METHANOGENESIS

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METHANOGENESIS

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EDITED BY

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Preface

The study of methane-producing microbes has witnessed unprecedented growth in its relatively short history. The stimulus for this interest originates from the broad impact methanogens have on the wider world of biological inquiry including the environment, early evolution, biochemistry, and molecular biology.

Methanogens are found in nearly every conceivable anaerobic environment—from the rumen and the intestines of humans to hot springs and the deep ocean floor, from sewage digestors and landfills to aquatic sediments. Methanogens grow at temperatures from 5 to 110°C and salinities ranging from freshwater to brine, a finding which has helped to redefine the environmental limits for life. Methanogens function in the global cycling of carbon; each year approximately 400 metric tons of methane are produced as an end product in the degradation of organic matter entering diverse anaerobic habitats. The methane is oxidized to CO_2 in aerobic zones, but an undetermined amount escapes into the upper atmosphere. Recent increases in the ruminant population and human activities have led to an increase in atmospheric methane, a major greenhouse gas.

Early studies on methane-producing microbes gave the first clue that led to the recent proposal of a new higher taxon for all life, which includes the Archaea, Bacteria, and Eucarya domains. The methanogens are the largest, most diverse, and best-studied group in the Archaea. The wealth of information on cell wall components, ultrastructure, and phylogenetics of methanogens has made a key contribution toward understanding the early origins and evolution of the Archaea.

The study of methanogens has also contributed to a broader understanding of the biochemical world. A plethora of novel cofactors has emerged from investigations on the pathway of methanogenesis, and new metalloenzymes have been discovered. An understanding of the bioenergetics is just beginning to unfold

but already has revealed uncommon mechanisms for the generation of chemical gradients. Biochemical studies clearly indicate that the transcriptional and translational apparatus of the methanogens resembles that of the Eucarya more than the Bacteria.

Although the field of methanogenesis has progressed from methods for the growth of these fastidious anaerobes to elegant studies of the novel biochemistry and into molecular biology, major interesting questions remain unanswered; for example, the mechanism of energy conservation and principles of gene expression. Today the field is at a threshold. The accomplishments of the past are pushing on the door to genetics. The development of a reliable genetic exchange system will soon catalyze another rapid expansion of research that will surely impact all aspects of methanogens. Events of the past predict that this new era will reveal still more processes that are unique to these microbes and afford new perspectives to the broader fields of biochemistry and molecular biology.

This is the first book exclusively on the subject of methanogenesis. It is intended to provide the reader with a comprehensive view of the field, past and present, and indicate future directions. For the experienced, it is anticipated that the contents will provide fresh insights and, for those new to the field, will stimulate interest and research in new directions. The chapters that follow reveal the unusual and diverse ecology, microbiology, physiology, biochemistry, and molecular biology which characterizes these microbes.

Finally, many factors have contributed to the success of this field not the least of which is a collegial scientifc community. I wish to thank the authors for their cooperation and advice.

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An Historical Overview of Methanogenesis

Ralph S. Wolfe

Discovery of "Combustible Air" from Sediments

Since ancient times combustible gas has been known to seep from geological fissures in certain areas of the world. However, the experiments of Alessandro Volta with combustible air obtained from sediments and marshy places created widespread interest and laid the scientific foundation for study of the biological formation of methane (Paoloni, 1976). Results of his experiments are recorded in a series of letters to his friend Father Carlo Campi. The experiments had their beginning when Father Campi observed bubbles rising near a spring and, finding that they were capable of catching fire, communicated this observation to his friend, Volta. Together they planned to return to the spring and conduct experiments, but this plan was abandoned when Volta became ill. Later, with the ideas of their proposed experiments in his mind, Volta explored the nature of bubbles from sediment in the shallow area of a lake. Volta wrote letter No. 1 to Father Campi which read, in part, as follows:

> So, on the 3rd of this month (Nov. 1776), with my head full of such ideas, and being in a little boat on Lake Maggiore, and passing close to an area covered with reeds, I started to poke and stir the bottom with my cane. So much air emerged that I decided to collect a quantity in a large glass container.
> . . .
> Now for some details. This air burns with a beautiful blue flame. To make it burn and to produce the flame, the mouth of the vessel must be wide. If it is too narrow, when one puts a little lighted candle close to it, one hears many slight explosions in rapid succession, so slight that they can barely be

Figure 1. For the publication of Volta's letters an artist presented salient features of Volta's initial experiments, the collection of bubbles released from sediment in a large inverted glass container, and, in the upper right, the experiment where the gas extinguishes a lighted candle when the candle is lowered into the vessel. (Although it is easy to repeat Volta's experiment today, it would represent a real challenge to repeat the experiment as depicted by the artist, i.e., to stand up in a small boat, hold a large inverted glass jug with one hand and stir the sediment with a pole held in the other hand—a sure formula for disaster.)

> heard. I usually use, for simple experiments, a little container made of glass, cylindrical in shape, three or four thumbs long, and the same width except at the mouth which is approximately ½ thumb wide. When a candle is put close to the mouth, it is pretty to see how it gets covered with a small blue flame, which descends slowly along the walls of the vessel, almost as if licking it, until it reaches the bottom. But the show is more beautiful and more curious if we put a bit of lighted candle inside the container, using a bent wire; then the blue flames enlarge and increase in vigor. If the candle is lowered too far, it goes out, while at the mouth the air continues to burn. Then if the candle is moved away from the bottom, it will light again as soon as it touches the flame at the rim.
>
> Isn't this the same thing that happens to the alcohol of wine? A torch immersed in such a fluid goes out, but when it approaches the surface it ignites and burns brightly. What better proof can there be that this flammable air, the same as any combustible substance, cannot burn unless it comes into contact with the ordinary air of the atmosphere? . . .

From the second letter 21 Nov. 1776 at Como:

> After having tested the soil that sleeps, as to say, under the water, it occurred to me to examine the soil near the water but not wet. For this, I chose a marshy soil left almost dry by the subsiding of our Lake, and got ready to make the test in two ways. The first was to dig a few little holes in the mud (others were already prepared by deep animal tracks) and when they had filled with water, I stirred the bottom as usual with my cane, and let the air escape. I carefully collected some of it, and it did not fail the test; it caught fire.
>
> The other test which gave me a more beautiful and charming sight was to push my cane in to the depth of about a foot in a place where the soil was softer and blacker, and covered with rotted grass, then pull it out all of a sudden and at once push a small lighted candle right next to the opening. It was just beautiful to see a blue flame appear at once and part of it fling itself upward while part of it went deep into the hole and touched the bottom.
>
> Then I hurriedly dug several more holes close together, and I could not get enough of watching the flame run from one to another, setting one and then another ablaze, then all of them burning and shining together. But if I pressed the ground with one foot, or trod upon it so that more air rushed out, only some of the holes would burn. . . .
>
> No, sir, no air is more combustible than the air from marshy soil. In the first place, we can deduce this from the extraordinary number of small explosions we can get from it. But a surer indication is that it transmits the property of flammability to the ordinary air with which it comes into contact, and in this respect it far surpasses other combustible air. The strongest of all these, obtained by dissolving iron filings in vitriolic acid, makes the loudest explosions when combined with a volume of ordinary air twice its own. The air of swamps, on the other hand, ignites and explodes most loudly, if to one part of it, we add 8 or 10 parts of ordinary air. If to one part of it, we add only 5 or 6 parts of ordinary air, it does not explode with its maximum flash and roar, but keeps flashing with a succession of small flames; finally, if we increase the proportion to twelve to one, the swamp air sets afire the whole mass.
>
> Now we can understand why this swamp air burns so lazily in containers and why it is imperative for their mouths to be wide. *No,* it is not *lack* of flammability; it is rather an indication of excess of flammability; since in order to burn more brightly, it must be diluted with ordinary air.

Another recorded observation of combustible gas from sediments was made on Nov. 5, 1783 by two unlikely persons, Thomas Paine and George Washington who were ignorant of Volta's discovery. After the Revolutionary War, Washington established his headquarters at Rockingham in New Jersey. He was trying, without apparent success, to get Congress to vote a pension for Paine whose writings had played an important role in the revolutionary cause. To make Paine more visible he invited him to his headquarters for a visit. Paine was interested

in the nearby sluggish Millstone River. In his book, *The Cause of the Yellow Fever,* Paine describes the experiments (Menzies, 1969):

> We had several times been told that the river or creek, that runs near the bottom of Rocky Hill, and over which there is a mill, might be set on fire, for that was the term the country people used; and as General Washington had a mind to try the experiment, General Lincoln, who was also there, undertook to make preparation for it against the next evening, November 5th. This was to be done, as we were told, by disturbing the mud at the bottom of the river, and holding something in a blaze, as paper or straw, a little above the surface of the water.
>
> Colonels Humphreys and Cobb were at that time Aids-de-Camp of General Washington, and those two gentlemen and myself got into an argument respecting the cause. Their opinion was that, on disturbing the bottom of the river, some bituminous matter arose to the surface, which took fire when the light was put to it; I on the contrary, supposed that a quantity of inflammable (flammable) air was let loose, which ascended through the water, and took fire above the surface. Each party held to his opinion, and the next evening the experiment was to be made.
>
> A scow had been stationed in the mill dam, and George Washington, General Lincoln, and myself and I believe Colonel Cobb (for Humphries was sick) and three or four soldiers with poles, were put on board the scow. General Washington placed himself at one end of the scow, and I at the other; each of us had a roll of cartridge paper, which we lighted and held over the water, about two or three inches from the surface, when the soldiers began disturbing the bottom of the river with the poles.
>
> As General Washington sat at one end of the scow, and I at the other, I could see better anything that might happen from his light, than I could from my own, over which I was nearly perpendicular. When the mud at the bottom was disturbed by the poles, the air bubbles rose fast, and I saw the fire take from General Washington's light and descend from thence to the surface of the water, in a similar manner as when a lighted candle is held so as to touch the smoke of a candle just as it is blown out, the smoke will take fire, and the fire will descend and light the candle. This was demonstrative evidence that what was called setting the river on fire was setting on fire the inflammable air that arose out of the mud.

Natural scientists of the day were intrigued by Volta's discovery. His letters describing the discovery of and experiments with combustible air were published in French and German. He corresponded with eminent scientists in several countries. By 1787, Lavoisier and others had obtained evidence that Volta's flammable air was "gas hidrogenium carbonatrum." The terms "carbonated hydrogen" and "carburetted hydrogen" were used in English. A detailed chronology of the many publications and authors is conveniently provided by Paoloni, pp. 258–262

(1976). By 1865 the term "methan" (methane) was proposed, and in 1892 this nomenclature was confirmed by an International Congress on Chemical Nomenclature.

Microbial Basis of Methanogenesis

Although Volta had noted the relationship of methane formation to water-saturated decaying plant material, and in the following years this relationship had been adequately confirmed by others, nearly a century elapsed before firm evidence was obtained that methane formation in such habitats was a microbial process. H. A. Barker summarized this era in his book, *Bacterial Fermentations* (Barker, 1956) which should be consulted for the specific references:

> The first definite indication that methane is formed by a microbiological process was obtained in 1868 by Béchamp, a student of Pasteur. Béchamp had previously studied the decomposition of sugar and starch that occurred when these substances were added to a simple inorganic medium containing chalk and incubated in the absence of oxygen. He ascribed the resulting fermentation to a living "ferment" which he thought was originally present in the chalk and therefore called *Microzyma cretae*. The description of this organism was remarkably vague; it was reported to be so small that even when examined under the highest magnification of the microscope it appeared only as a motile point. For some reason, now not altogether clear, Béchamp decided to find out whether the chalk and its microbe could also cause an anaerobic decomposition of ethyl alcohol. He was rewarded after some weeks by seeing the development of a vigorous fermentation which produced a large amount of methane, a little carbon dioxide, and a mixture of various fatty acids. Béchamp reported that when the gas evolution stopped the only "ferment" to be seen was *M. cretae!* Despite the naive interpretation of the microscopic observations, Béchamp's experiment clearly showed that methane can be formed from ethyl alcohol and calcium carbonate by a process probably caused by microorganisms. This was the first demonstration of biological methane formation from simple carbon compounds. Unfortunately, Béchamp's work was overlooked by most subsequent investigators.
>
> More adequate proof of the microbiological origin of methane was provided by Tappeiner in 1882. He set up three identical anaerobic cultures provided with plant materials as substrate and with considerable amounts of the intestinal contents of ruminants as an inoculum and a possible source of soluble catalysts. One of the three cultures was treated with an antiseptic to inhibit bacteria without inactivating soluble "ferments," the second was boiled to destroy both bacteria and "ferment," and the third was left untreated. The microbial nature of the fermentation was deduced from the observation that only the untreated culture produced methane.

During the last quarter of the 19th century, interest in the methane fermentation centered largely on the utilization of cellulose as a substrate. Since cellulose is the most abundant constituent of plants, it was reasonable to suppose that it must be a major source of methane in decomposing plant materials. Furthermore, methane had been shown to be formed in large amounts in the digestive tracts of herbivorous animals; therefore the possible role of methane-producing bacteria in cellulose digestion was of some concern to animal physiologists.

