greater than 90% homology, differing mainly in that the mitochondrial variant contains a mitochondrial leader peptide of 42 amino acids coded for in the first exon). Similar to the MTE-I gene, the second exon contains 202 base pairs. The putative active site serine is encoded in the third exon in both genes.

4.5. Promoter Analysis

Approximately 2.4kb of the promoter region of CTE-I and 2.8kb of the promoter region of MTE-I were sequenced in the 5′ direction from the ATG start site. The promoter sequences were analysed for the presence of various transcription factor sites, using TESS String Based Search, which identified putative peroxisome proliferator response elements (PPREs) in both promoters. These regions of the promoters have now been cloned into a luciferase expression vector, to be used in transfection experiments to examine for functional PPREs.

5. DISCUSSION

5.1 It is now well established that the acyl-CoA thioesterase activity in rat and mouse liver is strongly induced by peroxisome proliferators. The induced activity is due to induction of several enzymes belonging to two gene families, type I and type II thioesterases, each of which contains several members of structurally related enzymes. The type I thioesterases are localized to cytosol, mitochondria and peroxisomes,[4,11] which

are all immunologically related and are recognized by antibodies to MTE-I. In spite of the previous studies, the physiological functions of these acyl-CoA thioesterases have remained unknown. In an attempt to increase our understanding of these enzymes, we have studied the regulation of expression of the mitochondrial and cytosolic enzymes and also cloned the corresponding genes. The results show that both enzymes are highly, and very rapidly, regulated by peroxisome proliferators and by changes in nutritional conditions. The very rapid and strong upregulation by peroxisome proliferators and fasting, and the profound down-regulation of expression by feeding mice a fat-free diet, strongly suggest that these enzymes are tightly linked to lipid metabolism. The strong regulation by peroxisome proliferators and presence of putative PPREs in the promoters suggest that the transcription of these two genes may be regulated via PPAR. Recently it has been shown that possible ligands for PPARs include fatty acids.[12] Interestingly, it was recently shown that acyl-CoA thioesters are ligands of the hepatic nuclear factor-4α (HNF-4α).[13] Thus, a possible, and very intriguing function for the cytosolic (or putative nuclear) thioesterases may be in the regulation of transcription via PPAR and HNF-4α by modulation of acyl-CoA/free fatty acid ratios under various nutritional conditions.

REFERENCES

1. Reddy, J.K. & Mannaerts, G.P. (1994) *Annu. Rev. Nutr.* **14**, 343–370. Peroxisomal lipid metabolism.
2. Berge, R.K., Skrede, S. & Farstad, M. (1981) *Febs Lett.* **124**, 43–47. Effects of clofibrate on the intracellular localization of palmitoyl-CoA hydrolase and palmitoyl-L-carnitine hydrolase in rat liver.
3. Miyazawa, S., Furuta, S. & Hashimoto, T. (1981) *Eur. J. Biochem.* **117**, 425–430. Induction of a novel long-chain acyl-CoA hydrolase in rat liver by administration of peroxisome proliferators.
4. Svensson, L.T., Wilcke, M. & Alexson, S.E.H. (1995) *Eur. J. Biochem.* **230**, 813–820. Peroxisome proliferators differentially regulate long-chain acyl-CoA thioesterases in rat liver.
5. Katoh, H., Kawashima, Y, Watanuki, H., Kozuka, H. & Isono, H. (1987) *Biochim. Biophys. Acta.* **920**, 171–179. Effects of clofibric acid and tiadenol on cytosolic long-chain acyl-CoA hydrolase and peroxisomal β-oxidation in liver and extrahepatic tissues of rat.
6. Engberg, S.T., Aoyama, T., Alexson, S.E.H., Hashimotot, T. & Svensson, L.T. (1997) *Biochem. J.* **323**, 521–535. Peroxisome proliferator-induced acyl-CoA thioesterase from rat liver cytosol: molecular cloning and functional expression in Chinese hamster ovary cells.
7. Lindquist, P.J.G., Svensson, L.T. & Alexson, S.E.H. (1998) *Eur. J. Biochem.* **251**, 631–640. Molecular cloning of the peroxisome proliferator-induced 46-kDa cytosolic acyl-CoA thioesterase from mouse and rat liver.
8. Svensson, L.T., Engberg, S.T., Aoyama, T., Usuda, N., Alexson, S.E.H. & T. H. (1998) *Biochem. J.* **329**, 601–608. Molecular cloning and characterization of a mitochondrial peroxisome proliferator-induced acyl-CoA thioesterase from rat liver.
9. Broustas, C.G., Larkins, L.K., Uhler, M.D. & Hajra, A.K. (1996) *J. Biol. Chem.* **271**, 10470–10476. Molecular cloning and expression of cDNA encoding rat brain cytosolic acyl-cenzyme A thioester hydrolase.
10. Yamada, I, Furihata, T., Iida, N., Watanabe, T., Hosokawa, M., Satoh, T., Someya, A., Nagaoka, I. & Suga, T. (1997) *Biochem Biophys. Res. Commun.* **232**, 198–203. Molecular cloning and expression of cDNAs encoding rat brain and liver cytosolic long-chain acyl-CoA hydrolases.
11. Svensson, L.T., Alexson, S.E.H. & Hiltunen, J.K. (1995) *J. Biol. Chem.* **270**, 12177–12183. Very long chain and long chain acyl-CoA thioesterases in rat liver mitochondria. Identification, purification, characterization, and induction by peroxisome proliferators.
12. Kliewer, S.A., Sundseth, S.S., Jones, S.A., Brown, P.J., Wisely, G.B., Koble, C.S., Devchand, P., Wahli, W, Willson, T.M., Lenhard, J.M. & Lehmann, J.M. (1997) *Proc. Natl. Acad. Sci.* **94**, 4318–323. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferators-activated receptors α and γ.
13. Hertz, R., Magenheim, J., Berman, I. & Bar-Tana, J. (1998) *Nature* **392**, 512-516. Fatty acyl-CoA thioesters are ligands of hepatic nuclear factor-4α.

METABOLIC EFFECTS OF 3-THIA FATTY ACID IN CANCER CELLS

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1. INTRODUCTION

Gliomas are tumours of glial cell origin in the central nervous system (CNS). High grade malignant gliomas are very aggressive and almost always lethal. Although the treatment of neoplasms in other organ systems has radically improved during the last decade, improvements in glioma treatment have been marginal. Combinations of surgery, radiotherapy and chemotherapy may gain some weeks or months in survival time, however the median survival time after treatment is less than a year.[1] The aggressiveness of cancers depends on qualities such as proliferative rate, invasiveness and angiogenesis. Agents that influence one or more of these features may affect the tumour malignancy. Characteristic for the neuroepithelial tumours is their local invasive behavior. Neuroepithelial tumours are poorly demarcated and hardly ever properly encapsulated. This behavior makes complete surgical removal of the tumour difficult and they often recur with a fatal result.

It is largely accepted that a high dietary intake of poly-unsaturated fatty acids (PUFA) in the **ω-3** series has beneficial effects. Recently, cellular lipid metabolism has been suggested as a target for cancer therapy. Cancer cells, compared with normal cells, seem to be vulnerable to exposure of certain polyunsaturated fatty acids (PUFAs),[2] especially those in the **ω-3** series. Characteristic for these compounds are their poor ability to be oxidized in the cell due to multiple double bonds. They are however likely to be esterified to other lipids, and their incorporation into membrane phospholipids will influence membrane properties such as fluidity, protein interactions and susceptibility to lipid peroxidation. The hypolipidemic properties of some **ω-3** fatty acids, such as EPA, are probably explained by an induction of mitochondrial β-oxidation[3] that is not found after administration of the non-hypolipidemic **ω-3** PUFA docosahexaenoic acid (DHA).[4,5] However, both eicosapentaenoic acid (EPA) and DHA cause increased peroxisomal

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β-oxidation, and this effect may also be obtained by other drugs known as peroxisome proliferators. Peroxisomal β-oxidation is associated with H_2O_2 production, and H_2O_2 has to be detoxified by the cell to avoid cytotoxic oxidative stress. An intact antioxidant defense is thus important under these conditions.

Phenylacetate is a naturally occurring peroxisome proliferator, which is reported to be an antitumor agent.[6–8] Structurally, phenylacetate resembles the fibrate hypolipidemic drugs, and functionally they share several properties such as inhibition of cholesterogenesis, adipocyte differentiation and peroxisome proliferator activated receptor (PPAR) activation (reviewed[9]). Gliomas, but not mature normal brain cells, are highly dependent on mevalonate, which is an intermediate in cholesterogenesis.[8] Mevalonate is used for production of sterols and isoprenoid compounds vital for cell growth. Phenylacetate and lovastatin inhibit mevalonate pyrophosphate decarboxylase and HMG-CoA reductase, two enzymes in the mevalonate pathway, respectively. In combination a synergistic antitumor activity of these drugs is observed. Lovastatin is found to increase the transcription of PPAR.[6] PPAR is a transcription factor implicated in the control of lipid metabolism, cell growth and differentiation.

3-Thia fatty acids have been used as tools in experimental models to further penetrate the mechanisms of the lipid metabolism. These fatty acids are activated to their respective CoA-esters, but, they cannot be degraded by β-oxidation because of the sulfur substitution in 3-position (reviewed[10]). Tetradecylthioacetic acid (TTA), a representative member of the 3-thia family, has the chemical structure of palmitic acid (C16) in which a sulfur atom is located between the 2 and 3-carbon atoms ($COOH\text{-}CH_2\text{-}S\text{-}(CH_2)_{13}\text{-}CH_3$). TTA is a novel hypolipidemic drug which has been shown to combine several effects of ω-3 PUFAs, such as EPA, and structurally unrelated peroxisome proliferators, such as phenylacetate and fibrates. From this perspective we wanted to investigate if TTA shares the antitumor activity found for these functionally related compounds, and furthermore to study the metabolic effects of TTA in cancer cells in relation to the effects found in hepatocytes.

Since TTA is poorly oxidizable it is likely to be esterified into other lipids, primarily phospholipids. Thus, TTA readily enters the cell membrane in which it influences membrane properties. Moreover, TTA treatment influences the PUFA composition of membranes. As TTA changes the membrane PUFA content and possesses antioxidant properties, it may influence the susceptibility to lipid peroxidation. In addition to functioning as an antioxidant itself, TTA changes the antioxidant defense system in hepatocytes.[11,12] This indicates that TTA affects the cellular oxidative situation.

TTA induces peroxisomal and mitochondrial β-oxidation enzymes in addition to several other enzymes in lipid metabolism.[10] Furthermore, TTA functions as a ligand for PPAR-α and PPAR-γ[13] and it stimulates both peroxisomal and mitochondrial proliferation. An increasing amount of genes that are known to carry PPAR response elements (PPREs) is reported, and differential regulation by these receptors is likely to affect the growth potential. The result of TTA treatment is a reorganization of the cellular lipid metabolism towards an increased metabolic activity.

2. RESULTS AND DISCUSSION

Our objective was to study how TTA affect the growth and lipid metabolism in glioma cells. It is well established that normal brain tissue primarily oxidizes glucose as a source of energy, however, it is also capable of oxidizing fatty acids and ketone bodies.

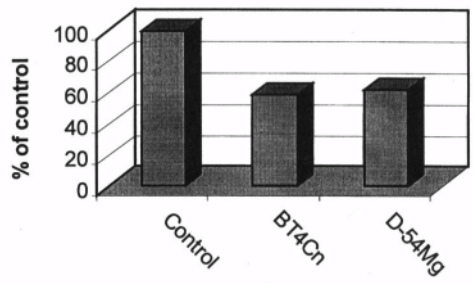

Figure 1. Growth of glioma cells exposed to 100 μM TTA. BT4Cn cells were grown for 6 days in monolayer cultures, and D-54 MG was grown for 14 days in spheroid cultures. The growth is given as cell number or spheroid diameter, as percentage of control. The results can be studied in more details in K. Berge *et al.* 1998 (this volume).

In these experiments cultured glioma cells were exposed to TTA, and key enzymes in peroxisomal (fatty acyl-CoA oxidase—FAO) and mitochondrial (carnitine palmitoyl transferase II—CPT II) fatty acid oxidation were studied.

TTA reduced the proliferation of both a rat (BT4Cn) and a human (D-54Mg) glioma cell line. Detailed results from the growth of these and other cell lines can be viewed in K. Berge *et al.* 1998 (this volume), however, the results from the previously mentioned glioma cells are summarized in Figure 1. TTA has previously been reported to reduce the growth of the breast cancer cell line MCF-7.[14,15]

To study the cellular lipid metabolism the human glioma line D-54 Mg was grown in monolayer cultures with TTA supplemented growth medium. A cell homogenate was prepared using a ball-bearing homogenizer,[16] and separated into subcellular fractions by differential centrifugation. CPT II activity and the FAO activity in the post-nuclear fraction were increased 3.4 and 2 fold, respectively, after TTA administration (Table 1). These data further suggest that the increases in both FAO and CPT II enzyme activities are primarily due to enrichments in the mitochondrial fraction. As expected, little or no FAO and CPT II activity were found in the microsomal and cytosolic fractions (data not shown).

These results suggest that TTA influences fatty acid catabolism in glioma cells in a similar pattern as in hepatocytes. Activities of key enzymes of both mitochondrial and peroxisomal β-oxidation were potentiated, and consequently TTA plays an active role in the glioma cell metabolism. The induction of FAO demonstrates that TTA is likely to induce peroxisome proliferation in D-54Mg. Previously, few observations indicating peroxisome proliferation in human cells have been reported. Furthermore, increased FAO activity is associated with increased production of H_2O_2 that must be detoxified by the antioxidant system. Thus it seems likely that the antioxidant systems will be affected in some way. Unpublished results demonstrate that TTA do affect both glutathione (GSH) content and activities of GSH associated enzymes.

Table 1. Effects of TTA on activities of key enzymes in mitochondrial and peroxisomal fatty acid oxidation in human glioma cells (D-54 Mg).

	CPT II			FAO		
Fraction	control	TTA	fold induction	control	TTA	fold induction
post-nuclear	0.5	1.6	3.2	2.7	5.5	2.0
mitochondria	2.8	5.5	2.0	3.2	7.1	2.2
peroxisomes	0.9	1.4	1.5	7.3	8.9	1.2

The cells were grown in monolayer cultures with or without 100 μM TTA supplemented in the growth medium. After homogenization and fractionation, activities of CPT II and FAO were measured. The values represent specific enzyme activities (nmol/mg/min).

Hopefully, further investigation will reveal more information about the TTA effects in glioma cells, and we will concentrate on the lipid metabolism, redox balance and PPAR-system.

REFERENCES

1. Brandes, A., Soesan, M. & Fiorentino, M.V. (1991). *Anticancer Res.* **11**:719–727. Medical treatment of high grade malignant gliomas in adults: an overview.
2. Begin, M.E., Ells, G., Das, U.N. & Horrobin, D.F. (1986). *J. Natl. Cancer Inst.* **77**:1053–1062. Differential killing of human carcinoma cells supplemented with n-3 and n-6 polyunsaturated fatty acids.
3. Willumsen, N., Skorve, J., Hexeberg, S., Rustan, A.C. & Berge, R.K. (1993). *Lipids* **28**:683–689. The hypotriglyceridemic effect of eicosapentaenoic acid in rats is reflected in increased mitochondrial fatty acid oxidation followed by diminished lipogenesis.
4. Willumsen, N., Hexeberg, S., Skorve, J., Lundquist, M. & Berge, R.K. (1993). *J. Lipid Res.* **34**:13–22. Docosahexaenoic acid shows no triglyceride-lowering effects but increases the peroxisomal fatty acid oxidation in liver of rats.
5. Willumsen, N., Vaagenes, H., Asiedu, D., Lie, Ø., Rustan, A.C. & Berge, R.K. (1996). *Lipids* **31**:579–592. Eicosapentaenoic acid but not docosahexaenoic acid (both as ethyl esters) increases mitochondrial fatty acid oxidation and upregulates 2,4-dienoyl-CoA reductase gene expression. A potential mechanism for the hypolipidemic action of fish oil in rats.
6. Prasaana, P., Thibault, A., Liu, L. & Samid, D. (1996). *J. Neurochem.* **66**:710–716. Lipid metabolism as a target for brain cancer therapy: Synergistic activity of lovastatin and sodium phenylacetate against human gliomas.
7. Ram, Z., Samid, D., Walbridge, S., Oshiro, E.M., Viola, J.J., Tao-Cheng, J.H., Shack, S., Thibault, A., Myers, C.E. & Oldfield, E.H. (1994). *Cancer Res.* **54**:2923–2927. Growth inhibition, tumor maturation, and extended survival in experimental brain tumors in rats treated with phenylacetate.
8. Samid, D., Ram, Z., Hudgins, W.R., Shack, S., Liu, L., Walbridge, S., Oldfield, E.H. & Myers, C.E. (1994). *Cancer Res.* **54**:891–895. Selective activity of phenylacetate against malignant gliomas: resemblance to fetal brain damage in phenylketonuria.
9. Pineau, T., Hudgins, R.W., Liu, L., Chen, L.C., Sher, X, Gonzalez, F.Z. & Samid, D. (1996). *Biochem. Pharmacol.* **52**:659–667. Activation of a human peroxisome proliferator-activated receptor by the antitumor agent phenylacetate and its analogs.
10. Berge, R.K. & Hvattum, E. (1994). *Pharmac. Ther.* **61(3)**:345–383. Impact of cytochrome P450 system on lipoprotein metabolism. Effect of abnormal fatty acids (3-Thia fatty acids).
11. Demoz, A., Svardal, A. & Berge, R.K. (1993). *Biochem. Pharmacol.* **45**:257–259. Relationship between peroxisome-proliferating sulfur-substituted fatty acid analogs, hepatic lipid peroxidation and hydrogen peroxide metabolism.
12. Demoz, A., Asiedu, O.K., Lie, Ø. & Berge, R.K. (1994). *Biochim. Biophys. Acta.* **1199**:238–244. Modulation of plasma and hepatic oxidative status and changes in plasma lipid profile by n-3 (EPA and DHA), n-6 (corn oil) and a 3-thia fatty acid in rats.
13. Formann, B., Chen, J. & Evans, R. (1997). *Proc. Natl. Acad. Sci.* **94**:4312–4317. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta.
14. Abdi-Dezfuli, F., Frøyland, L., Thorsen, T., Aakvag, A. & Berge, R.K. (1997). *Breast Cancer Res. Treat.* **45**:229–239. Eicosapentaenoic acid and sulphur substituted fatty acid analogues inhibit the proliferation of human breast cancer cells in culture.
15. Abdi-Dezfuli, F., Berge, R.K., Rasmussen, M., Thorsen, T. & Aakvag, A. (1994). *Ann. N. Y. Acad. Sci.* **744**:306–309. Effects of saturated and polyunsaturated fatty acids and their 3-thia fatty acid analogues on MCF-7 breast cancer cell growth.
16. Balch, W.E. & Rothman, J.E. (1985). *Arch. Biochem. Biophys.* **240**:413–425. Characterization of protein transport between successive compartments of the Golgi apparatus: asymmetric properties of donor and acceptor activities in a cell-free system.

POORLY OXIDIZABLE FATTY ACID ANALOGUES INHIBIT THE PROLIFERATION OF CANCER CELLS IN CULTURE

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1. INTRODUCTION

Tetradecylthioacetic acid (TTA) is a fatty acid analogue in which a sulfur atom substitutes the β-methylene group of the alkyl chain. The analogue closely resembles normal fatty acids except that it is unable to be metabolized by β-oxidation.[1] ω-3 fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are also poorly oxidized and it has been reported that they inhibit proliferation of cancer cells in culture.[2] Other similarities between TTA and ω-3 fatty acids are induction of several enzymes involved in mitochondrial and peroxisomal β-oxidation.[3–5] Moreover, they have hypotriglyceridemic effects, but TTA is much more potent than EPA.[6]

Both TTA and EPA have been shown to be inhibitors of growth of human breast cancer cells in culture.[7–8] TTA was found to have the most potent inhibitory effect on the estrogen responsive human cancer epithelial cell line MCF-7.

The aim of our study was to investigate whether TTA inhibits cell proliferation of different cancer cell lines cultured as monolayers or as spheroids. It was also of interest to examine the influence of TTA on tumour growth *in vivo*.

2. MATERIALS AND METHODS

Glioma cells (BT4Cn and D-54Mg) were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% newborn calf serum (heat denatured), L-glutamine (2mmol/l), 3% non-essential amino acids, penicillin (100IU/ml) and streptomycin (100µg/ml). HL-60 cells were grown in Iscove's medium supplemented with 10%

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heat-inactivated fetal calf serum (FCS), L-glutamine (2mmol/l) and gentamycin (0.1 mmol/1). CaCo2 cells were cultured in Dulbecco's modified Eagles medium (DMEM) with 20% FCS, insulin (10 μg/ml), 1% non-essential amino acids, L-glutamine (2 mmol/1) and antibiotics as for the glioma cells. MCF-7 cells were grown in DMEM to which had been added 10% newborn calf serum, 2% L-glutamine, penicillin (100IU/ml) and streptomycin (100 μg/ml). All cells were cultured at 37 °C, 95% air and 5% CO_2. Cell number was determined by cell counter or estimated by thymidine incorporation.

The multicellular spheroids were initiated by the agar overlay method described by Yuhas.[9] After 6 days in culture, spheroids with diameters between 200 μm and 300 μm were selected for growth experiments and transferred to 16mm multiwell culture dishes. The diameters of the spheroids were determined by using an inverted microscope with a calibrated graticule in the eyepiece. The wells were base-coated with 0.5ml 0.75% DMEM-agar and filled with 1 ml of medium.

Male brown rats, BD IX, were obtained from Gades Institute, Haukeland hospital, Bergen, Norway. They were housed in cages, in pairs, and maintained on a 12 h cycle of light and dark at a temperature of 20 ± 3 °C. During the experiments they weighed 250–400 g. They were fed a commercial standard pelleted food and provided with tap water *ad libitum.* Fatty acids were administered by oro-gastric intubation. TTA and palmitic acid were suspended in 0.5% (w/v) sodium carboxymethyl cellulose at a final stock solution of 75mg/ml. The animals were administered once a day with a dose of 300mg/kg body weight.

The tumour was implanted by stereotactical implantation. The rats were anaesthetized with 0.1ml Equithesin/100g body weight. A skin incision was made, and the skull was trepanned using a dental drill 2.5mm in diameter. 40,000 cells (20,000 cells/μl cell suspension) was injected with a Hamilton syringe with a cone-tipped 0.7mm needle at a depth of 2.5mm. The protocol was approved by the Norwegian State Comission for Animal Experimentation.

3. RESULTS

The effect of different concentrations of ERA and TTA on the growth of CaCo2 cells is shown in table 1. At 100μM, EPA reduced the proliferation of these cells by 34% and TTA by approximately 90%, compared to control, indicating that TTA is the most potent fatty acid. Indeed, at 50 μM, TTA reduced the growth by 36% whereas EPA was without any effect.

The incorporation of thymidine in the MCF-7 cells is shown in table 2. Compared to palmitic acid (control) this incorporation was reduced by 32% and 40% with EPA and

Table 1. Effect of EPA and TTA on cell growth (% of control) of human colon cancer cells (monolayer culture).

Cell line	Treatment (24 hours)	0μM	50μM	100μM
	Control	100		
CaCo2	EPA		102 ± 5	66 ± 5*
	TTA		74 ± 5*	9 ± 8*

Abbreviations: EPA, eicosapentaenoic acid; TTA, tetradecylthioacetic acid. Values represent mean percentage ± s.d.*$p < 0.05$ compared to control. Number of CaCo2 cells was estimated with thymidine incorporation.

Table 2. Effect of EPA and TTA on cell growth (% of control) of human breast cancer cells (monolayer culture).

Cell line	Treatment (72 hours)	64 μM
	Palmitic acid	100
MCF-7	EPA	68 ± 3*
	TTA	60 ± 3*

Abbreviations: EPA, eicosapentaenoic acid; TTA, tetradecylthioacetic acid. Values represent mean percentage ± s.d.*$p < 0.05$ compared to control. Number of cells was measured by cell counting. MCF-7 growth data has been published by Abdi-Dezfuli, F. *et al.* 1997.[8]

TTA, respectively. This, in agreement with earlier findings, suggests that TTA is more potent than EPA as an anticancer agent.

Figure 1 shows that TTA reduced the growth of human promyelocytic leukemic cells (HL-60) in a time-dependent manner. Significant growth inhibition by both TTA and palmitic acid was evident after 4 days in culture, compared to cells grown with no fatty acid supplementation (control). At this point, their anti-proliferative capacities were almost equal. However, after 8 days in culture, TTA was more effective than palmitic acid in reducing the cell growth, and the doubling time of the cells were 50, 56 and 80 hours for control, palmitic acid and TTA treatment, respectively.

It was also of interest to examine the effect of TTA on growth of human multicellular spheroids (D-54Mg). Figure 2 shows that the growth inhibitory effect of 100 μM TTA was evident after 7 days in culture. After 14 days in culture, TTA reduced the average spheroid diameter by approximately 38%, compared to control.

So far, we have focussed on the effect of fatty acids on human cancer cell proliferation. It was also of interest to examine whether the growth inhibitory effect of TTA on cancer cells was also evident on cancer cells from other species. Table 3 shows that 100 μM TTA inhibits the growth of rapid proliferating rat glioma cells (BT4Cn) by approximately 40% (after 6 days in culture). Interestingly, the same concentration of palmitic acid reduced the growth by approximately 23%.

During the last few years, cell cultures have been frequently used to test the effect

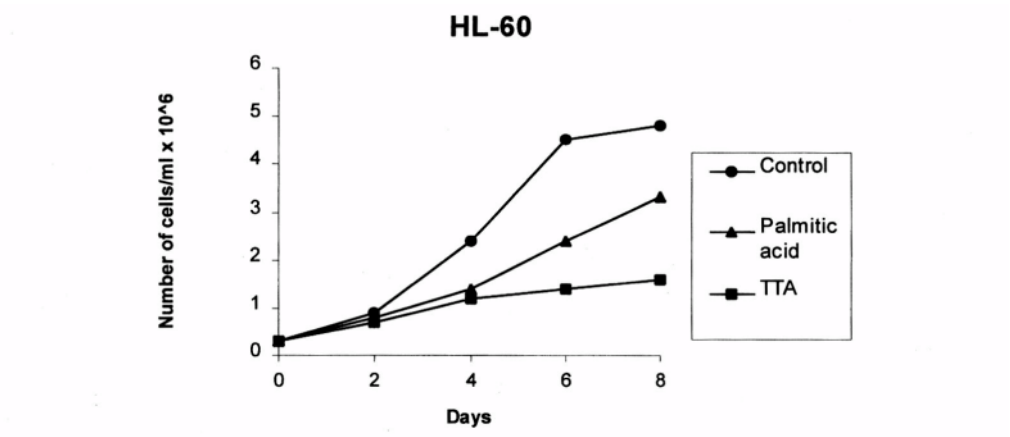

Figure 1. Effect of TTA on growth of human leukemic cells (cell suspension). Abbreviations: TTA, tetradecylthioacetic acid. Cell number was determined by cell counting.

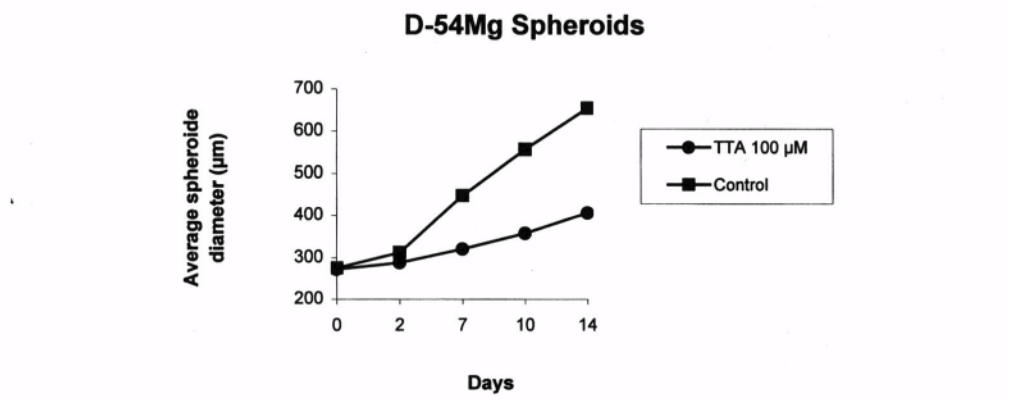

Figure 2. Effect of TTA on growth of human glioma spheroids. Abbreviations: TTA, tetradecylthioacetic acid.

of agents on cancer cell proliferation. Considering the general problems of using continous cultures of different cell lines, caution should be taken in extrapolating findings from cell culture experiments to the *in vivo* situation. However, we have performed an *in vivo* experiment with rats with intracranially implanted BT4Cn tumour. This experiment showed that the size of the tumour was decreased after TTA administration, compared to palmitic acid treatment (data not shown). Rats fed TTA showed prolonged survival time compared to rats fed palmitic acid (data to be published).

4. CONCLUSIONS

These data show that TTA and ERA inhibit growth of a variety of human cancer cells *in vitro.* In all these experiments TTA was more potent than EPA. However, cancer cells from different origins respond differently to poorly oxidizable fatty acids. Human colon cancer cells were more sensitive than human promyelocytic leukemic cells and human glioma cells.

Growth of rat cancer cells was also reduced by TTA. This suggests that cell lines from other species than human are sensitive to this sulfur substituted fatty acid analogue. Moreover, palmitic acid inhibited the proliferation of rat glioma cells (BT4Cn) and human leukemic cells (HL-60). However, TTA was more potent. On the growth of human breast cells, EPA was more potent than palmitic acid. Evidently, cancer cells may be more

Table 3. Effect of Palmitic acid and TTA on cell growth (% of control) of a rat glioma cell line (monolayer culture).

Cell line	Treatment (6 days)	0µM	100µM
	Control	100	
BT4Cn	Palmitic acid		77 ± 14*
	TTA		59 ± 12*

Abbreviations: TTA, tetradecylthioacetic acid. Cells were grown in tissue culture for 6 days. Values represent mean percentage ± s.d. *$p < 0.05$ compared to control. Number of cells was measured by cell counting.

sensitive to poorly oxidizable fatty acids than normal saturated fatty acids. Our experiments indicate that this effect may also occur *in vivo*.

The mechanism behind the reduced cell proliferation of cancer cells is not known, but several possible explanations exist. Several fatty acids, including TTA and EPA, serve as ligands for the peroxisomal proliferator activated receptor (PPAR).[10–11] The number of genes which have been found to carry the *cis*-binding site for this transcription factor, called the PPRE element, has been increasing the latest years. An examination of PPAR regulatable promoters suggests that this receptor family is intimately involved in fat metabolism, including fat breakdown,[12–13] storage[14] and synthesis.[15] Many of the enzymes involved in peroxisomal, microsomal and mitochondrial metabolism are regulated via PPAR. Preliminary data in our laboratory have shown that the gene expression and activity of several enzymes involved in lipid metabolism in the cancer cells are changed after TTA administration.

Details on enzyme distribution of CPT II and fatty acyl-CoA oxidase in both rat and human glioma cells are presented in "Metabolic effects of 3-thia fatty acids in cancer cells" by Tronstad KJ,. *et al.* 1998 (this issue).

REFERENCES

1. Lau, S., Brantley, R. & Thorpe, C. (1998). *Biochemistry* **27**:5089–5095. The reductive half-reaction in Acyl-CoA dehydrogenase from pig kidney: Studies with thiaoctanoyl-CoA and oxaoctanoyl-CoA analogues.
2. Finstad, H.S., Kolset, S.O., Holme, J.A., Wiger, R., Farrants, A.K., Blomhoff, R. & Drevon, C.A. (1994). *Blood* **84**:3799–3809. Effect of n-3 and n-6 fatty acids on proliferation and differentiation of promyelocytic leukemic HL-60 cells.
3. Skrede, S., Sørensen, H., Steineger, H.H., Høvik, K., Spydevold, Ø.S., Horn, R. & Bremer, J. (1997). *Biochim. Biophys. Acta* **1344**:115–131. Thia fatty acids, metabolism and metabolic effects.
4. Aarsland, A., Lundquist, M., Børretsen, B. & Berge, R.K. (1990). *Lipids* **25**:546–548. On the effect of peroxisomal β-oxidation and carnitine palmitoyltransferase activity by eicosapentaemoic acid in liver and heart from rats.
5. Willumsen, N., Hexeberg, S., Skorve, J., Lundquist, M. & Berge, R.K. (1993). *J. Lipid Res.* **34**:13–22. Docosahexaenoic acid shows no triglyceride-lowering effects but increases the peroxisomal fatty acid oxidation in liver of rats.
6. Frøyland, L., Vaagenes, H., Asiedu, D., Garras, A., Lie, Ø. & Berge, R.K. (1996). *Lipids* **31**:169–178. Chronic administration of eicosapentaenoic acid and docosahexaenoic acid as ethyl esters reduced plasma cholesterol and changed fatty acid composition in rat blood and organs.
7. Adbi-Dezfuli, F., Berge, R.K., Rasmussen, M., Thorsen, T. & Aakvaag, A. (1994). *Ann. N. Y. Acad. Sci.* **744**:306–309. Effects of saturated and polyunsaturated fatty acids and their 3-thia fatty acid analogues on MCF-7 breast cancer cell growth.
8. Adbi-Dezfuli, F, Frøyland, L., Thorsen, T., Aakvaag, A. & Berge, R.K. (1997). *Breast Cancer Res. Treat.* **45**:229–239. Eicosapentaenoic acid and sulpur substituted fatty acid analogues inhibit the proliferation of human breast cancer cells in culture.
9. Yuhas, J.M., Li, A.P., Martinez, A.O. & Ladman, A.J. (1977). *Cancer Res.* **37**:3639–3643. A simplified method for production and growth of multicellular tumor spheroids.
10. Kliewer, S.A., Sundseth, S.S., Jones, S.A., Brown, P.J., Wisely, O.B., Koble, C.S., Devchand, P., Wahli, W, Willson, T.M., Lenhard, J.M. & Lehmann, J.M. (1997). *Proc. Natl. Acad. Sci. U.S.A.* **94**:4318–4323. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma.
11. Forman, B.M., Chen, J. & Evans, R.M. (1997). *Proc. Natl. Acad. Sci. U.S.A.* **94**:4312–4317. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta.
12. Tugwood, J.D., Isseman, I., Anderson, R.G., Bundell, K.R., McPheat, W.L. & Green, S. (1992). *EMBO J.* **11**:433–439. The mouse peroxisome proliferator activated receptor recognizes a response element in the 5′ flanking sequence of the rat acyl CoA oxidase gene.

13. Zhang, B., Marcus, S.L., Sajjadi, F.G., Alvares, K., Reddy, J.K., Subramani, S., Rachubinski, R.A. & Capone, J.P. (1992). *Proc. Nad. Acad. Sci. U.S.A.* **89**:7541–7545. Identification of a peroxisome proliferator-responsive element upstream of the gene encoding rat peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase.
14. Tontonoz, P., Hu, E., Graves, R.A., Budavari, A.I. & Spiegelman, B.M. (1994). *Genes Dev.* **8**:1224–1234. mPar gamma 2:tissue-specific regulator of an adipocyte enhancer.
15. Rodriguez, J.C., Gil-Gomez, G, Hegardt, F.G. & Haro, D. (1994). *J. Biol. Chem.* **269**:18767–18772. Peroxisome proliferator-activated receptor mediates induction of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene by fatty acids.

THE ROLE OF PPARα AS A "LIPOSTAT" TRANSCRIPTION FACTOR

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1. INTRODUCTION

The ability of the cell to regulate fatty acid utilization and storage exquisitely is essential to maintain lipid homeostasis and normal functions. In children afflicted with enzymatic defects in the mitochondrial fatty acid oxidation pathway, cellular lipid utilization capacity is restrained leading to dramatic clinical consequences including hypoglycaemia, liver dysfunction, cardiomyopathy, and sudden death.[1–3] The PPARα is a ligand activated member of the nuclear receptor superfamilly of transcription factors.[4] Several lines of evidence suggest that PPARα plays a central role in the control of cellular lipid utilization pathways. Firstly, PPARα mediates the peroxisome proliferator pleiotropic response resulting from the actions of classical peroxisome proliferators.[4,5] Secondly, the majority of known PPARα target genes encode enzymes involved in cellular fatty acid (FA) oxidation.[6–11] Third, fatty acids and inhibitors of mitochondrial long-chain fatty acid (LCFA) import activate PPARα.[12–14] These facts suggest that PPARα serves as a cellular "lipostat", transducing changes in cellular lipid homeostasis to the transcription of target genes involved in fatty acid utilization. However, other than its recently defined role in the control of hepatic peroxisome proliferation,[5] little is known about the function of PPARα *in vivo*.

To investigate the role of PPARα in the control of cellular lipid homeostasis, we characterized the expression of target genes in response to a perturbation in FA oxida-

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tive flux. We hypothesized that in tissues with a high capacity for mitochondrial fatty acid β-oxidation such as heart and liver, inhibition of LCFA import would cause an accumulation of intracellular lipids or FA metabolites, inducing a PPARα-mediated feedback activation of target genes involved in alternate extra-mitochondrial oxidation pathways. Experiments were performed in which wild-type mice or mice lacking PPARα (PPARα –/–) were treated with etomoxir, an irreversible inhibitor of carnitine palmitoyltransferase I (CPT I), which catalyzes the import of LCFA into the mitochondrion. We found that inhibition of FA oxidative flux causes a feedback induction of PPARα target genes encoding peroxisomal, cytochrome P450, and mitochondrial FA oxidation enzymes. In PPARα –/– mice, a perturbation in cellular fatty acid flux causes massive hepatic and cardiac lipid accumulation, hypoglycaemia and death in 100% of male, but only 25% of female PPARα –/– mice. These results demonstrate a pivotal role for PPARα as a "lipid metabolic-stress" response factor.

2. MATERIALS AND METHODS

2.1. RNA Blot Analysis

Total cellular RNA was isolated from tissues using the RNA zol B technique.[15] The concentration of RNA was measured by A_{260nm}. Total RNA (15μg) was fractionated through a formaldehyde/agarose gel and transferred to a nylon membrane. Membranes were incubated with (α-^{32}P) dCTP-labelled cDNA. The mouse medium-chain acyl-CoA dehydrogenase (MCAD) EcoRI fragment of 1,500bp was provided by Dr Philip Wood, University of Alabama, Birmingham.[16] A mouse AGO cDNA of 559 bp was synthesized from total mouse liver RNA by reverse transcription/polymerase chain reaction (RT/PCR) (oligonucleotide primers; sens, 5′-CAATCACGCAATAGTTCTGGCTC-3′ and antisens 5′-AAGCTCAGGCAGTTCACTCAGG-3′). The AGO cDNA was then directly cloned into pCR™ II TA cloning vector according to the manufacturer's protocol (Invitrogen). Rat Cytochrome P450 4A1 (612bp) and Cytochrome P450 4A3 cDNAs (394 bp) were amplified by RT-PCR. Oligonucleotides complementary to sequence of cytochrome P450 4A1 and P450 4A3 cDNAs were chosen as ACCCTAGACACTGTCATGAAGTGT (at the 3′ end) and AGATGTGCTGAGTTCTCTGACAAT (at the 5′ end) and as CCTCCAGACTCCATCCCAGT (at the 3′ end) and CTCTCTACTGTTCTGTATCAGAAT (at the 5′ end), respectively. The PCR products obtained that were amplified from cDNA derived from rat liver were cloned directly into pCR™ II vector. Prehybridization and hybridization were performed in an hybridization oven at 65 °C, using the QuickHyb solution (Stratagene) following the manufacturer's instructions. The membranes were washed twice with 2 × SSC (1XSSC = 0.15M NaCl/0.015M sodium citrate) for 15min at room temperature, once with 2 × SSC and 1% SDS for 10min at room temperature and with 1 × SSC and 1% SDS for 30min at 62 °C. The signals were quantified by computerized densitometric analysis within the linear range of film sensitivity. The densitometric values were normalized to the signals obtained after hybridization of the blots with an 18S ribosomal cDNA probe to control for minor variations in RNA loading or integrity.

2.2 Animals Studies

All experiments were performed with mice ranging in age from 3–6 months (21–33g). Adult C57BL/6 × SJL/J mice were used for the experiments shown in Fig. 1. The

PPARα –/– and PPARα+/+ mice used for this study have been described.[5] For the metabolic inhibitor studies, etomoxir (50 μg/g body mass) or vehicle (sterile water) was given as a daily intraperitoneal injection at 09:00 a.m. for five days. At the time of harvest, animals were killed by CO_2 inhalation and liver and cardiac ventricle were rapidly dissected free, snap-frozen in liquid nitrogen, and stored at –80 °C until processed for isolation of RNA or lipid extraction. For histologic analyses, the tissue was processed at the time of harvest as described below.

Mouse tail vein blood glucose levels were determined by use of a standard clinical blood glucometer (One Touch II, Johnson and Johnson). This method allowed for serial glucose measurements without a significant drop in blood volume because reproducible measurements are possible using 5 μl samples.

All animal study protocols were approved by the Washington University School of Medicine Animal Studies Committee.

2.3. Tissue Histology Studies

For histologic analysis, organs were removed from the mice and quickly sliced into small pieces weighing less than 2 mg. The tissue was snap frozen in a cryomold and stored at –80 °C until it was prepared for cutting and oil red O staining.

2.4. Tissue Lipid Analysis

Tissue lipid extraction and thin layer chromatography (TLC) was performed using a modification of a previously described protocol.[17] In brief, a piece of liver was baked to a dry mass of approximately 100mg. The dessicated tissue was extracted with chloroform/methanol (2:1) × 2. The lipid layer was removed, dried and weighed. Percent lipid was calculated by dividing the extracted fat dry mass by the tissue sample dry mass (×100). For TLC separation, the lipid extract was resuspended in chloroform/methanol and separated in a two-step chromatograph in $CHCl_3$/methanol/acetic acid: 98/2/1 followed by hexane/ethyl ether/HCl: 94/6/0.2. The sample was run adjacent to a panel of standards (triglyceride, cholesterol, 1–2 diacylglyceride, and monoglyceride). After separation, the plate was stained with 3% copper acetate/8% phosphoric acid, followed by baking. The migration and quantity of sample species was compared with that of the standards.

2.5. Statistical Methods

Statistical comparisons were made using analysis of variance and Fisher's test, or Student's *t*-test. A statistically significant difference was defined as a p value <0.05.

3. RESULTS

To investigate the role of PPARα in the control of cellular lipid homeostasis *in vivo*, we analyzed the expression of known PPARα target genes encoding mitochondrial and extra-mitochondrial FA oxidation enzymes in response to a major perturbation in FA flux caused by pharmacologic inhibition of mitochondrial import of LCFA by etomoxir. The results obtained showed that the expression of peroxisomal acyl-CoA oxidase (ACO) gene was induced in heart and liver of mice receiving a five day course of etomoxir (Fig. 1). CPT I inhibition by etomoxir also led to a marked increase in the expression of

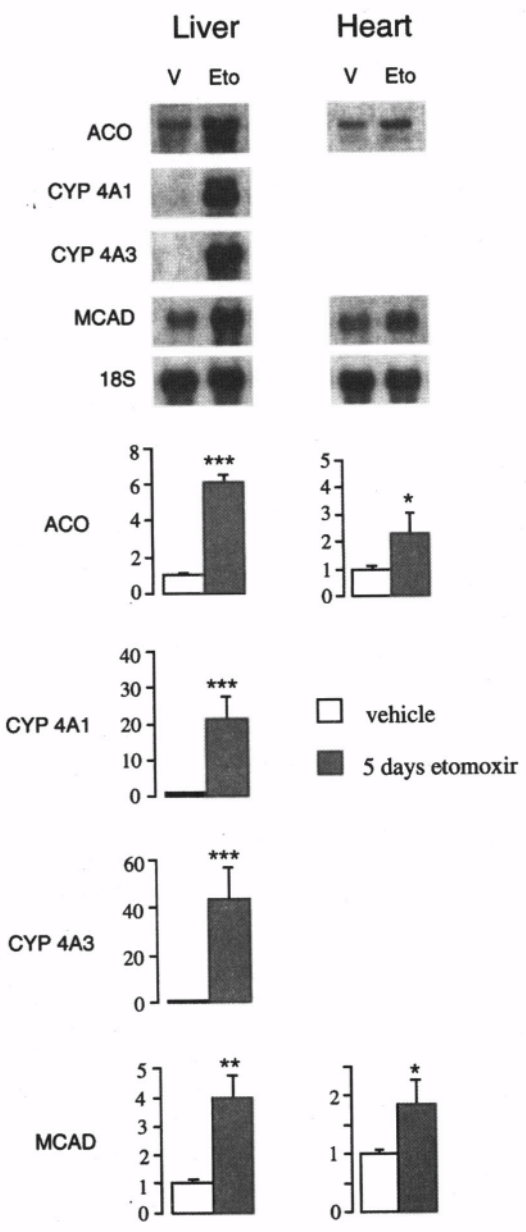

Figure 1. CPT I inhibition induces the expression of PPARα target genes involved in hepatic and cardiac fatty acid oxidation. Three-month-old male C57BL/6 × SJL/J mice were given daily intraperitoneal injections of vehicle (V) or etomoxir (Eto, 50 µg/g body mass) for 5 days. A representative autoradiogram is shown. ^{32}P-labeled cDNA probes used for the hybridizations encoded mouse acyl-CoA oxidase (ACO), rat cytochrome P450 4A1 (CYP 4A1) and P450 4A3 (CYP 4A3), mouse medium-chain acyl-CoA dehydrogenase (MCAD) and 18s ribosomal RNA (18s). CYP 4A1 and CYP 4A3 signals were only detected in liver. The signals on the Northern blot autoradiograms were quantified by laser densitometric analysis within the linear range of film sensitivity. Values were normalized to the signals obtained with 18s ribosomal cDNA probe. The bars represent the mean ± SE from at least 6 animals in each group normalized (= 1.0) to the results obtained with 5 days of vehicle administration. The asteriks denote a statistically significant difference (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) compared with the value obtained with vehicle alone using analysis of variance and Fisher's test.

PPARα target genes encoding hepatic cytochrome P450 oxidation enzymes (CYP 4A1 and CYP 4A3) and the expression of the gene encoding medium-chain acyl-CoA dehydrogenase (MCAD), which catalyses a rate-limiting step in the mitochondrial oxidation of medium-chain fatty acyl-thioesters produced by peroxisomal β-oxidation of LCFA. Thus, inhibition of mitochondrial long-chain fatty acid import induced the expression of PPARα target genes encoding cellular fatty acid oxidation enzymes. All of the mice tolerated the metabolic perturbation induced by etomoxir.

The CPT I inhibition experiments were repeated with mice homozygous for a targeted disruption of the PPARα gene (PPARα –/–). Western-blot analysis showed that

Table 1. CPT I inhibition results in gender-influenced death in PPARα –/– mice. The mice received a single daily intraperitoneal injection of vehicle (Veh, sterile water) or etomoxir (Eto, 50 μg/g body mass) for 5 consecutive days. Other abbreviations are M: male, F: female.

	Sex	Number of mice	Treatment	Survival rate %
PPARα +/+	M	5	Veh	100
		6	Eto	100
	F	5	Veh	100
		5	Eto	100
PPARα –/–	M	5	Veh	100
		9	Eto	0
	F	5	Veh	100
		8	Eto	75

the PPARα protein is absent in these mice.[5] These animals are viable, fertile, healthy and apparently normal; however, the expected hepatic peroxisome proliferation and the activation of genes encoding peroxisomal and mitochondrial enzymes in response to fibric acid derivatives are no longer observed. Groups of males and females wild-type (PPARα +/+) and PPARα–/– mice were given either vehicle or etomoxir daily for 5 consecutive days. All males PPARα–/– died (9/9) after receiving etomoxir, most of them within 24 h following the first injection (Table 1). In contrast, only two of eight female PPARα –/– died after etomoxir administration; they died after 2 and 3 injections respectively. All male and female age- and strain-matched PPARα +/+ control mice tolerated the 5 day treatment with etomoxir.

Analysis of the expression of PPARα target genes in the liver (Fig. 2) and heart (data not shown) of PPARα –/– female survivors demonstrated that etomoxir did not induce the gene expression, further supporting a role of this nuclear receptor in response to a perturbation of cellular lipid homeostasis.

Histologic analysis of hematoxylin and eosin stained sections of liver and heart from male PPARα –/– mice following a single injection of etomoxir revealed marked lipid accumulation in both organs with high fatty acid oxidative flux (Fig. 3A). The sections were also stained with oil red O, a method for staining of neutral lipids (Fig. 3B). The livers of PPARα–/– male and female vehicle-treated control mice contained patchy areas of small red droplets but not their heart, consistent with the existence of a mild

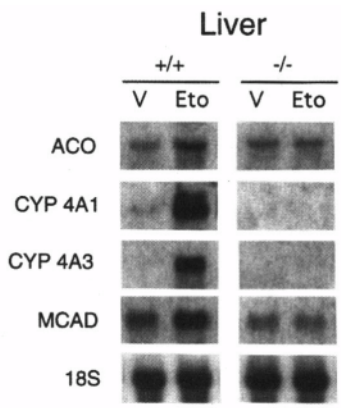

Figure 2. The feedback upregulation of PPARα target genes in response to CPTI inhibition is absent in female PPARα –/– mice. Northern blots performed with total RNA isolated from liver of vehicle (V) or etomoxir-treated (Eto, 5 days) PPARα +/+ (+/+) or PPARα –/– (–/–) female mice. The autoradiograms shown are representative of female PPARα –/– survivors and age-matched PPARα +/+ female mice.

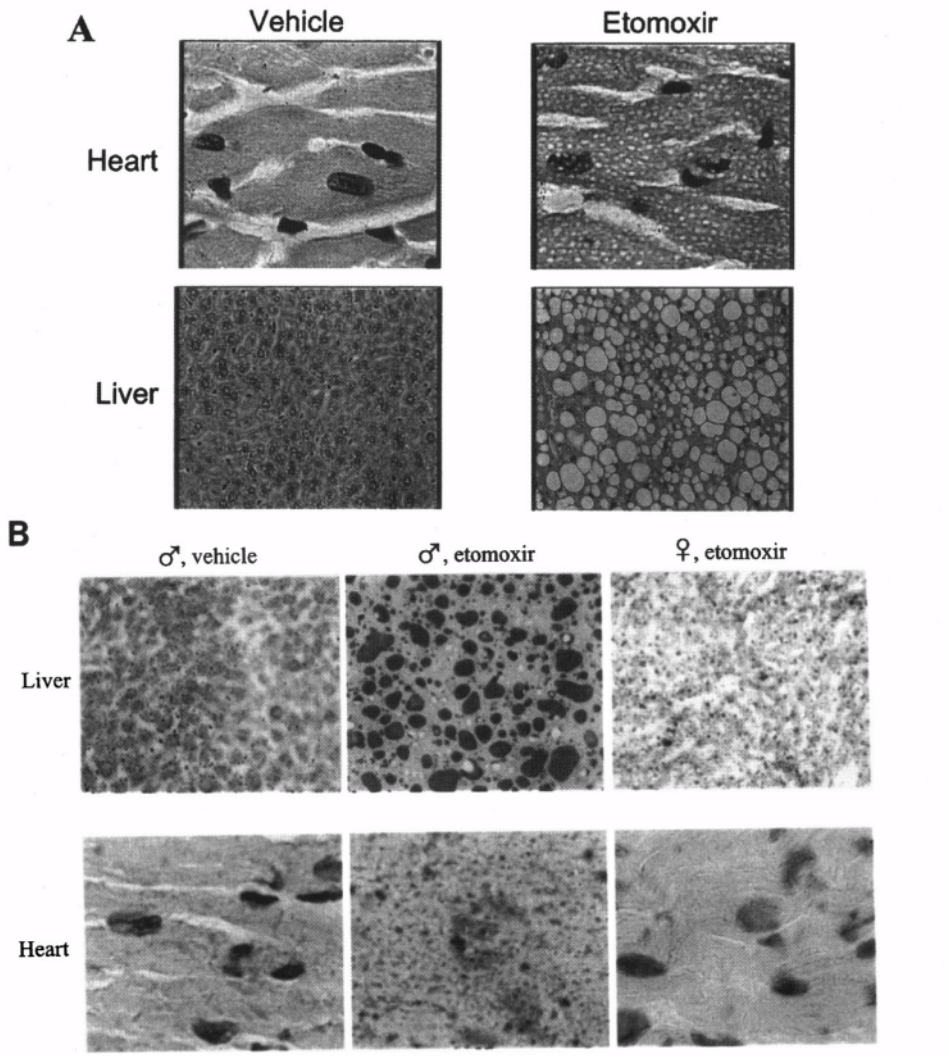

Figure 3. Hepatic and myocardial lipid accumulation in etomoxir-treated PPARα –/– mice parallels gender-related lethality. A. Representative hematoxylin/eosin stained samples of liver and heart from male PPARα –/– mice following a single dose of etomoxir or vehicle. B. Representative photomicrographs of oil red O stained sections of liver and heart prepared from tissues of a male PPARα –/– mouse that received vehicle (left); a male PPARα –/– mouse that died within 24 hours of the first dose of etomoxir (middle), and a female PPARα –/– mouse that survived three injections of etomoxir (right; representative of the hearts and livers of all female PPARα –/– surviors at 1, 3, or 5 days of etomoxir treatment). Magnification: liver at 300×; heart at 750×.

defect in hepatic fatty acid oxidation. After a single dose of etomoxir, **PPARα** –/– male mice developed massive micro- and macrovesicular hepatic steatosis and diffuse myocardial lipid accumulation. The sections obtained from the livers and hearts of the two female –/– mice that died in response to etomoxir appeared similar to that of organs from etomoxir-treated **PPARα** –/– males (data not shown). In striking contrast, the hearts of

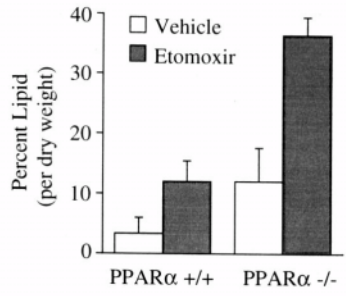

Figure 4. Lipid content of liver from male mice correlates with histologic evidence of fat accumulation. The bars represent mean (±SD) percent lipid (mg lipid/mg dry mass tissue ×100) from livers of male PPARα +/+ and male PPARα –/– mice given vehicle or a single injection of etomoxir (50 μg/g). Asteriks denote a statistically significant difference between values obtained from vehicle-compared to etomoxir-treated mice within the PPARα +/+ and PPARα –/– groups. *$p < 0.05$, Student's *t*-test.

the **PPARα** –/– females that survived had no detectable lipid accumulation, although mild hepatic steatosis was present (Fig. 3B). Thus lipid accumulation in heart and liver correlated with the gender-related sensitivity of **PPARα** –/– mice to etomoxir.

Lipids were extracted from liver of male **PPARα** +/+ or –/– mice treated either with vehicle or with one dose of etomoxir. Thin-layer chromatographic analysis of lipids confirmed that over 99% of accumulated lipid was triglyceride (data not shown). The liver lipid content was increased in both **PPARα** +/+ and **PPARα** –/– mice after etomoxir treatment, the increase being much more pronounced in the **PPARα**–/– animals (Fig. 4). Interestingly, when comparing the vehicle treated animals, even at baseline the lipid amount is higher in the **PPARα**–/– mice than in the **PPARα**+/+ confirming that a mild defect in hepatic fatty acid oxidation exists in the **PPARα** –/– mice, as already suggested from histologic analysis of the liver. These results demonstrated that liver lipid content paralleled the degree of triglyceride accumulation detected on histologic examination in the **PPARα** +/+ and **PPARα**–/– mice at baseline and in response to CPT I inhibition.

Fatty acids represent the main energy substrate in tissues such as heart and liver; but, when inhibition of fatty acid oxidation occurs, these tissues have to switch to glucose in order to meet their high energy demand. We therefore examined blood glucose levels to determine whether hypoglycaemia contributed to death of the **PPARα** –/– mice. In male **PPARα**–/– mice, mean blood glucose levels dropped from 80 to 26mg/dl within 20 hours following the first etomoxir injection (Table 2). In contrast, hypoglycaemia did not develop in etomoxir-treated male **PPARα** +/+ animals or the female **PPARα** –/– mice that survived the etomoxir treatment. These results parallel the gender-related abnormality in cellular lipid utilisation and identify a defect in glucose metabolism in the **PPARα** –/– mice.

Table 2. Male PPARα –/– mice develop hypoglycaemia in response to CPT I inhibition. The values are mean (±SD) blood glucose levels (mg/dl) of mice described in Table 1 at baseline and after etomoxir given as a single daily dose for 5 days. The timepoints indicate time elapsed after the initial injection. The asterisks denote a statistically significant difference ($p < 0.05$; paired *T*-test) compared to baseline levels. *M: male; F: female.*

Sex	Baseline	5 h	10 h	20 h	72 h	5 days
M PPARα –/–	80 ± 3	50 ± 11*	41 ± 15*	26 ± 6*	—	—
M PPARα +/+	90 ± 10	92 ± 3	—	91	84	84
F PPARα –/–	76 ± 7	64 ± 9	69 ± 9	71 ± 12	86	81 ± 16

4. DISCUSSION

Cellular fatty acid utilization rates are tightly controlled to maintain lipid homeostasis in tissues with high fatty acid oxidative flux such as heart and liver. Our results indicate that the lipid-activated nuclear receptor, PPARα, transduces changes in cellular fatty acid oxidative flux to the transcriptional control of genes involved in cellular fatty acid utilization *in vivo*. We propose that the expression of PPARα target genes encoding hepatic and cardiac mitochondrial, peroxisomal, and cytochrome P450 enzymes is orchestrated by PPARα in accordance with intracellular levels of fatty acid intermediates, several of which serve as activating PPARα ligands.[12–14]

Inborn errors in mitochondrial fatty acid β-oxidation enzymes are a relatively common cause of heritable metabolic disturbances during childhood.[1–3] Children afflicted with a defect in fatty acid oxidation are typically asymptomatic until a "crisis" is precipitated by a dietary or physiologic condition that dictates increased reliance on fatty acid oxidation for energy "production" such as fasting, prolonged exercise, or intercurrent illness. The clinical episodes in β-oxidation enzyme-deficient children are characterized by hypoglycaemia, cardiomyopathy, liver dysfunction, and a high incidence of sudden death. The molecular pathogenesis of the inherited defects in β-oxidation has not been characterized; potential contributing factors include energy deficiency or accumulation of toxic intracellular lipid intermediates. The latter is supported by the observation of myocardial and hepatic lipid accumulation in postmortem studies of children with β-oxidation defects.[1] The phenotype of the PPARα –/– mice in response to the metabolic stress imposed by CPT I inhibition is remarkably similar to that of humans with genetic defects in mitochondrial fatty acid oxidation. These results suggest that PPARα may serve as a lipid metabolic stress response factor. We speculate that PPARα is an important compensatory factor in human inborn errors in lipid metabolism and acquired diseases known to alter cardiac or hepatic lipid metabolism and that the PPARα –/– mouse should prove useful as an animal model of these diseases.

Our results demonstrate the importance of PPARα in cardiac as well as hepatic metabolism. Several lines of published evidence indicate that PPARα is involved in the regulation of liver metabolism. PPARα is required for the hepatic peroxisomal proliferative response to peroxisome proliferators.[4,5] The expression of enzymes involved in peroxisomal and mitochondrial fatty acid oxidation are reduced in liver of PPARα –/– mice.[5,18] Most PPARα target genes identified to date are expressed abundantly in heart, a tissue with high capacity for fatty acid oxidation. Our results define a role for PPARα in heart *in vivo*. The cardiac phenotype of the etomoxir-treated PPARα –/– mice strongly suggests that PPARα is involved in the control of myocardial lipid utilization pathways. Given that the postnatal mammalian heart relies primarily on fatty acid oxidation for energy production, PPARα likely plays a central role in the control of cardiac energy metabolism. It will be of interest to investigate the role of PPARα as a metabolic stress response factor in the context of cardiovascular disease states known to be associated with an alteration in myocardial lipid metabolism such as cardiac hypertrophy or ischaemia.

The development of hypoglycaemia in etomoxir-treated male PPARα –/– mice underscores the link between fatty acid oxidation and glucose homeostasis. The intracellular acetyl-CoA/long-chain acyl-CoA ratio is an important determinant of the activity of pyruvate carboxylase, which catalyzes a rate-limiting step in hepatic gluconeogenesis.[19,20] Accordingly, the diminished capacity for β-oxidation in the PPARα –/– mice would be expected to decrease the acyl-CoA/long-chain acyl-CoA ratio and, thus, place a constraint on gluconeogenic capacity. We propose that in etomoxir-treated male

PPARα –/– mice, a defect in glucose production coupled with increased glycogen utilization (resulting in diminished glycogen stores) results in a profound decrease in plasma glucose. These results indicate that in addition to promoting fatty acid oxidation, PPARα exerts an indirect positive regulatory effect on glucose production.

A surprising result of this study was the observation that the metabolic stress-induced phenotype of the PPARα –/– mice is strongly influenced by gender. Our results are consistent with the existence of a gender-influenced fatty acid utilization pathway that in addition to PPARα, play a role in cardiac and hepatic lipid homeostasis. The mechanism whereby gender influences regulates cellular lipid utilization is presently unknown. Preliminary data generated by our group suggests that estrogens control hepatic and cardiac lipid utilization pathways. It will be of considerable interest to determine whether cellular lipid metabolic pathways are controlled by sex hormones.

In summary, our results define an important role for PPARα in the maintenance of cellular lipid and glucose homeostasis *in vivo* via the transcriptional control of target genes encoding mitochondrial and extra-mitochondrial fatty acid oxidation enzymes. We also demonstrate that gender-related mechanisms are involved in hepatic and cardiac lipid metabolism. Lastly, we propose that the PPARα –/– mouse may prove useful as a model of human diseases due to inborn and acquired alterations in cellular lipid metabolism.

ACKNOWLEDGMENTS

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REFERENCES

1. Roe, C.R. &Coates, P.M. (1995); In *The metabolic and molecular bases of inherited disease,* (C.R. Scriver Beaudet, A.I., Sly, W.S., & Valle, D.), Me Graw-Hill, New York.
2. Kelly, D.P., Hale, D.E., Rutledge, S.L., Ogden, M.L., Whelan, A.J., Zhang, Z. & Strauss, A.W. (1992); *J. Inher. Metab. Dis.*; **15**, 171–180, Molecular basis of inherited medium-chain acyl-CoA dehydrogenase deficiency causing sudden child death.
3. Kelly, D.P. & Strauss, A.W. (1994); *N. Engl. J. Med.*; **330**, 913–919, Inherited Cardiomyopathies.
4. Issemann, I. & Green, S. (1990); *Nature;* **347**, 645–649, Activation of a member of the steroid hormone recpteur superfamily by peroxisome proliferators.
5. Lee, S.S.T., Pineau, T., Drago, J., J, L.E., W, O.J., Kroetz, D.L., Fernandez-Salguero, P.M., Westphal, H. & Gonzalez, F.J. (1995); *Mol. Cell. Biol.*; **15**, 3012–3022, Targeted disruption of the α isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators.
6. Aldridge, T.C., Tugwood, J.D. & Green, S. (1995); *Biochem. J.*; **306**, 473–479, Identification and characterization of DNA elements implicated in the regulation of CYP4A1 transcription.
7. Gulick, X, Cresci, S., Caira, T., Moore, D.D. & Kelly, D.P. (1994); *J. Biol. Chem.*; **91**, 11012–11016, The peroxisome proliferator-activated receptor regulates mitochondrial fatty acid oxidative enzyme gene expression.
8. Muerhoff, A.S., Griffin, K.J. & Johnson, E.F. (1992); *J. Biol. Chem.;* **267**, 19051–19053, The peroxisome proliferator-activated receptor mediates the induction of CYP4A6, a cytochrome P450 fatty acid omega-hydroxylase, by clofibric acid.

9. Rodriguez, J.C., Gil-Gomez, G., Hegardt, F.G. & Haro, D. (1994); *J. Biol. Chem.*; **269**, 18767–18772, Peroxisome proliferator-activated receptor mediates induction of mitochondrial 3-hydroxy-3methylglutaryl-CoA synthase gene by fatty acids.
10. Tugwood, J.D., Issemann, I., Anderson, R.G., Bundell, K.R., McPheat, W.L. & Green, S. (1992); *EMBO J.*; **11**, 433–439, The mouse peroxisome proliferator activated receptor recognizes a response element in the 5´ flanking sequence of the rat acyl CoA oxidase gene.
11. Zhang, B., Marcus, S.L., Sajjadi, F.G., Alvares, K.., Reddy, J.K., Subramani, S., Rachubinski, R.A. & Capone, J.P. (1992); *Biochem.*; **89**, 7541–7545, Identification of a peroxisome proliferator-responsive element upstream of the gene encoding rat peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase.
12. Göttlicher, M., Widmark, E., Li, Q. & Gustafsson, J.-A. (1992); *Proc. Natl. Acad. Sci. U.S.A*; **89**, 4653–4657, Fatty acids activate a chimera of the clofibric acid-activated receptor and the glucocorticoid receptor.
13. Keller, H., Dreyer, C, Medin, J., Mahfoudi, A., Ozato, K. & Wahli, W. (1993); *Proc. Natl. Acad. Sci. USA;* **90**, 2160–2164, Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor X receptor heterodimers.
14. Forman, B.C., Chen, J. & Evans, R.M. (1997);.*Proc. Natl. Acad. Sci.*; **94**, 4312–1317, Hypolipidemic drugs, polyunsaturated fatty acids and eicosanoids are ligands for PPAR α and δ.
15. Kelly, D.P., Gordon, J.I., Alpers, R. & Strauss, A.W. (1989); *J. Biol. Chem.*; **264**, 18921–18925, The tissue-specific expression and developmental regulation of two nuclear genes encoding rat mitochondrial proteins. Medium chain acyl-CoA dehydrogenase and mitochondrial malate dehydrogenase.
16. Tolwani, R.J., Farmer, S.C. & Wood, P.A. (1994); *Genomics;* **23**, 247-249, Molecular cloning and characterization of the mouse medium-chain acyl-Coa dehydrogenase cDNA.
17. Maxwell, R.J. & Marmer, W.N. (1983); *Lipids*; **18**, 453–459, Systematic protocol for the accumulation of fatty acid data from multiple tissue samples: tissue handling, lipid extraction and class separation, and capillary gas chromatographic analysis.
18. Aoyama, T., Peters, J.M., Iritani, N., Nakajima, T, Furihata, K., Hashimoto, T. & Gonzalez, F.J. (1998); *J. Biol. Chem.*; **273**, 5678–5684, Altered constitutive expression of fatty acid-metabolizing enzymes in mice lacking the peroxisome proliferator activated receptor α (PPARα).
19. Fanelli, C., Calderone, S., Epifano, L., De Vincenzo, A., Modarelli, F, Pampanelli, S., Perriello, G., De Feo, P., Brunetti, P., Gerich, J.E. & Bolli, G.B. (1993); *J. Clin. Invest.;* **92**, 1617–1622, Demonstration of a critical role for free fatty acids in mediating counterregulatory stimulation of gluconeogenesis and suppression of glucose utilisation in humans.
20. Gerich, l, Haymond, M., Rizza, R., Verdonk, C. & Miles, J. (1981); Hormonal and substrate determinants of hepatic glucose production in man. In The regulation of carbohydrate formation and utilization in mammals, (C. Veneziale), University Park Press, Baltimore, 419–45.

THE HYPOLIPIDAEMIC EFFECT OF EPA IS POTENTIATED BY 2- AND 3-METHYLATION

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1. INTRODUCTION

Fish oil, containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), possesses a number of beneficial effects, including the hypolipidaemic effect. It has been assumed that EPA and DHA both are hypotriglyceridaemic. However, recent animal studies[1–3] have shown that EPA, but not DHA, appears to be responsible for the triglyceride lowering effect of fish oil, possibly through increased **β-oxidation.** Results from a recent clinical study seem to support this pathway as the primary site for the hypolipidaemic effect of n-3 fatty acids.[4]

Earlier we fed EPA to rats at different doses, and found that high doses of EPA, at least 1,000mg/d/kg body mass, were necessary to obtain the hypolipidaemic effect.[1,5,6]

Introduction of methyl groups to fatty acids makes them more inert to fatty acid oxidation.[7] Recently, we have synthesized several methyl- or ethyl-derivatives of EPA, to make them more inert to fatty acid oxidation than EPA itself. Fatty acids that are difficult to metabolize are well known to increase the fatty acid oxidation, in addition to lowering plasma lipids, and the EPA-derivatives are therefore expected to influence the fatty acid oxidation system. However, different degrees of methylation, and the localization of the methyl group on EPA could have importance for the effects on lipid metabolism and plasma lipid level, and this was studied.

Tetradecylthioacetic acid (TTA) is a saturated fatty acid with a sulphur atom in the 3-position of the carbon chain. The sulphur atom in 3-position prevents TTA itself from being **β-oxidized**, but causes elevation of the general fatty acid oxidation. TTA was

Current Views of Fatty Acid Oxidation and Ketogenesis: From Organelles to Point Mutations
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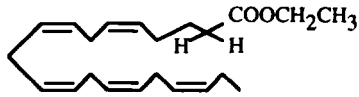
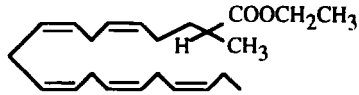
EPA



2-methyl EPA



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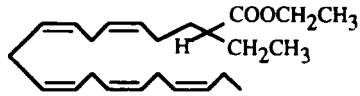

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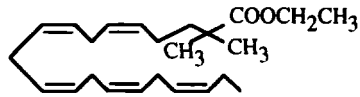
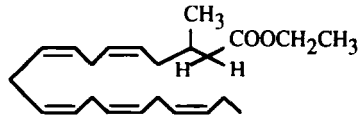


3-methyl EPA



TTA $CH_3\text{-}(CH_2)_{13}\text{-}S\text{-}CH_2\text{-}COOH$

2-methyl TTA $CH_3\text{-}(CH_2)_{13}\text{-}S\text{-}\underset{\displaystyle CH_3}{\underset{|}{C}}H\text{-}COOH$

Figure 1. Structural formulas of eicosapentaenoic acid (EPA) and different EPA-derivatives (all as ethyl esters), tetradecylthioacetic acid (TTA) and 2-methyl TTA.

methylated in 2-position, and we fed rats 2-methyl TTA at the dose 15mg/d/kg body mass. This is a very low dose, and usually we give TTA at a dose ten times this dose to obtain increased fatty acid oxidation. Its effects on fatty acid oxidation and plasma lipids were investigated.

2. RESULTS

In Table 1 we measured the plasma triglycerides in rats fed EPA and 2-methyl ERA at increasing doses.

As expected, EPA was without any effect at dose 600mg/d/kg body mass, or below, while 2-methyl EPA lowered the plasma triglycerides at this dose. Both EPA and 2-methyl EPA were hypotriglyceridaemic at the highest dose. We wanted to distinguish between the hypolipidaemic effects of the different EPA-derivatives, and found it necessary to use a low dose. The compounds were fed to rats at dose 250mg/d/kg body mass for 5 days. We measured the amount of plasma lipids (summation of plasma triglycerides, cholesterol and phospholipids) in these rats. As expected, EPA at this low dose did not have any hypolipidaemic effect compared to control. Also, 2-methyl EPA was without any

Table 1. Dose-dependent change in plasma triglycerides in rats treated with EPA or 2-methyl EPA.

	Dose (mg/day/kg body weight)			
Compound	0	250	600	1300
EPA	0.99 ± 0.18	1.04 ± 0.28	0.86 ± 0.13	0.69 ± 0.10*
2-methyl EPA	1.08 ± 0.13	0.99 ± 0.11	0.52 ± 0.03*	0.70 ± 0.08*

Values are presented as means ± SD from 6 animals, and *P < 0.05 compared to controls (Dose 0).

hypolipidaemic effect, while the other EPA-derivatives seemed to lower the plasma lipids (Fig. 2).

When measuring the mitochondrial β-oxidation in liver, 2-methyl EPA did not cause any effect compared to EPA or control, while the other EPA-derivatives increased the fatty acid oxidation in mitochondria. We measured the mitochondrial activity and gene expression of an enzyme involved in the oxidation of unsaturated fatty acids, the 2,4-dienoyl-CoA reductase. Both the activity and gene expression seemed to increase in rats fed 2,2-dimethyl EPA. We also measured the total activity of CPT in liver, and found an increased activity in rats fed 2,2-dimethyl EPA. The increase in total CPT-activity after administration of 2,2-dimethyl EPA seemed to be due to the observed increase in CPT-II transcription, as the mRNA level of CPT-I was unchanged (data to be published). The peroxisomal β-oxidation, the activity and gene expression of fatty acyl-CoA oxidase, the rate-limiting enzyme of peroxisomal β-oxidation, and the gene expression of the peroxisomal multifunctional protein were increased after administration of the EPA-derivatives, as shown in Table 2.

We measured the activity of some enzymes involved in lipogenesis, that is, acetyl-CoA carboxylase, the rate-limiting enzyme in fatty acid synthesis, and fatty acid synthase, another enzyme in fatty acid synthesis. The activities of these enzymes seemed to decrease after administration of the same EPA-derivatives as were found to increase the fatty acid oxidation.

Tetradecylthioacetic acid (TTA), a saturated fatty acid with a sulphur atom in the 3-position of the carbon chain, is blocked for β-oxidation, but causes elevation of the

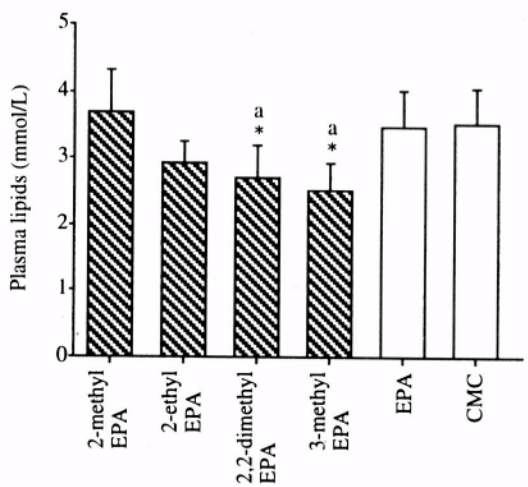


Figure 2. Plasma lipids from rats fed the different EPA-derivatives, EPA, or CMC (250 mg/day/kg body weight) for 5 days. The values of plasma lipids are given as the summation of plasma triglycerides, plasma cholesterol and plasma phospholipids, and are the means ± S.D. of 8 animals. *significantly different from CMC, and [a]significantly difference from EPA, P < 0.05.

Table 2. Effect of EPA-derivatives on peroxisomal fatty acid oxidation, activity and expression of fatty acyl-CoA oxidase, and expression of peroxisomal multifunctional protein.

TREATMENT	Peroxisomal β-oxidation (nmol/mg/min)	Fatty acyl-CoA oxidase		Peroxisomal multifunctional protein mRNA (fold increase)
		Activity (nmol/min/mg protein)	mRNA (fold increase)	
2-methyl EPA	1.17 ± 0.62	25.27 ± 5.86	1.04 ± 0.11	1.71 ± 0.28*[a]
2-ethyl EPA	2.01 ± 0.85	34.23 ± 4.96*	1.58 ± 0.19	2.02 ± 0.58*[a]
2,2-dimethyl EPA	4.37 ± 0.38*[a]	53.37 ± 7.82*[a]	2.75 ± 0.81*[a]	2.41 ± 0.58*[a]
3-methyl EPA	2.01 ± 0.27	40.05 ± 10.65*[a]	1.72 ± 0.52	2.08 ± 0.53*[a]
EPA	1.49 ± 0.25	23.15 ± 6.19	1.09 ± 0.17	1.11 ± 0.21
CMC	1.05 ± 0.59	20.05 ± 4.08	1.00 ± 0.24	1.00 ± 0.31

The values are given as means ± S.D. for eight animals. The rats were treated for 5 days with the different fatty acids at a dose of 250 mg/day/kg body weight. *significantly different ($P < 0.05$) from CMC, and [a]significantly different from EPA.

general fatty acid oxidation.[8,9] TTA was methylated in 2-position, and we fed rats 2-methyl TTA at the dose 15mg/d/kg body mass. This is a very low dose, and usually we give TTA at a dose ten times this dose to obtain increased fatty acid oxidation. As shown in Table 3, TTA and 2-methyl TTA at this low dose did not have any effect on the mitochondrial β-oxidation, CPT-I or CPT-II activity, and the plasma lipids were unchanged. However, the peroxisomal FAO activity increased significantly after administration of 2-methyl TTA compared to TTA. Thus, the increased peroxisomal β-oxidation (measured as FAO activity) in 2-methyl TTA fed rats did not cause any hypolipidaemic effect compared to TTA fed rats.