The experiments of Popoff, Tappeiner, and Hoppe-Seyler on the utilization of cellulose by crude enrichment cultures of bacteria obtained from soil or the digestive tracts of herbivorous animals demonstrated that cellulose is in fact decomposed under anaerobic conditions, frequently with the formation of methane and other products including carbon dioxide, hydrogen, and acetic and butyric acids. At first this was taken to mean that the bacteria which attacked cellulose also form methane. However, it was soon realized that another interpretation is possible, namely, that the methane is formed not by the cellulose-decomposing bacteria but by the action of other associated microorganisms on one or more of the products of the cellulose fermentation. This interpretation was supported by two lines of evidence. First, certain cellulose-fermenting cultures were found to produce carbon dioxide and hydrogen but no methane. This proved that cellulose fermentation is not necessarily associated with methane formation. Second, Hoppe-Seyler 1986, Omelianski 1906, and particularly Söhngen 1906 demonstrated that the products of cellulose fermentation, such as formate, acetate, butyrate, ethanol, and even hydrogen and carbon dioxide, can be readily used as substrates by methane-producing bacteria.

Although it appeared that methane formation from cellulose was likely a two-stage process, Omelianski presented evidence which he believed supported the existence of a sporeforming rod which converted cellulose to methane (Barker, 1956). The technology of the time was inadequate for resolution of the controversy, and no evidence to support Omelianski's contention has been obtained. The work of Söhngen reported in his thesis (Barker, 1956) and Söhngen (1910) was remarkable for its insight on the physiology of methanogenesis. He concluded from studies with enrichment cultures that 4 moles of H_2 were required to reduce 1 mole of CO_2 to CH_4 ($4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$). These experiments later provided a basis for the "carbon dioxide reduction theory" proposed by van Niel in 1934 (Barker, 1956). Since no pure cultures of methanogens were available, a definitive test of the hypothesis could not be performed. Söhngen also tested different substrates in his enrichment cultures with the curious result that the final products, CH_4 and CO_2, were the same whether acetone, ethanol, acetate, propionate or butyrate was the substrate. (It would be nearly 60 years before the role of acetogenic obligate proton-reducers in this process would be defined.)

Beginning of the Modern Era

Stephenson and Stickland (1933) reported the isolation of an organism that could oxidize hydrogen and reduce one-carbon compounds (carbon dioxide, carbon monoxide, formate, formaldehyde or methyl alcohol) to methane; however, it is difficult with present knowledge of the methanogens to assess the nature of their isolate. It is significant, however, that the culture did not yield methane from compounds of two or more carbon atoms. They observed that formate was converted to carbon dioxide and hydrogen at a rate more rapid than its conversion to methane, suggesting that formate was not an intermediate in the reduction of carbon dioxide to methane. In 1936, a year that marks the beginning of the modern era for study of methanogenesis, H. A. Barker first reported his pioneering experiments and methanogens (1936a,b). For the next 30 years his laboratory would play the leading role in providing a scientific basis for the physiology of methanogenesis. As a young man he had gone to Delft for postdoctoral study with A. J. Kluyver, a postdoctoral experience that yielded two important contributions to anaerobic microbial physiology. His enrichments for methanogens anaerobically produced methane in an ethanol-carbonate medium. From the medium which contained ethanol and calcium carbonate and which was inoculated with Delft canal sediment, he obtained *Clostridium kluyveri* as well as the culture known as *Methanobacillus omelianskii*. Barker used an adaptation of the agar shake method in which the cotton plug above the agar-deep was cut in half, and the lower sterile half was then pushed down into the tube. The upper half was pushed part way down the tube so as not to contact the sterile plug, pyrogallol and sodium carbonate being added to this plug before a solid rubber stopper was inserted to seal the tube. Alkaline pyrogallol absorbed oxygen in the head space as sodium sulfide in the agar medium created a reducing potential. By use of selective substrates growth of non-methanogens was kept to a minimum. Although growth of methanogens was slow, requiring several weeks to produce colonies large enough to pick, this procedure represented a major advance, a pivotal event in the study of methanogenesis. A standard aseptic technique could now be employed for obtaining colonies of methanogens (Barker, 1940).

Although *M. omelianskii* was believed at the time to represent the first pure culture of a methanogen, it now appears that Schnellen in Kluyver's laboratory documented in his thesis the first pure cultures, these being *Methanobacterium formicicum* and *Methanosarcina barkeri* (Schnellen, 1947). (In the late 1970s lyophilized cultures of these organisms prepared by Schnellen were discovered in Delft. Unfortunately, they were found to be nonviable, when tested in the laboratory of G. D. Vogel.) With Ruben and Kamen, Barker performed a pivotal experiment in 1940 which showed that $^{14}CO_2$ gave rise to $^{14}CH_4$ and that ^{14}C also was incorporated into cell carbon of *M. omelianskii* (Barker et al., 1940). Barker

then showed that the oxidation of ethanol to acetate by this organism was completely dependent on the presence of carbon dioxide; other terminal electron acceptors did not replace carbon dioxide (Barker, 1941), and he documented the relationship of carbon dioxide concentration to the rate of its reduction to methane with molecular hydrogen (Barker, 1943). Results of these experiments strongly supported van Niel's theory of carbon dioxide reduction to methane.

In 1947 Schnellen had also reported his studies on the conversion of methanol to methane by *Methanosarcina,* 4 moles of methanol being converted to 3 moles of methane and 1 mole of carbon dioxide. To achieve this stoichiometry it was believed at the time that all of the methanol was oxidized to carbon dioxide with the reduction of 3 moles of carbon dioxide to methane. Similarly the conversion of 1 mole of acetate to 1 mole of methane and 1 of carbon dioxide, shown by Söhngen (1910), was believed to involve first the complete oxidation of acetate to 2 moles of carbon dioxide and 8 reducing equivalents, 1 mole of carbon dioxide subsequently being reduced to methane by the reducing equivalents. However, Buswell and Sollo (1948) obtained an unusual result that altered the concept of methanogenesis from acetate. Working with acetate-fermenting crude cultures from sewage sludge to which they added ^{14}C-labeled carbon dioxide, only traces of label were found in methane, and the methyl group of acetate substituted for carbon dioxide with a unique labeling pattern. This was a damaging blow to the prevalent concept that carbon dioxide reduction was universally used by methanogens to produce methane. As the 1940s drew to a close, Thressa Stadtman and Barker, repeated, confirmed, and extended the observations of Buswell and Sollo (Stadtman and Barker, 1949). By addition of [^{14}C]-acetate to acetate-fermenting enrichment cultures which were mostly cocci, *Methanosarcina* not being observed, they found that methane was derived mainly from the methyl group of acetate and carbon dioxide from the carboxyl group. In addition they showed conclusively with the culture of *M. omelianskii* that the specific activity of the [^{14}C]-methane formed was equivalent to the specific activity of the [^{14}C]-carbon dioxide added; the carbons of ethanol or acetate were not precursors of methane. So it was now clear: there were *two routes* for the formation of methane, one by reduction of carbon dioxide and one by reduction of a methyl group. They postulated that each route led to a common precursor of methane.

Kluyver and Schnellen (1947) reported an extension of the studies of Stephenson and Stickland (1933) and of Barker (1943). They found that the culture of "*M. omelianskii*" could oxidize hydrogen and reduce carbon dioxide to methane, but could not do so with other one-carbon compounds. *M. barkeri* could not reduce formate and formaldehyde to methane in the presence of hydrogen. However, a mixture of hydrogen and carbon monoxide was converted to methane; carbon monoxide was first oxidized to carbon dioxide and hydrogen with three additional moles of hydrogen being required to reduce the carbon dioxide to methane. Likewise *Methanobacterium formicium* was found to convert carbon

monoxide and formate to methane but not formaldehyde or methanol. It was clear from these experiments that reduced one-carbon compounds (carbon monoxide, formate, formaldehyde, and methanol) were not intermediates in the reduction of carbon dioxide to methane.

1950s

As the 1950s began, the stage was set for a more definitive examination of methyl group conversion to methane in Barker's laboratory. For these experiments, Thressa Stadtman had obtained an acetate-fermenting enrichment culture of *Methanosarcina* (the organism, isolated by the agar-shake method, contained a motile rod as a contaminant). Cultures readily converted [methyl-^{14}C]-CH_3COOH to $^{14}CH_4$ and [carboxyl-^{14}C]-CH_3COOH to $^{14}CO_2$ (Stadtman and Barker, 1951b). Similarly $^{14}CH_3OH$ was converted to $^{14}CH_4$. With this organism, which resembled the *Methanosarcina barkeri* of Schnellen, it was now possible to ask a critical question about methyl group conversion to methane: *Is the methyl group transferred intact?* Pursuit of this question led to a series of definitive experiments, which remain as classics. To determine whether the three hydrogen atoms remained attached to the methyl carbon of acetate during fermentation, the methyl group of acetate was labeled with deuterium (Pine and Barker, 1956). When CD_3COOH was fermented to methane, mass analysis showed that the deuterium content of methane was CD_3H. When CH_3COOH acetate was fermented in the presence of D_2O, the methane formed had the mass of CH_3D, indicating one proton in methyl group reduction comes from water. So the methyl group of acetate was reduced intact to methane! Similar results were obtained when methanol was fermented; the hydrogen atoms of the methyl group remained associated with the methyl group as it was reduced to methane. These data had enormous implications for the terminal step of methanogenesis as well as for developing an understanding of how carbon dioxide was reduced to methane. The chemical constraints of carbon dioxide reduction are rigid, intermediates being equivalent to formate, formaldehyde and methanol. However, evidence had been obtained previously that these compounds were not free intermediates, but that the C_1 intermediates were bound. In his book, *Bacterial Fermentations,* Barker proposed a general scheme for methanogenesis that included the three major sources of methane (Barker, 1956): carbon dioxide as well as the methyl groups of acetate and methanol (Figure 2). Although Barker modestly disclaimed any unusual insight in developing this scheme, saying that it is simply dictated by chemistry, this model would be a valuable one for over 30 years as nature gradually yielded her closely guarded secrets. As he pointed out, the C_1 carrier X in the scheme very likely would be represented by more than one carrier.

Studies with cultures of "*Methanobacterium propionicum*" and "*Methanobac-*

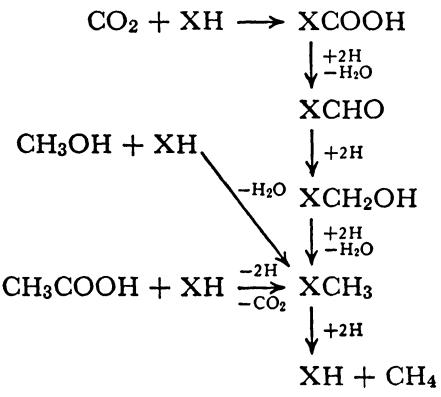

Figure 2. General scheme for methanogenesis proposed by Barker (1956).

terium suboxydans" (Stadtman and Barker, 1951a) as well as "*M. omelianskii*" (Stadtman and Barker, 1949) produced evidence that at the time not only supported the carbon dioxide reduction theory but was interpreted to indicate that a variety of compounds could be oxidized by methanogenic bacteria. So it seemed that the general equation for methanogenesis was well established; the electron donor $4H_2A$ would be any alcohol or fatty acid that could be oxidized by a dehydrogenase, A being the oxidized product:

$$4H_2A + CO_2 \rightarrow 4A + CH_4 + 2H_2O$$

Substrates (H_2A) such as propionate and butyrate were readily converted to acetate (A). Today these results are not repeatable with pure cultures of methanogens, and it is assumed that the cultures of these organisms that were used at the time were co-cultures and possibly contained some of the organisms reviewed by McInerney (1992).

Another important event of this decade was the isolation and characterization of *Methanobacterium ruminantium* from the rumen and its culture on hydrogen and carbon dioxide or formate (Smith and Hungate, 1958). The rumen had long been known to produce impressive quantities of methane, but there was a dearth of documentation on pure cultures of methanogens from the rumen and essentially no information which quantitated their numbers. *M. ruminantium* was isolated and quantitated by use of a new technique first described by Hungate (1950). This technique which would become universally known as the Hungate technique was an innovative approach, the purpose being to approximate the conditions of the natural habitat in a culture tube. By use of solid black rubber stoppers, resazurin as an O/R indicator, rumen fluid as a nutrient supplement, and a known

gas atmosphere, a *pre-reduced* sterile medium was prepared. Previous work (Mylroie and Hungate, 1954) had shown that a low oxidation-reduction potential was required for growth of *Methanobacterium formicicum* from sludge. Rumen fluid was found to have an E_h value of −346 to −335 mV. The major challenge now was to reproduce this routinely in the culture tube. Cysteine, cysteine and dithionite, as well as *Escherichia coli* and pyruvate were added to culture medium in attempts to produce a low E_h in the medium. [Later cysteine-sulfide would be found to supply a reproducible O/R buffer as the procedure became standardized (Hungate, 1969).] Development of the Hungate technique was a major event after 1936 in the culture of methanogens. The ability to culture cells in pre-reduced medium at an E_h below −330 mV in roll-tubes or liquid medium was a major advance over previous methods.

1960s

As the 1960s opened the major challenge to the field was to extend the whole-cell physiological studies of methanogens to cell extracts and the enzymology of methanogenesis. Johns and Barker (1960) reexamined the effect of hydrogen on the culture of *M. omelianskii*. With cell suspensions they studied the effect of hydrogen on the oxidation of ethanol to acetaldehyde and to acetate in the absence of carbon dioxide, finding that in the presence of hydrogen essentially no oxidation occurred. However, in the absence of hydrogen and carbon dioxide oxidation of ethanol to acetaldehyde, acetate and hydrogen could be detected.