3. DISCUSSION

This study shows that the methylation of EPA potentiated the hypolipidaemic effect of EPA, as low doses of the EPA-derivatives caused hypolipidaemic effect while EPA

Table 3. The effects of tetradecylthioacetic acid (TTA) and 2-methyl TTA on liver and plasma parameters.

	CMC	TTA	2-methyl TTA
CPT-I			
Activity (nmol/min/mg protein)	1.22 ± 0.07	1.39 ± 0.07	1.47 ± 0.24
mRNA (relative values)	1.00 ± 0.34	0.77 ± 0.11	0.46 ± 0.06*
CPT-II			
Activity (nmol/min/mg protein)	6.74 ± 1.40	7.60 ± 1.79	6.68 ± 1.44
mRNA (relative values)	1.00 ± 0.20	1.21 ± 0.26	1.14 ± 0.26
Mitochondrial β-oxidation			
Palmitoyl CoA	0.66 ± 0.08	0.56 ± 0.16	0.62 ± 0.21
Palmitoyl-L-carnitine	0.62 ± 0.14	0.52 ± 0.15	0.56 ± 0.16
Plasma lipids (mmol/L)			
Triglycerides	0.84 ± 0.21	0.54 ± 0.12	0.55 ± 0.10
Cholesterol	1.45 ± 0.30	1.51 ± 0.34	1.32 ± 0.11
Phospholipids	1.36 ± 0.09	1.42 ± 0.38	1.37 ± 0.21
Fatty acyl-CoA oxidase (nmol/min/mg protein)	9.03 ± 0.76	11.04 ± 1.49	17.16 ± 1.02*

The rats were fed TTA or 2-methyl TTA (15 mg/day/kg body weight) for 7 days. Rats fed CMC were used as control. The values are represented as means ± S.D. ($n = 4$) *denote significantly difference from CMC ($P < 0.05$).

itself did not have any effect on the plasma lipids at this low dose. This study shows that, at low doses (250mg/d/kg body mass), methylation of the EPA molecule potentiated the hypolipidaemic effect of EPA, as the order of lipid-lowering effects of the EPA derivatives was 3-methyl **EPA ≥ 2,2-dimethyl EPA > 2-ethyl EPA > 2-methyl EPA = EPA = CMC.** This was followed by increased activities and gene expressions of enzymes involved in peroxisomal and mitochondrial **β-oxidation.** The increased activity of total CPT in rats fed 2,2-dimethyl EPA seemed to be due to a transcriptional induction of CPT-II, as the CPT-I expression was unchanged. Obviously, the position and the length of the alkyl group attached to the EPA molecule was of vital importance for the hypolipidaemic effect of the EPA-derivatives. The data indicated that a single methyl or ethyl substituent at carbon atom number two was not sufficient to impart hypolipidaemic properties to EPA. However, there seemed to be a difference between 2-methyl EPA and 2-ethyl EPA, the last being more effective in affecting the plasma lipids. Two methylations in the 2-position, or one methylation in the 3-position, however, more profoundly potentiated the hypolipidaemic effect of EPA. The data suggest that the efficiency of the different EPA-derivatives as lipid lowering agents depend on the methylation and bulkiness. Thus, the length of the alkyl group (ethyl instead of methyl) and the bulkiness of the group (two methyl groups instead of one) attached to the EPA molecule in 2-position was of vital importance for the EPA derivatives as lipid lowering agents. 3-methyl EPA, with a methyl branching in 3 **(β)-position,** theoretically cannot be degraded by **β-oxidation.** As pure EPA at similar dose (250mg/d/kg body mass) did not affect the plasma lipid level, it is conceivable that the efficiency of these EPA-derivatives as hypolipidaemic agents may depend on poor susceptibility to undergo **β-oxidation,** e.g. steric hindrance, rather than polyunsaturation. The lipid-lowering effect also depends on the doses of these modified fatty acids, as we have recently shown that 2-methyl EPA reduced the plasma triglycerides at 600 and 1,300mg/d/kg body mass, but not at 250mg/d/kg body mass.[10]

An inhibition of one of the enzymes involved in fatty acid synthesis is consistent with retarded lipogenesis. The enzyme considered to be rate-limiting in fatty acid synthesis, i.e. acetyl-CoA carboxylase, and another fatty acid synthesis enzyme, i.e. fatty acid synthase, are both downregulated by hypolipidaemic EPA-derivatives. Thus, the hypolipidaemic effect observed by administration of the EPA-derivatives to rats could be due to both increased fatty acid oxidation and reduced lipogenesis. However, other results by our group indicate that the fatty acid oxidation is the major cause of the hypolipidaemic effect.

We found that methylated TTA increased the peroxisomal, but not the mitochondrial **β-oxidation,** and no hypolipidaemic effect was observed. Thus, the increased peroxisomal **β-oxidation** (measured as FAO activity) in rats fed 2-methyl TTA did not cause any hypolipidaemic effect compared to TTA fed rats. According to these findings, it is reasonable to believe that the peroxisomes are of minor importance for the lipid lowering effect. This study thereby supports the hypothesis by our group that increased mitochondrial **β-oxidation,** and not peroxisomal **β-oxidation,** is responsible for the triglyceride lowering effect.[3,11,12] This is in agreement with a recent study in humans.[13] In addition, we have recently obtained evidence that the mitochondrion is the principal target for nutritional and pharmacological control of triglyceride metabolism.[3] It should also be emphasized, that the mitochondria are the quantitatively dominating organelles in liver cells compared to the peroxisomes, implicating that increased mitochondrial **β-oxidation** might have a greater impact on the total fatty acid oxidation than an increase in peroxisomal **β-oxidation.**

In summary, we have demonstrated that methylation of EPA rendered EPA more

potent as a hypolipidaemic agent. The different degree of methylation, and the position of the methyl group on EPA had great impact on the ability to cause hypolipidemia. The hypothesis that the mitochondrion is the primary site for the hypolipidaemic effect was supported by results from 2-methyl TTA feeding. 2-methyl TTA, which induced the peroxisomal **β-oxidation,** but rendered the mitochondrial **β-oxidation** unchanged, caused no lipid lowering effect.

REFERENCES

1. Willumsen, N., Hexeberg, S., Skorve, J., Lundquist, M. & Berge, R.K. (1993) *J. Lipid Res.*; **34**, 13–22 Docosahexaenoic acid shows no triglyceride-lowering effects but increases the peroxisomal fatty acid oxidation in liver of rats.
2. Willumsen, N., Vaagenes, H., Lie, Ø., Rustan, A.C. & Berge, R.K. (1996) *Lipids*; **31**, 579–592 Eicosapentaenoic acid, but not docosahexaenoic acid, increases mitochondrial fatty acid oxidation and upregulates 2,4-dienoyl-CoA reductase gene expression in rats.
3. Frøyland, L., Madsen, L., Vaagenes, H., Totland, G.K., Auwerx, J., Kryvi, H., Staels, B. & Berge, R.K. (1997) *J. Lipid Res.*; **38**, 1851–1858 Mitochondrion is the principal target for nutritional and pharmacological control of triglyceride metabolism.
4. Pownall, H. (1997) *CVD/Lipids Dialog*; **January**, 8–9 Omega-3 fatty acids increase beta-oxidation?
5. Aarsland, A., Lundquist, M., Børretsen, B. & Berge, R.K. (1990) *Lipids*; **25**, 546–548 On the effect of peroxisomal **β-oxidation** and carnitine palmitoyltransferase activity by eicosapentaenoic acid in liver and heart from rats.
6. Willumsen, N., Skorve, J., Hexeberg, S., Rustan, A.C. & Berge, R.K. (1993) *Lipids*; **28**, 683–690 The hypotriglyceridaemic effect of eicosapentaenoic acid in rats is reflected in increased mitochondrial fatty acid oxidation followed by diminished lipogenesis.
7. Singh, H., Beckman, K. & Poulos, A. (1994) *J. Biol. Chem.;* **269,** 9514–9520 Peroxisomal **β-oxidation** of branched chain fatty acids in rat liver.
8. Berge, R.K., Kryvi, H., Aarsaether, N., Aarsland, A., Lillehaug, J.R. & Skorve, J. (1993) **Chapter 12,** 277-297 *in Peroxisomes, Biology and importance in toxicology and medicine.* Eds. Gibson, G., and Lake, B.
9. Skrede, S., Sørensen, H.N., Larsen, L.N., Steineger, H.H., Høvik, K., Spydevold, Ø.S., Horn, R. & Bremer, J. (1997) *Biochim. Biophys. Acta.*; **1344,** 115–131 Thia fatty acids, metabolism and metabolic effects.
10. Willumsen, N., Vaagenes, H., Holmsen, H. & Berge, R.K. (1998) *Biochim. Biophys. Acta*; **1369,** 193–203 On the effect of 2-deuterium- and 2-methyl-eicosapentaenoic acid derivatives on triglycerides, peroxisomal **β-oxidation** and platelet aggregation in rats.
11. Asiedu, D.K., Skorve, J., Willumsen, N., Demoz, A. & Berge, R.K. (1993) *Biochim. Biophys. Acta;* **1166,** 73–76 Early effects on mitochondrial and peroxisomal b-oxidation by the hypolipidaemic 3-thia fatty acids in rat livers.
12. Frøyland, L., Vaagenes, H., Asiedu, D.K., Garras, A., Lie, Ø. & Berge, R.K. (1996) *Lipids*; **31**, 169–178 Chronic administration of eicosapentaenoic acid and docosahexaenoic acid as ethyl esters reduced plasma cholesterol and changed the fatty acid composition in rat blood and organs.
13. Rambjør, G.S., Wålen, A.I., Windsor, S.L. & Harris, W.S. (1996) *Lipids*; **31**, S45–S49 Eicosapentaenoic acid is primarily responsible for hypotriglyceridaemic effect of fish oils in humans.

IS IT TIME TO RECONSIDER THE ROLE OF CPT I IN CONTROL OF HEPATIC KETOGENESIS?

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1. INTRODUCTION

Hepatic mitochondrial outer membrane carnitine palmitoyltransferase (CPT I, B.C. 2.3.1.21) is the initial step in a three-part system (comprising CPT I, the acylcarnitine/carnitine exchange carrier and CPT II) that transfers long-chain fatty acid-derived CoA-esters across the mitochondrial membrane into the matrix. Specifically, CPT I is a transmembrane protein which catalyses the first committed reaction in the pathways of long-chain fatty acid oxidation and ketogenesis and has, therefore, traditionally been considered as a site of potential control over these pathway fluxes. Here, we provide a brief overview of recent relevant experimental data and of frequently conflicting opinions relating to the contribution to control of hepatic ketogenesis by CPT I. In addition to reporting qualitative approaches, which have been used to explore control of these pathways, we shall also examine the recent use of metabolic control analysis (MCA).[1–4] This is a mathematical tool which allows us to make *quantitative* assessments of control distribution in complex, dynamic, metabolic pathways such as these.

Current Views of Fatty Acid Oxidation and Ketogenesis: From Organelles to Point Mutations
edited by Quant and Eaton, Kluwer Academic / Plenum Publishers, New York, 1999.

2. TRADITIONAL QUALITATIVE VIEWS

McGarry & Foster[5,6] established that octanoate, a medium-chain fatty acid, was oxidized independently of metabolic state, whereas, the oxidation rate of long-chain fatty acids was a function of metabolic status (e.g. fed, fasted, diabetic). Since octanoate bypasses CPT I these authors concluded that this enzyme was a prime candidate for control of oxidative fluxes, including ketogenesis, from long-chain fatty acids. This conclusion was supported by apparent correlations in changes of substrate supply, hormones and in development with parallel changes in ketogenesis and the activity and expression of CPT I.

In the fetus, the rates of hepatic **β-oxidation** and ketogenesis are low. However, following birth, the capacity of these metabolic pathways increases and results in a significant rise in the concentration of ketone bodies (increasing from 0.2 mM in the rat at birth to 2mM 24h later[7]). This physiological hyperketonaemia is maintained throughout the suckling period.[8] The changes are similar to those in CPT I, where the activity, protein concentration and level of mRNA encoding CPT I are low in the fetus and increase 5-fold during the first day of extrauterine life. The enzyme activity and gene expression remain high during the entire suckling period.[9,10] Furthermore, the inhibitory effect of malonyl-CoA (an intermediate compound in the biosynthetic pathway of fatty acids and a potent physiological reversible inhibitor of CPT I[11]) is decreased in the first 24h following birth. However, these changes are not seen in liver CPT II and the mRNA, immunoreactive protein and activity are not influenced by nutritional and hormonal changes in the postnatal period. CPT II activity is already high in fetal rat liver and does not change after birth. Thumelin *et al.*[9] cite their findings as evidence that CPT I has the potential to control hepatic long-chain fatty acid oxidation.

3. NEW QUANTITATIVE INSIGHTS

More recently, Drynan *et al.*[12] have used MCA to investigate the role of CPT I in controlling fluxes (from palmitate) through **β-oxidation,** ketogenesis and the Krebs cycle ($J_{\beta ox}$; J_{KB}; J_{Krebs}) in hepatocytes from adult rats in different metabolic states (fed, starved, starved/refed, starved/insulin treated). This enabled them to derive flux control coefficients ($C^{J_{\beta ox}}_{CPTI}$; $C^{J_{KB}}_{CPTI}$; $C^{J_{Krebs}}_{CPTI}$) which describe quantitatively the control exerted by CPT I over each of the respective pathway fluxes. (High values of flux control coefficients (close to one) would indicate that the control exerted by CPT I over pathway flux is high, similarly, a low value (close to zero) would indicate a trivial contribution to control. This quantitative scale has the benefit that in simple linear systems, where the sum of all the individual flux control coefficients of a defined pathway is $1.0 \left(\sum_{i=1}^{n} C^{J}_{E_i}\right)$, it is possible to state that, for example, when a step has a flux control coefficient of 0.2 it exerts 20% of the control over the pathway flux[13]). Under all of the conditions examined by Drynan *et al.*[12] in their hepatocytic system, the numerical value of the flux control coefficient for CPT I over ketogenesis ($C^{J_{KB}}_{CPTI}$) was high (within the range **0.75 ± 0.12 to 0.92 ± 0.57**) which provided quantitative support for the traditional view and suggested that CPT I could be a primary control site for hepatic ketogenesis in adult rats.

Indeed, similar conclusions can be drawn from the work of Spurway *et al.* [14] who have also used MCA. In their defined system consisting of cultured hepatocytes (from adult male rats) and using their two independent methods of bottom-up control analy-

sis (BUCA) to obtain values for flux control coefficients for CPT I over ketogenic flux ($C^{J_{KB}}_{CPTI}$), it was once more found that the numerical values where high (0.67 to 0.79) again providing further support for CPT I being a "rate-controlling" step of ketogenesis.

However, whilst these results indicate that under certain conditions CPT I may make a significant contribution to ketogenic flux control, results from traditional studies have also revealed that this enzyme is not the only one involved in hepatic ketogenesis which undergoes changes during the fetal-neonatal transition (nor indeed the suckling-weaning period) and thus may not be the only site with high control potential. The concentration of liver mitochondrial 3-hydroxy-3-methylglutaryl-CoA (mHMG-CoA) synthase, the second enzyme of the HMG-CoA cycle, increases markedly immediately following birth and remains elevated during the suckling period.[15,16] In addition, plasma glucagon levels (which increase the activity of mHMG-CoA synthase by lowering the concentration of succinyl-CoA and, therefore, decreasing the extent of succinylation and hence inactivation of the enzyme) transiently fall following birth, stimulating ketogenesis in neonatal rats.[17–19]

Again, the flux control potential of this mechanism has been investigated quantitatively using the top-down approach of MCA (TDCA).[20–22] TDCA operates on a different level from BUCA as complex pathways are conceptually broken down into "blocks" of reactions, each consuming or producing a common intermediate. This conceptual approach reduces the number and simplifies the nature of the experiments required to analyse flux control precisely in complex systems. Using this approach, Quant *et al.*[23] found that under certain experimental conditions, only approximately 28% of the control over ketogenesis was invested in the group of enzymes responsible for the production of acetyl-CoA (ie CPT I, the carnitine carrier, CPT II and the associated enzymes of β-oxidation and the respiratory chain) whilst 72% of the control resided with the enzymes of the HMG-CoA cycle. It was tentatively suggested that within this cycle control might be exerted at the level of mHMG-CoA synthase although this remains unconfirmed. This work therefore suggested that CPT I was unlikely to be the single, "rate-limiting" step for ketogenesis, although it did not rule out the possibility that CPT I may have significant control over entry of long-chain fatty acids into mitochondria.

The concept of CPT I being the "rate-limiting" step of fatty acid oxidation and ketogenesis has been further questioned by Krauss *et al.*[24] Application of TDCA in a defined, mitochondrial palmitoyl-CoA-oxidizing system enabled these workers to investigate the role of CPT I in the control over total carbon flux, although not specifically over ketogenesis. Numerical values of group flux control coefficients for CPT I over total oxidative carbon flux in adult rats ($^{*}C^{J_{carbon}}_{CPTI}$) were high, ranging from 0.87 to 1.00. This supported earlier conclusions, that in the adult rat, the proportion of control exerted by CPT I over the oxidative pathways was high. However, in the same defined system in suckling rats, the numerical values of the calculated group flux control coefficients were lower ($^{*}C^{J_{carbon}}_{CPTI}$ = 0.73 to 0.96), indicating a decreased level of control exerted by CPT I over total carbon flux in this age group. Therefore, at this stage of development control is shifted away from CPT I and, whilst the flux control coefficients are high supporting the idea that CPT I could be considered "rate-controlling" in suckling rats, it is unlikely to be the "rate-limiting" step. Recently we have redefined this top-down system[25,26] used by Krauss *et al* to reanalyse the data using BUCA to calculate individual flux control coefficients for CPT I over ketogenesis specifically. These new analyses support the conclusions drawn from the earlier work: in adult systems control coefficients are close to or above 1 and in the suckling systems below 1.

We have also recently begun to extend this work into a more "physiological" model

Figure 1. Distribution of flux control over ketogenesis is different in hepatocytes isolated from adult and suckling rats (grey segments) proportion of flux control exerted by CPT I (relative to summed flux control exerted by all the other pathway steps) over ketogenesis from palmitate; (white segments) summed flux control exerted over ketogenesis from palmitate by all the other pathway steps. Adult and suckling rat data are taken from Drynan *et al.*[12] and New *et al.*[25,26] respectively. The values for the flux control coefficients suggest that in adults, other steps of the pathways of fatty acid oxidation and ketogenesis (which would include, for example, all the steps of β-oxidation and HMG-CoA cycle) share only approximately 15% of the control over ketogenic flux, however, in the suckling rat, this proportion of control increases to approximately 45%.

system by using hepatocytes isolated from suckling rats. In the first stage of this work we have calculated individual flux control coefficients for CPT I over ketogenesis specifically and compared them with equivalent coefficients calculated by similar analysis in adult rats[12,25,26] Here, as in the mitochondrial systems, we found that CPT I exerts significantly

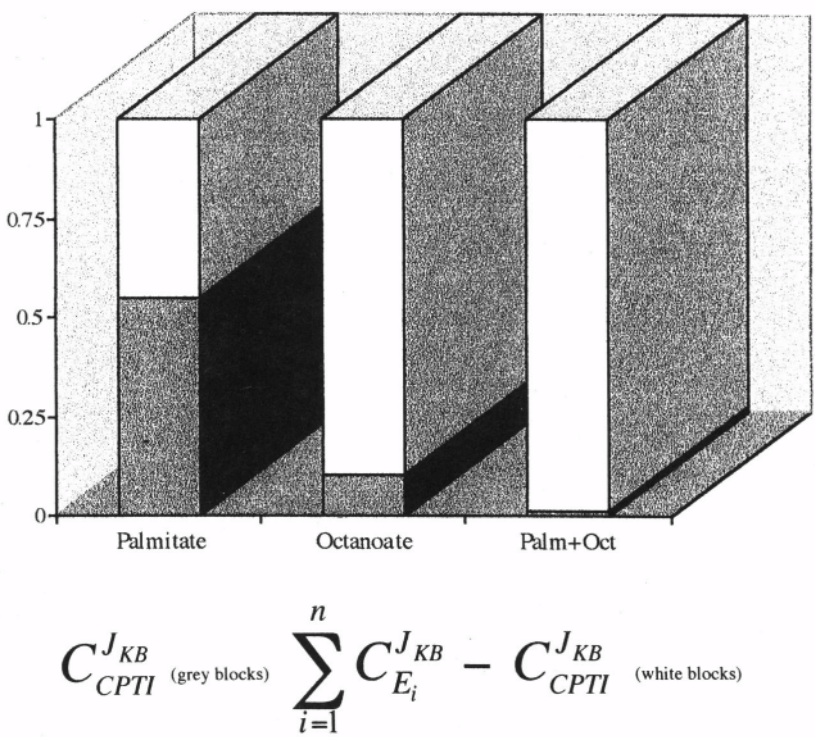

Figure 2. Potential flux control of CPT I over ketogenesis from a range of substrates (grey blocks) flux control coefficient for CPT I over ketogenesis; (white blocks) sum of flux control coefficients of other pathway steps over ketogenesis.

less control over ketogenesis in hepatocytes isolated from suckling rats than those from adult rats (Fig. 1). Values of less than 0.6 are not consistent with the enzyme being the "rate-limiting" step of ketogenesis. Such low values for flux control coefficients refute the traditional concept of a single "rate-limiting" step where control is situated at one site and substantiate theoretical tenets of MCA, which suggest that such cases are rare and that control is multisite and distributed throughout all the steps of the pathway.[27,28]

Results from our preliminary work in the second stage of our more "physiological" system using different fatty acid substrates[29,30] (Fig. 2) support earlier work. Very low values for the flux control coefficients suggest that CPT I cannot be considered *either* "rate-limiting" *or* "rate-controlling" for ketogenesis at this stage of development.

In conclusion, it appears to be over simplistic to assume that control distribution in a metabolic pathway remains the same regardless of development, nutritional supply or hormones or that large levels of control are situated at a single "rate-limiting" step in a pathway. In this specific case, MCA has supported the traditional view that CPT I does have a significant contribution to control over ketogenic flux in adult rats. However, it has also demonstrated that control distribution changes: in suckling systems control over ketogenesis is distributed throughout the whole pathway. We strongly suggest that it might be time to reconsider the role of CPT I as the major control site for ketogenesis in all metabolic states and developmental stages.

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REFERENCES

1. Kacser, H. & Burns, J.A. (1973) *Symp. Soc. Exp. Biol.* **27**, 65–104. The control of flux.
2. Heinrich, R. & Rapoport, T.A. (1974) *Eur. J. Biochem.* **42**, 89–95. A linear steady-state treatment of enzymatic chains: General properties, control and effector strength.
3. Heinrich, R. & Rapoport, T.A. (1974) *Eur J Biochem* **42**, 97–105. A linear steady-state treatment of enzymatic chains: Critique of the crossover theorem and a general procedure to identify interaction sites with an effector.
4. Fell, D.A. (1992) *Biochem. J.* **286**, 313-330. Metabolic control analysis: a survey of its theoretical and experimental development.
5. McGarry, J.D. & Foster, D.W. (1971) *J. Biol. Chem.* **246**, 1149–1159. The regulation of ketogenesis from octanoic acid.
6. McGarry, J.D. & Foster, D.W. (1980) *Annu. Rev. Biochem.* **49**, 395–420. Regulation of hepatic fatty acid oxidation and ketone body production.
7. Ferré, P., Pégorier, J.-P., Williamson, D.H. & Girard, J. (1978) *Biochem. J.* **176**, 759–765. The development of ketogenesis at birth in the rat.
8. Girard, J., Ferré, P., Pégorier, J. & Duée, P. (1992) *Physiol. Rev.* **72**, 507-562. Adaptations of glucose and fatty acid metabolism during perinatal period and suckling-weaning transition.
9. Thumelin, S., Esser, V, Charvy, D., Kolodziej, M., Zammit, V.A., McGarry, D., Girard, J. & Pégorier, J. (1994) *Biochem. J.* **300**, 583-587. Expression of liver carnitine palmitoyltransferase I and II genes during development in the rat.
10. Asins, G., Serra, D., Arias, G. & Hegardt, F.G. (1995) *Biochem. J.* **306**, 379-384. Developmental changes in carnitine palmitoyltransferases I and II gene expression in intestine and liver of suckling rats.
11. Fraser, F., Corstorphine, C.G. & Zammit, V.A. (1997) *Biochem. J.* **323**, 711–718. Topology of carnitine palmitoyltransferase I in the mitochondrial membrane.

12. Drynan, L., Quant, P.A. & Zammit, VA. (1996) *Biochem. J.* **317**, 791–795. Flux control exerted by mitochondrial outer membrane carnitine palmitoyltransferase over **β-oxidation**, ketogenesis and tricarboxylic acid cycle activity in hepatocytes isolated from rats in different metabolic states,
13. Brand, M.D., Vallis, B.P.S. & Kesseler, A. (1994) *Eur. J. Biochem.* **226**, 819–829. The sum of flux control coefficients in the electron transport chain of mitochondria.
14. Spurway, T.D., Sherratt, H.S.A., Pogson, C.I. & Agius, L. (1997) *Biochem. J.* **323**, 119–122. The flux control coefficient of carnitine palmitoyltransferase I on palmitate **β-oxidation** in rat hepatocytes.
15. Decaux, J-F., Robin, D., Robin, P., Ferré, P. & Girard, J. (1988) *FEBS Lett.* **232**, 156–158. Intramitochondrial factors controlling hepatic fatty acid oxidation at weaning in the rat.
16. Thumelin, S., Forestier, M, Girard, J. & Pégorier, J-P. (1992) *Biochem. J.* **292**,493–496. Developmental changes in mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene expression in rat liver, intestine and kidney.
17. Quant, P.A., Tubbs, P.K. & Brand, M.D. (1989) *Biochem. J.* **262**, 159-164. Treatment of rats with glucagon or mannoheptulose increases mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase activity and decreases succinyl-CoA content in liver.
18. Quant, P.A., Tubbs, P.K. & Brand, M.D. (1990) *Eur. J. Biochem.* **187**, 169–174. Glucagon activates mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase *in vivo* by decreasing the extent of succinylation of the enzyme.
19. Quant, P.A., Robin, D., Robin, P., Ferré, P., Brand, M.D. & Girard, J. (1991) *Eur. J. Biochem.* **195**, 449–454. Control of hepatic mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase during the foetal/neonatal transition, suckling and weaning in the rat.
20. Quant, P.A. (1993) *Trends Biochem. Sci.* **18**, 26–30. Experimental application of top-down control analysis to metabolic systems.
21. Brown, G.C., Hafner, R.P. & Brand, M.D. (1990) *Eur. J. Biochem.* **188**, 321-325. A top down approach to the determination of control coefficients in metabolic control theory.
22. Brand, M.D. (1996) *J. Theor. Biol.* **182**, 351–360. Top down metabolic control analysis.
23. Quant, PA., Robin, D., Robin, P., Girard, J. & Brand, M.D. (1993) *Biochim. Biophys. Acta* **1156**, 135–143. A top-down control analysis in isolated rat liver mitochondria: can the 3-hydroxy-3-methylglutaryl-CoA pathway be rate-controlling for ketogenesis?
24. Krauss, S., Lascelles, C.V., Zammit, V.A. & Quant, P.A. (1996) *Biochem. J.* **319,** 427–433. Flux control exerted by overt carnitine palmitoyltransferase over palmitoyl-CoA oxidation and ketogenesis is lower in suckling than in adult rats.
25. New, K.J., Elliott, K.R.F. & Quant, P.A. (1997) *Biochem. Soc. Trans.* **25**, 418S. Quantitative analysis of control exerted by mitochondrial outer membrane carnitine palmitoyltransferase over carbon fluxes from palmitate in hepatocytes isolated from suckling rats.
26. New, K.J., Krauss, S., Elliott, K.R.F. & Quant, P.A. (1999) *Eur. J. Biochem.* **259**, 684–691. Comparisons of flux control exerted by mitochondrial outer membrane carnitine palmitoyltransferase over ketogenesis in hepatocytes and mitochondria isolated from suckling or adult rats.
27. Fell, D.A. & Thomas, S. (1995) *Biochem. J.* **311**, 35–39. Physiological control of metabolic flux—the requirement for multi-site modulation.
28. Fell, D.A. (1999) *Biochemist* (*in press*). Traditional concepts of metabolic control mislead more than enlighten.
29. New, K.J., Elliott, K.R.F. & Quant, P.A. (1998) *Biochem Soc Trans* **26**, S88. Flux control exerted by mitochondrial outer membrane carnitine palmitoyltransferase over ketogenic flux in hepatocytes isolated from suckling rats.
30. New, K.J., Eaton, S., Elliott, K.R.F. & Quant, P.A. (1998) in *BioThermoKinetics in the post genomic era* **8**, 113–116 (eds. Larsson, C, Påhlman, I.-L. & Gustafsson, L.) ISBN 91-630-7048-0 Chalmers Reproservice, Göteborg. CPT I: a reassessment of its role in control over ketogenic flux.

DEVELOPMENTAL COMPARISON OF HUMAN AND RAT HEPATIC MITOCHONDRIAL 3-HYDROXY-3-METHYLGLUTARYL-COA SYNTHASE

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1. INTRODUCTION

The capacity of the liver to produce ketone bodies, acetoacetate and β-hydroxybutyrate, by β-oxidation and subsequent ketogenesis of stored or dietary fatty acids varies greatly.[1] Elevated levels of ketone bodies, which show the largest physiological variation of circulating metabolites, are beneficial under certain conditions when they act as precursors for myelinogenesis or alternate oxidizable chemical fuels for the brain and peripheral tissues thus sparing glucose. However, in certain uncontrolled disease states, pathological ketoacidosis may lead to coma and/or death. Ketone body metabolism is highly integrated with that of other metabolic substrates, e.g. glucose, amino acids etc. in health as well as in disease and during development.[2]

Mitochondrial 3-hydroxy-3-methylglutaryl-CoA (mHMG-CoA) synthase, the second enzyme of the mHMG-CoA pathway of ketogenesis, is regarded as an important locus of regulation and control of hepatic ketone body production in some metabolic states.[3–13] The role of this enzyme in exerting control over ketogenesis has been investigated in animal models, where the absolute amounts and relative activities appear to

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change in parallel with ketogenic capacity influenced by physiological or pathological development, levels of hormones and nutrition.[4–8,14] However, due to inaccessibility of human tissue, little is known about the expression and activity of the human hepatic mitochondrial enzyme.[15]

Our recent research has initiated a profile for human hepatic mHMG-CoA synthase and demonstrated that it may be inactivated by a mechanism characteristic of succinylation observed in other species.[15–17] This allows us to make tentative comparisons of the similarities and differences in enzyme expression and activity in human and rat during development in this paper.

2. GESTATIONAL AND NEONATAL EXPRESSION AND ACTIVITY OF MHMG-COA SYNTHASE

The human fetus has a low capacity for fatty acid oxidation, ketogenesis and gluconeogenesis. Although the major fetal substrate is glucose, supplied by the mother via the umbilical cord, concentrations of ketone bodies in maternal blood increase during the last trimester of pregnancy, with good correlations found between the concentrations in maternal and fetal blood. These elevated levels may serve as an alternate substrate for the fetus during any periods of sustained maternal fasting.

There is, however, an immediate and substantial postnatal requirement for ketone bodies by the human neonate, which initiates the rapid development and onset of fatty acid β-oxidation, ketogenesis and gluconeogenesis, to meet the energy demands during the first few days after birth. We have hypothesized that these processes would be accompanied by the induction/activation of the ketogenic enzymes such as mHMG-CoA synthase.

During the fetal-neonatal transition in rats ketone body production correlates with changes in hepatic mHMG-CoA synthase.[5,18] Protein expression increases from 18d gestation and doubles over the last two days to reach adult levels at term. Ketogenic capacity does not increase, however, due to increasing inhibition of the enzyme (~50%) by succinylation. Rapid activation (desuccinylation) of the enzyme, due to the birth-stress-induced glucagon surge, occurs shortly after birth (Fig. 1.).

Little is known about the control of ketogenesis during human fetal and neonatal development but, as in rat, its onset is also linked to hormonal and metabolic changes accompanying birth. In healthy appropriate-birthweight-for-gestational-age (AGA) infants, transient self-limiting hypoglycaemia occurs and a counter-regulatory response to low glucose is met by increased ketogenesis within the first postnatal day.[19,20] This marked ketogenic response results in high plasma ketone body concentrations being observed 2-3 days after birth. The energy requirement of the human neonate is high because of the presence of the important, relatively large brain mass. In the first few days of extra-uterine life ketone bodies can account for 25% of the neonatal basal energy requirement protecting neurological function and acting as glucose-sparing cerebral fuels.[21] They are reported to be the major source of carbon skeletons for sterol synthesis during brain development having a particular role in sythesis of brain structures during myelinogenesis.[22,23]

Expression of human fetal hepatic mHMG-CoA synthase, which is apparent at 16 weeks gestation, increases from 50% to 100% at term during the period from ~20 to 40 weeks gestation (Fig. 2). Therefore, although the same pattern of induction of hepatic

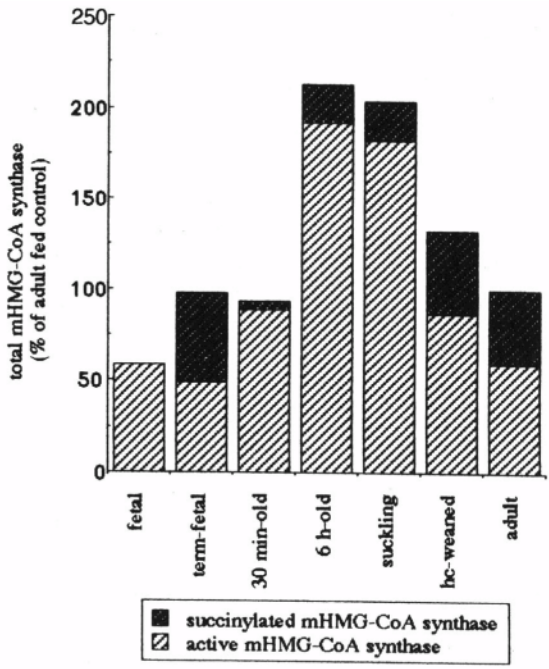

Figure 1. Total, active and succinylated rat hepatic mHMG-CoA synthaseTotal, active and succinylated rat hepatic mHMG-CoA synthase were measured in isolated mitochondria (methods and full data in Quant *et al.*[5,18]).

mHMG-CoA synthase occurs in human as in rat, it does so over a relatively longer period of gestation. The low capacity for ketogenesis in the human fetus over this period is also occasioned by increased enzyme inactivation, similar to and characteristic of succinylated enzyme in rat. Throughout gestation, inhibition of up to 25% of total enzyme lowers enzyme activity despite increased levels of expression.

Activation of the enzyme appears to occur at birth. In live-born fetuses and neonates, less inhibited enzyme is found relative to those still-born, despite immaturity, or in infants and children (Table 1).

Whilst in the rat, enzyme activation at birth and increased neonatal expression contribute to the significant and rapid ketogenic response, it is likely in human that enzyme

Table 1. Inactive human hepatic HMG-CoA synthase.
Homogenates isolated from *postmortem* human frozen livers were assayed spectrophotometrically for inactive HMG-CoA synthase monitoring the disappearance of acetoacetyl-CoA (ΔA_{303nm}).[15] Inactive HMG-CoA synthase is expressed as a percentage of total enzyme: mean value, mean obtained from each of a number of human samples (n as shown) ± S.E.M.; maximum value, maximum value achieved for each human sample ± S.E.M. where n is the number of samples.

HMG-CoA synthase Inactive enzyme (% of total enzyme)	Source of Frozen Liver Samples		
	Stillborn Fetuses (16–20 wk gestation)	Liveborn Neonates (27–36 wk gestation)	Children (6–12 y)
mean value	12.69 ± 2.95	1.56 ± 2.21	19.28 ± 2.85
	n = 4	n = 2	n = 8
maximum value	26.91 ± 6.24	2.56 ± 3.75	28.90 ± 1.18
	n = 4	n = 2	n = 8

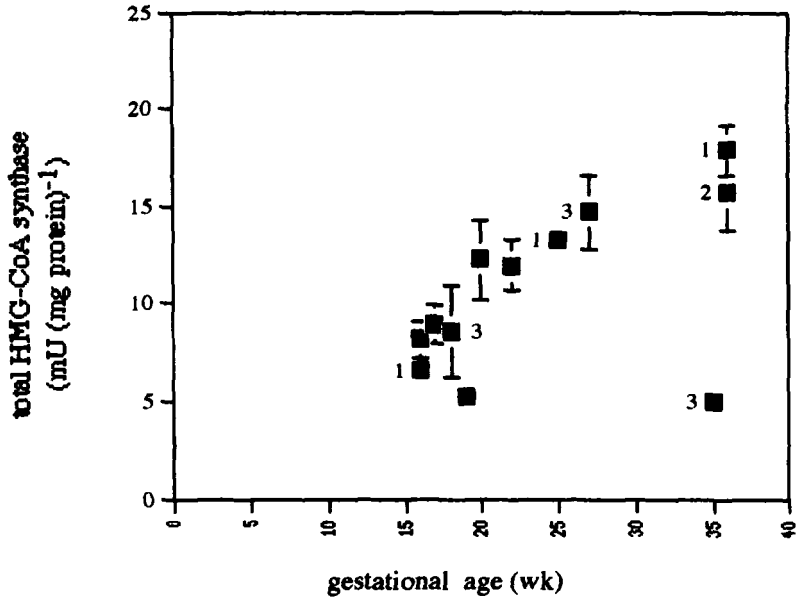

Figure 2. Developmental profile of total human hepatic HMG-CoA synthase Homogenates isolated from *post-mortem* human fresh or frozen liver were assayed spectrophotometrically for total HMG-CoA synthase (inactive + active; mitochondrial + cytoplasmic isoenzymes) by monitoring the disappearance of acetoacetyl-CoA (ΔA_{303nm}).[15] Total activity is expressed as mU (mg total protein)$^{-1}$: mean (4 assays from 1 preparation) or mean (mean of 4 assays from each of a number of preparations from each sample) ± S.E.M.; n = 4 or as indicated next to closed squares. Data taken from Quant *et al.*[16]

expression during gestation and activation after birth allow sufficient capacity to produce the peak plasma ketone body concentrations observed during the second or third neonatal day.

Plasma ketone body concentrations are found to be significantly lower in preterm neonates than those born at fullterm (>36wk gestation).[19] The lack of ketogenesis observed in these preterm or small-for-gestational-age (SGA) infants appears to be related to immaturity, despite appropriate metabolic responses to delivery-stress during the first day and irrespective of plasma glucose, non-esterified fatty acids and hormone concentrations. It has been suggested that it may be accounted for by reduced expression of mHMG-CoA synthase.[24]

3. INFANCY AND CHILDHOOD

In human it might be hypothesized that ketogenesis would play an important role during early infancy and childhood (<6y) when metabolic systems are highly active maintaining energy balance whilst promoting marked growth and maturation.

In rat the equivalent period would be during suckling and early weaning (between 14 and 20 days after birth). Expression of mHMG-CoA synthase increases over the neonatal period from ~1–6 hours and results in a 2–3-fold increase perinatally which, with maintained activation throughout suckling, allows persistent physiological keton-

aemia until weaning (Fig. 1). This is maintained further by weaning on to a high-fat low-carbohydrate diet but falls rapidly with weaning on to the normal high-carbohydrate diet.[5,18]

Little evidence has been found in human, due to lack of availability of samples, to indicate altered mHMG-CoA synthase expression and activity with the onset and duration of suckling or during the suckling-weaning transition similar to that seen in rat.

However, during this transition there are significant nutritional and metabolic differences between human and rat. In rat plasma fatty acid substrates for ketogenesis come mainly from hydrolysis of maternal milk triacylglycerols, due to lack of white adipose tissue at birth. In human neonates there is significant accumulated triacylglycerol in the liver, rapidly mobilized for utilization *in situ* after birth. Significant amounts of fat are stored in the human fetus in white adipose tissue comprising 16–20% body mass, mainly as triacylglycerides containing high proportions of palmic (C16) and oleic (C18: 1) acids. Plasma free fatty acids start to rise soon after birth from lipolysis of this store. Once lactation is established, fatty acids from intestinal hydrolysis of milk triacylglycerols are directly absorbed and transported to the liver as free fatty acids. Human milk differs from rat milk as it comprises more carbohydrate as lactose, less fat, of which a higher proportion is unsaturated long-chain fatty acids and a lower proportion is of medium-chain fatty acids.[6] For the remainder of this period in human, there is the introduction and establishment of a mixed diet predominated by carbohydrates where fats might only assume a primary metabolic role during prolonged periods of starvation and/or illness.

During early infancy and childhood human hepatic mHMG-CoA synthase expression appears to remain at levels similar to those at term, suggesting that the potential capacity for its use is maintained despite a relatively slower maturation rate over a period when there might be more flexible nutritional conditions than in the rat. Ketogenesis in human infants is extremely important particularly during the initial neonatal period and for meeting energy shortfalls during periods of metabolic stress later during infancy and childhood.

In rat there is an almost total reliance, for the majority of the period of maturation and growth, on energy and nutritional needs being met by the maternal milk supply. Ketogenesis in rat during this equivalent stage might be significantly more important as a continuous and ongoing metabolic process due to nutritional rigidity.

4. LATER CHILDHOOD AND ADULTHOOD

By adulthood, in both human and rat, enzyme expression is similar to that observed at term.

In human, despite maintained enzyme expression, increased inactivation occurs during later childhood and adulthood relative to that seen in the neonatal period and early infancy, resulting in activity (at >6 years) being 30+% lower than at term during a life stage when generally there might be more stable metabolic and nutritional states (Table 2).

In the adult rat following weaning, expressed enzyme returns to a level similar to that seen at term. Whilst there is inhibition of the enzyme, it is not to the degree found prior to birth at term and as a result active enzyme remains slightly higher. Induction of the enzyme can be demonstrated following prolonged starvation, during high-fat-feeding and in experimentally-induced alloxan-diabetes.[5,18,24]

Although ketone body production has been shown to increase under equivalent

Table 2. Human hepatic active HMG-CoA synthase.
Homogenates isolated from *postmortem* human frozen livers were assayed spectrophotometrically for active HMG-CoA synthase monitoring the disappearance of acetoacetyl-CoA (ΔA_{303nm}).[15] Active HMG-CoA synthase is expressed as mU (mg total protein)$^{-1}$: mean ± S.E.M. (n = as indicated).

	Source of Frozen Liver Samples		
HMG-CoA synthase	Liveborn Neonates (27–36 wk gestation)	Stillborn Term-Fetuses, Infants & Children <6 y	Children (6–12 y)
Active enzyme (mU (mg total protein)$^{-1}$)	15.60 ± 0.62 n = 2	13.59 ± 1.23 n = 7	10.64 ± 0.92 n = 8

metabolic conditions in human, lack of samples for this research did not allow us to confirm whether enzyme induction would occur in a similar method. Despite the multiplicity of sources of human liver samples used and metabolic conditions which might have prevailed when obtained, there was little variation seen in enzyme expression over later childhood and early adulthood.

This pattern of enzyme expression and activation may suggest that activation and inactivation of the enzyme, rather than altered expression during changing metabolic conditions seen in the rat may be more important as a mechanism for regulating and controlling ketogenesis subsequent to birth in human.

REFERENCES

1. Zammit, V.A. (1981) *Trends Biochem. Sci.* **6**, 46–49. Intrahepatic regulation of ketogenesis.
2. Zammit, V.A. (1994) *Diabet. Rev.* **2**, 135–155. Regulation of ketone body metabolism. A cellular perspective.
3. Dashti, N. & Ontko, J.A. (1979) *Biochem. Med.* **22**, 365–374. Rate-limiting function of 3-hydroxy-3-methylglutaryl-CoA synthase in ketogenesis.
4. Quant, P.A., Tubbs, P.K., & Brand, M.D. (1990) *Eur. J. Biochem.* **187**, 169–174. Glucagon activates mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase activity *in vivo* by decreasing the extent of succinylation of the enzyme.
5. Quant, P.A., Robin, D., Roin, P., Ferré, P., Brand, M.D. & Girard, J. (1991) *Eur. J. Biochem.* **195**, 449–454. Control of hepatic mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase during the foetal-neonatal transition, suckling and weaning in the rat.
6. Girard, J., Ferré, P., Pégorier, J.-P. & Duée, P.-H. (1992) *Physiol. Rev.* **72**, 507–562. Adaptions of glucose and fatty acid metabolism during the perinatal period and suckling weaning transition.
7. Quant, P.A., Robin, D., Robin, P., Girard, J. & Brand, M.D. (1993) *Biochim. Biophys. Acta* **1156**, 135–143. A top-down control analysis in isolated rat liver mitochondria: can the 3-hydroxy-3-methylglutaryl-CoA pathway be rate-controlling for ketogenesis?
8. Serra, D., Casals, N., Asins, G., Royo, T., Cuidada, C.J. & Hegardt, F.G. (1993) *Arch. Biochem. Biophys* **307**, 40–45. Regulation of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase protein by starvation, fat feeding and diabetes.
9. Asins, G., Rosa, J.L., Serra, D., Gil-Gomez, G., Ayte, J., Bartrons, R., Tauler, A. & Hegardt, F.G. (1994) *Biochem. J.* **299**, 65–69. Gene expression of enzymes regulating ketogenesis and fatty acid metabolism in regenerating rat liver.
10. Quant, P.A. & Makins, R.A. (1994) *Biochem. Soc. Trans.* **22**, 441–446. Metabolic control analysis of hepatic β-oxidation: the top-down approach.
11. Valera, A., Pelegrin, M., Asins, G, Filiat, C., Sabater, J., Pujolt, A. & Hegardt, F.G. (1994) *J. Biol. Chem.* **269**, 6267–6270. Overexpression of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase in transgenic mice causes hepatic hyperketogenesis.

12. Zammit, V.A. (1994) *Diabet. Rev.* **2**, 135–155. Regulation of ketone body metabolism A cellular perspective.
13. Zammit, V.A. (1996) *Biochem. J.* **314**, 1–14. Role of insulin in hepatic fatty acid partitioning: emerging concepts.
14. Arias, G., Asins, G., Hegardt, F.G., Serra, D. (1997) *Arch. Biochem. Biophys.* **340**, 287–298. The effect of fasting/refeeding and insulin treatment on the expression of the regulatory genes of ketogenesis in intestine and liver of suckling rats.
15. Lascelles, C.V. & Quant, P.A. (1997) *Clin. Chim. Acta* **260**, 85–96. Investigation of human hepatic mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase in postmortem or biopsy tissue.
16. Quant, P.A., Lascelles, C.V., New, K.J., Patil, K.K., Azzouzi, N., Eaton, S. & Elliott, K.R.F. (1998) *Biochem. Soc. Trans.* **26**, 125–130. Impaired neonatal ketogenesis.
17. Lascelles, C.V. & Quant, P.A. (1998) *Biochem. Soc. Trans.* **26,** S89. Gestational and neonatal expression and activity of human hepatic mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase.
18. Quant, P.A. (1994) in *Essays in Biochemistry* **28,** 13–25 (Tipton, K. ed.) Portland Press, London. The role of mitochondrial HMG-CoA synthase in regulation of ketogenesis.
19. Hawdon, J.M., Ward Platt, M.P., Aynsley-Green, A. (1992) *Arch. Dis. Child.* **67**, 357–365. Patterns of metabolic adaption for preterm and term infants in the first neonatal week.
20. Hawdon, J.M., Ward Platt, M.P., Aynsley-Green, A. (1994) *Arch. Dis. Child.* **70**, 59–64. Prevention and management of neonatal hypoglycaemia.
21. Hamosh, M. (1991) in *Neonatal nutrition and metabolism* (Hay, W.W. ed.) Mosby Year Book Inc., St. Louis, U.S.A. Lipid metabolism.
22. Robinson, A.M. & Williamson, D.H. (1980) *Physiol. Rev.* **60**, 143–187. Physiological roles of ketone bodies as substrates and signals in mammalian tissue.
23. Nehlig, A., & Pereira de Vasconcelos, A. (1992) *Prog. Neurobiol.* **40**, 163–221. Glucose and ketone body utilization by the brain of neonatal rats.
24. Quant, P.A., Henson, J.N., Williams, A., Jeffrey, I., Taylor, P. & Carter, N.D. (1993) *J. Physiol.* **467**, 367P.

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REGULATION OF THE KETOGENIC ENZYME MITOCHONDRIAL 3-HYDROXY-3-METHYLGLUTARYL-COA SYNTHASE IN ASTROCYTES AND MENINGEAL FIBROBLASTS

Implications in Normal Brain Development and Seizure Neuropathologies

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1. PHYSIOLOGICAL BASIS OF KETOGENESIS

1.1. Carbon-Carbon Bond Energy as a Fuel Source

Metabolic fuels may be defined as complex carbon molecules that can be exploited for the energy released upon breakage of their carbon-carbon bonds. Fatty acids and hexoses, principally glucose, represent the two major classes of such complex carbon molecules that are used for fuel by eukaryotes. Typically, glucose is first metabolized anaerobically (glycolysis) to three-carbon molecules (pyruvate, phosphoenolpyruvate) which then provide the precursors of the tricarboxylic acid or Krebs cycle, ie: oxaloacetate and acetyl-CoA. The cycling of such intermediates via the Krebs cycle then effects the con-

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tinuous release of CO_2, resulting in the full exploitation of glucose carbon-carbon bond energy. Fatty acids are similarly exploited via the Krebs cycle, after having been catabolised to acetyl-CoA by extensive rounds of β-oxidation. However, eukaryotic cells cannot directly convert such acetyl-CoA to oxaloacetate and, thus, under conditions of high rates of fatty acid catabolism oxaloacetate becomes limiting. Fatty acid-catabolising prokaryotic cells circumvent this via the glyoxylate shunt, a short cut in the Krebs cycle, that converts isocitrate directly to malate and, hence, oxaloacetate. Fatty acid-catabolising eukaryotic cells, however, do not possess a glyoxylate shunt and, thus, under high fatty acid/low glucose conditions they generate high levels of acetyl-CoA that cannot be further metabolized by the Krebs cycle. To obviate this problem, acetyl-CoA molecules are fused together to generate the four-carbon "ketone bodies" acetoacetate and β-hydroxbutyrate which then diffuse out of the cell as "lost carbon-carbon bond fuel" (Fig. 1a).

1.2. Ketogenesis Versus Ketolysis

The process of converting fatty acids to ketone bodies is called ketogenesis whilst the process of exploiting such ketone bodies is variously described as ketolysis or ketone body utilisation.[1] The situation described in Fig. 1a is presumably not tolerable in a single cell procaryote whose sole fuel source is fatty acids. However, in a hypothetical "symbiotic" 2-cell-type eukaryotic system (Fig. 1b), carbon skeletons could still be conserved provided that, a "ketogenic" cell-type produces ketone bodies because its sole energy source is fatty acids and the ketolytic cell-type consumes such ketone bodies via an obligate consumption of glucose (to generate the requisite oxaloacetate). In practice, this is what occurs in multi-organ eukaryotes (Fig. 1c). Thus, organs such as liver, that can use fatty acids as a virtual sole carbon source (hence producing large concentrations of ketone bodies), can do so without loss of carbon skeletons to the environment (via urine/breath etc.) provided that other organs such as brain, muscle etc. have sufficient

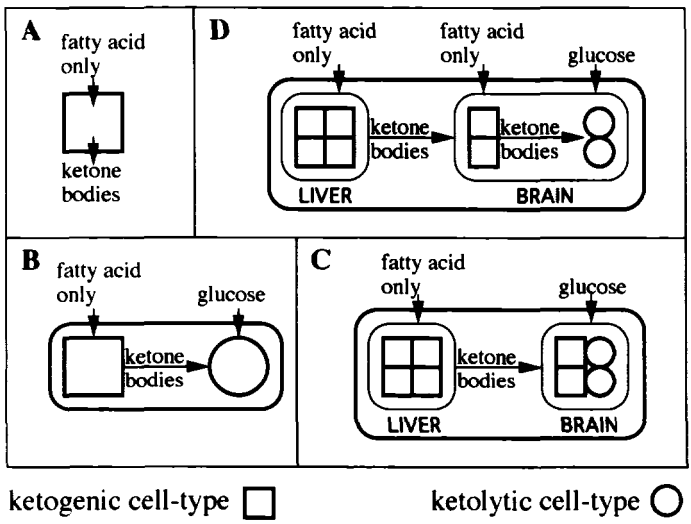

Figure 1. Ketogenic/ketolytic systems: from single cell to organism.

glucose (and hence oxaloacetate) supply to metabolise fully such ketone bodies via conversion back to acetyl-CoA and entry into an active Krebs cycle.

1.3. Ketogenic/Ketolytic Systems: Inter-Organ and Inter-Cellular Relationships

The processes of ketogenesis and ketolysis have been defined at the organ level[1] ie: ketogenic organs (eg. liver), supplying ketone bodies to ketolytic organs (eg. brain, muscle etc). However, following on from Fig. 1b, there is no theoretical reason why different cell-types within the same tissue should not exhibit a mutual "symbiotic" ketogenic/ketolytic relationship (Fig. 1d), nested within classical inter-organ ketogenic/ketolytic relationships, provided that the "ketogenic" cell-type in such an organ can access and catabolise fatty acids. It is becoming increasingly clear that this may be the case in organs such as kidney and brain but to understand this we must digress into the details of the key steps of ketogenesis from fatty acids, ie the conversion of fatty acid-derived acetyl-CoA to the ketone body acetoacetate.

2. THE HMG-CoA CYCLE OF KETOGENESIS

2.1. Mitochondrial 3-Hydroxy-3-Methylglutaryl-CoA Synthase as an Indicator of Ketogenic Potential

Ketogenesis is accomplished by the three mitochondrially-located enzymes of the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) cycle (Fig. 2). The enzymes are as follows[2]: mitochondrial acetoacetyl-CoA thiolase (T2), mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (mHS) and 3-hydroxy-3-methylglutaryl-CoA lyase (HL). Of the three enzymes, only mHS functions solely in the conversion of acetyl-CoA to acetoacetate, since both T2 and HL have alternate essential functions in the cell related to ketolysis and amino-acid turnover.[2] As such, the abundances of mHS mRNA and/or protein are closely related to cellular ketogenic potential.[1] The suckling liver is the archetypal ketogenic organ and this is duly reflected in the detection of high levels of mHS mRNA and protein.[3,4] Furthermore, we have quantified, by the highly sensitive RNase protection assay, the absolute abundance of mHS mRNA in 11 d-old suckling liver at

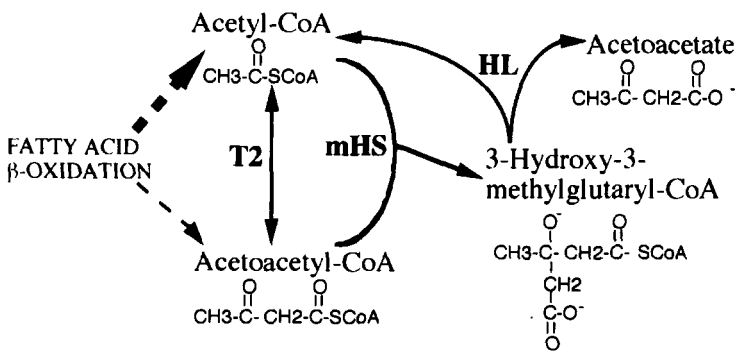

Figure 2. Enzymes of the ketogenic HMG-CoA cycle.

4,300 ± 950 molecules of mRNA/ng total RNA.[5] However, other organs such as suckling kidney have been shown to exhibit ketogenesis[3] and this is also reflected in detection of significant abundances of mHS mRNA[3,4] that we have quantified at 520 ± 120 molecules of mRNA/ng total RNA[5] (ie: 8% of that found in suckling liver). In the case of suckling brain, we have demonstrated levels of mHS mRNA between 40–80 molecules of mRNA/ng total RNA[5] (ie: 1–2% of that found in suckling liver), suggesting a very limited ketogenic capacity for the brain as a whole.

2.2. Mitochondrial HS mRNA is Enriched in Certain Brain Cell-Types

Although whole brain may have a nominal ketogenic capacity, we reasoned that different brain cell-types might exhibit greatly varying abundances of mHS mRNA and, thus, we prepared highly pure primary cultures of a variety of neural cell-types.[5,6] Resulting from these studies we have identified primary cultures of neonatal cortical astrocytes and meningeal fibroblasts containing 264 ± 51 and 337 ± 65 molecules of mHS mRNA/ng total RNA respectively when cultured in serum-free media.[6] Such data imply that cortical astrocytes/meningeal fibroblasts *in vivo* may exhibit abundances of mHS mRNA of at least 8% of that found in hepatocytes of suckling liver. In this article we will discuss how these abundances may further increase under the influence of physiological concentrations of certain hormones. In combination with previous studies demonstrating astrocytic ketogenesis from fatty acids[7] and leucine,[8] such data indicate that cortical astrocytes and, perhaps meningeal fibroblasts, may be major sources of ketone body fuels within brain. Thus, according to the model we suggested in Figs. 1b and 1d for the "brain system", astrocytes (or meningeal fibroblasts) could constitute the "ketogenic" cell-type, providing ketone bodies on behalf of other glucose-dependent "ketolytic" cell-types, such as neurons.

3. FATTY ACIDS AS A POTENTIAL PHYSIOLOGICAL BRAIN FUEL

3.1. Fatty Acid Supply and Accessability

We mentioned that for a given cell-type, eg. astrocyte, to execute ketogenesis it would need access to a supply of fatty acids and a capacity to metabolise those fatty acids. To answer the first point, we can say that such a supply is available from the plasma in the form of free fatty acids but is not equally available to all cells in the body. This is in part due to differences in extraction capacity by different organs and also because an organ such as brain has a directional arrangement of cell-types with respect to the blood supply. Thus, for example, whilst astrocytes and meningeal fibroblasts (or, for that matter hepatocytes; the classical ketogenic cell-type) are in direct contact with blood capillaries containing plasma metabolites, neurons, for example, are only indirectly in contact via their contact with astrocytes. Neurons would, therefore, not appear to be obvious "ketogenic cell-type" candidates unless astrocytes were to convey significant concentrations of un-metabolised fatty acids directly to neurons. The brain has a well recognised barrier to certain blood-borne metabolites, chiefly water-soluble, formed by the interface between capillary endothelial cells and astrocytes.[9] However, evidence indicates that saturated, mono-unsaturated and poly-unsaturated fatty acids exhibit facile transfer across the blood brain barrier[10] and are thus able to access brain cell-types such as astrocytes.

3.2. Fatty Acid Catabolism

To address the second point regarding the capacity of the brain to metabolise such fatty acids to ketone bodies, we must assess both the evidence for the existence of fatty acid catabolising enzymes and the capacity for those enzymes to be appropriately induced. In the case of the former, there is evidence[11] to suggest that the brain is not distinct from other organs of the body in lacking any of the basic enzymes of fatty acid catabolism responsible for generating fatty acid-derived acetyl-CoA. Obviously, the capacity of certain brain cell-types to generate ketone bodies subsequently from such acetyl-CoA moieties is a matter of some debate and the subject of this review. The latter point, with regard to the inducibilty of such of fatty acid catabolising enzymes is, however, of prime importance since it has recently become clear that fatty acid-activated transcription factors can co-ordinate induction of expression of many of the enzymes of fatty acid catabolism, including mHS. Such coordinate induction of fatty acid catabolizing enzymes in liver results in profound amplifications in flux of fatty acids to ketone bodies. The prime transcription factor responsible for mediating fatty acid/retinoid-induced upregulation of expression of genes encoding enzymes of fatty acid catabolism is the peroxisome proliferator-activated receptor (PPAR):retinoid X receptor (RXR) heterodimer (Fig. 3) and we must now briefly digress into some molecular biology to understand its mode of action.

4. PPAR:RXR HETERODIMERS AND LIPID HOMEOSTASIS

4.1. PPAR:RXR Heterodimers and PPREs

PPAR:RXR heterodimers regulate target gene transcription by binding to cognate DNA elements (Fig. 3), termed peroxisome proliferator response elements (PPREs), in the promoter regions of PPAR:RXR target genes.[12] To complicate matters somewhat, three forms of each receptor exist, thus generating nine possible combinations of heterodimer (Fig.3). In the case of the mHS gene, most if not all combinations can bind the mHS PPRE (Fig. 3)[13] and the PPARα:RXRα combination has been shown to mediate fatty acid/retinoid induction of mHS gene expression.[14] The significance of this is that different heterodimer combinations may have different effects on gene transcription, for example transcriptional upregulation of the gene encoding acyl-CoA oxidase by PPARα:RXRα heterodimers may be confounded by the presence of PPAR β:RXRα het-

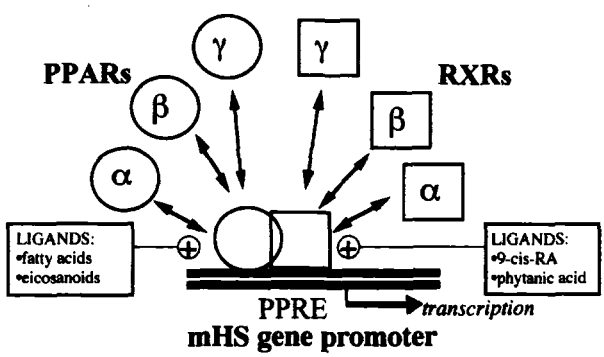

Figure 3. Mitochondrial HS PPRE is promiscuous for different PPAR/RXR combinations.

erodimers.[15] Thus, for a particular cell-type, a potentially complex level of transcriptional control may be exerted on a PPRE-containing gene such as mHS according to the levels of different PPAR:RXR heterodimer combinations that compete for binding to the PPRE.

4.2. PPAR:RXR Heterodimers in the Brain

Using the RNase protection assay, we have shown that the mRNAs encoding PPARα, RXRα, PPARβ, and RXRβ are readily detectable in primary cultures of both cortical astrocytes and meningeal fibroblasts.[16] Such data predict the presence of up to four PPAR:RXR heterodimers in such cells, namely PPARα:RXRα, PPARα:RXRβ, PPARβ:RXRα, and PPARβ:RXRβ. The predicted presence of PPARα:RXRα and PPARα:RXRβ favours the notion that fatty acid-induced activation of mHS gene expression by incoming fatty acids occurs (in an analogous fashion to that observed in liver hepatocytes) in cortical astrocytes and meningeal fibroblasts, given that such activation may, in part, be repressed by the effects of PPARβ:RXRα. The action of PPARβ:RXRβ on gene expression is, as yet, undefined. In summary, there is evidence to suggest that both cortical astrocytes and meningeal fibroblasts may have the ability *in vivo* to access and rapidly utilise (via PPAR:RXR-mediated activation of enzymes of β-oxidation and ketogenesis) fatty acid supplies derived from the circulatory system. Metabolic hormones such as insulin and glucocorticoids are involved in the regulation of PPAR, RXR and mHS gene expression and, with this in mind, we now turn to the potential involvement of such hormones in long-term upregulation of neural ketogenic systems.

5. HORMONAL REGULATION OF NEURAL MHS GENE EXPRESSION

5.1. Glucocorticoids Selectively Increase mHS mRNA Abundances

In vitro experiments have shown that regulation of hepatic mHS gene transcription initiation by the hormones insulin, hydrocortisone and glucagon is mediated at the level of cis-acting elements in the mHS gene promoter.[17] Furthermore, the absolute amounts of mHS mRNA in liver are influenced by levels of circulating hormones such as glucocorticoids, insulin and glucagon.[18] Based on this, we investigated the effects of addition of insulin, hydrocortisone and dibutyryl cAMP (as a model for glucagon action) on mHS mRNA abundances in primary cultures of neonatal cortical astrocytes and meningeal fibroblasts maintained in a serum-free/hormone-free medium. Whilst physiological concentrations of insulin or dibutyryl cAMP did not exert significant effects,[6] 1 μM of the glucocorticoid hydrocortisone resulted in 4-fold increases over control in mHS mRNA abundance, with values of 1,095 ± 138 and 1,177 ± 46 molecules of mRNA/ng total RNA for neonatal cortical astrocytes and meningeal fibroblasts, respectively.[6] Similar increases were not observed for either T2 or HL mRNAs, thus implying a selective effect by hydrocortisone on the levels of the mHS mRNA that encodes the key ketogenic enzyme of the HMG-CoA cycle. Such data imply that, under the influence of glucocorticoids, cortical astrocytes and meningeal fibroblasts *in vivo* may exhibit abundances of mHS mRNA up to 25% of that found in hepatocytes of suckling liver. How might glucocorticoids exert their effects on mHS mRNA abundances in astrocytes and meningeal fibroblasts?

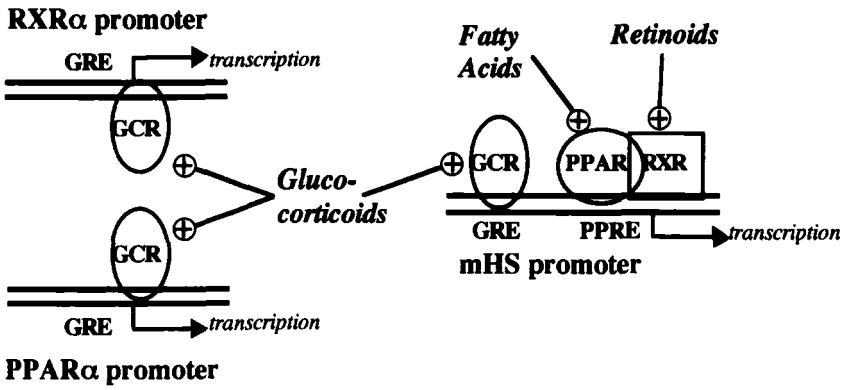

Figure 4. Direct and indirect actions of glucocorticoids on mHS gene expression.

5.2. Glucocorticoid Action on mHS Gene Transcription Initiation

The increases in mHS mRNA observed in response to glucocorticoids are likely to occur as a result of their direct action on the rate of transcription initiation of the mHS gene, since glucocorticoid response elements (GREs) that bind the glucocorticoid receptor (GR) have been identified in the mHS gene promoter (Fig. 4).[17] In addition, glucocorticoids may act indirectly on mHS gene expression via the PPARα:RXRα heterodimer, since the promoter regions of the genes encoding PPARα and RXRα also contain GREs (Fig. 4).[19,20] Such direct and indirect co-operative effects by glucocorticoids and fatty acids on mHS gene expression may thus permit the development of a potent and sustained increase in ketogenic flux from fatty acids.

6. POSSIBLE FUNCTIONS OF BRAIN-LOCALISED KETOGENESIS

6.1. Glucocorticoids and Fat Mobilisation: a Response to Stress

We can more easily understand the logic of the synergistic interactions of the GR and PPAR:RXR signal pathways on mHS gene expression, when we realise that one of the functions of glucocorticoid release into the blood is in the response of starvation stress.[21] Starvation partly parallels the situations of the suckling neonate or the adult on a high fat/low carbohydrate diet in that all three situations result in elevated plasma free fatty acid concentrations.[22,23] In the case of starvation, plasma free fatty acid concentrations increase as a result of fat mobilization from adipose tissue, in an attempt by the body to compensate for low blood glucose fuel availability. Such fatty acids are predictably converted to ketone bodies, via the hepatic HMG-CoA cycle, resulting in a significant hyperketonaemia that can provide up to 70% of the fuel requirements of the brain during starvation.[22,23] Glucocorticoids released from the adrenal cortex act to effect such increases in plasma free fatty acids, making sense of their synergistic action with fatty acid "hormones" on hepatic mHS gene expression.

6.2. Glucocorticoid/Fatty Acid-Induced Ketogenesis in Brain

If a similar response exists in cortical astrocytes and meningeal fibroblasts, such cell-types might also alter their ketogenic rate in response to synchronised changes in plasma concentrations of fatty acid fuels and glucocorticoids. For example, this might be important during the mid-suckling period of the neonate, where milk-derived plasma free fatty acids and glucocorticoid levels are steadily rising.[22] Brain cell-types such as astrocytes could then provide increasing supplies of ketone body fuels to surrounding energy-demanding cell-types within the developing brain, eg. post-natally developing neurons undergoing synaptogenesis. In addition, such ketone bodies could provide vital precursors for myelin production by oligodendrocytes, during the post-natal period of intense myelination.[23] Differences in ketogenic fatty acid availability in formula milks and breast milk might thus have important consequences for the developing brain.

6.3. Ketone Body Concentrations within Brain

Since the liver can achieve a plasma ketone body concentration of between 1–2mM[24] when exposed to a high fat diet, what might be the advantage of an organ such as brain in possessing an endogenous ketogenic capacity? Firstly, it might provide a compensatory mechanism when, for reasons of reduced hepatic ketogenesis, plasma ketone body concentrations fall to lower limits (eg. 0.5 mM). This would hold true if there was an absolute requirement for a given concentration of ketone body within brain eg. 1 mM. However, there is some evidence to suggest that the brain as a whole uses ketone bodies facultatively, rather than having an obligate requirement for a minimum plasma concentration.[25] Secondly, however, possessing an endogenous ketogenic capacity might expose ketolytic cell-types in the brain to ketone body concentrations that are simply unattainable in the plasma as a result of hepatic ketogenesis. This is potentially very important in the brain, since ketone body concentrations in the range of 2–5 mM cause significant perturbations in concentrations of key amino acid neurotransmitters.[26,27] Such changes may have important consequences for the normal functioning of nerve cell discharge threshholds and, as a consequence of this, in the aetiology of epileptic seizure disorders.

6.4. Ketone Bodies and Seizure Disorders

Epileptic seizures affect approximately 1% of children and constitute one of the most common paediatric neurological disorders. An increasingly popular epilepsy treatment is the so-called ketogenic diet (KD),[28,29] a high fat diet with associated high plasma concentrations of free fatty acids, ketone bodies and glucocorticoids,[24] which can result in prolonged seizure remission in up to 30% of intractable infant epilepsies.[28,29] Although the biochemical basis behind many seizure-types remains to be defined, certain anti-epileptic drugs (AEDs) (eg. vigabatrin) appear to enhance the activity of the seizure-suppressing inhibitory neurotransmitter γ-aminobutyric acid (GABA), whilst others (eg. lamotrigine, felbamate) appear to inhibit the activity of seizure-inducing excitatory amino-acids (EAAs) such as aspartate and glutamate.[30] Similarly, the mode of action of the KD appears to involve alterations in amino-acid neurotransmitter concentrations, since, as previously mentioned, addition of ketone bodies to synaptosomes causes perturbations in the concentrations of GABA and EAAs.[26] GABA/EAAs are by-products of the Krebs cycle and thus excess ketone bodies are postulated to alter their concentrations via perturbations in the concentrations of Krebs cycle intermediates eg. acetyl-

CoA.[26,27] Compared to liver, the archetypal ketogenic organ, cortical astrocytes and meningeal fibroblasts thus represent a site of action for the KD much closer to the locality of seizure-susceptible neurons.

7. CONCLUSIONS

The identification of the components of the HMG-CoA cycle in certain brain cell-types opens up a hitherto unrealised area neural intermediary metabolism namely ketogenesis. Studying changes in neural ketogenesis may have important consequences for understanding processes of brain development such as myelination and the metabolism of certain neurotransmitters whose concentrations are closely linked to the rate of operation of the KREBS. Furthermore, establishing the hormonal, nutritional, and pharmacological conditions under which cells directly adjacent to neurons *in vivo*, ie: astrocytes/meningeal fibroblasts, produce the concentrations of ketone body required to effect perturbations in neuronal synapse GABA/EAA concentrations may assist in:

a. the development of novel AEDs that perturb neuronal neurotransmitter concentrations via alterations in glial fatty acid metabolism. An example of this might be the identification of novel glucocorticoids, since they have been shown to be seizure-suppressing both clinically[31] and in hippocampal slice cultures[32] and
b. the understanding and improvement of the KD since, despite its clinical efficacy, there are few animal/cell culture models for the KD.[33] A drawback of the KD with certain patients is intolerance of the high fat/low carbohydrate diet and the resultant systemic hyperlipedaemia. Identifying the components of the KD that act as ketogenic precursors for astrocytes/meningeal fibroblasts (ie: fatty acids) and that are preferentially absorbed by the brain, would assist in the development of a KD with reduced side-effects. An example of this might be the addition to the KD of polyunsaturated fatty acids such as docosahexaenoic acid which are preferentially and avidly extracted from the circulation by the brain.[34]

REFERENCES

1. Quant, P.A. (1994) in *Essays in Biochemistry* **28**, 13–24: (Tipton, K.F., ed.). Portland Press The role of mitochondrial HMG-CoA synthase in regulation of ketogenesis.
2. Mitchell, G.A., Kassovska-Bratinova, S., Boukaftane, Y., Robert, M.F., Wang, S.P., Ashmarina, L., Lambert, M., Lapierre, P. & Potier, E. (1995) *Clin. Invest. Med.* **18**, 193–216. Medical aspects of ketone body metabolism.
3. Thumelin, S., Forestier, M., Girard, J. & Pegorier, J.P. (1993) *Biochem. J.* **292**, 493–496. Developmental-changes in mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene-expression in rat-liver, intestine and kidney.
4. Serra, D., Asins, G. & Hegardt, F.G. (1993) *Arch. of Biochem. Biophys.* **301**, 445–448. Ketogenic mitochondrial 3 hydroxy 3-methylglutaryl-CoA synthase gene expression in intestine and liver of suckling rats.
5. Cullingford, T.E., Dolphin, C.T., Bhakoo, K.K., Peuchen, S., Canevari, L. & Clark, J.B. (1998) *Biochem. J.* **329**, 373–381. Molecular cloning of rat mitochondrial 3-hydroxy-3-methylglutaryl CoA lyase and detection of the corresponding mRNA and of those encoding the remaining enzymes comprising the ketogenic HMG-CoA cycle in CNS of suckling rat.