In 1961 Sidney Elsden and I obtained a culture of *M. omelianskii* from H. A. Barker and began a study of amino acid synthesis from [^{14}C]-carbon dioxide and [^{14}C]-ethanol. The labeling patterns in the amino acids were definitive, and M. Knight continued these studies in his thesis (Knight et al., 1966). I returned to Urbana convinced that *M. omelianskii* could be mass cultured in sufficient quantities to prepare cell extracts (Wolfe, 1991). In 1963 we reported the formation of methane from pyruvate and serine by extracts of *M. omelianskii* (Wolin et al., 1963b).

Methyl Cobalamin Becomes an Important Tool

Blaylock and Stadtman (1963) showed that in the presence of pyruvate the methyl group of methylcobalamin was reduced to methane by *Methanosarcina barkeri*. These experiments were based on the work of D. D. Woods and coworkers (Guest et al., 1962). The discovery that methylcobalamin could donate the methyl group on the upper axial ligand for reduction to methane was a pivotal event, and provided a tool for studying the enzymology of methyl group reduction to methane. These studies were extended to extracts

of *M. omelianskii* to show that in a hydrogen atmosphere the requirement for pyruvate could be replaced by ATP (Wolin et al., 1963a), and later the cobalamin product of methyl group reduction to methane was found to be B_{12r} (Wolin et al., 1964b). Subsequently, the inhibition of this reaction by ADP and AMP was shown, and the cell extract was resolved into two protein fractions, each of which was required to effect methanogenesis from methylcobalamin (Wood and Wolfe, 1966b). The potent inhibition of methanogenesis by viologen dyes was also discovered at this time by M. J. Wolin (Wolin et al., 1964a). Although it had been known for sometime that methanogenic bacteria contained high quantities of vitamin B_{12}, sewage sludge being an industrial source of the vitamin, it was not until the work of Lezius and Barker (1965) that the corrinoid of *M. omelianskii* was identified as Factor III, the 5-hydroxy-benzimidazolecobamide derivative. Experiments by Wood et al. (1966) showed that the methyl group of methyl-factor III or methyl factor B (the cobinamide derivative) was readily reduced to methane by extracts of *M. omelianskii*. Subsequently, Wood and Wolfe (1966a) reported the alkylation of an enzyme in the methane-forming system of *M. omelianskii*, and these techniques were used to propylate with a ^{14}C-label a B_{12}-enzyme that was involved in methanogenesis. The radio label was used to follow purification of the protein and finally the label was removed by photolysis. Blaylock and Stadtman (1966) reported a study of a soluble enzyme system from *M. barkeri*. Later Blaylock reported a cobamide-dependent methanol-cyanocob(I)alamin methyltransferase from *M. barkeri* (Blaylock, 1968) and also resolved the methanogenic system into three protein fractions: a B_{12}-protein, an uncharacterized protein, and ferredoxin, as well as an unusual cofactor. The nature of the factor is unknown, but the fact that it also was found in extracts of *Clostridium sticklandii* makes it unlikely that it was any of the known coenzymes of methanogenesis.

The methylcobamide era was an exciting one and led to the discovery in the late 1960s of coenzyme M (McBride and Wolfe, 1971a). After this event it appeared that methylcobalamin, as such, was not a free intermediate in methanogenesis from CO_2, and interest in the methylreductase shifted to methyl coenzyme M. For the methanogenic pathway it would be more than 15 years before the role of cobamide enzymes in methyltransfer would be studied in greater detail.

Serine

With the discovery that L-serine served as a substrate for methanogenesis (Wolin et al., 1963), it was soon discovered that carbon-3 of serine was the source of methane and that folate derivatives, N^5,N^{10}-methylenetetrahydrofolate and N^5-methyltetrahydrofolate, served as C_1 carriers for methanogenesis (Wood et al., 1965; Wood and Wolfe, 1965). So in 1965 it appeared as if tetrahydrofolate was involved as a major C_1 carrier in methanogenesis for *M. omelianskii*. This

was curious, for Stadtman was unable to show any activity for folate derivatives in extracts of *Methanosarcina*. At the time we assumed that these two very different organisms had very different methanogenic pathways.

Resolution of Methanobacillus omelianskii

By 1966 considerable evidence on the physiology of the culture *M. omelianskii* had accumulated. Ferredoxin had been found in extracts of *M. omelianskii* to be involved in acetaldehyde oxidation (Brill and Wolfe, 1966), and it seemed logical that this was the electron carrier that linked the oxidative steps with the reductive steps, reduction of carbon dioxide to methane being thermodynamically more favorable than the oxidation of ethanol to hydrogen (Stadtman, 1967). But within the cell it appeared that these two processes cooperated rather poorly, and this was reflected in the poor cell yields, which were equivalent to about 0.85 g per mol of ethanol fermented; whereas with *Methanosarcina* cell yields were 3.3 g per mol of methanol fermented (Stadtman, 1967).

Although the culture of *M. omelianskii* had been maintained for 30 years and was considered to be one of the better understood methanogens, its status changed dramatically in 1967, when Bryant et al. (1967) reported that the culture was an association of two organisms. In the ethanol-carbonate medium of Barker (1936) this association was actually a symbiotic one, for neither organism could grow without the other. Oxidation of ethanol to acetate and hydrogen was thermodynamically possible by the "S" organism only when the methanogen removed the hydrogen by oxidizing it to reduce carbon dioxide to methane. The events leading to resolution of the culture into the "S" organism and the hydrogen-oxidizing methanogen, *Methanobacterium* M.o.H. (*Methanobacterium bryantii*) have been related previously (Wolfe, 1991). The "S" organism opened up the concept of interspecies hydrogen transfer (Wolin and Miller, 1982) that not only led to the resolution of *M. omelianskii* but much later to the isolation in Bryant's laboratory of *Syntrophomonas wolfei* (McInerney, et al., 1981), which anaerobically oxidizes butyrate to acetate and hydrogen in the presence of a methanogen or other hydrogen-oxidizing anaerobe (McInerney, 1992), as well as to *Syntrophobacter wolinii*, which anaerobically oxidizes propionate to acetate, carbon dioxide and hydrogen in the presence of appropriate hydrogen-oxidizing anaerobes (Boone and Bryant, 1980; McInerney, 1992). Thus, the cultures of the 1950s known as *Methanobacterium propionicum*, and *Methanobacterium suboxydans* which were reported to use fatty acids higher than acetate (Barker, 1956) were the result of inadequate technology, the cultures very likely being syntrophic associations. In addition, the cultures of *Methanosarcina mazei* and *Methanothrix sohngenii* at that time also must have contained syntrophs, since they were reported to use butyrate.

For cultivation of *Methanobacterium* M.o.H. a technology for growing anaer-

obes on hydrogen and carbon dioxide was soon developed (Bryant et al., 1968). Electron microscopy of hydrogen-grown methanogens was documented (Langenberg et al., 1968). Cultures were scaled up to the 10-liter stage and eventually to the 200-liter stage from which kilogram quantities of cells could be obtained. Extracts of hydrogen-grown methanogens did use methylcobalamin as a substrate for methanogenesis in a hydrogen atmosphere, but pyruvate and serine were ineffective, and the C_1-folate derivatives showed no activity. Folic acid was not, in fact, a C_1 carrier in methanogens, and the results obtained with *M. omelianskii* were due to enzymes from both the "S" organism and the methanogen.

The 1960s represented a pivotal period in the history of methanogenesis. The door on the enzymology of methanogens was cracked open, mass culture of methanogens on hydrogen-carbon dioxide and on methanol was developed, and a number of curious observations due to mixed cultures were cleared up. As the 1960s drew to a close, experiments leading to the discovery of coenzyme M were underway by McBride. Roberton documented ATP synthesis and ATP pools in hydrogen-grown methanogens (Roberton and Wolfe, 1969, 1970). Since the major energy-yielding step in methanogenesis was methyl group reduction to methane (Stadtman, 1967), we assumed in 1968 that a possible substrate level phosphorylation step occurred there. This seemed plausible as methyl group reduction was catalyzed by soluble enzymes, vessicles not being required. It would be 15 years before convincing evidence for a chemiosmotic basis for ATP synthesis by methanogens was presented.

1970s

Because chapters of this book focus on recent events only some of the contributions are listed here, details being found in the chapters which follow. Study of methanogenesis may be considered to have been largely in the lag phase in the 1970s with discovery of coenzymes, development of a pressurized atmosphere for growing methanogens, and discovery of the archaebacteria.

1970–79: Some Historical Events in the Biochemistry of Methanogenesis

1970

ATP, ADP, AMP pools were documented in *Methanobacterium* (Roberton and Wolfe, 1970)

1971

Discovery of coenzyme M (McBride and Wolfe, 1971)

Synthesis of dimethylarsine by extracts of *Methanobacterium* (McBride and Wolfe, 1971)

1972

Isolation of *Methanobacterium thermoautotrophicum* (Zeikus and Wolfe, 1972)
Isolation of F_{420} (Cheesman et al., 1972)
1974
Structure of coenzyme M (Taylor and Wolfe, 1974a,b)
Coenzyme M is the growth factor required by *M. ruminantium* (Taylor et al., 1974)
1975
F_{420} is a coenzyme for formate dehydrogenase and hydrogenase (Tzeng et al., 1975a,b)
Growth of methanogens in Petri plates (Edwards and McBride, 1975)
1976
Growth of methanogens in a pressurized atmosphere (Balch and Wolfe, 1976)
1977
Methanogens and discovery of the archaeobacteria (Fox et al., 1977, Woese and Fox, 1977)
Methyl-coenzyme M stimulates CO_2 reduction to CH_4, the RPG effect (Gunsalus and Wolfe, 1977)
Fluorescence of individual cells is reported (Mink and Dugan, 1977)
1978
Structure of F_{420} (Eirich et al., 1978)
Factors F_{342} and F_{430} are reported (Gunsalus and Wolfe, 1978)
Successful growth of *Methanosarcina* on acetate (Mah et al., 1978; Weiner and Zeikus, 1978)
Bromoethansulfonate (BES) is a potent inhibitor of the methylreductase (Gunsalus et al., 1978)
Yellow fluorescent compound (YFC) is reported (Daniels and Zeikus, 1978)
1979
Structure of F_{420} is confirmed (Ashton et al., 1979)
Coenzyme M is found only in methanogens (Balch and Wolfe, 1979)
Methanogens are reevaluated (Balch et al., 1979)
Cytochromes are found in methanol-grown cells (Kuhn et al., 1983)
Trimethylamine and other methyl-compounds serve as substrates (Hippe et al, 1979)

1980s

In the 1980s study of methanogenesis moved out of the lag phase into the log phase. Publications increased dramatically as a critical mass of investigators began to report results.

1980–89; Some Historical Events in the Biochemistry of Methanogenesis

1980

A procedure for anaerobic column chromatography is developed (Gunsalus et al., 1980)

Methylcoenzyme M reductase is resolved into components A, B, and C (Gunsalus and Wolfe, 1980)

F_{430} contains nickel (Whitman and Wolfe, 1980; Diekert et al., 1980b); and a tetrapyrrole is proposed (Diekert et al., 1980a)

Syntrophic associations of methanogens and other organisms are grown in a chemostat (Winter and Wolfe, 1980)

1981

Homogeneous component C of the methylreductase is yellow and has an $\alpha_2\beta_2\gamma_2$ subunit composition (Ellefson and Wolfe, 1981)

1982

Carbon dioxide reduction factor is discovered (Romesser and Wolfe, 1982)

F_{430} is the chromophore of the methylreductase (Ellefson and Wolfe, 1982)

Synthetic medium for *Methanococcus voltae* (Whitman et al., 1982)

1983

b-Type cytochromes are found in acetate-grown cells (Kuhn et al., 1983)

Specific methyltransferases are found in methanol-grown cells (van der Meijden et al., 1983)

Resolution of component A of the methylreductase system into four components (Nagle and Wolfe, 1983)

Plating efficiency of methanococci is reported (Jones et al., 1983)

F_{342} is identified as a pterin derivative (methanopterin) (Keltjens et al., 1983)

1984

A trimethylamine:HS-CoM methyltransferase is described (Naumann et al., 1984)

Structure of methanopterin (van Beelen et al., 1984)

Structure of methanofuran, carbon dioxide reduction factor (Leigh et al., 1984)

Tetrahydromethanopterin is a C_1 carrier at the methenyl, methylene, and methyl levels (Escalante-Semerena et al., 1984)

A methanol:cobamide methyltransferase is described (van der Meijden et al., 1984)

1985

Methanogen genes function in aerobically grown *E. coli* (Hamilton and Reeve, 1985)

Methanofuran is a formyl carrier in methanogenesis (Leigh et al., 1985)

Component A2 is purified to homogeneity (Rouvière et al., 1985)

F_{420} is a coenzyme for methylene-H_4MPT dehydrogenase (Hartzell et al., 1985)

N_5-formyl-H_4MPT is the product of methenyl-H_4MPT cyclohydrolase (Donnelly et al., 1985)

1986

Isolation of CODH enzyme complex, which contains a corrinoid protein, from acetate-grown *Methanosarcina* (Terlesky et al., 1986)

Cloning, expression, and regulation by molybdenum of formate dehydrogenase (Shuber et al., 1986).

Oxidation of CO is coupled to proton-motive-force-driven ATP synthesis in acetate-grown cells (Bott et al., 1986)

5,10-Methenyl-H_4MPT cyclohydrolase is purified to homogeneity (DiMarco et al., 1986)

Structure of component B is 7-mercaptoheptanoylthreonine phosphate (HS-HTP) (Noll et al., 1986).