6. Cullingford, T.E., Bhakoo, K.K. & Clark, J.B. (1998) *J. Neurochem.* **71**, 1804–1812. Hormonal regulation of the mRNA encoding the ketogenic enzyme mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase in neonatal primary cultures of cortical astrocytes and meningeal fibroblasts.
7. Auestad, N., Korsak, R.A., Morrow, J.W. & Edmond, J. (1991) *J. Neurochem.* **56**, 1376–1386. Fatty acid oxidation and ketogenesis by astrocytes in primary culture.
8. Bixel, M.G. & Hamprecht, B. (1995) *J. Neurochem.* **65**, 2450–2461. Generation of ketone bodies from leucine by cultured astroglial cells.
9. Pardridge, W.M. (1998) *J. Neurochem.* **70**, 1781–1792. CNS drug design based on principles of blood-brain barrier transport.
10. Anderson, G.J., Tso, P.S. & Connor, W.E. (1994) *J. Clin. Invest.* **93**, 2764–2767. Incorporation of chylomicron fatty acids into the developing rat brain.
11. Chang, M.C., Grange, E., Rabin, O. & Bell, J.M. (1997) *J. Lipid Res.* **38**, 295–300. Incorporation of [U-^{14}C]palmitate into rat brain: effect of an inhibitor of beta-oxidation.
12. Green, S. (1995) *Mutat. Res.* **333**, 101–109. PPAR: a mediator of peroxisome proliferator action.
13. Juge-Aubry, C., Pernin, A., Favez, T., Burger, A.G., Wahli, W., Meier, C.A. & Desvergne, B. (1997) *J. Biol. Chem.* **272**, 25252–25259. DNA binding properties of peroxisome proliferator-activated receptor subtypes on various natural peroxisome proliferator response elements. Importance of the 5′-flanking region.
14. Rodriguez, J.C., Gil-Gomez, G., Hegardt, F.G. & Haro, D. (1994) *J. Biol. Chem.* **269**, 18767–18772. Peroxisome proliferator-activated receptor mediates induction of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene by fatty acids.
15. Jow, L. & Mukherjee, R. (1995) *J. Biol. Chem.* **270**, 3836–3840. The human peroxisome proliferator-activated receptor PPAR subtype NUC1 represses the activation of hPPAR alpha and thyroid hormone receptors.
16. Cullingford, T.E., Bhakoo, K.K., Peuchen, S., Dolphin, C.T., Patel, R. & Clark, J.B. (1998) *J. Neurochem.* **70**, 1366–1375. Distribution of mRNAs encoding the peroxisome proliferator-activated receptor (PPAR) -a, -b, and -g and the retinoid X receptor (RXR) -a, -b, and -g in rat central nervous system.
17. Gil-Gomez, G., Ayte, J. & Hegardt, G. (1993) *Eur. J. Biochem.* **213**, 773–779. The rat mitochondrial 3-hydroxy-3-methylglutaryl-coenzyme-A-synthase gene contains elements that mediate its multihormonal regulation and tissue specificity.
18. Casals, N., Roca, N., Guerrero, M., Gil-Gomez, G., Ayte, J., Ciudad, C.J. & Hegardt, F.G. (1992) *Biochem. J.* **283**, 261–264. Regulation of the expression of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene. Its role in the control of ketogenesis.
19. Lemberger, T., Staels, B., Saladin, R., Desvergne, B., Auwerx, J. & Wahli, W. (1994) *J. Biol. Chem.* **269**, 24527–24530. Regulation of the peroxisome proliferator-activated receptor alpha gene by glucocorticoids.
20. Steineger, H.H., Arntsen, B.M., Spydevold, O. & Sorensen, H.N. (1997) *Biochimie* **79**, 107–110. Retinoid X receptor (RXR alpha) gene expression is regulated by fatty acids and dexamethasone in hepatic cells.
21. Dallman, M.F., Strack, A.M., Akana, S.F., Bradbury, M.J., Hanson, E.S., Scribner, K.A. & Smith, M. (1993) *Front. Neuroendocrinol.* **14**, 303–347. Feast and famine: critical role of glucocorticoids with insulin in daily energy flow.
22. Girard, J., Ferré, P., Pégorier, J-P. & Duée, P-H. (1992) *Physiol. Rev.* **72**, 507–562. Adaptations of glucose and fatty acid metabolism during perinatal period and suckling-weaning transition.
23. Nehlig, A. & Pereira de Vasconcelos, A. (1992) *Prog. Neurobiol.* **40**, 163–221. Glucose and ketone body utilization by the brain of neonatal rats.
24. Langfort, J., Pilis, W., Zarzeczny, R., Nazar, K. & Kaciuba-Uscilko, H. (1996) *J. Physiol. Pharmacol.* **47**, 361–371. Effect of low-carbohydrate-ketogenic diet on metabolic and hormonal responses to graded exercise in men.
25. Auestad, N., Fisher, R., Chiappelli, F., Korsak, R.A. & Edmond, J. (1990) *Proc. Soc. Exp. Biol. Med.* **195**, 335–344. Growth and development of brain of artificially reared hypoketonemic rat pups.
26. Erecinska, M., Nelson, D., Daikhin, Y. & Yudkoff, M. (1996) *J. Neurochem.* **67**, 2325–2334. Regulation of GABA level in rat brain synaptosomes: fluxes through enzymes of the GABA shunt and effects of glutamate, calcium, and ketone bodies.
27. Yudkoff, M., Daikhin, Y., Nissim, I., Grunstein, R. & Nissim, I. (1997) *J. Neurochem.* **69**, 682–692. Effects of ketone bodies on astrocyte amino acid metabolism.
28. Tallian, K.B., Nahata, M.C. & Tsao, C.Y. (1998)*Ann. Pharmacother.* **32**, 349–361. Role of the ketogenic diet in children with intractable seizures.
29. Freeman, J.M., Kelly, M.T. & Freeman, J.B. (1994) *The epilepsy diet t reatment* in New York: Demos.

30. Emilien, G. & Maloteaux, J.M. (1998) *Int. J. Clin. Pharmacol. Ther.* **36**, 181–194. Pharmacological management of epilepsy. Mechanism of action, pharmacokinetic drug interactions, and new drug discovery possibilities.
31. Schlumberger, E. & Dulac, O. (1994) *Dev. Med. Child. Neurol.* **36**, 863–872. A simple, effective and well-tolerated treatment regime for West syndrome.
32. Duport, S., Stoppini, L. & Correges, P. (1997) *Life Sci.* **60**:PL. Electrophysiological approach of the antiepileptic effect of dexamethasone on hippocampal slice culture using a multirecording system: the Physiocard.
33. Hori, A., Tandon, P., Holmes, G.L. & Stafstrom, C.E. (1997) *Epilepsia*, **38**, 750–758. Ketogenic diet: Effects on expression of kindled seizures and behavior of adult rats.
34. Martinez, M. (1992) *J. Pediatr.* **120**, S129–S138. Tissue levels of polyunsaturated fatty acids during early human development.

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STUDIES ON REGULATION OF THE PEROXISOMAL ß-OXIDATION AT THE 3-KETOTHIOLASE STEP

Dissection of the Rat Liver Thiolase B Gene Promoter

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1. SUMMARY

The peroxisomal 3-oxoacyl-CoA thiolase (thiolase) is the last enzyme involved in the β-oxidation of fatty acids. The enzyme cleaves long chain fatty acyl-CoA to generate acetyl-CoA and shortened acyl-CoA. The enzyme is nuclear encoded, synthesized in the cytoplasm and transported into peroxisomes. The thiolase B gene is inducible by the peroxisome proliferator compounds, like other genes involved in β-oxidation of fatty acids in peroxisomes.

The importance of studying thiolase is that it generates acetyl-CoA which is the precursor for the synthesis of molecules like cholesterol and fatty acids. The structural and functional analysis of thiolase at molecular level may add to the knowledge of fatty acid metabolism and further the obesity phenomenon. It is known that several genes mediate lipid homeostasis in target organs like liver, adipose tissue and are regulated by peroxisome proliferator activated receptors (PPARα and PPARγ). To elucidate the mechanism of induction of rat liver thiolase B gene, an upstream 2.8kb fragment containing promoter element has been subcloned and partially sequenced. The sequence analysis revealed a putative PPRE (Peroxisome Proliferator Response Element) of AGACCT T TGAACC sequence at –681 to –668 [Kliever *et al.* (1992) Nature 358:771–774]. By transient expression of a luciferase reporter gene in HeLa cells, we conclude that the identified

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PPRE could be functional in induction of thiolase B gene, but other sequences of genes might be involved.

2. INTRODUCTION

The peroxisomal 3-oxoacyl-CoA thiolase (thiolase) is involved in the final reaction of fatty acids β-oxidation. The enzyme cleaves long chain fatty acyl-CoA to generate acetyl-CoA and chain-shortened acyl-CoA. The importance of studying thiolase is that it generates acetyl-CoA which is the precursor for the synthesis of molecules like cholesterol and fatty acids. It is now established that several genes mediate lipid metabolism in target organs like liver and adipose tissue, and are thus regulated by several Peroxisome Proliferator-Activated Receptors (PPARs).

In rat liver at least 3 genes encode for peroxisomal thiolase of which thiolase B is inducible by peroxisome proliferators.[1] To elucidate the mechanism of induction of thiolase B, an upstream 2.8kb fragment containing the promoter element has been sub-cloned and partially sequenced. The sequence analysis reavealed a putative PPRE (Peroxisome Proliferator Response Element) AGACCT T TGAACC at –681 to –668.[2,3]

To analyzes the functional elements in the 2.8 kb fragment, several deletions were made in the 5′ and the 3′ region in a plasmid containing TK promoter and the sequence encoding luciferase. Transfection assays were performed with these various deleted constructs in HeLa cells. Preliminary transfection results seem to suggest that this localized PPRE element is not the only one in controlling thiolase B expression by PPARα.

3. MATERIALS AND METHODS

3.1. Structure of PTBTK luc Plasmid and Deleted Constructs

a. Tool (Fig. 1): a 2.6kb genomic DNA fragment of the 5′-upstream region of PTB gene was inserted between HindIII and Af1II sites in a pBLluc vector. (Fig. 1)
b. Preparation of 5′ or 3′ deleted upstream constructs in pB luc vector (Table I see also Fig. 2)
c. Use of HeLa cell line (human cervical carcinoma) as host cell for transient expression.
d. Techniques of transfection of 2×10^5 cells/dish of HeLa cells in transient expression [6μg of luciferase reporter plasmid and co-transfected with pCMV-βgal(4μg) and +/– pCMV-PPARα or pCMV Not (2μg).

Luciferase activity has been normalized with β-galactosidase activity.

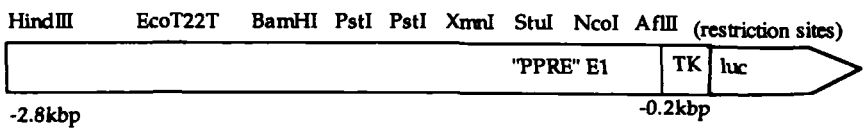

Figure 1.

Table I

#	Plasmid	construct name	deletion	size of upstream region
1	pPTBTKluc	original construct	wild sequence	~2.6 kb
2	pPTB(-2 kb/-190)TKluc	p«EcoT»	-5′ del	~1800 bp
3	pPTB(-1113/-190)TKluc	p«BamHI/5»	-5′ del	923 bp
4	pPTB(-788/-190)TKluc	p«PstI»	-5′ del	598 bp
5	pPTB(-613/-190)TKluc	p«StuI»	-5′ del	423 bp
6	pPTB(-487/-190)TKluc	p«NcoI 5»	-5′ del	297 bp
7	pPTB(-2.8 kb/-1113)TKluc	p«BamHI/3»	-3′ del	1687 bp
8	pPTB(-2 kb/-669)TKluc	p«XmnI»	-3′ del	2131 bp
9	pPTB(-2.8 kb/-487)TKluc	p«NcoI 3»	-3′ del	2313 bp
10	pPTB(-750/-487)TKluc	p«PstI/NcoI»	5′ and 3′ del	263 bp
11	pPTB(-750/-613)TKluc	p«PstI/StuI»	5′ and 3′ del	137 bp
12	pPTB(-750/-669)TKluc	p«PstI/XmnI»	5′ and 3′ del	81 bp

4. RESULTS

4.1. The Structure of the Deleted Constructs in pBluc are Reported in the Figure 1 (Figure 2)

4.2. Influence of Promoter Strength in Luciferase Gene Expression (Table II)

Conclusion: in HeLa cells, the PTB gene promoter works equally as the TK gene promoter in luciferase gene expression.

4.3. Influence of the PTB 5′-upstream Sequence on luc Gene Trancient Expression in HeLa Cells (Table III)

Conclusion: the presence of the PTB 5′-upstream region strongly enhances the luc gene expression. The enhancement is more than 2 times in HeLa cells.

4.4. Effect of 5′ or 3′ Deletions in the PTB 5′-Upstream Sequence in luc Gene Transient Expression in HeLa Cells (Table IV)

Conclusion: The 5′ side of the 5′-upstream region from the StuI site is inhibitory (or down regulating), especially between BamHI and StuI sites. In contrast, the sequence between StuI and NcoI sites containing the consensus El motif (2), «GGGAC C CAGGA» (conserved in peroxisomal acyl-CoA oxidase, bifunctional enzyme and thiolase B genes) is an enhancer region.

The 3′ side of the PPRE is important for gene expression especially the sequence between XmnI and StuI sites.

4.5. Effect of mPPAR α

results in HeLa cells (Table V)

Conclusion: the presence of PPAR α in HeLa cells (in the absence of exogenous activator) has no activating effect on luc gene reporter expression and rather shows a slight inhibitory effect.

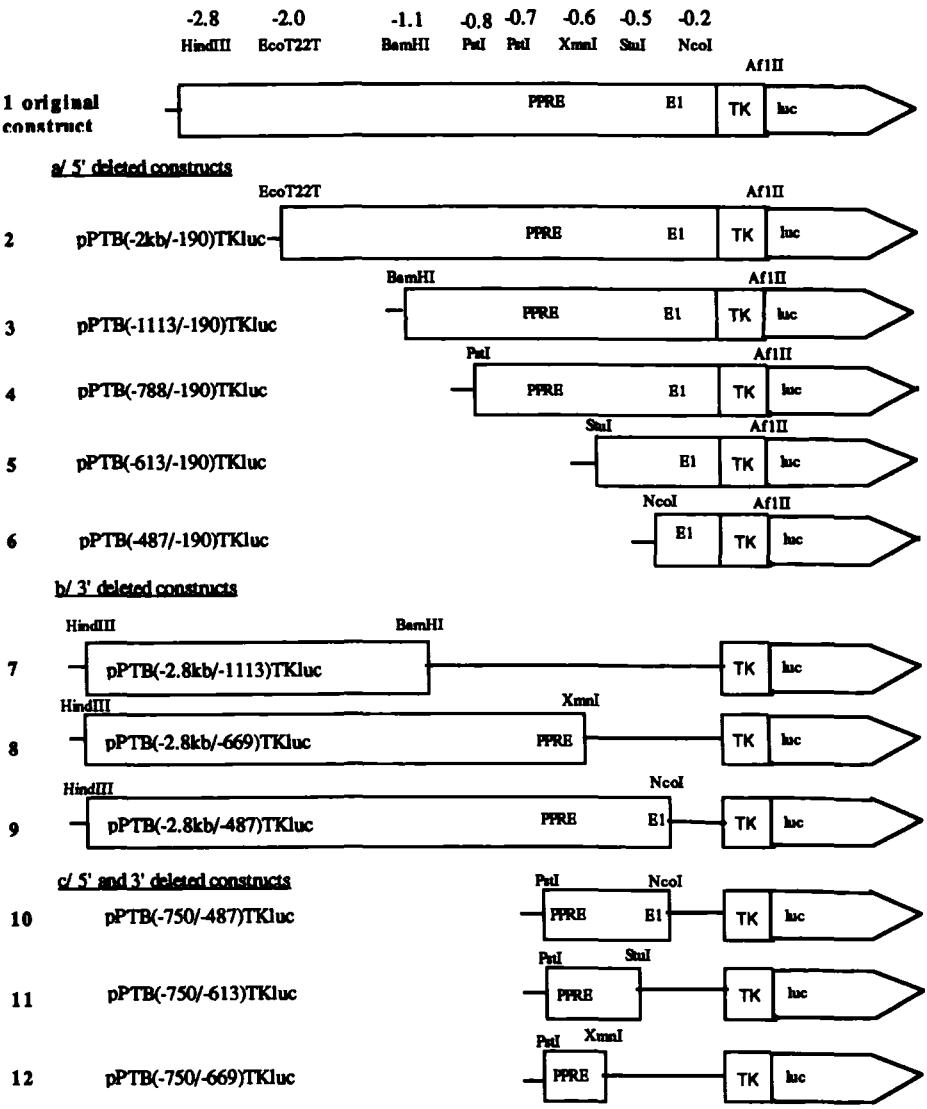

Figure 2. approximative position of the restriction sites

4.6. Effect of Ciprofibrate at 0.5 mM for 2.5 Days on PPAR α-transfected HeLa Cells. The Results are Expressed in the Table VI as the Ratio

construct + PPARα + Cipro in % / construct + PPARα—Cipro in % (Table VI)

Conclusion: ciprofibrate, a strong peroxisome proliferator, in presence of exogenous PPARα, shows only a slight or no inducing effect on the luc gene reporter expression even when the construct contains the DR1.

«AGACCT T TGAAGG» (the putative PPRE as suggested by the PPAR/RXR binding study (3).

Table II

Promoter	relative expression (mean M)	(nb of experiments)
PTB promot.	100%	(2)
TK promot.	106% ± 11%	(2)

Table III

5′ upstream sequence	relative expression (mean M)	(nb of experiments)
pTK luc	100%	(5)
pPTBK luc	270% ± 50%	(5)

Table IV

# as Table I	contructs	relative expression (mean M)	(nb of experiments)
1	pPTBTKluc	100%	(4)
2	p «EcoT»-5′ del	435 ± 102%	(2)
3	p «BamHI»-5′ del	322 ± 61%	(3)
5	p «StuI»-5′ del	598 ± 95%	(4)
6	p «NcoI»-5′ del	4 ± 3%	(3)
7	p «BamHI»-3′ del	42 ± 1%	(2)
8	p «XmnI»-3′ del	20 ± 3%	(2)
9	p «NcoI»-3′ del	67 ± 12%	(2)
11	p «PstI/StuI» 5′ and 3′ del	66%	
12	p «PstI/XmnI» 5′ and 3′ del	18%	

Table V

Transfecting plasmids	relative expression (mean M)	(nb of experiments)
pPTBTKluc	100%	(4)
pPTBTKluc + pCMVPPAR α	82 ± 2%	(4)

Table VI

# as Table I	Construct	relative expression (effect of ciprofibrate)
	pPTBluc	143%
1	pPTBTKluc	110 ± 4%
3	p «BamHI»-5′ del	70%
4	p «PstI»-5′ del	80%
5	p «StuI»-5′ del	70%
6	p «NcoI»-3′ del	100%
7	p «BamHI»-3′ del	147%
8	p «XmnI»-3′ del	178%
10	p «PstI/NcoI» 5′ and 3′ del	92%
11	p «PstI/StuI» 5′ and 3′ del	122%
12	p «PstI/XmnI» 5′ and 3′ del	177%

5. DISCUSSION AND STATE OF THE ART

Figure 3 summarizes the location of the different DNA regions of the 5′ upstream promoters fragment which appears to be involved in the modulation of the rat liver thiolase B gene expression. (Fig. 3)

The lack of peroxisome proliferator-dependent transactivation in HeLa cells would not be due to a peroxisome proliferator-responsive enhancer but due to other types of enhancer elements, but not peroxisome proliferator-dependent ones.

Open questions remaining at this moment are:

— How does ciprofibrate stimulate PTB—gene expression (mRNA level is strongly increased)?
— Is the putative PPRE DR1 at –669/–681 involved in the *in vivo* gene regulation?
— Is there any other functional DR1 far upstream of the 2.8 kbp fragment?
— What is the role of the El region and what are the DNA-binding proteins involved?
— Are there any cis-regulatory elements downstream of the PTB-gene promoter?
— Would the results be the same with other gene reporter constructs (ex. CAT constructs)? For instance, is the TK promoter not a too strong one to see any regulatory effects?

ACKNOWLEDGMENTS

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ABBREVIATIONS

— pPTBTKluc = plasmid containing the upstream promoter sequence of rat liver peroxisomal thiolase B gene, the promoter thymidine kinase of simplex herpes virus and the luciferase gene as reporter gene.
— **pCMV-βgal** = **β**-galactosidase reporter gene downstream from a cytomegalovirus promoter.
— pCMV **PPARα** = mouse **PPARα-cDNA** downstream from a cytomegalovirus promoter.
— pCMV NOT = same plasmid as above without PPAR gene.
— pPTBluc = plasmid containing 5′ upstream region and the natural promoter of the rat peroxisomal thiolase B gene.

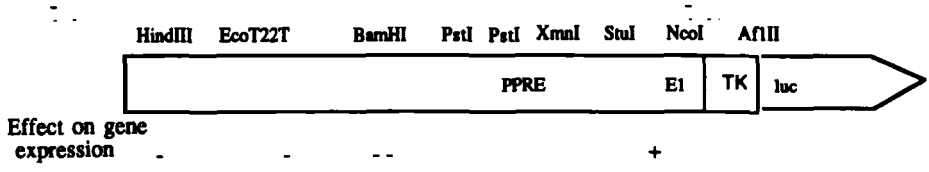

Figure 3.

REFERENCES

1. Miyazawa S., Osumi T. & Hashimoto T. (1980) The presence of a new 3-oxoacyl-CoA thiolase in rat liver peroxisomes. *Eur. J. Biochem.* 103:589–596.
2. Hijikata M., Wen J.K., Osumi T. & Hashimoto T. (1990) Rat peroxisomal 3-ketoacyl-CoA thiolase gene *J. Biol. Chem.* 265:4600–4606.
3. Kliewer S.A., Umesono K., Noonan D.J., Heyman R.A. & Evans R.M. (1992) Convergence of 9-cis retinoic acid and peroxisome proliferator signaling pathways through heterodimer formation and their receptors. *Nature* 358:771–774.

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ROLE AND ORGANIZATION OF PEROXISOMAL β-OXIDATION

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1. ABSTRACT

In mammals, peroxisomes are involved in breakdown of very long chain fatty acids, prostanoids, pristanic acid, dicarboxylic fatty acids, certain xenobiotics and bile acid intermediates. Substrate spectrum and specificity studies of the four different β-oxidation steps in rat and/or in man demonstrate that these substrates are degraded by separate β-oxidation systems composed of different enzymes. In both species, the enzymes acting on straight chain fatty acids are palmitoyl-CoA oxidase, an L-specific multifunctional protein (MFP-1) and a dimeric thiolase. In liver, bile acid intermediates undergo one cycle of β-oxidation catalyzed by trihydroxycoprostanoyl-CoA oxidase (in rat), or branched chain acyl-CoA oxidase (in man), a D-specific multifunctional protein (MFP-2) and SCP_x-thiolase. Finally, pristanic acid is degraded in rat tissues by pristanoyl-CoA oxidase, the D-specific multifunctional protein-2 and SCP_x-thiolase. Although in man a pristanoyl-CoA oxidase gene is present, so far its product has not been found. Hence, pristanoyl-CoA is believed to be desaturated in human tissues by the branched chain acyl-CoA oxidase. Due to the stereospecificity of the oxidases acting on 2-methyl-branched substrates, an additional enzyme, 2-methylacyl-CoA racemase, is required for the degradation of pristanic acid and the formation of bile acids.

2. INTRODUCTION

Following the discovery of peroxisomal long chain fatty acid β-oxidation in rat liver and its induction by hypolipidemic drugs,[1] the involved enzymes were characterized mainly by Hashimoto, Osumi and coworkers in liver from induced rats.[2] This resulted in the following picture. After activation, the CoA-esters are desatured by an FAD-dependent acyl-CoA oxidase. The formed 2-*trans*-enoyl-CoA is hydrated to a 3-L-hydroxyacyl-CoA that is subsequently dehydrogenated to a 3-oxoacyl-CoA. These reactions are

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catalysed by a single protein, called initially bifunctional protein, that displays enoyl-CoA hydratase and NAD-dependent 3-hydroxyacyl-CoA dehydrogenase activities. Subsequent work showed that this protein also contained a Δ^3,Δ^2-enoyl-CoA isomerase activity (which is involved in the degradation of unsaturated fatty acids),[3] hence, it was renamed multifunctional protein. The 3-oxoacyl-CoA is cleaved by a dimeric thiolase. In man similar enzymes were found. However, in contrast to the situation in rodents, in man these enzymes are not induced by peroxisome proliferators.

Besides long chain fatty acids, peroxisomes can degrade a variety of other lipophilic carboxylates. Physiological substrates include very long chain fatty acids (VLCFA), arachidonic acid derivatives (leukotrienes, prostaglandins, thromboxanes), polyunsaturated fatty acids, dicarboxylic fatty acids, pristanic acid, and the bile acid intermediates di- and trihydroxycoprostanic acid.[4,5] Given the structural variation in these substrates, the question emerged whether all these are handled by one set of enzymes or by different enzymes. A first indication for the likelihood of the latter possibility was found in 1987 during our studies on peroxisomal bile acid formation. We found that the oxidase acting on trihydroxycoprostanoyl-CoA was not induced upon treatment of rats with clofibrate and was only detectable in liver.[6] In contrast, the palmitoyl-CoA oxidase activity was inducible and present in all tissues. In the subsequent years we have systematically analysed each step of the peroxisomal β-oxidation sequence; we have characterized the involved enzymes with regard to substrate spectrum and stereospecificity and we have isolated and/or cloned these enzymes in both rat and man. In this paper we have focused on the use of three classes of substrates, namely straight chain fatty acids, synthetic 2-methyl-branched fatty acids (as substitutes for pristanic acid) and bile acid intermediates. In the following paragraphs, our findings are summarized. When appropriate, references are also given to work of other groups who reached independently similar conclusions or that confirmed our findings.

3. PEROXISOMAL REACTIONS RELATED TO β-OXIDATION

3.1. Activation

For reasons that are not clear yet, substrates for peroxisomal β-oxidation are activated at different subcellular sites. Long straight[7,8] and 2-methyl-branched chain fatty acids[9,10] (but also 3-methyl-branched chain fatty acids; see next chapter) are activated by mitochondria, peroxisomes and endoplasmic reticulum (ER). Based on indirect data, the peroxisomal activities seem to reside in different proteins,[11,12] but this idea was recently challenged.[13] The CoA-esters of VLCFAs are formed in the ER and peroxisomes.[14,15] A separate synthetase (70.7kDa) is involved in this process.[16,17] The catalytic sites of the peroxisomal synthetases acting long chain fatty acids (palmitoyl-CoA synthetase)[8,18] and, most likely, VLCFAs (lignoceroyl-CoA synthetase)[18,19] face the cytosol. Hence, to be further -degraded, a process that takes place in the peroxisomal matrix, the formed acyl-CoAs have to pass the membrane. This occurs presumably via integral membrane proteins containing an ATP binding cassette motif (ABC transporters).[20] In broken systems, however, no latency of palmitoyl-CoA oxidation is observed.[21]

Dicarboxylic fatty acids,[22] proistaglandins[23] and bile acid intermediates[2425] are activated by ER enzymes. To date how the corresponding CoA-esters reach the peroxisomal matrix has not been studied. It is highly probable that, as in the transloca-

tion of straight chain acyl-CoAs, ABC transporters are involved in their transmembrane movement.

3.2. Desaturation

In rat liver peroxisomes three acyl-CoA oxidases were found that were named according to their substrate specificity.[26–28] The acyl-CoA oxidase isolated initially by Hashimoto and coworkers[2] (often named palmitoyl-CoA oxidase although palmitoyl-CoA is not considered to be a physiologial substrate[28]) is active on the CoA esters of straight chain mono- and dicarboxylic fatty acids, prostaglandins, VLCFA and xenobiotics.[28] Pristanoyl-CoA oxidase, initially discovered as a non-inducible palmitoyl-CoA oxidase,[26] is active on 2-methyl-branched chain acyl-CoAs such as pristanoyl-CoA but can also handle long and very long straight chain acyl-CoAs.[28] The presence of pristanoyl-CoA oxidase in rat liver was later confirmed by others.[29] Finally, trihydroxycoprostanoyl-CoA oxidase (THCCox) is most active on the CoA-esters of the bile acid intermediates, di- and trihydroxycoprostanic acid, but can desaturate 2-methyl-branched acyl-CoAs. THCCox is a liver specific enzyme, while the two others are found in all tissues. For unknown reasons, only palmitoyl-CoA oxidase is inducible.[26,27] Due to a splicing event, two isoforms of this enzyme are present.[30,31] The bacterially expressed isoforms do not show major differences in affinities for straight chain monocarboxylic acyl-CoAs.[32] Both rat pristanoyl-CoA oxidase[33] and THCCox[34] have been isolated and their cDNAs have been cloned.[35,36] More information with regard to the molecular properties of the three acyl-CoA oxidases is given in Table 1.

Two other oxidase activities have been described in rat liver peroxisomes, one acting on glutaryl-CoA,[37] the other on valproyl-CoA.[38] The glutaryl-CoA oxidase activity, however, co-purifies with the inducible palmitoyl-CoA oxidase.[28,39] The valproyl-CoA oxidase was claimed to differ from the above described acyl-CoA oxidases, but in our hands this activity was recovered mainly in the cytosol (Casteels, M. & Van Veldhoven P. P., unpublished data).

Based on a report by Bennett *et al.*,[47] human peroxisomes might contain also a glutaryl-CoA oxidase. The nature of this enzyme activity remains to be proven (see above).

In man, at the protein level, two oxidases were found.[40,41] The first one is palmitoyl-CoA oxidase, the counterpart of the rat enzyme with regard to substrate spectrum and

Table 1. Molecular properties of acyl-CoA oxidases.

species	name	number of amino acids	deduced molecular mass (dalton)	meric structure	size mRNA (kb)	C-terminus	gene
rat	palmitoyl-CoA oxidase*						
	type I	661	74,678	$A_2 + B_2C_2$ + ABC ?	3.7	SKL	
	type II	661	74,690		3.7	SKL	
	trihydroxycoprostanoyl-CoA oxidase	681	76,411	2 × 76.4 kDa	2.6	HKM**	
	pristanoyl-CoA oxidase	700	78,445	8 × 78.5 kDa	3.8	SQL	
man	palmitoyl-CoA oxidase*						
	type I	661	74,600	$A_2 + B_2C_2$ + ABC ?	3.7	SKL	17q25.1
	type II	660	74,494				
	branched chain acyl-CoA oxidase	681	76,739	2 × 76.4	2.6	SKL	3p14.3
	pristanoyl-CoA oxidase	700	77,552	?	?	SKL	4p15.3

* After import, the 74 kDa subunit is cleaved into 53 kDa (subunit B) and 21 kDa (subunit C) fragments.

** This sequence, although known to be a glycosomal import signal, is apparently not functional in mammals.

molecular characteristics. Also in man, two isoforms are present due to a splicing event.[42] After a detailed substrate spectrum analysis, the second oxidase was named branched chain acyl-CoA oxidase (BRCACox).[41] It is active on 2-methyl-branched compounds such as pristanoyl-CoA and trihydroxycoprostanoyl-CoA, but also on straight chain acyl-CoAs (VLCFAs, dicarboxylic fatty acids). The presence of a second oxidase in human liver was confirmed by others but, using only pristanoyl-CoA as substrate, the enzyme was named pristanoyl-CoA oxidase.[43] Cloning of its cDNA revealed that BRCACox is the counterpart of rat THCCox.[44] In contrast to the rat enzyme, BRCACox is found in most tissues. It has been mapped to chromosome 3p14.3.[44,45] Somewhat confusing is the recent finding in man of the gene for the counterpart of rat pristanoyl-CoA oxidase.[46] Apparently, it is a functional gene, located at chromosome 4pl5.3, that is translated into mRNA that, at least in a bacterial expression system, gives rise to a full length oxidase containing a typical peroxisome targeting signal.[46] So far, based on enzyme activity measurements or immunoblotting, the protein has not been shown to be present, however. One hypothesis is that is might be expressed at certain developmental stages or in certain tissues not yet investigated.

3.3. Hydratation and Dehydrogenation

Investigation of the 3-hydroxyacyl-CoA dehydrogenase activities in purified rat liver peroxisomes, using the 3-hydroxyacyl-CoAs of straight chain fatty acids, of 2-methyl-branched chain fatty acids and of trihydroxycoprostanic acid as substrates, revealed initially 5 different enzymes (named I to V).[48] Enzyme IV was a monomeric 78 kD protein, possessed crotonase activity, was induced by clofibrate, and was identified as the inducible multifunctional protein.[48] Interestingly, enzyme III, a monomeric 80 kD protein, also hydrated crotonyl-CoA. In contrast to enzyme IV, enzyme III was not induced by clofibrate.[48] This was the first indication, published in 1994, that peroxisomes contained a second multifunctional protein. It was named multifunctional protein 2 (MFP-2) (the inducible, firstly isolated protein is referred to as MFP-1). Based on its substrate spectrum, the newly identified multifunctional protein was postulated to be involved in bile acid formation.[48]

Using pure 3-hydroxyacyl-CoA isomers, the stereospecificity of these MFPs was investigated. As predicted, MFP-1 was L-specific, but MFP-2 turned out to be D-specific.[49] Both MFPs act on the enoyl-CoAs and the 3-hydroxyacyl-CoAs of straight and 2-methyl-branched fatty acids and of trihydroxycoprostanic acid (the latter compound is called varanoyl-CoA). The configuration of the 3-hydroxy derivative of 2-methyl-branched enoyl-CoAs formed by MFP-1 and of the derivative dehydrogenated by MFP-1 are, however, not identical. MFP-2 on the other hand, forms and uses the same 3-hydroxy-2-methylacyl-CoAs. This is the 3R,2R-isomer in the case of pristanoyl-CoA or the 24R,25R-isomer in the case of varanoyl-CoA. Hence, only MFP-2 can be involved in degradation of bile acid intermediates and pristanic acid.[49,50] Based on the reactions of MFP-1 with the varanoyl-CoA isomers, Xu and Quebas[51] concluded also that bile acids could not be formed via MFP-1.

Isolation and cloning of rat MFP-2[49] revealed its identity to an enzyme called type IV 17β-estradiol dehydrogenase. Independently, Hiltunen and coworkers,[52] who also confirmed the D-specificity of the protein, and Gustafsson and coworkers[53] also obtained the rat cDNA. Type IV 17β-estradiol dehy drogenase was isolated initially from porcine endometrium and subsequently cloned by Adamski and coworkers.[54] The recombinant porcine enzyme displays crotonase and 3-hydroxybutyryl-CoA dehydrogenase activities[55]

Table 2. Molecular properties of multifunctional proteins.

species	name	number of amino acids	deduced molecular mass (dalton)	meric structure	size mRNA (kb)	C-terminus	gene
rat	MFP-1	721	78,526	1 × 78 kDa	4.7	SKL	
	MFP-2	735	79,331	1 × 80 kDa*	2.8	AKL	
man	MFP-1	722	79,208	1 × 79 kDa	4.7	SKL	3q26–28
	MFP-2	736	79,686	1 × 80 kDa*	2.8	SKL	5q2.3

* Proteolytically cleaved, presumably after import, in a 44 kDa hydratase and 40 kDa dehydrogenase fragment. Both fragments apparently form homodimers.

and rat MFP-2 dehydrogenates estradiol.[49,52] Compared to the β-oxidation activities of MFP-2, the estradiol dehydrogenase activity is very low,[49,52] and most probably has no physiological consequence. By homology, the cDNAs of the mouse,[56] man[57] and guinea pig[58] counterparts have been obtained in the meantime. These enzymes are multidomain proteins.[59] The N-terminus contains the 3-hydroxyacyl-CoA dehydrogenase (and estradiol dehydrogenase) domain, the central part possesses enoyl-CoA hydratase activity and the C-terminus shows homology to sterol carrier protein-2 (SCP2). Interestingly, the first two domains are homologous to those found in the yeast multifunctional proteins[60,61] that are also D-specific.[61]

MFP-2 is subject to a proteolytic cleavage that seems to be physiological.[49,52,54] This results in an active D-specific hydratase of 44kDa[62] and an active D-specific 3-hydroxyacyl-CoA dehydrogenase of 40 kD.[49] The 44kDa hydratase might give rise to a 33kDa D-specific hydratase that was discovered and further characterized by Hiltunen and coworkers.[52]

The molecular properties of the peroxisomal multifunctional proteins are given in Table 2.

Also in man the presence of a second, D-specific, MFP was proven enzymically and immunologically in liver[63,64] and in fibroblasts.[65] The gene for MFP-2 was mapped to chromosome 5q2.3.[63,66] Interestingly, in fibroblasts the MFP-2 activity is severalfold higher than that of MFP-1[65] and the protein seems to play a role in the breakdown of very long chain fatty acids as well.[65]

3.4. Thiolytic Cleavage

In peroxisomes purified from liver of control rats, two thiolase activities were found and their corresponding proteins were isolated.[67] Based on N-terminal amino acid sequencing, the first one was identified as thiolase A. This is a constitutively expressed protein, that had not been isolated before but whose presence was postulated previously on the basis of mRNA analysis.[68,69] It is rather similar to thiolase B, which had been purified by other groups from clofibrate treated animals. The substrate spectra of thiolase A and B are almost identical. Interestingly, thiolase A is more stable than B (Antonenkov V., Mannaerts G. P. and Van Veldhoven P. P., unpublished data). The other thiolase activity resided in SCP_X, a 58 kDa protein identified initially by the presence of an SCP2 domain at its C-terminus.[70] Based on the amino acid sequence deduced from its cDNA, a thiolase activity was postulated for SCP_X and subsequently demonstrated with the recombinant protein.[70,71] In an active form, SCP_x-thiolase had not been purified before. Like thiolase A and B, SCP_x-thiolase cleaves straight 3-oxoacyl-CoAs, but only SCP_x-thiolase acts on the 3-oxoacyl-CoA derivatives of 2-methyl-branched fatty acids

and trihydroxycoprostanic acid.[67] Hence, SCP_x-thiolase is indispensable for the degradation of pristanic acid and trihydroxycoprostanic acid. Based on studies with recombinant SCP_X, its role in pristanic acid removal was confirmed by others.[73]

3.5. Racemisation

The synthesis of optically pure 2-methylacyl-CoA allowed us to investigate the stereospecificity of the acyl-CoA oxidases. Rat pristanoyl-CoA oxidase and THCCox (and most likely BRCACox) desaturate only the 2S-forms.[74] Naturally occurring pristanic acid is racemic at position 2.[75] To degrade the 2R-isomer, it needs to be transformed to the 2S-isomer and this happens at the level of the CoA ester.[76] The responsible enzyme is 2-methylacyl-CoA racemase.[76] It is present in mitochondria and in peroxisomes, both in rat and man.[77,78] Presumably, the mitochondrial racemase activity is required for the racemisation of the shortened pristanic acid metabolites formed by peroxisomal β-oxidation and that contain 2R-methyl branches. Mitochondrial oxidation of branched fatty acids, like the peroxisomal one, is stereospecific for the 2S-isomers.[79]

Due to a stereospecific hydroxylation step during the ω-oxidation of cholesterol, only 25R-trihydroxycoprostanic acid is formed.[80,81] After activation it needs to be converted to 25S-THC-CoA since THCCox acts only on the 25S- isomer,[74] a finding confirmed by others.[82] Most likely, racemisation of THC-CoA and pristanoyl-CoA is catalyzed by the same enzymes.[76] Recently, the cloning of the (putatively peroxisomal) rat and mouse racemase was reported.[83] Apparently, it is identical to 2-aryl-propionyl-CoA epimerase.[84]

4. SEPARATE β-OXIDATION SYSTEMS

Based on the substrate specificity and stereoselectivity of the above described enzymes we have proposed the presence of separate β-oxidation systems handling a limited set of substrates (Fig. 1).[5] Bile acid intermediates can only be degraded via racemase, THCCox (in rat liver) or BRCACox (in human liver), MFP-2 and SCP_x-thiolase. Pristanic acid breakdown relies on the action of racemase (for the 2R-isomer), pristanoyl-CoA oxidase (in rat tissues) or BRCACox (in human tissues), MFP-2, and SCPx-thiolase. Finally, palmitoyl-CoA oxidase, MFP-2 and thiolase take care of the straight chain fatty acids. Although not yet experimentally proven, it is likely that these enzymes are associated with each other or kept together by an extra protein, such as SCP_2, to form a multi-enzyme complex, to allow channelling of intermediates.

Schematically shown are two separate β-oxidation systems, one involved in degradation of straight chain fatty acid derivatives (left side), the other handling 2-methyl-branched compounds (right side), each of them consisting of four steps that are catalyzed by an acyl-CoA oxidase, a multifunctional protein (MFP) (containing two or more activities) and a thiolase. Only the proteins belonging to the left system are (at least in rodents) induced by peroxisome proliferators and correspond to those initially discovered and characterized in rat liver. In rat, the CoA-esters of bile acid intermediates and pristanic acid are desaturated by separate enzymes (highlighted in italic), while in man only one oxidase appears to be involved. Before the naturally occurring 2R-pristanic acid and 25R-bile acid intermediates can be desaturated, a racemisation reaction is required. The 2-methyl-2-enoyl-CoAs, generated by the oxidases, are thought to be hydrated by the same MFP-2. Under normal conditions, the intermediates are believed to be channeled from

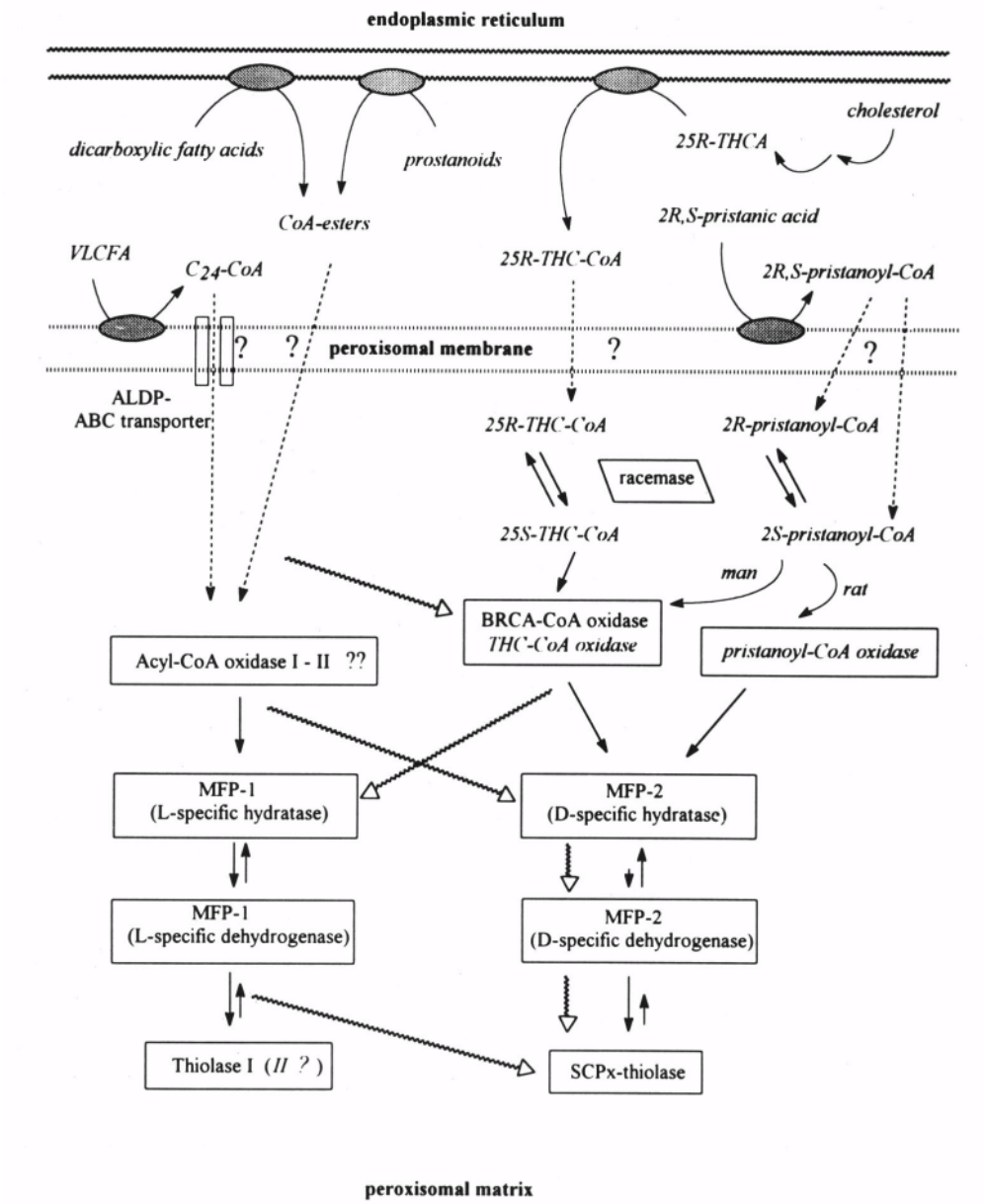

Figure 1. Proposed organisation of peroxisomal β-oxidation in mammals.

enzyme to enzyme in the separate systems (solid arrows). In case of deficiencies, shuttling of intermediates between the two systems can occur at different levels (zigzag arrows) although not all intermediates produced under these conditions can be further degraded. One should keep in mind that the enzymes involved in the degradation of 2-methyl-branched compounds can also act on straight chain compounds (and *in vitro* even better than the corresponding enzymes of the left system when the chain lenght of the substrates is increasing). The mechanism of membrane translocation of the CoA-esters (dashed arrows), most likely mediated by ABC-transporters, awaits further investigation.

The exact role and substrates of the two acyl-CoA oxidase isoforms in rat and in man and of the two dimeric thiolases in rat are not known.

ALDP: adrenoleukodystrophy protein; BRCA-: branched chain acyl-; C_{24}: lignoceric acid; THCA: trihydroxycoprostanic acid; THC-: trihydroxycoprostanoyl-.

Such model can also explain most findings in patients with peroxisomal β-oxidation defects, and showing various degrees of accumulation of VLCFA, pristanic acid and bile acid intermediates, or combinations thereof.[85] Under pathological conditions where channelling is interrupted, one can imagine that the intermediate will leave the system and be further degraded by the other system (see Fig. 1). This can lead either to a non-physiological dead-end intermediate or to a competition with the normal intermediates. In this respect, it is worthwhile to remember that 2-methyl-enoyl-CoAs can be hydrated both by MFP-1 and MFP-2. However, the 3-hydroxy-2-methyl intermediate made by MFP-1 cannot be further metabolized.

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REFERENCES

1. Lazarow, P.B. & de Duve, C. (1976) *Proc. Natl. Acad. Sci.* **73**, 2043–2046. A fatty acyl-CoA oxidizing system in rat liver peroxisomes; enhancement by cloflbrate, a hypolipidemic drug.
2. Hashimoto, T. (1996) *Ann. N.Y. Acad. Sci.* **804**, 86–98. Peroxisomal β-oxidation. Enzymology and molecular biology.
3. Palosaari, P.M. & Hiltunen, J.K. (1990) *J. Biol. Chem.* **265**, 2446–2449. Peroxisomal bifunctional protein from rat liver is a trifunctional enzyme possessing 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and Δ3,Δ2-enoyl-CoA isomerase activities.
4. Kunau, W.H., Dommes, V. & Schulz, H (1995) *Prog. Lipid Res.* **34**, 267–342. β-Oxidation of fatty acids in mitochondria, peroxisomes, and bacteria: a century of continued progress.
5. Mannaerts, G.P. & Van Veldhoven, P.P. (1996) *Ann. N. Y. Acad. Sci.* **804**, 99–115. Functions and organization of peroxisomal β-oxidation.
6. Casteels, M., Schepers, L., Van Eldere, J., Eyssen, H.J. & Mannaerts, G.P. (1988) *J Biol-Chem.* **263**, 4654–4661. Inhibition of 3α,7α,12α-trihydroxy-5β-cholestanoic acid oxidation and of bile acid secretion in rat liver by fatty acids.
7. Krisans, S.K., Mortensen, R.M. & Lazarow, P.B. (1980) *J. Biol. Chem.* **255**, 9599–9607. Acyl-CoA synthetase in rat liver peroxisomes. Computer-assisted analysis of cell fractionation experiments.
8. Mannaerts, G.P, Van Veldhoven, P., Van Broekhoven, A., Vandebroek, G. & Debeer, L.J. (1982) *Biochem. J.* **204**, 17–23. Evidence that peroxisomal acyl-CoA synthetase is located at the cytoplasmic side of the peroxisomal membrane.
9. Vanhove G., Van Veldhoven P.P., Vanhoutte F., Parmentier G., Eyssen H.J. & Mannaerts G.P. (1991) *J. Biol. Chem.* **266**, 24670–24675. Mitochondrial and peroxisomal β-oxidation of the branched chain fatty acid 2-methylpalmitate in rat liver.
10. Wanders, R.J.A., Denis, S., van Roermund, C.W., Jakobs, C. & ten Brink, H.J. (1992) *Biochim. Biophys. Acta.* **1125**, 274–279. Characteristics and subcellular localization of pristanoyl-CoA synthetase in rat liver.

11. Vanhooren, J.C., Asselberghs, S., Eyssen, H.J., Mannaerts, G.P. & Van Veldhoven, P.P. (1994) *Int. J. Biochem.* **26**, 1095–1101. Activation of 3-methyl-branched fatty acids in rat liver.
12. Pahan, K., Cofer, J., Baliga, P. & Singh, I. (1993) *FEES Lett.* **322**, 101–104. Identification of phytanoyl-CoA ligase as a distinct acyl-CoA ligase in peroxisomes from cultured human skin fibroblasts.
13. Watkins, P.A., Howard, A.E., Gould, S.J., Avigan, J. & Mihalik, S.J. (1996) *J. Lipid Res.* **37**, 2288–2295. Phytanic acid activation in rat liver peroxisomes is catalyzed by long chain acyl-CoA synthetase.
14. Singh, H. & Poulos, A. (1988) *Arch. Biochem. Biophys.* **266**, 486–495. Distinct long chain and very long chain fatty acyl CoA synthetases in rat liver peroxisomes and microsomes.
15. Lazo, O., Contreras, M., Yoshida, Y., Singh, A.K., Stanley, W., Weise, M. & Singh, I. (1990) *J. Lipid Res.* **31**, 583–595. Cellular oxidation of lignoceric acid is regulated by the subcellular localization of lignoceroyl-CoA ligases.
16. Uchida, Y., Kondo, N., Orii, T. & Hashimoto, T. (1996) *J. Biochem. Tokyo.* **119**, 565–571. Purification and properties of rat liver peroxisomal very-long-chain acyl-CoA synthetase.
17. Uchiyama, A., Aoyama, T., Kamijo, K., Uchida, Y., Kondo, N., Orii, T. & Hashimoto, T. (1996) *J. Biol. Chem.* **271**, 30360–30365. Molecular cloning of cDNA encoding rat very long-chain acyl-CoA synthetase.
18. Lageweg, W., Tager, J.M. & Wanders, R.J.A. (1991) *Biochem. J.* **276**, 53–56. Topography of very-long-chain-fatty-acid-activating activity in peroxisomes from rat liver.
19. Lazo, O., Contreras, M. & Singh, I. (1990) *Biochemistry* **29**, 3981–3986. Topographical localization of peroxisomal acyl-CoA ligases: differential localization of palmitoyl-CoA and lignoceroyl-CoA ligases.
20. Hettema, E.H., van Roermund, C.W., Distel, B., van den Berg, M., Vilela, C., Rodrigues-Pousada, C., Wanders, R.J.A. & Tabak, H.F. (1996) *EMBO J.* **15**, 3813–3822. The ABC transporter proteins Pat1 and Pat2 are required for import of long-chain fatty acids into peroxisomes of *Saccharomyces cerevisiae.*
21. Thomas, J., Debeer, L.J., De Schepper, P.J. & Mannaerts, G.P. (1980) *Biochem. J.* **190**, 485–94. Factors influencing palmitoyl-CoA oxidation by rat liver peroxisomal fractions. Substrate concentration, organelle integrity and ATP.
22. Vamecq, J., de Hoffman, E. & Van Hoof, F. (1985) *Biochem. J.* **230**, 683–693. The microsomal dicarboxylyl-CoA synthetase.
23. Schepers, L., Casteels, M., Vamecq, J., Parmentier, G., Van Veldhoven, P.P. & Mannaerts, G.P. (1988) *J. Biol. Chem.* **263**, 2724–2731. β-Oxidation of the carboxyl side chain of prostaglandin E_2 in rat liver peroxisomes and mitochondria.
24. Prydz, K., Kase, B.F., Björkhem, I. & Pedersen, J.L. (1988) *J. Lipid Res.* **29**, 997–1004. Subcellular localization of 3α,7α-dihydroxy- and 3α,7α,12α-trihydroxy-5β-cholestanoyl-coenzyme A ligase(s) in rat liver.
25. Schepers L., Casteels M., Verheyden K., Parmentier G., Asselberghs S., Eyssen H.J. & Mannaerts G.P. (1989) *Biochem. J.* **257**, 221–229. Subcellular distribution and characteristics of trihydroxycoprostanoyl-CoA synthetase in rat liver.
26. Schepers L., Van Veldhoven P.P., Casteels M., Eyssen H.J. & Mannaerts G.P. (1990) *J. Biol. Chem.* **265**, 5242–5246. Presence of three acyl-CoA oxidases in rat liver peroxisomes. An inducible fatty acyl-CoA oxidase, a non-inducible fatty acyl-CoA oxidase and a non-inducible trihydroxycoprostanoyl-CoA oxidase.
27. Van Veldhoven P.P., Vanhove G., Vanhoutte F., Dacremont G., Parmentier G., Eyssen H. & Mannaerts G.P. (1991) *J. Biol. Chem.* **266**, 24676–24683. Identification and purification of a branched chain fatty acyl-CoA oxidase.
28. Van Veldhoven P.P., Vanhove G., Asselberghs S., Eyssen H.J. & Mannaerts G.P. (1992) *J. Biol. Chem.* **267**, 20065–20074. Substrate specificities of rat liver peroxisomal acyl-CoA oxidases: Palmitoyl-CoA oxidase (inducible acyl-CoA oxidase), pristanoyl-CoA oxidase (non-inducible acyl-CoA oxidase) and trihydroxycoprostanoyl-CoA oxidase.
29. Wanders, R.J.A., Denis, S., Jakobs, C. & ten Brink, H.J. (1992) *Biochim. Biophys. Acta.* **1124**, 199–202. Identification of pristanoyl-CoA oxidase as a distinct, clofibrate non-inducible enzyme in rat liver peroxisomes.
30. Miyazawa, S., Hayashi, H., Hijikata, M., Ishii, N., Furuta, S., Kagamiyama, H., Osumi, T. & Hashimoto, T. (1987) *J. Biol. Chem.* **262**, 8131–8137. Complete nucleotide sequence of cDNA and predicted amino acid sequence of rat acyl-CoA oxidase.
31. Osumi, T., Ishii, N., Miyazawa, S. & Hashimoto, T. (1987) *J. Biol. Chem.* **262**, 8138–8143. Isolation and structural characterization of the rat acyl-CoA oxidase gene.

32. Setoyama, C., Tamaoki, H., Nishina, Y., Shiga, K. & Miura, R. (1995) *Biochem. Biophys. Res. Commun.* **217**, 482–487. Functional expression of two forms of rat acyl-CoA oxidase and their substrate specificities.
33. Van Veldhoven, P.P., Van Rompuy, P., de Béthune, B. & Mannaerts G.P. (1994) *Eur. J. Biochem* **222**, 795–801. Large scale purification and further characterization of rat pristanoyl-CoA oxidase.
34. Van Veldhoven, P.P., Van Rompuy, P., Vanhooren, J.C.T. & Mannaerts, G.P. (1994) *Biochem. J.* **303**, 195–200. Purification of further characterization of peroxisomal trihydroxycoprostanoyl-CoA oxidase from rat liver.
35. Van Hooren, J.C.T., Fransen, M., de Béthune, B., Baumgart, E., Torrekens, S., Baes, M., Van Leuven, F., Mannaerts, G.P. & Van Veldhoven, P.P. (1996) *Eur. J. Biochem.* **239**, 302–309. Rat pristanoyl-CoA oxidase: cDNA cloning and recognition of its C-terminal (SQL) by the PTS1-receptor.
36. Baumgart, E., Vanhooren, J.C.T., Fransen, M., Van Leuven, F., Fahimi, H.D., Van Veldhoven, P.P. & Mannaerts, G.P. (1996) *Biochem. J.* **320**, 115–121. Molecular cloning and further characterization of rat peroxisomal trihydroxycoprostanoyl-CoA oxidase.
37. Vamecq, J. & Van Hoof, F. (1984) *Biochem. J.* **221**, 203–211. Implication of a peroxisomal enzyme in the catabolism of glutaryl-CoA.
38. Vamecq, J., Vallee, L., Fontaine, M., Lambert, D., Poupaert, J. & Nuyts, J.P. (1993) *FEBS Lett.* **322**, 95–100. CoA esters of valproic acid and related metabolites are oxidized in peroxisomes through a pathway distinct from peroxisomal fatty and bile acyl-CoA β-oxidation.
39. Poosch, M.S. & Yamazaki, R.K. (1989) *Biochim. Biophys. Acta.* **1006**, 291–298. The oxidation of dicarboxylic acid CoA esters via peroxisomal fatty acyl-CoA oxidase.
40. Casteels, M., Schepers, L., Van Veldhoven, P.P., Eyssen, H.J. & Mannaerts G.P. (1990) *J. Lipid Res.* **31**, 1865–1872. Separate peroxisomal oxidases for fatty acyl-CoAs and trihydroxycoprostanoyl-CoA in human liver.
41. Vanhove, G.F., Van Veldhoven, P.P., Fransen, M., Denis, S., Eyssen, H.J., Wanders, R.J.A. & Mannaerts, G.P. (1993) *J. Biol. Chem.* **268**, 10335–10344. The CoA esters of 2-methyl-branched chain fatty acids and of the bile acid intermediates di- and trihydroxycoprostanic acids are oxidized by one single peroxisomal branched chain acyl-CoA oxidase in human liver and kidney.
42. Fournier, B., Saudubray, J.M., Benichou, B., Lyonnet, S., Munnich, A., Clevers, H. & Poll-The, B.T. (1994) *J. Clin. Invest.* **94**, 526–531. Large deletion of the peroxisomal acyl-CoA oxidase gene in pseudoneonatal adrenoleukodystrophy.
43. Wanders, R.J.A., ten Brink, H.J., van Roermund, C.W., Schutgens, R.B., Tager, J.M. & Jakobs, C. (1990) *Biochem. Biophys. Res. Commun.* **172**, 490–495. Identification of pristanoyl-CoA oxidase activity in human liver and its deficiency in the Zellweger syndrome.
44. Baumgart, E., Vanhooren, J.C.T., Fransen, M., Marynen, P., Pyupe, M., Vandekerckhove, J., Leunissen, J.A.M., Fahimi, H.D., Mannaerts, G.P. & Van Veldhoven, P.P. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 13748–13753. Molecular characterization of the human peroxisomal branched chain acyl-CoA oxidase. cDNA cloning, chromosomal assignment, tissue distribution and evidence for the absence of the protein in Zellweger syndrome.
45. Mograbi, N.N., Naylor, S.L., Van Veldhoven, P.P., Baumgart, E., Dawson, D.B. & Bennett, M.J. (1997) *Biochem. Biophys. Res. Commun.* **231**, 767–769. Assignment of the human branched chain acyl-CoA oxidase to chromosome 3pl4.3–p21.1 by rodent/human somatic cell hybridization.
46. Vanhooren, J.C.T, Marynen, P., Mannaerts, G.P. & Van Veldhoven, P.P. (1997) *Biochem. J.* **325**, 593–599. Evidence for the presence of pristanoyl-CoA oxidase gene in man.
47. Bennett, M.J., Pollitt, R.J., Goodman, S.I., Hale, D.E. & Vamecq, J. (1991) *J. Inherit. Metab. Dis.* **14**, 165–73. Atypical riboflavin-responsive glutaric aciduria, and deficient peroxisomal glutaryl-CoA oxidase activity: a new peroxisomal disorder.
48. Novikov, O.K., Vanhove, G., Carchon, H., Asselberghs, S., Eyssen, H.J., Van Veldhoven, P.P. & Mannaerts, G.P. (1994) *J. Biol. Chem.* **269**, 27125–27135. Peroxisomal β-oxidation: Purification of four novel 3-hydroxyacyl-CoA dehydrogenases from rat liver peroxisomes.
49. Dieuaide-Noubhani, M., Novikov, O.K., Baumgart, E., Vanhooren, J.C.T, Fransen, M., Goethals, M., Vandekerckhove, J., Van Veldhoven, P.P. & Mannaerts, G.P. (1996) *Eur. J. Biochem* **240**, 660–666. Further characterization of the peroxisomal 3-hydroxyacyl-CoA dehydrogenases from rat liver: relationship between the different dehydrogenases and evidence that fatty acids and the C_{27} bile acids di- and trihydroxycoprostanic acids are metabolized by separate multifunctional proteins.
50. Dieuaide-Noubhani, M., Mannaerts, G.P. & Van Veldhoven, P.P. (1997) *Biochem. J.* **325**, 367–373. Evidence that multifunctional protein-2 and not multifunctional protein-1 is involved in pristanic acid degradation.

51. Xu, R. & Cuebas, D.A. (1996) *Biochem. Biophys. Res. Commun.* **221**, 271–278. The reactions catalyzed by the inducible bifunctional enzyme of rat liver peroxisomes cannot lead to the formation of bile acids.
52. Qin, Y.M., Poutanen, M.H., Helander, H.M., Kvist, A.P., Siivari, K.M., Schmitz, W., Conzelmann, E., Hellman, U. & Hiltunen, J.K. (1997) *Biochem. J.* **321**, 21–28. Peroxisomal multifunctional enzyme of β-oxidation metabolizing D-3-hydroxyacyl-CoA esters in rat liver: molecular cloning, expression and characterization.
53. Corton, J.C., Bocos, C., Moreno, E.S., Merritt, A., Cattley, R.C. & Gustafsson, J.A. (1997) *Biochimie* **79**, 151–162. Peroxisome proliferators alter the expression of estrogen-metabolizing enzymes.
54. Leenders, F., Adamski, J., Husen, B., Thole, H.H. & Jungblut, P.W. (1994) *Eur. J. Biochem.* **15**, 222, 221–227. Molecular cloning and amino acid sequence of the porcine 17 β-estradiol dehydrogenase.
55. Normand, T., Husen, B., Leenders, F., Pelczar, H., Baert, J.L., Begue, A., Flourens, A.C., Adamski, J. & de Launoit, Y. (1995) *J. Steroid. Biochem. Mol. Biol.* **55**, 541–548. Molecular characterization of mouse 17 β-hydroxysteroid dehydrogenase IV.
56. Adamski, J., Normand, T., Leenders, F., Monte, D., Begue, A., Stehelin, D., Jungblut, P.W. & de Launoit, Y. (1995) *Biochem. J.* **311**, 437–43. Molecular cloning of a novel widely expressed human 80 kDa 17 β-hydroxysteroid dehydrogenase IV.
57. Caira, F, C1emencet, M.-C, Cherkaoui-Malki, M., Dieuaide-Noubhani, M., Pacot, C., Van Veldhoven, P.P. & Latruffe, N. (1998) *Biochem. J.* **330**, 1361–1368. Differential regulation by a peroxisome proliferator of the different multifunctional proteins in guinea pig. cDNA cloning of the guinea pig D-specific multifunctional protein 2.
58. Leenders, F., Husen, B., Thole, H.H. & Adamski, J. (1994) *Mol. Cell. Endocrinol.* **104**, 127–131. The sequence of porcine 80kDa 17 β-estradiol dehydrogenase reveals similarities to the short chain alcohol dehydrogenase family, to actin binding motifs and to sterol carrier protein 2.
59. Nuttley, W.M., Aitchison, J.D. & Rachubinski, R.A. (1988) *Gene* **69**, 171–180. cDNA cloning and primary structure determination of the peroxisomal trifunctional enzyme hydratase-dehydrogenase-epimerase from the yeast Candida tropicalis pK233.
60. Hiltunen, J.K., Wenzel, B., Beyer, A., Erdmann, R., Fossa, A. & Kunau, W.H. (1992) *J. Biol. Chem.* **267**, 6646–6653. Peroxisomal multifunctional β-oxidation protein of Saccharomyces cerevisiae. Molecular analysis of the fox2 gene and gene product.
61. Leenders, F., Tesdorpf, J.G., Markus, M., Engel, T., Seedorf, U. & Adamski. J. (1996) *J. Biol. Chem.* **271**, 5438–5442. Porcine 80-kDa protein reveals intrinsic 17 β-hydroxysteroid dehydrogenase, fatty acyl-CoA-hydratase/dehydrogenase, and sterol transfer activities.
62. Dieuaide-Noubhani, M., Novikov, D, Vandekerckhove, J., Van Veldhoven, P.P. & Mannaerts, G.P. (1997) *Biochem. J.* **321**, 253–259. Identification and characterization of the 2-enoyl-CoA hydratases involved in peroxisomal β-oxidation in rat liver.
63. Novikov, D., Dieuaide-Noubhani, M., Vermeesch, J.R., Fournier, B., Mannaerts, G.P. & Van Veldhoven, P.P. (1997) *Biochim. Biophys. Acta.* **1360**, 229–240. The human peroxisomal multifunctional protein involved in bile acid synthesis: activity measurement, deficiency in Zellweger syndrome and chromosome mapping.
64. Jiang, L.L., Kobayashi, A., Matsuura, H., Fukushima, H. & Hashimoto, T. (1996) *J. Biochem. Tokyo* **120**, 624–632. Purification and properties of human D-3-hydroxyacyl-CoA dehydratase: medium-chain enoyl-CoA hydratase is D-3-hydroxyacyl-CoA dehydratase.
65. Jiang, L., Kurosawa, T., Sato, M., Suzuki, Y., Hashimoto, T. (1997) *J. Biochem. Tokyo.* **121**, 506–513. Physiological role of D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein.
66. Leenders, F., Prescher, G., Dolez, V., Begue, A., de Launoit, Y. & Adamski, J. (1996) *Genomics* **37**, 403–404. Assignment of human 17 β-hydroxysteroid dehydrogenase IV to chromosome 5q2 by fluorescence in situ hybridization.
67. Antonenkov, V., Van Veldhoven, P.P., Waelkens, E. & Mannaerts, G.P. (1997) *J. Biol. Chem.* **272**, 26023–26031. Substrate specificities of 3-oxoacyl-CoA thiolase A and sterol carrier protein2/3-oxoacyl-CoA thiolase purified from normal rat liver peroxisomes. Sterol carrier protein 2/3-oxoacyl-CoA thiolase is involved in the metabolism of 2-methyl-branched fatty acids and bile acid intermediates.
68. Hijikata, M., Wen, J.K., Osumi, T. & Hashimoto, T. (1990) *J. Biol. Chem.* **265**, 4600–4606. Rat peroxisomal 3-ketoacyl-CoA thiolase gene. Occurrence of two closely related but differentially regulated genes.
69. Bodnar, A.G. & Rachubinski, R.A. (1990) *Gene* **91**, 193–199. Cloning and sequence determination of cDNA encoding a second rat liver peroxisomal 3-ketoacyl-CoA thiolase.
70. Ossendorp, B.C. & Wirtz, K..W. (1993) *Biochimie* **75**, 191–200. The non-specific lipid-transfer protein (sterol carrier protein 2) and its relationship to peroxisomes.

71. Ossendorp, B.C., Van Heusden, G.P., De Beer, A.L., Bos, K., Schouten, G.L. & Wirtz, K.W. (1991) *Eur. J. Biochem.* **201**, 233–239. Identification of the cDNA clone which encodes the 58-kDa protein containing the amino acid sequence of rat liver non-specific lipid-transfer protein (sterol-carrier protein 2). Homology with rat peroxisomal and mitochondrial 3-oxoacyl-CoA thiolases.
72. Seedorf, U., Brysch, P., Engel, T., Schrage, K. & Assmann, G. (1994) *J. Biol Chem.* **269**, 21277–21283. Sterol carrier protein X is peroxisomal 3-oxoacyl coenzyme A thiolase with intrinsic sterol carrier and lipid transfer activity.
73. Wanders, R.J.A., Denis, S., Wouters, F., Wirtz, K.W. & Seedorf, U: (1997) *Biochem. Biophys. Res. Commun.* **236**, 565–569. Sterol carrier protein X (SCPx) is a peroxisomal branched-chain β-ketothiolase specifically reacting with 3-oxo-pristanoyl-CoA: a new, unique role for SCPx in branched-chain fatty acid metabolism in peroxisomes.
74. Van-Veldhoven, P.P., Croes, K., Asselberghs, S., Herdewijn, P. & Mannaerts, G.P. (1996) *FEBS Lett.* **388**, 80–84. Peroxisomal **β-oxidation** of 2-methyl-branched acyl-CoA esters: stereospecific recognition of the 2S-methyl compounds by trihydroxycoprostanoyl-CoA oxidase and pristanoyl-CoA oxidase.
75. Lough, A.K. (1973) *Prog. Chem. Fats Other Lipids* **14**, 1–48. The chemistry and biochemistry of phytanic, pristanic and related acids.
76. Schmitz, W., Fingerhut, R. & Conzelmann, E. (1994) *Eur. J. Biochem.* **222**, 313–323. Purification and properties of an **α-methylacyl-CoA** racemase from rat liver.
77. Schmitz, W., Albers, C., Fingerhut, R. & Conzelmann, E. (1995) *Eur. J. Biochem.* **231**, 815–822. Purification and characterization of an α-methylacyl-CoA racemase from human liver.
78. Van Veldhoven, P.P., Croes, K., Casteels, M. & Mannaerts, G.P. (1997) *Biochim. Biophys. Ada.* **1347**, 62–68. 2-Methylacyl racemase: a coupled assay based on the use of pristanoyl-CoA oxidase/peroxidase and reinvestigation of its subcellular distribution in rat and human liver.
79. Schmitz, W. & Conzelmann, E. (1997) *Eur. J. Biochem.* **244**, 434–440. Stereochemistry of peroxisomal and mitochondrial **β-oxidation** of **α-methylacyl-CoAs.**
80. Gustafsson, J. & Sjöstedt, S. (1978) *J. Biol. Chem.* **253**, 199–201. On the stereospecificity of microsomal 26-hydroxylation in bile acid biosynthesis.
81. Shefer, S., Cheng, F.W., Batta, A.K., Dayal, B., Tint, G.S. & Salen, G. (1978) *J. Clin. Invest.* **62**, 539–545. Biosynthesis of chenodeoxycholic acid in man: stereospecific side-chain hydroxylations of 5ß-cholestane-3α,7α-diol.
82. Pedersen, J.I., Veggan, T. & Bjorkhem, I. (1996) *Biochem. Biophys. Res. Commun.* **224**, 37–42. Substrate stereospecificity in oxidation of (25S)-**3α,7α, 12α-trihydroxy-5β-cholestanoyl-CoA** by peroxisomal trihydroxy- **5β-cholestanoyl-CoA** oxidase.
83. Schmitz, W., Helander, H.M., Hiltunen, J.K.. & Conzelmann, E. (1997) *Biochem. J.* **326**, 883–889. Molecular cloning of cDNA species for rat and mouse liver α-methylacyl-CoA racemases
84. Reichel, C., Brugger, R., Bang, H., Geisslinger, G. & Brune, K. (1997) *Mol. Pharmacol.* **51**, 576–582. Molecular cloning and expression of a 2-arylpropionyl-coenzyme A epimerase: a key enzyme in the inversion metabolism of Ibuprofen.
85. Wanders, R.J.A., Schutgens, R.B.H. & Barth, P.O. (1995) *J. Neuropathol. Exp. Neural.* **54**, 726–739. Peroxisomal disorders: a review.

HEPATIC α-OXIDATION OF PHYTANIC ACID

A Revised Pathway

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1. ABSTRACT

Synthetic 3-methyl-branched chain fatty acids were used to decipher the breakdown of phytanic acid. Based on results obtained in intact or permeabilized rat hepatocytes, rat liver homogenates or subcellular fractions, a revised α-oxidation pathway is proposed which appears to be functioning in man as well. In a first step, the 3-methyl-branched chain fatty acid is activated by an acyl-CoA synthetase. This reaction requires CoA, ATP and Mg^{2+}. Subsequently, the acyl-CoA ester is hydroxylated at position 2 by a peroxisomal dioxygenase. This step is dependent on α-oxoglutarate, ascorbate (or glutathione), Fe^{2+} and O_2. The 2-hydroxy-3-methylacyl-CoA intermediate is cleaved by a peroxisomal lyase to formyl-CoA and a 2-methyl-branched fatty aldehyde. Formyl-CoA is (partly enzymically) hydrolyzed to formate, which is then converted, most likely in the cytosol, to CO_2. In the presence of NAD^+, the aldehyde is dehydrogenated to a 2-methyl-branched fatty acid, presumably by a peroxisomal aldehyde dehydrogenase. This acid can—after activation—be degraded via a D-specific peroxisomal β-oxidation system.

2. INTRODUCTION

In 1963, Klenk and Kahlke[1] identified the lipid accumulating in Refsum's disease as phytanic acid (3,7,11,15-tetramethylhexadecanoic acid). As a result, phytanic acid is probably the best known 3-methyl-branched chain fatty acid. Over the subsequent 30 years, many groups have tried to elucidate the breakdown of phytanic acid. The presence of the 3-methyl group in phytanic acid prevents its degradation via β-oxidation, the

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normal catabolic pathway for straight or 2-methyl-branched fatty acids. The pioneering work of Steinberg and associates showed that the major route for phytanic acid removal is via α-oxidation.[2] In this process phytanic acid is shortened by one carbon atom to pristanic acid, which is further degraded by peroxisomal β-oxidation.[3,4] Until recently, the actual α-oxidation steps were not well characterized. A lot of controversy can be found in the literature with regard to reaction intermediates, cofactor requirements and subcellular localization. In most text books a scheme, depicting a hydroxylation in the 2-position followed by an oxidative decarboxylation generating CO_2 and pristanic acid, is shown.[2] Nevertheless, the existence of a 2-hydroxy intermediate was not generally accepted.[5–9] Also the occurrence of 2-oxophytanic acid, the putative oxidation product of 2-hydroxyphytanic acid,[2] was subject to dispute.[6,10,11,12] This oxo-derivative can be formed by the long chain α-hydroxy acid oxidase,[13] but this enzyme was assumed to be present only in renal peroxisomes. Apparently, in rat and human liver a low activity is detectable.[12]

As for the cofactors required, even more confusion existed. The simplest combination reported was ATP-Mg^{2+};[14,15] the most complex mixtures included ATP, NAD^+, NADPH or nicotinamide, O_2, Mg^{2+}, and Fe^{3+} or Fe^{2+}.[6,16]

Needless to say that depending on the actual assay conditions, the α-oxidation process has been localised to various subcellular sites: mitochondria,[6,7,9,14,17] mitochondria plus cytosol,[16] mitochondria plus peroxisomes[15,18,19] or microsornes.[20] Also, depending on the species investigated, different localizations were reported: mitochondrial in man, peroxisomal in rodents.[21]

In our studies, described below, we have relied on the use of synthetic 3-methyl-branched chain fatty acids (3-MBFA) such as 3-methylheptadecanoic and 3 -methylhexadecanoic acid. In rat hepatocytes[20] and human skin fibroblasts[22,23,24] these substrates have been shown to be valid substitutes for phytanic acid. The advantages of this approach are several fold. Obviously, these fatty acids, as well as the intermediary products, are easier to synthesize and the introduction of a label at a certain position is less cumbersome. Since these compounds are not endogenous, the identification of possible intermediates and products is greatly facilitated. Furthermore, the validity of our findings is underlined by reports from other groups[25,26] that used phytanic acid and reached similar conclusions.

In retrospect, following major findings have contributed to the elucidation of phytanic α-oxidation.

1. Phytanic acid accumulation is not only seen in Refsum's disease, but also in several peroxisomal disorders[27,28] suggesting a link between α-oxidation and peroxisomes.
2. Compared to intact cells, α-oxidation rates were severely depressed in broken systems,[20] a finding that was later confirmed by others.[15] This fact casted doubt about the validity of earlier results obtained in broken preparations.
3. As shown by Poulos and coworkers,[24] in addition to CO_2, formate is produced by fibroblasts supplied with phytanic acid.