Growth of methanogens on 2-propanol and other alcohols as electron donors (Widdel, 1986)

Formylmethanofuran:H_4MPT formyltransferase is required for methanogenesis from CO_2 (Donnelly and Wolfe, 1986)

F_{430} is required for methylreductase activity (Hartzell and Wolfe, 1986)

Localization of component C (Ossmer et al., 1986)

Molyodopterin is a cofactor for formate dehydrogenase (May et al., 1986)

1987

Structure and expression of methylcoenzyme M reductase genes, mcr operon (Cram et al., 1987)

Regulation of hydrogenase is found in *M. barkeri* (Bhatnagar et al., 1987)

Ni EPR signal of CODH from *Methanosarcina* is similar to that from *Clostridium thermoaceticum* (Terlesky et al., 1987)

HS-HTP is the electron donor for the methylreductase (Noll and Wolfe, 1987)

Acetyl-CoA is an intermediate in aceticlastic methanogenesis (Grahame and Stadtman, 1987)

Chemical synthesis of HS-HTP (Noll et al., 1987)

HS-HTP functions as component B in an ATP-independent system (Ankel-Fuchs et al., 1987)

Characterization, kinetics, and hydrogen transfer of the F_{420}-reducing hydrogenase (Livingston et al., 1987, Fox et al., 1987)

Stereochemical course of methyl transfer from methanol (Zydowsky et al., 1987)

CoM-S-S-HTP is a product of the methylreductase reaction (Bobik et al., 1987)

1988

CoM-S-S-HTP is the coenzyme product of the methylreductase (Ellerman et al., 1988)

Ferredoxin is reduced by CODH from acetate-grown cells (Terlesky and Ferry, 1988)

Methylation of a corrinoid protein occurs during aceticlastic methanogenesis (van der Wijingaard, 1988)
Electrochemical gradient of Na^+ is produced during methanol oxidation (Muller et al., 1988)
CoM-S-S-HTP replaces methylcoenzyme M for the RPG effect (Bobik and Wolfe, 1988)
Cloning and characterization of methylcoenzyme M reductase genes (Bokranz et al., 1988)
Oxygen causes the conversion of F_{420} to F_{390} and $F_{390}G$ (Kiener et al., 1988)
Different methyltransferases are formed during methanol or acetate fermentation (Grahame, 1989)
CoM-S-S-HTP has a specific reductase (Hedderich et al., 1988)
Methanococcus voltae has an autotrophic acetyl-CoA biosynthetic pathway (Shieh et al., 1988)
The methanoreductosome is defined (Mayer et al., 1988)
New EPR signals are assigned to nickel in the methylreductase (Albracht et al., 1988)

1989

Identification of a hydrogenase-linked gene which encodes a polyferredoxin (Reeve et al., 1989)
Methyl-H_4MPT is an intermediate in aceticlastic methanogenesis (Fischer and Thauer, 1989)
CODH from acetate-grown *Methanosarcina* has 4Fe-4S centers and an atypical Fe-S center (Krzycki et al., 1989)
F_{420} is a coenzyme for alcohol dehydrogenase (Widdel and Wolfe, 1989)
Dimethylsulfide is a substrate for a methylotrophic methanogen (Oremland et al., 1989)
Alternative synthesis of CoM-S-S-HTP by fumarate reductase (Bobik and Wolfe, 1989)
Formaldehyde reduction to CH_4 generates a primary electrochemical potential of Na^+ (Kaesler and Schönheit, 1989)
Growth of methanogens on cyclopentanol and CO_2 (Bleicher et al., 1989)
Formylmethanofuran dehydrogenase (Borner et al., 1989; Karrasch, Börner, et al., 1989)
P-type ATPase is characterized in *M. voltae* (Dharmavaran and Konisky, 1989)
Oxygen-stable CODH from *Methanothrix* (Jetten et al., 1989)
ATP synthesis is coupled to methylreductase in vesicles (Pinemann et al., 1989)
Carbonic anhydrase is found in *Methanosarcina* (Karrasch, Bott, et al., 1989)
Component A3 is resolved into two components (Rouvière and Wolfe, 1989)
ATPase subunits of *M. barkeri* are similar to F_0F_1-ATPases of eukaryotes (Inatomi et al., 1989)

1990–91: Some Historical Events in the Biochemistry of Methanogenesis

1990

Cloning and expression of genes encoding the F_{420}-reducing hydrogenase from *M. thermoautotrophicum* (Alex et al., 1990).

Two genetically distinct methylcoenzyme M reductases are identified in *M. thermoautotrophicum* (Rospert et al., 1990)

ATP synthesis is coupled to CoM-S-S-HTP reduction (Pinemann et al., 1990)

CODH from acetate-grown cells catalyzes both synthesis and cleavage of acetyl-CoA as well as CO exchange (Abbanat et al., 1990)

Reduction of CoM-S-S-HTP drives proton translocation (Deppenmeier et al., 1990)

Cloning and expression of enzymatically active formylmethanofuran: H_4MPT formyltransferase (DiMarco et al., 1990)

5,10-Methylene-H_4MPT reductase is purified to homogeneity (te Brommelstroet et al., 1990; Ma and Thauer, 1990)

A complex cofactor contains HS-HTP (Sauer et al., 1990)

CoM-S-S-CoM reductase is described (Smith and Rouvière, 1990)

5,10-Methylene-H_4MPT dehydrogenase with hydrogenase activity (Zirngibl et al., 1990)

Spectrochemical studies reveal fine structural conformations of F_{430} (Olson et al., 1990; Fruenlid et al., 1990; Zimmer and Crabtree, 1990; Won et al., 1990)

Molybdopterin quanine dinucleotide is a cofactor for formylmethanofuran dehydrogenase (Karrasch et al., 1990)

1991

Cloning and expression of genes for acetyl-CoA synthetase and carbon monoxide dehydrogenase from *Methanothrix* (Eggen et al., 1991a,b)

H_4MPT accepts a methyl group from the CODH-corrinoid complex during acetyl-CoA cleavage (Grahame, 1991)

Resolution of the corrinoid iron-sulfur component from CODH and its reduction to the CO^{1+} state (Abbanat and Ferry, 1991)

Methylene-H_4MPT dehydrogenase has two genetically distinct forms (von Bünau et al., 1991)

CODH high-spin system in *Methanotrix* (Jetten et al., 1991)

Photoactivation of the methylreductase (Olson et al., 1991)

Detection of a methyl-nickel (II) F_{430} (Lin and Jaun, 1991)

F_{430} configuration assignments, coordination chemistry, models (Hamilton et al., 1991; Kaplan et al., 1991; Farber et al., 1991; Renner et al., 1991)

Methylreductase preparations with a specific activity that approximates whole cells (Rospert et al., 1991)

Formylmethanofuran dehydrogenase contains molybdopterin dinucleotide and molybdopterin hypoxanthine dinucleotide (Börner et al., 1991)

Some Thoughts on the Present and the Future

The era of discovery of coenzymes and enzymes of the methanogenic pathway is now ending as it is very likely that most of the actors in this process have been identified. There undoubtedly are surprises left, for Nature is always reluctant to yield readily, methanogens being one of the last microbial groups to have their metabolic pathways elucidated. Methanogens in many ways are now at the stage where *E. coli* was in the 1950s. A major challenge is to develop convenient and reliable genetic systems so that studies on control, regulation, and molecular biology of methanogens may assume their rightful places in the future. The recent finding of Schönheit that methanogens can be grown under non-methanogenic conditions has enormous implications for the future! Control and regulation in methanogens were once considered to be unlikely areas of promise. It now appears that in addition to substrate variation that sophisticated regulation occurs during growth on hydrogen and carbon dioxide; so far two fruitful systems have been identified with two methylcoenzyme M reductases and two methylene-H_4MPT dehydrogenases being discovered in *M. thermoautotrophicum*. Undoubtedly this is just the beginning. Although enzymes have been identified, we know nothing about the reaction mechanism of any of them. Because of its uniqueness F_{430} has attracted most attention. Analysis by X-ray crystallography of the methylreductase is not far away and it will be exciting to discover the position of each F_{430} molecule in the methylreductase and the active site. Of all microbial groups methanogens provide one of the best opportunities for an in depth study of evolution; their unique biochemistry delineates a point of reference.

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I

MICROBIOLOGY

1

Diversity and Taxonomy of Methanogens

David R. Boone, William B. Whitman, and Pierre Rouvière

1.1 Introduction

The production of methane is a ubiquitous, defining characteristic of methanogens. The production of methane or any other hydrocarbon as a major catabolic product is unique to this group of microbes, which share many other characteristics that are not common among other microbes. Phylogenetically, methanogens are *Archaeobacteria* (Woese et al., 1978), a group of microbes that are distinguished from true bacteria by a number of characteristics, including the possession of membrane lipids composed of isoprenoids ether-linked to glycerol or other carbohydrates (De Rosa and Gambacorta, 1988; Jones et al., 1987; Langworthy, 1985), a lack of peptidoglycan containing muramic acid (Kandler and Hippe, 1977), and distinctive ribosomal RNA sequences (Balch et al., 1979; Woese, 1987). This group also includes some extreme halophiles and some extremely thermophilic, sulfur-dependent microbes (Woese, 1987) and is phylogenetically distinct from eukaryotes and true bacteria (Figure 1.1).

The catabolic pathways of methanogens can be divided into three groups: CO_2-reducing, methylotrophic, and aceticlastic pathways. The CO_2-reducing pathways use a series of four two-electron reductions to convert CO_2 or bicarbonate to methane (Rouvière and Wolfe, 1988). Most methanogens can grow by using H_2 as a source of electrons via hydrogenase; for some methanogens this is the sole catabolic pathway (Whitman et al., 1991). Although the source of H_2 for methanogenesis may be geologic, H_2 is more commonly obtained from other bacteria that form it as a catabolic product. In many methanogenic environments, this H_2 is utilized rapidly even when it is present at very low concentration (Wolin,

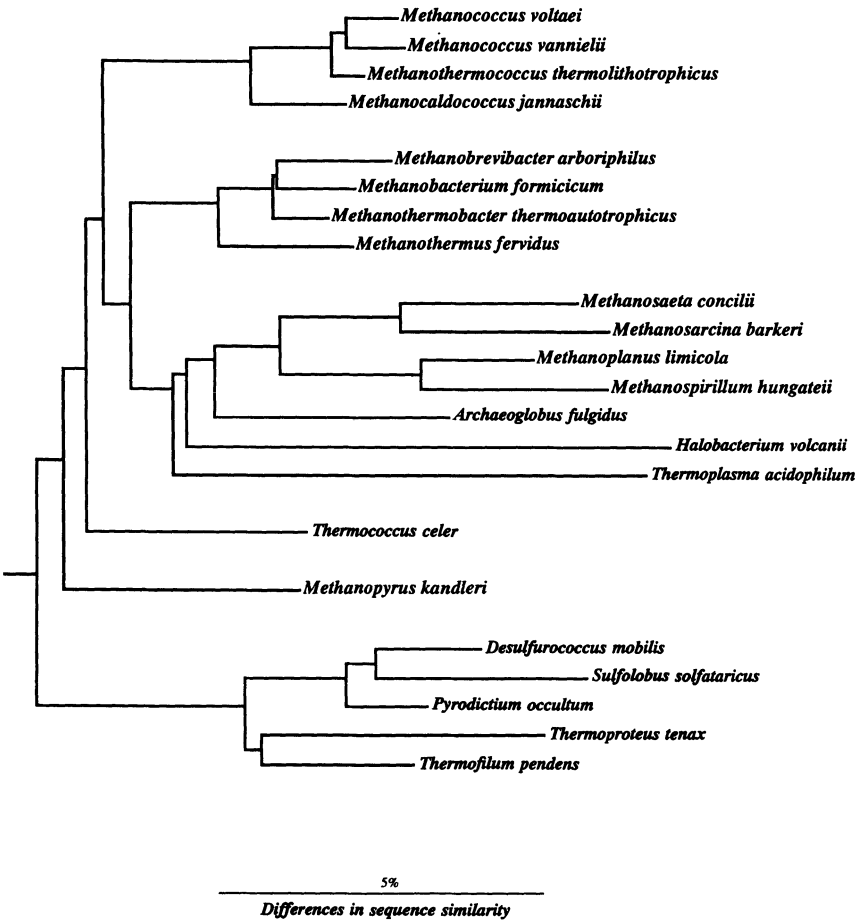

Figure 1.1. Phylogenetic relationships of microbes based on partial sequences of 16S rRNA.

1976). Thus, H_2 may never accumulate even though it is an important extracellular intermediate.

Many H_2-using methanogens can also use formate as an electron donor for the reduction of CO_2 to CH_4. Like H_2, formate may be an important substrate for methanogenesis even though its concentration in methanogenic environments is low, because it is rapidly produced and consumed (Boone et al., 1989; Hungate et al., 1970; Thiele and Zeikus, 1988). A limited number of methanogens can also oxidize secondary alcohols for CO_2 reduction to methane, and an even

smaller number can use some primary alcohols (Bleicher et al., 1989; Maestrojuán et al., 1990; Widdel, 1986; Widdel et al., 1988; Zellner and Winter, 1987a).

Methylotrophic pathways catabolize compounds that contain methyl groups, such as methanol (Schnellen, 1947), trimethylamine (Hippe et al., 1979), and dimethyl sulfide (Kiene et al., 1986; Mathrani et al., 1988; Oremland et al., 1989). Typically the methyl group is transferred to a methyl carrier (ultimately to coenzyme M) and reduced to methane. Electrons for this methyl reduction may be obtained by oxidizing a fraction of the methyl groups to CO_2 or by using H_2 as an electron donor.

Acetate is degraded by many methylotrophic methanogens and by some rod-shaped methanogens. This catabolic pathway (called the aceticlastic pathway) splits acetate, oxidizes the carboxyl group to CO_2 and reduces the methyl group to methane.

1.2 Taxonomy of Methanogens

Prior to the publication of the eighth edition of *Bergey's Manual* in 1974 (see Bryant, 1974), species of methanogenic bacteria were classified together with nonmethanogens, based mainly on morphological criteria. Recognizing the physiological unity of methanogens, Bryant reorganized their taxonomy by bringing them together into a single group (Bryant, 1974). The validity of this idea was later confirmed by phylogenetic analysis, especially the cataloging and sequencing of 16S rRNA (Balch et al., 1979; Woese, 1987), which documented the unity of methanogens and their evolutionary relationship with some extreme halophiles and extremely thermophilic sulfur-dependent organisms. These organisms are members of the kingdom *Archaeobacteria* (Woese et al., 1978). The phylogeny of *Archaeobacteria*, as indicated by 16S rRNA sequences and taken together with other unique features (e.g., chemistry of membrane lipids and outer envelopes) described elsewhere in this book, led to the concept that *Archaeobacteria* are one of three major lines of descent, the other two being eukaryotes and bacteria (Balch et al., 1979).

Later, a new taxonomic level higher than kingdom was proposed, with the methanogens and other *Archaeobacteria* classified in the urkingdom "*Archaea*" (Woese et al., 1990). At present neither the taxonomic rank of urkingdom nor the name "*Archaea*" has been adopted by the International Committee for Systematic Bacteriology. Therefore, the name is informal and enclosed in quotes. The concept of urkingdoms is justified because the differences among the groups *Archaeobacteria*, true bacteria, and eukaryotes are more profound than differences between the eukaryotic kingdoms (plants, animals, fungi, and protists). With the establishment of the urkingdom "*Archaea*," two archaeobacterial kingdoms were proposed (Winkler and Woese, 1991; Woese et al., 1990). The

methanogens are included in the "*Euryarchaeota*," which also contains the extreme halophiles, *Thermoplasma*, and some of the nonmethanogenic extreme thermophiles.

Although the methanogens are a phylogenetically coherent group, their diversity is very great. This diversity has caused some confusion in their taxonomy, and the phylogenetic depth of many taxa is not uniform either within the methanogens or with the common usage in the taxonomy of other bacteria. Historically, this situation developed because the last major revision of methanogens's taxonomy created taxa whose phylogenetic depth was much greater than common usage in other groups of bacteria (Balch et al., 1979). While this strategy was justifiable considering the small number of strains known at that time and the general absence of quantitative phylogenetic data for the eubacteria, in the following decade a large number of new taxa were described. Because many of these taxa were created following the conventions commonplace in other bacterial groups, the result was a taxonomy that was no longer uniform.

While there are few clear, quantitative rules to determine taxonomic rank from the phylogeny or genetic relatedness, consistency of the taxonomy between major groups of organisms has obvious advantages. Foremost of these is that it allows the relationships between organisms to be inferred from taxonomy. As a practical matter, this feature avoids confusion when investigators schooled in other areas of bacteriology use the taxonomy. Within the methanogens, this problem is illustrated by the biochemical studies of the methylreductase systems of *Methanobacterium thermoautotrophicum*, where detailed investigations of two different strains, ΔH and Marburg, were compared. While the results from the two strains were largely in agreement, some important differences were noted (Ellermann et al., 1988; Rouvière et al., 1988). These differences are understood more fully only with recognition of the substantial genetic differences between these strains, which have DNA reassociation values of only 46% (determined by the spectrophotometric method) or 31–35% (determined by the S-1 nuclease method) (Brandis et al., 1981; Touzel et al., 1992). For most groups of bacteria, reassociation values this low are sufficient to warrant placement of the strains into separate species (Wayne et al., 1987). Thus, the taxonomy of methanogens greatly overstated the relatedness of these strains.

A more profound consequence of a phylogenetically consistent taxonomy is that it is predictive. This property can be explained by the following metaphor. Imagine a huge library in which every volume is a bacterial species or group, and the volumes are organized according to content. If the goal is to learn the contents of every volume in the library, two strategies are available. The first is to read every volume. The second is to read only selected volumes and discover the organizing principles of the library. From this information, the contents of the remaining volumes can then be inferred. The phylogeny provides some of the organizing principles for the "library." An understanding of the relationships

between phenotype or the "contents" of the taxa and the phylogeny is also required.

The taxonomy of methanogens presented below is an attempt to form taxa of consistent depth within the methanogens, and whose depth is also consistent with that of equal ranks used in taxonomy of other bacteria. For species-level distinctions, the recommendations of the International Committee on Systematic Bacteriology are followed (Wayne et al., 1987). Thus, we distinguish organisms as separate species if their DNA reassociation is less than 70%, the change in the melting temperature of their hybrid DNA is greater than 5°C, and substantial phenotypic differences exist. Because the S-1 nuclease method of DNA:DNA reassociation systematically underestimates relatedness when compared to other reassociation methods, values obtained by the S-1 nuclease method were corrected as proposed by Grimont et al. (1980). When 16S rRNA sequence data were available, a similarity of less than 98% was considered evidence for separate species (Devereux et al., 1990). However, sequence similarities of greater than 98% were not considered sufficient evidence that two organisms belonged to the same species (Martinez-Murcia et al., 1992). For genera, interspecies DNA reassociation values of less than 20–30% were considered indicative of separate genera (Johnson, 1984). When 16S rRNA sequence data were available, a similarity of less than about 93–95% was considered evidence for separate genera (Devereux et al., 1990). This usage is consistent with the proposal of Fry et al. (1991) for *Legionella*.

The usage of the rank of family has varied greatly in methanogen taxonomy (cf. Balch et al., 1979; Zellner et al., 1989c). In general, this situation reflects the ambiguous usage in other bacterial groups. For the taxonomy proposed below, the rank of family was used to indicate closely related genera with low DNA reassociation values and 16S ribosomal RNA sequence similarities greater than 88–93%. Similarly, in other bacterial groups families include organisms with sequence similarities greater than 92–95% and exclude organisms with sequence similarities less than 89–93% (Dewhirst et al., 1989, 1992). These values of sequence similarity are consistent with the rRNA clusters defined by DeLey and coworkers for rRNA hybridizations, where families include organisms whose hybrids have a $\Delta T_{m(e)}$ of 5–8°C and exclude organisms where the hybrids have a $\Delta T_{m(e)}$ of 10–14°C (Colwell et al., 1986; De Smedt and De Ley, 1977; De Smedt et al., 1980; Gillis and De Ley, 1980; Rossau et al., 1989). Likewise, family limits for catalog data are proposed by Fox and Stackebrandt to be S_{ab} values of 0.5–0.6, which correspond to sequence similarities of 88–91% (Fox and Stackebrandt, 1987). This usage was chosen to avoid under-representation of the phylogenetic diversity in the taxonomy even though it leads to recognition of some families for which only a single species has been described. As emphasized by others, phylogenetic family definitions must also be consistent with phenotypic differences and hence must be fairly flexible (Fox and Stackebrandt, 1987).

The rank of order was used to recognize deep phylogenetic differences between families. Higher ranks of class and division were not used because their meaning is not well established. Because some nonmethanogenic halophilic archaeobacteria are specifically related to certain groups of methanogens (Burggraf, Ching, et al., 1991), it is also likely that these higher ranks would include nonmethanogens.

1.3 Taxa of Methanogens

The proposed taxonomy is presented in Table 1.1. Methanogens are classified into five orders within the kingdom *Archaeobacteria*. Three of these, *Methanobacteriales*, *Methanococcales*, and *Methanomicrobiales*, were described in *Bergey's Manual of Systematic Bacteriology* (Boone and Mah, 1989). Subsequently, the methylotrophic and aceticlastic methanogens were separated from *Methanomicrobiales* to form *Methanosarcinales* (Rouviére et al., 1991). In addition, a novel organism was discovered and placed in a new order, *Methanopyrales* (Burggraf, Stetter, et al., 1991).

Changes in taxonomy and orthographic variations have also given rise to different names being used for the same species. To aid in literature searches and to minimize the confusion, these synonyms and spelling variations are compiled in Table 1.2.

1.3.1 Methanobacteriales

Methanobacteriales is an order of mainly rod-shaped methanogens which grow by CO_2 reduction; members of one of its genera (*Methanosphaera*) are cocci that grow only by using H_2 to reduce methanol to methane. *Methanobacteriales* strains contain pseudomurein cell walls that often cause Gram-stain results to be positive. The order *Methanobacteriales* comprises two families, *Methanobacteriaceae* and *Methanothermaceae*.

Methanothermaceae contains a single genus, *Methanothermus*, of extremely thermophilic methanogens (Table 1.3). *Methanothermus* strains can be distinguished from other members of this order by their high temperature optima of 83–88°C. These rod-shaped methanogens grow on CO_2 and H_2. The two species of this genus, *Methanothermus fervidus* and *Methanothermus sociabilis*, are physiologically similar (Table 1.3) although they are phylogenetically distinct (Lauerer et al., 1986).

Methanobacteriaceae is a diverse family, which includes the genera *Methanobacterium*, *Methanothermobacter* gen. nov., *Methanobrevibacter*, and *Methanosphaera*. In general, *Methanobacterium* and *Methanothermobacter* species require few if any organic nutrients and grow at pH values near neutrality, but some species are alkaliphilic or acidophilic (Tables 1.4–1.6). Species of

Table 1.1 Taxonomy of methanogenic bacteria

Order Family Genus Species
Methanobacteriales
Methanobacteriaceae
Methanobacterium
M. formicicum, M. bryantii,
M. uliginosum, M. alcaliphilum,
M. ivanovii, M. thermoalcaliphilum,
M. thermoaggregans, M. espanolae,
M. thermophilum, "M. palustre"
Methanothermobacter gen. nov.
M. thermoautotrophicus comb. nov.,
M. wolfeii comb. nov
Methanobrevibacter
M. arboriphilicus, M. ruminantium,
M. smithii
Methanosphaera
M. stadtmaniae, M. cuniculi
Methanothermaceae
Methanothermus
M. fervidus, M. sociabilis
Methanococcales
Methanococcaceae
Methanococcus
M. vannielii, M. voltaei,
M. maripaludis, "M. aeolicus"
Methanothermococcus gen. nov.
M. thermolithotrophicus comb. nov.
Methanocaldococcaceae fam. nov.
Methanocaldococcus gen. nov.
M. jannaschii comb. nov.
Methanoignis gen. nov.
M. igneus
Methanomicrobiales
Methanomicrobiaceae
Methanomicrobium
M. mobile
Methanolacinia
M. paynteri
Methanogenium
M. cariaci, M. organophilum,
M. liminatans

Order Family Genus Species
Methanomicrobiaceae (Continued)
Methanoplanus
M. limicola, M. endosymbiosus
Methanoculleus
M. olentangyi, M. marisnigri,
M. thermophilicus
Methanofollis gen. nov.
M. tationis comb. nov.
Methanocorpusculaceae
Methanocorpusculum
M. parvum, M. labreanum,
M. bavaricum, M. sinense
Methanospirillaceae fam. nov.
Methanospirillum
M. hungateii
Methanosarcinales
Methanosarcinaceae
Methanosarcina
M. barkeri, M. mazeii,
M. thermophila, M. acetivorans,
M. vacuolata
Methanolobus
M. tindarius, M. siciliae, M. vulcani,
M. oregonensis comb. nov.
Methanococcoides
M. methylutens
Methanohalophilus
M. mahii, M. halophilus
Methanohalobium
M. evestigatum
Methanosalsus gen. nov.
M. zhilinaeae comb. nov.
Methanosaetaceae fam. nov.
Methanosaeta
M. concilii, M. thermophila comb. nov.
Methanopyrales ord. nov.
Methanopyraceae fam. nov.
Methanopyrus
M. kandleri

Table 1.2 Synonyms of names of methanogenic bacteria

Name	Objective synonyms[a]	Subjective synonyms[b]	Alternate spellings[c]
Methanothermobacter wolfeii	*Methanobacterium wolfeii*		*M. wolfei*
Methanothermobacter thermoautotrophicus	*Methanobacterium thermoautotrophicum*	*Methanobacterium thermoformicicum*	*Methanobacterium thermoautotrophicus*
Methanobrevibacter ruminantium	*Methanobacterium ruminantium*		
Methanobrevibacter smithii		*Methanobacterium ruminantium*	
Methanosphaera stadtmaniae			*M. stadtmanae*
Methanococcus voltaei			*M. voltae*
Methanothermococcus thermolithotrophicus	*Methanococcus thermolithotrophicus*		
Methanocaldococcus jannaschii	*Methanococcus jannaschii*		
Methanoignis igneus	*Methanococcus igneus*		
Methanomicrobium mobile	*Methanobacterium mobilis*		*Methanobacterium mobile*
Methanolacinia paynteri	*Methanomicrobium paynteri*		
Methanoculleus olentangyi	*Methanogenium olentangyi*	*Methanoculleus bourgensis*	*Methanogenium bourgense*
Methanoculleus marisnigri	*Methanogenium marisnigri*		
Methanoculleus thermophilicus	*Methanogenium thermophilicum*		
Methanofollis tationis	*Methanogenium tationis*		
Methanocorpusculum parvum		*M. aggregans*	
Methanospirillum hungateii			*M. hungatei, M. hungatii*
Methanosarcina mazeii	*Methanococcus mazei*	*M. frisia, Methanococcus frisius*	*M. mazei*
Methanolobus oregonensis	*Methanohalophilus oregonensis*		*Methanohalophilus oregonense*

Continued

Table 1.2 *Continued*

Name	Objective synonyms[a]	Subjective synonyms[b]	Alternate spellings[c]
Methanohalophilus mahii		*M. halophilus, Halomethanococcus doii*[d]	*Methanococcus halophilus*
Methanohalophilus halophilus	*Methanococcus halophilus*	*M. mahii, Halomethanococcus doii*[d]	
Methanohalobium evestigatum		*Halomethanococcus doii*[d]	*Methanohalobium evestigatus*
Methanosalsus zhilinaeae	*Methanohalophilus zhilinaeae*		*Methanohalophilus zhilinae*
Methanosaeta concilii	*Methanothrix concilii*	*Methanothrix soehngenii*	
Methanosaeta thermophila	*Methanothrix thermophila*	*Methanothrix thermoacetophila, Methanosaeta thermoacetophila*	

[a]Two names are objective synonyms when they have the same type strain.
[b]Two names are subjective synonyms when they have different type strains which may be considered to belong to a single species.
[c]The same name has been spelled in different ways, though the alternate spellings are the name of a single taxon.
[d]*Halomethanococcus doii* may be synonymous with *Methanohalophilus mahii*, *Methanohalophilus halophilus*, or *Methanohalobium evestigatum*; however the type strain of *H. doii* is not available to do further comparisons.