3. STUDIES ON α-OXIDATION IN RAT LIVER

In Fig. 1, a revised pathway for the α-oxidative degradation of phytanic acid is shown. The different reactions depicted are based on experimental data obtained in rat

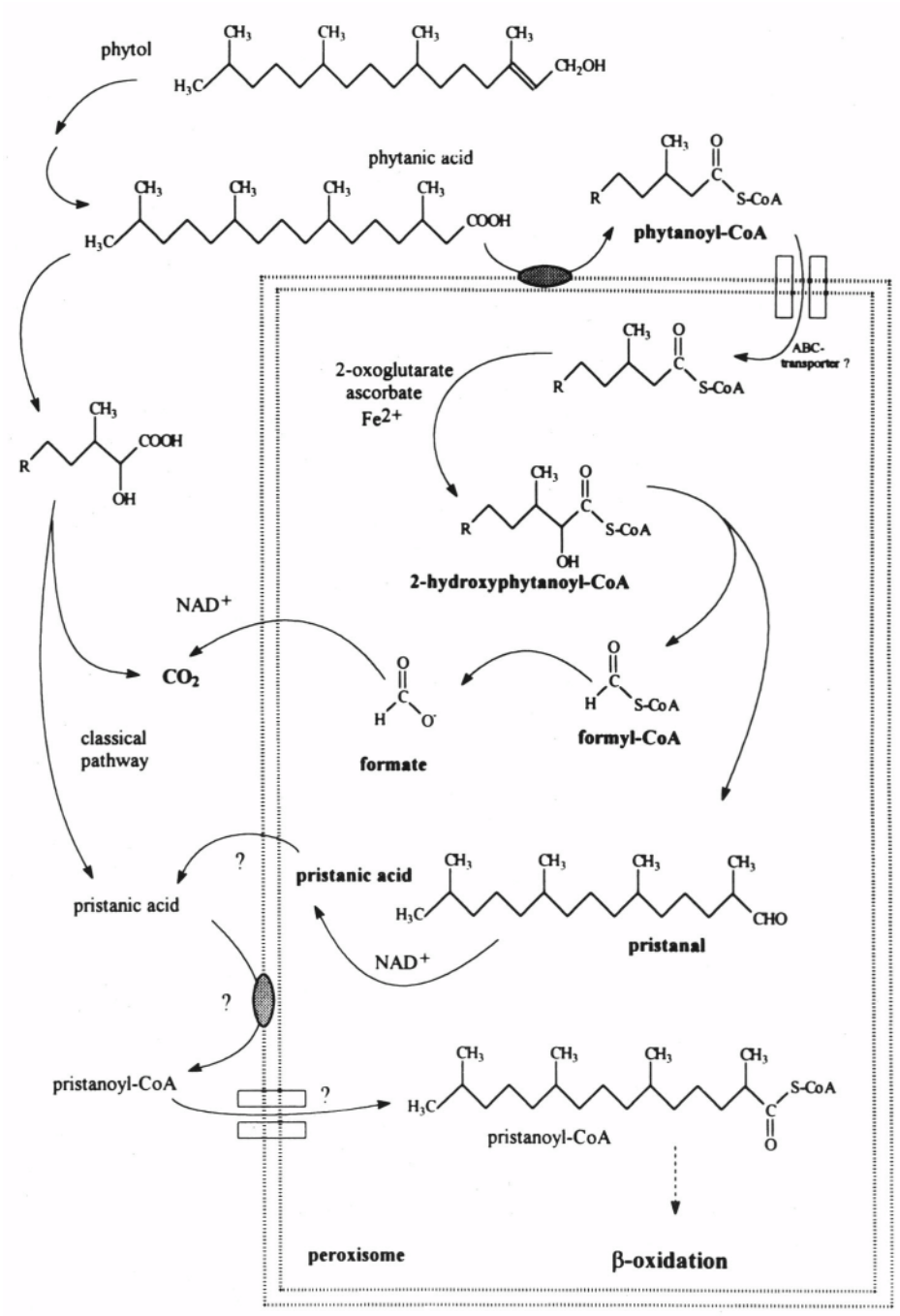

Figure 1. Classical versus revised pathway for α-oxidation of phytanic acid.

hepatocytes, either intact or permeabilized, and in homogenates, subcellular fractions or purified peroxisomes from rat liver. Some of the different steps are now described in more detail.

On the left, the intermediates formed during a-oxidation of phytanic acid accord-

ing to the classical pathway as proposed by Steinberg *et al.*[2] are shown. On the right, a revised pathway which is confined to peroxisomes (boxed area), is presented. Intermediates and products, the occurrence of which was experimentally proven using synthetic 3-methyl-branched analogues, are indicated in bold. Presumably, phytanic acid is activated at the cytosolic side of the peroxisomal membrane. For the translocation of the formed CoA-ester, in analogy to the situation for (very) long chain acyl-CoAs,[29] the involvement of an ABC-transporter is postulated. Hydroxylation seem to be a membrane bound process, whereas the cleavage reaction, giving rise to formyl-CoA and pristanal, is localized in the matrix. Formyl-CoA is hydrolysed to formate that will leave the peroxisomes to be further oxidized in the cytosol to CO_2. Pristanal is oxidized to pristanic acid by a (presumably peroxisomal) NAD-dependent aldehyde dehydrogenase, but the sequence of further steps leading to pristanoyl-CoA is not clear yet.

3.1. Formate is the Precursor of CO_2

As found initially in human skin fibroblasts,[24] formate is also produced in other cell types such as rat hepatocytes,[30] human HepG2 hepatoma cells and canine MDCK cells during the degradation of 3-MBFAs (unpublished data). The ratio of formate to CO_2 production can differ, however, being high in flbroblasts (about 10)[24] and low in rat hepatocytes (about 0.3–0.4).[30] As a consequence, both products should be determined in order to obtain valid data. Addition of unlabelled formate to isolated rat hepatocytes incubated with 3-methyl-[1-^{14}C]heptadecanoate decreased the generation of radioactive CO_2. This was accompanied by a compensatory increase in radioactive formate.[31] This clearly indicates that formate is formed first and subsequently converted to CO_2.

Two pathways have been described for formate metabolism: a peroxidatic pathway via catalase and a folate dependent one carbon pathway.[32] Treatment of rats with aminotriazole, an irreversible inhibitor of catalase, severely depressed α-oxidation activity but only slightly decreased the production of CO_2 from exogenously added formate.[30] Whether the effect of aminotriazole is (solely) linked to the inhibition of catalase is unclear (see further). In addition to these above mentioned pathways our data point to a cytosolic NAD^+-dependent dehydrogenase activity that acts on the formate produced during **α-oxidation**. In permeabilized cells or broken systems, supplied with the appropriate cofactors (see further), almost no CO_2 is formed, during **α-oxidation** unless NAD^+ is added.[31]

3.2. Iron Plays a Role in the α-Oxidation Process

In various studies in broken systems iron ions have been implicated as a cofactor,[6,16,20] although the effective concentrations cannot be considered as physiological. Treatment of intact rat hepatocytes with the iron chelators, desferrioxamine or *o*-phenanthroline, caused a suppression of **α-oxidation,** but mitochondrial or peroxisomal **β-oxidation** were not affected.[33] The effect on **α-oxidation** could be reversed, but only partially, by the addition of Fe^{3+}.[33] It is likely that Fe^{3+} ions do not readily permeate the hepatocyte membranes, explaining the incomplete restoration. This is in agreement with the almost complete restitution of **α-oxidation** rates by fortifying the intact cells with iron-saturated chicken ovotransferrin.[33] Addition of Fe^{3+} to permeabilized cells resulted in a virtually complete reversion of the chelator dependent inhibition.[33] As discussed below, the dependency of **α-oxidation** on iron ions is due to a hydroxylation step.

3.3. Activation and Hydroxylation are Required for α-Oxidation

Knowing that damaging the integrity of cells impaired the α-oxidation process,[20] a search for possible cofactors was initiated in rat hepatocytes permeabilized with *Staphylococcus aureus* toxin. In such systems, the intracellular environment can be varied experimentally but the integrity of the intracellular membranes is conserved.[34]

Addition of ATP, Mg^{2+}, CoA and Fe^{2+} to permeabilized cells resulted in α-oxidative rates comparable to those in intact cells.[31] In the presence of reducing agents, such as glutathione (a compound needed to keep the permeabilized hepatocytes viable), Fe^{3+} could replace Fe^{2+}.[31] Given the reports about a possible 2-hydroxy intermediate[5,8] and the presence of γ-butyrobetaine hydroxylase in peroxisomes,[35] the role of cofactors (such as ascorbic acid and 2-oxoglutarate) for dioxygenases catalyzing hydroxylation reactions[36] was verified. By replacing the glutamate (a normal constituent of the incubation medium of the permeabilized cells), by mannitol the extra requirement for 2-oxoglutarate became evident.[31] In rat liver homogenates, the dependency on ascorbate (or glutathione)—in addition to 2-oxoglutarate, ATP, Mg^{2+}, CoA, and Fe^{2+}—was revealed.[31] Apparently, this complex mixture represents the cofactors for two different reactions. An activation step requiring ATP, CoA and Mg^{2+} and a hydroxylation step depending on Fe^{2+}, 2-oxoglutarate and ascorbate (or another reducing agent). In order to oxidize the formed formate NAD^+ is also required.[31]

Analysis of reaction intermediates showed, as expected, the formation of a 3-methyl-branched chain acyl-CoA (3-MBA-CoA) in the presence of ATP—Mg^{2+}—CoA.[31] No hydroxylated fatty acid could be found when only the cofactors for hydroxylation were present. However, if both sets of cofactors were present, in addition to 3-MBA-CoA, another CoA-derivative was formed. Further purification and GC-MS analysis identified this intermediate as a 2-hydroxy-3-methyl-branched acyl-CoA.[31] This was consistent with the findings of Mihalik *et al.*[25] who showed that 2-hydroxyphytanoyl-CoA was formed in purified rat liver peroxisomes in the presence of the mentioned cofactors. With the activated substrate, only the hydroxylation cofactors were needed to form the hydroxylated intermediate and formate.[31]

Hence, activation precedes hydroxylation. Although 3-MBFA can be activated at different cellular sites—mitochondria, peroxisomes and endoplasmic reticulum[37,38]—only the peroxisomal activity seems to supply a precursor for the hydroxylation reaction.[31] Whether the acyl-CoA synthetase acting on phytanic acid is the same as the one activating palmitic acid and pristanic acid is a matter of controversy.[37–39] Anyway, the requirement for an activation step explains the inhibition of α-oxidation by fenoprofen,[31] an inhibitor of long chain fatty acid activation,[40] and by different fatty acids (unpublished data), presumably due to competitive inhibition or by depletion of the available CoA.

The role of a hydroxylation step is consistent with the effects of iron chelators[33] and anti-oxidants such as propylgallate[31] seen intact cells. So far, the hydroxylated 3-MBA-CoA intermediate has been demonstrated only in broken systems and was never seen in intact cells. This could suggest that this intermediate is accumulating and not further degraded. Control experiments however showed that racemic 2-hydroxy-3-methyl-[1-^{14}C]hexadecanoyl-CoA, when incubated with rat liver homogenates, gives rise to formate and that this process is peroxisomal.[41] Pretreatment of the rats with aminotriazole did not affect the formate production from 2-hydroxy-3-methyl-[1-^{14}C]hexadecanoyl-CoA in liver homogenates.[41] However, when 3-methyl-[1-^{14}C]hexadecanoic acid was used

as substrate in the presence of the above mentioned cofactors, the formation of 2-oxidation products and of 2-hydroxy-3-methyl acyl-CoA was suppressed by 49 and 63%, respectively.[30] This indicates that aminotriazole inhibits the hydroxylation reaction. Perhaps this can be explained by the inhibition of catalase since this enzyme seems to play a role in other dioxygenase-catalyzed hydroxylation reactions.[36]

3.4. The Intermediary 2-Hydroxy-3-Methyl-Branched Acyl-CoA is Cleaved into Formyl-CoA and a 2-Methyl-Branched Fatty Aldehyde

Although in loading studies formation of 2-hydroxyphytanic acid has been shown,[42] no evidence for the presence or generation of a hydroxylated 3-MBFA was obtained in our studies. This fact, together with the apparent absence of CoA-esters other than 3-methylacyl-CoA and its hydroxylated derivative, led us to analyse in more detail the water soluble α-oxidation products in order to reveal CoA-containing compounds. Indeed, upon incubation of purified peroxisomes with 2-hydroxy-3-methyl-[1-^{14}C]hexadecanoyl-CoA, an unknown labeled alkali-sensitive compound, eluting on reversed phase columns between CoA and acetyl-CoA, was discovered.[43] This compound was identified as formyl-CoA.[43] Control experiments revealed that the formyl-CoA did not arise by activation of formate, and that formyl-CoA is hydrolysed, partly actively, in peroxisomes to formate.[43] So far formyl-CoA hydrolases have been described only in micro-organisms.[44]

The finding of formyl-CoA, and not formate, as end-product of the α-oxidation process directly suggested that the second product might be a fatty aldehyde and not a fatty acid as assumed till then. GC-analysis of the lipophilic products formed by peroxisomes incubated with 2-hydroxy-3-methylhexadecanoyl-CoA showed an unknown metabolite, co-eluting with synthetic 2-methylpentadecanal.[43] If NAD^+ was present, the aldehyde was converted almost completely into 2-methylpentadecanoic acid,[45] presumably by a peroxisomal aldehyde dehydrogenase.[46]

4. STUDIES ON α-OXIDATION IN HUMAN LIVER

Similarly to the studies in rat liver, α-oxidation in human liver homogenates depended on the presence of cofactors for activation and for hydroxylation.[47] In agreement with other reports,[26] formation of 2-hydroxy-3-methyl-branched chain acyl-CoA was confined to subcellular fractions enriched in peroxisomes.[47] Verhoeven *et al.*[48] showed that pristanal is formed in human liver homogenates incubated with phytanic acid, confirming our findings in rat liver peroxisomes.[45]

Since peroxisomal phytanoyl-CoA synthetase is not affected in Refsum's disease patients,[38,49] the basic defect must be further downstream, most likely at the level of the phytanoyl-CoA hydroxylase. Enzyme measurements revealed a hydroxylase deficiency, not only in Refsum's disease[50] but also in Zellweger syndrome[26] and rhizomelic chondrodysplasia punctata.[51] Recently, the cloning of this enzyme, together with mutation analysis in some Refsum's patients, was reported.[52,53] The presence of a peroxisome targeting signal 2 in the hydroxylase explains its deficiency in rhizomelic chondrodysplasia punctata. Whether the reported presence of 2-hydroxyphytanic acid in plasma of patients with Zellweger syndrome and rhizomelic chondrodysplasia punctata[11] is due to residual activity of the mislocalized hydroxylase or reflects the existence of some alternative pathway, awaits further investigation.

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REFERENCES

1. Klenk, E. & Kahlke, W. (1963) *Hoppe-Seyler's Z. Physiol. Chem.* **333**, 133–139. Uber das Vorkommen der 3,7,11,15-Tetramethylhexadecansaure (Phytansaure) in den Cholesterinestern und andern Lipoidfraktionen der Organe bei einem Kjankheitsfall unbekannter Genese (Verdacht auf Heredopathia atactica polyneuritiformis, Refsum's syndrom).
2. Steinberg, D. (1989). *In*: The metabolic basis of inherited disease (Scriver, C.R., Beaudet, A.L., Sly, W.S. & Valle, D., Eds.) Me Graw-Hill, New York. pp. 1533–1150. Refsum's Disease.
3. Mannaerts, G.P. & Van Veldhoven, P.P. (1996) *Ann. N. Y. Acad. Sci.* **804**, 99–115. Functions and organization of peroxisomal β-oxidation.
4. Van Veldhoven, P.P. & Mannaerts. G.P. (1998) (next chapter). Role and organisation of peroxisomal β-oxidation systems in mammals.
5. Tsai, S.-C, Herndon, J.H.Jr., Uhlendorf, B.W., Fales, H.M. & Mize, C.E. (1967) *Biochem. Biophys. Res. Commun.* **28**, 571–577. The formation of α-hydroxyphytanic acid from phytanic acid in mammalian tissues.
6. Tsai, S.-C., Avigan, J. & Steinberg, D. (1969) *J. Biol. Chem.* **244**, 2682–2692. Studies of the α-oxidation of phytanic acid by rat liver mitochondria.
7. Tsai, S.-C., Steinberg, D., Avigan, J. & Fales, H.M. (1973) *J. Biol. Chem.* **248**, 1091–1097. Studies on the stereospecificity of mitochondrial oxidation of phytanic acid and of α-hydroxyphytanic acid,
8. ten Brink, H.J., Schor, D.S.M., Kok, R.M., Stellaard, J.K., Kneer, J., Poll-The, B.T., Saudubray, J.M. & Jakobs, C. (1992) *Pediatr. Res.* **32**, 566–570. *In vivo* study of phytanic acid α-oxidation in classic Refsum's disease and chondrodysplasia punctata.
9. Skjeldal, O.H. & Stokke, O. (1988) *Scand J. Clin. Lab. Invest.* **48**, 97–102. Evidence against α-hydroxyphytanic acid as an intermediate in the metabolism of phytanic acid.
10. Mize, C.E., Avigan, J., Steinberg, D., Pittman, R.C., Fales, H.M. & Milne, G.W.A. (1969) *Biochim. Biophys. Acta* **176**, 720–739. A major pathway for the mammalian oxidative degradation of phytanic acid.
11. ten Brink, H.J., Schor, D.S.M., Kok, R.M., Poll-The, B.T., Wanders, R.J.A. & Jakobs, C. (1992) *J. Lipid Res.* 33, 1449–1457. Phytanic acid α-oxidation: accumulation of 2-hydroxyphytanic acid and absence of 2-oxophytanic acid in plasma from patients with peroxisomal disorders.
12. Wanders, R.J.A., van Roermund, C.W., Schor, D.S., ten Brink, H.J. & Jakobs, C. (1994) *Biochim. Biophys. Acta* **1227**, 177–182. 2-Hydroxyphytanic acid oxidase activity in rat and human liver and its deficiency in the Zellweger syndrome.
13. Draye, J.-P., Van Hoof, F., de Hoffmann, E. & Vamecq, J. (1987) *Eur. J. Biochem.* **167**, 573–578. Peroxisomal oxidation of L-2-hydroxyphytanic acid in rat kidney cortex.
14. Skjeldal, O.H. & Stokke, O. (1987) *Biochim. Biophys. Acta* **921**, 38–42. The subcellular localisation of phytanic acid oxidase in rat liver.
15. Wanders, R.J.A. & van Roermund, C.W. (1993) *Biochim. Biophys. Acta* **1167**, 345–350. Studies on phytanic acid α-oxidation in rat liver and cultured human skin fibroblasts.
16. Muralidharan, V.B. & Kishimoto, Y. (1984) *J. Biol. Chem.* **259**, 13021–13026. Phytanic acid α-oxidation in rat liver: Requirement for a cytosolic factor.
17. Watkins, P.A. & Mihalik, S.J. (1990) *Biochem. Biophys. Res. Commun.* **167**, 580–586. Mitochondrial oxidation of phytanic acid in human and monkey liver: implication that Refsum's disease is not a peroxisomal disorder.
18. Mihalik, S.J., Solimúan, T.M., Day, R.F. & Watkins, P.A. (1992) *Prog. Clin. Biol. Res.* **375**, 239–244. Involvement of both peroxisomes and mitochondria in the α-oxidation of phytanic acid. In: New developments in fatty acid oxidation (Coates, P.M. & Tanaka, K., Eds.) Wiley-Liss, New York.
19. Fingerhut, R., Schmitz, W. & Conzelmann, E. (1993) *J. Inher. Metab. Dis.* **16**, 591–594. Accumulation of phytanic acid α-oxidation intermediates in Zellweger fibroblasts.

20. Huang, S., Van Veldhoven, PP., Vanhoutte, F., Parmentier, G., Eyssen, H.J. & Mannaerts, G.P. (1992) *Arch. Biochem. Biophys.* **296**, 214–223. α-Oxidation of 3-methyl-substituted fatty acids in rat liver.
21. Singh, I., Pahan, K., Dhaunsi, G.S., Lazo, O. & Ozand, P. (1993) *J. Biol. Chem.* **268**, 9972–9979. Phytanic acid α-oxidation. Differential subcellular localization in rat and human tissues and its inhibition by Nycodenz.
22. Singh, H., Usher, S., Johnson, D. & Poulos, A. (1990) *J. Inker. Metab. Dis.* **13**, 387–389. Metabolism of branched chain fatty acids in peroxisomal disorders.
23. Van Veldhoven, P.P., Huang, S., Eyssen, H.J. & Mannaerts, G.P. (1993) *J. Inker. Metab. Dis.* **16**, 381–391. The deficient degradation of synthetic 2- and 3-methyl-branched fatty acids in fibroblasts from patients with peroxisomal disorders.
24. Poulos, A., Sharp, P., Singh, H., Johnson, D.W., Carey, W.F. & Easton, C. (1993) *Biochem. J.* **292**, 457–461. Formic acid is a product of the α-oxidation of fatty acids by human skin fibroblasts: deficiency of formic acid production in peroxisome-deficient fibroblasts.
25. Mihalik, S.J., Rainville, A.M. & Watkins, P.A. (1995) *Em J. Biochem.* **232**, 545–551. Phytanic acid α-oxidation in rat liver peroxisomes. Production of a-hydroxyphytanoyl-CoA and formate is enhanced by dioxygenase cofactors.
26. Jansen, G.A., Mihalik, S.J., Watkins, P.A., Moser, H.W., Jakobs, C., Denis, S. & Wanders, R.J.A. (1996) *Biochem. Biophys. Res. Commun.* **229**, 205–210. Phytanoyl-CoA hydroxylase is present in human liver, located in peroxisomes, and deficient in Zellweger syndrome: direct, unequivocal evidence for the new, revised pathway of phytanic acid α-oxidation in humans.
27. Poulos, A., Sharp, P., Fellenberg, A.J. & Danks, D.M. (1985) *Hum. Genet.* **70**, 172–177. Cerebro-hepato-renal (Zellweger) syndrome, adrenoleukodystrophy, and Refsum's disease: plasma changes and skin fibroblast phytanic acid oxidase.
28. Poulos, A., Sharp, P., Fellenberg, A.J. & Johnson, D.W. (1988) *Eur. J. Pediatr.* **147**, 143–147. Accumulation of pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) in the plasma of patients with generalised peroxisomal dysfunction.
29. Hettema, E.H., van Roermund, C.W., Distel, B., van den Berg, M., Vilela, C., Rodrigues-Pousada, C., Wanders, R.J.A. & Tabak, H.F. (1996) *EMBO J.* **15**, 3813–3822. The ABC transporter proteins Patl and Pat2 are required for import of long-chain fatty acids into peroxisomes of *Saccharomyces cerevisiae*.
30. Casteels, M., Croes, K., Van Veldhoven, P.P. & Mannaerts, G.P. (1994) *Biochem. Pharmacol.* **48**, 1973–1975. Aminotriazole is a potent inhibitor of α-oxidation of 3-methyl-substituted fatty acids in rat liver.
31. Croes, K., Casteels, M., de Hoffmann, E., Mannaerts, G.P. & Van Veldhoven, P.P. (1996) *Eur. J. Biochem.* **240**, 674–683. α-Oxidation of 3-methyl-substituted fatty acids in rat liver. Production of formic acid instead of CO_2, cofactor requirements, subcellular localization and formation of a 2-hydroxy-3-methylacyl-CoA intermediate.
32. Palese, M. & Tephly, T.R. (1975) *J. Toxicol. Environ. Health* **1**, 13–24. Metabolism of formate in the rat.
33. Croes, K., Casteels, M., Van Veldhoven, P.P. & Mannaerts, G.P. (1995) *Biochim. Biophys. Acta.* **1255**, 63–67. Evidence for the importance of iron in the α-oxidation of 3-methyl-substituted fatty acids in the intact cell.
34. McEwen, B.F. & Arion, W.J. (1985) *J. Cell Biol.* **100**, 1922–1929. Permeabilization of rat hepatocytes with *Staphylococcus aureus* α-toxin.
35. Paul, H.S., Sekas, G. & Adibi, S.A. (1992) *Eur. J. Biochem.* **203**, 599–605. Carnitine biosynthesis in hepatic peroxisomes. Demonstration of γ-butyrobetaine hydroxylase activity.
36. Hayaishi, O., Nozaki, M. & Abbott, M.T. (1975) In: The Enzymes (Boyer, P.O., Ed.) Academic Press, New York. pp. 119–189. Oxygenases: dioxygenases.
37. Pahan, K., Cofer, J., Baliga, P. & Singh, I. (1993) *FEBS Lett.* **322**, 101–104. Identification of phytanoyl-CoA ligase as a distinct acyl-CoA ligase in peroxisomes from cultured human skin fibroblasts.
38. Vanhooren, J.C., Asselberghs, S., Eyssen, H.J., Mannaerts, G.P. & Van Veldhoven, P.P. (1994) *Int. J. Biochem.* **26**, 1095–1101. Activation of 3-methyl-branched fatty acids in rat liver.
39. Watkins, P.A., Howard, A.E., Gould, S.J., Avigan, J. & Mihalik, S.J. (1996) *J. Lipid Res.* **37**, 2288–2295. Phytanic acid activation in rat liver peroxisomes is catalyzed by long chain acyl-CoA synthetase.
40. Lageweg, W. & Wanders, R.J.A. (1993) *Biochem. Pharmacol.* **46**, 79–85. Studies on the effect of fenoprofen on the activation and oxidation of long chain and very long chain fatty acids in hepatocytes and subcellular fractions from rat liver.

41. Croes, K. (1998) *Acta Biomedica Lovaniensia* 165 (Ph.D. thesis—Leuven University Press). α-Oxidation of 3-methyl-branched fatty acids: A revised pathway.
42. Verhoeven, N.M., Schor, D.S.M., Previs, S.F., Brunengraber, H. & Jakobs, C. (1997) *Eur. J. Pediatr.* **156**, S83–S87. Stable isotope studies of phytanic acid α-oxidation: *in vivo* production of formic acid.
43. Croes, K., Van Veldhoven, P.P., Mannaerts, G.P. & Casteels, M. (1997) *FEBS Lett.* **407**, 197–200. Production of formyl-CoA during peroxisomal α-oxidation of 3-methyl-branched fatty acids.
44. Sly, W.S. & Stadtman, E.R. (1963) *J. Biol. Chem.* **238**, 2632–2638. Formate metabolism. I. Formyl coenzyme A, an intermediate in the formate-dependent decomposition of acetyl-phosphate in *Clostridium kluyveri.*
45. Croes, K., Casteels, M., Asselberghs, S., Herdewijn, P., Mannaerts, G.P. & Van Veldhoven, P.P. (1997) *FEBS Lett.* **412**, 643–645. Formation of a 2-methyl-branched fatty aldehyde during peroxisomal α-oxidation.
46. Antonenkov, V.D., Pirozhkov, S.V. & Panchenko, L.F. (1985) *Eur. J. Biochem.* **149**, 159–167. Intraparticulate localization and some properties of a clofibrate-induced peroxisomal aldehyde dehydrogenase from rat liver.
47. Casteels, M., Croes, K., Van Veldhoven, P.P. & Mannaerts, G.P. (1997) *J. Inker. Metab. Dis.* **20**, 665–673. Peroxisomal localization of α-oxidation in human liver.
48. Verhoeven, N.M., Schor, D.S., ten Brink, H.J., Wanders, R.J.A. & Jakobs, C. (1997) *Biochem. Biophys. Res. Commun.* **237**, 33–36. Resolution of the phytanic acid α-oxidation pathway: Identification of pristanal as product of the decarboxylation of 2-hydroxyphytanoyl-CoA.
49. Pahan, K., Khan, M. & Singh, I. (1996) *J. Lipid Res.* **37**, 1137–1143. Phytanic acid oxidation: normal activation and transport yet defective α-hydroxylation of phytanic acid in peroxisomes from Refsum disease and rhizomelic chondrodysplasia punctata.
50. Jansen, G.A., Wanders, R.J.A., Watkins, P.A. & Mihalik, S.J. (1997) *New Engl. J. Med.* **337**, 133–134. Phytanoyl-coenzyme A hydroxylase deficiency—the enzyme defect in Refsum's disease.
51. Jansen, G.A., Mihalik, S.J., Watkins, P.A., Moser, H.W., Jakobs, C., Heijmans, H.S.A. & Wanders, R.J.A. (1997) *J. Inker. Metab. Dis.* **20**, 444–446. Phytanoyl-CoA hydroxylase is not only deficient in classical Refsum disease but also in rhizomelic chondrodysplasia punctata.
52. Mihalik, S.J., Morrell, J.C., Kim, D., Sacksteder, K.A., Watkins, P.A. & Gould, S.J. (1997) *Nature Genet.* **17**, 185–189. Identification of PAHX, a Refsum disease gene.
53. Jansen, G.A., Oftnan, R., Ferdinandusse, S., Ijlst, L., Muijsers, A.O., Skjeldal, O.K., Stokke, O., Jakobs, C., Besley, G.T.N., Wraith, J.E. & Wanders, R.J.A. (1997) *Nature Genet.* **17**, 190–193. Refsum disease is caused by mutations in the phytanoyl-CoA hydroxylase gene.

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FUNCTIONS AND DYSFUNCTIONS OF PEROXISOMES IN FATTY ACID α- AND β-OXIDATION

New Insights

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1. INTRODUCTION

Peroxisomes are essential subcellular organelles catalyzing a number of different metabolic functions especially related to lipid metabolism. Studies in the last few years have clearly established the indispensable role of peroxisomes in fatty acid β-oxidation, fatty acid α-oxidation, etherphospholipid synthesis, biosynthesis of cholesterol and other isoprenoids and the biosynthesis of polyunsaturated fatty acids. Much of our knowledge about peroxisomes has come from studies on a group of inherited diseases in man in which there is an impairment in one or more peroxisomal functions. The prototype of this group of disorders is the cerebro-hepato-renal syndrome of Zellweger, also called Zellweger syndrome. In these patients morphologically identifiable peroxisomes are missing due to mutations in different genes which are involved in peroxisome biogenesis. Genetic analyses have shown that mutations in at least 10 different genes called *PEX*-genes, can give rise to Zellweger syndrome or any of the other peroxisome biogenesis disorders.[1]

The finding by Brown and coworkers[2] that there was accumulation of very-long-chain fatty acids (notably C26:0) in plasma from Zellweger patients whereas levels of

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other fatty acids including long-chain fatty acids were normal, provided the first clue that the mitochondrial and peroxisomal β-oxidation systems might have different substrate specificities. Subsequent studies indeed revealed that peroxisomes are the primary site of oxidation of very-long-chain fatty acids whereas oxidation of long-chain fatty acids occurs predominantly in mitochondria. Later studies showed that oxidation of pristanic acid and the bile acid intermediates di- and trihydroxycholestanoic acid is also primarily peroxisomal.

The finding that there is accumulation of phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) in plasma from Zellweger patients, suggested that peroxisomes might also play a major role in phytanic acid α-oxidation. The pathway of phytanic acid α-oxidation was unknown, however, and the role of peroxisomes disputed. Studies from different laboratories including our own have now led to a full resolution of the pathway and the role of peroxisomes therein.[3–8] In this paper we will discuss our current state of knowledge about fatty acid α- and β-oxidation in peroxisomes with particular emphasis on the enzymology, transport of metabolites across the membrane and dysfunction in patients suffering from certain peroxisomal disorders.

2. SUBSTRATE SPECIFICITY OF THE PEROXISOMAL FATTY ACID β-OXIDATION SYSTEM: CLUES FROM PATIENTS WITH INHERITED DEFECTS

The conclusion that peroxisomes are involved in the β-oxidation of a distinct set of fatty acids and fatty acid derivatives has largely been derived from studies in patients affected by inherited diseases in which either mitochondrial or peroxisomal β-oxidation is impaired.

Fatty acids oxidized in peroxisomes include:

2.1. Very-Long-Chain Fatty Acids

Oxidation of very-long-chain fatty acids is predominantly if not exclusively peroxisomal, although C26:0-CoA can be oxidized by the mitochondrial β-oxidation system provided the mitochondrial membrane is disrupted. The most likely explanation for this apparent discrepancy is that carnitine palmitoyltransferase I (CPTI) is not reactive with C26:0-CoA so that C26:0 simply can not enter the mitochondrial space.

2.2. Pristanic Acid (2,6,10,14-Tetramethylpentadecanoic Acid)

Pristanic acid is a 2-methyl fatty acid which undergoes exclusive oxidation in the peroxisome. Indeed, pristanic acid oxidation is strongly deficient in Zellweger cells lacking peroxisomes. On the other hand, normal oxidation rates were found in patients with an inherited deficiency of acyl-CoA oxidase, suggesting the existence of a different acyl-CoA oxidase (see later).

Pristanic acid is either derived directly from dietary sources or is produced from phytanic acid via α-oxidation (see later). Studies by Singh *et al.*[9] have clearly shown that pristanoyl-CoA is not a substrate for mitochondrial CPTI.

2.3. Di- and Trihydroxycholestanoic Acid

Di- and trihydroxycholestanoic acid are intermediates in the complex formation of bile acids from cholesterol. The two cholestanoic acids are activated to their CoA-esters at the endoplasmic reticulum membrane[10,11] and undergo β-oxidation to chenodeoxycholoyl-CoA and choloyl-CoA in the peroxisome. This is followed by conjugation with either taurine or glycine to produce chenodeoxytauro- or glycocholate and tauro- or glycocholate, respectively, which can pass the canalicular membrane to end up in the bile fluid.

2.4. Prostaglandins, Leukotrienes and Thromboxanes

Studies by Diczfalusy *et al.*[12] have shown that oxidation of prostaglandin F2α is primarily peroxisomal. This is concluded from both *in vitro* and *in vivo* studies which showed that the major urinary metabolite of prostaglandin F2α i.e. 5α, 7α-dihydroxy-11-keto-tetranorprosta-l,16-dioic acid was virtually absent in urine from Zellweger patients. Similar studies have resolved the major role of peroxisomes in the β-oxidation of N-acetyl-LTE_4[13] and thromboxane B2.[14,15]

2.5. Dicarboxylic Acid Oxidation

Peroxisomes are probably also the main site of dicarboxylic acid oxidation.[16] Dicarboxylic acids are formed from mono-carboxylic acids via initial ω-hydroxylation followed by oxidation of the C-OH-group to an aldehyde and finally an acid. The resulting dicarboxylic acid is activated at the ER-membrane,[17] transported to the peroxisome and chain-shortened in the mitochondria.

Peroxisomes are also involved in the β-oxidation of other fatty acids not described here.

3. D-BIFUNCTIONAL PROTEIN AND STEROL-CARRIER-PROTEIN X (SCPX): NEW INSIGHTS INTO THEIR FUNCTIONAL ROLE FROM STUDIES ON PATIENTS AND MUTANT MICE

3.1. D-Bifunctional Protein

Recent studies from different laboratories have led to the identification of a new peroxisomal multifunctional β-oxidation protein with both enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activity. This protein was first identified by Adamski and coworkers[18] in a systematic study to identify 17β-hydroxysteroid dehydrogenases. The enzyme turned out to be localized in peroxisomes.[19] Subsequent cloning of the cDNA led to the surprising discovery that the encoded protein appeared to have 3 domains, including an enoyl-CoA hydratase, 3-OH-acyl-CoA dehydrogenase and sterol-carrier-protein domain. Studies by Leenders *et al.*[20].established that the 78 kDa protein indeed catalyzes all 3 partial reactions.

Independent studies from several laboratories also led to the identification of this enzyme.[21–26] In 1994 Novikov *et al.*[27] had already found that peroxisomes contain multiple 3-hydroxyacyl-CoA dehydrogenases of which one turned out to be the newly recognized D-bifunctional protein.[21,22] Systematic studies by the group of Hiltunen[25,26] into the

various enoyl-CoA hydratases in rat liver, and the group of Hashimoto[23,24] also led to the identification of this multifunctional protein which has different names including multifunctional protein 2 (MFP 2),[21,22] multifunctional enzyme II (MFE II)[25,26] and D-bifunctional protein.[23,24] A problem with the names multifunctional enzyme or multifunctional protein is that there are many multifunctional enzymes including a mitochondrial multifunctional enzyme involved in β-oxidation.[28] In order to avoid this confusion, we have suggested the name peroxisomal multifunctional β-oxidation protein 2 (pMOP 2).[29]

However, until there is a unified nomenclature we will use the term D-bifunctional protein as suggested by Hashimoto *et al.*[23,24] next to L-bifunctional protein since the name refers to one of the most striking aspects of this enzyme which catalyzes the formation of a D-3-hydroxyacyl-CoA intermediate and not a L-3-hydroxyacyl-CoA ester.

Substrate specificity studies have shown that the two enzymes are quite different although they both accept *trans*-2-enoyl-CoA esters of straight-chain-fatty acids.[20–26] The enoyl-CoA esters of pristanic acid and the bile acid intermediate trihydroxycholestanoic acid are only handled by the D-specific multifunctional enzyme although Qin *et al.*[26] suggested that pristenoyl-CoA might be handled by both enzymes. The recent identification of patients with defects in only the D-bifunctional protein show that D-BP indeed plays a major role in peroxisomal fatty acid oxidation in man. In the patient described by Suzuki *et al.*[30] the D-bifunctional protein was completely lacking whereas in our patient (see Van Grunsven *et al.*[31]) the protein was normally present but functionally inactive at the level of the D-3-hydroxyacyl-CoA dehydrogenase component.

Interestingly, fatty acid oxidation studies in fibroblasts from our patient revealed deficient oxidation of both straight-chain as well as 2-methyl-branched fatty acids (C26 :0 and pristanic acid, respectively). These data suggest that the D-bifunctional protein may also be the primary hydratase/dehydrogenase involved in C26:0 β-oxidation. This is now under active study.

3.2. Sterol-Carrier-Protein X (SCPx)

Until recently it was thought that a single thiolase, identified by Hashimoto and coworkers,[32,33] was responsible for the thiolytic cleavage of all 3-ketoacyl-CoA esters derived from both straight-chain (e.g. C26:0) and 2-methyl branched-chain (e.g. pristanic acid and di- and trihydroxycholestanoic acid) fatty acids. This conclusion was based on the finding that a genetic deficiency of the classic 41 kDa thiolase identified by Miyazawa *et al.*[32,33] as observed in the patient described by Goldfischer *et al.*[34] and Schram *et al.*,[35] was associated with accumulation of both very-long-chain fatty acids and trihydroxycholestanoic acid. In an effort to establish whether there is a distinct thiolase for the 3-ketopristanoyl-CoA esters of pristanic acid and di- and trihydroxycholestanoic acid, we measured the 3-ketopristanoyl-CoA thiolase activity in peroxiomes from control and clofibrate-treated rats.[29] As expected 3-keto-palmitoyl-CoA thiolase activity was greatly stimulated in these peroxisomes whereas there was no stimulation of 3-ketopristanoyl-CoA thiolase activity.[29] In fact, 3-ketopristanoyl-CoA thiolase activity was even reduced in peroxisomes from treated rats. These results argued against a single thiolase accepting both straight- and 2-methyl branched-chain 3-ketoacyl-CoA esters. The question arose then which thiolase catalyzes the thiolytic cleavage of 3-ketopristanoyl-CoA. Apparently it had to be a thiolase not stimulated by clofibrate. A likely candidate was the peroxisomal thiolase identified earlier,[36] as a result of studies on sterol-carrier-protein 2 (SCP 2), alternatively called non-specific lipid-transfer-protein (nsLTP). SCP 2 (nsLTP) is a small

protein of 143 amino acids which undergoes processing to the mature form of SCP 2 containing 123 amino acids. Northern blot analysis using the SCP 2—cDNA as a probe revealed the existence of an additional, much longer, mRNA. This larger transcript codes for a 58kDa protein containing a thiolase domain (amino acids 1–404) plus, a sterol-carrier-protein domain (amino acids 405–547) (see[37] for review). The carboxyl terminus of SCPx ends in alanine-lysine-leucine which is an established peroxisome-targeting-signal Type 1 (CPT 1 (see[38] for review). After import into peroxisomes the 58 kDa may be proteolytically cleaved to produce a 46 kDa thiolase plus 123 amino acid SCP 2. The human SCPx-gene has been characterised at the molecular level,[39,40] spans 80kb and contains 16 exons. Transcription can start at two sites controlled by specific promoters in intron 1 and 12, respectively[40] (Fig. 1). The larger transcript codes for the complete 58 kDa thiolase/SCP (SCPx) whereas the smaller transcript codes for the 143 amino acid SCP 2. Seedorf *et al.*[36] showed that the 58kDa SCPx expressed in *E. Coli* indeed displayed thiolase activity. Remarkably, substrate specificity studies showed that SCPx was comparable to the classic Hashimoto-thiolase[32,33] although studies were restricted to the 3-ketoacyl-CoA esters of straight-chain fatty acids. Our recent studies[29] and those of others,[41,42] however, clearly show that the two thiolases are quite distinct when the reactivity with 3-ketoacyl-CoA esters of 2-methyl branched-chain fatty acids is concerned since the classic 41 kDa thiolase is practically inactive with these substrates in contrast to SCPx which readily acts on the 3-ketoacyl-CoAs of pristanic acid and trihydroxycholestanoic acid. Since all these studies are *in vitro* studies making use of isolated enzymes and/or cell homogenates, it is important to verify these conclusions by independent studies. In this respect it is very important that Seedorf *et al.*[43] recently succeeded in creating a SCPx knock-out mouse. Several lines of evidence indicate that disruption of the SCPx-gene leads to a deficient oxidation of 2-methyl fatty acids whereas oxidation of straight-chain fatty acids appeared normal. This is concluded from (i) fatty acid oxidation studies in fibroblasts from SCPx (–/–) mice, (ii) measurement of metabolites in plasma from these mice and (iii) loading studies with phytol. Taken together, these data show that SCPx plays an indispensible role in the peroxisomal oxidation of branched-chain fatty acids and that there are separate pathways for the oxidation of straight-chain and branched-chain fatty acids in peroxisomes (see Fig. 2).

4. PEROXISOMES: A CLOSED COMPARTMENT IMPLYING THE EXISTENCE OF METABOLITE TRANSPORTERS

Although long disputed, current evidence suggests that peroxisomes are closed compartments under *in vivo* conditions which requires the existence of metabolite transporters as described for other organelles. Another consequence of the peroxisome being a closed compartment is that the peroxisome must have a system allowing continued reoxidation of NADH to NAD^+. Ultimately, the NADH has to end up in the mitochondria for aerobic oxidation in the respiratory chain with molecular oxygen as terminal acceptor of reducing equivalents. Transport of cytosolically formed NADH into mitochondria is mediated by so-called NAD(H) redox shuttles which include the malate/aspartate shuttle and the glycerolphosphate/dihydroxyacetonephosphate shuttle. Recent studies in the yeast *S. cerevisiae* have revealed the existence of such a NAD(H) redox shuttle (see Van Roermund *et al.*[44] which remains incompletely understood but at least involves the cytosolic and peroxisomal forms of malate dehydrogenase. Baumgart and coworkers[45] have suggested another NAD(H) redox shuttle in rat liver peroxisomes.

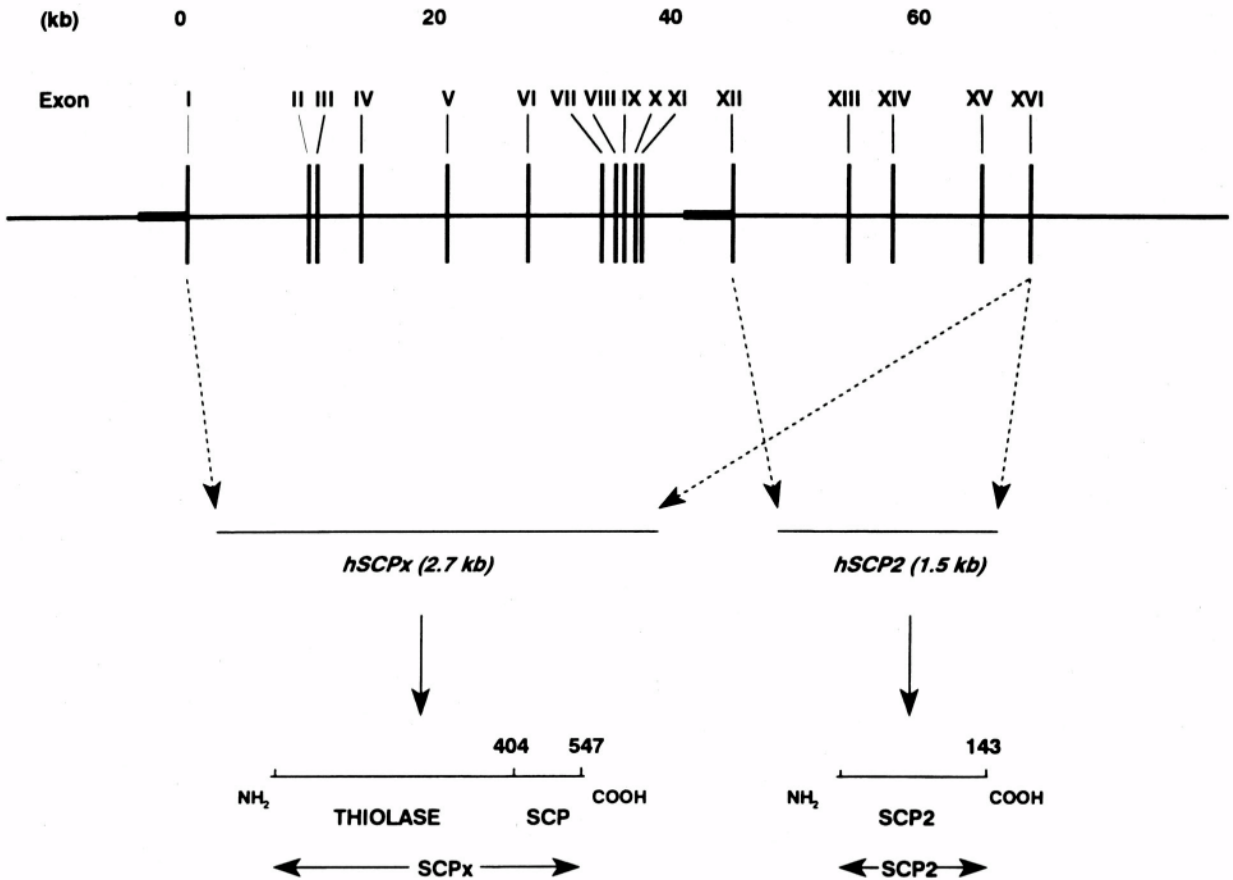

Figure 1. Structure of the human SCPx/SCP2 gene. Exons are respresented by vertical lines. The two promotor regions which drive formation of the 2,8 kb mRNA encoding SCPx and a 1.5 kb mRNA encoding SCP2, are indicated by thick horizontal lines. See Ohba *et al.*[39,40] for detailed information.

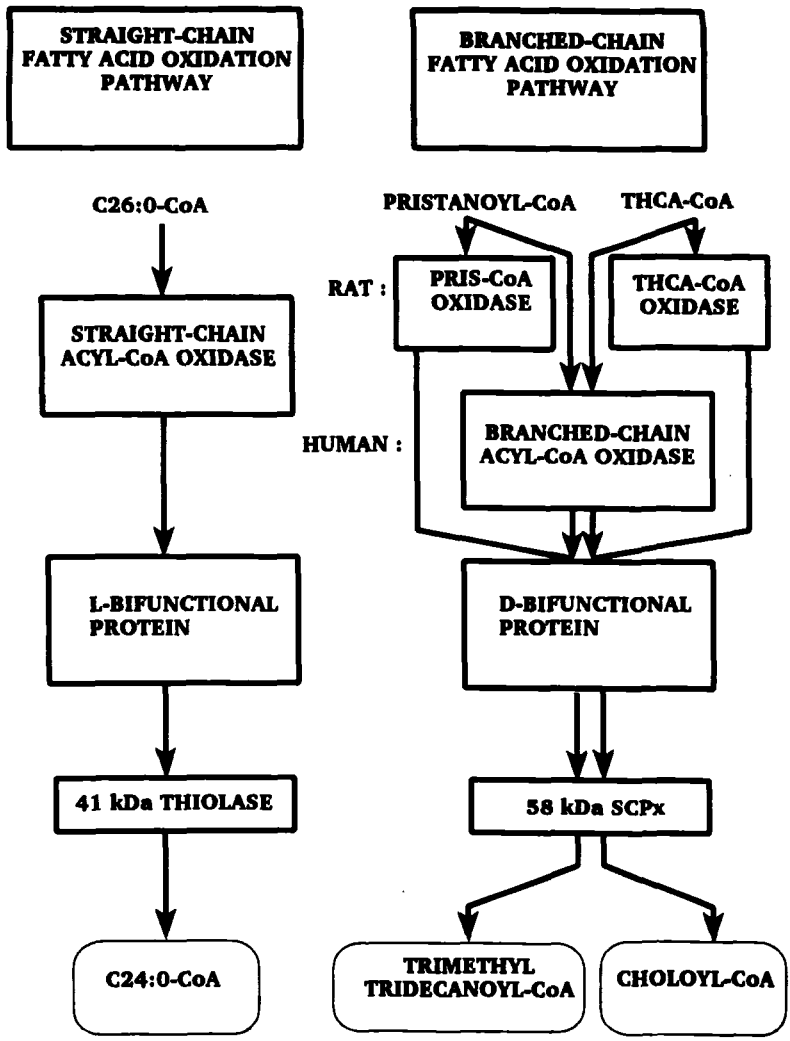

Figure 2. Proposed structure of the peroxisomal fatty acid β-oxidation machinery with separate enzyme systems for the oxidation of straight-chain and branched-chain fatty acids. See text for more detailed information.

Peroxisomes in the yeast *S. cerevisiae* are the only site of β-oxidation and furthermore, this yeast species is able to degrade a range of polyunsaturated fatty acids with double bonds at both even and uneven positions which requires the participation of 2,4-dienoyl-CoA reductase, an NADPH-requiring reaction. Oxidation of polyunsaturated fatty acids thus requires continued reduction of NADP to NADPH. Independent studies by ourselves (Van Roermund *et al.*[46]) and others (Henke *et al.*[47]) have identified the peroxisomal isoform of NADP-linked isocitrate dehydrogenase as an essential component of such a NADP(H) redoxshuttle which requires full elucidation in the future.

The concept of a peroxisome being a closed compartiment also requires transport systems for fatty acids which undergo oxidation in the peroxisome. Since oxidation of fatty acids in peroxisomes is incomplete, the peroxisome must also have systems to allow export of chain-shortened fatty acids. Recent evidence notably in yeast suggests that

uptake of long-chain fatty acids into peroxisomes is mediated by Pat 1p and Pat 2p,[48] alternatively named Pxa 1/2[49,50] or Pal 1/2.[51] These proteins are so-called half-transporters belonging to the family of ABC-transporters. Since peroxisomes lack the essential elements of a carnitine cycle such as carnitine: long-chain acyltransferase activity, transport probably does not involve the carnitine-esters. Our recent studies[52] suggest that it is the acyl-CoA ester which is transported by the Pat 1p/Pat 2p-complex, which most likely operates as a heterodimer.[49,50] Based on these findings we suggest that the ALD-protein which also belongs to the family of ABC-transporters, transports very-long-chain fatty acids in their CoA ester form (see Verleur *et al.*[52]).

5. DISORDERS OF FATTY ACID OXIDATION

As discussed above, the peroxisomal fatty acid β-oxidation system is specifically involved in the degradation of a specific group of fatty acids including very-long-chain fatty acids, pristanic acid and di- and trihydroxycholestanoic acid. Several inherited diseases in man have been described in which peroxisomal β-oxidation is impaired at some level as reflected in the differential accumulation of very-long-chain fatty acids, pristanic acid and the bile acid intermediates in plasma from patients. The following disorders can be distinguished:

5.1. X-Linked Adrenoleukodystrophy (X-ALD)

X-ALD has a very variable expression even within the same kindred. At least six phenotypic variants can be distinguished. Classification of patients is somewhat arbitrary and is based upon the age of onset and the principal organs involved (Moser *et al.*[53]). The most devastating form is childhood cerebral ALD (CC ALD) which is characterized by rapidly progressive cerebral demyelination.[54] Age of onset is between 3 and 10 years of age. Early neurologic symptoms frequently include behavioural disturbances, diminished school performance, deterioration of vision and impaired auditory discrimination. There is a rapid downhill progression and seizures, spastic tetraplegia and dementia develop within months (see[53] for more information). The diagnostic hallmark in X-ALD patients is the accumulation of very-long-chain fatty acids in plasma. VLCFA-measurement is generally regarded to be conclusive in all clinical forms of X-ALD although one or two occasional misdiagnoses have been described. In case of ambiguous results one should always perform more detailed studies in fibroblasts. It is generally accepted that the accumulation of VLCFA in X-ALD patients is due to the defective oxidation of these fatty acids in peroxisomes.[55] It was originally thought that the molecular defect had to be at the level of the gene coding for the peroxisomal C26:0 acyl-CoA synthase, since this enzyme activity was found to be deficient in X-ALD fibroblasts.[56,57] The X-ALD gene was identified by the teams of Aubourg and Mandel[58] and the deduced ALD-protein did not show any homology to known acyl-CoA synthases. Instead, the ALD-protein turned out to belong to the family of ABC-proteins which also includes the *C*ystic *F*ibrosis *T*ransmembrane *R*egulator (CFTR)-protein and the *M*ulti *D*rug *R*esistance (MDR)-proteins (see[59]). The ALD-protein is a so-called half transporter with six transmembrane spanning elements and probably functions as a homo- or heterodimer. In analogy with the findings in *S. cerevisiae*[52] we believe that the ALD-protein transports the C26:0-CoA ester across the peroxisomal membrane. This would make sense since according to our data[60] but not those of others,[61] the catalytic site of the peroxisomal

C26:0 acyl-CoA synthetase faces the cytosol and not the peroxisomal interior. Analysis of the ALD-gene in X-ALD patients both at the cDNA and genomic level, have revealed a large variety of different mutations including deletions, insertions and point mutations.[62–64] Interestingly, most mutations render the ALD-protein unstable resulting in the absence of any cross-reactive immunological material upon immunofluorescence or immunoblot analysis (71% in our hands; see[63]).

5.2. Straight-Chain Acyl-CoA Oxidase Deficiency

Following the initial report by Poll-Thé *et al.*[65] of two patients with a clinical presentation strongly resembling neonatal adrenoleukodystrophy, very few additional patients have been described.[66,67] In these patients the straight-chain acyl-CoA oxidase is deficient resulting in the deficient oxidation of C26:0 only, with normal oxidation of branched-chain fatty acids which explains the normal levels of pristanic acid and the bile acid intermediates in these patients.

5.3. Bifunctional Protein Deficiency

Bifunctional protein deficiency was first described by Watkins *et al.*[68] in a patient presenting with hypotonia, macrocephaly and a number of other abnormalities strongly reminiscent of a disorder of peroxisome biogenesis such as neonatal adrenoleukodystrophy. Subsequent studies did show a peroxisomal involvement (elevated levels of C26:0 and trihydroxycholestanoic acid) although peroxisomes were clearly present. These data suggested an isolated defect in the peroxisomal β-oxidation pathway. Immunoblot analyses indeed revealed abnormalities since the bifunctional protein was found to be completely absent. It should be emphasized that this is the original bifunctional protein identified by Hashimoto and coworkers (Furuta *et al.*[69]) which now goes by the name L-bifunctional protein (L-BP).[23,24] Detailed studies at the molecular level have failed to identify any mutations in the gene coding for L-bifunctional protein (Van Roermund, Van Grunsven, Hoefler and Wanders, unpublished). We are currently reinvestigating this patient. Our preliminary data show that the primary defect in this patient is *not* at the level of the L-BP, but at the level of D-BP. Molecular analysis of D-bifunctional protein at the cDNA-level shows clear and unambigous mutations (Van Grunsven *et al.*, in preparation). The original immunoblot data of Watkins *et al.*[68] remain unexplained. These new data also shed light on the results obtained in patients with an unknown defect in peroxisomal β-oxidation (see below).

5.4. Peroxisomal Thiolase Deficiency

So far only a single patient first described by Goldfischer *et al.*[34] has been identified with peroxisomal thiolase deficiency.[35] The patient displayed severe clinical abnormalities resembling those described in Zellweger syndrome which led to the name pseudo-Zellweger syndrome.

5.5. Patients with Defects in Peroxisomal β-Oxidation of Unknown Etiologies

In the past many patients have been described with defects in peroxisomal β-oxidation of unknown origins. In order to identify the underlying defect in these patients,

several groups have performed complementation analysis.[70–76] In these studies use was made of two reference cell lines: (i) fibroblasts from the patient described by Poll-Thé *et al.*[65] with acyl-CoA oxidase deficiency and (ii) cells from the patient by Watkins *et al.*[68] with bifunctional protein deficiency. All studies showed that most patients belong to the bifunctional protein deficiency group simply because cells from these patients *did* show complementation if the acyl-CoA oxidase deficient cells were used but not if the bifunctional protein deficient cells were used. We have studied our bifunctional protein deficiency patients at the molecular level by performing mutation analysis of L-bifunctional protein cDNA and found no mutations. However, we then discovered that cells from the patient with an established defect in the D-bifunctional protein at the level of the 3-OH-acyl-CoA dehydrogenase component (see Van Grunsven *et al.*[31]), also showed *no* complementation with the cells from the patient described by Watkins *et al.*[68] with presumed L-BP deficiency. This led to a joint effort to resolve the Watkins-case which now appears to be D-bifunctional protein deficient. This explains all the hitherto puzzling observations.

6. FATTY ACID ALPHA-OXIDATION: ENZYMOLOGY AND DEFICIENCIES IN MAN

6.1. Phytanic Acid Degradation

The presence of a methyl group at the third carbon atom of any fatty acid prevents its direct β-oxidation. In principle oxidation of such 3-methyl branched clain fatty acids may then proceed via 2 mechanisms which include (i) ω-oxidation or (ii) α-oxidation.

In the case of phytanic acid it has been established that ω-oxidation does occur but only to a limited extent implying that α-oxidation is the major pathway. Steinberg and coworkers[77] performed a series of studies to establish the structure of the phytanic acid oxidation pathway. These studies revealed that 2-hydroxyphytanic acid is an obligatory intermediate.[78] This was concluded from the fact that phytanic acid was found to be oxidized to CO_2 both in total homogenates as well as in mitochondria from rat liver if ATP, NAD^+ and NADPH were present. Gaschromatographic analysis of the reaction mixture revealed the time-dependent accumulation of 2-hydroxyphytanic, pristanic, Δ2-pristenic and 4,8,12-trimethyltridecanoic acid.[78] These data led to the scheme proposed by Tsai *et al.*,[78] shown in Figure 3A. Tsai *et al.*[78] also found that the conversion of phytanic acid to 2-hydroxyphytanic acid required molecular oxygen which led them to suggest that the first step in the pathway is mediated by a mitochondrial cytochrome P_{450} type of hydroxylase.

It is now firmly established that the pathway proposed by Tsai *et al.*[78] is incorrect. This is concluded from the following key observations. Firstly, Poulos and coworkers[79] made the important observation that formic acid and not CO_2 is the primary product of α-oxidation. Secondly, Watkins *et al.*[3] presented convincing evidence showing that phytanoyl-CoA and not phytanic acid is the true substrate for phytanic acid α-oxidation.

Thirdly, Mihalik *et al.*[4] discovered a new enzyme which converts phytanoyl-CoA into 2-hydroxyphytanoyl-CoA in a reaction involving 2-oxoglutarate, Fe^{++} and ascorbate. The existence of this enzyme was confirmed in subsequent studies both in rat[5] and man.[6] Interestingly, phytanoyl-CoA hydroxylase is localized in peroxisomes. This has been

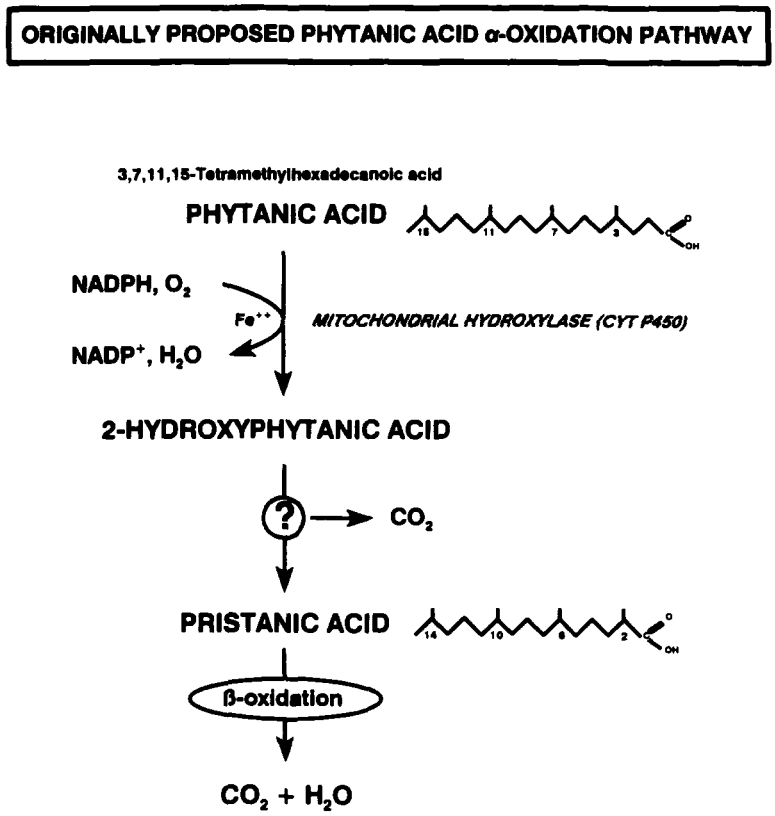

Figure 3A. Schematic representation of the originally proposed pathway for the α-oxidation of phytanic acid by Tsai *et al.*.[78] See text for detailed information.

established both in rat liver[5] as well as in human liver[6] which is hard to reconcile with data in the literature[80] suggesting a different localization of phytanic acid α-oxidation in rat (mitochondrial) and man (peroxisomal). As discussed in detail elsewhere, all studies with homogenates or isolated organelles previous to the discovery of phytanoyl-CoA hydroxylase by Mihalik *et al.*[4] which also includes our own studies,[81,82] suffer from the fact that the wrong cofactor combination was used lacking 2-oxoglutarate, Fe^{++} and ascorbate. The lack of appropriate cofactors in studies on fatty acid α-oxidation also explains the very low rates of phytanic acid α-oxidation described in homogenates and isolated organelles (see[82]).

Recent studies have led to a full resolution of the structure of the phytanic acid α-oxidation pathway. Indeed independent studies by Croes *et al.*[7] and Verhoeven[83] revealed that 2-hydroxyphytanoyl-CoA undergoes cleavage to produce formyl-CoA and pristanal respectively, which is than oxidized to pristanic acid (Fig. 3B). The pristanic acid is now ready for β-oxidation after its activation to its CoA-ester.

6.2. Disorders of Phytanic Acid α-Oxidation

Phytanic acid has been found to accumulate in different types of peroxisomal disorders including:

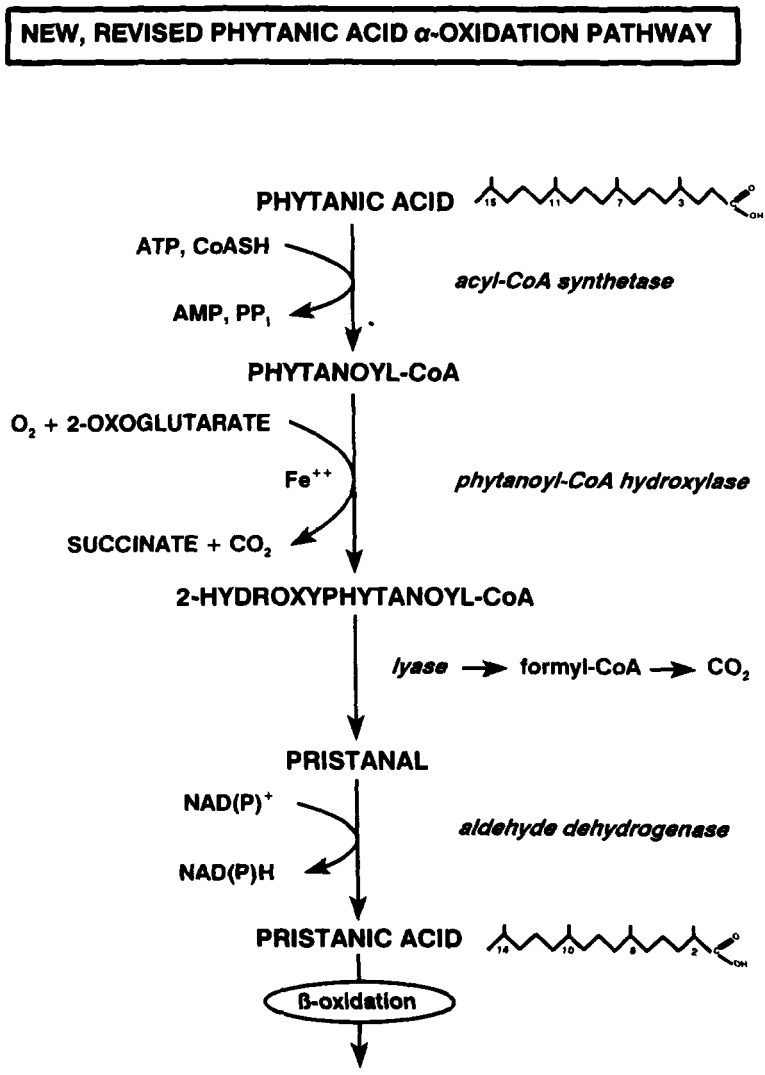

Figure 3B. New, revised phytanic acid α-oxidation pathway. See text for detailed information.

(i) Zellweger syndrome and the other disorders of peroxisome biogenesis,
(ii) Rhizomelic chondrodysplasia punctata type 1 and
(iii) Classical Refsum disease.

6.2.1. Zellweger Syndrome and Other Disorders of Peroxisome Biogenesis. We have found that phytanoyl-CoA hydroxylase (PhyH) is deficient in liver[6] and fibroblasts (Jansen *et al.*, in preparation) from patients suffering from a defect in peroxisome biogenesis. This follows logically from the fact that PhyH is a peroxisomal matrix enzyme which is targeted to peroxisomes via the PTS2 import route.

Indeed, all phytanoyl-CoA hydroxylases studied by us thus far, which includes human PhyH[84] all show a perfect PTS2-signature in the NH2-terminal portion of the protein.

6.2.2. Rhizomelic Chondrodysplasia Punctata Type I (RCDP Type I). Patients suffering from RCDP type I which is due to a non-functional PTS2-receptor,[85–87] also show deficient oxidations of phytanic acid. Recent data show that this results from a deficiency of phytanoyl-CoA hydroxylase.[88] The deficiency of PhyH probably results from its mis-localization to the cytosol as a consequence of the non-functional PTS2-receptor and the apparent instability of the PhyH-protein in the cytosol. Pulse/chase experiments are underway to resolve this.

6.2.3. Classical Refsum Disease. In patients suffering from classical Refsum disease PhyH is functionally inactive due to mutations in the structural gene for PhyH. Indeed, molecular studies by ourselves[84] and others[89] have shown a variety of distinct mutations in affected patients.

REFERENCES

1. Fujiki, Y. (1997) *Biochim. Biophys. Acta* **1361**, 235–250. Molecular defects in genetic diseases of peroxisomes.
2. Brown, F.R., 3d, McAdams, A.J., Cummins, J.W., Konkol, R., Singh, I., Moser, A.B. & Moser, H.W. (1982) *Johns Hopkins Med. J.* **151**, 344–351. Cerebro-hepato-renal (Zellweger) syndrome and neonatal adrenoleukodystrophy: similarities in phenotype and accumulation of very long chain fatty acids.
3. Watkins, P.A., Howard, A.E. & Mihalik, S.J. (1994) *Biochim. Biophys. Acta* **1214,** 288–294. Phytanic acid must be activated to phytanoyl-CoA prior to its alpha-oxidation in rat liver peroxisomes.
4. Mihalik, S.J., Rainville, A.M. & Watkins, P.A. (1995) *Eur. J. Biochem.* **232**, 545–551. Phytanic acid alpha-oxidation in rat liver peroxisomes. Production of alpha-hydroxyphytanoyl-CoA and formate is enhanced by dioxygenase cofactors.
5. Croes, K., Casteels, M., de Hoffmann, E., Mannaerts, G.P. & Van Veldhoven, P.P. (1996) *Eur. J. Biochem.* **240**, 674–683. alpha-Oxidation of 3-methyl-substituted fatty acids in rat liver. Production of formic acid instead of CO2, cofactor requirements, subcellular localization and formation of a 2-hydroxy-3-methylacyl-CoA intermediate.
6. Jansen, G.A., Mihalik, S.J., Watkins, P.A., Moser, H.W., Jakobs, C., Denis, S. & Wanders, R.J.A. (1996) *Biochem. Biophys. Res. Commun.* **229**, 205–210. Phytanoyl-CoA hydroxylase is present in human liver, located in peroxisomes, and deficient in Zellweger syndrome: direct, unequivocal evidence for the new, revised pathway of phytanic acid alpha-oxidation in humans.
7. Croes, K., Casteels, M., Asselberghs, S., Herdewijn, P., Mannaerts, G.P. & Vanveldhoven, P.P. (1997) *FEBS. Lett.* **412**, 643–645. Formation of a 2-methyl-branched fatty aldehyde during peroxisomal alpha-oxidation.
8. Verhoeven, N.M., Schor, D.S., ten Brink, H.J., Wanders, R.J.A. & Jakobs, C. (1997) *Biochem. Biophys. Res. Commun.* **237**, 33–36. Resolution of the phytanic acid alpha-oxidation pathway: identification of pristanal as product of the decarboxylation of 2-hydroxyphytanoyl-CoA.
9. Singh, H., Beckman, K. & Poulos, A. (1994) *J. Biol. Chem.* **269**, 9514–9520. Peroxisomal beta-oxidation of branched chain fatty acids in rat liver. Evidence that carnitine palmitoyltransferase I prevents transport of branched chain fatty acids into mitochondria.
10. Schepers, L., Casteels, M., Verheyden, K., Parmentier, G., Asselberghs, S., Eyssen, H.J. & Mannaerts, G.P. (1989) *Biochem. J.* **257**, 221–229. Subcellular distribution and characteristics of trihydroxycoprostanoyl-CoA synthetase in rat liver.
11. Prydz, K., Kase, B.F., Bjorkhem, I. & Pedersen, J.I. (1988) *J. Lipid. Res.* **29**, 997–1004. Subcellular localization of 3 alpha, 7 alpha-dihydroxy- and 3 alpha,7 alpha, 12 alpha-trihydroxy-5 beta-cholestanoyl-coenzyme A ligase(s) in rat liver.
12. Diczfalusy, U., Kase, B.F., Alexson, S.E. & Bjorkhem, I. (1991) *J. Clin. Invest.* **88**, 978–984. Metabolism of prostaglandin F2 alpha in Zellweger syndrome. Peroxisomal beta-oxidation is a major importance for in vivo degradation of prostaglandins in humans.
13. Jedlitschky, G., Huber, M., Volkl, A., Muller, M., Leier, I., Muller, J., Lehmann, W.D., Fahimi, H.D. & Keppler, D. (1991) *J. Biol. Chem.* **266**, 24763–24772. Peroxisomal degradation of leukotrienes by beta-oxidation from the omega-end.

14. Diczfalusy, U., Vesterqvist, O., Kase, B.F., Lund, E. & Alexson, S.E. (1993) *J. Lipid. Res.* **34**, 1107–1113. Peroxisomal chain-shortening of thromboxane B2: evidence for impaired degradation of thromboxane B2 in Zellweger syndrome.
15. de Waart, D.R., Koomen, G.C. & Wanders, R.J.A. (1994) *Biochim. Biophys. Acta* **1226,** 44–48. Studies on the urinary excretion of thromboxane B2 in Zellweger patients and control subjects: evidence for a major role for peroxisomes in the beta-oxidative chain-shortening of thromboxane B2.
16. Suzuki, H., Yamada, J., Watanabe, T. & Suga, T. (1989) *Biochim. Biophys. Acta* **990**, 25–30. Compartmentation of dicarboxylic acid beta-oxidation in rat liver: importance of peroxisomes in the metabolism of dicarboxylic acids.
17. Vamecq, J., de Hoffmann, E. & Van Hoof, F. (1985) *Biochem. J.* **230**, 683–693. The microsomal dicarboxylyl-CoA synthetase.
18. Adamski, J., Husen, B., Marks, F. & Jungblut, P.W. (1992) *Biochem. J.* **288**, 375–381. Purification and properties of oestradiol 17 beta-dchydrogenase extracted from cytoplasmic vesicles of porcine endometrial cells.
19. Markus, M., Husen, B., Leenders, F., Jungblut, P.W., Hall, P.F. & Adamski, J. (1995) *Eur. J. Cell. Biol.* **68**, 263–267. The organelles containing porcine 17 beta-estradiol dehydrogenase are peroxisomes.
20. Leenders, F., Tesdorpf, J.G., Markus, M., Engel, T., Seedorf, U. & Adamski, J. (1996) *J. Biol. Chem.* **271**, 5438–5442. Porcine 80-kDa protein reveals intrinsic 17 beta-hydroxysteroid dehydrogenase, fatty acyl-CoA-hydratase/dehydrogenase, and sterol transfer activities.
21. Dieuaide-Noubhani, M., Novikov, D., Baumgart, E., Vanhooren, J.C., Fransen, M., Goethals, M., Vandekerckhove, J., Van Veldhoven, P.P. & Mannaerts, G.P. (1996) *Eur. J. Biochem.* **240**, 660–666. Further characterization of the peroxisomal 3-hydroxyacyl-CoA dehydrogenases from rat liver. Relationship between the different dehydrogenases and evidence that fatty acids and the C27 bile acids di- and trihydroxycoprostanic acids are metabolized by separate multifunctional proteins.
22. Dieuaide-Noubhani, M., Asselberghs, S., Mannaerts, G.P. & Van Veldhoven, P.P. (1997) *Biochem. J.* **325**, 367–373. Evidence that multifunctional protein 2, and not multifunctional protein 1, is involved in the peroxisomal beta-oxidation of pristanic acid.
23. Jiang, L.L., Kobayashi, A., Matsuura, H., Fukushima, H. & Hashimoto, T. (1996) *J. Biochem. (Tokyo).* **120**, 624–632. Purification and properties of human D-3-hydroxyacyl-CoA dehydratase: medium-chain enoyl-CoA hydratase is D-3-hydroxyacyl-CoA dehydratase.
24. Jiang, L.L., Kurosawa, T., Sato, M., Suzuki, Y. & Hashimoto, T. (1997) *J. Biochem. (Tokyo).* **121**, 506–513. Physiological role of D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein.
25. Qin, Y.M., Haapalainen, A.M., Conry, D., Cuebas, D.A., Hiltunen, J.K. & Novikov, D.K. (1997) *Biochem. J.* **328**, 377–382. Recombinant 2-enoyl-CoA hydratase derived from rat peroxisomal multifunctional enzyme 2: role of the hydratase reaction in bile acid synthesis.
26. Qin, Y.M., Poutanen, M.H., Helander, H.M., Kvist, A.P., Siivari, K.M., Schmitz, W., Conzelmann, E., Hellman, U. & Hiltunen, J.K. (1997) *Biochem. J.* **321**, 21–28. Peroxisomal multifunctional enzyme of beta-oxidation metabolizing D-3-hydroxyacyl-CoA esters in rat liver: molecular cloning, expression and characterization.
27. Novikov, D.K., Vanhove, G.F., Carchon, H., Asselberghs, S., Eyssen, H.J., Van Veldhoven, P.P. & Mannaerts, G.P. (1994) *J. Biol. Chem.* **269**, 27125–27135. Peroxisomal beta-oxidation. Purification of four novel 3-hydroxyacyl-CoA dehydrogenases from rat liver peroxisomes.
28. Uchida, Y., Izai, K., Orii, T. & Hashimoto, T. (1992) *J. Biol. Chem.* **267**, 1034–1041. Novel fatty acid beta-oxidation enzymes in rat liver mitochondria. II. Purification and properties of enoyl-coenzyme A (CoA) hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein.
29. Wanders, R.J.A., Denis, S., Wouters, F., Wirtz, K.W. & Seedorf, U. (1997) *Biochem. Biophys. Res. Commun.* **236**, 565–569. Sterol carrier protein X (SCPx) is a peroxisomal branched-chain beta-ketothiolase specifically reacting with 3-oxo-pristanoyl-CoA: a new, unique role for SCPx in branched-chain fatty acid metabolism in peroxisomes.
30. Suzuki, Y., Jiang, L.L., Souri, M., Miyazawa, S., Fukuda, S., Zhang, Z., Une, M., Shimozawa, N., Kondo, N., Orii, T. & Hashimoto, T. (1997) *Am. J. Hum. Genet.* **61**, 1153–1162. D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein deficiency: a newly identified peroxisomal disorder.
31. van Grunsven, E.G., van Berkel, E., Ulst, L., Vreken, P., de Klerk, J.B., Adamski, J., Lemonde, H., Clayton, P.T., Cuebas, D.A. & Wanders, R.J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 2128–2133. Peroxisomal D-hydroxyacyl-CoA dehydrogenase deficiency: resolution of the enzyme defect and its molecular basis in bifunctional protein deficiency.