Table 1.3 Characteristics of *Methanothermus* species[a]

Property	Species	
	fervidus	*sociabilis*
Morphology	Rod	Rod
Cell width (μm)	0.3–0.4	0.3–0.4
Cell length (μm)	1–3	3–5
Forms flocs	–	+
Gram stain	+	+
Optimum temp (°C)	77–83	88
Temperature range (°C)	60–97	55–97
Optimum pH	6.5	6.5
pH range	<7.0	5.5–7.5
Substrates	H_2	H_2
Autotrophic growth	NR[b]	+
Growth factors[c]	YE	None
mol% G+C	33 (Tm, Lc)[d]	33 (Tm), 32 (Lc)
Type strain	V245	Kf1-F1
Culture collections[e]	DSM 2088, OCM 153	DSM 3496, OCM 173

[a]References: Lauerer et al., 1986; Stetter et al., 1981.
[b]NR = not reported.
[c]YE = yeast extract.
[d]Tm = determined by melting point, Lc = determined by liquid chromatography.
[e]Culture collections: DSM = Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany); OCM = Oregon Collection of Methanogens (Beaverton, OR, USA).

Methanobacterium are mesophiles or thermophiles. However, we propose a new genus, *Methanothermobacter*, and it is possible that future phylogenetic analysis will lead to the transfer of the current thermophilic species of *Methanobacterium* into this new genus. If that occurs, the circumscription of *Methanobacterium* should be modified to exclude thermophilic organisms.

The physiology of mesophilic strains of *Methanobacterium* is shown in Table 1.4. *Methanobacterium formicicum* is the oldest described species of *Methanobacterium*. *Methanobacterium bryantii* has some physiological and morphological differences from *M. formicicum*, including the inability of the latter to catabolize formate. The phylogenetic distinction between these two strains was documented by DNA reassociation of 22% (Zellner et al., 1989a). The phylogenetic distinction of *Methanobacterium uliginosum* is based on low DNA reassociation with *M. bryantii* and on a much lower mole percent guanine plus cytosine than *M. formicicum* (König, 1984). *Methanobacterium alcaliphilum* is differentiated by its growth at high pH and high mole percent guanine plus cytosine (Boone et al., 1986; Worakit et al., 1986). The differentiation of *Methanobacterium*

Table 1.4 Characteristics of mesophilic *Methanobacterium* species[a]

Property	Species						
	formicicum	*bryantii*	*ivanovii*	*uliginosum*	*alcaliphilum*	*palustre*	*espanolae*
Morphology	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Cell width (μm)	0.4–0.8	0.5–1.0	0.5–0.8	0.2–0.6	0.5–0.6	0.5	0.8
Cell length (μm)	2–15	1.5	1.2	2–4	2–25	2.5–5	3–22
Filaments (μm)	Yes	10–15	2–15	NR[b]	Short	65	Yes[c]
Gram stain	+	var	+	+	−	+	+
pH: optimum	6.6–7.8	6.9–7.2	7.0–7.4	6.0–8.5	8.1–9.1	7.0	5.6–6.2
range	NR	NR	6.5–8.2	NR	7.0–9.9	NR	NR
Substrates[d]	H_2, for, (iP,iB)	H_2, (iP,iB)	H_2	H_2	H_2	H_2, for, iP,iB	H_2
Autotrophy	+	+	+	NR	−	+	NR
Growth factors[e]	(ac, cys)	(ac, cys, B-vit)	(ac)	NR	Peptone	NR	vit
mol% G+C[f]	41–42 (Bd)	31 (Tm), 33 (Bd)	37 (Tm)	29 (Tm), 34 (Lc)	57 (Bd)	34 (Tm)	34 (Tm)
Type strain	MF	M.o.H.	31 (= Ivanov)	P2St	WeN4	F	GP9
Culture collections[g]	DSM 1535, OCM 55	DSM 863, OCM 110	DSM 2611, OCM 140	DSM 2956, OCM 176	DSM 3387, OCM 11	DSM 3108, OCM 238	OCM 178

[a]References: Belyaev et al., 1983, 1986; Boone, 1987; Boone et al., 1986; Bryant and Boone, 1987b, Bryant et al., 1971; Bryant et al., 1967; Jain et al., 1987; Kneifel et al., 1986; König, 1984; Patel et al., 1990; Worakit et al., 1986; Zellner and Winter, 1987; Zellner et al., 1989a.

[b]NR = not reported.

[c]Short filaments contain one to three cells.

[d]Substrates for methanogenesis: H_2 = hydrogen gas; for = formate; iP = isopropanol; iB = isobutanol. Parentheses indicate that activity toward these substrates were reported for some strains.

[e]Growth factors: ac = acetate; cys = cysteine; B-vit = B vita mins; vit = vitamins. Parentheses indicates that factors are stimulatory but not required for growth.

[f]Bd = determined by buoyant density; Tm = determined by melting point; Lc = determined by liquid chromatography.

[g]Culture collections: DSM, Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany); OCM, Oregon Collection of Methanogens (Beaverton, OR, USA).

Table 1.5 Characteristics of *Methanothermobacter* and thermophilic *Methanobacterium* species[a]

	Methanothermobacter		*Methanobacterium*		
Property	*thermoautotrophicus*	*wolfeii*	*thermoaggregans*	*thermoalcaliphilum*	*thermophilum*
Morphology	Rod	Rod	Rod	Rod	Rod
Cell width (μm)	0.3–0.6	0.4	0.4–0.6	0.3–0.4	0.36
Cell length (μm)	2–7	2.4–2.7	6–8	3–4	1.4–6.5
Filaments (μm)	10–120	Yes	>10	>10	<30
Gram stain	+	+	−	−	−
pH:optimum	7.0–8.0	7.0–7.5	7.0–7.5	8.0–8.5	7.6
range	NR[b]	6.0–8.2	6.5–9.0	6.5–10.0	7–8.5
T(°C):optimum	55–70	55–65	65	58–62	57
range	50–75	37–74	40–75	40–69	45–65
Substrates[c]	H_2, (for)	H_2	H_2	H_2	H_2
Autotrophy	+	+	+	+	+
Growth factors[d]	None	(YE)	(YE)	(YE)	(CoM)
mol% G+C[e]	50–52 (Bd), 46–49 (Tm), 49 (Lc)	61 (Tm)	42 (Tm)	39 (Tm)	45
Type strain	ΔH	DSM 2970	DSM 3266	AC60	M
Culture collections[f]	DSM 1053	DSM 2970, OCM 154	DSM 3266, OCM 141	DSM 3267, OCM 142	

[a]References: Blotevogel and Fischer, 1985; Blotevogel et al., 1985; Derikx et al., 1989; Touzel et al., 1992; Winter et al., 1984; Yamamoto et al., 1989; Zeikus and Wolfe, 1972; Zhao et al., 1986; Zhilina and Ilarionov, 1985.

[b]NR = not reported.

[c]Substrates for methanogenesis: H_2 = hydrogen gas; for = formate. Parentheses indicates that activity toward this substrate was reported for some strains.

[d]Growth factors: YE = yeast extract; CoM = coenzyme M. Parenthe ses indicate that the factor is stimulatory but not re quired.

[e]Bd = determined by buoyant density; Tm = determined by melting point; Lc = determined by liquid chromatography.

[f]Culture collections: DSM = Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany); OCM = Oregon Collection of Methanogens (Beaverton, USA).

Table 1.6. Characteristics of *Methanobrevibacter* species[a]

Properties	Species		
	ruminantium	*arboriphilicus*	*smithii*
Morphology	Short rod	Short rod	Short rod
Cell width (μm)	0.7	0.6	0.6–0.7
Cell length (μm)	0.8–1.7	1.2–1.4	1.0–1.5
Chains	+	−	+
Gram stain	+	+	+
pH:optimum	NR[b]	7.0–8.0	NR
range	NR	6–8.5	NR
T(°C):optimum	37–39	33–40	37–39
range	NR	20–45	NR
Inhibition by bile salts	+	+	−
Substrates[c]	H_2, for	H_2 (for)	H_2, for
Autotrophy	−	+	+
Growth factors[d]	ac, B-vit, (CoM, 2 MB, aa)	B-vit	(ac), B-vit
mol% G+C[e]	31 (Bd)	28–32 (Tm, Bd)	30 (Tm), 31 (Bd)
Type strain	M1	DH1	PS
Culture collections[f]	DSM 1093	DSM 1125, OCM 147	DSM 861, OCM 144

[a]References: Bryant et al., 1971; Lovley et al., 1984; Miller, 1989; Miller et al., 1982, 1986; Smith and Hungate, 1958; Zeikus and Hennig, 1975.

[b]NR = not reported.

[c]Substrates for methanogenesis: H_2 = hydrogen gas; for = formate. Parentheses indicate that activity towards this substrate was reported for some strains.

[d]Growth factors: ac = acetate; B-vit = B vitamins; CoM = coenzyme M; 2-MB = 2-methylbutyrate; aa = amino acids. Parentheses indicate that the factors are required for only some strains or are stimulatory.

[e]Bd = determined by buoyant density; Tm = determined by melting point.

[f]Culture collections: DSM = Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany); OCM = Oregon Collection of Methanogens (Beaverton, USA).

ivanovii from *M. bryantii* is based on a large number of physiological differences (Jain et al., 1987), although many of the physiological differences are small and most of these were not determined at the same time and in the same laboratory. The justification of *M. ivanovii* as a separate species would also benefit from a sharper contrast to *M. uliginosum*. *Methanobacterium espanolae* is an acidophilic species, and its taxonomic separation from other species is based in part on its growth at low pH (Patel et al., 1990). This species appears to be most closely related to *M. bryantii*, *M. formicicum*, and *M. uliginosum*. It has only 47% DNA reassociation with *M. bryantii* (Patel et al., 1990), but no reassociation experiments between *M. espanolae* and other *Methanobacterium* species have

been done. These are needed to confirm *M. espanolae* as a separate species. "*Methanobacterium palustre*" has been proposed as a new species of methanogen, although the name has not yet been validated. Phylogenetically, it has been distinguished from *M. formicicum* and *M. bryantii* by DNA reassociation studies, but separation from other mesophilic species of *Methanobacterium* is based on physiological studies and polyamine content. The establishment of "*M. palustre*" as a distinct species of *Methanobacterium* should be corroborated by further phylogenetic analysis.