32. Miyazawa, S., Osumi, T. & Hashimoto, T. (1980) *Eur. J. Biochem.* **103**, 589–596. The presence of a new 3-oxoacyl-CoA thiolase in rat liver peroxisomes.
33. Miyazawa, S., Furuta, S., Osumi, T., Hashimoto, T. & Ui, N. (1981) *J. Biochem. (Tokyo).* **90**, 511–519. Properties of peroxisomal 3-ketoacyl-coA thiolase from rat liver.
34. Goldfischer, S., Collins, J., Rapin, I., Neumann, P., Neglia, W., Spiro, A.J., Ishii, T., Roels, F., Vamecq, J. & Van Hoof, F. (1986) *J. Pediatr.* **108**, 25–32. Pseudo-Zellweger syndrome: deficiencies in several peroxisomal oxidative activities.
35. Schram, A.W., Goldfischer, S., van Roermund, C.W., Brouwer-Kelder, E.M., Collins, J., Hashimoto, T., Heymans, H.S., van den Bosch, H., Schutgens, R.B., Tager, J.M. & *et al.* (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 2494–2496. Human peroxisomal 3-oxoacyl-coenzyme A thiolase deficiency.
36. Seedorf, U., Brysch, P., Engel, T., Schrage, K. & Assmann, G. (1994) J. *Biol. Chem.* **269**, 21277–21283. Sterol carrier protein X is peroxisomal 3-oxoacyl coenzyme A thiolase with intrinsic sterol carrier and lipid transfer activity.
37. Wirtz, K.W. (1997) *Biochem. J.* **324**, 353–360. Phospholipid transfer proteins revisited.
38. Subramani, S. (1997) *Nat. Genet.* **15**, 331–333. PEX genes on the rise.
39. Ohba, T., Rennert, H., Pfeifer, S.M., He, Z., Yamamoto, R., Holt, J.A., Billheimer, J.T. & Strauss, J.F. (1994) *Genomics.* **24**, 370–374. The structure of the human sterol carrier protein X/sterol carrier protein 2 gene (SCP2).
40. Ohba, T., Holt, J.A., Billheimer, J.T. & Strauss, J.F., 3rd. (1995) *Biochemistry.* **34**, 10660–10668. Human sterol carrier protein x/sterol carrier protein 2 gene has two promoters.
41. Antonenkov, V.D., Van Veldhoven, P.P., Waelkens, E. & Mannaerts, G.P. (1997) *J. Biol. Chem.* **272**, 26023–26031. Substrate specificities of 3-oxoacyl-CoA thiolase A and sterol carrier protein 2/3-oxoacyl-CoA thiolase purified from normal rat liver peroxisomes. Sterol carrier protein 2/3-oxoacyl-CoA thiolase is involved in the metabolism of 2-methyl-branched fatty acids and bile acid intermediates.
42. Bunya, M., Maebuchi, M., Kamiryo, T., Kurosawa, T, Sato, M., Tohma, M, Jiang, L.L. & Hashimoto, T. (1998) *J. Biochem. (Tokyo).* **123**, 347–352. Thiolase involved in bile acid formation.
43. Seedorf, U., Raabe, M., Ellinghaus, P., Kannenberg, F., Fobker, M., Engel, T, Denis, S., Wouters, F., Wirtz, K.W., Wanders, R.J.,Maeda,N.& Assmann, G. (1998) Genes. *Dev.* **12**, 1189–1201. Defective peroxisomal catabolism of branched fatty acyl coenzyme A in mice lacking the sterol carrier protein-2/sterol carrier protein-x gene function.
44. van Roermund, C.W., Elgersma, Y, Singh, N., Wanders, R.J. & Tabak, H.F. (1995) *EMBO. J.* **14**, 3480–3486. The membrane of peroxisomes in Saccharomyces cerevisiae is impermeable to NAD(H) and acetyl-CoA under in vivo conditions.
45. Baumgart, E., Fahimi, H.D., Stich, A. & Volkl, A. (1996) *Journal of Biological Chemistry* **271**, 3846–3855. L-lactate dehydrogenase A4- and A3B isoforms are bona fide peroxisomal enzymes in rat liver. Evidence for involvement in intraperoxisomal NADH reoxidation.
46. van Roermund, C.W.T., Hettema, E.H., Kal, A.J., van den Berg, M., Tabak, H.F. & Wanders, R.J.A. (1998) *EMBO. J.* **17**, 677–687. Peroxisomal beta-oxidation of polyunsaturated fatty acids in Saccharomyces cerevisiae: isocitrate dehydrogenase provides NADPH for reduction of double bonds at even positions.
47. Henke, B., Girzalsky, W., Berteaux-Lecellier, V. & Erdmann, R. (1998) *J. Biol. Chem.* **273**, 3702–3711. IDP3 encodes a peroxisomal NADP-dependent isocitrate dehydrogenase required for the beta-oxidation of unsaturated fatty acids.
48. Hettema, E.H., van Roermund, C.W., Distel, B., van den Berg, M., Vilela, C., Rodrigues-Pousada, C., Wanders, R.J. & Tabak, H.F. (1996) *EMBO. J.* **15**, 3813–3822. The ABC transporter proteins Patl and Pat2 are required for import of long-chain fatty acids into peroxisomes of Saccharomyces cerevisiae.
49. Shani, N., Watkins, P.A. & Valle, D. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 6012–6016. PXA1, a possible Saccharomyces cerevisiae ortholog of the human adrenoleukodystrophy gene.
50. Shani, N. & Valle, D. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 11901–11906. A Saccharomyces cerevisiae homolog of the human adrenoleukodystrophy transporter is a heterodimer of two half ATP-binding cassette transporters.
51. Swartzman, E.E., Viswanathan, M.N. & Thorner, J. (1996) *J. Cell. Biol.* **132**, 549–563. The PAL1 gene product is a peroxisomal ATP-binding cassette transporter in the yeast Saccharomyces cerevisiae.
52. Verleur, N., Hettema, E.H., van Roermund, C.W.T., Tabak, H.F. & Wanders, R.J.A. (1997) *Eur. J. Biochem.* **249**, 657–661. Transport of activated fatty acids by the peroxisomal ATP-binding-cassette transporter Pxa2 in a semi-intact yeast cell system.
53. Moser, H.W., Smith, K.D. & Moser, A.B. (1995) *in* The metabolic and molecular bases of inherited

disease (Scriver, C.R., Beaudet, A.L., Sly, W.S., & Valle, D. eds), 2325–2349. McGraw-Hill, New York. X-linked adrenoleukodystrophy.
54. Powers, J.M. (1985) *Clin. Neuropathol.* **4**, 181–199. Adreno-leukodystrophy (adreno-testiculo-leukomyelo-neuropathic-complex).
55. Singh, I., Moser, A.E., Goldfischer, S. & Moser, H.W. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 4203–4207. Lignoceric acid is oxidized in the peroxisome: implications for the Zellweger cerebro-hepato-renal syndrome and adrenoleukodystrophy.
56. Lazo, O., Contreras, M., Hashmi, M., Stanley, W., Irazu, C. & Singh, I. (1988) *Proc. Natl. Acad Sci. U. S. A.* **85**, 7647–7651. Peroxisomal lignoceroyl-CoA ligase deficiency in childhood adrenoleukodystrophy and adrenomyeloneuropathy.
57. Wanders, R.J., van Roermund, C.W., van Wijland, M.J., Schutgens, R.B., van den Bosch, H., Schram, A.W. & Tager, J.M. (1988) *Biochem. Biophys. Res. Commun.* **153**, 618–624. Direct demonstration that the deficient oxidation of very long chain fatty acids in X-linked adrenoleukodystrophy is due to an impaired ability of peroxisomes to activate very long chain fatty acids.
58. Mosser, J., Douar, A.M., Sarde, C.O., Kioschis, P., Feil, R., Moser, H., Poustka, A.M., Mandel, J.L. & Aubourg, P. (1993) *Nature.* **361**, 726–730. Putative X-linked adrenoleukodystrophy gene shares unexpected homology with ABC transporters.
59. Higgins, C.F. (1992) *Ann. Rev. Cell. Biol.* **8**, 67–113. ABC transporters: from microorganisms to man.
60. Lageweg, W., Tager, J.M. & Wanders, R.J.A. (1991) *Biochem. J.* **276**, 53–56. Topography of very-long-chain-fatty-acid-activating activity in peroxisomes from rat liver.
61. Lazo, O., Contreras, M. & Singh, I. (1990) *Biochemistry.* **29**, 3981–3986. Topographical localization of peroxisomal acyl-CoA ligases: differential localization of palmitoyl-CoA and lignoceroyl-CoA ligases.
62. Feigenbaum, V., Lombard-Platet, G., Guidoux, S., Sarde, C.O., Mandel, J.L. & Aubourg, P. (1996) *Am. J. Hum. Genet.* **58**, 1135–1144. Mutational and protein analysis of patients and heterozygous women with X-linked adrenoleukodystrophy.
63. Kemp, S., Mooyer, P.A., Bolhuis, P.A., van Geel, B.M., Mandel, J.L., Barth, P.G., Aubourg, P. & Wanders, R.J. (1996) *J. Inherit. Metab. Dis.* **19**, 667–674. ALDP expression in flbroblasts of patients with X-linked adrenoleukodystrophy.
64. Watkins, P.A., Gould, S.J., Smith, M.A., Braiterman, L.T., Wei, H.M., Kok, F., Moser, A.B., Moser, H.W. & Smith, K..D. (1995) *Am. J. Hum. Genet.* **57**, 292–301. Altered expression of ALDP in X-linked adrenoleukodystrophy.
65. Poll-The, B.T., Roels, F, Ogier, H., Scotto, J., Vamecq, J., Schutgens, R.B.H., Wanders, R.J.A., van Roermund, C.W.T., van Wijland, M.J., Schram, A.W & *et al.* (1988) *Am. J. Hum. Genet.* **42**, 422–434. A new peroxisomal disorder with enlarged peroxisomes and a specific deficiency of acyl-CoA oxidase (pseudo-neonatal adrenoleukodystrophy).
66. Wanders, R.J., Schelen, A., Feller, N., Schutgens, R.B., Stellaard, F., Jakobs, C., Mitulla, B. & Seidlitz, G. (1990) *J. Inherit. Metab. Dis.* **13**, 371–374. First prenatal diagnosis of acyl-CoA oxidase deficiency.
67. Watkins, P.A., McGuinness, M.C., Raymond, G.V., Hicks, B.A., Sisk, J.M., Moser, A.B. & Moser, H.W. (1995) *Ann. Neural.* **38**, 472–477. Distinction between peroxisomal bifunctional enzyme and acyl-CoA oxidase deficiencies.
68. Watkins, P.A., Chen, W.W., Harris, C.J., Hoefler, G, Hoefler, S., Blake, D.C., Jr., Balfe, A., Kelley, R.I., Moser, A.B., Beard, M.E. & *et al.* (1989) *J. Clin. Invest.* **83**, 771–777. Peroxisomal bifunctional enzyme deficiency.
69. Furuta, S., Miyazawa, S., Osumi, T, Hashimoto, T. &Ui, N. (1980) *J. Biochem. (Tokyo).* **88**,1059–1070. Properties of mitochondria and peroxisomal enoyl-CoA hydratases from rat liver.
70. McGuinness, M.C., Moser, A.B., Poll-The, B.T. & Watkins, P.A. (1993) *Biochem. Med. Metab. Biol.* **49**, 228–242. Complementation analysis of patients with intact peroxisomes and impaired peroxisomal beta-oxidation.
71. Wanders, R.J., van Roermund, C.W., Brul, S., Schutgens, R.B. & Tager, J.M. (1992) *J. Inherit. Metab. Dis.* **15**, 385–388. Bifunctional enzyme deficiency: identification of a new type of peroxisomal disorder in a patient with an impairment in peroxisomal beta-oxidation of unknown aetiology by means of complementation analysis.
72. Moser, A.B., Rasmussen, M., Naidu, S., Watkins, P.A., McGuinness, M., Hajra, A.K., Chen, G., Raymond, G., Liu, A., Gordon, D. & *et al.* (1995) *J. Pediatr.* **127**, 13–22. Phenotype of patients with peroxisomal disorders subdivided into sixteen complementation groups.
73. Wanders, R.J., Jansen, G.A., van Roermund, C.W., Denis, S., Schutgens, R.B. & Jakobs, B.S. (1996) *Ann. N. Y. Acad. Sci.* **804**, 450–460. Metabolic aspects of peroxisomal disorders.

74. Paton, B.C., Sharp, P.C., Crane, D.I. & Poulos, A. (1996) *J. Clin. Invest.* **97**, 681–688. Oxidation of pristanic acid in fibroblasts and its application to the diagnosis of peroxisomal beta-oxidation defects.
75. van Grunsven, E.G. & Wanders, R.J. (1997) *J. Inherit. Metab. Dis.* **20**, 437–440. Genetic heterogeneity in patients with a disorder of peroxisomal beta-oxidation: a complementation study based on pristanic acid beta-oxidation suggesting different enzyme defects.
76. van Grunsven, E.G., van Roermund, C.W., Denis, S. & Wanders, R.J. (1997) *Biochem. Biophys. Res. Commun.* **235**, 176–179. Complementation analysis of fibroblasts from peroxisomal fatty acid oxidation deficient patients shows high frequency of bifunctional enzyme deficiency plus intragenic complementation: unequivocal evidence for differential defects in the same enzyme protein.
77. Steinberg, D. (1995) *in* The metabolic and molecular bases of inherited disease (Scriver, C.R., Beaudet, A.L., Sly, W.S., & Valle, D. eds), 2351–2369. McGraw-Hill, New York. Refsum disease.
78. Tsai, S.C., Avigan, J. & Steinberg, D. (1969) *J. Biol. Chem.* **244**, 2682–2692. Studies on the alpha oxidation of phytanic acid by rat liver mitochondria.
79. Poulos, A., Sharp, P., Singh, H., Johnson, D.W., Carey, W.F. & Easton, C. (1993) *Biochem. J.* **292**, 457–461. Formic acid is a product of the alpha-oxidation of fatty acids by human skin fibroblasts: deficiency of formic acid production in peroxisome-deficient fibroblasts.
80. Singh, I., Pahan, K., Dhaunsi, G.S., Lazo, O. & Ozand, P. (1993) *J. Biol. Chem.* **268**, 9972–9979. Phytanic acid alpha-oxidation. Differential subcellular localization in rat and human tissues and its inhibition by nycodenz.
81. Wanders, R.J., van Roermund, C.W., Jakobs, C. & ten Brink, H.J. (1991) *J. Inherit. Metab. Dis.* **14**, 349–352. Identification of pristanoyl-CoA oxidase and phytanic acid decarboxylation in peroxisomes and mitochondria from human liver: implications for Zellweger syndrome.
82. Wanders, R.J. & van Roermund, C.W. (1993) *Biochim. Biophys. Acta* **1167**, 345–350. Studies on phytanic acid alpha-oxidation in rat liver and cultured human skin fibroblasts.
83. Verhoeven, N.M., Roe, D.S., Kok, R.M., Wanders, R.J.A., Jakobs, C. & Roe, C. (1998) *J. Lipid. Res.* **39**, 66–74. Phytanic acid and pristanic acid are oxidized by sequential peroxisomal and mitochondrial reactions in cultured fibroblasts.
84. Jansen, G.A., Ofman, R., Ferdinandusse, S., IJlst, L., Muijsers, A.O., Skjeldal, O.H., Stokke, O., Jakobs, C., Besley.G.T., Wraith, J.E. & Wanders, R.J.A. (1997) *Nat. Genet.* **17**,190–193. Refsum disease is caused by mutations in the phytanoyl-CoA hydroxylase gene.
85. Braverman, N., Steel, G, Obie, C., Moser, A.B., Moser, H.W., Gould, S.J. & Valle, D. (1997) *Nat. Genet.* **15**, 369–376. Human PEX7 encodes the peroxisomal PTS2 receptor and is responsible for rhizomelic chondrodysplasia punctata.
86. Motley, A.M., Hettema, E.H., Hogenhout, E.M., Brites, P., ten Asbroek, A.L., Wijburg, F.A., Baas, F., Heijmans, H.S.A., Tabak, H.F., Wanders, R.J.A. & Distel, B. (1997) *Nat. Genet.* **15**, 377–380. Rhizomelic chondrodysplasia punctata is a peroxisomal protein targeting disease caused by a non-functional PTS2 receptor.
87. Purdue, P.E., Zhang, J.W., Skoneczny, M. & Lazarow, P.B. (1997) *Nat. Genet.* **15**, 381–384. Rhizomelic chondrodysplasia punctata is caused by deficiency of human PEX7, a homologue of the yeast PTS2 receptor.
88. Jansen, G.A., Mihalik, S.J., Watkins, P.A., Moser, H.W., Jakobs, C., Heijmans, H.S. & Wanders, R.J. (1997) *J. Inherit. Metab. Dis.* **20**, 444–446. Phytanoyl-CoA hydroxylase is not only deficient in classical Refsum disease but also in rhizomelic chondrodysplasia punctata.
89. Mihalik, S.J., Morrell, J.C., Kim, D., Sacksteder, K.A., Watkins, P.A. & Gould, S.J. (1997) *Nat. Genet.* **17**, 185–189. Identification of PAHX, a Refsum disease gene.

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ENZYMOLOGY OF β-OXIDATION OF (POLY)UNSATURATED FATTY ACIDS

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1. INTRODUCTION

Most unsaturated fatty acids which enter our body via the alimentary tract (or arise endogenously via microsomal chain elongation/desaturation systems) have their double bonds in *cis* configuration in either odd or even numbered positions. The main metabolic pathway to degrade them is β-oxidation. Experiments with isolated subcellular organelles or organs have demonstrated that the β-oxidation of unsaturated fatty acyl-CoA esters is not just chain-shortening but can also pass over pre-existing carbon double bonds. β-oxidation, whether it takes place in mitochondria or peroxisomes, has only one unsaturated intermediate, namely *trans*-2-enoyl-CoA. Therefore, β-oxidation alone is not sufficient for the oxidation of (poly)unsaturated fatty acyl-CoA esters and auxiliary enzyme systems are required to link their carbon chain to the β-oxidation pathway (Fig. 1).

2. ENOYL-CoA ISOMERASE

Δ^3,Δ^2-Enoyl-CoA isomerase activity (EC 5.3.3.8) was first demonstrated in isolated rat liver mitochondria by Stoffel and coworkers.[1] The enzyme catalyzes the conversion of both *cis* and *trans*-3-enoyl-CoA esters to their *trans-2* counterparts with the catalytic rate being about ten times higher for *cis*-enoyl-CoA substrates.[2,3] Originally, isomerase was regarded as an obligatory enzyme for the metabolism of monounsaturated and polyunsaturated fatty acids with double bonds at odd-numbered carbon atoms, e.g. oleic, linolenic and arachidonic acids.[1–3] However, according to recent data, isomerase also participates in the β-oxidation of unsaturated fatty acids with double bonds at even-numbered position, since Δ^3-intermediates arise from double bonds at the Δ^4-position *via* a 2,4-dienoyl-CoA reductase-dependent pathway[4] (see below).

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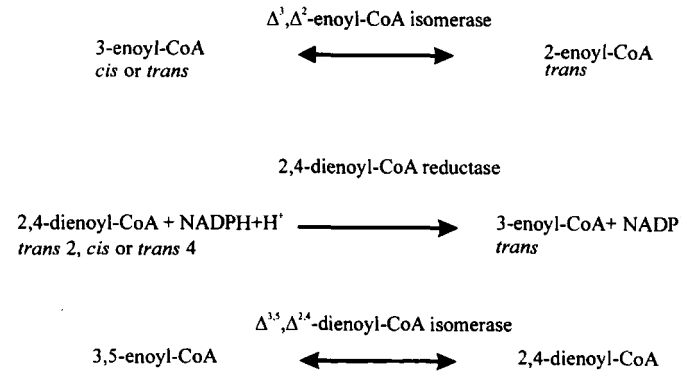

Figure 1. Auxiliary enzymes required in β-oxidation of (poly)unsaturated fatty acids.

Two mitochondrial isoforms of rat Δ^3,Δ^2-enoyl-CoA isomerase have been described, the first one being a mitochondrial short-chain isomerase with a preferred chain-length specificity of C_6–C_{12}.[3,5] This heat-stable enzyme has a subunit size of 30kDa and a pI value of about 9.5. The second isoform found in rat liver is also mitochondrial, but its catalytic rate is greatest for C_{10}–C_{12} enoyl-CoA substrates and it is not clofibrate-inducible, unlike the rat liver short-chain specific enzyme.[6] The cDNA sequence of the mitochondrial short-chain isomerase has been published and its amino acid sequence shows similarity to the mitochondrial 2-enoyl-CoA hydratase 1.[7] Currently the hydratase/isomerase family has about 30 known eukaryotic and prokaryotic members and the structure for two members has been published (rat mitochondrial 2-enoyl-CoA hydratase 1[8] and *Pseudomonas* sp. 4-chlorobenzoyl-CoA dehalogenase[9]). They all possess a conserved fingerprint of Val-Ser-X-Ile-Asn-Gly-X-X-X-Ala-Gly-Gly-X-Leu-X-X-X-X-Cys-Asp-Tyr, potentially have similar main chain folding, and catalyze reactions with carboxylic CoA esters as substrates.[8–11]

Sequence aligments of hydratase/isomerase proteins show that Glu165 of the mitochondrial short-chain isomerase and Glu164 of the mitochondrial hydratase from rat are conserved throughout the family.[10,12] It has been shown by site-directed mutagenesis analysis[10,12] that the replacement of these residues with glutamine leads to reduced activity. The results of crystallization studies of hydratase 1 are in line with this observation since the data showed that Glu 164 provides an α-proton during hydration reactions.[8]

Rat peroxisomal multifunctional enzyme 1 (MFE-1) which is a monomeric protein with a molecular mass of 79 kDa possessing 2-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities was purified as early as 1979.[13] Since then the rat peroxisomal isomerase was purified and the protein turned out to be identical to MFE-1.[14] Based on the kinetic properties of MFE-1 from rat liver it has been suggested that substrate channelling occurs between sites catalyzing hydration and dehydrogenation.[15] The observation that addition of purified hydratase 1 and 3-hydroxyacyl-CoA dehydrogenase to the assay did not increase the observed isomerization rates lead to the proposal that substrates are also channelled from the active site of isomerization to that of dehydration without release in the bulk phase.[14] This proposal was further supported by a comparison of the amino acid sequence of the amino terminal half of MFE-1 with mitochondrial hydratase 1[16] and isomerase[7] and also by structural data on hydratase 1[8] and 4-chlorobenzoyl-CoA dehalogenase.[9] The observed amino acid sequence similarity and

the size of the polypeptide suggested that both isomerase and hydratase 1 activities are catalyzed by the amino terminal half of the multifunctional polypeptide, occupying only one substrate binding site.[17]

Two Δ^3,Δ^2-enoyl-CoA isomerases have been characterized in humans: a mitochondrial monofunctional enzyme and a peroxisomal activity as part of a multifunctional Δ^3,Δ^2-enoyl-CoA isomerase/2-enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase enzyme.[18] The mitochondrial isoform has the same subunit size as the rat short-chain isomerase (30kDa), although it does not have any clear chain-length specificity and its pI value is 6.5.[18,19] The human mitochondrial Δ^3,Δ^2-enoyl-CoA isomerase exhibits a sequence identity of 74% and 78% to the rat counterpart at the amino acid and nucleotide levels, respectively. Many basic amino acid residues in the rat isomerase have been changed to acidic or neutral residues in the human enzyme, explaining in part the differences observed between these proteins.

It has been shown that an isomerase from fat-degrading plant tissue was entirely restricted to the peroxisomes and the isomerase activity could be attributed to a homodimer with a molecular mass of 50kDa.[20] Interestingly, Erdmann and co-workers have recently demonstrated a peroxisome-associated isomerase activity in *Saccharomyces cerevisiae* cells grown on oleic acid as carbon source,[21] however the properties of the corresponding protein have not been characterized.

3. DIENOYL-CoA REDUCTASE

2,4-Dienoyl-CoA reductase (EC 1.3.1.34) catalyses the NADPH-dependent reduction of *trans*-2 and *cis/trans*-4 double bonds in unsaturated fatty acyl-CoAs to *trans*-3 in eukaryotes and to *trans*-2 in bacteria.[3,22,23] 2,4-Dienoyl-CoA reductase activity has been reported to increase in liver mitochondria and peroxisomes from clofibrate-treated rats. Further investigations have shown separate inducible isoforms in both organelles.[24–28] In addition to a 120kDa mitochondrial reductase characterized earlier, a novel 60kDa isoform has been reported in rat liver mitochondria that represents 80% and 50% of the total reductase activity in clofibrate-treated rat heart and liver homogenates, respectively, as measured with 2,4-hexedienoyl-CoA, the rest of the activity being in the 120kDa mitochondrial isoform and the peroxisomal isoform.[29] The mitochondrial 120kDa reductase isoform was induced 6-fold in clofibrate-treated rat liver, as measured with 2,4-hexedienoyl-CoA, whereas the 60kDa isoform was induced 3.5-fold.[30]

Only rat and human 120kDa mitochondrial 2,4-dienoyl-CoA reductase, *S. cerevisiae* peroxisomal reductase (*SPS19*) and bacterial reductase have been characterized at the DNA level.[31–33] Spsl9p has an overall 24% identity to rat mitochondrial reductase. It lacks the mitochondrial targeting sequence, and instead has Ser-Lys-Leu that targets it to peroxisomes.[33] **Sps19Δ** cells did not grow on petroselinic acid supporting the proposal that there exists only one 2,4-dienoyl-CoA reductase in *S. cerevisiae*. The promoter region of *SPS19* contains an ORE which is sufficient for oleate-dependent induction.[33]

2,4-Dienoyl-CoA reductase belongs to the short-chain dehydrogenase/reductase superfamily[34] in which a highly conserved Tyr-Xaa-Xaa-Xaa-Lys motif is important for catalysis.[35,36] The same protein family has a pattern of an NAD(H) or an NADP(H) binding site in which Gly-Xaa-Xaa-Xaa-Gly-Xaa-Gly common in the amino-terminal part of the protein, represents the βαβ structure found in several dinucleotide binding proteins.[37,38] According to the original proposal, unsaturated fatty acids with

even-numbered double bonds were assumed to be degraded via the so called "epimerase" pathway,[1] but it is now clear that this metabolism proceeds via a reductase-dependent pathway. It has been shown that both *E. coli*[39] and *S. cerevisiae*,[33] strains deficient in reductase activities, were unable to grow on petroselinic acid as the sole carbon source and that a reductase deficiency is lethal to humans.[40]

4. DIENOYL-CoA ISOMERASE

The discovery of Δ^3-Δ^2-enoyl-CoA isomerase gave rise to the proposal that metabolites with odd-numbered double bonds underwent only a 3-*cis* to 2-*trans* isomerization before re-entering the β-oxidation spiral (Fig. 2). However, an additional pathway that reduces the double bond in an NADPH-dependent manner was recently described by Tserng and Jin.[41] The starting metabolite in this reaction sequence is *trans*-2-*cis*-5-dienoyl-CoA, which can either complete the β-oxidation cycle to yield *cis*-3-enoyl-CoA or can be converted to *trans*-3,*cis*-5-dienoyl-CoA by Δ^3,Δ^2-enoyl-CoA isomerase. A novel enzyme, $\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA isomerases then catalyzes the shift of both double bonds to produce a *trans*-2,4-*trans*-dienoyl-CoA[42–45] which is a substrate of 2,4-dienoyl-CoA reductase and, hence, can be reduced to *trans*-3-enoyl-CoA. This is then converted to *trans*-2-enoyl-CoA by Δ^3,Δ^2-enoyl-CoA isomerase (Fig. 2).

Two $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerases have been isolated and purified from rat liver to apparent homogeneity by the Schulz[43] and Tserng[44] groups and both enzymes are located in mitochondria. Schulz *et al.*[43] described a 126kDa enzyme with a subunit size 32kDa, whereas Tserng *et al.*[44] showed a native molecular mass of 200kDa with four subunits of 55 kDa. The relationship between these two $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerases is still unknown.

Additionally, another rat liver $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase (DECI) has been characterized at the molecular level by Filppula *et al.*[45] cDNA of the rat DECI encodes a protein with a predicted molecular mass of 36 kDa which belongs to the hydratase/isomerase superfamily.[45,46] It has a potential N-terminal mitochondrial targeting signal as well as a C-terminal PST1. Apparently, both targeting signals are functional *in vivo* since subcellular fractionation and immunoelectron microscopy using antibodies to a synthetic polypeptide derived from the C-terminus of DECI1 showed that DECI is located in the matrix of both mitochondria and peroxisomes. Transport of the protein into the mitochondria with cleavage of the mitochondrial targeting signal results in a mature mitochondrial form with a subunit size of 32 kDa but transport to peroxisomes yields a subunit of 36 kDa.[45]

5. COMMENTS ON THE DIENOYL-CoA ISOMERASE-REDUCTASE PATHWAY *VS.* ENOYL-CoA ISOMERASE PATHWAY

The elucidation of this novel pathway raised the question as to whether unsaturated fatty acids with odd-numbered double bonds are metabolized via one or both branches of the pathway (Fig. 2). Tserng and co-workers[47–49] concluded that in rat the novel $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase → 2,4-dienoyl-CoA reductase-dependent pathway is the dominant route, especially in liver mitochondria. Conversely, Shoukry and Schulz[50] have shown by concentration-dependent and time-dependent measurements that the flux through the reductase-dependent branch (20%) was much slower than through the

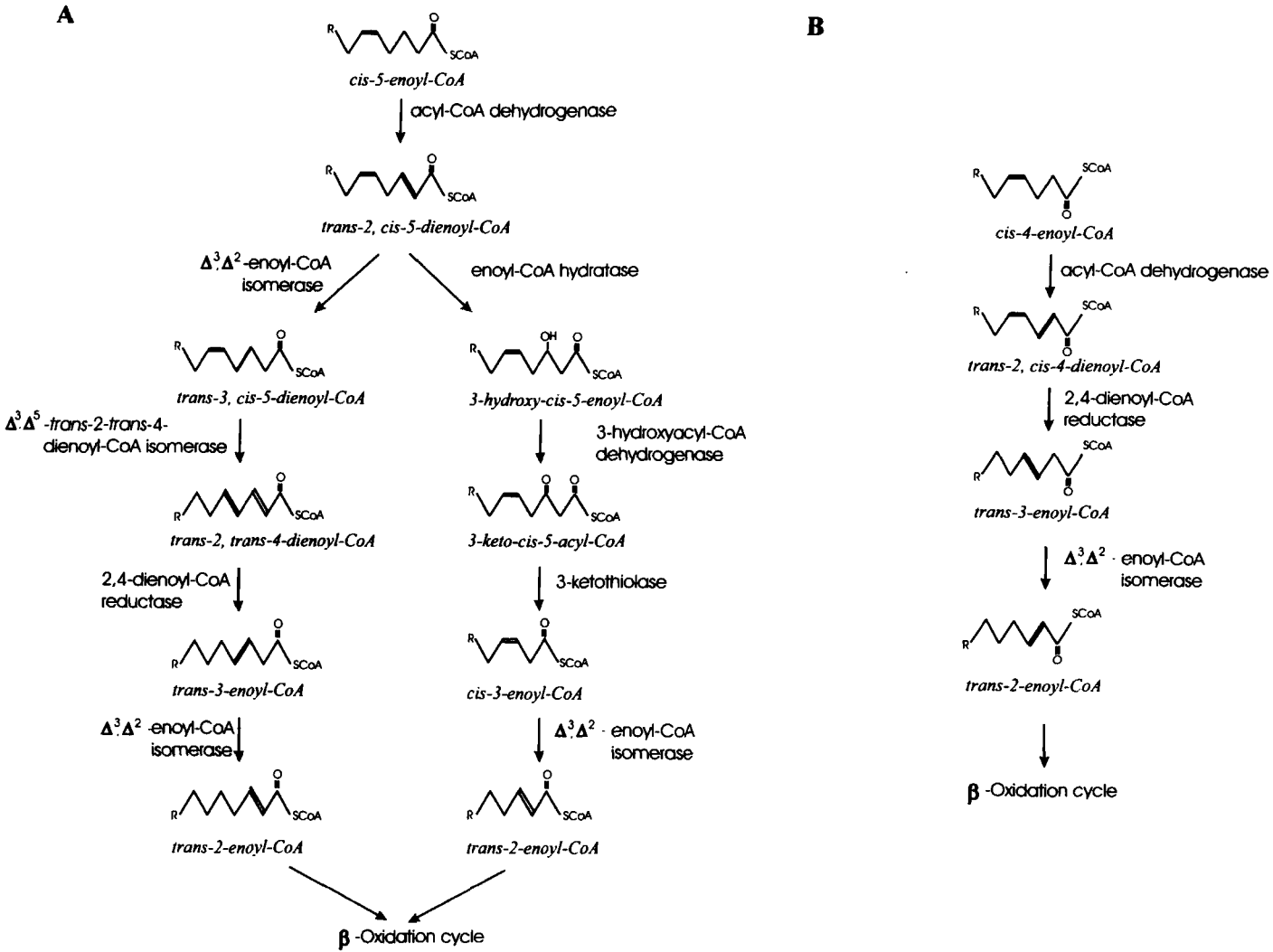

Figure 2. Metabolic pathways for *cis*-5 (A) and *cis*-4 (B) fatty acid oxidation.

isomerase-dependent branch (80%). The main reason for the slow degradation of 2,5-octadienoyl-CoA via the reductase-dependent pathway is the low activity of enoyl-CoA isomerase towards 2,5-dienoyl-CoAs relative to the activity of 3-hydroxyacyl-CoA dehydrogenase with 3-hydroxy-5-enoyl-CoAs as a substrate in rat liver mitochondria.[50] However, if 3,5-octadienoyl-CoA is formed from 2,5-octadienoyl-CoA it can be metabolized at a significant rate only by its conversion to 2,4-octadienoyl-CoA. The major reason for this situation is the greater thermodynamic stability of 3,5-octadienoyl-CoA as compared with the 2,5-isomer. An experiment with a system reconstituted from β-oxidation enzymes in the absence of $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase and 2,4-dienoyl-CoA reductase demonstrated that the metabolism of 3,5-octadienoyl-CoA via the isomerase-dependent pathway is very slow even when this compound is present at a high concentration. However, the fluxes through both pathways were almost equal at [NADH]/[NAD$^+$] ratios of 0.5 and I^{50}. Thus, the relative contribution of the reductase-dependent pathway to the degradation of unsaturated fatty acids with odd-numbered double bonds appears to be more significant when the intramitochondrial [NADH]/[NAD$^+$] ratio is high, e.g. under conditions of restricted energy utilization.

6. CONCLUDING REMARKS

Our understanding of the β-oxidation of unsaturated fatty acyl-CoA esters has expanded rapidly during the last few years. It is now clear that both mitochondria and peroxisomes in mammals have the complete enzymatic machinery to β-oxidise unsaturated fatty acids at rates dependent on the chain length of the fatty acids and the number and configuration of the double bonds. Most unsaturated fatty acids are oxidized in peroxisomes faster than their saturated counterparts, with the exception being of fatty acids with the initial double bond at the Δ^5-position, e.g. arachidonic acid. Similar to other enzymes of lipid metabolism, the auxiliary enzymes are present as isoforms and can be found in both peroxisomes and mitochondria, while some of the activities are an integral part of multifunctional enzymes of β-oxidation systems. Currently, the physiological role of these isoforms is not always understood, and the contribution of alternative pathways (Fig. 2) to the degradation of unsaturated fatty is partially open.

The known human inborn errors affecting auxiliary enzymes of β-oxidation consists of one identified case of 2,4-dienoyl-CoA reductase deficiency and a possible case of peroxisomal MFE-1 deficiency. Once the auxiliary enzymes required for the metabolism on unsaturated fatty acids have been identified and characterized also at molecular level, it may be possible to identify more inborn errors belonging to this category.

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REFERENCES

1. Stoffel, W., Ditzer, R. & Caesar, H. (1964) *Hoppe-Seyler's Z. Physiol. Chem.*, **339,** 167–181, Der Stoffwechsel der ungesättigten Fettsäuren. III. Zur β-Oxidation der Mono- und Poly enfettsauren. Der Mechanismus der enzymatischen Reaktionen an 3-*cis*-Enoyl-CoA-Verbindugen.
2. Stoffel, W. & Ecker, W. (1969) *Methods Enzymol.*, **14**, 99–105, Δ^3-*cis*,Δ^2- trans-Enoyl-CoA isomerase from rat liver mitochondria.
3. Stoffel, W. & Grol, M. (1978) *Hoppe-Seyer's Z. Physiol. Chem.*, **359**, 1777–1782, Purification and

properties of 3-*cis*-2-*trans*-enoyl-CoA isomerase (dodecenoyl-CoA delta-isomerase) from rat liver mitochondria.

4. Kunau, W.-H. & Dommes, P. (1978) *Eur. J. Biochem.*, **91**, 533–544, Degradation of unsaturated fatty acids. Identification of intermediates in the degradation of *cis*-4-decenoyl-CoA by extracts of beef liver mitochondria.
5. Palossari, P.M., Kilponen, J.M., Sormunen, R.T., Hassinen, I.E. & Hiltunen, J.K. (1990) *J. Biol. Chem.,* **265**, 3347–3353, **Δ^3-Δ^2-Enoyl-CoA** isomerases. Characterization of the mitochondrial isoenzyme in the rat.
6. Kilponen, J.M., Palossari, P.M. & Hiltunen, J.K. (1990) *Biochem. J.*, **269**, 223–226, Occurrence of a long-chain **Δ^3-Δ^2-enoyl-CoA** isomerase in rat liver.
7. Palosaari, P.M., Vihinen, M., Mäntsälä, P.I., Alexson, S.E.H., Pihlajaniemi, T. & Hiltunen, J.K. (1991) *J. Biol. Chem.*, **266**, 10750–10753, Amino acid sequence similarities of the mitochondrial short chain **Δ^3,Δ^2-enoyl-CoA** isomerase, 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase enzyme in rat liver. The proposed occurrence of isomerization and hydration in the same catalytic domain of the multifunctional enzyme.
8. Engel, C.K, Mathieu, M., Zeelen, J.P., Hiltunen, J.K. & Wierenga, R.K. (1996) *EMBO. J.*, **15**, 5135–5145, Crystal structure of enoyl-coenzyme A (CoA) hydratase at 2.5 angstroms resolution: a spiral fold defines the CoA-binding pocket.
9. Benning, M.M., Taylor, K.L., Liu, R.-Q., Yang, G., Xiang, H., Wesenberg, G., Dunaway-Mariano, D. & Holden, H.M. (1996) *Biochemistry*, **35**, 8103–8109, Structure of 4-chlorobenzoyl coenzyme A dehalogenase determined to 1.8 A resolution: an enzyme catalyst generated via adaptive mutation.
10. Muller-Newen, G. & Stoflel, W. (1993) *Biochemistry,* 32, 11405–11412, Site-directed mutagenesis of putative active-site amino acid residues of 3,2-*trans*-enoyl-CoA isomerase, conserved within the low-homology isomerase/hydratase enzyme family.
11. Engel, C.K., Kiema, T.R., Hiltunen, J.K. & Wierenga, R.K. (1998) *J. Mol. Biol.*, **275**, 847–859, The crystal structure of enoyl-CoA hydratase complexed with octanoyl-CoA reveals the structural adaptations required for binding of a long chain fatty acid-CoA molecule.
12. Muller-Newen, G., Janssen, U. & Stoffel, W. (1995) *Eur. J. Biochem.*, **228**, 68–73, Enoyl-CoA hydratase and isomerase form a superfamily with a common active-site glutamate residue.
13. Osumi, T. & Hashimoto, T. (1979) *Biochem. Biophys. Res. Commun.*, **89**, 580–584, Peroxisomal **β-oxidation** system of rat liver. Copurification of enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase.
14. Palosaari, P.M. & Hiltunen, J.K. (1990) *J. Biol. Chem.*, **265**, 2446–2449, Peroxisomal bifunctional protein from rat liver is a trifunctional enzyme possessing 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and **Δ^3-Δ^2-enoyl-CoA** isomerase activities.
15. Yang, S.-Y., Cuebas, D. & Schulz, H. (1986) J. *Biol. Chem.*, **261**, 15390–15395, Channeling of 3-hydroxy-4-*trans*-decenoyl coenzyme A on the bifunctional **β-oxidation** enzyme from rat liver peroxisomes and on the large subunit of the fatty acid oxidation complex from *Escherichia coli.*
16. Minami-Ishii, N., Taketani, S., Osumi, T. & Hashimoto, T. (1989) *Eur. J. Biochem.*, **185**, 73–78, Molecular cloning and sequence analysis of the cDNA for rat mitochondrial enoyl-CoA hydratase. Structural and evolutionary relationships linked to the bifunctional enzyme of the peroxisomal **β-oxidation** system.
17. Palosaari, P.M., Vihinen, M. & Hiltunen, J.K. (1992) In *New Developments in Fatty Acid Oxidation* (Coates, P.M. & Tanaka, K. eds.), Wiley-Liss, New-York, 41–46, Peroxisomal multifunctional **Δ^3,Δ^2-**enoyl-CoA isomerase, 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase enzyme from rat liver. Identity with peroxisomal bifunctional protein and proposed domain structure.
18. Kilponen, J.M. & Hiltunen, J.K. (1993) *FEBS Lett.*, **322**, 299–303, **β-Oxidation** of unsaturated fatty acids in humans.
19. Kilponen, J.M., Häyrinen, H.M., Rehn, M. & Hiltunen, J.K. (1994) *Biochem. J.*, **300**, 1–5, cDNA cloning and amino acid sequence of human mitochondrial **Δ^3-Δ^2-enoyl-CoA** isomerase: comparison of the human enzyme with its rat counterpart, mitochondrial short-chain isomerase.
20. Engeland, K. & Kindl, H. (1991) *Eur. J. Biochem.*, **196**, 699–705, Purification and characterization of a plant peroxisomal **Δ^3,Δ^2-enoyl-CoA** isomerase acting on 3-*cis*-enoyl-CoA and 3-*trans*-enoyl-CoA.
21. Henke, B., Girzalsky, W., Berteaux-Lecellier, V. & Erdmann, R. (1998) *J. Biol. Chem.*, **273**, 3702–3711, IDP3 encodes a peroxisomal NADP-dependent isocitrate dehydrogenase required for the beta-oxidation of unsaturated fatty acids.
22. Mizugaki, M., Kimura, C, Nishimaki, T, Kawaguchi, A., Okuda, S. & Yamanaka, H. (1983) *J. Biochem.*, **94**, 409–413, Studies on the metabolism of unsaturated fatty acids. XII. Reaction catalyzed by 2,4-dienoyl-CoA reductase of *Escherichia coli.*

23. Dommes, V. & Kunau, W-H. (1984) *J. Biol. Chem.*, **259**, 1781–1788, 2,4-Dienoyl-CoA reductases from bovine liver and *Escherichia coli.* Comparison of properties.
24. Borrebaek, B., Osmundsen, H. & Bremer, J. (1980) *Biochem. Biophys. Res. Commun.*, **93**, 1173–1180, *In vivo* induction of 4-enoyl-CoA reductase by clofibrate in liver mitochondria and its effect on pent-4-enoate metabolism.
25. Mizugaki, M., Nishimaki, T., Yamamoto, H., Sagi, M. & Yamanaka, H. (1982) *J. Biochem.*, **92**, 2051–2054, Studies on the metabolism of unsaturated fatty acids. XI. Alterations in the activities of enoyl-CoA hydratase, 3-hydroxyacyl-CoA epimerase and 2,4-dienoyl-CoA reductase in rat liver mitochondria and peroxisomes by clofibrate.
26. Dommes, V, Baumgart, C. & Kunau, W.-H. (1981) *J. Biol. Chem.*, **256**, 8259–8262, Degradation of unsaturated fatty acids in peroxisomes. Existence of a 2,4-dienoyl-CoA reductase pathway.
27. Kimura, C., Kondo, A., Koeda, N., Yamanaka, H. & Mizugaki, M. (1984) *J. Biochem.*, **96**, 1463–1469, Studies on the metabolism of unsaturated fatty acids. XV. Purification and properties of 2,4-dienoyl-CoA reductase from rat liver peroxisomes.
28. Hiltunen, J.K., Kärki, T., Hassinen, I.E. & Osmundsen, H. (1986) *J. Biol. Chem.*, **261**, 16484–16493, β-Oxidation of polyunsaturated fatty acids by rat liver peroxisomes. A role for 2,4-dienoyl-coenzyme A reductase in peroxisomal β-oxidation.
29. Hakkola, E.H. & Hiltunen, J.K. (1993) *Eur. J. Biochem.*, **215**, 199–204, The existence of two mitochondrial isoforms of 2,4-dienoyl-CoA reductase in the rat.
30. Hakkola, E.H., Hiltunen, J.K. & Autio-Harmainen, H.I. (1994) *J. Lipid Res.*, **35**, 1820–1828, Mitochondrial 2,4-dienoyl-CoA reductases in the rat: differential responses to clofibrate treatment.
31. Hirose, A., Kamijo, K., Osumi, T., Hashimoto, T. & Mizugaki, M. (1990) *Biochim. Biophys. Acta*, **1049**, 346–349, cDNA cloning of rat liver 2,4-dienoyl-CoA reductase.
32. Koivuranta, K.T., Hakkola, E.H. & Hiltunen, J.K. (1994) *Biochem. J.*, **304**, 787–792, Isolation and characterization of cDNA for human 120kDa mitochondria! 2,4-dienoyl-coenzyme A reductase.
33. Gurvitz, A., Rottensteiner, H., Kilpeläinen, S.H., Hartig, A., Hiltunen, J.K., Binder, M., Dawes, I.W. & Hamilton, B. (1997) *J. Biol. Chem.*, **272**, 22140–22147, The *Saccharomyces cerevisiae* peroxisomal 2,4-dienoyl-CoA reductase is encoded by the oleate-inducible gene *SPS*19.
34. Baker, M.E. (1995) *Biochem. J.*, **309**, 1029–1030, Enoyl-acyl-carrier-protein reductase and *Mycobacterium tuberculosis* InhA do not conserve the Tyr-Xaa-Xaa-Xaa-Lys motif in mammalian 11β-and 17β-hydroxysteroid dehydrogenases and Drosophila alcohol dehydrogenase.
35. Jörnvall, H., Persson, M. & Jeffery, J. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 4226–4230, Alcohol and polyol dehydrogenases are both divided into two protein types, and structural properties cross-relate the different enzyme activities within each type.
36. Persson, B., Krook, M. and Jörnvall, H. (1991) *Eur. J. Biochem.*, **200**, 537–543, Characteristics of short-chain alcohol dehydrogenases and related enzymes.
37. Jörnvall, H., Persson, B., Krook, M., Atrian, S., Gonzalez-Duarte, R., Jeffery, J. & Ghosh, D. (1995) *Biochemistry*, **34**, 6003–6013, Short-chain dehydrogenases/reductases (SDR).
38. Wierenga, R.K., De Maeyer, M.C.H. & Hol, W.G.J. (1985) *Biochemistry*, **24**, 1346–1357, Interaction of pyrophosphate moieties with α-helixes in dinucleotide binding proteins.
39. You, S.Y., Cosloy, S. & Schulz, H. (1989) *J. Biol. Chem.*, **264**, 16489–16495, Evidence for the essential function of 2,4-dienoyl-coenzyme A reductase in the β-oxidation of unsaturated fatty acids *in vivo.*
40. Roe, C.R., Millington, D.S., Norwood, D.L., Kodo, N., Sprecher, H., Mohammed, B.S., Nada, M., Schulz, H. & McVie, R. (1990) *J. Clin. Invest.*, **85**, 1703–1707, 2,4-Dienoyl-coenzyme A reductase deficiency: a possible new disorder of fatty acid oxidation.
41. Tserng, K.-Y. & Jin, S.J. (1991) *J. Biol. Chem.*, **266**, 11614–11620, NADPH-dependent reductive metabolism of *cis*-5 unsaturated fatty acids. A revised pathway for the beta-oxidation of oleic acid.
42. Smeland, T.E., Nada, M., Cuebas, D. & Schulz, H. (1992) *Proc. Natl. Acad Sci. USA*, **89**, 6673–6677, NADPH-dependent β-oxidation of unsaturated fatty acids with double bonds extending from odd-numbered carbon atoms.
43. Luo, M.J., Smeland, T.E., Shoukry, K. & Schulz, H. (1994) *J. Biol. Chem.*, **269**, 2384–2388, $\Delta^{3,5}$,$\Delta^{2,4}$-Dienoyl-CoA isomerase from rat liver mitochondria.
44. Chen, L.-S., Jin, S.-J. & Tserng, K.-Y. (1994) *Biochemistry*, **33**, 10527-10534, Purification and mechanism of Δ^{3},Δ^{5}-*t*-2,*t*-4-dienoyl-CoA isomerase from rat liver.
45. Filppula, S.A., Yagi, A.I., Kilpeläinen, S.H., Novikov, D., FitzPatrick, D.R., Vihinen, M., Valle, D. & Hiltunen, J.K. (1998) *J. Biol. Chem.*, **273**, 349–355, $\Delta^{3,5}$-$\Delta^{2,4}$-Dienoyl-CoA isomerase from rat liver: molecular characterization.

46. FitzPatrick, D.R., Germain-Lee, E. & Valle, D. (1995) *Genomics*, **27**, 457–466, Isolation and characterization of rat and human cDNAs encoding a novel putative peroxisomal enoyl-CoA hydratase.
47. Tserng, K.-Y., Chen, L.-S. & Jin, S.J. (1995) *Biochem. J.*, **307**, 23–28, Comparison of metabolic fluxes of *cis*-5-enoyl-CoA and saturated acyl-CoA through the **β-oxidation** pathway.
48. Tserng, K.-Y. & Jin, S.-J. (1995) *Biochem. J.*, **308**, 39–44, Oxidation of *cis*-5-unsaturated fatty acids in intact rat liver mitochondria: the operation of reduction pathways.
49. Tserng, K.-Y, Jin, S.-J. & Chen, L.-S. (1996) *Biochem. J.*, **313**, 581–588, Reduction pathway of *cis*-5 unsaturated fatty acids in intact rat-liver and rat-heart mitochondria: assessment with stable-labelled substrates.
50. Shoukry, K. & Schulz, H. (1998) *J. Biol. Chem.*, **273**, 6892–6899, Significance of the reductase-dependent pathway for the **β-oxidation** of unsaturated fatty acids with odd-numbered double bonds.

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THE EFFECT OF β-OXIDABLE AND NON-β-OXIDABLE THIA FATTY ACIDS ON FATTY ACID METABOLISM

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1. INTRODUCTION

Thia fatty acids are fatty acid analogues where a methyl group of a normal fatty acid is substituted with sulphur. Such fatty acids can be categorised in two groups with distinct biological and metabolic properties depending on the position of the sulphur-atom in the fatty acid. In 3-thia fatty acids the β-methyl group is substituted with sulphur and, hence, they are blocked and cannot undergo β-oxidation. As they are substrates for CPT-I and CPT-II they can enter the mitochondria and further metabolism is blocked because they are not substrates for acyl-CoA-dehydrogenase. The only method of metabolising the 3-thia fatty acids is, consequently, ω-oxidation to a dicarboxylic acid, followed by β-oxidation from the ω-end.

The 3-thia, non-β-oxidable thia fatty acids (as exemplified by TTA (tetradecylthioacetic acid, $HOOC\text{-}CH_2\text{-}S\text{-}CH_2(13)\text{-}CH_3$) decrease plasma triglycerides and cholesterol levels when administered to rats.[1] At the same time TTA increased the hepatic fatty acid oxidation capacities. Recently we have demonstrated that stimulation of mitochondrial β-oxidation, but not peroxisomal fatty acid oxidation, decreases hepatic triglyceride formation.[2] This has also been shown with ω-3 fatty acids and fibrates in different animal models (rats, rabbits and dogs). Altogether the mitochondrion seems to be the principal target for the plasma triglycerid lowering effect.

The other category of thia-fatty acids is the β-oxidable variant with sulphur in the 4th position as exemplified by TTP (tetradecylthiopropanoic acid, $HOOC\text{-}CH_2\text{-}CH_2\text{-}S\text{-}CH_2(13)\text{-}CH_3$). Since the β-carbon is available for oxidation, these fatty acid analogues can undergo one cycle of mitochondrial β-oxidation, but the alkyl-thioacryloyl-CoAs then formed are poor substrates for both mitochondrial hydratase[3] and for CPT-II. Consequently they will accumulate in the mitochondrial matrix where they inhibit normal fatty acid oxidation.[4] In contrast to the effect obtained with TTA, repeated

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Table 1. List of thia fatty acids.

Compound	Formula	Comments
TTA	CH_3-$(CH_2)_{13}$-S-CH_2-COOH	Non-β-oxidable
5-S	CH_3-$(CH_2)_{11}$-S-$(CH_2)_3$-COOH	Available for 1 round of β-oxidation.
7-S	CH_3-$(CH_2)_9$-S-$(CH_2)_5$-COOH	Available for 2 rounds of β-oxidation.
9-S	CH_3-$(CH_2)_7$-S-$(CH_2)_7$-COOH	Available for 3 rounds of β-oxidation.

administration of TTP increased the hepatic level of triglycerides, while the concentrations of cholesterol and phospholipids remained unchanged.[5] The TTP tended to enlarge the mitochondria and did not promote proliferation of mitochondria and peroxisomes when compared to the **non-β-oxidable** fatty acid. The most striking effect of the TTP was the formation of numerous fat droplets in the liver cells. The volume fraction of lipid droplets increased 23-fold after TTP feeding.[6] Thus, TTP feeding appears to cause a pathological condition associated with fat droplets and high triglyceride levels in the liver. The reason for this is that the hepatic mitochondrial fatty acid oxidation is strongly inhibited. The 4-thia fatty acids have also been reported to increase plasma levels of triglycerides and cholesterol when administered to rats.[5,7]

2. RESULTS AND DISCUSSION

To elucidate more of the so far not fully understood mechanisms behind the effect of treatment with the thia fatty acids, we compared the effect of treatment with thia acids with the sulphur in different positions (Table 1). The main difference between these 16 carbon (17 including the sulphur) fatty acids is their availability for mitochondrial **β-oxidation.** As already mentioned TTA is blocked for **β-oxidation,** but can enter the mitochondria, as it is substrate for CPT-I and CPT-II. However, 5-S, 7-S and 9-S can both enter the mitochondria and undergo 1, 2, or 3 cycles of **β-oxidation** respectively, before they are metabolised into chain-shortened 3-thia fatty acids which cannot be **β-oxidised.** Evidently, the metabolised forms of 5-S, 7-S and 9-S will accumulate in the mitochondria. In agreement with earlier findings TTA administration lowered plasma triglycerides, 5-S tended to lower plasma triglycerides, but 7-S and 9-S did not effect the plasma triglyceride levels (data not shown). As shown previously, TTA administration lowered plasma cholesterol levels, while none of the **β-oxidable** fatty acids (5-S, 7-S and 9-S) had any effect on plasma cholesterol (Fig. 1). Hence, we see that the administered **β-oxidable** thia-fatty acids are less (if at all) hypolipidaemic than TTA. The same **β-oxidable** thia fatty acids are also weaker inducers of mitochondrial (Fig. 2) and peroxisomal (data not shown) **β-oxidation** than TTA.

It has been shown previously that chain-shortened 3-thia fatty acids have less hypolipidemic effect than TTA[8] (Table 2). Thus the less biological potency by the **β-**oxidable fatty acids compared to TTA could be due to chain shortening of the administered thia fatty acids by mitochondrial **β-oxidation.** Another possibility is the formation of an inhibitory metabolite during **β-oxidation** of these **β-oxidable** thia fatty acids as is the case for 4-thia fatty acids.

In order to elucidate that, we compared the effect of treating rats with the **β-**oxidable thia fatty acid 5-S and the **non-β-oxidable** 3-thia fatty acid DTA (dodecanylthio acetic acid, **HOOC-CH_2-S-CH_2(11)-CH_3).** Theoretically DTA is formed when 5-S has undergone 1 cycle of **β-oxidation.** In agreement with earlier findings (Table 2),

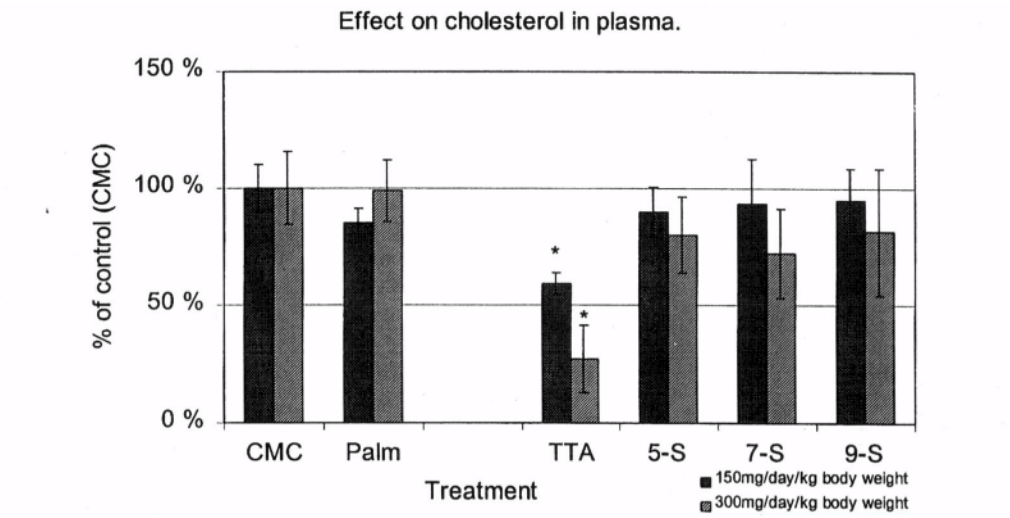

Figure 1. Effect of thia fatty acids on rat plasma cholesterol. The values represent the mean ±SD for four animals in each experimental group. Measurements performed in rat EDTA plasma. All values given as percentage of control (CMC). The rats were treated with a dose of 150 or 300 mg/d/Kg body mass for 7 days.

administration of DTA lowered the plasma triglycerides and plasma cholesterol levels (data not shown). 5-S administration decreased plasma triglycerides and plasma cholesterol significantly less than dodecanylthio acetic acid (DTA) at the same dosages. These results are in agreement with formation of an inhibitory metabolite during β-oxidation of 5-S. Whether increased intrahepatic direction of fatty acids towards esterification, as

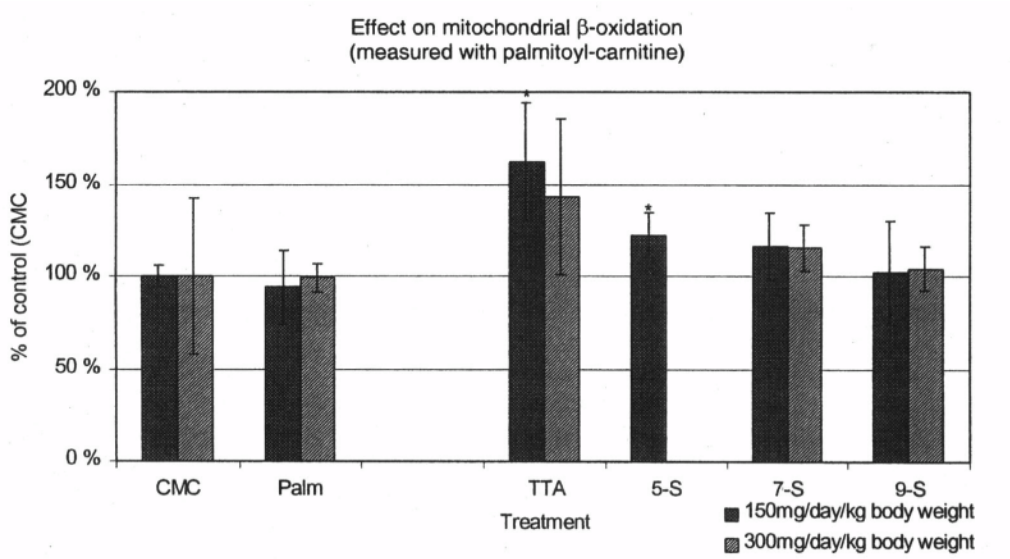

Figure 2. Effect of treatment with thia fatty acids on mitochondrial β-oxidation. The values represent the mean ±SD for four animals in each experimental group. Measurements performed in E-fractions of rat liver tissue. All values given as percentage of control (CMC). The rats were treated with a dose of 150 or 300 mg/d/Kg body mass for 7 days.

Table 2. Effects of 3-thia fatty acids with different chain-length and palmitic acid (control) on plasma triacylglycerol and phospholipid levels after 7 days of treatment. Results are expressed as means ± SD of five different animals in each group. [a]Significantly different from control: $P < 0.05$. From.[8]

	Plasma	
Treatment	Triacylglycerol	Phospholipids
	mmol/l	
Control	0.8 ± 0.1	1.3 ± 0.2
C8-S-acetic acid	0.7 ± 0.3	1.3 ± 0.2
C11-S-acetic acid	0.7 ± 0.1	1.2 ± 0.2
C12-S-acetic acid	0.5 ± 0.2[a]	0.8 ± 0.2[a]
C13-S-acetic acid	0.4 ± 0.2[a]	0.6 ± 0.1[a]
C14-S-acetic acid	0.4 ± 0.2[a]	0.7 ± 0.2[a]
C15-S-acetic acid	0.8 ± 0.3	0.9 ± 0.2
C16-S-acetic acid	1.0 ± 0.2	1.1 ± 0.1

opposed to mitochondrial **β-oxidation,** may effect the hepatic lipid metabolism and plasma lipid lowering effect are also to be considered.

REFERENCES

1. Berge, R.K., Aarsland, A., Kryvi, H., Bremer, J. & Aarsæther, N. 1989. *Biochem. Pharmacol.* **38**:3969–3979. Alkylthio acetic acids (3-thia fatty acids)-A new group of **non-β-oxidizable** peroxisome-inducing fatty acids analogues.II. Dose-response studies on hepatic peroxisomal- and mitochondrial changes and long-chain fatty acid metabolizing enzymes in rats.
2. Asiedu, D.K., Al-Shurbaji, A., Rustan, A.C., Björkhem, I., Berglund, L., Berge, R.K. 1995. *Eur. J. Biochem.* **227**:715–722. Hepartic fatty acid metabolism as a determinant of plasma and liver triacyl-glycerol levels.
3. Lau, S.M., Brantley, R.K. & Thorpe, C. 1989. *Biochemistry* **28**:8255–8262.4-Thia-trans-2-alkenoyl-CoA derivatives: properties and enzymatic reactions.
4. Hovik, R., Osmundsen, H., Berge, R., Aarsland, A. & Bergseth, S. 1990. *Biochem. J.* **270**:167–173. Effects of thia-substituted fatty acids on mitochondrial and peroxisomal **β-oxidation.**
5. Aarsland, A., Aarsæther, N., Bremer, J., Berge, R.K. 1989. *J. Lipid Res.* **30**:1711–1718. Alkylthioacetic acids (3-thia fatty acids) as **non-β-oxidizable** fatty acid analogues: a new group of hypolipidemic drugs.III. Dissociation of cholesterol- and triglyceride-lowering effects and the induction of peroxisomal **β-oxidation.**
6. Kryvi, H., Aarsland, A. & Berge, R.K. 1990. *J. Struct. Biol.* **103**:257-265. Morphologic effects of sulphur-substituted fatty acids on rat hepatocytes with special reference to proliferation of peroxisomes and mitochondria.
7. Berge, R.K., Aarsland, A., Kryvi, H., Bremer, A. & Aarsæther, N. 1989 *Biochim. Biophys. Acta* **1004**:345–356. Alkylthioacetic acids (3-thia fatty acids)-A new group of **non-βh-oxidizable** peroxisome-inducing fatty acid analogues.I. A study on the structural requirements for proliferation of peroxisome s and mitochondria in rat lliver.
8. Frøyland, L., Sjursen, W., Garras, A., Lie, ø., Songstad, J., Rustan, A.C. & Berge, R.K. 1997. *J. Lipid Res.* **38**:1522–1534. Effect of 3-thia acids in the lipid composition of rat liver, lipoproteins, and heart.

EPA AND DHA POSSESS DIFFERENT METABOLIC PROPERTIES

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It is generally accepted that high levels of plasma cholesterol represent an important risk factor for coronary artery disease. However, more resent research suggests that high serum levels of triglyceride (TG)-rich lipoproteins, i.e, very low density lipoprotein (VLDL) and its remnants are also important risk factors.[1,2] Serum lipids can be lowered either by dietary treatment with fish oil[3,4] or by pharmacological treatment with drugs of the fibrate class.[5] Fibrate action has been partly ascribed to increased fatty acid oxidation, decreased TG synthesis and secretion, and enhanced clearance of VLDL from serum due to a down-regulation of hepatic apoC-III gene-expression.[6,7]

Besides lowering plasma lipids and the apparent protection in thrombosis, dietary supplements enriched in omega-3 fatty acids have proved to lower blood pressure, alter lipoprotein metabolism and dampen platelet aggregation among other beneficial effects in humans.[8–10] The two components of fish oil attracting the most attention, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are still often referred to as omega-3, or n-3 fatty acids without any further distinction. Nearly all studies that have been conducted have used a mixture of EPA and DHA. It has, however, been reported that DHA are retroconverted to EPA in cultured hepatocytes,[11] rat[12] and man[13] and it is, therefore, conceivable that EPA and DHA possess different metabolic properties.[14]

The availability of TG is a major driving force in the secretion of VLDL by the liver. Evidently, factors influencing the balance between TG biosynthesis and/ or fatty acid oxidation may ultimately influence plasma lipoprotein levels and metabolism.[15] We have demonstrated this mechanism of action with sulfur-substituted fatty acids (3-thia fatty acids). In rats, the TG-lowering effect of the 3-thia fatty acid tetradecylthioacetic acid (C14-S-acetic acid), was established within hours of feeding and this was mainly due to stimulated mitochondrial fatty acid oxidation, thereby reducing hepatic TG synthesis and secretion.[16,17] The mechanism has been further evaluated using cell cultures.[18]

Treatment of rats with the peroxisomal- and mitochondrial- proliferating C14-S-acetic acid, caused a drop in the hepatic content of ERA.[19] Conversion of EPA to

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docosahexaenoic acid (DHA) can not explain the decreased content of EPA, as the hepatic content of DHA also decreased.[19] The decreased hepatic levels of n-3 fatty acids may result from a selective increased **β-oxidation** as the 2.4-dienoyl-CoA reductase activity, which is necessary to oxidize polyunsaturated fatty acids, was increased in both the mitochondrial- and the peroxisomal fraction after 3-thia fatty acid treatment.[19]

Activation of the fatty acids to their respective CoA esters, is a necessary step prior to oxidation. DHA was converted to its CoA ester in all subcellular fractions, but was a poor substrate for oxidation in both the mitochondrial- and the peroxisomal fractions.[20] Compared to EPA-CoA, DHA-CoA was more effectively synthesized in the peroxisomal than the mitochondrial fraction, especially in animals treated with 3-thia fatty acids.[20] It has been reported that very long chain fatty acyl-CoA synthases as lignoceryl-CoA are absent in mitochondria[21] and DHA-CoA synthase activity measured in the mitochondrial fraction, might also be due to contamination of peroxisomes.[20]

DHA-CoA was a substrate for peroxisomal fatty acyl-CoA oxidase and the activity of DHA-CoA oxidase increased several folds after treatment with 3-thia fatty acids (Table 1). Noteworthy, in contrast to EPA-CoA, DHA-CoA was a very poor substrate, if at all, for mitochondrial carnitine acyltransferase (CAT) -I and CAT-II (Table 1). In addition, neither mitochondrial CAT-I nor CAT-II activity increased after 3-thia fatty acid treatment (Table 1) when DHA-CoA was used as a substrate. It might, therefore, be considered that DHA can not be oxidized by the mitochondria.

The different metabolism of EPA and DHA is consistent with our findings that the oxidation of EPA and not DHA in cultured hepatocytes was strongly inhibited by the CAT-II inhibitor aminocarnitine (Fig. 1). The overall kinetics suggest that EPA can be oxidized in both peroxisomes and mitochondria, but is oxidized in preference by mitochondria.

3-Thia fatty acids increased oxidation of the different fatty acids, but the oxidation of palmitic-, oleic acid and EPA in the peroxisomal fraction was only 5–20% of the mitochondrial fatty acid oxidation.[20] Thus, the capacity of the mitochondria to oxidize long-chain fatty acids is of quantitative major importance. Seen as a whole, our results suggest that EPA is preferentially oxidized by mitochondria, while DHA is preferentially oxidized by peroxisomes. This might explain why the EPA level was decreased 40–80% after 3-thia fatty acid treatment whereas the DHA level was decreased only 17–24%.[19]

Table 1. Effect of C14-S-acetic acid treatment on mitochondrial carnitine acyltransferase-I and -II and peroxisomal fatty acyl-CoA oxidase, using EPA-CoA and DHA-CoA as substrates.

		Fatty acid treatment (300 mg/day/kg body weight)	
Variables	Substrate	Palmitic acid	C14-S-acetic acid
Mitochondrial carnitine acyltransferase-I (mmol/min/mg protein)	DHA-CoA	0.11 ± 0.05	0.10 ± 0.07
	EPA-CoA	1.69 ± 0.10	2.35 ± 0.21*
Mitochondrial carnitine acyltransferase-II (mmol/min/mg protein)	DHA-CoA	11.5 ± 5.1	9.1 ± 1.5
	EPA-CoA	32.2 ± 8.2	99.7 ± 27.7*
Peroxisomal fatty acyl-CoA oxidase (nmol/min/mg proten)	DHA-CoA	4.9 ± 2.1	57.1 ± 25.0*
	EPA-CoA	7.3 ± 1.1	37.2 ± 12.3*

Values represent means ± SD of four rats treated for 7 days. *$p < 0.05$ between control (palmitic acid) and treated rats (C14-S-acetic acid)

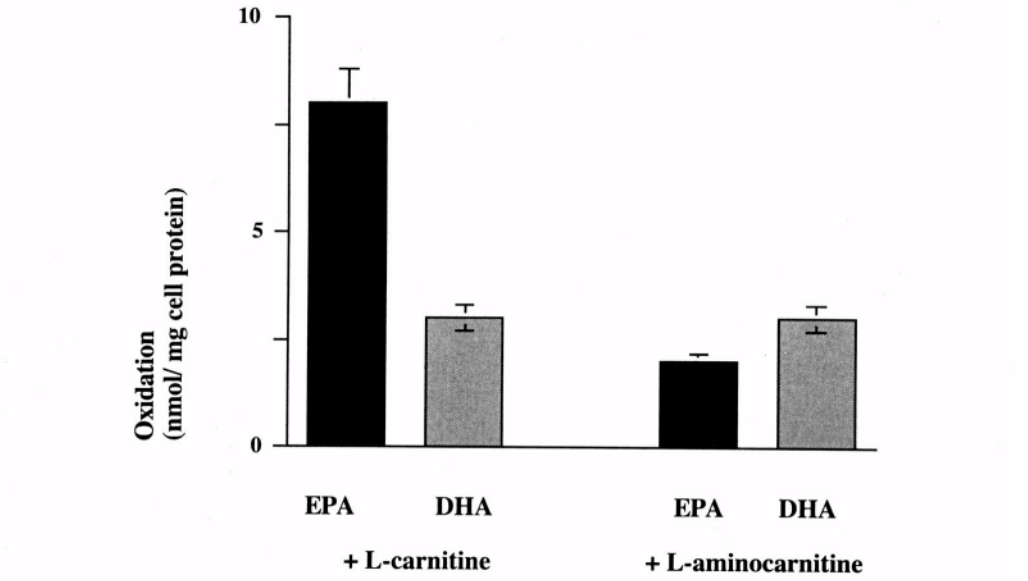

Figure 1. Oxidation of 14C EPA and 14C DHA by coultured rat hepatocytes. The cells were plated and incubated in media containing 0.5 mM L-carnitine or 50 μM L-aminocarnitine (an CPT-II inhibitor).

When EPA or DHA are fed to rats, they accumulate in different organs.[12] However, DHA feeding also leads to an accumulation of EPA.[12] As DHA seems difficult to metabolize, one might speculate that DHA needs to be converted to EPA for further metabolism.

In rats fed a single dose of EPA the plasma TG concentration was decreased within hours, accompanied by an increased hepatic fatty acid oxidation.[22] Table 2 shows after 10 days of repeated administrations of EPA, the plasma concentration of TG was decreased almost 40% and both the mitochondrial and peroxisomal oxidation was increased. On the other hand, administration of DHA increased the peroxisomal fatty acid oxidation, but both the plasma TG levels and mitochondrial fatty acid oxidation were unchanged (Table 2). These results among others[23] suggest that the hypotriglyceridaemic effect of n-3 fatty acids is independent of the peroxisomal fatty acid oxidation.

After long-term feeding (12 weeks) with EPA an increased volume fraction of mitochondria was seen with a concomitant reduction in plasma VLDL-TG (Table 3). In

Table 2. Effect of dietary EPA, DHA and palmitic acid on serum triglycerides and fatty acid oxidation in mitochondria and peroxisomes.

	Fatty acid treatment (1,000 mg/day/kg body weight)		
Variables	Palmitic acid	DHA	EPA
Serum triglycerides (mmol/L)	1.36 ± 0.25	1.31 ± 0.32	0.85 ± 0.25*
Peroxisomal fatty acid oxidation (nmol/min/mg proten)	4.8 ± 0.7	8.9 ± 0.4*	9.3 ± 1.8*
Mitochondrial fatty acid oxidation (nmol/min/g liver)	250 ± 16	258 ± 18	296 ± 10*

Values represent means ± SD of five rats treated for 10 days. *$p < 0.05$ between control (palmitic acid) and treated rats (EPA or DHA)

Table 3. Effect of dietary EPA, DHA and palmitic acid on serum very-low density lipoprotein triglycerides (VLDL-TG) and hepatic volume fractions of mitochondria and peroxisomes.

Fatty acid treatment (1g/day/kg body weight)	VLDL-TG (mmol/L)	Mitochondria (%)	Peroxisomes (%)
Palmitic acid	1.35 ± 0.17	15.7 ± 1.4	1.1 ± 0.1
DHA	1.48 ± 0.23	17.2 ± 1.6	1.9 ± 0.2*
EPA	0.95 ± 0.31*	21.8 ± 2.3*	1.2 ± 0.1

Values represent means ± SD of five rats treated for 12 weeks *$p < 0.05$ between control (palmitic acid) and treated rats (EPA or DHA)

contrast, DHA had no effect on plasma VLDL-TG and did not affect the mitochondria but increased the volume fraction of peroxisomes (Table 3). In addition, the volume fraction of hepatic fat droplets decreased after EPA, but not after DHA treatment.[24] The mitochondria are much more abundant than peroxisomes in most animal cells and under normal conditions they oxidize more than 90% of long-chain fatty acids.[25] Thus, the mitochondria would, therefore, be a more useful target than peroxisomes for nutritional and pharmacological control of triglyceride metabolism.

We have suggested that 3-thia fatty acids[26,27] and EPA which we have demonsrated is the hypotriacylglycerolidaemic component of fish oil[12,28] may mediate their hypotriacylglycerolidaemic effect by increasing mitochondrial β-oxidation. In addition, we have obtained evidence that the mitochondria are the principal target for nutritional and pharmacological control for the hypolipidaemic effect of EPA, fibrates and C14-S-acetic acid in rats and rabbits.[24]

Both EPA and C14-S-acetic acid are converted to their respective CoA esters in mitochondria.[20] Furthermore, in contrast to DHA-CoA, EPA-CoA and C14-S-acetyl-CoA are easily transferred into the mitochondria by the CAT system.[20] EPA is more difficult to oxidize than saturated and monounsaturated fatty acids, due to the double bonds and C14-S-acetic acid is non-oxidizable by β-oxidation, due to the sulfur atom in 3-position. Thus, accumulation of their respective CoA esters in the mitochondria might give an "fatty acid overload" signal leading to increased mitochondrial fatty acid oxidation. C14-S-acetic acid mimics the effects of peroxisome proliferators such as the fibrates and it was recently shown that it may be a ligand for the peroxisome proliferating activated receptor (PPAR) α.[29] As administration of the 3-thia fatty acids seem to force EPA to the mitochondria, an additional "fish oil effect" might be seen.

In conclusion, we have demonstrated that the polyunsaturated n-3 fatty acids, EPA and DHA are differently metabolized in rat liver. As palmitic- and oleic acid, EPA is oxidized by both peroxisomes and mitochondria, however, during mitochondria- and peroxisome proliferation, EPA seems to be preferentially oxidized by the mitochondria whereas DHA is most likely oxidized by the peroxisomes. Thus, different metabolic properties of these polyunsaturated n-3 fatty acids also imply different effects i.e. they affect organelle proliferation in relation to the substrate preference.

REFERENCES

1. Austin, M.A. 1991. *Atheroscler. Thromb.* **11**, 2–14. Plasma triglyceride and coronary heart disease.
2. Hodis, H.N., Mack, W.J., Azen, S.P., Alaupovic, P., Pogoda, J.M., LaBree, L, Hemphill, L.C., Kramsch, D.M. & Blankenhorn, D.H. 1994. *Circulation,* **90**, 42–49. Triglyceride- and cholesterol-rich lipoproteins

have a different effect on mild/ moderate and sereve lesion progression as assayed by quantitative coronary angiography in a controlled trial of lovastatin.

3. Bang, H.O., Dyerberg, J. & Nielsen, A.B. 1971. *Lancet*, **710**, 1143–1145. Plasma lipid and lipoprotein pattern in Greenlandic West-coast Eskimos.
4. Nestel, P.J., Connor, W.E., Reardon, M.F., Connor, S.W.S. & Boston, R. 1984. *J. Clin. Invest*., **74**, 82–89. Suppression by diets rich in fish oil of very low density lipoprotein production in man.
5. Shepherd, J. 1994. *Atherosclerosis*, **110**, S55–S63. The flbrates in clinical practice: focus on micronised fenofibrate.
6. Staels, B., Vu-Dac, N., Kosykh, V.A., Saladin, R., Fruchart, J.-C, Dallongeville, J. & Auwerx, J. 1995. *J. Clin. Invest*, **95**, 705–712. Fibrates downregulate apolipoprotein C-III expression independent of induction of peroxisomeal acyl coenzyme A oxidase. A potential mechanism for the hypolipidemic action of fibrates.
7. Hertz, R., Bishara-Shieban, J. & Bar-Tana, J. 1995. *J. Biol. Chem*., **270**, 13470–13475. Mode of action of peroxisome proliferators as hypolipidemic drugs.
8. Leaf, A. & Kang, J. 1996. *J. Intern. Med.*, **240**, 5–12. Prevention of cardiac sudden death by N-3 fatty acids: a review of the evidence.
9. Harris, W. 1997. *Am. J. Clin. Nutr*., **65**, 1645S–1654S. n-3 fatty acids and serum lipoproteins: human studies.
10. Blok, W., Katan, M. & van der Meer, J.W. 1996. *J. Nutr*., **126**, 1515–1533. Modulation of inflammation and cytokine production by dietary (n-3) fatty acids.
11. Grønn, M., Christensen, E., Hagve, T.A. & Christophersen, B.O. 1992. *Biochim. Biophys. Acta*, **1125**, 35–43. Effects of dietary purified eicosapentaenoic acid (20:5(n-3)) and docosahexaenoic acid (22:6(n-3)) on fatty acid desaturation and oxidation in isolated rat liver cell.
12. Frøyland, L., Vaagenes, H., Asiedu, D., Garras, A., Lie, ø. & Berge, R.K. 1996. *Lipids*, **31**, 169–178. Chronic administration of eicosapentaenoic acid and docosahexaenoic acid as ethyl esters reduced plasma cholesterol and changed the fatty acid composition in rat blood and organs.
13. Von Schacky, C. & Weber, P.C. 1985. *J. Clin. Invest*., **76**, 2446–2450. Metabolism and effects of platlet function of the purified eicosapentaenoic and docosahexaenoic acid in humans.
14. Berge, R.K. 1993. *Omega-3 News*, **1**, 5–7. The triglyceride-lowering effect of omega-3 fatty acids. Is it all in eicosapentaenoic acid.
15. Soutar, A. 1978. *Nature*, **273**, 11–12. Does dietary fat influence plasma lipiorotein structure?
16. Asiedu, D.K., Skorve, J., Willumsen, N., Demoz, A. & Berge, R.K. 1993. *Biochim. Biophys. Acta*, **1166,** 73–76. Early effects on mitochondrial and peroxisomal β-oxidation by the hypolipidemic 3-thia fatty acids in rat livers.
17. Asiedu, D.K., Demoz, A., Skorve, J., Grav, H.J. & Berge, R.K. 1994. *Biochem. Pharmacol*., **49**, 1013–1022. Acute modulation of rat hepatic lipid metabolism by sulphur-substituted fatty acid analogues.
18. Skorve, J., Rustan, A.C. & Berge, R.K. 1995. *Lipids*, **30**, 987–994. Effects of non-β-oxidable sulfur-substituted fatty acid analogues on synthesis and secreation of triacylglycerol and cholesterol in cultured rat hepatocytes.
19. Frøyland, L., Madsen, L., Sjursen, W, Garras, A., Lie, ø., Songstad, J., Rustan, A.C. & Berge, R.K. 1997. *J. Lipid Res*., **38**, 1522–1534. Effect of 3-thia fatty acids on the lipid composition of rat liver, lipoproteins, and heart.
20. Madsen, L., Frøyland, L., Dyrøy, E., Helland, K. & Berge, R.K. 1998. *J. Lipid Res*., **39**, 583–593. Docosahexaenoic- and eicosapentaenoic acid are differently metabolized in rat liver during mitochondria- and peroxisome proliferation.
21. Lazo, O., Contreras, M., Yoshida, Y., Singh, A.K., Stanley, W., Weise, M. & Singh, I. 1990. *J. Lipid Res*., **31**, 583–595. Cellular oxidation of lignoceric acid is regulated by the subcellular localization of lignoceroyl-CoA ligases.
22. Willumsen, N.,Vaagenes, H., Asiedu, D., Lie, ø., Rustan, A.C. & Berge, R.K. 1996. *Lipids*, **31**, 579–592. Eicosapentaenoic acid but not docosahexaenoic acid (both as ethyl esters) increases mitochondrial fatty acid oxidation and upregulates 2,4-dienoyl-CoA reductase gene expression. A potential mechanism for the hypolipidemic action of fish oil in rats.
23. Willumsen, N., Hexeberg, S., Skorve, J., Lundquist, M. & Berge, R.K. 1993. *J. Lipid Res*., **34**, 13–22. Docosahexaenoic acid shows no triglyceride-lowering effects but increases the peroxisomal fatty acid oxidation in liver of rats.
24. Frøyland, L., Madsen, L., Vaagenes, H., Totland, G.K., Auwerx, J., Kryvi, H., Staels, B. & Berge, R.K. 1997. *J. Lipid Res*., **38**, 1851–1858. Mitochondrion is the principal target for nutritional and pharmacological control of triglyceride metabolism.

25. Mannaerts, G.P., Debeer, L.J., Thomas, J. & DeShepper, P.J. 1979. *J. Biol. Chem.*, **254**, 4585–4595. Mitochondrial and peroxisomal fatty acid oxidation in liver homogenates and isolated hepatocytes from control and clofibrate treated rats.
26. Asiedu, D.K., Demoz, A., Skorve, J., Grav, H.J. & Berge, R.K. 1995. *Biochem. Pharmacol.*, **49**, 1013–1022. Acute modulation of rat hepatic lipid metabolism by sulphur-substituted fatty acid analogues.
27. Asiedu, D.K., Al-Shurbaji, A., Rustan, A.C., Björkhem, I., Berglund, L. & Berge, R.K. 1995. *Em J. Biochem.*, **227**, 715–722. Hepatic fatty acid metabolism as a determinant of plasma and liver triacylglycerol levels.
28. Willumsen, N., Skorve, J., Hexeberg, S., Rustan, A.C. & Berge, R.K. 1993. *Lipids*, **28**, 683–689. The hypotriglyceridemic effect of eicosapentaenoic acid in rats is reflected in increased mitochondrial fatty acid oxidation followed by diminished lipogenesis.
29. Foreman, B.M., Chen, J. & Evans, R.M. 1996. *Ann. N. Y. Acad. Sci*, **804**, 266–275. The peroxisome proliferatoe activated receptors: ligands and activators.

THE USE OF [9,10-^{3}H]MYRISTATE, [9,10-^{3}H]PALMITATE AND [9,10-^{3}H]OLEATE FOR THE DETECTION AND DIAGNOSIS OF MEDIUM AND LONG-CHAIN FATTY ACID OXIDATION DISORDERS IN INTACT CULTURED FIBROBLASTS

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The release of ^3H_2O from [9,10-^{3}H]myristate and/or [9,10-^{3}H]palmitate has been used extensively for detecting medium- and long-chain fatty acid oxidation defects, both in cultured fibroblasts[1,2] and in fresh lymphocytes.[3] Over the past 10 years we have used both substrates to screen routinely for fatty acid oxidation defects in over 1,200 patients and have identified 113 individuals with specific fatty acid oxidation disorders (Table 1). More recently we have examined the use of a third substrate, [9,10-^{3}H]oleate, to improve discrimination of long-chain defects.[4]

1. ASSAYS WITH LABELLED MYRISTATE AND PALMITATE

Comparison of the absolute and relative activities obtained with these two substrates can often pinpoint the nature of the fatty acid oxidation defect. Medium chain acyl-CoA dehydrogenase deficiency (MCAD) is characterised by a much decreased oxidation of myristate and a high palmitate/myristate (P/M) ratio, mean percentage activity relative to simultaneous controls being 11.5 ± 5.1 $(n = 18)$ with a corresponding P/M ratio of 4.6 ± 1.7 (Fig. 1).

The long chain defects, very long-chain acyl-CoA dehydrogenase deficiency (VLCAD) and long-chain 3-hydroxacyl-CoA dehydrogenase deficiency (LCHAD), generally give a low P/M ratio. The mean P/M ratio for LCHAD is 0.60 ± 0.07 $(n = 18)$ and

Current Views of Fatty Acid Oxidation and Ketogenesis: From Organelles to Point Mutations edited by Quant and Eaton, Kluwer Academic / Plenum Publishers, New York, 1999.

Table 1. Fatty acid oxidation defects detected using ^3H_2O release assays, 1988–1998.

Defect	No. of cases
Primary carnitine deficiency	6
Carnitine palmitoyltransferase type I deficiency	4
Carnitine-acylcarnitine translocase deficiency	2
Carnitine palmitoyltransferase type II deficiency	
Infantile form	6
Adult onset	1
Very long chain acyl-CoA dehydrogenase deficiency	13
Long chain 3-hydroxyacyl-CoA dehydrogenase deficiency	25
Mitochondrial trifunctional protein deficiency	1
Medium chain acyl-CoA dehydrogenase deficiency	41
Glutaric aciduria type II—severe	8
Glutaric aciduria type II—mild	6

the mean percentage activity with palmitate of **34.0 ± 10.6** (Fig. 2), giving good discrimination between LCHAD and controls.[2] However, discrimination is rather poor for VLCAD, particularly "mild" variants, where both absolute activities and the P/M ratio may show overlap with the control range (Fig. 3).

2. ASSAYS WITH [9,10-^{3}H]OLEATE: VERY LONG-CHAIN ACYL CoA DEHYDROGENASE DEFICIENCY

The introduction of our [9,10-^{3}H]oleate assay has given improved discrimination in the detection of long chain defects especially enabling us to confidently detect all cases of both LCHAD and VLCAD.[4]

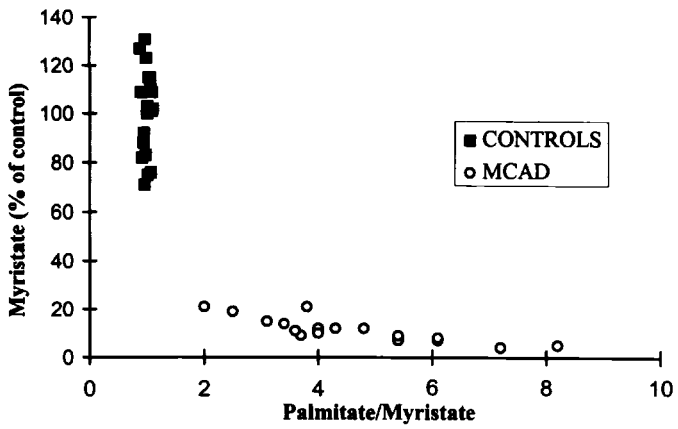

Figure 1. Individual results of the ^3H_2O-release assays of 18 patients with MCAD as compared to 20 controls. This figure combines the results of 18 assays. In each case the release of ^3H_2O from [9,10-^{3}H]myristate and [9,10-^{3}H]palmitate was determined in parallel with at least 3 unaffected cell lines (not all control data are plotted). All determinations were in duplicate and the activities for the individual cell lines are expressed as a proportion of the assay mean.

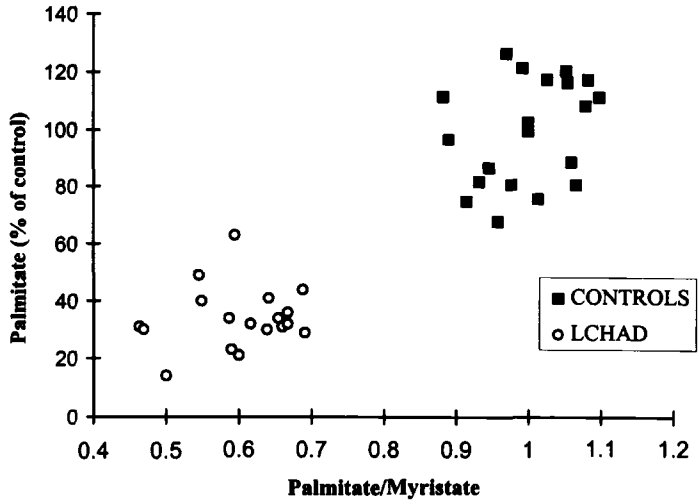

Figure 2. Individual results of the ^3H_2O-release assays of 18 patients with LCHAD as compared to controls. Details as for Figure 1.

Clinically, VLCAD can be divided into "mild" and "severe" variants.[5] In our series, the "severe" group comprised patients with evidence of cardiomyopathy at presentation or cardiomyopathy in a previously affected sibling and/or early presentation (3 days to 3 months of life) with generally a poor clinical outcome. The "mild" group contained two adult presentations with predominantly skeletal muscle involvement and four children (two pairs of affected siblings from two families) surviving with relatively little clinical intervention in the early years.

Mean activities with [9,10-^{3}H]oleate for "mild" and "severe" VLCAD (as a percentage of mean control activity) were 16.0 ± 6.1 and 15.0 ± 5.8, respectively, and the

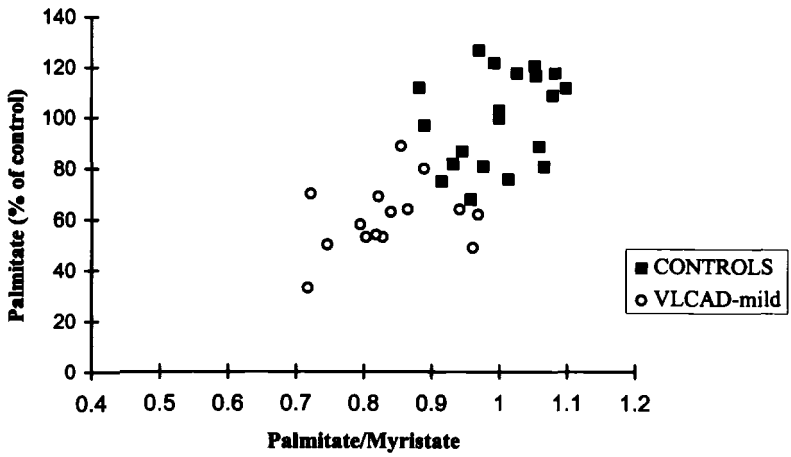

Figure 3. Overlap of controls and "mild" VLCAD patients when only [9,10-^{3}H]myristate and [9,10-^{3}H]palmitate are used as substrates. Details as for Figure 1. The data from the "mild" VLCAD group is comprised of results from six patients, each patient cell line having been assayed two or three times. Details as for Figure 1.

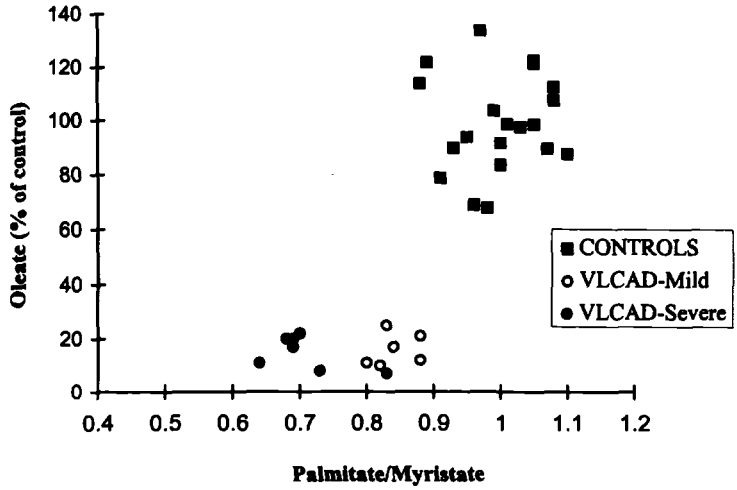

Figure 4. Improved discrimination achieved between controls and VLCAD cell lines with the introduction of the [9,10-^{3}H]oleate assay. The VLCAD data are from 6 "mild" and 7 "severe" VLCAD patients, each data point being the mean of between two and six assays.

corresponding myristate/oleate (M/O) ratios were 4.4 ± 1.1 (n = 6) and 4.4 ± 2.0 (n = 7) (Fig. 4). However, comparing the activity with all three substrates may indicate the degree of clinical severity: palmitate oxidation is less markedly impaired in the milder variants, the mean P/M ratio being 0.84 ± 0.03 (n = 6) and 0.71 ± 0.06 (n = 7) for "mild" and "severe" variants respectively.

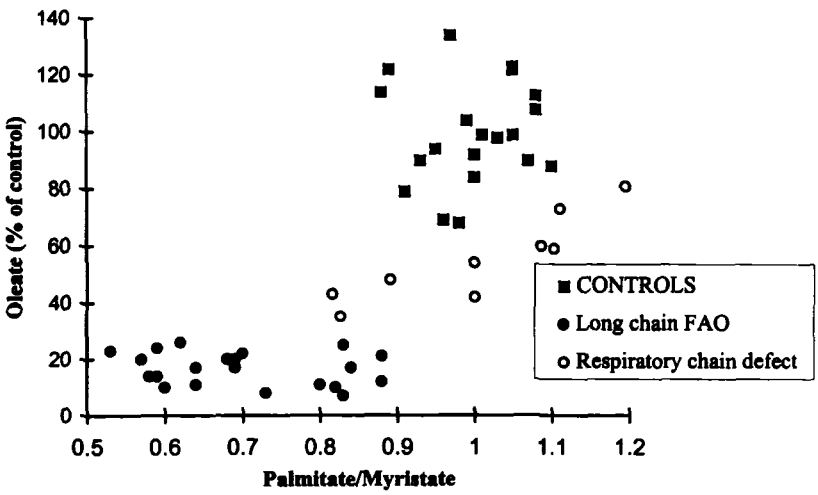

Figure 5. The data show how patients with confirmed or suspected defects of the mitochondrial respiratory chain can be separated from patients with confirmed long chain fatty acid oxidation defects (data from Figure 4 plus 8 cases of LCHAD). In each case the release of ^3H_2O from [9,10-^{3}H]myristate, [9,10-^{3}H]palmitate and [9,10-^{3}H]oleate was determined in parallel with at least 3 unaffected cell lines (not all control data plotted). All determinations were in duplicate and the activities for the individual cell lines are expressed as a proportion of the assay mean.

3. RESPIRATORY CHAIN DISORDERS

In recent years we have increasingly encountered patients whose clinical and biochemical presentations both suggest a fatty acid oxidation disorder but who do not meet the criteria for any of the recognised **β-oxidation** defects. These patients may have mild to moderate reductions in fibroblast oxidation of both [9,10-^{3}H]myristate and [9,10-^{3}H]palmitate, often into the VLCAD and LCHAD ranges, but show only a moderate reduction in oxidation of [9,10-^{3}H]oleate (Fig. 5). A number of such patients have presented with hepatic and/or skeletal muscle symptomatology but have subsequently proved to have a definite or probable mitochondrial respiratory chain disorder.

REFERENCES

1. Manning, N.J., Olpin, S.E., Pollitt, R.J. & Webley, J. (1990) *J. Inherited Metab. Dis.* **13**, 58–68. Comparison of [9,10-^{3}H]palmitic acid and [9,10-^{3}H]myristic acids for the detection of fatty acid oxidation defects in intact cultured fibroblasts.
2. Olpin, S.E., Manning, N.J., Carpenter, K.., Middleton, B. & Pollitt, R.J. (1992) *J. Inherited Metab. Dis.* **15**, 883–890. Differential diagnosis of hydroxydicarboxylic aciduria based on release of ^3H_2O from [9,10-^{3}H]myristic and [9,10-^{3}H]palmitic acids by intact cultured fibroblasts.
3. Brivet, M., Slama, A., Saudubray, J-M. & Lemonnier, A. (1995). *Ann. Clin. Biochem.* **32**, 293–297. Rapid diagnosis of long and medium chain fatty acid oxidation disorders using lymphocytes.
4. Olpin, S.E., Manning, N.J., Pollitt, R.J. & Clark, S. (1997). *J. Inherited Metab. Dis.* **20**, 415–419. Improved detection of long-chain fatty acid oxidation defects in intact cells using [9,10-^{3}H]oleate.
5. Vianey-Saban, C., Divry, C., Brivet, M., Nada, M., Zabot, M.T., Mathieu, M. & Roe, C. (1998) *Clin. Chim. Acta* , **269**, 43–62. Mitochondrial very-long-chain acyl-coenzyme A dehydrogenase deficiency: clinical characteristics and diagnostic considerations in 30 patients.

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RAPID DIAGNOSIS OF ORGANIC ACIDEMIAS AND FATTY-ACID OXIDATION DEFECTS BY QUANTITATIVE ELECTROSPRAY TANDEM-MS ACYL-CARNITINE ANALYSIS IN PLASMA

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1. ABSTRACT

The analysis of circulating free carnitine and acyl-carnitines provides a powerful selective screening tool for genetic defects in mitochondrial fatty acid oxidation and defects in the catabolism of branched chain amino acids. Using electrospray tandem mass spectrometry (ESI/MS/MS) we developed a sensitive quantitative analysis of free carnitine and acyl-carnitines in plasma and/or serum. This method was evaluated by analyzing 250 control samples and 103 samples of patients suffering from twelve different defects in either mitochondrial fatty acid oxidation or the catabolism of branched chain amino acids. The reproducibility of the method was acceptable with a day-to-day coefficient of variation ranging from 6–15% for free carnitine and the different acyl-carnitines. Except for one patient with a mild form of short chain acyl CoA dehydrogenase (SCAD) deficiency and a single sample from a patient with a mild form of multiple acyl CoA dehydrogenase (MAD) deficiency all patient samples were clearly abnormal under a wide variety of clinical conditions, illustrating the high sensitivity and specificity of the method.

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2. INTRODUCTION

Organic acidemias caused by mitochondrial enzyme defects in the catabolism of branched-chain amino acids and defects in mitochondrial fatty acid oxidation comprise a group of more than 20 distinct disorders. In all these disorders there is a accumulation of potentially toxic acyl-CoA esters in mitochondria which are converted into acyl-carnitine esters, releasing coenzyme A.[1–4] This results in an increased concentration of circulating acyl-carnitines, increased excretion of acyl-carnitines in urine and secondary carnitine deficiency. Therefore the analysis of circulating free carnitine and acyl-carnitines provides a powerful selective screening tool for these disorders.[5–9]

Electrospray-tandem-mass-spectrometry (ESI/MS/MS) analysis has provided a significant technological advance for the rapid and accurate analysis of acyl-carnitines with high sensitivity and specificity, allowing the analysis of large numbers of samples.[5,6,9] Moreover, ESI/MS/MS analysis of acyl-carnitines does not require time-consuming separation techniques like GC or HPLC before mass-spectrometry because separation occurs during MS/MS analysis allowing high specificity even with complex mixtures like plasma and urine. The basic principle of this technique is that analytes are ionized by electrospray, a soft ionization technique that minimizes fragmentation of the molecules. Molecular ions are selected in the first mass-spectrometer and undergo collision induced fragmentation, after which the daughter ions are selected in the second mass-spectrometer. By selecting appropriate scanning functions (e.g. parent-ion scan for butylated acyl-carnitines or neutral-loss scan for several butylated amino acids) specific multiple analytes of different chemical classes can be detected and quantified.[6,7,10]

ESI/MS/MS of acyl-carnitines has been successfully applied in newborn screening for defects in fatty acid oxidation and organic acidemias.[5,6,9,11] In addition, acyl-carnitine analysis has also been applied for the selective screening for these disorders,[12,13,14,15] for postmortem diagnosis using bile fluid[16] and for prenatal diagnosis of organic acidemias and defects of fatty acid oxidation.[17,18] In the current study we developed a quantitative acyl-carnitine analysis in plasma and included the analysis of free carnitine in the same assay. The results show that this analysis is highly sensitive and reproducible and therefore suitable for selective screening of fatty acid oxidation defects, organic acidemias and secondary carnitine deficiency.

3. MATERIALS AND METHODS

3.1. Blood Samples

Blood samples were collected by venous puncture, centrifuged and the resulting plasma or serum was stored at –20°C until analysis. Samples were collected from patients (n = 103) either suspected of a defect in fatty acid oxidation or an organic acidemia, or from patients known to suffer from any of these defects. All diagnoses were confirmed either enzymatically or using molecular analysis. Reference samples (n = 250) were obtained from hospitalized children in the age from 1 month to 10 years not suspected or suffering from one of the above mentioned disorders.

3.2. Sample Preparation

In this study, to 50 µl plasma or serum 50 µl standard 1 (23.5 µmol/L $^2H_3$-free carnitine in H_2O) and 50 µl standard 2 (10 µmol/L $^2H_3$-C2, 2 µmol/L $^2H_3$-C8 and 2 µmol/L

$^2H_3$-C16 carnitine in acetonitrile) were added. Samples were mixed and subsequently deproteinized with 500 µl acetonitrile and centrifuged. The resulting supernatant was dried under nitrogen at 45 °C, and subsequently derivatized in 100 µl butanolic-HCl for 15 min at 60 °C. Samples were dried under nitrogen at 45 °C and redissolved in 300 µl acetonitrile. Prior to injection, 70 µl of the acetonitrile containing the acyl-carnitines was mixed with 30 µl H_2O.

3.3. Sample Introduction and ESI-MS/MS Analysis

Free carnitine and acyl-carnitines were measured using scanning for precursor ions of mass 85 from 200–550 Da during 2 minutes on a Micromass Quattro II triple quadrupole mass spectrometer, using a Gilson 231 XL autosampler and a Hewlett-Packard HP-1100 HPLC pump essentially as described previously.[5,6]

3.4. Calibration

Calibration curves were obtained for free carnitine in the range of 5–100 µmol/L, 2–40 µmol/L for acetyl-carnitine and from 0.25–6 µmol/L for all other available acyl-carnitines by adding standards to a normal plasma pool. All calibration curves were linear ($r > 0.99$, data not shown). For unsaturated and hydroxylated acyl-carnitines an identical response as for their saturated counterparts was assumed.

4. RESULTS AND DISCUSSION

Since all butylated acyl-carnitine species share a similar fragmentation pattern with a common $m/z = 85$ fragment, acyl-carnitines can be detected with a parent-ion scan for this fragment.[5,6,11] In normal controls the plasma or serum acyl-carnitine profile shows only substantial amounts of free carnitine and acetyl-carnitine, whereas other acyl-carnitine species are only present in trace amounts (Fig. 1). In contrast to acyl-carnitine profiles obtained from blood-spots, long chain acyl-carnitines (C14–C18) are virtually absent in plasma or serum acyl-carnitine profiles obtained from normal controls, suggesting that the long-chain acyl-carnitines observed in blood-spot samples are derived from intracellular sources.[5,6] This facilitates the visual inspection of acyl-carnitine profiles, especially when profiles are printed in a standard way, with the $^2H_3$-C8-carnitine internal standard at 50% of full scale, allowing an easy detection of defects in the metabolism of long-chain acyl-carnitines (Fig. 1).

Since we wanted to establish a quantitative analysis of acyl-carnitines the assay was calibrated with authentic standards for free and saturated acyl-carnitines *(cf.* Fig. 1). All calibration curves were linear in the physiological and pathological range ($r > 0.99$). Since most unsaturated and hydroxylated acyl-carnitines are not commercially available, no calibration curves for these acyl-carnitines were constructed and an identical response as for their saturated counterparts was assumed. Reproducibility was acceptable with a day-to-day-coefficient of variation of 8.6% for free carnitine and 6–15% for the different acyl-carnitines (table 1).

The analysis of samples from patients suffering from various defects in mitochondrial beta-oxidation shows, in each case, a characteristic acyl-carnitine profile in which the specific accumulation of acyl-carnitine species can be observed. For instance, in short-chain acyl-CoA dehydrogenase (SCAD) deficiency there is a specific accumulation of

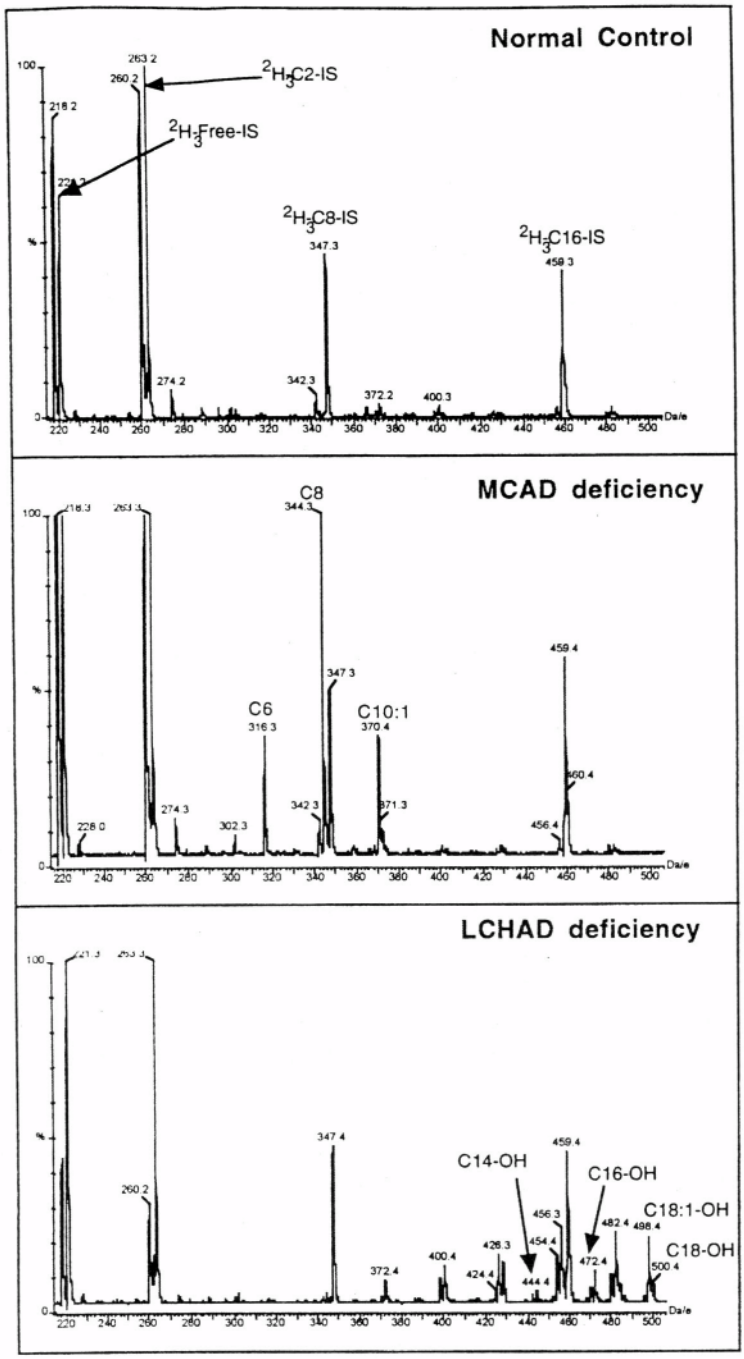

Figure 1. Acyl-carnitine profiles in fatty acid oxidation defects. Acyl-carnitine profiles were obtained from plasma or serum samples as described in materials and methods. In the upper left panel "normal control" the positions of the deuterated internal standards (IS) are indicated. For comparison the profiles were printed with the $^2H_3$-C8-IS at 50% of full scale in all panels. The ion at m/z 218 corresponds to free carnitine; m/z 260, acetyl-carnitine; m/z 274, propionyl-carnitine; m/z 288, butyryl-carnitine; m/z 302, iso-C5-carnitine (isovaleryl-carnitine); m/z 316, hexanoyl-carnitine; m/z 344, octanoyl-carnitine; m/z 370, C10:1-carnitine; m/z 400, C12-carnitine; m/z 426, C14:1-carnitine; m/z 444, hydroxy-C14-carnitine; m/z 456, C16-carnitine; m/z 472, hydroxy-C16-carnitine; m/z 482, C18:1-carnitine; m/z 498, hydroxy-C18:1-carnitine.

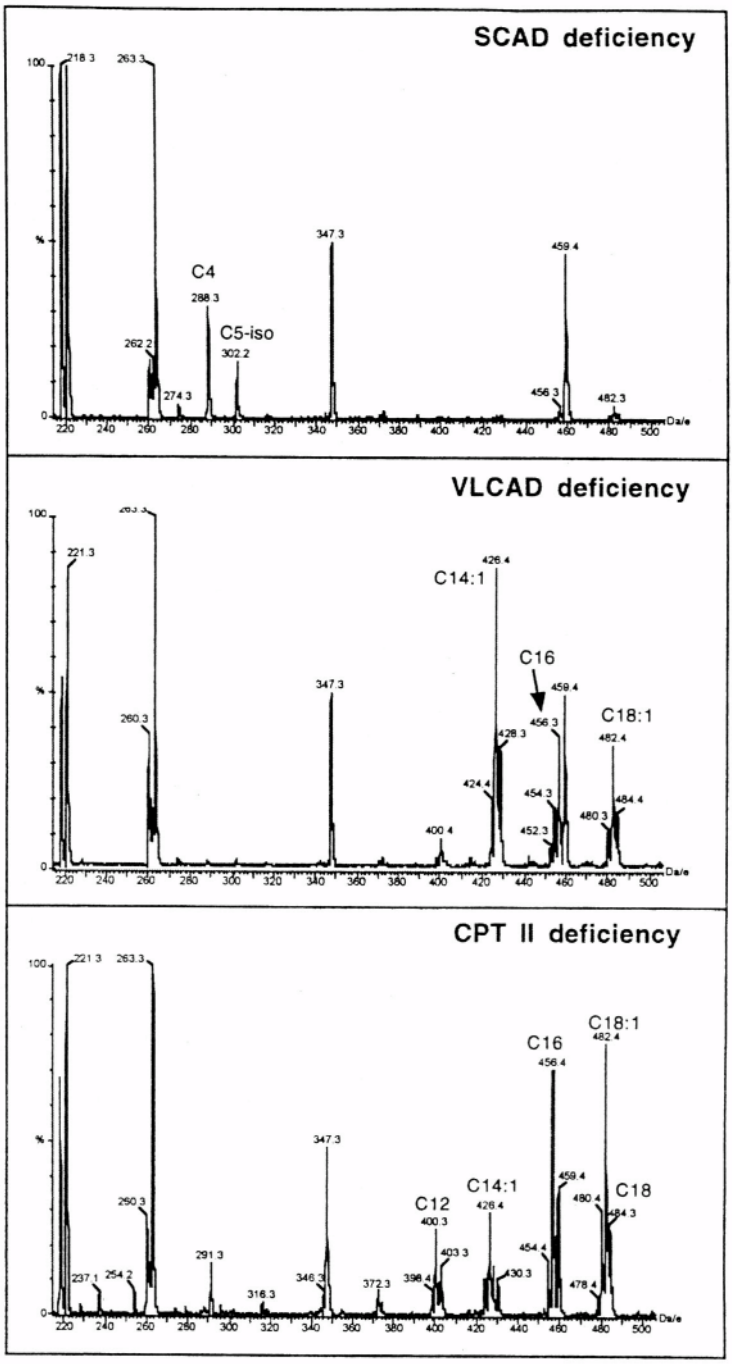

Figure 1. *Continued.*

Table 1. Reproducibility of free carnitine and acylcarnitine analysis.

carnitine[a]	concentration µmol/L	CV (%) within-run[b]	CV (%) day-to-day[b]
free	53.6	5.2	8.6
C2	8.4	4.2	15.3
C3	6.3	5.6	10.5
C4	3.5	3.3	9.3
C5	3.7	6.0	8.6
C5:1	6.2	4.1	8.9
C6	2.2	4.6	6.8
C8	2.2	5.6	7.9
C10	2.2	6.8	7.4
C12	3.9	5.4	11.6
C14	5.4	3.5	8.0
C16	3.3	3.9	7.1
C18	4.1	3.0	6.1

[a]free carnitine and acyl-carnitine standard solutions were added to pooled plasma in order to achieve the desired concentration. [b]n = 10.

butyryl-carnitine and in some, but not all, cases an accumulation of C5-carnitine; in medium-chain acyl-CoA dehydrogenase (MCAD) deficiency a specific accumulation of hexanoyl-, octanoyl- and decenoyl-carnitine is observed, similarly to the reported blood-spot acyl-carnitine profiles in samples from MCAD patients.[5,6,7,11] In very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency a marked accumulation of C14:1-carnitine, with less prominent elevations of C16- , C16:1, C18-, C18:1 and C18:2-carnitine are observed. In carnitine palmitoyl transferase II (CPTII) deficiency there is a marked accumulation of C16-, C16:1, C18- and C 18:1-carnitine, with less prominent accumulations of C14:1- and C12-carnitine (Fig. 1). When the acyl-carnitine profiles of VLCAD and CPT II patients are compared, it is striking that in VLCAD deficiency, the concentration of C 14:1-carnitine is always higher than the concentration of other long-chain acyl-carnitines, whereas in CPT II deficiency C18:1- and C16-carnitine concentrations are always higher than the C14:1-carnitine concentration, suggesting that acyl-carnitine analysis enables discrimination between these two disorders. In long-chain 3-hydrox yacyl-CoA dehydrogenase (LCHAD) deficiency a specific accumulation of long chain acyl-carnitines and their hydroxylated analogues (C16-OH, C18-OH and C 18:1-OH) is observed, whereas in multiple acyl-CoA dehydrogenase (MAD or glutaric acidemia type II (GAII)) deficiency there is a general accumulation of acyl-carnitines from C4 to C18 (Fig. 2). In addition, characteristic acyl-carnitine profiles are observed in several organic acidemias including glutaric acidemia type I (GA I) in which a prominent accumulation of an ion m/z 388 corresponding to glutaryl-carnitine can be observed, and in isovaleric acidemia (IVA) in which a prominent accumulation of an ion m/z 302 corresponding to iso-C5 (isovaleryl)-carnitine is observed (Fig. 2). In methylmalonic acidemia (MMA) and propionic acidemia (PA) an accumulation of m/z 274 corresponding to propionyl-carnitine is always observed, whereas in most samples of patients with methylmalonic acidemia an additional ion at m/z 374, corresponding to methylmalonyl-carnitine can be detected. In defects of ketone body metabolism, 3-hydroxy-3-methyl-glutaryl-CoA lyase (HMG-CoA lyase) deficiency ions at m/z 318 and m/z 402 corresponding to hydroxy-C5- and 3-methylglutaryl-carnitine respectively accumulate. Finally in 2-methylacetoacetyl-CoA thiolase deficiency (beta-keto thiolase deficiency), an ion at m/z 300, corresponding to tiglyl-carnitine characteristically accumulates (Fig. 2).

In this study, the analysis of plasma samples from patients suffering from fatty acid oxidation defects or organic acidemias **(n = 103)** showed abnormal acyl-carnitine profiles in all cases when the samples were taken under stress (e.g. hypoglycemic episodes). In clinically stable episodes an essentially normal acyl-carnitine profile was obtained for only one patient with a mild form of SCAD deficiency and for a patient with a mild form of multiple acyl-CoA dehydrogenase deficiency (data not shown). For all other defects abnormal profiles were observed under a wide variety of clinical conditions illustrating the high sensitivity of the method. In table 2, the upper reference limit, defined as the

Table 2. Diagnostic m/z ratios and acylcarnitine concentrations in various fatty acid oxidation defects and organic acidemias.

disorder (no.)	diagnostic m/z/ratio	acylcarnitines[d]	reference (n = 250) controls(95th perc.[a]) µmol/L[c]	patients (range[b]) µmol/L[c]
SCAD/EMA (2)	288/274	C4/C3	0.98[e]	0.71–9.0[e]
	288/347	C4/C8-d3	0.32	0.62–1.28
	302/260	C5/C3	0.80[e]	0.19–4.52[e]
	302/347	C5/C8-d3	0.22	0.16–0.64
MCAD(23)	316/347	C6/C8-d3	0.12	0.12–2.14
	344/347	C8/C8-d3	0.22	1.28–12.24
	344/342	C8/C8:1	2.32[e]	6.49–46.49[e]
	370/347	C10:1/C8-d3	0.22	0.26–1.84
LCHAD (18)	456/342	C16/C8:1	2.89	10.8–258.96
	472/459	C16OH/C16-d3	0.02	0.12–0.60
	498/459	C18:1OH/C16-d3	0.01	0.14–0.86
	500/459	C18OH/C16-d3	0.04	0.12–0.78
	498/342	C18:1OH/C8:1	0.23[e]	0.31–2.49[e]
VLCAD(5)	426/459	C14:1/C16:d3	0.18	0.76–13.28
	426/342	C14:1/C8:1	1.48[e]	8.26–427.05[e]
	424/459	C14:2/C16-d3	0.08	0.30–3.48
CPT-II(4)	426/342	C14:1/C8:1	1.48[e]	2.50–42.79[e]
	456/342	C16/8:1	2.89[e]	101.24–221.65[e]
	456/459	C16/C16-d3	0.24	2.06–3.94
	454/459	C16:1/C16-d3	0.08	0.50–0.86
	484/459	C18/C16-d3	0.10	0.64–1.44
	482/459	C18:1/C16-d3	0.28	1.56–4.32
	482/342	C18:1/C8:1	2.86[e]	78.98–149.88[e]
	480/459	C18:2/C16-d3	0.18	0.54–1.66
GA-I (2)	388/347	glutaryl/C8-d3	0.06	0.46–1.34
GA-II/MAD(3)	288/274	C4/C3	0.98[e]	0.70–8.19[e]
	302/274	C5/C3	0.80[e]	1.39–2.01[e]
	316/347	C6/C8-d3	0.12	0.04–3.54
	344/347	C8/C8-d3	0.22	0.04–7.96
	372/347	C10/C8-d3	0.30	0.08–5.88
	388/347	glutaryl/C8-d3	0.06	0.04–0.08
HMG-CoA-lyase(16)	318/347	hydroxy-C5/C8-d3	0.06	0.08–1.42
	402/347	methylglutaryl/C8-d3	0.02	0.08–0.62
Beta-ketothiolase (3)	300/347	C5:1/C8-d3	0.04	0.14–0.72
	318/347	hydroxy-C5/C8-d3	0.06	0.12–0.30
PA(10)	274/263	C3/C2-d3	1.30	6.50–60.10
MMA(15)	274/263	C3/C2-d3	1.30	13.00–70.50
	374/347	methylmalonyl/C8-d3	0.06	0.12–0.94
IVA(2)	302/274	C5/C3	0.80[e]	52.96–60.47[e]
	302/347	C5/C8-d3	0.22	15.52–18.38

[a]the 95th percentile of the reference range; [b]range of values found in patient samples, not all concentrations and/or ratios were abnormal in each case; [c]expressed as ratio; [d]"-d3" depicts a labeled internal standard. For explanation see text

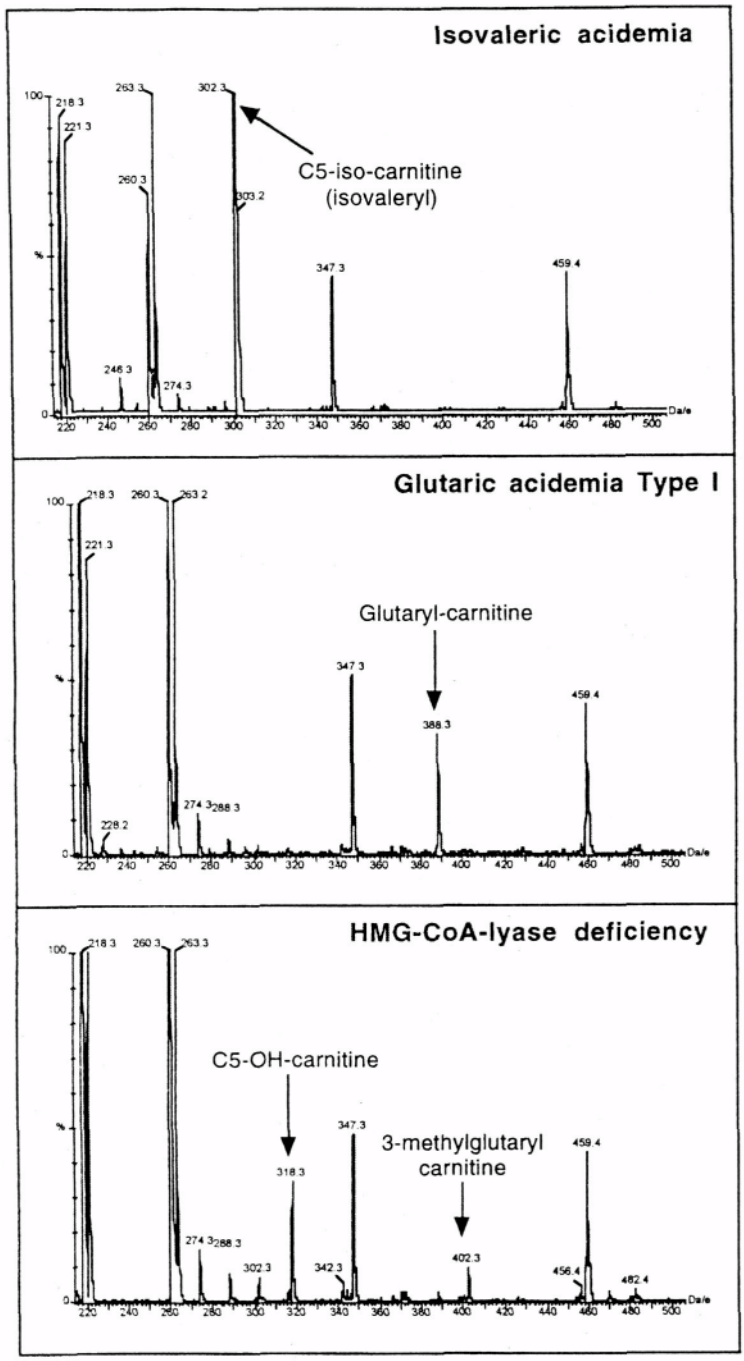

Figure 2. Acyl-carnitine profiles in organic acidemias. Acyl-carnitine profiles were obtained from plasma or serum samples as described in materials and methods. For comparison the profiles were printed with the $^{2}H_{3}$-C8-IS at 50% of full scale in all panels. The ion at m/z 374 corresponds to methylmalonyl-carnitine; m/z 388, glutaryl-carnitine; m/z 318, hydroxy-C5-carnitine; m/z 402, 3-methylglutaryl-carnitine; m/z 300, tiglyl-carnitine. For other ions see the legend of figure 1.

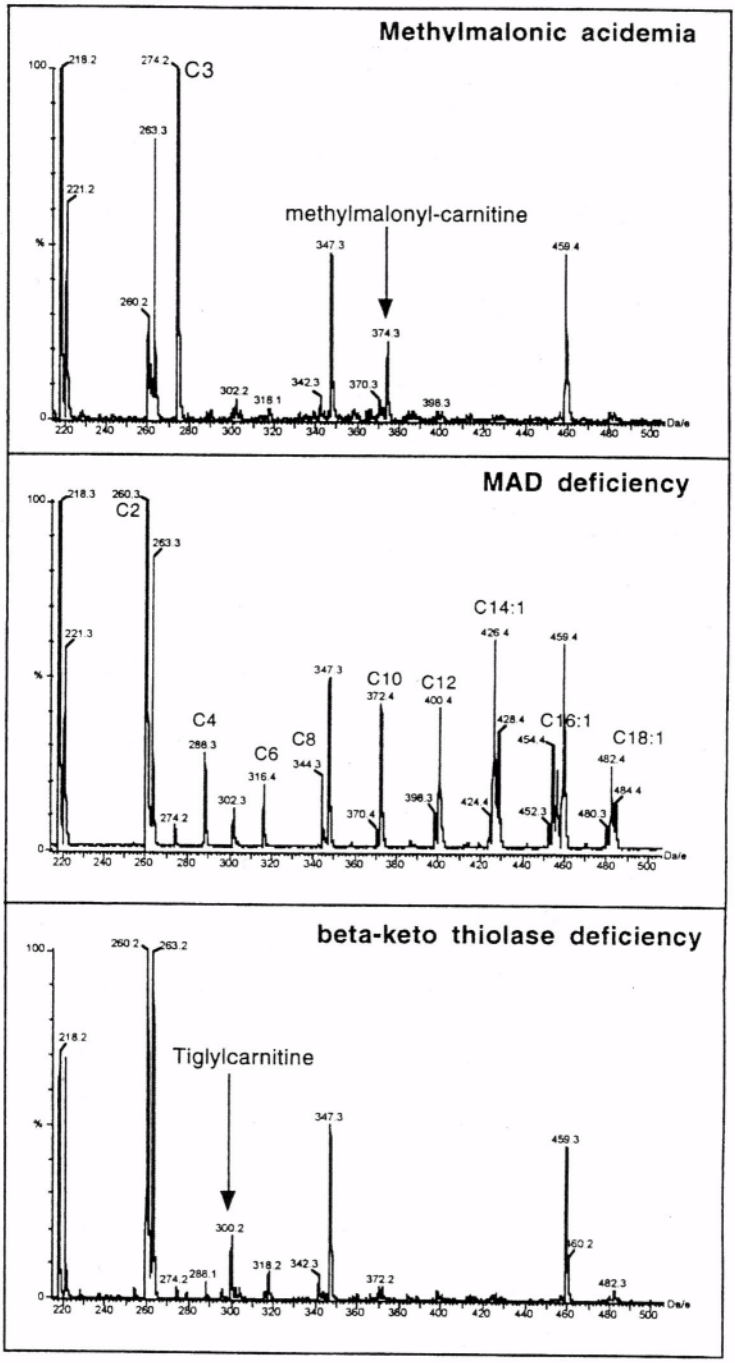

Figure 2. *Continued.*

95th percentile is compared to the concentrations (or ratios) observed in patient samples. The results show that in all defects a clear discrimination can be made between controls and affected individuals based on a combination of diagnostic m/z ratios. However, the individual concentrations of the different acyl-carnitines varied considerably between patients and between different samples of the same patient, which might reflect the metabolic condition of the patient and thus be important in the therapy control of patients suffering from one of these defects (details will be published elsewhere). Most patients affected with defects in the mitochondrial fatty-acid oxidation suffered from a secondary carnitine deficiency at the time of diagnosis (data not shown), although free carnitine concentrations varied considerably depending on metabolic state, type of disease and during treatment. Therefore, free carnitine is not included in table 2 as diagnostic parameter.

The data presented in this study show that we have developed a quantitative analysis of free carnitine and acyl-carnitine species in plasma or serum. The quantitative nature of this analysis enables good discrimination between normal and abnormal profiles and accurate therapy monitoring, although one should realize that mild cases exist which may have normal profiles in clinically stable episodes. Compared to other methods for quantitative acyl-carnitine analysis like HPLC or GC/MS, the method described here is less laborious and allows determination of a wide variety of acyl-carnitine species, including hydroxylated acyl-carnitine species, which is not the case for the (laborious) GC/MS method.[19] Since for selective screening for inborn errors of metabolism, apart from urine samples, mostly serum or plasma samples are send in, this method allows direct comprehensive acyl-carnitine analysis in such materials, obviating the need for a separate blood-spot for acyl-carnitine analysis which would allow qualitative or semi-quantitative acyl-carnitine analysis.[6,8,11]

In conclusion, ESI/MS/MS of plasma or serum samples is a suitable and rapid technique for detecting defects in the catabolism of branched-chain amino acids and defects in mitochondrial fatty-acid oxidation with a high sensitivity and accuracy.

REFERENCES

1. Sewell, A.C. & Bohles, H.J. (1995) *Eur. J. Ped.* **154**, 871–877. Acyl-carnitines in intermediary metabolism.
2. Nada, M.A., Rhead, W.J., Sprecher, H., Schulz, H. & Roe, C.R. (1995) *J. Biol. Chem.* **270**, 530–535. Evidence for intermediate channeling in mitochondrial beta-oxidation.
3. Nada, M.A., Chace, D.H., Sprecher, H. & Roe, C.R. (1995) *Biochem. Mol. Med.* **54**, 59–66. Investigation of beta-oxidation intermediates in normal and MCAD-deficient human fibroblasts using tandem mass spectrometry.
4. Pourfarzam, M., Schaefer, J., Turnbull, D.M. & Bartlett, K. (1994) *Clin. Chem.* **40**, 2267–2275. Analysis of fatty acid oxidation intermediates in cultured fibroblasts to detect mitochondrial oxidation disorders.
5. Rashed, M.S., Bucknall, M.P., Little, D., Awad, A., Jacob, M., Alamoudi, M. & Ozand, P.T. (1997) *Clin. Chem.* **43**, 1129–1141. Screening blood spots for inborn errors of metabolism by electrospray tandem mass spectrometry with a microplate batch process and a computer algorithm for automated flagging of abnormal profiles.
6. Rashed, M.S., Ozand, P.T., Bucknall, M.P. & Little, D. (1995) *Ped. Res.* **38**, 324–331. Diagnosis of inborn errors of metabolism from blood spots by acyl-carnitines and amino acids profiling using automated electrospray tandem mass spectrometry.
7. van Hove, J.L., Zhang, W., Kahler, S.G., Roe, C.R., Chen, Y.T., Terada, N., Chace, D.H., Iafolla, A.K., Ding, J.H. & Millington, D.S. (1993) *Am. J. Hum. Genet.* **52**, 958–966. Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency: diagnosis by acylcarnitine analysis in blood.

8. Vianey-Saban, C., Guffon, N., Delolne, F., Guibaud, P., Mathieu, M. & Divry, P. (1997) *J. Inher. Metab. Dis.* **20**, 411–414. Diagnosis of inborn errors of metabolism by acyl-carnitine profiling in blood using tandem mass spectrometry.
9. Millington, D.S., Kodo, N., Norwood, D.L. & Roe, C.R. (1990) *J. Inher. Metab. Dis.* **13**, 321–324. Tandem mass spectrometry: a new method for acylcarnitine profiling with potential for neonatal screening for inborn errors of metabolism.
10. Sweetman, L. (1996) *Clin. Chem.* **42**, 345–346. Newborn screening by tandem mass spectrometry (MS-MS).
11. Chace, D.H., Hillman, S.L., van Hove, J.L. & Naylor, E.W. (1997) *Clin. Chem.* **43**, 2106–2113. Rapid diagnosis of MCAD deficiency: quantitatively analysis of octanoylcarnitine and other acyl-carnitines in newborn blood spots by tandem mass spectrometry.
12. Shimizu, N., Yamaguchi, S., Orii, T., Previs, S.F. & Rinaldo, P. (1991) *Biol. Mass Spectr.* **20**, 479–483. Mass spectrometric analysis of metabolite excretion in five Japanese patients with the late-onset form of glutaric aciduria type II.
13. van Hove, J.L., Rutledge, S.L., Nada, M.A., Kahler, S.G. & Millington, D.S. (1995) *J. Inher. Metab. Dis.* **18**, 592–601. 3-Hydroxyisovalerylcarnitine in 3-methylcrotonyl-CoA carboxylase deficiency.
14. Fontaine, M., Briand, G., Ser, N., Armelin, I., Rolland, M.O., Degand, P. & Vamecq, J. (1996) *Clin. Chim. Acta* **255**, 67–83. Metabolic studies in twin brothers with 2-methylacetoacetyl-CoA thiolase deficiency.
15. Matsumoto, M., Matsumoto, I., Shinka, T., Kuhara, T., Imamura, H. & Shimao, S. (1990) *Acta Paed. Japonica* **32**, 76–82. Organic acid and acyl-carnitine profiles of glutaric aciduria type I.
16. Rashed, M.S., Ozand, P.T., Bennett, M.J., Barnard, J.J., Govindaraju, D.R. & Rinaldo, P. (1995) *Clin. Chem.* **41**, 1109–1114. Inborn errors of metabolism diagnosed in sudden death cases by acyl-carnitine analysis of postmortem bile.
17. Shigematsu, Y., Hata, I., Nakai, A., Kikawa, Y., Sudo, M., Tanaka, Y. & Yamaguchi, S. (1996) *Ped. Res.* **39**, 680–684. Prenatal diagnosis of organic acidemias based on amniotic fluid levels of acyl-carnitines.
18. Nada, M.A., Vianey-Saban, C., Roe, C.R., Ding, J.H., Mathieu, M., Wappner, R.S. & McGlynn, J.A (1996) *Prenatal Diagnosis* **16**, 117–124. Prenatal diagnosis of mitochondrial fatty acid oxidation defects.
19. Costa, C.G., E.A. Struys, A. Bootsma, H. J. ten Brink, L. Dorland, I. Tavares deAlmeida, M. Duran & Jakobs, C. (1997) *J. Lipid Res.* **38**:173–182. Quantitative analysis of plasma acyl-carnitines using gas chromatography chemical ionization mass fragmentography.

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GENETICS OF CARNITINE PALMITOYLTRANSFERASE II DEFICIENCIES

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1. INTRODUCTION

β-Oxidation of long chain fatty acids is the major source of energy supply for the skeletal muscle, especially during prolonged exercise and fasting. The carnitine palmitoyltransferase enzyme system facilitates the entry of long chain fatty acid into the mitochondrial matrix for β-oxidation. Two distinct enzymes can be differentiated by their sensitivity to inhibition by malonyl-CoA, the product of the acetyl-CoA carboxylase reaction. Carnitine palmitoyltransferase I (CPT I), located on the outer membrane, catalyses the transfer of acyl groups from coenzyme A to carnitine, the acylcarnitine so formed then traversing the inner membrane by means of a specific transporter. Carnitine palmitoyltransferase II (CPT II), on the matrix side of the inner membrane, reverses the transacylation reaction, regenerating acyl-CoA.[1] cDNA cloning of CPT I and CPT II from rat liver and human liver has recently established that CPT I and CPT II are distinct proteins. CPT I exists as tissue-specific isoforms. Conversely, CPT II is expressed as the same protein bodywide in both rat and human as well.[2–9]

Whereas adults with deficiency of CPT II have a disorder characterised by exercise intolerance and myoglobinuria, the same deficiency in new-borns is a generalised lethal disease with reduced CPT II activity in multiple organs. Distinct genetic variations have been described for both disorders. In this review article we will report the current knowledge of the molecular genetic background of both types of CPT II deficiency.

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2. CLINICAL MANIFESATIONS

2.1. Muscle Form (adult onset)

Adult onset CPT II deficiency (muscle type) is the most common disorder of lipid metabolism affecting skeletal muscle. Since its first description in 1973 by DiMauro and DiMauro about 70 patients are published in the literature. It is a clinically as well as biochemically heterogeneous disease. Only 20% of these patients have been females. In more than two-thirds of the cases, the first intermittent symptom began during the first or second decade of life, but in other patients they appeared as late as the fifth decade. In the majority of cases, the biochemical diagnosis was established in the second decade. The symptoms typically consist of attacks of myalgia, cramps, muscle stiffness or tenderness with weakness, and—in severe instances—progress to myoglobinuria. The frequency of these attacks in individual patients ranges from one or two during a lifespan to several attacks per week. The severity of attacks can vary from mild myalgias lasting for only a few hours or mild pigmenturia without other symptoms to severe muscle weakness and rhabdomyolysis lasting for several days. The myoglobinuria may lead to acute renal failure. Involvement of the respiratory muscles may cause a respiratory insufficiency requiring assisted ventilation. In the symptom free intervals, there is usually no muscle weakness and the neurological examination is entirely normal. The symptoms are most commonly precipitated by prolonged exertion. The ability to perform short, intense exercise is not impaired. Myalgia, cramps muscle tightness, and weakness develop during prolonged exertion, e.g. long distance walking or running, hiking, mountain climbing sustained standing, wrestling or swimming. The first symptoms may occur several hours after the period of heavy exercise and may involve any muscle group or may be generalised. Fasting, exposure to cold or a high fat intake can precipitate rhabdomyolysis even without exercise or can contribute to the effects stress can provoke. Sometimes, however, there is no apparent cause for the rhabdomyolysis in CPT deficiency.

In spite of the primarily muscular presentation, their enzyme defect is not restricted to muscle and is expressed in other tissues, such as liver, fibroblasts and leukocytes.

2.2. Hepatic Form (lethal-infantile)

The second form presents as a lethal neonatal multiorgan deficiency of carnitine palmitoyltransferase II. Reduced CPT II activity in multiple organs is observed and reduced concentrations of total and free carnitine and increased concentrations of lipids and long chain acylcarnitine can be found. The accumulation of long-chain acylcarnitines has an arrhythmogenic effect on the heart. The patients presented with hypoketotic hypoglycaemia, seizures, tachycardia, cardiomegaly, hepatomegaly, liver failure and died within the first two years.

Since its first description in 1989 only a few cases have been reported.[10–13] It was pointed out that the difference in clinical severity of CPT II deficiency in adult and newborns is not determined by the degree of reduction in CPT II activity nor in the tissue distribution of the deficiency since isoforms of the enzyme do not exist and the magnitude of the reduction is similar in both forms of the disease.

In 1995 an exceptional case of neonatal CPT II deficiency was published, which presented as a lethal myopathy. The common metabolic features of the lethal infantile form were absent.[14]

3. BIOCHEMISTRY

The CPT system consists not only of the translocase but also of two functionally separate forms of CPTs. CPT I which catalyses the formation of acylcarnitine from carnitine and Acyl-CoA, and CPT II which catalyses the formation of Acyl-CoA from acylcarnitine and CoA.

Within a species CPT II, in contrast to CPT I, is conserved across tissue lines. It is a homotetrameric enzyme of approximately 68 kDa molecular weight. The human cDNA predicted a nascent product of 658 amino acids and is reduced by 25 residues upon mitochondrial import.[15]

CPT II is loosely associated with the inside of the inner mitochondrial membrane and can easily be solubilized in an active form by mild detergent treatment, such as 1% Tween-20.[16] In line with these findings is the absence of any amino acid sequence indicative of a membrane spanning domain.[8] The matrix location of CPT II ¥s catalytic centre is confirmed by its insensitivity to protease treatment of intact mitochondria, whereas after exposure of the matrix face by freeze/thawing, the enzyme is readily proteolysed.[17]

Widely different results have been obtained on CPT II levels in normal tissues and in tissues of patients suffering form CPT deficiency. This might be partly due to the use of different assays and assay conditions for CPT.[18] All assays are based on the reaction

palmitoyl-CoA + carnitine <---> palmitoylcarnitine + CoA

Depending on the assay system used, CPT levels in muscle in muscle CPT deficiency range from not detectable to about 30% of normal. Partial muscle CPT deficiency with activities ranging form 40% to 60% of normal have also been reported. In most cases CPT deficiency in muscle was established either by the isotope exchange assay or by the backward assay. However, in several of these patients, normal CPT activity was detected when the forward assay was performed under optimal conditions.[19–21]

In a large group of 18 patients using the isotope forward assay all had normal total **(= CPT I + II)** enzyme activity. After inhibition of the enzyme by malonyl-CoA and Triton X-100 there was a significant difference in enzyme activity in patients and controls. In controls we observed a residual activity of 40.2% (malonyl-CoA) and 57.7% (Triton X-100) respectively. In contrast had our patients a mean residual activity of 8.9% (malonyl-CoA) and 7.1% (Triton X-100).[22] We, therefore, speculate that the pathologic mechanism in adult onset CPT II deficiency is not a reduction of CPT activity in general but maybe a misregulation of the enzyme. This might have pathophysiological implications since malonyl-CoA is the natural "regulator" of this enzyme system.

4. GENETICS

4.1. The CPT II Gene

Finocchiario *et al.* cloned and sequenced a cDNA encoding human liver carnitine palmitoyltransferase in 1991 after mapping it to chromosome 1p32 by fluorescence *in situ* hybridisation. Two years later the structure of the gene was completely resolved. It contains an open reading frame of 1974 basepairs that encodes a protein of 658 amino acid

residues including 25 residues of an NH2-terminal leader peptide. It spans approximately 20kb and is composed of five exons, ranging from 81 to 1,305bp in length separated by introns ranging from 1.5kb to 87kb in length.[23] The exon-intron boundaries conform to the consensus splice junction sequences. The 5-prime and 3-prime untranslated regions of exons 1 and 5 were also determined, including the ploydenylation signal and the ploydenylation site.[6,23,24]

There are some unusual features in this gene. Exon four is with 1,305bp exceptionally long and accounts for 66% of the translated portion of the mRNA. In vertebrates only approximately 0.5% of internal exons are more than 550bp in length, the average size being 137 bp.[23,25] Intron three is with 8 kb also particularly long. Less than 8% of vertebrate introns are more than 3 kb in size.

Searching sequence databases with FASTA turned up no other proteins homologous to CPT II except other carnitine acyl transferases such as carnitine acetyltransferase, choline acetyltransferase or carnitine octanoyltransferase.

Two polymorphisms are present in the gene. It is a G1992 --> A transition, leading to a Valin to Isoleucin substitution at amino acid position 368 and a C2040 --> T transition resulting in a Methionin substitution through a Valin at amino acid position 647 with a frequency of 0.5 and 0.25 in the normal population respectively exhibiting Hardy-Weinberg equilibrium.

4.2. Mutation analysis in Patients

4.2.1. *Adult onset (muscular) form.* Soon after identification of the gene disease causing mutations could be detected in the patients.

Particularly common among Europeans with the classical adult muscular form of the disorder is a C --> T transition at nucleotide 439 which changes amino acid 113 from serine to leucine. It was first described by Taroni et al in 1993 and in subsequent studies it was shown that this mutation can be found in approximately 60% of all alleles. It is therefore called the "common" CPT II deficiency mutation.[23]

Screening the S113L negative alleles revealed two more mutations. A C --> A transversion at nucleotide position 665 in exon one leading to an amino acid exchange of Prolin to Histidin at amino acid position 50 (P50H) and a G --> A transition at position 2,173 of exon 5 resulting in an amino acid exchange of Aspartic acid to Asparagin at position 553 (D553N).[23] Notably, two patients were heterozygous for the R631C mutation, a nucleotide exchange C --> T in position 1,992 leading to an exchange of Arginin to Cystein at amino acid position 631, which was hitherto assigned to be associated with the lethal infantile form of the disease. They have an yet unidentified mutation on their second allele.

Of our sample of 18 patients 14 were available for genetic analysis. Seven were homozygous for the "common" S1 13L mutation. Six patients were heterozygous for the this mutation and one patient had the common alteration on neither allele. This patient was homozygous for the rare mutation P50H, which was overall present in two patients. The D553N and R631C mutations were not present in our collective. Direct sequencing of the entire coding region could identify three novel mutations. The first is a T --> C transition in position 742 leading to a substitution of the amino acid Methionin to Threonin in position 214 (M214T). The second is a T --> C transition in position 1,442, resulting in the amino acid exchange of Phenylalanin to Leucin in position 448 (F448L). This mutation was observed in two patients. In two brothers a A to T transition in position

1,547 could be observed. This exchange of nucleic acids leads to the substitution of a Tyrosin to a Phenylalanin at amino acid position 479 (Y479F).[26]

To address the question whether the presence of the polymorphisms exacerbates the effects of certain mutations or maybe mitigates deleterious effects, Taroni *et al.* Expressed wild-type and mutant CPT II cDNA constructs in transfected COS-cells. It was shown that they do not affect enzyme activity by themselves. In combination with the S113L, P50H and D553N mutations they are functionally inert and no synergistic effect could be observed. This is not the case with the R631C mutation associated with the lethal-infantile form of the disease, were an exacerbating effect is described.[23] For the other mutations information regarding this aspect is not yet available.

4.2.2. *Lethal infantile (hepatic) form.* The first mutation associated with the hepatic form was the before mentioned R631C mutation found homozygously in a boy with an acute episode with seizures, coma and respiratory distress after a history of recurrent episodes of vomiting, sweating and lethargy. Three more mutations associated with the lethal infantile form were identified subsequently either homozygously or compound heterozygously, namely F383Y, Q174L and Y628S.[27,28] All patients displayed the above mentioned metabolic features and died within a few month.

The child with the lethal myopathic form of the disease described in the chapter "clinical manifestations" underwent no genetic analysis.

5. CONCLUSION

CPT II deficiency is a clinically as well as biochemically heterogeneous disease. Phenotypic expression ranges from mild myalgia without myoglobinuria to severe exercise-induced attacks leading eventually to renal failure and death. Obviously it is rare disease, but might be immensely underdiagnosed, considering that: (i) the investigation of a muscle biopsy specimen is necessary to establish the diagnosis and (ii) expression of potentially mild phenotypes.

As more and more patients get investigated it shows that the marked clinical heterogeneity corresponds to a as marked genetic heterogeneity. Due to small numbers and only limited information on the clinical status of the patients it is difficult to draw strict phenotype/genotype correlations. A further problem is the different test methods used by different groups. Using the isotope forward assay all our patients had an overall CPT activity of 100%, but showed a different pattern after inhibition by malonyl-CoA and Triton X-100 as controls.

The range of residual CPT II activity in our patient sample was from 1.8% to 20%. The patient with the lethal infantile form who lead to the identification of the R631C mutation showed CPT II activity of 6.6%. Unaffected parents, which are heterozygous for disease causing mutations, obviously have 50% enzyme activity. In a very interesting experiment Bonnefont *et al.* compared the metabolic consequences of a lethal-infantile associated mutation (Y628S) with the common adult-onset S113L mutation, whereby they demonstrated a similar residual activity of CPT II between 10% and 15% of control mean values after expression in transfected COS-cells.[27] Both the adult and infantile cases hitherto studied have been shown to be associated with a decreased amount of steady-state CPT II protein.[10,23,24,29] All together these data do not suggest any threshold effect to be responsible for the two distinct clinical features of this disease, even bearing in mind that comparison of the data is difficult due to the different test methods used.

In summary, to date there is neither a plausible concept of how different mutations in the same gene cause such disparate clinical symptomatology of the disease on one hand (lethal-neonatal versus adult-onset) nor how such different residual activities of this enzyme lead to comparably similar phenotypes (adult-onset muscular) on the other hand. A variable degree of reduction of CPT activity, variable posttranslational modifications of the enzyme in different tissues, a disturbance of regulatory properties of the CPT system or a variable efficiency of further distal components of the **β-oxidation** machinery by an pathologically altered enzyme might determine the difference in clinical severity of the different forms of CPT II deficiency.

REFERENCES

1. McGarry, J.D.; Woeltje, K.F.; Kuwajima, M. & Foster, D.W. (1989) *Diabetes Metab. Rev.* **5**, 271–284; Regulation of ketogenesis and the renaissance of carnitine palmitoyltransferase.
2. Britton, C.H.; Schultz, R.A.; Zhang, B.; Esser, V.; Foster, D.W. & McGarry, J.D. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 1984–1988; Human liver mitochondrial carnitine palmitoyltransferase I: characterization of its cDNA and chromosomal localization and partial analysis of the gene.
3. Brown, N.F.; Esser, V.; Foster, D.W. & McGarry, J.D. (1994) *J. Biol. Chem.* **269**, 26438–26442; Expression of a cDNA for rat liver carnitine palmitoyltransferase I in yeast establishes that catalytic activity and malonyl-CoA sensitivity reside in a single polypeptide.
4. Demaugre, F.; Bonnefont, J.P.; Cepance, C; Scholte, J.; Saudubray, J.M. & Leroux, J.P. (1990) *Pediatr. Res.* **27**, 497–500; Immunoquantitative Analysis of Human Carnitine Palmitoyltransferase **I** and **II** Defects.
5. Esser, V.; Britton, C.H.; Weis, B.C.; Foster, D.W. & McGarry, J.D. (1993) *J. Biol. Chem.* **268**, 5817–5822; Cloning, sequencing, and expression of a cDNA encoding rat liver carnitine palmitoyltransferase I. Direct evidence that a single polypeptide is involved in inhibitor interaction and catalytic function.
6. Finocchiaro, G.; Taroni, F.; Rocchi, M.; Martin, A.L.; Colombo, I.; Tarelli, G.T. & DiDonato, S. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 661–665; cDNA cloning, sequence analysis, and chromosomal localization of the gene for human carnitine palmitoyltransferase.
7. Weis, B.C.; Esser, V.; Foster, D.W. & McGarry, J.D. (1994) *J. Biol. Chem.* **269**, 18712–18715; Rat heart expresses two forms of mitochondrial carnitine palmitoyltransferase I. The minor component is identical to the liver enzyme.
8. Woeltje, K.F.; Esser, V.; Weis, B.C.; Sen, A.; Cox, W.F.; McPhaul, M.J.; Slaughter, C.A.; Foster, D.W. & McGarry, J.D. (1990) *J. Biol. Chem.* **265**, 10720–10725; Cloning, sequencing, and expression of a cDNA encoding rat liver mitochondrial carnitine palmitoyltransferase II.
9. Woeltje, K.F.; Esser, V.; Weis, B.C.; Cox, W.F.; Schroeder, J.G.; Liao, S.T.; Foster, D.W. & McGarry, J.D. (1990) *J. Biol. Chem.* **265**, 10714–10719; Inter-tissue and inter-species characteristics of the mitochondrial carnitine palmitoyltransferase enzyme system.
10. Demaugre, F.; Bonnefont, J.P.; Colonna, M.; Cepanec, C.; Leroux, J.P. & Saudubray, J.M. (1991) *J. Clin. Invest.* **87**, 859–864; Infantile Form of Carnitine Palmitoyltransferase II Deficiency with Hepatomuscular Symptoms and Sudden Death.
11. Hug, G. & Soukoup, S. (1991) *New Engl& J. Med.* **325**, 1862–1864; Lethal neonatal multiorgan deficiency of carnitine palmitoyltransferase II.
12. Taroni, F; Verderio, E.; Garavaglia, B.; Fiorucci, S.; Finocchiaro, G.; Uziel, G. & DiDonato, S. (1992) *New Development in Fatty Oxidation* (Coates, P.M. & Tanaka, K. eds) pp. 521–531, Wiley-Liss. New York.
13. Hug, G.; Soukup, S.; Berry, H. & Bove, K.E. (1989). *Pediatr. Res.* **25**, 115A (Abstract); Carnitine palmitoyl transferase (CPT): deficiency of CPT II but not of CPT I with reduced total and free carnitine but increased acylcarnitine.
14. Land, J.M.; Mistry, S.; Squier, M.; Hope, P.; Ghadiminejad, I.; Orford, M. & Saggerson, D. (1995) *Neuromuscular Disorders* **5**, 129–137; Neonatal Carnitine Pamitoyltransferase-2 Deficiency; a Case Presenting with Myopathy.
15. Brown, N.F.; Esser, V.; Gonzalez, A.D.; Evans, C.T.; Slaughter, C.A.; Foster, D.W. & McGarry, J.D. (1991) *J. Biol. Chem.* **266**, 15446–15449; Mitochondrial import and processing of rat liver carnitine

palmitoyltransferase II defines the amino terminus of the mature protein. Possibility of differential modification of the rat and human isoforms.
16. Woeltje, K.F.; Kuwajima, M.; Foster, D.W. & McGarry, J.D. (1987) *J. Biol. Chem.* **262**, 9822–9827; Characterization of the Mitochondrial Carnitine Palmitoyltranferase Enzyme System.
17. McGarry, J.D., Brown, N.F., Inthanousay, P.P., Park, D.I., Cook, B.A., & Foster, D.W. (1992) Insights into the topography of mitochondrial carnitine Palmitoyltransferase gained from the use of proteases. *New developments in fatty acid oxidation* (Coates, P.M. & Tanaka, K. eds) pp. 47–61, Wiley-Liss. Inc, New York.
18. Bieber, L.L. & Farrell, S. (1983) Carnitine acyltransferases. *The Enzymes* (Boyer, P.D. ed) pp. 627–644, Academic Press, New York.
19. Trevisan, C.P.; Angelini, C.; Freddo, L.; Isaya, G. & Martinuzzi, A. (1984) *Neurology* **34**, 353–356; Myoglobinuria and carnitine palmitoyltransferase (CPT) deficiency: studies with malonyl-CoA suggest absence of only CPT-II.
20. Scholte, H.R.; Jennekens, F.G. & Bouvy, J.J. (1979) *J. Neurol. Sci.* **40**, 39–51; Carnitine palmitoyltransferase II deficiency with normal carnitine palmitoyltransferase I in skeletal muscle and leukocytes.
21. Meola, G.; Bresolin, N.; Rimoldi, M.; Velicogna, M.; Fortunate, F. & Scarlato, G. (1987) *J. Neural.* **235**, 74–79; Recessive carnitine palmitoyl transferase deficiency: biochemical studies in tissue cultures and platelets.
22. Zierz, S. & Engel, A.G. (1985) *Eur. J. Biochem.* **149**, 207–214; Regulatory properties of a mutant carnitine palmitoyltransferase in human skeletal muscle.
23. Verderio, E.; Cavadini, P.; Montermini, L.; Wang, H.; Lamantea, E.; Finocchiaro, G.; DiDonato, S.; Gellera, C. & Taroni, F. (1995) *Hum. Mol. Genet.* **4**, 19–29; Carnitine palmitoyltransferase II deficiency: structure of the gene and characterization of two novel disease-causing mutations.
24. Taroni, F.; Verderio, E.; Dworzak, F.; Willems, P.J.; Cavadini, P. & DiDonato, S. (1993) *Nature Genetics* **4**, 314–319; Identification of a common mutation in the carnitine palmitoyltransferase II gene in familial recurrent myoglobinuria patients.
25. Hawkins, J.D. (1988) *Nucleic Acid Research* **16,** 9893–9908; A survey on intron and exon lengths.
26. Wieser, T; Deschauer, M. & Zierz, S. (1997) *J. Mol. Med.* 75, B51 (Abstract); Carnitine Palmitoyltransferase II Deficiency: Three Novel Mutations.
27. Bonnefont, J.P.; Taroni, F.; Cavadini, P.; Cepanec, C.; Brivet, M.; Saudubray, J.M.; Leroux, J.P. & Demaugre, F. (1996) *Am. J. Hum. Genet.* **58**, 971–978; Molecular analysis of carnitine palmitoyltransferase II deficiency with hepatocardiomuscular expression.
28. Yamamoto, S.; Abe, H.; Kohgo, T; Ogawa, A.; Ohtake, A.; Hayashibe, H.; Sakuraba, H.; Suzuki, Y; Aramaki, S.; Takayanagi, M.; Hasegawa, S. & Niimi, H. (1996) *Hum. Genet.* 98, 116–118; Two novel gene mutations **(Glu174→Lys, Phe383→Tyr)** causing the "hepatic" form of carnitine palmitoyltransferase II deficiency.
29. Taroni, F.; Verderio, E.; Fiorucci, S.; Cavadini, P.; Finocchiaro, G.; Uziel, G.; Lamantea, E.; Gellera, C. & DiDonato, S. (1992) *Proc. Nat. Acad. Sci., U.S.A.* **89**, 8429–8433; Molecular characterization of inherited carnitine palmitoyltransferase II deficiency.