Analysis of 16S rRNA sequences indicates that the branching of *Methanobacterium thermoautotrophicum* and *Methanobacterium wolfeii* from mesophilic *Methanobacterium* species is sufficiently deep (Figure 1.2) that these thermophilic species should be separated into a new genus. Therefore, we propose to transfer them to *Methanothermobacter* gen. nov., with *Methanothermobacter thermoau-*

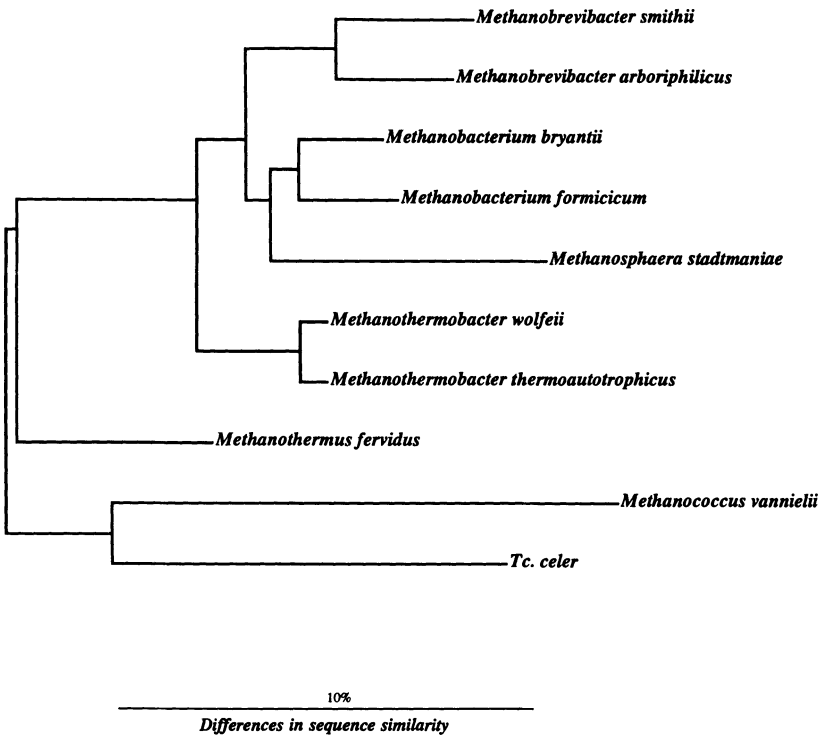

Figure 1.2. Phylogenetic relationships of *Methanobacteriales* based on partial sequences of 16S rRNA.

totrophicus comb. nov. as the type species and *Methanothermobacter wolfeii* comb. nov. as the second species of this new genus of thermophilic, hydrogenotrophic, rod-shaped methanogens.

Methanobacterium thermoformicicum was proposed as a species of thermophilic *Methanobacterium*, distinguished from *M. thermoautotrophicus* mainly by its ability to catabolize formate (Zhilina and Ilarionov, 1985). However, this species was later found to be a synonym of *Methanothermobacter thermoautotrophicus* (= *Methanobacterium thermoautotrophicum*) (Touzel et al., 1992). *Methanobacterium thermoalcaliphilum* and *Methanobacterium thermoaggregans* were also proposed as thermophilic species of *Methanobacterium*, and their separation from *Methanothermobacter thermoautotrophicus* and *Methanothermobacter wolfeii* was based on physiological differences (see Table 1.5) and a difference in mole percents of guanine plus cytosine. However, the comparisons of mole percents of guanine plus cytosine were not based on values obtained in the same laboratory and may not be reliable. Also, when physiological characteristics such as pH optima are used as distinguishing characteristics, new organisms should be compared to type strains examined at the same time (although this practice is not common at present). More phylogenetic data are needed to ensure that these species are distinct from each other and from *Methanothermobacter thermoautotrophicus* and to determine whether they should be transferred to the genus *Methanothermobacter*. *Methanobacterium thermophilum* is another species of thermophilic methanogen which might also belong in the genus *Methanothermobacter*. The major characteristic which differentiates this organism is a lower temperature optimum (57°C) (Laurinavichyus et al., 1988). This organism was also reported to require coenzyme M, although the growth rate in mineral medium with Ni added was similar to the rate in medium with coenzyme M (Laurinavichyus et al., 1988). As with *M. thermoalcaliphilum* and *M. thermoaggregans*, more phylogenetic data are needed to determine whether the species is phylogenetically distinct and whether it should be transferred to *Methanothermobacter*. DNA reassociation experiments further suggest that this genus should contain at least one additional species, represented by strain Marburg, which has sometimes been classified within *M. thermoautotrophicus*.

Methanobrevibacter strains use H_2 or formate to reduce CO_2 to CH_4. They are very short rods or cocco-bacilli, mesophilic, and sometimes have complex organic requirements (Table 1.6). The species (*Methanobrevibacter ruminantium*, *M. arboriphilicus*, and *M. smithii*) are well distinguished by DNA reassociation experi ments (Miller and Wolin, 1986). Commonly found in the gastrointestinal tract or feces of mammals, a number of isolates have been described whose taxonomy is uncertain (Lovley *et al.*, 1984; Miller and Wolin, 1986; Miller *et al.*, 1986; Misawa *et al.*, 1986).

Methanosphaera are cocci which occur singly or in small groups. They grow

only by using H_2 to reduce CH_3OH to CH_4. The two species (*Methanosphaera stadmaniae* and *M. cuniculi*) are similar in their characteristics (Table 1.7) but phylogenetically distinct (Biavati et al., 1988).

1.3.2 Methanococcales

Methanococcales is an order of coccoid, marine methanogens. They are slightly halophilic, and most are chemolithotrophic, growing by using H_2 or formate to reduce CO_2 to CH_4. Previously, a single family (*Methanococcaceae*) and genus (*Methanococcus*) comprised all species in *Methanococcales*. Most strains are mesophilic, and we propose to transfer the three thermophilic species to separate genera, leaving only mesophilic organisms in *Methanococcus* (Table 1.8). This reorganization is justified by the low 16S rRNA sequence similarity between the mesophilic and thermophilic species (Figure 1.3). In addition, the mesophilic species are related at the genus level by the criteri on of DNA reassociation values (Keswani and Whitman, unpublished data).

Methanococcus thermolithotrophicus grows most rapidly at 65°C (Table 1.9). We propose to transfer this species to *Methanothermococcus* gen. nov. as *Methanothermococcus thermolithotrophicus* comb. nov. *Methanothermococcus* is a

Table 1.7 Characteristics of *Methanosphaera* species[a]

	Species	
Property	*stadtmaniae*	*cuniculi*
Morphology	Coccus	Coccus
Cell diameter	1.0	0.6–1.2
Gram stain	+	+
pH:optimum	6.5–6.9	6.8
range	NR[b]	NR
T(°C) optimum	37	35–40
Substrate[c]	H_2 + Me	H_2 + Me
Growth factors[d]	ac, ile, leu, thiamine, biotin	ac, YE, Trypticase
mol% G+C[e]	26 (Tm)	23 (Tm)
Type strain	MCB–3	1R7
Culture collection[f]	DSM 3091	DSM 4103, OCM 183

[a]References: Biavati et al., 1988; Miller and Wolin, 1983, 1985.
[b]NR = not reported.
[c]Substrates for methanogenesis are H_2 plus methanol.
[d]Growth factors: ac = acetate; ile = isoleucine; leu = leucine; YE = yeast extract.
[e]Tm = determined by melting point.
[f]Culture Collection: DSM = Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany); OCM = Oregon Collection of Methanogens (Beaverton, USA).

Table 1.8 Characteristics of mesophilic *Methanococcus* species[a]

	Species			
Property	*vannielii*	*voltaei*	*maripaludis*	*"aeolicus"*
Morphology	Coccus	Coccus	Coccus	Coccus
Diameter (μm)	1.3	1.5	1.0	1.7
Gram stain	−	−	−	−
Motility	+	+	+	+
pH: optimum	NR[b]	6.0–7.0	6.8–7.2	NR
range	7–9	5.8–8.0	6.4–8.2	NR
T(°C):optimum	35–40	35–40	35–39	NR
range	20–40	20–45	18–47	NR
Substrates[c]	H_2, for	H_2, for	H_2, for	H_2, for
Autotrophy	+	−	+	+
Growth factors[d]	None	ac, ile, leu, (PL)	(ac, CA)	None
NaCl (M):optimum	−	0.4	0.4	−
range	0.06–0.8	0.1–1.1	0.06–0.8	0.2–0.8
mol% G+C[e]	31 (Bd), 33 (Lc)	30 (Lc), 31 (Bd)	33 (Bd, Lc)	30 (Lc)
Type strain	SB[f]	PS	JJ	PL-15/H
Culture collections[g]	DSM 1224, OCM 148	DSM 1537, OCM 70	DSM 2067	−

[a]References: Corder et al., 1983; Jarrell and Koval, 1989; Jones and Stadtman, 1975; Jones et al. 1983b, Stadtman and Barker, 1951; Ward et al., 1989; Whitman, 1989; Whitman et al., 1982, 1986, 1987.
[b]NR = not reported.
[c]Substrates for methanogenesis: H_2 = hydrogen gas; for = formate.
[d]Growth factors: ac = acetate; ile = isoleucine; leu = leucine; PL = pantoyl lactone; CA = Casamino acids. Parentheses indicate that factors were stimulatory but not required.
[e]Bd = determined by buoyant density; Lc = determined by liquid chromatography.
[f]Unnamed by original authors, but called "strain SB" by other authors.
[g]Culture collections: DSM = Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany); OCM = Oregon Collection of Methanogens (Beaverton, USA).

thermophilic genus of marine cocci, placed within the family *Methanococcaceae*, and *Methanothermococcus thermolithotrophicus* is the type species.

Methanococcus jannaschii is a second thermophilic former member of the genus *Methanococcus*, growing most rapidly at 85 °C (Table 1.9). We propose to transfer *Methanococcus jannaschii* to a new genus, *Methanocaldococcus* gen. nov., as *Methanocaldococcus jannaschii* comb. nov. *Methanocaldococcus* is thus a genus of extremely thermophilic marine methanogens, with *Methanocaldococcus jannaschii* as the type species. This genus is the type genus of *Methanocaldococcaceae* fam. nov., in the order *Methanococcales*. These new taxa are justified

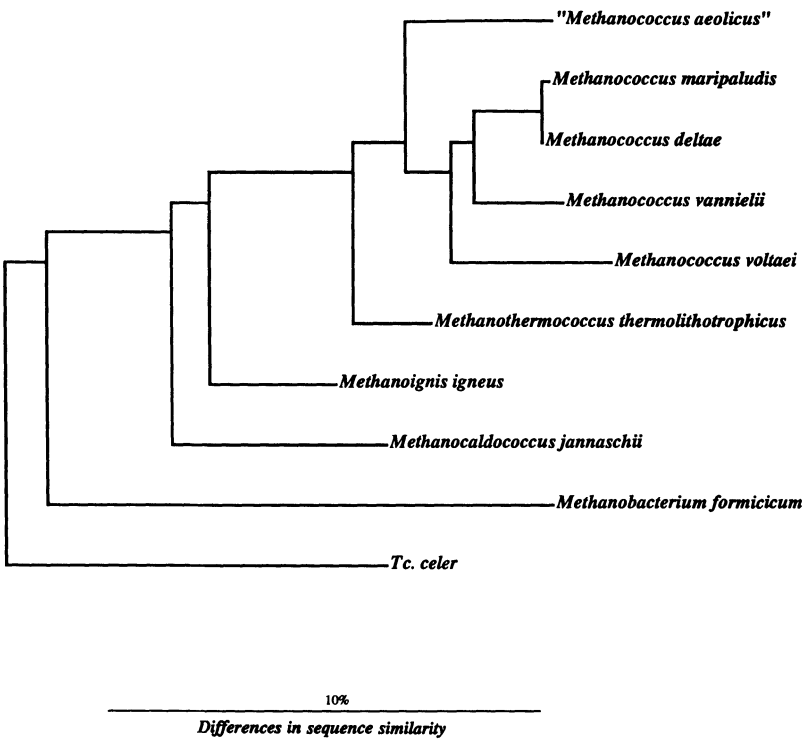

Figure 1.3. Phylogenetic relationships of *Methanococcales* based on partial sequences of 16S rRNA.

by the physiological and phylogenetic differences (Figure 1.3) between *Methanocaldococcus jannaschii* and other methanococci.

Methanococcus igneus (Table 1.9) is a third species previously classified in the genus *Methanococcus*, which we propose to transfer as the type species of a new genus, *Methanoignis*, as *Methanoignis igneus* comb. nov. Although 16S rRNA sequence analysis does not indicate a specific relationship to either *M. jannaschii* or the *Methanococcaceae* (Burggraf et al., 1990), *M. igneus* is an extreme thermophile with measurable DNA reassociation with *M. jannaschii*. Thus, it is classified within the *Methanocaldococcaceae* at present.

1.3.3 Methanomicrobiales

Until recently, the order *Methanomicrobiales* was considered to contain at least three families, *Methanomicrobiaceae*, *Methanosarcinaceae*, and *Methanocor-*

Table 1.9 Characteristics of thermophilic *Methanococcales* species[a]

Property	*Methanothermococcus thermoautotrophicus*	*Methanoignis igneus*	*Methanocaldococcus jannaschii*
Morphology	Coccus	Coccus	Coccus
Diameter (μm)	1.0	1.3–1.8	1.0
Gram stain	–	–	–
Motility	+	(+)[b]	+
pH: optimum	6.5–7.5	5.7	6.0–6.5
range	6.5–8	5–7.5	5.2–7.6
T(°C): optimum	65	88	85
range	30–70	45–91	48–94
Substrates[c]	H_2, for	H_2	H_2, (for)
Autotrophy	+	+	+
Growth factors[d]	None	None	(YE)
NaCl:(M) optimum	0.7	0.3	0.3–0.7
range	0.2–1.4	0.08–1.2	0.1–0.85
mol% G+C[e]	31 (Tm), 34 (Lc)	31 (Tm)	31 (Bd,Lc), 33 (Tm)
Type strain	SN1	Kol5	JAL-1
Culture collection[f]	DSM 2095, OCM 138	DSM 5666	DSM 2661, OCM 168

[a]References: Burggraf et al., 1990; Hatchikian et al., 1989; Huber et al., 1982; Jones et al., 1983a, 1989; Zhao et al., 1988.

[b]Flagella observed by electron microscopy, motility not observed.

[c]Substrates for methanogenesis: H_2 = hydrogen gas; for = formate. Parentheses indicate that activity towards this substrate was reported for some strains.