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IDENTIFICATION OF A MISSENSE MUTATION IN A PATIENT WITH LETHAL CARNITINE ACYL-CARNITINE CARRIER DEFICIENCY

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1. INTRODUCTION

Mitochondrial β-oxidation of long-chain fatty acids is the major source of energy production in man. The mitochondrial inner membrane is impermeable to long chain fatty acids or their CoA esters whereas acylcarnitines are transported. Three different gene products are involved in this carnitine dependent transport shuttle: carnitine palmitoyl transferase I (CPT I), carnitine acyl-carnitine carrier (CAC) and carnitine palmitoyl transferase II (CPT II). The first enzyme (CPT I) converts fatty acyl-CoA esters to their carnitine esters which are subsequently translocated across the mitochondrial inner membrane in exchange for free carnitine by the action of the carnitine acyl-carnitine carrier (CAC). Once inside the mitochondrion, CPT II reconverts the carnitine ester back to the CoA ester which can then serve as a substrate for the β-oxidation spiral.

Since the first report of CAC deficiency (OMIM entry 212138) by Stanley *et al.*,[1] 7 cases have been reported. Most patients suffer from hypoketotic hypoglycaemia and

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hyperammonemia due to deficient fatty acid oxidation. They show a severe phenotype characterised by generalised muscle weakness, cardiomyopathy, hepatomegaly and reduced liver function usually with fatal outcome, although two cases have been reported with a milder phenotype.[2,3] Among the different fatty acid β-oxidation disorders, long chain fatty acid β-oxidation is usually most impaired in case of CAC deficiency (< 5% control activity) which can be demonstrated in fibroblasts or lymphocytes.

The cDNA encoding rat CAC was cloned in 1997 by Indiveri *et al.*[2] and the human homologue was cloned shortly after.[4] The latter also described the first mutation in the cDNA of a CAC deficient patient with mild presentation.

We now report the resolution of the molecular defect in a patient with enzymatically proven CAC deficiency with a severe phenotype.

2. MATERIALS AND METHODS

Case report: The patient was first described by Niezen-Koning *et al.*[5] She presented at 36 hours of age with sudden cardiorespiratory insufficiency and extreme hypoglycaemia. Despite treatment with low-fat diet supplemented with medium-chain triglycerides and carnitine she died at 24 month of age.

Cell culture conditions were exactly as described before.[6]

Fatty acid β-oxidation of [9,10-^{3}H]-myristate and palmitate in fibroblasts was performed essentially as described before.[7]

Synthesis of [1-^{14}C]acetylcarnitine: [l-^{14}C]Acetylcarnitine was prepared enzymatically from [1-^{14}C]acetyl-CoA in an incubation mixture (final volume 1ml) containing: 25mM HEPES pH 7.6, 2mM N-ethylmaleimide (NEM), 10mM EDTA, 40 μM carnitine (Sigma Chemical Co., St. Louis, MO), 44 μM (600,000dpm) [1-^{14}C]acetyl-CoA (Amersham Life Science) and 50 μg (4U) carnitine acetyltransferase (Boehringer Mannheim). The reaction was allowed to proceed for 1 hour at 25 °C. Thereafter excess N-ethylmaleimide was neutralised by addition of 10 μl 200 mM cysteine. The reaction mixture was applied to a Dowex AG-1-X8 anion exchanger (1ml bed volume, Cl^- form, 200–400 mesh) to remove unreacted [l-^{14}C]acetyl-CoA from the reaction mixture (recovery typically >90%). The elvant was used directly as substrate for activity measurements.

Carnitine acylcarnitine carrier activity measurements: The reaction was performed in a glass tube with a rubber septum. This tube contained two smaller tubes, one containing the reaction mixture and the other containing 0.5ml 2M NaOH. The assay mixture (final volume, 500 μl, pH 7.4) contained 25mM Tris, 150mM KCl, 2mM EDTA, 10mM KP_i, l0mg/ml BSA (bovine serum albumin), 40 μg/ml digitonin, 0.1 mM acetylcarnitine, 60,000dpm [1-^{14}C]acetyl-carnitine and 200μg fibroblast suspension. Reactions were allowed to proceed for 30min at 25 °C. Reactions were subsequently stopped by addition of 100 μl 2.6 M perchloric acid. $^{14}CO_2$ was trapped overnight at 4°C and the radiolabelled product was measured in the NaOH fraction using a liquid scintillation counter.

RNA isolation and cDNA synthesis. Total RNA was isolated from cultured skin fibroblasts using the acid guanidinium thiocyanate-phenol-chloroform extraction procedure[8] and used to prepare cDNA.[9]

Sequence analysis: The complete cDNA encoding CAC was amplified in two overlapping fragments using the following M13-tagged primers:

Fragment A: -43CACf-$_{21M13}$ 5′-tgt aaa acg acg gcc agt GGC AGG TCG AGA ACT

Table 1. Fatty acid β-oxidation and CAC activity measurements in fibroblasts from the index patient and control subjects.

parameter measured	patient	controls
rate of fatty acid oxidation: (nmol/h/mg)		
• [9,10-^{3}H]-myristate (C14)	<0.1	5.9 ± 2.4
• [9,10-^{3}H]-palmitate (C16)	<0.1	7.8 ± 3.2
CAC activity: (pmol/min/mg)	ND*	51 ± 15

*not detectable

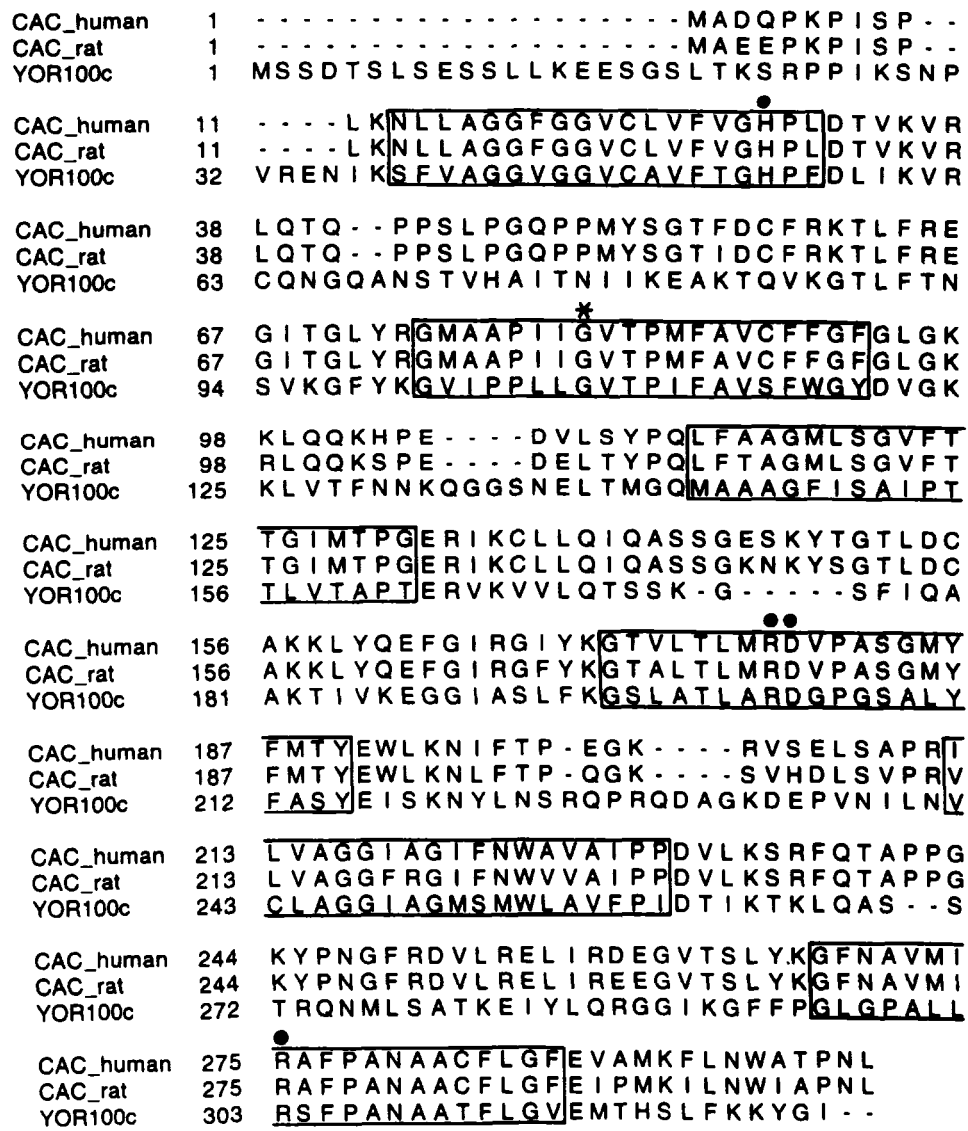

Figure 1. Comparison of different CAC proteins from human (accession no. Y10319), rat (accession no. X97831) and *S. cerevisiae* (hypothetical ORF YOR100c). The predicted membrane spanning segments are boxed. Basic and acidic residues in these membrane spanning segments are indicated by a period (•) and the mutated glycine at position 81 is indicated by an asterisk (*).

GAC AG-3′ and 468CACr$_{M13rev}$ 5′-cag gaa aca gct atg acc TGC ACA GTC CAA GGT ACC AG-3′; Fragment B: 368CACf$_{-21M13}$ 5′-tgt aaa acg acg gcc agt CAC CAC AGG AAT CAT GAC TC-3′ and 967CACr$_{M13rev}$ 5′-cag gaa aca gct atg acc TAC TCC TTC TCC TCA ACG AC-3′. Sequence analysis of these PCR fragments using BigDye fluorescent labelled M13 primers was performed on an Applied Biosystems 377A automated DNA sequencer following the manufacturer's (PE Applied Biosystems, Foster City, CA) protocols.

3. RESULTS AND DISCUSSION

Biochemical studies in fibroblasts from the index patient showed that the rate of β-oxidation of myristate and palmitate was severely decreased to less than 5% of the controls (Table 1). Enzyme measurements of the very-long chain acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, thiolase, carnitine palmitoyltransferase I and II showed normal activities (not shown). The severe deficiency of long chain fatty acid oxidation was explained by the complete deficiency of CAC activity (Table 1).

To clarify the genetic defect in this patient we sequenced the complete cDNA encoding CAC. We found a G241A missense mutation, hereby changing the codon for glycine at position 81 into arginine. This glycine is located in the second predicted membrane spanning segment and is likely to cause inactivation of the carrier.

Comparison of human, rat and yeast CAC homologues of CAC reveals that this glycine is conserved (Fig. 1). The six membrane spanning segments contain 3 basic residues (H in segment 1 and R in segments 4 and 6) and one acidic residue (D in segment 4). All of these changed residues are conserved and may play an important role in carrier function. Introduction of a fourth basic residue as identified in the patient's cDNA (G81R) probably inactivates CAC. On the other hand, the rat sequence as published before[2] contains in addition a third arginine in the fifth membrane spanning segment. Comparison with 4 mouse sequences in the EST data base revealed at this position an alanine as found in the human and yeast sequence. We suspect that arginine 219 in the rat sequence represents a sequence error.

Expression studies of the mutant CAC hope to reveal the influence of the G81R mutation on the protein function. Such expression studies in yeast are now underway.

REFERENCES

1. Stanley, C.A., Hale, D.E., Berry, G.T., DeLeeuw, S., Boxer, J. & Bonnefont, J.P. (1992) *N. Eng. J. Med.,* **327**, 19–23. A deficiency of carnitine-acylcarnitine translocase in the inner mitochondrial membrane.
2. Indiveri, C., lacobazzi, V, Giangregorio, N. & Palmieri, F. (1997) *Biochem. J.*, **321**, 713–719. The mitochondrial carnitine carrier protein: cDNA cloning, primary structure and comparison with other mitochondrial transport proteins.
3. Olpin, S.E., Bonham, J.R., Downing, M., Manning N.J., Pollitt R.J., Sharrard, M.J. & Tanner, M.S. (1995) *J. Inher. Metab. Dis.,* **20**, 714–715. Carnitine-acylcarnitine translocase deficiency—a mild phenotype.
4. Huizing, M., lacobazzi, V, IJ1st, L., Savelkoul, P., Ruitenbeek, W., van den Heuvel, L., Indiveri, C., Smeitink, J., Trijbels, F., Wanders, R.J.A. & Palmieri, F. (1997) *Am. J. Hum. Genet.*, **61**, 1239–1245. Cloning of the human carnitine-acylcarnitine carrier cDNA and identification of the molecular defect in a patient.
5. Niezen-Koning, K.E., van Sprongen, F.J., IJ1st, L., Wanders, R.J.A., Brivet, M., Duran, M., Reijngoud,

D.J., Heymans, H.S.A. & Smit, G.P. (1995) *J. Inher. Metab. Dis.,* **18**, 230–232. A patient with lethal cardiomyopathy and a carnitine-acylcarnitine translocase deficiency.

6. Wanders, R.J.A., van Roermund, C.W.T., van Wijland, M.J.A., Schutgens, R.B.H., Heikoop, J., van der Bosch, H., Schram, A.W. & Tager, J.M. (1987) *J. Clin. Invest.,* **80**, 1778–1783. Peroxisomal fatty acid beta-oxidation in relation to the accumulation of very long chain fatty acids in cultured skin fibroblasts from patients with Zellweger syndrome and other peroxisomal disorders.
7. IJ1st, L., Mandel, H., Oostheim, W., Ruiter, J.P.N., Gutman, A. & Wanders, R.J.A. (1998) *J. Clin. Invest.,* in press. Molecular basis of hepatic carnitine palmitoyltransferase I deficiency.
8. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.*, **162**, 156–159. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction.
9. IJ1st, L., Wanders, R.J.A., Ushikubo, S., Kamijo, T. & Hashimoto, T. (1994) *Biochim. Biophys. Acta.*, **1215**, 347–350. Molecular basis of long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency: identification of the major disease-causing mutation in the α-subunit of the mitochondrial trifunctional protein.

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MCAD DEFICIENCY

Acylcarnitines (AC) by Tandem Mass Spectrometry (MS-MS) are Useful to Monitor Dietary Treatment

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1. INTRODUCTION

Medium chain acyl-CoA-dehydrogenase (MCAD) deficiency is one of the most common inborn errors of metabolism.[1] Classically the disease appears in infants with vomiting, lethargy, hypotonia and slight liver enlargement. Prolonged fasting and/or an intercurrent illness usually trigger this presentation. Eighteen percent of patients with MCAD deficiency present with sudden death and total mortality rate before diagnosis is about 24%.[2] Children who survive the first episode may have severe sequelae.[2] During the acute presentation, common laboratory findings are hypoketotic hypoglycemia, slight metabolic acidosis and increased uric acid, ammonium and liver enzymes, but biochemical diagnosis is based on the analysis of organic acids (OA), acylcarnitines (AC) and/or acylglycines.[3,4,5] A common mutation, G985, accounts for the vast majority of the alleles in northern Europeans,[6] but is less prevalent in other populations.[7] Recently the use of tandem mass spectrometry (MS-MS) for AC analysis has allowed neonatal screening for MCAD deficiency.[7,8] Typical profiles show a marked increase of octanoylcarnitine (C8), less prominent increase of hexanoyl (C6), decenoyl (C10: 1) and decanoylcarnitines (C10) and an abnormal C8 to C10 ratio.[5,7,8]

The recommended treatment of MCAD deficiency includes carnitine and a low fat (15–25%), high carbohydrate (CHO) (65–75%) diet, avoiding long periods of fasting. The use of uncooked cornstarch has also been suggested.[4,9,10]

The increased diagnosis and survival of patients with MCAD deficiency highlights the need for biochemical tests to determine the metabolic status of the patient, allowing

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dietary modifications to optimise treatment. However monitoring of the long term treatment is difficult, as hypoglycaemia, increased uric acid or abnormal organic acids may only appear under stress.[3–4]

2. AIM

To assess the biochemical response of two patients with MCAD deficiency to different diets and to evaluate the usefulness of AC to monitor these treatments.

3. PATIENTS

Two patients with MCAD deficiency were studied. **Patient 1**: This female was normal until 15m of age when she presented with a Reye-like syndrome. Diagnosis of MCAD deficiency was established. She survived the initial episode but a severe seizure disorder and cerebral palsy developed. Since diagnosis the patient has been on a diet with 65–67% of total calories given as CHO, 25–27% as fat and 7.5–8% as protein. Due to a partial lactose intolerance, diet was prepared using a lactose free formula (LK® Kasdorf, Argentina) mixed with glucose polymers (GP) (Polimerosa®—Kasdorf, Argentina) or GP plus uncooked corn starch (CS) and was given by nasogastric feeds every 4–6 h. Her studies were performed at age 2y 10m as follows:

Test No 1: (GP)

The patient was fed her usual amount of formula ($1/6^{th}$ of her 24 h intake), containing 1 g/kg of GP. Blood samples were obtained 2, 4 and 6h after finishing the feed.

Test No 2: (GP + CS)

CS at a dose of 1 gr/kg, was added to the previous preparation (total amount of CHO added = 2 g/kg). Blood sampling was similar to test No 1.

Patient 2

This female was born full term to a non-consanguineous couple. Her physical examination at birth was normal. A Guthrie card sample was obtained on the 2^{nd} day of life and sent for routine newborn screening with MS-MS. Diagnosis was established on the 4^{th} day of life. She was breast fed, supplemented with an infant formula (Similac® Abbot, Argentina) every 3–4 h until 5 months of age. From then on, her diet consisted of the same formula mixed with GP or GP + CS, and semisolids, providing 65–70% of total calories as CHO, 20% as fat and 10–15% as protein. Maximum interval allowed between feeds was 4 h until one year of age and 6 h thereafter. The patient is currently 2 years old, has had no decompensations, her weight, length and head circumference are at the 25^{th}–50^{th} percentile and she is physically normal. Her studies were performed at age 18m as follows:

Test No 1: (GP)

The patient was given her usual amount of formula, mixed with 3 g/kg of GP. Blood samples were obtained 2, 4, 6 and 8h after finishing the feed.

Test No 2: (GP + CS)

The test was similar to the previous one except that out of the 3g/kg of CHO, 1.5g/kg (50%) were given as CS and the remainder 1.5g/kg as GP.

Both patients have been treated with carnitine (~Albicar, Casasco, Argentina) (100mg/kg/day) since diagnosis.

4. METHODS

The protocol was approved by the ethics committee of the *"Fundación para el Estudio de las Enfermedades Neurometabólicas"* where all the studies were performed. An informed consent was obtained from the parents prior to each test. Both patients were in stable condition, without any sign of intercurrent illness and had normal liver enzymes and creatine kinase when the tests were performed. A heparin lock was maintained throughout each test and blood was obtained for AC, non-esteritied fatty acids, insulin, glucose, β-hydroxybutyrate, amino acids, carnitine (total and free) and uric acid levels. For acylcarnitine analysis, blood samples were spotted in a filter paper (Schleicher & Schuell 903) and allowed to dry at room temperature. Sample preparation and analysis were performed as described[11,12] with a VG Quattro II, triple quadrupole mass spectrometer (Micromass, UK) with electrospray injection, an HPLC pump and autosampler (Hewlett Packard series 1050). Acquisition was performed scanning for ions parent of m/z 85. The signals in the profiles correspond to the [M^+] ions of the acylcarnitine butyl esters. Free, acetyl, octanoyl and hexadecanoylcarnitines were quantitated with their corresponding stable isotopes. Labelled internal standards were obtained from Dr. Herman J. Ten Brink (Academic Hospital V. U. Amsterdam). Normal values were obtained from 58 children in whom fatty acid oxidation defects were excluded by our usual protocol of amino acids, AC and carnitine in blood, and organic acids in urine. None of the controls received a special diet and their blood samples were obtained after an overnight fast (children) or at the end of their usual fasting period (infants). When enough sample was available, levels of octanoic acid were measured in plasma by GC/MS, as described.[13] Mutation analysis was performed by Drs. B. Storstein Andersen and N. Gregersen at the Centre for Medical Molecular Biology, University of Aarhus, Denmark.

5. RESULTS

The initial AC profile obtained in each patient was typical of MCAD, with marked elevation of C8 and milder elevations of C6, C10:1 and C10 (Fig. 1).

Mutation analysis confirmed the biochemical diagnosis: Both patients were compound heterozygotes, with the prevalent G985 mutation in one of their MCAD alleles. The second allele was a 1-bp-insertion of a T in exon 11, corresponding to cDNA position 1190 in case No 1 (patient number 13, in reference 14), and an A to T transversion in exon 7, corresponding to cDNA position 503 in case No 2.

Results of the tests performed with GP in both patients showed that C8 was elevated in all samples, and increased progressively with fasting (Table 1, Figs. 2 and 3). The use of GP + CS allowed a marked improvement of C8 levels at 4 and 6h in both patients but failed to prevent a C8 increase at 8 h in patient No 2 (Table 1, Figs. 2 and 3).

Values of NEFA followed a pattern similar to C8-carnitine (Table 1). Accordingly, there was a significant positive correlation between the C8-carnitine and NEFA values

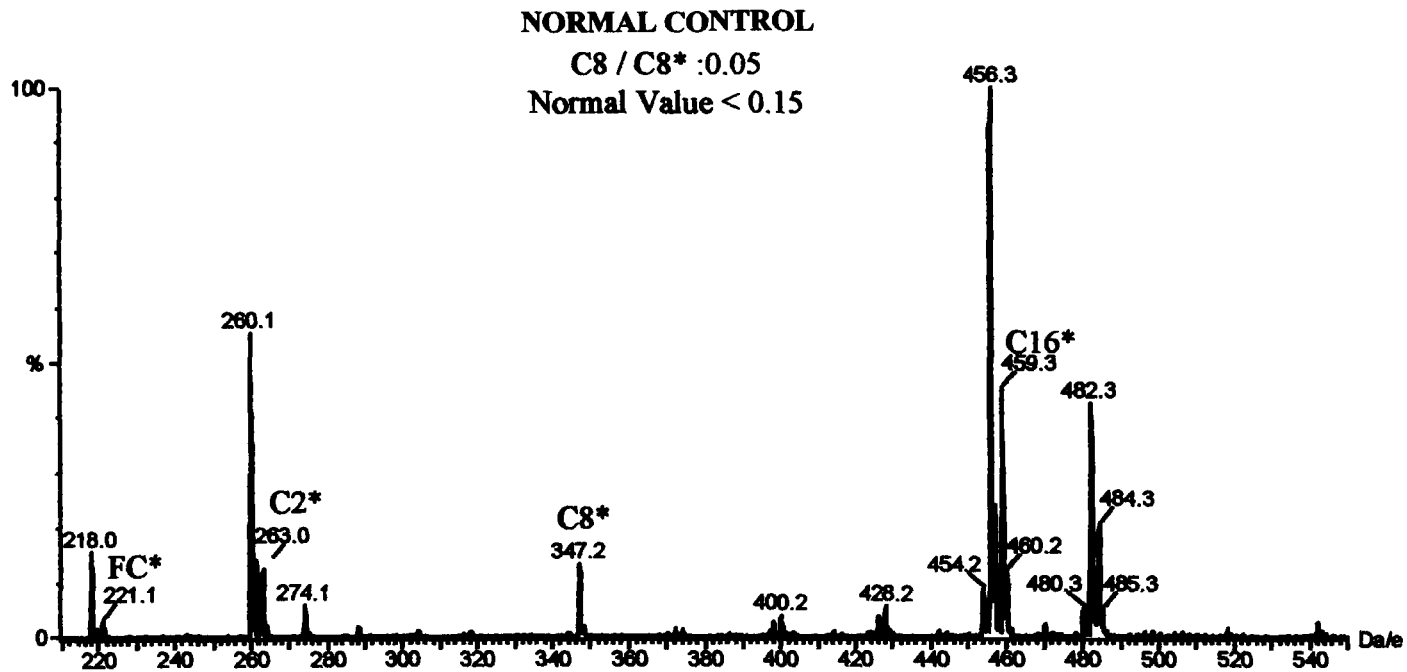

Figure 1. Acylcarnitine profile of the initial (diagnostic) samples. Note increased levels of C6, C8 and C10:1 compared to normal control.

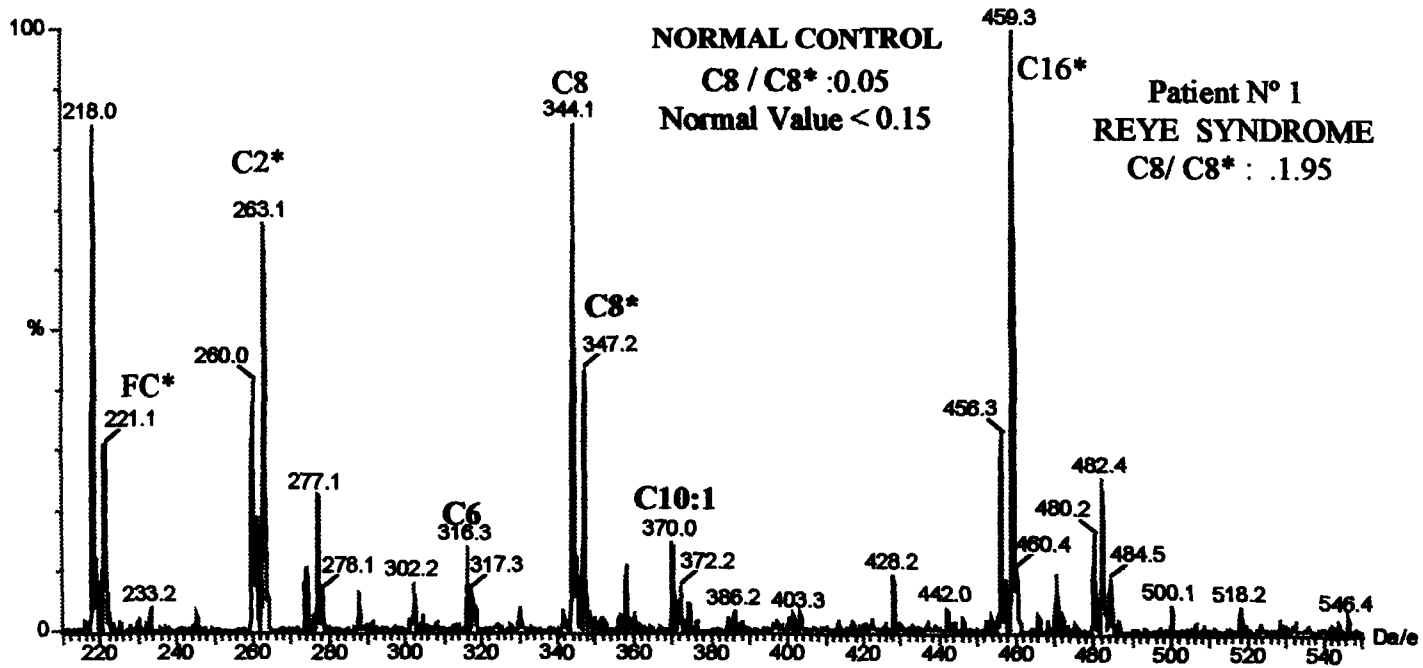

Figure 1. (Continued)

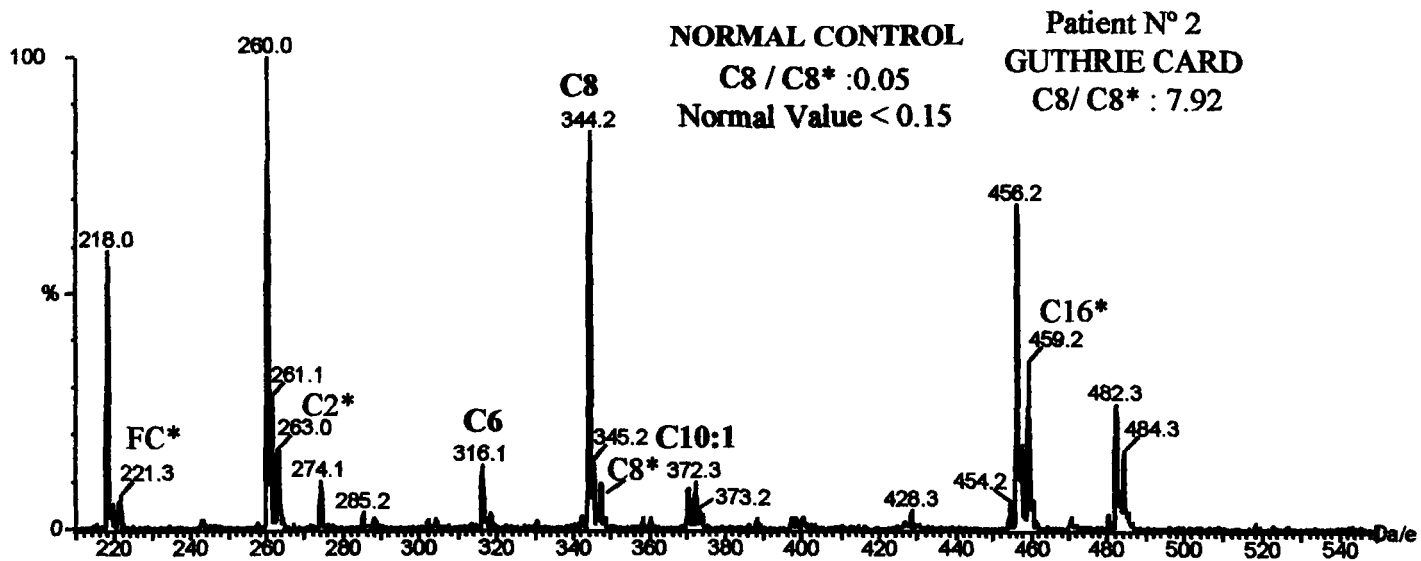

Figure 1. (Continued)

Table 1. BIOCHEMICAL PARAMETERS MEASURED DURING THE TESTS.

	Patient No 1						Patient No 2							
	GP			GP + CS			GP				GP + CS			
	2 h	4 h	6 h	2 h	4 h	6 h	2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h
Octanoylcarnitine (NI = <0.15 uMol)	0,36	1,84	2,88	0,24	0,44	0,45	0,50	0,77	5,27	5,33	0,72	0,73	1,39	6,39
NEFA (mMol)	0,04	NA	0,45	0,07	0,15	0,27	0,05	0,19	0,6	0,72	0,1	0,06	0,17	0,43
Octanoic Acid (nl 0.1–10*)	6,80	NA	NA	NA	NA	NA	7,75	NA	85,3	77,4	11,75	NA	14,8	NA
Insulin (uU/mL)	46,80	30,20	24,70	NA	NA	13,80	25,70	7,40	4,10	4,70	17,00	11,00	9,00	7,00
B-OH-Butyrate (mMol)	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	<0,1	0,14	<0,1	<0,1	<0,1	<0,1
Glucose (mMol)	3,30	3,80	4,90	3,90	4,50	4,70	6,50	4,20	4,30	4,00	5,60	4,70	4,50	4,30
Uric Acid (NI = 2.0–7.6 mg/dl)	1,80		1,60	0,80		2,60	2,10			2,00	2,80			2,30
Carnitine Total (uMol)	42,1			42,9			41,9				111,2			
Carnitine Free (uMol)	25,4			39,4			39,6				94,6			
Free, % of total	59			92			80				85			

ND: Not Detectable. NA: Not Available

*From reference 13

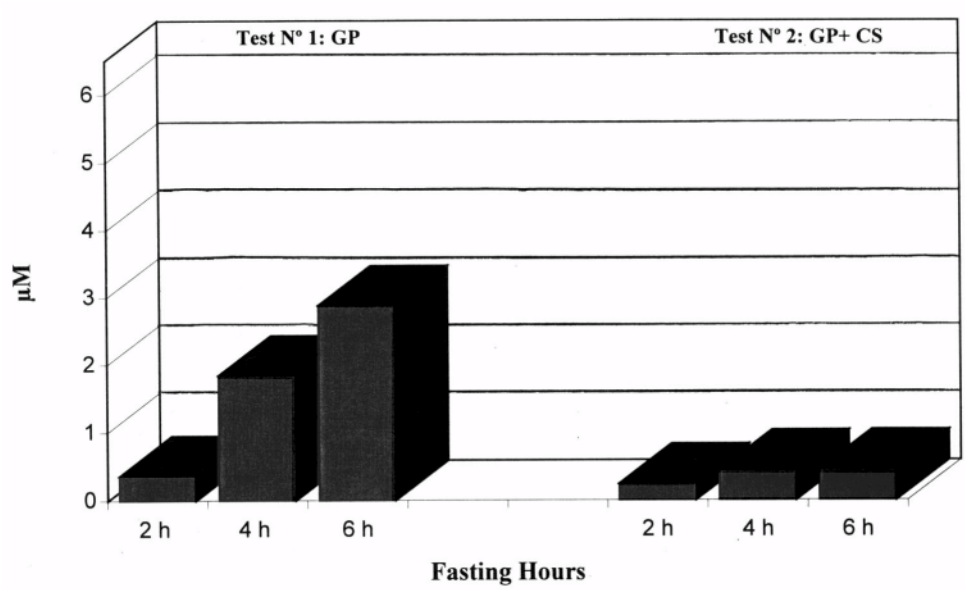

Figure 2. Levels of Octanoylcarnitine obtained in patient 1 during the tests.

($r = 0.87$, $p < 0.05$, $n = 13$). Values of octanoic acid (Table 1) showed also a significant correlation with C8-carnitine ($r = 0.99$, $p < 0.001$, $n = 6$) and NEFA ($r = 0.98$, $p < 0.001$, $n = 6$). In patient No 2, insulin levels in the GP test were higher at 2h and lower at 4, 6 and 8 h than those in the GP + CS test (Table 1). The patients' clinical condition remained unchanged during the tests. Blood glucose and uric acid values remained within normal

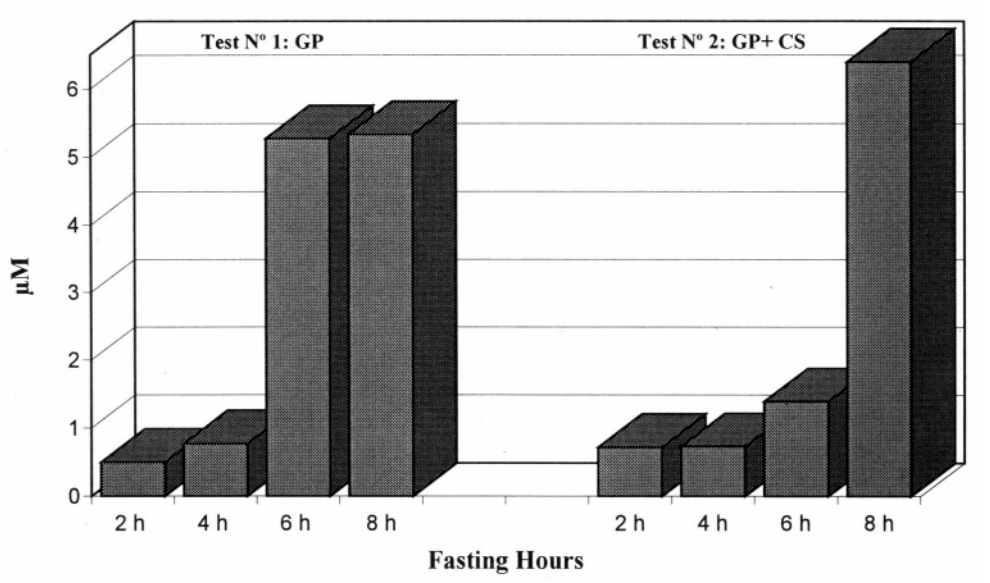

Figure 3. Levels of Octanoylcarnitine obtained in patient 2 during the tests.

limits (Table 1) as did serum amino acids. Levels of free carnitine were low in patient 1 during the GP test (Table 1).

6. DISCUSSION

MCAD deficiency is one of the most common inborn errors of metabolism. It is generally believed that treatment of this condition is simple and prognosis is good once diagnosis is established. A study of 120 cases showed that no patient died after diagnosis, however there were significant sequelae in those patients who survived the initial episode of decompensation.[2] Early recognition through newborn screening programs aimed to prevent mortality and reduce sequelae of the disease. However, two patients that had been diagnosed by newborn screening died suddenly, one after an immunisation and the other during an intercurrent illness.[7]

The diagnosis of an increasing number of patients with MCAD deficiency, together with its morbidity and mortality, even in patients under treatment, highlight the need for its optimal management. Even though a good dietary treatment is essential for this condition, monitoring of the diet is difficult as the usual biochemical markers of the disease (hypoglycemia, increased uric acid or abnormal organic acids) only appear under stress. The purpose of our study was to assess the biochemical response of two patients with MCAD deficiency to different diets and to evaluate the usefulness of AC to monitor these treatments.

In our patients, C8-carnitine levels were always elevated, increased markedly during short periods of fasting and improved at 4 and 6h when complex carbohydrates were used. These results suggest that C8-carnitine is indeed a very sensitive marker of the metabolic status in these children.

Patients with MCAD deficiency may be encephalopathic despite normoglycemia[15] and it is believed that the accumulated octanoic acid is the toxic compound.[1,16] The significant correlation found in our study between octanoic acid and C8-carnitine, validates the significance of measuring the later as a marker of the metabolic status of the patient. To our knowledge, this is the first time that the levels of octanoic acid and C8-carnitine have been measured simultaneously in MCAD deficient patients. While further studies, with a larger number of samples may be needed to confirm these findings, the possibility of using blood spots on filter paper, together with a simple and rapid sample preparation and analysis, make the C8-carnitine the method of choice for the follow-up of these patients.

Dietary treatment of MCAD deficiency is aimed to decrease the use of fatty acids as a fuel, which due to the block in fatty acid oxidation, can result in the accumulation of toxic compounds. For that reason it is essential to avoid long periods of fasting, which would mobilize free fatty acids from adipose tissue stores and activate the lipolytic pathway. A high CHO diet will increase endogenous insulin secretion, suppressing lipolysis and stimulating lipogenesis. Our results show that the use of glucose polimers produces a short lasting increase of insulin levels and, when the levels of insulin drop, C8-carnitine, octanoic acid and NEFA rise, even during a short period of fasting. The rationale for the use of CS is to obtain a CHO release that will last for longer periods of time, achieving a more even and prolonged insulin secretion. Our studies show that CS mixed with GP in the formula, improves C8-carnitine levels at 4 and 6h of fasting, but the amount used (1.5g/kg) was not enough to prevent an increase at 8 h of fasting in patient 2. To our knowledge this is the first time that the usefulness of CS for manage-

ment of MCAD patients is documented. Further studies are necessary to determine the optimal amount and composition of CHO in the diet of these patients.

We conclude that C8-carnitine levels measured by MS-MS, are very useful to monitor the metabolic status in patients with MCAD deficiency. This marker will allow determining the best dietary treatment and fasting tolerance for these patients. This will probably be true as long as normal levels of free carnitine are maintained and valproic acid, which can increase C8-carnitine levels and is contraindicated in patients with MCAD deficiency, is not given.[17] Our results also highlight the importance of carefully recording the hours of fasting when values of AC are measured in patients with fatty acid oxidation disorders.

ACKNOWLEDGMENTS

To Dr. B. S. Andresen and Dr. N. Gregersen , from The Center for Molecular Biology, University of Aarhus, Denmark, who performed the molecular studies.

REFERENCES

1. Roe, C.R. & Coates, P. (1995) *In* The metabolic and molecular bases of inherited disease. 1501–1534 Mitochondrial fatty acid oxidation disorders (Scriver, C., Beaudet, A.L., Sly, W.S. & Valle, D. eds.) 7th ed. MGraw-Hill, New York.
2. Iafolla, A.K., Thompson, R.J. & Roe, C.R. (1994) *J Pediatr.* **124**, 409–415 Medium chain acyl-CoA-dehydrogenase deficiency: clinical course in 120 affected children.
3. Rinaldo, P. (1994) *In* Liver disease in Children. 295–308 Laboratory diagnosis of inborn errors of metabolism. (Suchy, F.J. ed.) Mosby, St. Louis.
4. Stanley, C.A. (1995) *In* Inborn Metabolic Diseases. Diagnosis and Treatment. 133–146 Disorders of fatty acid oxidation. (Fernandes, J., Saudubray, J.M. & Van den Berghe G. eds). 2nd, Ed. Springer-Verlag. Berlin.
5. Van Hove, J.L.K., Zhang, W., Kahler, S.G., Roe, C.R., Chen, Y.T., Terada, N., Chase, D., Iafolla, K., Ding, J.H. & Millington, D. (1993) *Am J Hum Genet.* **52**, 958–66 Medium chain Acyl-CoA dehydrogenase (MCAD) deficiency: Diagnosis by acylcarnitine in blood.
6. Gregersen, N., Winter, V. & Curtis, D., et al. (1993) *Hum Hered* **43**, 342–350 Medium Chain Acyl-CoA Dehydrogenase deficiency: Theprevalent mutation G985 (K304E) is subject to a strong founder effect from Northwestern Europe.
7. Ziadeh, R., Hoffman, E.P., Finegold, D.N., Hoop, R.C., Brackett, J.C., Strauss, A.W. & Naylor, E. (1995) *Pediatr Res.* **37**, 675–678 Medium chain Acyl-CoA dehydrogenase deficiency in Pennsylvania: Neonatal screening shows high incidence and unepected mutation frequencies.
8. Chace, D.H., Hillman, S.L., Van Hove, J.L.K. & Naylor, E.W. (1997) *Clinical Chemistry* **43**, 2106–2113. Rapid diagnosis of MCAD deficiency: quantitative analysis of octanoylcarnitine and other acylcarnitines in newborn blood spots by tandem mass spectrometry.
9. Treem, W.R. (1994) *In* Liver disease in Children. 852–887 Inborn defects in mitochondrial fatty acid oxidation (Suchy, F.J. ed.) Mosby, St. Louis.
10. (1997) *In* The Ross metabolic formula system nutrition support protocols. 373–390. Mitochondrial fatty acid oxidation defects. (Acosta PB & Yannicelli S. eds.) 3rd ed.
11. Millington, D.S., Terada, N., Chace, D.H., Chen, Y.T., Ding, J.H., Kodo, N. & Roe, C. (1992) *In* New developments in fatty acid oxidation. 339–354 The role of tandem mass spectrometry in the diagnosis of fatty acid oxidation disorders (Tanaka K. ed.)
12. Millington, D.S., Chace, D.H., Hillman, S.L., Kodo, N. & Terada, N. (1994) *In* Biological mass spectrometry: present and future 559–579 Diagnosis of Metabolic disease (Matsuo, T., Caprioli, R.M., Gross, M.L. & Sevama. Y. eds.) John Wiley & Sons.
13. Costa, C.G., Dorland, L., Holwerda, U, Tavares de Almeida, I., Poll-The, W.T., Jacobs, C. & Duran, M. (1998) *Clin Chem.* **44**, 463–471 Simultaneous analysis of plasma free fatty acids and their 3-hydroxy analogs in fatty acid B-oxidation disorders.

14. Andresen, B.S, Bross, P. & Udvari, S. et al. (1997) *Human Molecular Genetics* **6**, 695–707 The molecular basis of medium-chain acyl-CoA-dehydrogenase (MCAD) deficiency in compound heterozygous patients: is there correlation between genotype and phenotype?
15. Stanley, C.A., Hale, D.E. & Coates, P.M. et al. (1983) *Pediatr Res.* **17**, 877 Medium chain acyl-CoA-dehydrogenase deficiency in children with non-ketotic hypoglycemia and low carnitine levels.
16. Trauner, D.A. & Adams, H. (1981) *Pediatr Res.* **15**, 1097 Intracraneal pressure elevations during octanoate infusion in rabbits: and experimental model of Reye's syndrome.
17. Njolstad, P.R., Skjeldal, O.H. & Agsteribbe, E. et al. (1997) *Pediatric Neurology,* **16**, 160–162 Medium chain Acyl-CoA-dehydrogenase deficiency and fatal valproate toxicity.

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D-HYDROXYACYL-COA DEHYDROGENASE DEFICIENCY

Identification of a New Peroxisomal Disorder with Implications for Other Disorders of β-Oxidation

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1. ABSTRACT

The second and third steps of peroxisomal β-oxidation are catalysed by two multifunctional enzymes: D-bifunctional protein and L-bifunctional protein. Here we show that fibroblasts of a patient described as being deficient in the 3-hydroxyacyl-CoA dehydrogenase component of D-bifunctional protein and fibroblasts of a patient described as being deficient in L-bifunctional protein do not complement one another. Using a newly developed method to measure the activity of D-bifunctional protein in fibroblast homogenates, we found that the activity of the D-bifunctional protein was completely deficient in the patient with presumed L-bifunctional protein deficiency.

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2. INTRODUCTION

Until recently, β-oxidation of different fatty acids and fatty acid derivatives in peroxisomes was thought to be mediated by a single bifunctional protein containing both 2-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities.[1–3] Recent studies have shown that there is an additional bifunctional protein alternatively called multifunctional protein 2,[4] multifunctional enzyme 2,[5,6] or D-bifunctional protein.[7–9] This newly identified bifunctional protein generates a D-3-hydroxyacyl-CoA, whereas the other bifunctional protein generates L-3-hydroxyacyl-CoA intermediates.[4,5,7,8] Because of this remarkable difference in stereospecificity of the two bifunctional proteins, Hashimoto and coworkers suggested the names D-bifunctional protein (D-BP) and L-bifunctional protein (L-BP), respectively.[5,7,8]

Available evidence suggests that D-BP is involved in the β-oxidation of straight-chain fatty acids (e.g. C26:0), 2-methyl-branched chain fatty acids (e.g. pristanic acid), and bile acid intermediates (e.g. THCA), whereas L-BP is involved in the β-oxidation of straight-chain fatty acids only and *not* in the β-oxidation of 2-methyl-branched chain fatty acids and bile acid intermediates.[5,6,9]

We recently identified a new peroxisomal disorder in a patient showing signs and symptoms comparable to those observed in Zellweger syndrome. In the patients' plasma, very-long-chain fatty acids, pristanic acid, and di- and trihydroxycholestanoic acid were elevated suggesting a defect in the peroxisomal β-oxidation system. Subsequent studies identified the defect in this patient at the level of the 3-hydroxyacyl-CoA dehydrogenase component of the newly identified D-bifunctional protein.[10]

Remarkably, no complementation was found when cells from the newly identified patient were fused with cells from the patient described by Watkins and coworkers[11] with L-bifunctional protein deficiency. We now present data shedding some light on this apparent enigma.

3. MATERIALS AND METHODS

3.1. Case Reports

Patient 1 suffered from an isolated deficiency of the 3-hydroxyacyl-CoA dehydrogenase component of D-bifunctional protein and has been fully described elsewhere.[10] The clinical and biochemical characteristics of patient 2 have been described previously by Watkins and coworkers.[11]

3.2. Complementation Analysis

Fibroblasts were grown using standard conditions in Dulbecco Modified Eagles Medium with fetal calf serum and fused or cocultivated according to Brul *et al.*[12] The fused cells were cultured for three days in Dulbecco's Modified Eagle Medium without fetal calf serum after which overall pristanic acid β-oxidation was measured as described before.[13]

3.3. Measurement of the Activity of D-bifunctional Protein

The combined activity of the 2-enoyl-CoA hydratase and D-3-hydroxyacyl-CoA dehydrogenase components of D-bifunctional protein was measured as described in.[10]

4. RESULTS AND DISCUSSION

Recently we described a patient with a defect in the 3-hydroxyacyl-CoA dehydrogenase component of the D-bifunctional protein.[10] We have now performed complementation studies and fused fibroblasts from this patient (patient 1) with fibroblasts from the L-bifunctional protein deficient patient known from literature (patient 2)[11] followed by measurement of pristanic acid β-oxidation in the fused cells (Table 1). As a control, cells were not fused but only cocultivated after which pristanic acid β-oxidation was measured. Remarkably, pristanic acid β-oxidation was not restored after fusion, indicating that cells from patient 1 and patient 2 did *not* complement one another. As expected, both cell lines did show complementation when fusions were performed with fibroblasts from a Zellweger patient (Table 1).

The results of the complementation studies suggest that in both patient 1 *and* patient 2 the defect is in the same gene although patient 1 was described as being D-bifunctional protein deficient and patient 2 was described as being L-bifunctional protein deficient. Importantly, both patients showed elevated levels of C26:0 and accumulation of bile acid intermediates (DHCA, THCA) in plasma.[10,11] The accumulation of THCA in plasma and the deficient β-oxidation of pristanic acid in fibroblasts are hard to reconcile with the fact that L-bifunctional protein has been found *not* to be involved in the β-oxidation of 2-methyl branched-chain fatty acids, like pristanic acid, and bile acid intermediates, like THCA. In fact, recent studies have shown that it is the D-bifunctional protein which is involved in the β-oxidation of these substrates.[5,6,9] This led us to measure the activity of D-bifunctional protein in fibroblasts from patient 2.

To this end fibroblasts of patient 2 were incubated with the enoyl-CoA ester of THCA and formation of 24-hydroxy-THC-CoA and 24-keto-THC-CoA was measured (Fig. 1). The results in Fig.1 clearly show that patient 1 is deficient at the level of the 3-hydroxyacyl-CoA dehydrogenase component of D-bifunctional protein because of the increased formation of 24-hydroxy-THC-CoA and lack of 24-keto-THC-CoA formation (Fig. 1B). In contrast, there was only very little formation of 24-hydroxy-THC-CoA and no formation of 24-keto-THC-CoA in patient 2 (Fig. 1C).

These data strongly suggest that it is the D-bifunctional enzyme which is functionally inactive in patient 2. Subsequent studies which include analysis at the molecular level, have revealed distinct mutations in the gene coding for D-bifunctional protein. These results will be described elsewhere (Van Grunsven *et al.*, in preparation).

Taken together, our results resolve the puzzling finding that cells from our patient 1 with a defect in the 3-hydroxyacyl-CoA dehydrogenase component of the D-bifunctional protein failed to show complementation with cells from the patient with presumed L-bifunctional protein deficiency which we have now found to be deficient in the D-specific enzyme. The finding that C26:0 is strongly increased in plasma from both

Table 1. Results of complementation analyses.

	Pristanic acid β-oxidation activity (pmol/h/mg protein)	
	fused	cocultivated
patient 1[a] × patient 2[b]	0	0
patient 1[a] × ZS[c]	506	0
patient 2[b] × ZS[c]	443	0

[a]D-hydroxyacyl-CoA dehydrogenase deficient patient[10]
[b]L-bifunctional protein deficient patient[11]
[c]patient suffering from Zellweger Syndrome (ZS)

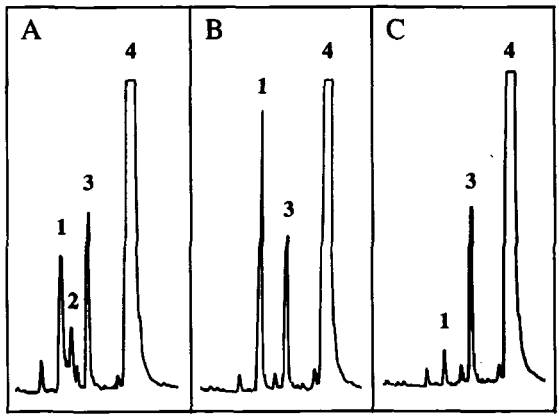

Figure 1. D-Bifunctional protein activity measurement. Fibroblast homogenates were incubated with 24-ene-THC-CoA and the products 24-hydroxy-THC and 24-keto-THC-CoA were separated by HPLC. (A) control, (B) patient 1, (C) patient 2. Peaks are numbered as follows: 1, 24-hydroxy-THC; 2, 24-keto-THC-CoA; 3, contaminant; 4, (24*E*)-24-ene-THC-CoA.

patient 1 and patient 2, suggests that D-bifunctional protein is also involved in C26:0 β-oxidation.

5. REFERENCES

1. van den Bosch, H., Schutgens, R.B.H., Wanders, R.J.A. & Tager, J.M. (1992) *Ann. Rev. Biochem.* **61**, 157–197. Biochemistry of peroxisomes.
2. Reddy, J.K. & Mannaerts, G.P. (1994) *Ann. Rev. Nut.* **14**, 343–370. Peroxisomal lipid metabolism.
3. Wanders, R.J.A., Schutgens, R.B.H. & Barth, P.G. (1995) *J. Neuropathol. Exp. Neurol.* **54**, 726–739. Peroxisomal disorders: a review.
4. Dieuaide-Noubhani, M., Novikov, D., Baumgart, E., Vanhooren, J.C., Fransen, M., Goethals, M., Vandekerckhove, J., Van Veldhoven, P.P. & Mannaerts, G.P. (1996) *Eur. J. Biochem.* **240**, 660–666. Further characterization of the peroxisomal 3-hydroxyacyl-CoA dehydrogenases from rat liver. Relationship between the different dehydrogenases and evidence that fatty acids and the C27 bile acids di- and tri-hydroxycoprostanic acids are metabolized by separate multifunctional proteins.
5. Qin, Y.M., Poutanen, M.H., Helander, H.M., Kvist, A.P., Siivari, K.M., Schmitz, W., Conzelmann, E., Hellman, U. & Hiltunen, J.K. (1997) *Biochem. J.* **321**, 21–28. Peroxisomal multifunctional enzyme of beta-oxidation metabolizing D-3-hydroxyacyl-CoA esters in rat liver: molecular cloning, expression and characterization.
6. Qin, Y.M., Haapalainen, A.M., Conry, D., Cuebas, D.A., Hiltunen, J.K. & Novikov, D.K. (1997) *Biochem. J.* **328**, 377–382. Recombinant 2-enoyl-CoA hydratase derived from rat peroxisomal multifunctional enzyme 2: role of the hydratase reaction in bile acid synthesis.
7. Jiang, L.L., Kobayashi, A., Matsuura, H., Fukushima, H. & Hashimoto, T. (1996) *J. Biochem. (Tokyo)* **120**, 624–632. Purification and properties of human D-3-hydroxyacyl-CoA dehydratase: medium-chain enoyl-CoA hydratase is D-3-hydroxyacyl-CoA dehydratase.
8. Jiang, L.L., Miyazawa, S., Souri, M. & Hashimoto, T. (1997) *J. Biochem. (Tokyo)* **121**, 364–369. Structure of D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein.
9. Jiang, L.L., Kurosawa, T., Sato, M., Suzuki, Y. & Hashimoto, T. (1997) *J. Biochem. (Tokyo)* **121**, 506–513. Physiological role of D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein.
10. van Grunsven, E.G., van Berkel, E., Ijlst, L., Vreken, P., de Klerk, J.B.C., Adamski, J., Lemonde, H., Clayton, P.T., Cuebas, D.A. & Wanders, R.J.A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 2128–2133. Peroxisomal D-Hydroxyacyl-CoA dehydrogenase deficiency: Resolution of the enzyme defect and its molecular basis in bifunctional protein deficiency.

11. Watkins, P.A., Chen, W.W., Harris, C.J., Hoefler, G., Hoefler, S., Blake, D.C., Jr., Balfe, A., Kelley, R.I., Moser, A.B., Beard, M.E. & Moser, H.W. (1989) *J. Clin. Invest.* **83**, 771–777. Peroxisomal bifunctional enzyme deficiency.
12. Brul, S., Westerveld, A., Strijland, A., Wanders, R.J.A., Schram, A.W., Heymans, H.S., Schutgens, R.B.H., van den Bosch, H. & Tager, J.M. (1988) *J. Clin. Invest.* **81**, 1710–1715. Genetic heterogeneity in the cerebrohepatorenal (Zellweger) syndrome and other inherited disorders with a generalized impairment of peroxisomal functions. A study using complementation analysis.
13. Wanders, R.J.A., Denis, S., Ruiter, J.P., Schutgens, R.B.H., van Roermund, C.W.T. & Jakobs, B.S. (1995) *J. Inherit. Metab. Dis.* **18 Suppl 1**, 113–124. Measurement of peroxisomal fatty acid beta-oxidation in cultured human skin fibroblasts.

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PHYTANOYL-COA HYDROXYLASE DEFICIENCY

Enzymological and Molecular Basis of Classical Refsum Disease

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1. INTRODUCTION

Refsum disease (RD) was first described as a distinct entity in the 1940s by Sigvald Refsum.[1] Most patients show a tetrad of abnormalities which include retinitis pigmentosa, peripheral neuropathy, cerebellar ataxia and elevated protein levels in the cerebrospinal fluid with a normal cell count as main manifestations. However, subsequently several patients have been described who lack this tetrad of abnormalities (for review see[2]). A major breakthrough with respect to the identification of the metabolic defect in RD was the finding by Klenk and Kahlke in the 1960s of the accumulation phytanic acid (3,7,11,15-tetramethylhexadecanoic acid), an unusual branched chain fatty acid, in body fluids and tissues of patients with RD.[3] Since phytanic acid contains a methyl group at the third carbon atom, it can not be degraded via β-oxidation. Instead, the first carboxyl group is removed by α-oxidation.

We recently found the enzyme defect in RD to be at the level of phytanoyl-CoA hydroxylase (PhyH), a peroxisomal enzyme catalysing the first step of the α-oxidation of phytanic acid, which involves conversion of phytanoyl-CoA to 2-hydroxyphytanoyl-CoA.[4,5] Furthermore we identified the human *PHYH* cDNA after purification of PhyH

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from rat liver peroxisomes.[6] The human *PHYH* cDNA encodes a peroxisomal protein containing a cleavable leader sequence with a Peroxisome Targeting Signal type 2 (PTS2). We now report the development of an enzyme assay allowing PhyH activity measurement in human skin fibroblasts. In addition, we performed *PHYH* mutation analysis at the cDNA level in a series of patients with RD, and we developed an expression system in bakers yeast *S. cerevisiae* in order to verify whether the mutations found in the *PHYH* cDNA from patients with RD affect PhyH activity.

2. MATERIALS AND METHODS

2.1. Phytanoyl-CoA Hydroxylase Activity Measurement in Human Liver and Skin Fibroblast Homogenates

Measurement of PhyH activity in human liver homogenates is based on the conversion of [l-^{14}C]phytanoyl-CoA to [1-^{14}C]2-hydroxyphytanoyl-CoA followed by separation of the CoA esters by means of a radio-HPLC method (see[7] for details).

PhyH activity in skin fibroblast homogenates was determined using a gas chromatography / mass spectrometry (GC-MS) method (Jansen *et al.*, manuscript in preparation). Briefly, skin fibroblasts were homogenised and incubated in the presence of all substrates and cofactors as described for human liver homogenates.[7] The product of the PhyH reaction, 2-hydroxyphytanoyl-CoA was hydrolysed to form 2-hydroxyphytanic acid which was measured by stable isotope dilution GC-MS essentially as described by ten Brink *et al.*[8]

2.2. Mutation Analysis of the *PHYH* cDNA

cDNA was obtained from skin fibroblasts by RT-PCR and subsequent *PHYH* mutation analysis was carried out exactly as described before.[6]

2.3. *PHYH* Expression in *Saccharomyces cerevisiae*

The *PHYH* open reading frame was amplified from control human fibroblast cDNA using XbaI and HindIII tagged primers and cloned into the XbaI and HindIII sites of a yeast expression plasmid containing the oleate inducible *CTA1* promoter.[9] *S. cerevisiae* strain BJ1991 was transformed as described.[10] Yeast cells were harvested and resuspended in 250 µl buffer containing 20mM Tris pH 7.5, 5mM dithiotreitol, 1 µg ml^{-1} leupeptin, 2 mg ml^{-1} Pefabloc and 10% (v/v) glycerol. After addition of 200 µl glass beads the suspension was vortexed for 30min at 4°C, centrifuged for 2min at 12,000 × *g* at 4°C, and the clear lysate was removed and used for PhyH activity measurements, carried out as described above for human liver homogenates.

3. RESULTS AND DISCUSSION

3.1. PhyH Activity Measurement in Human Liver and Skin Fibroblast Homogenates

Initially, we used the method developed by Mihalik *et al.*[11] to measure PhyH activ-

ity in human liver. In that assay [1-^{14}C]phytanic acid was added as substrate to purified peroxisomes in the presence of ATP, Mg^{2+} and CoASH, which resulted in rapid conversion of the radiolabeled phytanic acid to phytanoyl-CoA, the substrate for PhyH. This conversion was catalysed by long chain acyl-CoA synthetase present in the peroxisomal membrane.[12] When applied to human liver homogenates, this assay system did not give satisfactory results because of relatively low long chain acyl-CoA synthetase activity in human liver homogenates yielding only low amounts of phytanoyl-CoA. In addition, a much higher hydrolase activity was present in human liver homogenates, causing a hydrolysis of phytanoyl-CoA to form the free fatty acid again. These findings led us to develop an assay system using phytanoyl-CoA rather than phytanic acid as substrate in the presence of ATP, Mg^{2+} and CoASH.[7] This HPLC based assay allows detection of both the substrate, phytanoyl-CoA, and the product, [1-^{14}C]2-hydroxyphytanoyl-CoA (Fig. 1). PhyH activity measurement in a liver homogenate from a patient with RD showed no detectable amounts of 2-hydroxyphytanoyl-CoA, demonstrating that PhyH is deficient in RD (Table 1; see also[4]).

Since liver (biopsy) material is not very convenient for diagnosis we have now set up an assay allowing PhyH activity measurement in skin fibroblast homogenates. This assay is based on a gas chromatography / mass spectrometry (GC-MS) detection method (Jansen *et al.*, manuscript in preparation). Using this assay we have measured PhyH activ-

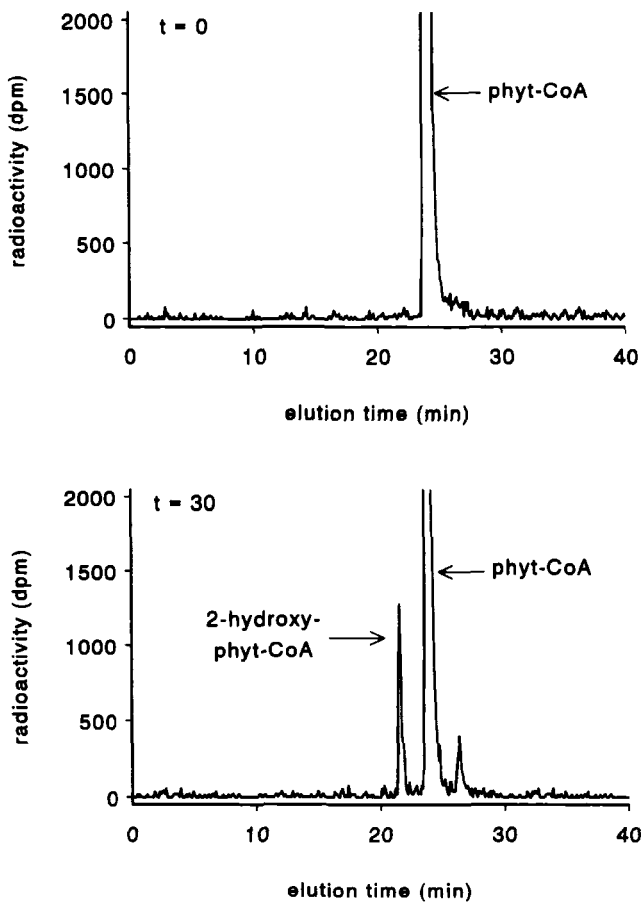

Figure 1. PhyH activity measurement in liver homogenate. At the start of the incubation (t = 0, upper panel) the HPLC trace shows one single peak of the substrate [1-^{14}C]phytanoyl-CoA with a retention time of 24 min. After 30 min incubation (t = 30, lower panel) a large second peak appears, which is the reaction product 2-hydroxyphytanoyl-CoA (retention time 22 min). The activity measurements are carried out exactly as described.[7]

Table 1. PhyH activity measurements in human liver- and skin fibroblast homogenates.

Patient	PhyH activity (nmol × h^{-1} × mg^{-1}) Liver homogenate*		Fibroblast homogenate	
Controls	2.45 ± 0.88	(n = 11)	0.13 ± 0.02	(n = 10)
Refsum disease	≤0.05	(n = 1)	0.01 ± 0.01	(n = 6)
Zellweger syndrome	≤ 0.05	(n = 3)	0.03 ± 0.01	(n = 4)

*See[7] for details.

ity in fibroblast homogenates from patients with RD, as well as from patients with Zellweger syndrome (ZS) in which peroxisomes are deficient due to a defect in peroxisome biogenesis.[13] Both types of patients are clearly deficient in PhyH activity (Table 1), which corresponds exactly to our previous findings in liver material from patients with RD and ZS.[5,7] Measurement of PhyH activity in fibroblasts from patients with classical RD revealed a complete deficiency of PhyH activity, which is due to mutations in the *PHYH* gene.[6] In the case of ZS the deficiency of PhyH activity is a secondary consequence of the absence of peroxisomes in these patients. The PhyH protein is probably normally synthesised, but will not function properly or will be broken down in the cytosol as found for other peroxisomal proteins.[14]

3.2. Mutation Analysis of the *PHYH* cDNA

Mutation analysis of the *PHYH* cDNA has revealed that all patients with RD analysed contain mutations confirming that *PHYH* is the defective gene in RD. The mutations found include: 1) a single nucleotide deletion which causes a premature stop codon resulting in a truncated protein, 2) a 111 nucleotide deletion resulting in a protein lacking 37 internal amino acids, and 3) several different point mutations resulting in the substitution of a single amino acid (Table 2; see also[15]).

3.3. *PHYH* Expression in *Saccharomyces cerevisiae*

It is evident that the single nucleotide deletion (del T164) resulting in a truncated protein which contains only 16% of the PhyH amino acid sequence will cause PhyH deficiency. In the case of the 111 nucleotide deletion it is very likely that the protein lacking

Table 2. Mutations in the phytanoyl-CoA hydroxylase cDNA in 8 patients with Refsum disease.

Patient	Mutation		effect on coding sequence
1[a]	del T164	homozygous	frameshift[b]
2[a]	del T164	homozygous	frameshift[b]
3[a]	del 111 bp (135–246)	homozygous	del aa[c] 46–82
4[a]	del 111 bp (135–246)	homozygous	del aa[c] 46–82
5[a]	del 111 bp (135–246)	heterozygous	del aa[c] 46–82
	A805C	heterozygous	$Asn^{269}His$
6	C823T	homozygous	$Arg^{274}Trp$
7	C823T	homozygous	$Arg^{274}Trp$
8	G610A	homozygous	$Gly^{204}Ser$

[a]the mutations in patients 1–5 have been described in,[6] for patients 6–8 see.[15]
[b]termination after codon 66.
[c]aa, amino acid.

an internal fragment of 37 amino acids will be misfolded or lack a part of the protein which is important for enzymatic activity such as substrate- or cofactor binding sites, which explains the deficiency of PhyH activity. However, it is difficult to predict the consequences of a single amino acid substitution on the activity of the PhyH protein. In order to verify the effect of the G610A transition (giving rise to a $Arg^{275}Trp$ substitution, as found in patient 8, Table 2), we have cloned this mutation in the *PHYH* open reading frame and used the yeast *S. cerevisiae* to express the mutant protein. Activity measurements in yeast lysate preparations containing the expression vehicle without the *PHYH* open reading frame showed no detectable PhyH activity. When the correct human *PHYH* open reading frame was cloned into this expression system, a high PhyH activity was measured. When the *PHYH* open reading frame containing the G610A transition was expressed, no PhyH activity could be detected in the yeast lysate. This finding provides the final proof that this point mutation is causing the loss of PhyH activity in this patient with RD.

REFERENCES

1. Refsum, S. (1946) *Acta Psychiatr. Scand. (suppl.)* **38**, 9–303. Heredopathia atactica polyneuritiformis.
2. Steinberg, D. (1995) *in* The metabolic and molecular bases of inherited disease (Scriver, C.R., Beaudet, A.L., Sly, W.S. & Valle, D. eds), 2351–2369. McGraw-Hill, New York. Refsum disease.
3. Klenk, E. & Kahlke, W. (1963) *Hoppe Seylers. Z. Physiol. Chem.* **333**, 133–139. Ueber das Vorkommen der 3,7,11,15-Tetramethylhexadecansaure (phytansaure) in den Cholesterinestern und anderen Lipoidfraktionen der Organe bei einem Krankheitsfall unbekanter Genese (Verdacht auf Heredopathia atactica polyneuritiformis, Refsum's syndrome).
4. Jansen, G.A., Wanders, R.J.A., Watkins, P.A. & Mihalik, S.J. (1997) *N. Engl. J. Med.* **337**, 133–134. Phytanoyl-Coenzyme A hydroxylase deficiency: the enzyme defect in Refsum's disease.
5. Jansen, G.A., Mihalik, S.J., Watkins, PA., Moser, H.W., Jakobs, C., Denis, S. & Wanders, R.J.A. (1996) *Biochem. Biophys. Res. Commun.* **229**, 205–210. Phytanoyl-CoA hydroxylase is present in human liver, located in peroxisomes, and deficient in Zellweger syndrome: direct, unequivocal evidence for the new, revised pathway of phytanic acid alpha-oxidation in humans.
6. Jansen, G.A., Ofman, R., Ferdinandusse, S., IJlst, L., Muijsers, A.O., Skjeldal, O.H., Stokke, O., Jakobs, C., Besley, G.T.N., Wraith, J.E. & Wanders, R.J.A. (1997) *Nature Genet.* **17**, 190–193. Refsum disease is caused by mutations in the phytanoyl-CoA hydroxylase gene.
7. Jansen, G.A., Mihalik, S.J., Watkins, P.A., Jakobs, C., Moser, H.W. & Wanders, R.J.A. (1998) *Clin. Chim. Acta* **271**, 203–211. Characterization of phytanoyl-Coenzyme A hydroxylase in human liver and activity measurements in patients with peroxisomal disorders.
8. ten Brink, H.J., Schor, D.S.M., Kok, R.M., Poll-The, B.T., Wanders, R.J.A. & Jakobs, C. (1992) *J. Lipid Res.* **33**, 1449–1457. Phytanic acid alpha-oxidation: accumulation of 2-hydroxyphytanic acid and absence of 2-oxophytanic acid in plasma from patients with peroxisomal disorders.
9. Elgersma, Y., Van Den Berg, M., Tabak, H.F. & Distel, B. (1993) *Genetics* **135**, 731–740. An efficient positive selection procedure for the isolation of peroxisomal import and peroxisome assembly mutants of *Saccharomyces cerevisiae.*
10. Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *in* Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.
11. Mihalik, S.J., Rainville, A.M. & Watkins, P.A. (1995) *Eur. J. Biochem.* **232**, 545–551. Phytanic acid alpha-oxidation in rat liver peroxisomes. Production of alpha-hydroxyphytanoyl-CoA and formate is enhanced by dioxygenase cofactors.
12. Watkins, P.A., Howard, A.E., Gould, S.J., Avigan, J. & Mihalik, S.J. (1996) *J. Lipid Res.* **37**, 2288–2295. Phytanic acid activation in rat liver peroxisomes is catalyzed by long-chain acyl-CoA synthetase.
13. Lazarow, P.B. & Moser, H.W. (1995) *in* The metabolic and molecular bases of inherited disease (Scriver, C.R., Beaudet, A.L., Sly, W.S. & Valle, D. eds), 2287–2324. McGraw-Hill, New York. Disorders of peroxisome biogenesis.

14. Schram, A.W., Strijland, A., Hashimoto, T., Wanders, R.J.A., Schutgens, R.B.H., van den Bosch, H. & Tager, J.M. (1986) *Proc. Natl. Acad Sci. U. S. A.* **16**, 6156–6158. Biosynthesis and maturation of peroxisomal beta-oxidation enzymes in fibroblasts in relation to the Zellweger syndrome and infantile Refsum disease.
15. Jansen, G.A., Ferdinandusse, S., Skjeldal, O.H., Stokke, O., De Groot, C.J., Jakobs, C. & Wanders, R.J.A. (1998) *J. Inher. Metab. Dis.* (in press).

RATIONALE FOR A CONDITIONAL KNOCKOUT MOUSE MODEL TO STUDY CARNITINE PALMITOYLTRANSFERASE I DEFICIENCIES

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1. ABSTRACT

Several severe congenital cardiomyopathies are known to be associated with deficiencies in long-chain fatty acid transport and oxidation. Our studies are focused on a key enzyme in the regulation of intracellular long-chain fatty acid transport: carnitine palmitoyltransferase 1. Of this enzyme, two isoforms are expressed in the neonatal heart: L-CPT1 (the "liver-type" isoform) and M-CPT1 (the "muscle-type" isoform). It is known from studies in rats that chemical inhibition of both CPT1 isoforms results in hypertrophy of the cardiomyocytes, leading to an increase in heart-weight of up to 25%. With the aid of expressed sequence tag database analyses, cDNA- and genomic sequence information, we analysed the human gene for M-CPT1 in detail, and obtained partial clones of the murine genes for both CPT1 isoforms. We now started the development of a conditional knockout model to analyse and dissect deficiencies in these genes.

While of the other mitochondrial components of the carnitine system deficiencies are known, some with severe cardiac consequences, M-CPT1 deficiencies have never been described. This suggests that M-CPT1 deficiency either (1) has not been recognised within the pool of congenital disorders, (2) is detrimental in an early stage of reproduction or embryogenesis, or (3) does not lead to physiological problems, probably due to the existence of a rescue system. If (1) is the case, the phenotypic effects of M-CPT1 deficiency have to be studied in order to generate criteria for clinical decision making and diagnosis. Option (2) demonstrates the necessity to use novel vector systems to create condi-

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tional gene disruptions. Hypothesis (3) implies a possible role for L-CPT1, and a knockout model allows a study of the interaction between the genes for L-CPT1 and M-CPT1. Applicable strategies to develop such a model system will be discussed.

2. INTRODUCTION

Long-chain fatty acids are the major substrates for energy generation in the heart.[1] Before entering the mitochondrial β-oxidation cycle, long-chain fatty acids have to be transported across the mitochondrial membranes as carnitine esters. The machinery needed for transport of long-chain acylcarnitines includes three membrane-associated components:[2] the enzyme carnitine palmitoyltransferase 1 (CPT1), the carrier carnitine acylcarnitine-translocase (CACT), and the enzyme carnitine palmitoyltransferase 2 (CPT2). Of CPT1, two isoforms are expressed in the neonatal heart: L-CPT1 (the "liver-type" isoform) and M-CPT1 (the "muscle-type" isoform).[3] These isoforms are encoded by two separate genes,[4,5,6] *CPT1A* and *CPT1B*, respectively.

Several familial cardiomyopathies are known to be associated with deficiencies in long-chain fatty acid transport and oxidation.[7,8,9] Patients with adult[10,11] and juvenile presentations of CACT- and CPT2 deficiencies have been described, of which the acute juvenile forms are accompanied by severe cardiac problems.[12,13,14,15,16] Of CPT1 deficiencies, only defects in the liver-type enzyme are documented.[17,18,19,20] In these patients hepatic problems dominate, i.e., hypoketotic hypoglycemia under circumstances where an increase in β-oxidation is called upon. These primary metabolic consequences in L-CPT1 defects can lead to hepatomegaly, encephalopathy, coma, and sudden death. Apart from malfunctioning of other organ systems, transient cardiac arrhythmia,[20] tachycardia[21] and cardiomegaly[21,22] in cases of L-CPT1 deficiency have been observed. These cardiac deviances, and also the sudden death phenomenon, may be caused by the metabolic state of the whole body, but the direct effect of a lack of L-CPT1 in the heart itself may play a role as well. If a L-CPT1 deficiency could be limited to the heart in a model system, this would allow a better understanding of the role of L-CPT1 in heart metabolism, and of the cardiac contribution to sudden death in some cases of L-CPT1 deficiency.

Whereas the "hepatic" CPT deficiency refers to a L-CPT1 defect, the "muscular" CPT deficiency is the adult presentation of a CPT2 defect, while the acute juvenile CPT2 malfunctioning has been designated "hepatocardiomuscular" (see also McKusick's Online Mendelian Inheritance in Man accession numbers 255110, 255120, 600528, 600649, 600650, 601987; http://www3.ncbi.nlm.nih.gov/omim/). All known CPT1 deficiencies are L-CPT1 defects, and M-CPT1 deficiencies are not known. However, it is clear that M-CPT1 plays a functional role in long-chain fatty acid metabolism in heart muscle,[3] e.g., it has been shown by studies in rats that chemical inhibition with etomoxir, which acts irreversibly on both CPT1 isoforms,[3] results in hypertrophied cardiomyocytes, leading to an increase in heart-weight of up to 25%.[23,24]

3. WHAT CAN BE EXPECTED? POSSIBLE EXPLANATIONS FOR THE ABSENCE OF M-CPT1 DEFICIENT PATIENTS

Since L-CPT1 deficiency is compatible with life, it is anticipated that a *CPT1A* knockout mouse model will be a viable model system. The value of such an animal model will increase if conditional (spatiotemporal) control of L-CPT1 function can be achieved

(see above). For a model of M-CPT1 deficiency the conditional aspect may even be of more importance. The main question to be answered is why there are no M-CPT1 deficient patients known. Possible explanations for this can be divided in three groups, which we will discuss below. It is of clinical and social importance which of these explanations will hold true. Not only will a *CPT1B* knockout study be relevant even if a probably low number of patients suffer from such a recessively inherited disorder, it may also improve the differential diagnosis of metabolic disease[9] in a large population of patients[8] when M-CPT1 deficiency can be excluded as a possible cause of metabolic disorder. Furthermore, the social impact and psychological consequences for the direct environment of cases of unexplained sudden death are hardly quantifiable.

The outcome of a *CPT1B* knockout study will provide answers to existing questions. It can be expected that new questions will arise as well, and that basic scientific insight may gain considerably. One of the major reasons to develop a conditional knockout mouse model for both *CPT1* genes is to investigate the phenotype of a M-CPT1 defect as a single entity, as well as in conjunction with a L-CPT1 defect.

The possible explanations we have been considering are the following:

(1) M-CPT1 deficiency could be structurally misdiagnosed. It may not have been recognised within the pool of congenital disorders due to the specific gene expression pattern of *CPT1B*. The genetic regulation of expression of the two CPT1 isoforms in skin fibroblasts, the default tissue material for diagnosis of metabolic disorders, permits only the enzymatic measurement of L-CPT1, and not M-CPT1.[2,22] For the accurate measurement of M-CPT1 much more invasive methods would be needed to obtain biopsies in stead of the relatively mild approach used to obtain skin fibroblasts. Although justified in obvious cases of severe (cardio)muscular problems, it is certainly not routine to obtain muscle- or fat biopsies from young children, let alone heart biopsies. It is, therefore, easily conceivable that the specific expression pattern of the *CPT1B* gene has limited the probability to accurately diagnose possible patients. If this is the case, the phenotypic effects of M-CPT1 deficiency have to be studied in detail in order to determine clear criteria for diagnostics and clinical decision making. A proper understanding of the onset of the disorder will improve genetic counselling and advice. Therefore, possible disease-related problems during late stages of embryogenesis and near-term fetal development should also be within the scope of this study.

(2) M-CPT1 deficiency could be detrimental in an early stage of reproduction or embryogenesis. If this explanation is true, the accuracy of diagnosing cardiomuscular disorders will improve as there would be one possible metabolic disorder less to take into account. If early problems due to inaccurate expression of *CPT1B* occur, it is of importance to identify the developmental stage at which these are initiated and effectuated. In that respect, the expression pattern of *CPT1B* is intriguing since it is known that abundant levels of its mRNA are present in rat testis.[25] We know from dbEST (expressed sequence tag database) analysis that a substantial number of mouse and human *CPT1B* clones in this database originate from testis as well.

At first sight, obligatory post-meiotic expression of *CPT1B* could explain why M-CPT1 defects never occur congenitally, when proper expression from a single allele is a prerequisite for the viability and fertility of spermatozoa. This would mean that, although heterozygous germ-line cells could exist due to a *de novo* mutation in *CPT1B*, the mutant allele would not pass through the haploid stage, and even heterozygotes would never be born. However, this thought is contradicted by experimental evidence that haploid sperm cells actually behave as diploids, probably due to cellular interconnections.[26] Moreover, there is important circumstantial evidence that the complete lack of an active gene for

M-CPT1 can be overcome during haploid stages in humans: several patients are known with subtelomeric deletions of chromosome 22q,[27,28,29] the chromosomal site which contains the *CPT1B* gene.[5,6] These reported cases are hemizygous for *CPT1B,* and none of them appear to be mosaic (H. E. McDermid, personal communication). Since the parents have normal karyotypes,[28] the *de novo* rearrangements of 22qter must have arisen in germline cells of one of the parents, most likely during meiosis I. This strongly suggests that gametes without an active locus for M-CPT1 are fertile. Some of the 22qter deletions are of paternal origin,[27] i.e., the gametes were spermatozoa. Thus, if *CPT1B* malfunctioning would lead to problems in early stages of reproduction or development, these problems can only become manifest in diploid cell stages in cases of homozygosity or compound heterozygosity. This could be relevant either in pre-meiotic stages of germ cell development, or in early stages of embryo development. It will be clear that information on the importance of *CPT1* expression in reproduction is crucial for the development of a viable animal model.

(3) M-CPT1 deficiency may not be a cause of physiological problems, since there may exist a rescue system. Again, this would be relevant for the spectrum of defects to count with in the diagnosis of long-chain fatty acid metabolic disorders. It would also mean that the M-CPT1 enzyme is not necessary for life, which would prompt us to the subsequent question why there exist different CPT1 isoforms at all. The conceivable explanation for the existence of at least two CPT1 isoforms, is that body homeostasis is too complex to be covered by a single gene product at a crucial entry site in long-chain fatty acid oxidation. The two known isoforms are similar, but have different properties with respect to a.o. inhibitor sensitivity and carnitine affinity,[2] e.g., rat L-CPT1 shows a much higher affinity for carnitine than rat M-CPT1 (K_ms of 32 µM and 500 µM, respectively).[30] In the healthy rat heart, the expression of the CPT1 isoforms shift from L-CPT1 to M-CPT1 during the fetal-neonatal transition.[3] The teleological explanation that in the late fetal / early neonatal heart, L-CPT1 is expressed when free carnitine levels are too low for optimal M-CPT1 activity, and that in the neonatal heart M-CPT1 takes over CPT1 function after free carnitine levels increase,[3] seems plausible. Therefore, if a rescue system appears, this implies a candidate role for L-CPT1, in which L-CPT1 function prolongs after the level of free carnitine increases in the heart. Other enzymes with acylcarnitine transferase properties may also be involved such a rescue system. A binary knockout model with disrupted genes of both isoforms is needed to study the possible interaction between the genes for L-CPT1 and M-CPT1.

4. MOLECULAR GENETIC CONSIDERATIONS

In our opinion, the model of choice to study a genetic deficiency is a knockout model. This implies that the model animal has to be a mouse,[31] since only in murine embryonic stem cells it has been possible to efficiently create the desired genetic alterations while pertaining these cells pluripotent, a prerequisite for proper embryonic development. Thus, in contrast to transgenic model systems, which in principle can be generated in any mammalian species by means of oocyte microinjection, the choice of animal for knockout studies is limited to the mouse. Techniques to analyse cardiovascular function are well developed for larger animal models, and some of these are now modified for the small size of the mouse heart and for the animal's rapid cardiac cycle.[32]

As discussed in the previous section, the possibility that a *CPT1B* gene knockout can be detrimental in pre-meiotic or post-zygotic stages of reproduction cannot be

excluded. This necessitates the use of novel vector systems to create conditionally controllable gene disruptions, in order to circumvent possible complications in a classic knockout model, and in order to obtain a viable model system. An analogous approach for the *CPT1A* gene knockout enables the dissection of cardiac and hepatic metabolic consequences of a L-CPT1 deficiency.

Novel knockout techniques allow the specific initiation of gene-disruption events by the use of the bacteriophage P1-derived *Cre-LoxP* system.[33,34,35] This system consists of two elements: short DNA sequences called *LoxP* sites, and a specific recombinase which causes rearrangements of DNA by its action on these sites. The recombinase is encoded by the *Cre* gene, and in most applications it functions through deleting a DNA fragment between two *LoxP* sites (the "floxed" DNA). By introducing *LoxP* sequences in introns which flank important exons, silent gene-disruptions are created, which only become apparent after the action of the Cre-recombinase. Mice resembling classic knockouts can be obtained by breeding, after crossing of the *LoxP*-tagged mice with "deleter" mice which express *Cre* body-wide.[36] Other transgenics which express *Cre* later in development, or specifically in one tissue, can be used in order to create the desired spatiotemporal disruption of the target gene. Transgenic and knock-in mice expressing *Cre* in various specific manners are in development at several laboratories. Useful information about promoter specificity in conjunction to *Cre* expression can be found on the internet at http://www.mshri.on.ca/develop/nagy/Cre.htm. We refer to ref.[37] for a recent example of a knock-in strategy, which was used for ventricular myocardium-specific gene targeting at early stages of ventricular specification.

Not only by crossing with mice which express *Cre*, but also by infection with Cre-adenovirus vectors the tissue-specific control of the knockout event can be achieved.[38] This is a simple and attractive approach which reduces time, labour, and the number of animals needed for breeding. Adenoviral delivery of Cre in adult ventricular cardiomyocytes *in vivo* has been shown effective.[39]

In man, and presumably also in mouse, the *CPT1A* and *CPT1B* genes are asyntenic (i.e., the loci are on different chromosomes).[4,5,6] In the human genome, *CPT1A* is located on chromosome 11q13,[5] whereas *CPT1B* is located very closely to the distal end of chromosome 22q (22q telomere, 22qter, also called 22q13.32)[5,6] (see also Genbank accession number U62317). The equivalent of human 22qter is a region of chromosome 15 in mouse, and for 11q13 this is a region of chromosome 19. Mice with defined mutations in these particular chromosomal regions are not known. The genomic sequences of the chromosomal region surrounding human[6,40] and rat[41] *CPT1B* genes are known in detail, but of the larger *CPT1A* gene no complete genomic sequence has been reported yet. Partial genomic sequences from the human[4] and rat[42] *CPT1A* gene point to the existence of several large introns in this gene in both species. If in the mouse the *CPT1A* gene contains large introns as well, the introduction of *loxP* sites will probably be less a problem than for *CPT1B*. The *CPT1B* gene is relatively compact, spanning less than 10kb in man and rat.[6,40,41] For normal activity of the floxed gene, the small size of the *CPT1B* gene may not allow even small alterations in most of the introns, as this could affect proper splicing. Moreover, the first introns may contribute to the regulation of transcription and should be avoided in cloning strategies for the insertion of *LoxP* sites.

As discussed above, an attractive strategy to create a target site for Cre is to introduce *LoxP* sequences in introns. To lower the risk that the activity of the floxed gene or its product is negatively affected, two *loxP* sites should be used, without any additional alterations like selection—or reporter genes. However, it is tempting to introduce at least one reporter system in either gene-targeting experiment, as this may provide a means to

follow gene-inactivation after Cre-mediated excision. Even if normal function of the floxed or fused gene is altered, this may give rise to an informative animal model. Several reporter systems can be used in these types of experiments,[43] but success seems to depend largely on specific conditions which differ from gene to gene. In our current studies we focus on the jellyfish green fluorescent protein (GFP) reporter system,[44] because exogenous substrates are not required for GFP, which may be useful to monitor gene expression in living cells. The GFP system, just as any other reporter, can be used in two ways:

— An off-on reporter switch after Cre-mediated excision, i.e., Cre-mediated inactivation of the floxed gene results in expression of GFP. In this case the *GFP* gene should be cloned outside the floxed sequence, and should become activated either by in-frame fusion to the first region of the CPT1 peptide sequence, or by replacement of the complete coding sequence. An in-frame fusion may be the best approach, as the 5´ *LoxP* site still would be situated distant from the promoter and first introns. Coding sequence replacement requires a *LoxP* site between the promoter and the start codon. Off-on expression switching is a dominant trait, and therefore allows expression of the reporter in homozygous and heterozygous mice. Gene-activity of those floxed genes which escaped to Cre-mediated inactivation cannot be assessed this way.
— An on-off reporter switch allows a better estimate of the remaining amount of gene product after Cre-mediated excision. This situation can be reached by placing the reporter between the *loxP* sites, in-frame with the CPT1 protein sequence, either N- or C-terminal. Again, a prerequisite for an optimal analysis is that the gene fusion does not interfere with normal gene function. The on-off switch is of recessive nature, just as the target gene alteration itself, and therefore better reflects the level of residual activity after Cre-mediated inactivation.

Both these strategies create knock-in situations: the off-on switch results in a reporter gene knock-in behind the endogenous CPT1 promoter *after* inactivation of CPT1, while the on-off approach attaches the reporter to active CPT1 and allows gene expression studies in the first generation of mice already. For these reasons our first efforts focus on the latter strategy. Much will depend on the flexibility of CPT1 enzyme function, and also on the applicability and sensitivity of GFP in these studies.

5. CONCLUDING REMARKS

Genetic defects in transport and oxidation of fatty acyl derivatives regularly cause cardiomyopathies and produce dramatic symptoms under circumstances where an increase in β-oxidation is called upon. Our aim is to create a binary (*CPT1A* and *CPT1B*) and conditional (*Cre-LoxP*) knockout mouse model to study deficiencies with expected consequences for the regulation and action of intracellular long-chain fatty acid transport to the mitochondrial matrix. Depending on the viability of the animals, mice with complete or partial knockout genotypes will be analysed. The emphasis will be on the analysis of the phenotypes of homozygotes (LLmm and llMM) and double-homozygotes (llmm) at the macroscopic-, microscopic-, molecular- and biochemical level.

Once the above described system has been developed, a broad range of intriguing questions can be approached efficiently. Our primary interests are focused on pediatric

cardiology, lipid metabolism, and inherited metabolic disorders. However, it is clear that a conditional CPT1 knockout system will provide a valuable model for a wide range of research fields, including those of diabetes, obesity, apoptosis, muscular dystrophy and brain metabolism. The application of reporter systems like *GFP* enable the combination of gene knockout and reporter knock-in. Much will depend on the functional feasibility of the various approaches.

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REFERENCES

1. Neely, J.R., Rovetto, M.J. & Oram, J.F. (1972). *Prog Cardiovasc Dis* **15**:289–329. Myocardial utilization of carbohydrate and lipids.
2. McGarry, J.D. & Brown, N.F. (1997). *Eur J Biochem* **244**:1–14. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis.
3. Brown, N.F., Weis, B.C., Husti, J.E., Foster, D.W. & McGarry, J.D. (1995). *J Biol Chem* **270**:8952–8957. Mitochondrial carnitine palmitoyltransferase I isoform switching in the developing rat heart.
4. Britton, C.H., Schultz, R.A., Zhang, B., Esser, V., Foster, D.W. & McGarry, J.D. (1995). *Proc Natl Acad Sci U S A* **92**:1984–1988. Human liver mitochondrial carnitine palmitoyltransferase I: characterization of its cDNA and chromosomal localization and partial analysis of the gene.
5. Britton, C.H., Mackey, D.W., Esser, V., Foster, D.W., Burns, D.K., Yarnall, D.P., Froguel, P. & McGarry, J.D. (1997). *Genomics* **40**:209–211. Fine chromosome mapping of the genes for human liver and muscle carnitine palmitoyltransferase I (CPT1A and CPT1B).
6. van der Leij, F.R., Takens, J., van der Veen, A.Y., Terpstra, P. & Kuipers, J.R.G. (1997). *Biochim Biophys Acta* **1352**:123–128. Localization and intron usage analysis of the human CPT1B gene for muscle type carnitine palmitoyltransferase I.
7. Coates, P.M. & Tanaka, K. (1992). *J Lipid Res* **33**:1099–1110. Molecular basis of mitochondrial fatty acid oxidation defects.
8. Kelly, D.P. & Strauss, A.W. (1994). *N Engl J Med* **330**:913–919. Inherited cardiomyopathies.
9. Schwartz, M.L., Cox, G.F., Lin, A.E., Korson, M.S., Perez-Atayde, A., Lacro, R.V. & Lipshultz, S.E. (1996). *Circulation* **94**:2021–2038. Clinical approach to genetic cardiomyopathy in children.
10. Olpin, S.E., Bonham, J.R., Downing, M., Manning, N.J., Pollitt, R.J., Sharrard, M.J. & Tanner, M.S. (1997). *J Inherit Metab Dis* **20**:714–715. Carnitine-acylcarnitine translocase deficiency—a mild phenotype.
11. DiMauro, S. & DiMauro, P.M. (1973). *Science* **182**:929–931. Muscle carnitine palmityltransferase deficiency and myoglobinuria.
12. Stanley, C.A., Hale, D.E., Berry, G.T., Deleeuw, S., Boxer, J. & Bonnefont, J.P. (1992). *N Engl J Med* **327**:19–23. Brief report: a deficiency of carnitine-acylcarnitine translocase in the inner mitochondrial membrane.
13. Pande, S.V., Brivet, M., Slama, A., Demaugre, F., Aufrant, C. & Saudubray, J.M. (1993). *J Clin Invest* **91**:1247–1252. Carnitine-acylcarnitine translocase deficiency with severe hypoglycemia and auriculo ventricular block. Translocase assay in permeabilized fibroblasts.
14. Niezen-Koning, K.E., van Spronsen, F.J., Ijlst, L., Wanders, R.J., Brivet, M., Duran, M., Reijngoud, D.J., Heymans, H.S. & Smit, G.P. (1995). *J Inherit Metab Dis* **18**:230–232. A patient with lethal cardiomyopathy and a carnitine-acylcarnitine translocase deficiency.
15. Demaugre, F., Bonnefont, J.P., Colonna, M., Cepanec, C., Leroux, J.P. & Saudubray, J.M. (1991). *J Clin Invest* **87**:859–64. Infantile form of carnitine palmitoyltransferase II deficiency with hepatomuscular symptoms and sudden death. Physiopathological approach to carnitine palmitoyltransferase II deficiencies.
16. Hug, G., Bove, K.E. & Soukup, S. (1991). *N Engl J Med* **325**:1862–1864. Lethal neonatal multiorgan deficiency of carnitine palmitoyltransferase II.

17. Bougneres, P.F., Saudubray, J.M., Marsac, C., Bernard, O., Odievre, M. & Girard, J. (1981). *J Pediatr* **98**:742–746. Fasting hypoglycemia resulting from hepatic carnitine palmitoyl transferase deficiency.
18. Demaugre, F., Bonnefont, J.P., Mitchell, G., Nguyen, H.N., Pelet, A., Rimoldi, M., Di, D.S. & Saudubray, J.M. (1988). *Pediatr Res* **24**:308–311. Hepatic and muscular presentations of carnitine palmitoyl transferase deficiency: two distinct entities.
19. Haworth, J.C., Demaugre, F., Booth, F.A., Dilling, L.A., Moroz, S.P., Seshia, S.S., Seargeant, L.E. & Coates, P.M. (1992). *J Pediatr* **121**:553–557. Atypical features of the hepatic form of carnitine palmitoyltransferase deficiency in a Hutterite family.
20. Bergman, A.J., Donckerwolcke, R.A., Duran, M., Smeitink, J.A., Mousson, B., Vianey-Saban, C. & Poll-The, B.T. (1994). *Pediatr Res* **36**:582–588. Rate-dependent distal renal tubular acidosis and carnitine palmitoyltransferase I deficiency.
21. Schaefer, J., Jackson, S., Taroni, F., Swift, P. & Turnbull, D.M. (1997). *J Neurol Neurosurg Psychiatry* **62**:169–176. Characterisation of carnitine palmitoyltransferases in patients with a carnitine palmitoyltransferase deficiency: implications for diagnosis and therapy.
22. Tein, I., Demaugre, F., Bonnefont, J.P. & Saudubray, J.M. (1989). *J Neurol Sci* **92**:229–245. Normal muscle CPT1 and CPT2 activities in hepatic presentation patients with CPT1 deficiency in fibroblasts. Tissue specific isoforms of CPT1?
23. Hulsmann, W.C., Peschechera, A., Schneijdenberg, C.T. & Verkleij, A.J. (1994). *Cardioscience* **5**:193–197. Comparison of the effects of carnitine palmitoyltransferase-1 and -2 inhibitors on rat heart hypertrophy.
24. Rupp, H., Elimban, V. & Dhalla, N.S. (1992). *Faseb J* **6**:2349–2353. Modification of subcellular organelles in pressure-overloaded heart by etomoxir, a carnitine palmitoyltransferase I inhibitor.
25. Brown, N.F., Hill, J.K., Esser, V., Kirkland, J.L., Corkey, B.E., Foster, D.W. & McGarry, J.D. (1997). *Biochem J* **327**:225–31. Mouse white adipocytes and 3T3-L1 cells display an anomalous pattern of carnitine palmitoyltransferase (CPT) I isoform expression during differentiation. Inter-tissue and interspecies expression of CPT I and CPT II enzymes.
26. Braun, R.E., Behringer, R.R., Peschon, J.J., Brinster, R.L. & Palmiter, R.D. (1989). *Nature* **337**:373–376. Genetically haploid spermatids are phenotypically diploid.
27. Phelan, M.C., Thomas, G.R., Saul, R.A., Rogers, R.C., Taylor, H.A., Wenger, D.A. & McDermid, H.E. (1992). *Am J Med Genet* **43**:872–876. Cytogenetic, biochemical, and molecular analyses of a 22q13 deletion.
28. Nesslinger, N.J., Gorski, J.L., Kurczynski, T.W., Shapira, S.K., Siegel-Bartelt, J., Dumanski, J.P, Cullen, R.F., Jr., French, B.N. & McDermid, H.E. (1994). *Am J Hum Genet* **54**:464–472. Clinical, cytogenetic, and molecular characterization of seven patients with deletions of chromosome 22ql3.3.
29. Doheny, K.F., McDermid, H.E., Harum, K., Thomas, G.H. & Raymond, G.V. (1997). *J Med Genet* **34**:640–644. Cryptic terminal rearrangement of chromosome 22q13.32 detected by FISH in two unrelated patients.
30. McGarry, J.D., Mills, S.E., Long, C.S. & Foster, D.W. (1983). *Biochem J* **214**:21–28. Observations on the affinity for carnitine, and malonyl-CoA sensitivity, of carnitine palmitoyltransferase I in animal and human tissues. Demonstration of the presence of malonyl-CoA in non-hepatic tissues of the rat.
31. Thomas, K.R., Folger, K.R. & Capecchi, M.R. (1986). *Cell* **44**:419–428. High frequency targeting of genes to specific sites in the mammalian genome.
32. James, J.F., Hewett, T.E. & Robbins, J. (1998). *Circ Res* **82**:407–415. Cardiac physiology in transgenic mice.
33. Sauer, B. & Henderson, N. (1988). *Proc Natl Acad Sci U S A* **85**:5166–5170. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1.
34. Gu, H., Marth, J.D., Orban, PC., Mossmann, H. & Rajewsky, K. (1994). *Science* **265**:103–106. Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting.
35. Barinaga, M. (1994). *Science* **265**:26–28. Knockout mice: round two.
36. Schwenk, F., Baron, U. & Rajewsky, K. (1995). *Nucleic Acids Res* **23**:5080–5081. A cre-transgenic mouse strain for the ubiquitous deletion of loxP- flanked gene segments including deletion in germ cells.
37. Chen, J., Kubalak, S. & Chien, K. (1998). *Development* **125**:1943–1949. Ventricular muscle-restricted targeting of the RXR(alpha) gene reveals a non-cell-autonomous requirement in cardiac chamber morphogenesis.
38. Wang, Y., Krushel, L.A. & Edelman, G.M. (1996). *Proc Natl Acad Sci U S A* **93**:3932–3936. Targeted DNA recombination in vivo using an adenovirus carrying the cre recombinase gene.
39. Agah, R., Frenkel, P.A., French, B.A., Michael, L.H., Overbeek, P.A. & Schneider, M.D. (1997). *J Clin Invest* **100**:169–179. Gene recombination in postmitotic cells. Targeted expression of Cre recombinase provokes cardiac-restricted, site-specific rearrangement in adult ventricular muscle in vivo.

40. Yamazaki, N., Yamanaka, Y., Hashimoto, Y., Shinohara, Y., Shima, A. & Terada, H. (1997). *FEBS Lett* **409**:401–406. Structural features of the gene encoding human muscle type carnitine palmitoyltransferase I.
41. Wang, D., Harrison, W., Buja, L.M., Elder, F.F. & McMillin, J.B. (1998). *Genomics* **48**:314–323. Genomic DNA sequence, promoter expression, and chromosomal mapping of rat muscle carnitine palmitoyltransferase I.
42. Park, E.A., Steffen, M.L., Song, S., Park, V.M. & Cook, G.A. (1998). *Biochem J* **330**:217–24. Cloning and characterization of the promoter for the liver isoform of the rat carnitine palmitoyltransferase I (L-CPT I) gene.
43. Torres, R.M. & Kühn, R. *Laboratory protocols for conditional gene targeting.* Oxford University Press, Oxford, New York and Tokyo. (1997).
44. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W. & Prasher, D.C. (1994). *Science* **263**:802–805. Green fluorescent protein as a marker for gene expression.

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BIOCHEMICAL CHARACTERISATION OF MUTATIONS OF HUMAN MEDIUM-CHAIN ACYL-COA DEHYDROGENASE

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1. INTRODUCTION

Medium chain acyl-CoA dehydrogenase (MCAD; EC 1.3.99.3) is one of the family of enzymes that catalyse the first of the four reactions that comprise the β-oxidation cycle, the oxidation of fatty acyl-CoA to enoyl-CoA[1] with the insertion of an α-β double bond. MCAD is specific for 6–12 carbon long, straight chain fatty acyl-CoAs. The human enzyme consists of 421 amino acids[2] 25 of which are cleaved upon entry into mitochondria.[3] In the mitochondria, MCAD folding is assisted by heat shock protein 60.[4] Subsequently the monomers are assembled to the active tetramer, incorporating one FAD molecule per subunit.[5,6]

MCAD deficiency is the most common defect of β-oxidation in humans.[7,8] It is an autosomal, recessively-inherited, potentially fatal disease, usually manifesting within the first years of life.[9] The clinical manifestations can be quite diverse, but usually include fasting-induced non-ketotic hypoglycaemia, lethargy, even coma. Up to 25% of the first of such attacks can be fatal, although affected individuals who remain symptom free for years have also been reported.[10,11] The wide clinical spectrum is partially due to the fact that fasting, sometimes in connection with infection and fever, is required to trigger the disease.

The high incidence of MCAD-deficiency largely reflects the high frequency of one predominant mutation, A985G, found in almost 90% of all affected alleles.[12–13]

The product of this common mutation is a lysine to glutamate change at position 304 in the mature protein. The K304E mutant was found to be unstable in patient fibroblasts[14] and upon overexpression in COS-7 cells,[15] whereas in *E. coli* most of the protein

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was found in insoluble aggregates,[16] indicating misfolding and/or hampered tetramer assembly. The chaperonin cpn60 (GroEL in *E. coli*) and co-chaperonin cpn-10 (GroES in *E. coli*) cooperate in the folding procedure and GroEL together with GroES, when co-overexpressed with the K304E mutant, aid the folding and correct assembly of the mutant enzyme.[17]

Over the last few years, a large number of other MCAD mutations have also been discovered, usually in heterozygous combination with A985G.[11] Although the G985 homozygotes may display the entire clinical spectrum, it could be speculated that a more distinct correlation between clinical phenotype and genotype is possible in patients with the rare mutations. These rare mutations, have now been recreated by site-directed mutagenesis by the megaprimer PCR procedure[18] or by subcloning from plasmids harboring patient MCAD cDNAs. The pWt plasmid[19] has been used as a template in the megaprimer procedure. The plasmids containing the mutations have been transformed into *E. coli* JM109 cells, overexpressed with and without the co-overexpression of GroEL and GroES, and investigated with respect to their ability to fold and assemble into a stable, active tetramer.[11,20] The main aims of our current study were to characterise biochemically these naturally occurring mutations, and establish possible correlation between the severity of the mutation, as established from the *in vitro* measurement of enzyme activity, and the clinical representation in the affected patients. This involves the overexpression in the presence of chaperonins, purification and characterisation of the mutant proteins and this has been undertaken for several of the rarer mutations. In this paper we present results for the mutation G242R.

2. PATIENT HISTORY OF THE G242R MUTATION

One patient, a 4-month old Danish boy, is previously described as case 1.[21] This patient died suddenly at the age of four months and was characterized as a borderline SIDS. β-Oxidation activity in fibroblasts was decreased relative to controls, but was nevertheless higher than levels usually observed for MCAD patients.

All exons of the MCAD gene from this patient's genomic DNA were amplified and sequenced as previously described.[11] No changes other than heterozygosity for the G799A mutation in exon 9, corresponding to the G242R change in the protein, were observed in the genomic DNA from this patient. The only known gross rearrangement in the MCAD gene, a deletion involving exons 11 and 12,[22] was excluded on the basis that both patients were heterozygous for the 1161A > G polymorphism.[23] In conclusion, our results strongly suggest that this patient is only heterozygous for the G242R mutation, and has no other disease-causing mutations in the MCAD gene.

3. PURIFICATION OF WILD TYPE AND G242R MCAD

For purification, 10 of the non-G985 mutations were subcloned into the pTrc-MCAD vector to allow expression of the mutant protein in higher yields, and transformed into *E. coli* TG1 cells. For the G799A mutation, the ECoRI/HindIII fragment (position 689-1315) of the pG242R plasmid was ligated into the pTrc-MCAD plasmid, digested with the same enzymes.

The wild type and the mutant G242R have successfully been purified. The enzymes were purified from 6L of culture. The G242R mutant was co-overexpressed with the

Table 1. Purification of wild type and G242R MCAD.

	Protein (mg)		Total activity (μmol/min)		Specific Activity (μmol/min/mg)	
Stage	WT	*G242R*	WT	*G242R*	WT	*G242R*
Cellular supernatant	3,380	*4,380*	2,690	*1,560*	0.796	*0.356*
40–70% Ammonium Sulphate	1,410	*1,000*	2,560	*1,620*	1.82	*1.62*
DE52 gradient	400	*466*	2,390	*1,360*	5.98	*2.92*
Calcium Phosphate	113	*58*	1,770	*1,160*	15.7	*20.0*
Octyl-Sepharose	82	*31.5*	1,520	*880*	18.5	*27.8*

chaperonins GroEL and GroES. After induction with 0.1 mM IPTG at $OD_{532} = 0.8$, the cultures were grown at 28 °C for 16 hours.[24] The cells were broken up by a large-scale version of a lysozyme/sonication protocol.[17] The purification procedure was modified from,[25] with the following differences: The DE52 buffer contained 5μM FAD, the gel filtration step was omitted and the AH-Sepharose column was replaced by octyl-sepharose.

The octyl sepharose column bound porcine kidney MCAD in 1M ammonium sulphate in 250 mM KPi, but both the human wild type and G242R enzymes bound only in 250mM KPi and 40% ammonium sulphate (1.75M). The human enzymes were eluted in 250nM KPi and 15% ammonium sulphate (0.65 M).

After purification the enzymes were precipitated by 80% ammonium sulphate in 250mM potassium phosphate, (containing 0.3mM EDTA) pH 7.6 and stored at 4°C in an approximately 5-fold excess, of FAD. Prior to use, the enzyme was dissolved in 250mM KPi, 0.3 mM EDTA, pH 7.6, and run through a Sephadex G-25 column to remove the ammonium sulphate and excess FAD. The enzyme concentration was measured from the visible spectrum, taking $\varepsilon_{448} = 14{,}800 l.mol^{-1}.cm^{-2}$.[24] Total protein was measured by the Bradford microassay and enzyme activity was measured by the ferricenium assay.[26]

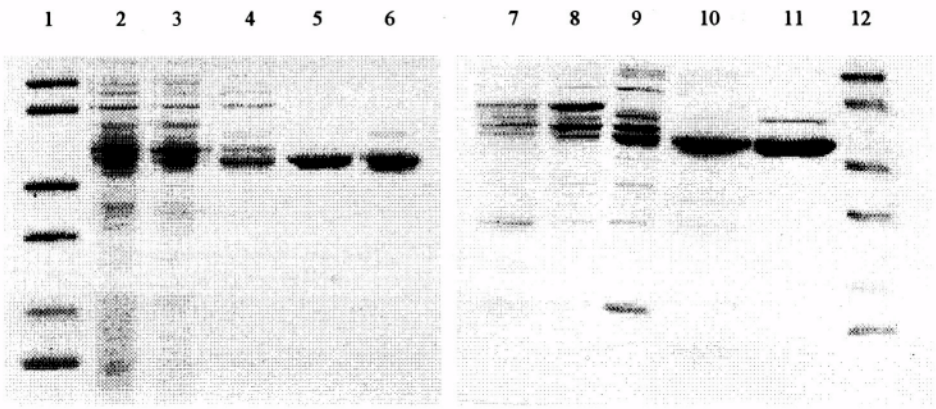
The purity of the enzyme was estimated from the A280: A450 ratio[25] and by SDS-PAGE gel, followed by Coomassie staining. The following table shows the absorbance maxima and ratios for the wild type and the G242R mutant enzymes.

A280: A450 ratio of purified enzymes:
Wild type 5.34
G242R 5.24

The purification was also monitored by running SDS-PAGE gels with samples taken after each purification step. In case of the G242R mutant, 5 μg protein was loaded in each lane. For the wild type enzyme 7.5μg of the supernatant, 5μg of the ammonium sulphate and 2.5 μg of the other steps were loaded. The results for the wild type and G242R mutants are shown in figure 1.

Table 2. Spectral characteristics of the purified wild type and G242R MCAD enzymes.

Wild type		G242R	
peak (nm)	ratio	peak (nm)	ratio
271	5.8	271	5.7
371	0.74	372	0.70
447	1	447	1



Lanes 1 and 12: molecular weight markers, 2-6 wild type, 7-11 G242R; 2: supernatant 3: ammonium sulphate 4: DE52 5: calcium phosphate 6: Octyl Sepharose 7: supernatant 8: ammonium sulphate 9: DE52 10: calcium phosphate 11: Octyl Sepharose.

Figure 1. SDS-PAGE showing purification of wild type and G242R MCAD.

4. THERMAL STABILITY OF THE WILD TYPE AND G242R MCAD

The thermal stability of the partially active MCAD mutants has so far been determined only on the cellular lysates after co-overexpression with GroEL and GroES.[11,23] The G242R mutant has been shown to have a slightly reduced stability compared to the wild type. However, the presence of chaperonins and other proteins may have a significant influence on the results, so temperature stability of the purified proteins was also measured. The enzymes were incubated at 37 °C and residual activity was measured over a 100 minute period. The results are shown in figure 2.

5. CONCLUSIONS

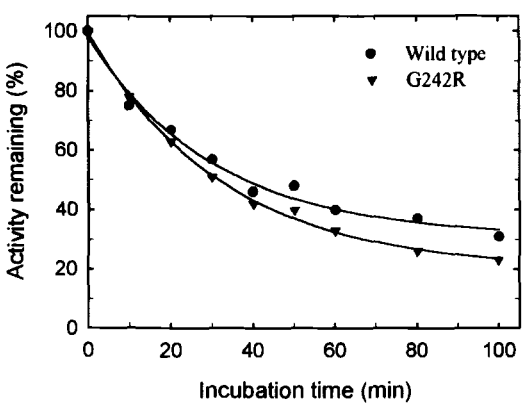


Figure 2. Thermal stability of wild type and G242R MCAD at 37 °C.

In previous experiments, wild type and mutant MCAD enzymes were overexpressed on a small scale, using the plasmid pWt and mutant derivatives, for studies on the cellular lysate.[11,20] However, the yields for large scale purification purposes were very low. To obtain larger amounts of pure protein, the plasmid pTrc has been obtained, which in the case of the wild type increased the yield by about 5-fold (data not shown).

All but one of 10 partially active mutations have now been subcloned into the pTrc expression vector. The first of the 10 partially active mutant proteins, G242R, has been successfully purified to a level similar to the wild type enzyme. The purification of the others is in progress.

As a first approach we chose to characterize the G242R mutation as it is the most interesting of the mutations. The G242R mutation is one of the two most frequent non-G985 mutations and our preliminary results had shown that it is partially active, and that it shows an abnormal behaviour in native gels.[11] From a patient point of view, the G242R mutation is of particular interest, since, as described in the previous sections a patient, affected by this mutation appeared to be only heterozygous for the G242R mutation in one allele and to have a wild type sequence in the other allele, suggesting a dominant effect of this mutation under certain circumstances. This would be highly unusual, as MCAD deficiency is traditionally considered an autosomal recessive inherited disease.

The purified MCAD wild type and G242R mutant enzymes show no obvious spectral differences. The visible peak was at 447 nm for both the wild type and the G242R mutant. The near UV peak was at 371/372nm, and the far UV peak at 271 nm for both enzymes. The ratios between the peak absorbances are also closely similar, and the slight differences may be due to slight impurities. They were both purified to >90% purity, as seen from the SDS-PAGE gels, which indicate one minor contaminating band for both enzymes. The A280:A450 ratios have been reported for the purified porcine kidney enzymes as 5.25.[25] This suggests, that approximately the same level of purity has been achieved with the human wild type and G242R enzymes. Slightly better purification was achieved in case of the G242R mutant.

The specific activity of the G242R mutant was found to be 50% higher than that of the wild type. Previously[11] it was shown that the specific activity of the G242R, as measured in the *E. coli* cellular lysate is 30–40% of the wild type, when the enzyme is co-overexpressed with the bacterial chaperonins GroEL and GroES, but the native PAGE was inconclusive about the amount of tetramer present. The G242R mutant behaves in a very unusual manner on the native gel, the band is upshifted and much less intense than would be expected from the measured activity, a property in which it is unique among all MCAD mutations so far investigated.[11,20] The present data show that the amount of active tetramers formed in case of the G242R is only 20–25% of the wild type, but once formed, the mutant enzyme has a specific activity approximately 150% of that of the wild type enzyme. However, the conditions have been selected to minimise the effect of the impaired folding mechanism by the co-overexpression of chaperonins. In the human tissues, the amount of active tetramers is likely to be considerably lower than under the experimental conditions, as G242R is a disease-causing mutation. Nevertheless it was shown that the G242R mutant, can yield levels of active tetramer under optimised conditions that are not dramatically decreased when compared to the wild type enzyme. Also, the specific activity of this mutant showed a 50% increase, indicating that reduced activity of the correctly folded enzyme is not likely to be responsible for symptoms of MCAD-deficiency in the patients.

The thermal stability of the purified wild type protein and G242R has been compared. In a previous study, using higher temperatures and shorter incubation times the G242R showed a slight reduction in stability compared to the wild type.[11] In this study,

it was shown, that at the physiological temperature, the G242R shows a detectable, but not drastic reduction in stability. The wild type and the G242R proteins show 50% inactivation after 40 minutes and 33 minutes respectively. It is noteworthy though, that patients usually have fever (patient 1 had a slight cold prior to death), so the temperature might be more relevant *in vivo* than in the case of *in vitro* experiments. Unfortunately, our results from the biochemical characterization of the G242R mutant protein have not explained how the G242R mutant protein should be able to exert a dominant effect. So, it is still a mystery, whether the disease in the described patient is a result of a dominant effect of the G242R protein (perhaps mediated through the tetrameric structure of the enzyme), compound-heterozygosity for the G242R mutation and a undetected mutation, or true heterozygosity for the G242R mutation in MCAD combined with unknown genetic factors.

In conclusion, the evidence from this work still supports the theory that it is not the stability of the enzyme already formed, but the impaired folding and/or tetramer assembly, that is the most significant factor in the disease-causing nature of this mutation.

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REFERENCES

1. Beinert H. (1963) Acyl-CoA dehydrogenases, Boyer PD, Lardy H. & Myrback K. eds. The Enzymes, Academic Press New York. **7**:447–66.
2. Kelly D.P., Kim J.-J.P., Billadello J.J., Hainline B.E., Chu T.W. & Strauss A.W. (1987) Nucleotide sequence of medium-chain acyl-coenzyme A dehydrogenase mRNA and its expression in enzyme-deficient human tissue. Proc Natl Acad Sci **84**:4068–72.
3. Matsubara Y., Krauss J.P., Ozasa H., Glassberg R., Finocchiaro G., Ikeda Y. & Mole J. *et al.* (1987) Molecular cloning and nucleotide sequence of cDNA encoding the entire precursor of rat liver medium-chain acyl-CoA dehydrogenase. J Biol Chem **262**:10104–8.
4. Saijo T., Welch W.J. & Tanaka K. (1994) Intramitochondrial folding and assembly of medium-chain acyl-CoA dehydrogenase. J Biol Chem **269**:4401–8.
5. Hall C.L. & Kamin H. (1975) The purification and some properties of electron transfer flavoprotein and general acyl-CoA dehydrogenase from pig liver mitochondria. J Biol Chem **250**:3476–86.
6. Ikeda Y., Hale D.E., Keese S.M., Coates P.M. & Tanaka K. (1986) Biosynhesis of variant medium-chain acyl-CoA dehydrogenase in cultured fibroblasts from patients with medium-chain acyl-CoA dehydrogenase deficiency. Ped Res **20**:843–7.
7. Gregersen N., Andresen B.S., Bross P., Bolund L. & Kolvraa S. (1994) Disorders of mitochondrial fatty acid oxidation especially medium-chain acyl-CoA dehydrogenase (MCAD) deficiency. In Farriaux J.P. & Dhondt J.L. eds; New Horizons in Neonatal Screening. Elsevier Science BV, pp 247–55.
8. Roe C.R. & Coates P.M. (1995) Mitochondrial fatty acid oxidation disorders. In Scriver C.R., Beaudet A.L., Sly W.S. & Valle D. (eds) The Metabolic and Molecular Bases of Inherited Disease. McGraw-Hill, New York, 1501–33.
9. Iafolla A.K., Thompson R.J. & Roe C.R. (1994) Medium-chain acyl-CoA dehydrogenase deficiency: clinical course in 120 affected children. J Pediatr **124**:409–15.
10. Andresen B.S., Jensen T.G., Bross P., Knudsen I., Winter V., Kolvraa S., Bolund L., Ding J.-H., Chen Y.-T, van Hove J.L.K., Curtis D., Yokota I., Tanaka K., Kim J.-J.P. & Gregersen N. (1994) Disease

causing mutations in exon 11 of the medium-chain acyl-CoA dehydrogenase (MCAD) gene. Am J Hum Genet **54**:975–88.

11. Andresen B.S., Bross P., Udvari S.Z., Kirk J., Gray G., Kmoch S., Chamoles N., Knudsen I., Winter W., Wilcken B., Yokota I., Hart K., Packman S., Harpey J.P., Saudabray J.M., Hale D.E., Bolund L., Kolvraa S. & Gregersen N. (1997) The molecular basis of medium-chain acyl-CoA dehydrogenase (MCAD) deficiency in compound heterozygous patients: is there correlation between genotype and phenotype? Hum Mol Genet **6**:695–707.
12. Gregersen N., Andresen B.S., Bross P., Winter V., Rüdiger N., Engst S., Christensen E., Kelly D., Strauss A.W., Kolvraa S., Bolund L. & Ghisla S. (1991) Molecular characterisation of medium-chain acyl-CoA dehydrogenase (MCAD) deficiency: identification of a lys^{329} to glu mutation in the MCAD gene, and expression of inactive mutant enzyme protein in *E. coli.* Hum Genet **86**:545–51.
13. Blakemore A.I.F., Singleton H., Pollitt R.J., Engel P.C., Kolvraa S., Gregersen N. & Curtis D. (1991) Frequency of the G985 mutation in the general population. Lancet **337**:298–9.
14. Coates P.M., Indo Y., Young D., Hale D.E. & Tanaka K. (1992) Immunochemichal characterisation of variant medium-chain acyl-CoA dehydrogenase in fibroblasts from patients with medium-chain acyl-CoA dehydrogenase deficiency. Pediatr Res **31**:34–8.
15. Jensen T.G., Andresen B.S., Bross P., Jensen U.B., Helme E., Kolvraa S., Gregersen N. & Bolund L. (1992) Expression of wild type and mutant medium-chain acyl-CoA dehydrogenase (MCAD) cDNA in eukaryotic cells. Biochym Biophys Acta **1180**:65–72.
16. Bross P., Jensen T.G., Krautle F., Winter V., Andresen B.S., Engst S., Bolund L., Kolvraa S., Ghisla S., Rasched I. & Gregersen N. (1992) Characterisation of medium-chain acyl-CoA dehydrogenase (MCAD) with a point mutation associated with MCAD deficiency. In New Developments in Fatty Acid Oxidation, Coates P.M. & Tanaka K. eds, pp 473–8. Wiley-Liss, New York.
17. Bross P., Andresen B.S., Winter V., Kräutle F., Jensen T.G., Nandy A., Kolvraa S., Ghisla S., Bolund L. & Gregersen N. (1993) Co-overexpression of bacterial GroESL chaperonins partly overcomes non-productive folding and tetramer assembly of E. coli expressed human medium-chain acyl-CoA dehydrogenase (MCAD) carrying the prevalent disease-causing K304E mutation. Biochym Biophys Acta **1182**:264–74.
18. Kuipers O.P., Boot H.J. & DeWos W.M. (1991) Improved site-directed mutagenesis method using PCR Nucleic Acids Res **19**:45581991.
19. Bross P., Jespersen C., Jensen T.G., Andresen B.S., Kristensen M.J., Winter A., Nandy A., Krautle F., Ghisla S., Bolund L., Kim J.-J.P. & Gregersen N. (1995) Effects of two mutations detected in medium-chain acyl-CoA dehydrogenase (MCAD)-deficient patients on folding, oligomer assembly and stability of MCAD enzyme. J Biol Chem **270**:10284–90.
20. Andresen B.S., Udvari S.Z., Bross P., Knudsen I., Moore R., Ijlst L., Wanders R.J.A., deKlerk J.B.C., Bolund L. & Gregersen N. No obvious genotype-phenotype correlation in 62 unrelated patients with MCAD deficiency not caused by homozygosity for the prevalent G985 mutation. Compromised folding is the main molecular defect of the mutant MCAD protein. (In preparation).
21. Lundemose J.B., Kolvraa S., Gregersen N., Christensen E. & Gregersen M. (1997) Fatty acid oxidation disorders as primary cause of sudden and unexpected death in infants and young children: an investigation performed on cultured fibroblast from 79 children who died aged 0–4 years. Mol Pathol **50**:212–7.
22. Morris A.A.M., Taylor R.W., Lightowlers R.N., Aynsley-Green A., Bartlett K. & Turnbull D.M. (1995) Medium-chain acyl-CoA dehydrogenase deficiency caused by a deletion of exons 11 and 12. Hum Mol Genet **4**:747–9.
23. Andresen B.S., Kolvraa S., Bross P., Bolund L., Curtis D., Eiberg H. & Zhang Z. (1993) A silent A to G mutation in exon 11 of the medium-chain acyl-CoA dehydrogenase (MCAD) gene. Hum Mol Genet **2**:488.
24. Nandy A., Kieweg V., Krautle F.-G, Vock P., Kuchler B., Bross P., Kim J.-J., Rasched I. & Ghisla S. (1996) Medium-long-chain chimeric acyl-CoA dehydrogenase: medium-chain enzyme with the active center base arrangement of long-chain acyl-CoA dehydrogenase. Biochemistry **35**:12402–11.
25. Thorpe C. (1981) Acyl-CoA dehydrogenase from pig kidney. Methods in Enzymology **71**:366–74.
26. Lehman T.C., Hale D.E., Bhala A. & Thorpe C. (1990) An acyl-Coenzyme A dehydrogenase assay utilizing the ferricenium ion. Anal Biocham **186**:280–4.

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LESSONS LEARNED FROM THE MOUSE MODEL OF SHORT-CHAIN ACYL-COA DEHYDROGENASE DEFICIENCY

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1. INTRODUCTION

Short-chain acyl-CoA dehydrogenase (SCAD) is one of four similar acyl-CoA dehydrogenases that catalyze the first reaction in the mitochondrial β-oxidation of fatty acids. As the nae implies, the substrates for this enzyme are short-chain acyl-CoAs (C_4–C_6). A little over a decade ago, the first spontaneous animal model for an inherited defect in β-oxidation of fatty acids was discovered and characterized, the BALB/cByJ mutant mouse with inherited short-chain acyl-CoA dehydrogenase (SCAD = enzyme, *Acads* = gene) deficiency. At that time SCAD was called butyryl-CoA dehydrogenase (BCD = enzyme, *Bcd-1* = gene), thus referring to its primary substrate. Although these mutant mice have no clinically overt abnormal phenotype, they do have important biochemical and pathological abnormalities. This mouse model has since been extensively characterized, progressing from the molecular basis of its SCAD deficiency to the evaluation of germ-line genetic correction with an SCAD transgene. It has been a unique model to study *in vivo* the metabolic bases of fatty acid β-oxidation deficiency disease.

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2. BACKGROUND

The mouse model of SCAD deficiency was discovered by a combination of processes. The first clue was revealed by a cellulose acetate electrophoresis assay for the genetic marker butyryl-CoA dehydrogenase (BCD).[1] The *Bcd-1* locus, located on mouse chromosome 5, encodes for BCD, now called SCAD. There were two electrophoretic allozyme variants known in the various inbred strains.[1] The fast anodally migrating band, representing the *Bcd-1*^{a} allele, is found in A/J, C57BL/6J, SJL/J, SM/J, and 101/H strains. Likewise, the slow anodally migrating band, representing the *Bcd-1*^{b} allele, is found in BALB/c, CBA/H, NZB/B1, NZC/B1, and 129/J mice.[1] In 1986, Prochazka and Leiter[2] reported that BALB/cByJ mice had a null activity allele at the *Bcd-1* locus. Based on this interesting genetic marker variant, our gas chromatography-mass spectrometry analysis of urinary organic acids, revealed in the BALB/cByJ mice had not only an off-scale peak of butyrylglycine, but also a very large peak of ethylmalonic acid and a significant peak of methylsuccinic acid.[3] This pattern, except for the butyrylglycine, all fit well with the report by Rhead and colleagues of two human patients with short-chain acyl-CoA dehydrogenase deficiency.[4] In 1989, both Wood, *et al.*,[3] and Schiffer, *et al.*,[5] independently reported the deficiency of short-chain acyl-CoA dehydrogenase (SCAD) and the biochemical abnormalities associated with this defect in BALB/cByJ mice. Schiffer *et al.*[5] also demonstrated that the SCAD deficiency mapped to the *Bcd-1* locus on chromosome 5.

The BALB/cByJ mice are direct descendents of the BALB/cBy strain maintained originally by Donald Bailey at the Jackson Laboratory through a subline removed from his research colony in 1975 and put in the Jackson Laboratory production colony;[2] these became the BALB/cByJ subline, now known to have SCAD deficiency. In later studies based on PCR analysis of the BALB/cByJ-*fld* mutants,[6] it was determined that the *Acads* mutation occurred sometime between 1981 and 1982. This study also established that the mutation for fatty liver dystrophy *(fld)* was not the same as SCAD deficiency, although fatty liver is found in both. Having been separated since 1935,[7] BALB/cByJ are more distantly related to the other common BALB/cJ mouse strain also available from the production colony of the Jackson Laboratory. BALB/cJ mice have a very aggressive, hyperactive behavior as compared to the relatively reserved BALB/cByJ mice. We initially speculated that the BALB/cByJ mice were mentally retarded as a result of SCAD deficiency, but the BALB/cBy mice also have placid behavior. Therefore, we recommend that the best SCAD-normal control subline to use is the BALB/cBy mice, since they are virtually coisogenic.

3. CLINICAL AND PATHOLOGIC CHARACTERIZATION

In our initial paper[3] describing this mutant mouse model, we reported that they had undetectable SCAD activity, which agreed metabolically with the urinary organic acid results and the genetic marker results. In addition, upon fasting the blood glucose of these mice would drop to approximately half of normal control values and their serum glycine concentrations would also decrease to two thirds of normal.[3] We speculated that the glycine was being lost in the urine due to the abnormally high amounts of butyrylglycine being excreted. We also did carnitine loading experiments and found that these mice were not carnitine deficient, perhaps because they were excreting excess butyryl-CoA as butyrylglycine via glycine conjugation rather than as butyrylcarnitine.[3] We did, however, find excessive muscle butyrylcarnitine in the mutants and trace to no detectable amounts

of muscle butyrylcarnitine in the controls. The mutant mice develop severe fatty liver with fasting, as well as excessive fat in the liver without fasting as compared to normal controls. Immunoprecipitation studies later showed that there was no detectable SCAD antigen produced in multiple tissues.[8]

Clinically, these mutants have remained consistently normal.[3] We have challenged them with fasting,[3] medium-chain triglyceride loading,[3] as well as, sodium butyrate loading, sodium benzoate and salicylate[9] loading in an attempt to overload the glycine conjugation pathway. We have also fed them a high fat diet (40% fat) composed of butter fat with a relatively high short-chain fatty acid content. Even after consuming this diet for over a month, these mice showed no clinical signs of disease with fasting. Pathological characteristics include predominately fatty liver and kidney[3,9] ultrastructural studies demonstrated swollen, disorganized mitochondria in hepatocytes from fasted mutants with the appearance of a grade II change as described for children with swollen mitochondria associated with Reye Syndrome.[9] There was also depletion of hepatocyte glycogen stores.

4. MOLECULAR GENETICS OF MOUSE SCAD AND SCAD DEFICIENCY

4.1. Mouse SCAD cDNA Characteristics

We cloned and reported the complete sequence of the cDNA for mouse precursor SCAD.[10] The full length mouse SCAD cDNA is 1,792 base pairs; the mouse SCAD cDNA coding sequence covers 1,239 base pairs. The coding sequence contains a 24-amino acid leader peptide and a 388-amino acid mature peptide. Comparison of this sequence to reported rat and human SCAD cDNA sequences revealed a high degree of homology between the three species. Comparison of the amino acid sequence to that of the other acyl-CoA dehydrogenases, medium-chain acyl-CoA dehydrogenase (MCAD) and long-chain acyl-CoA dehydrogenase (LCAD), also showed a high degree of similarity.

4.2. Mouse SCAD Genomic Characteristics

To further understand the similarities between the members of this gene family, to characterize how this gene is regulated, and to determine if there is coordinate regulation between these related genes, we isolated genomic clones containing the mouse *Acads* gene.[11] We showed that *Acads* is a compact, single-copy gene approximately 5,000bp in size. We sequenced the entire coding portion of the gene, all of the intron/exon junctions, and an 850bp segment upstream of the translation start site. We determined that the gene consists of 10 exons ranging in size from 56bp to 702bp, and 9 introns ranging in size from 80bp to approximately 700bp. The 5′ region of the mouse *Acads* gene lacks a TATA box or a CAAT box, is GC rich, and also lacks any similarity to the related gene, medium-chain acyl-CoA dehydrogenase. This was the first report of the gene structure and 5′ regulatory sequence of the short-chain acyl-CoA dehydrogenase gene in any species.

4.3. Mutation Characterization

We also demonstrated the molecular basis of this defect by DNA and RNA analyses, comparing these mice with the wild-type predecessor strain BALB/cBy,[12] see

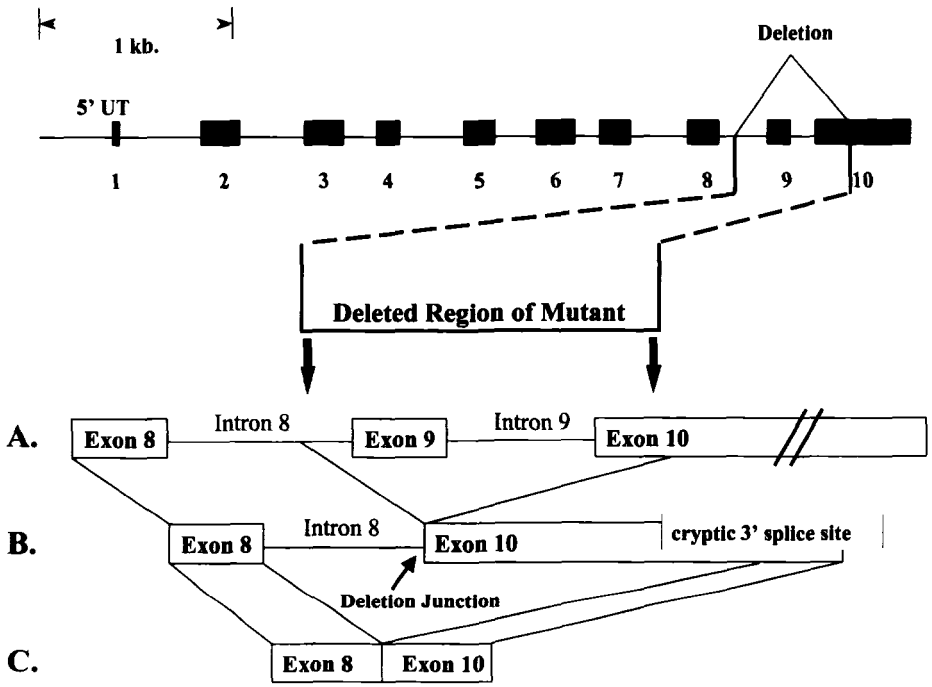

Figure 1. Molecular structure of the mouse short-chain acyl-CoA dehydrogenase gene including the deletion mutation found in BALB/cByJ mice. Adapted and redrawn from previous publications.[11,12] A. The fine genomic structure of the deletion mutation. b. Molecular structure of the approximately normal size *Acads* mRNA from the mutant allele. This consists of an RNA copy of the deleted gene sequence with no splicing. c. Molecular structure of the short *Acads* mRNA from the mutant allele. This consists of a misspliced mRNA from exon 8 to a cryptic splice site in exon 10.

Figure 1. The mutant strain has a 278 bp deletion in the 3′ end of the structural gene for SCAD, and reduced steady state levels of SCAD mRNA. The deletion begins in intron 8 and extends into exon 10. Two major transcripts are produced from this allele in the mutant. One contains intronic sequence due to the absence of splicing, and the second transcript results from missplicing of a normal splice donor site to a cryptic splice acceptor site in the 3′ terminal exon. Both abnormal transcripts have aberrant stop codons, thus confirming the molecular basis of SCAD deficiency in this unique mouse model. Based on the cloning and molecular characterization of this mutant allele,[12] we also devised a polymerase chain reaction (PCR) assay[13] to distinguish the *Acads*^{o} null allele from the other two functional alleles, *Acads*$^{a \text{ or } b}$. This assay is technically much simpler than the allozyme electrophoresis assay, and it provides for a rapid method of testing BALB/c stocks for genetic contamination by the BALB/cByJ subline. BALB/cByJ mice are otherwise genetically indistinguishable from the commonly used, but behaviorally and biologically different BALB/cJ mice. In a recent study, Cordydon and colleagues reported the genomic characteristics of human SCAD gene *(ACADS).*[14] The human *ACADS,* like the mouse *Acads,* is composed of 10 exons, but is 13kb in size, making it more than twice the size of the mouse *Acads* gene. This same group then characterized several variants in the human *ACADS* gene frequently associated with varying degrees of SCAD deficiency and of ethylmalonic aciduria in particular.[15] Earlier descriptions of human

ACADS mutations were also point mutations, but were very rare and associated with severe neonatal forms of disease,[16] in contrast with the mouse SCAD gene deletion mutation with misspliced SCAD mRNA.[12] Missplicing, however, may be an important mechanism to study further in the mouse model, since this has been a common finding in human patients with medium-chain acyl-CoA dehydrogenase deficiency (MCAD).[17,18]

5. STUDIES TO INVESTIGATE MECHANISMS OF DISEASE

In human patients with SCAD or MCAD deficiency the common features include hypoglycemia, hyperammonemia, metabolic acidosis, organic acidemia and a fatty change of liver.[4,15,16,17] As stated previously there are no overt clinical signs reported in these mice; however, they do show several biochemical and pathological features. We have found that upon fasting SCAD deficient mice under 8 weeks of age that the blood glucose will drop in half as compared to normal controls.[3] In our experience, normal mouse blood glucose concentrations run approximately 150-200 mg/dl and the fasted mutants will drop to 70–90 mg/dl; this is apparently not low enough induce any clinical signs of hypoglycemia. Yamanaka and colleagues have confirmed the hypoglycemia in the mutant mice,[19] and studies using perfused liver from these mice also showed glucose production was lower in the mutants, as well as a failure to increase ketone body production using either butyrate, octanoate, or oleate as substrates.[19] Surprisingly, liver acetyl-CoA was not deficient even in the fasted SCAD deficient mutants.

6. EFFECTS OF SCAD DEFICIENCY ON OTHER METABOLIC PATHWAYS

As mentioned, human patients with acyl-CoA dehydrogenase deficiencies share the disease features of hypoglycemia, hyperammonemia, tissue fatty change, hypoketonemia, carnitine deficiency and organic acidemia due to apparent disruption of normal fatty acid, glucose and urea metabolism. Most of the acute clinical episodes occur in young children. These episodes are precipitated by fasting and are often fatal with the *in vivo* mechanisms essentially unknown. Since the genes of the rate controlling enzymes of these pathways are tissue and developmentally regulated at the transcriptional level, we measured, throughout neonatal development, the steady-state mRNA levels of long-chain (LCAD), medium-chain (MCAD), short-chain (SCAD) acyl-CoA dehydrogenases, pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), carbamyl phosphate synthetase I (CPS), ornithine transcarbamylase (OTC), and argininosuccinate synthetase (AS) in fed or fasted-SCAD deficient BALB/cByJ mice, as compared to BALB/cBy normal controls.[20] Overall our results showed no major effects on steady state mRNA expression of acyl-CoA dehydrogenases due to SCAD deficiency, regardless of age or fasting. In SCAD deficient mice, we found depressed mRNA expression and enzyme activity for the urea cycle enzymes CPS and AS at 6 days old, and found no apparent effects on expression of gluconeogenesis enzymes PC or PEPCK. In the mouse there was a period of overall lower gene expression for most of these metabolic genes at 6 and 15 days of age which appears to parallel the developmental period when human children with these diseases are most severely affected.

Qureshi and colleagues have investigated several aspects of SCAD deficiency using the BALB/cByJ mouse model. SCAD deficient mice were shown by these investigators to

have significantly lower concentrations of acetyl-CoA in both liver and cerebrum, while cerebrum had significantly higher concentrations of lactate, as compared to BALB/cJ normal controls.[21] Fasting aggravated these abnormalities. Fasted SCAD deficient mutants also had signficantly reduced concentrations of liver free carnitine, as well as brain long-chain acyl-carnitines.[21] L-carnitine treatment was associated with increased cerebral CoA-SH concentrations, as well as both hepatic and cerebral acetyl-CoA concentrations.[21]

They also investigated the effects of combining SCAD and ornithine transcarbamylase (OTC) deficiency[22] in the same mouse. Double mutants were produced by crossing the SCAD deficient mice with sparse fur *(spf)* mice that have X-linked OTC deficiency. By combining these mutations, the double mutant offspring (male or female) often did not survive to breeding age. These investigators postulate that this double mutant mouse may serve as a model to study ammonia: fatty acyl-CoA synergism in secondary hyperammonemic syndromes.

In other studies by this group, riboflavin deficiency was induced in the SCAD deficient BALB/cByJ mice by feeding them a riboflavin deficient diet for three weeks.[23] Since SCAD and the other acyl-CoA dehydrogenases are riboflavin dependent enzymes requiring FAD, they hypothesized that inducing a multiple acyl-CoA dehydrogenase deficiency via FAD depletion would potentiate the SCAD deficiency. Interestingly, they found that SCAD deficiency combined with riboflavin deficiency enhanced the excretion of butyrylglycine, ethylmalonic, methylsuccinic acids, and other dicarboxylic acids. Furthermore, they reported increased hepatic ammonia levels, but not increased cerebral ammonia or glutamine concentrations. Acetyl-carnitine treatment restored most if not all of these measures to normal.

7. TRANSGENIC CORRECTION STUDIES *IN VIVO*: THE POTENTIAL FOR GENE THERAPY

We have explored the potential therapeutic effects of liver specific expression of a short-chain acyl-CoA dehydrogenase (SCAD) transgene in the SCAD deficient mouse model.[24] Transgenic mice were produced with a rat albumin promoter/enhancer controlling a mouse SCAD minigene (ALB-SCAD) on both the SCAD normal genetic background (C57BL/6J x SJL/J-F_1) and an SCAD deficient background. In three transgenic lines produced on the SCAD deficient background, recombinant SCAD activity and antigen in liver mitochondria increased up to 7-fold of normal control values. All three lines showed a markedly reduced organic aciduria and fatty liver, which are sensitive indicators of the metabolic abnormality seen in this disease found in children. We found no detrimental effects of high liver SCAD expression in transgenic mice on either background. These studies provide important basic and practical therapeutic information for the potential gene therapy of nuclear-encoded mitochondrial enzyme deficiencies, as well as insights into the mechanisms of this specific disease.

8. ROLE OF FATTY ACID OXIDATION IN OTHER DISEASES

We and others have recently become interested in the roles aberrant fatty acid metabolism may play in the pathogenesis of more common dyslipidemic syndromes such as diabetes, obesity and development of cardiovascular disease. Recent studies reported by Park and coworkers,[25] attempted to identify different genetic backgrounds in mice that

influence the development of these more chronic disease processes. They found that the BALB/cByJ mice became significantly heavier and had significantly higher total serum cholesterol, HDL cholesterol, and triglyceride concentrations in the fed state than C57BL/6J mice. These authors did not mention the fact that these mice have SCAD deficiency. As more is learned by genetically manipulating lipid metabolism in mouse models, we can more fully understand the roles aberrant fatty acid metabolism plays in these complex, chronic disease processes.

9. SUMMARY

The SCAD deficient mouse model has been useful to investigate mechanisms of deficient fatty acid oxidation disease in human patients. This mouse model has been thoroughly characterized and is readily available from the Jackson Laboratory. Using the new technologies of gene-knockout mouse modeling, we envisage developing additional members of the acyl-CoA dehydrogenase family of enzyme deficiencies in mice and furthering our understanding of fatty acid metabolism in health and disease.[26]

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REFERENCES

1. Seeley, T.-L. & Holmes, R.S. (1981) *Biochem. Genet.,* **19**, 333–345 Genetics and ontogeny of butyryl CoA dehydrogenase in the mouse and linkage of *Bcd-1* with *Dao-1*.
2. Prochazka, M. & Leiter, E.H. (1986) *Mouse News Lett.,* **78**, 31. A null activity variant found at the butyryl CoA dehydrogenase *(Bcd-*1*)* locus in BALB/cByJ mice.
3. Wood, P.A., Amendt, B.A., Rhead, W.J., Millington, D.S., Inoue, F. & Armstrong, D. (1989) *Pediatr. Res.,* **25**, 38–43. Short-chain acyl-coenzyme A dehydrogenase deficiency in mice.
4. Amendt, B.A., Greene, C, Sweetman, L., Cloherty, J., Shih, V., Moon, A., Teel, L. & Rhead, W.J. (1987) *J. Clin. Invest.,* **79**, 1303–1309. Short-chain acyl-coenzyme A dehydrogenase deficiency.
5. Schiffer, S.P., Prochazka, M., Jezyk, P.F., Roderick, T.H., Yudkoff, M. & Patterson, D.F. (1989) *Biochem. Genet.,* **27**, 47–58. Organic aciduria and butyryl CoA dehydrogenase deficiency in BALB/cByJ mice.
6. Reue, K. & Cohen, R.D. (1996) *Mammalian Genome,* 7, 694–695. *Acads* gene deletion in BALB/cByJ mouse strain occurred after 1981 and is not present in BALB/cByJ-*fl*d mutant mice.
7. Les, E.P. (Fall 1990) *JAX Notes* **No.443**, 2–4. A brief history of the two substrains of BALB/c, BALB/cJ and BALB/cByJ available from the Jackson Laboratory.
8. Amendt, B.A., Freneaux, E., Reece, C., Wood, P.A. & Rhead, W.J. (1992) *Pediatr. Res.,* **31**, 552–556. Short-chain acyl-coenzyme A dehydrogenase activity, antigen and biosynthesis are absent in the BALB/cByJ mouse.
9. Armstrong, D.L., Masiowski, M.L. & Wood, P.A. (1993) *Am. J. Med. Genet,* **47**, 884–892. Pathologic characterization of short-chain acyl-CoA dehydrogenase deficiency in BALB/cByJ mice.
10. Kelly, C.L., Hinsdale, M.E. & Wood, P.A. (1993) *Genomics* **18**, 137–140. Cloning and characterization of mouse short-chain acyl-CoA dehydrogenase cDNA.
11. Kelly, C.L. & Wood, P.A. (1996) *Mammalian Genome,* **7**, 262–264. Cloning and characterization of the mouse short-chain acyl-CoA dehydrogenase gene.
12. Hinsdale, M.E., Kelly, C.L. & Wood, P.A. (1993) *Genomics* **16**, 605–611. Null allele at bcd-1 locus in BALB/cByJ mice is due to a deletion in the short-chain acyl-CoA dehydrogenase gene and results in missplicing of mRNA.

13. Wood, P.A., Hinsdale, M.E. & Kelly, C.L. (1993) Mouse *Genome* **91**, 342–344. Molecular detection of the *Bcd-1* null allele in BALB/cByJ mice by polymerase chain reaction: A simple assay for genetic monitoring.
14. Corydon, M.J., Andresen, B.S., Bross, P., Kjeldsen, M., Andreasen, P.H., Eiberg, H., Kolvraa, S. & Gregerson, N. (1997) *Mammalian Genome* **8**, 922–926. Structural organization of the human short-chain acyl-CoA dehydrogenase gene.
15. Gregersen, N., Winter, VS., Corydon, M.J., Corydon, T.J., Rinaldo, P., Ribes, A., Martinez, G, Bennett, M.J., Vianeysaban, C, Bhala, A., Hale, D.E., Lehnert, W, Kmoch, S., Roig, M., Riudor, E., Eiberg, H., Andresen, B.S., Bross, P., Bolund, L.A. & Kolvraa, S. (1998) *Hum. Mol. Genet.,* **7**, 619–627. Identification of four new mutations in the short-chain acyl-CoA dehydrogenase (SCAD) gene in two patients-One of the variant alleles 511C- > T is present at an unexpectedly high frequency in the general population, as was the case for 625G- > A, together conferring susceptibility to ethylmalonic aciduria.
16. Naito, E., Indo, Y. & Tanaka, K. (1990) *J. Clin. Invest.,* **85**, 1575–1582. Identification of two variant short chain acyl-coenzyme A dehydrogenase alleles, each containing a different point mutation in a patient with short chain acyl-coenzyme A dehydrogenase deficiency.
17. Kelly, D.P., Whelan, A.J., Ogden, M.L., Alpers, R., Zhang, Z., Bellus, G, Gregersen, N., Dorland, L. & Strauss, A.W. (1990) *Proc. Natl. Acad. Sci. USA,* **87**, 9236–9240. Molecular characterization of inherited medium-chain acyl-CoA dehydrogenase deficiency.
18. Zhang, Z.F., Kelly, D.P., Kim, J.J., Zhou, Y.Q., Ogden, M.L., Whelan, A.J. & Strauss, A.W. (1992) *Biochemistry,* **31**, 81–89. Structural organization and regulatory regions of the human medium-chain acyl-CoA dehydrogenase gene.
19. Yamanaka, H., Ueshima, Y., Nakajima, T., Yoshida, N., Inoue, F, Kodo, N., Kinugasa, A. & Sawada, T. (1992) *J. Inherited Metab. Dis.,* **15**, 353–355. Gluconeogenesis and ketogenesis in perfused livers from short-chain acyl-CoA dehydrogenase-deficient mice.
20. Hinsdale, M.E., Hamm, D.A. & Wood, PA. (1996) *Biochem. Mol. Med.,* **57**, 106–115. Effects of short-chain acyl-CoA dehydrogenase deficiency on developmental expression of metabolic enzyme genes in the mouse.
21. Qureshi, I.A., Ratnakumari, L., Michalak, A., Giguere, R., Cyr, D. & Butterworth, R.F. (1993) *Biochem. Med. Metab. Biol.,* **50**, 145–158. A profile of cerebral and hepatic carnitine, ammonia, and energy metabolism in a model of organic aciduria:BALB/cByJ mouse with short-chain acyl-CoA dehydrogenase deficiency.
22. Qureshi, I.A., LeBlanc, D., Cyr, D., Giguere, R. & Mitchell, G. (1993) *Biochem. Biophys. Res. Commun.* **191**, 744–749. Breeding experiments to combine the X-linked sparse-fur (spf) mutation with the autosomal recessive BALB/cByJ strain: Testing the biochemical phenotype of double-mutant mice as a model for ammonia:fatty acyl CoA synergism.
23. Rao, K.V.R. & Qureshi, I.A. (1997) *Can. J. Physiol. Pharmacol.,* **75**, 423–4360. Decompensation of hepatic and cerebral acyl-CoA metabolism in BALB/cByJ mice by chronic riboflavin deficiency: restoration by acetyl-L-carnitine.
24. Kelly, C.L., Rhead, W.J., Kutschke, W.K., Brix, A.E., Hamm, D.A., Pinkert, C.A., Lindsey, J.R. & Wood, PA. (1997) *Hum. Mol. Genet.,* **6**, 1451–1455. Functional correction of short-chain acyl-CoA dehydrogenase deficiency in transgenic mice: Implications for gene therapy of human mitochondrial enzyme deficiencies.
25. Park, E.I., Paisley, E.A., Mangian, H.J., Swartz, D.A., Wu, M., O'Morchoe, P.J., Behr, S.R., Visek, W.J. & Kaput, J. (1997) *J. Nutrition,* **127**, 566–573. Lipid level and type alter stearoyl CoA desaturase mRNA abundance differently in mice with distinct susceptibilities to diet-influenced diseases.
26. Wood, P.A., Farmer, S.C., Tolwani, R.J., Warren, J.R., Steinkampf, M.P., Johnson, L.W., Mountz, J.D. & Kelly, D.P. (1992) *"in" New developments in fatty acid oxidation,* (Coates, P.M. & Tanaka, K. eds.) Wiley-Liss, New York, pp. 151–160. Molecular studies of mouse medium and long-chain acyl-CoA dehydrogenase genes for-site-directed mutagenesis of embryonic stem cells.

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