[d](YE), yeast extract was stimulatory to growth.

[e]Bd = determined by buoyant density; Tm = determined by melting point; Lc = determined by liquid chromatography.

[f]Culture collections: DSM = Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany); OCM = Oregon Collection of Methanogens (Beaverton, USA).

pusculaceae. Another proposal recognized the family *Methanoplanaceae*, containing the genus *Methanoplanus*. However, phylogenetic data showed that species of *Methanoplanus* (the genus which is the sole member of *Methanoplanaceae*) are not phylogenetically separate from the family *Methanomicrobiaceae*. Thus, our taxonomy classifies the genus *Methanoplanus* as a member of the family *Methanomicrobiaceae*, and does not recognize the family *Methanoplanaceae*.

The family *Methanosarcinaceae* was originally classified as a family of *Methanomicrobiales*, but it is now separated into a fourth distinct order of methanogens, the *Methanosarcinales*.

The remaining members of *Methanomicrobiales*, like members of the orders *Methanobacteriales* and *Methanococcales*, contain mainly organisms which grow by using H_2 to reduce CO_2 to CH_4. Formate is a common electron donor for CO_2

reduction, and alcohols are used by some species (Tables 10–14). Almost all species of *Methanomicrobiales* require acetate as a source of cell carbon, and many have additional, complex nutritional requirements. Cultures are mesophilic or thermophilic, and they are generally slightly halophilic. The cell wall of *Methanomicrobiales* is usually only a protein layer (S-layer), but an external sheath is present in one species (*Methanospirillum hungateii*). The protein cell wall makes many *Methanomicrobiales* osmotically sensitive: they are lysed by dilute detergents or hypotonic shock. The shape is usually irregularly coccoid, but they may appear as plate-shaped or rod-shaped cells. The shape of the sheathed species, *Methanospirillum hungateii*, is a gentle α-helical spiral which is conferred by the sheath.

The family *Methanocorpusculaceae* contains a single genus of coccoid, hydrogenotrophic methanogens (*Methanocorpusculum*). These are coccoid methanogens which oxidize H_2, formate, or alcohols and reduce CO_2 to methane (Table 1.10). DNA reassociation studies (Xun et al., 1988) were the basis of the transfer of some coccoid species to this genus (Maestrojuán et al., 1990), but we consider *Methanocorpusculum parvum* and *M. aggregans* to be members of the same species. DNA reassociation studies which use S1 nuclease tend to give lower values than membrane-filter methods, so that even though Xun et al. (1988) found reassociation values between these strains of about 70%, these would likely have greater than 70% similarity if determined by membrane-filter methods. *Methanocorpusculum labreanum* has only 31% sequence similarity to the type strain of *M. parvum* (determined by S1 nuclease [Xun et al., 1989]) and only 48% with *M. parvum* MSt (formerly *M. aggregans* MSt) (Xun et al., 1989), supporting the segregation of *M. labreanum* as a species separate from *M. parvum*.

Methanocorpusculum bavaricum and *Methanocorpusculum sinense* were described later (Zellner et al., 1989). Whole-cell protein electrophoresis and other characteristics (Zellner et al., 1989) suggest that these two species are distinct and different from *M. parvum*, but no comparison was made to the other *Methanocorpusculum* species, *M. labreanum*.

Methanomicrobiaceae, the other family of *Methanomicrobiales*, contains several genera that are diverse in morphology, physiology, and phylogeny. The evolutionary deepness of the branching of *Methanospirillum* (Figure 1.4) from other members of this family supports its separation into a new family within *Methanomicrobiales*, the *Methanospirillaceae*. This family contains a single genus and species (*Methanospirillum hungateii*) (Table 1.11). The family *Methanomicrobiaceae* contains the genera *Methanomicrobium*, *Methanolacinia*, *Methanogenium*, *Methanoplanus*, *Methanoculleus*, and *Methanofollis* gen. nov.

The genera *Methanomicrobium* and *Methanolacinia* each contain a single species (Table 1.11). We propose to transfer *Methanogenium tationis* as the type and only species of the new genus *Methanofollis* as *Methanofollis tationis*.

Table 1.10 Characteristics of *Methanocorpusculum* species[a]

Property	Species			
	parvum	*labreanum*	*sinense*	*bavaricum*
Morphology	Irregular coccus	Irregular coccus	Irregular coccus	Irregular coccus
Diameter (μm)	0.5–2.0	0.4–2.0	<1	<1
Gram stain	–	NR[b]	–	–
Motility[c]	±	–	(+)	(+)
pH:optimum	6.5–7.5	7.0	7.0	7.0
range	6.0–8.0	6.5–7.5	NR	NR
T(°C):optimum	37	37	30	37
range	20–40	25–40	20–40	>15–<45
NaCl:range (M)	<0.8	<0.5	NR	NR
Substrates[d]	H_2, for, iP, iB	H_2, for	H_2, for	H_2, for, iP, iB
Autotrophy	–	NR	–	–
Growth factors[e]	ac, YE or peptones	(ac), peptone	rf, YE	rf, YE
mol% G+C[f]	49–51 (Tm), 50 (Lc), 52 (Bd)	50 (Bd)	52 (Tm), 50 (Lc)	48 (Tm), 51 (Lc)
Type strain	XII	Z	CHINAZ	SZSXXZ
Culture collections[g]	DSM 3823, OCM 63	DSM 4855, OCM 1	DSM 4274, OCM 128	DSM 4179, OCM 127

[a]References: Ollivier et al., 1985; Xun et al., 1989; Zellner et al. 1987, 1989c; Zhao et al., 1989.
[b]NR = not reported.
[c]Motility: – = nonmotile; + = motile; (+) = weakly motile.
[d]Substrates for methanogenesis: H_2 = hydrogen gas; for = formate; iP = isopropanol; iB = isobutanol.
[e]Growth factors: ac = acetate; YE = yeast extract; rf = rumen fluid. Parentheses indicate that factors were stimulatory but not required.
[f]Bd = determined by buoyant density; Tm = determined by melting point; Lc = determined by liquid chromatography.
[g]Culture collections: DSM = Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany); OCM = Oregon Collection of Methanogens (Beaverton, OR, USA).

Table 1.11 Characteristics of *Methanolacinia, Methanomicrobium, Methanospirillum* and *Methanofollis* species[a]

Property	*Methanolacinia paynteri*	*Methanomicrobium mobile*	*Methanospirillum hungateii*	*Methanofollis tationis*
Morphology	Pleomorphic rods	Rod	Spirillum	Irregular coccus
Cell width (μm)	0.6	0.7	0.5	1–2.5
Cell length (μm)	1.5–2.5	1.5–2.0	7.4	–
Gram stain	–	–	–	NR[b]
Motility[c]	(+)	(+)	+	+
Flagellation	Yes	One polar	Two polar tufts	Peritrichous
pH: optimum	7.0	6.1–6.9	NR	7
range	NR	5.9–7.7	NR	6.3–8.8
T(°C): optimum	40	40	35–40	37–40
range	20–45	30–45	NR	25–45
NaCl (M): optimum	<0.15	NR	<0.1	<1.2
Substrates[d]	H_2, iP, iB, cPe	H_2, for	H_2, for, (iP, iB)	H_2, for
Autotrophy	–	–	(+)[e]	–
Growth factors[f]	ac (YE, Tryp)	complex[a]	(ac, vit, peptone)	ac (YE, peptone)
mol% G+C[g]	45 (Bd), 38 (Tm)	49 (Bd)	46–50 (Tm)	54 (Tm)
Type strain	G-2000	1[h]	JF-1	DSM 2702
Culture collections[i]	DSM 2545	DSM 1539	DSM 864, OCM 16	DSM 2702, OCM 43

[a]References: Ferry and Wolfe, 1977; Ferry et al., 1974; Kuhner et al., 1991; Patel and Roth, 1977; Patel et al., 1976; Paynter and Hungate, 1968; Rivard et al., 1983; Tanner and Wolfe, 1988; Widdel et al., 1988; Zabel et al., 1984; Zellner et al., 1989b.

[b]NR = not reported.

[c]Motility: + = motile; (+) = weakly motile.

[d]Substrates for methanogenesis: H_2 = hydrogen gas; iP = isopropanol; iB = isobutanol; cPe = cyclopentanol; for = formate. Parentheses indicate substrates utilized by same strains.

[e]Some strains do not require an organic carbon source.

[f]Growth factors: ac = acetate; YE = yeast extract; Tryp = Trypticase peptones; vit = vitamins. Complex growth requirements include: acetate, isobutyrate, isovalerate, 2-methybutyrate, tryptophan (or indole), pyridoxine, thiamine, biotin, cobalamin, 7-mercaptoheptonylt hreonine phosphate. *p*-Aminobenzoic acid was stimulatory. Parentheses indicate that factors are stimulatory or required by some strains.

[g]Bd = determined by buoyant density; Tm = determined by melting point.

[h]Later authors have called this strain "BP."

[i]Culture collections: DSM = Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany); OCM = Oregon Collection of Methanogens (Beaverton, OR, USA).

Table 1.12 Characteristics of *Methanogenium* species[a]

Property	Species			
	cariaci	*organophilum*	*"frittonii"*	*liminatans*
Morphology	Irregular coccus	Irregular coccus	Irregular coccus	Irregular coccus
Diameter (μm)	1.0–3.0	0.5–1.5	1–2.5	1.5
Gram stain	–	NR[b]	—[c]	NR
Motility	–	–	–	+
Flagellation	Peritrichous	NR	none	yes
pH:optimum	6.8–7.3	6.4–7.3	7–7.5	7
range	6.0–8.3	NR	6–8.25	NR
T(°C):optimum	37–45	30–35	57	40
range	15–<50	15–39	26–62	25–45
NaCl:range (M)	<1.5M	(0.34)[d]	<0.5	<0.8
Substrates[e]	H_2, for, iP, iB, iPe	H_2, for, iP, B, E, np	H_2, for	H_2, for, iP, iB, cPe
Autotrophy	–	–	+	–
Growth factors[f]	ac, YE	ac, PABA, biotin, B12	(YE, CA, Tryptose)	ac
mol% G+C[g]	52 (Bd)	47 (Tm)	49 (Bd)	59 (Tm), 61 (Lc)
Type strain	JR1	CV	FR-4	GKZPZ
Culture collections[h]	DSM 1497, OCM 49	DSM 3596, OCM 72	DSM 2832, OCM 200	DSM 4140

[a]References: Harris et al., 1984; Maestrojuán et al., 1990; Romesser et al., 1979; Widdel, 1986; Widdel et al., 1988; Xun et al., 1989; Zellner et al., 1990.

[b]NR = not reported.

[c]Gram stain inconclusive due to fragile cell envelope.

[d]Good growth at 0.34 M NaCl, but the range was not reported.

[e]Substrates for methanogenesis: H_2 = hydrogen gas; for = formate; iP = isopropanol; iB = isobutanol; iPe = isopentanol; E = ethanol; nP = 1-propanol; cP = cyclopentanol.

[f]Growth factors: ac = acetate; YE = yeast extract; PABA = *p*-aminobenzoate; B_{12} = vitamin B_{12}; CA = Casamino acids. Parentheses indicate factors that are stimulatory but not required for growth.

[g]Bd = determined by buoyant density; Tm = determined by melting point; Lc = determined by liquid chromatography.

[h]Culture collections: DSM = Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany); OCM = Oregon Collection of Methanogens (Beaverton, OR, USA).

Table 1.13 Characteristics of *Methanoplanus* species[a]

Property	Species	
	limicola	*endosymbiosus*
Morphology	Plate	Disc
Dimensions	1–3×1–2×0.1–0.25	1.6–3.4
Gram stain	–	NR[b]
Motility[c]	(+)	–
Flagellation	Polar tuft	Peritrichous
pH:optimum	6.5–7.5	6.8–7.3
T(°C):optimum	40	32
range	17–41	16–36
NaCl (M):optimum	0.17	0.25
range	0.07–0.92	<0.75
Substrates[d]	H_2, for	H_2, for
Autotrophy	–	NR
Growth factors[e]	ac (YE)	*p*-cresol, (YE)
mol% G+C[f]	48 (Tm)	39 (Tm)
Type strain	M3	MC1
Culture collections[g]	DSM 2279	DSM 3599, OCM 102

[a]References: van Bruggen et al., 1986; Wildgruber et al., 1982.
[b]NR = not reported.
[c]Motility: (+) = weakly motile; – = nonmotile.
[d]Substrates for methanogenesis: H_2 = hydrogen gas; for = formate.
[e]Growth factors: ac = acetate; YE = yeast extract. Parentheses indicate factors that are stimulatory but not required for growth.
[f]Tm = determined by melting point.
[g]Culture collections: DSM = Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany); OCM = Oregon Collection of Methanogens (Beaverton, OR, USA).

Methanogenium is a genus of marine methanogens, comprising three species, *Methanogenium cariaci*, *Methanogenium organophilum*, and *Methanogenium liminatans*; "*Methanogenium frittonii*" has been described but never validated (Table 1.12).

Methanoplanus comprises two species, *Methanoplanus limicola* and *Methanoplanus endosymbiosus* (Table 1.13).

The genus *Methanoculleus* contains four species, *Methanoculleus bourgensis*, *M. marisnigri*, *M. thermophilicus*, and *M. olentangyi* (Table 1.14). Just as *M. parvum* and *M. aggregans* should be considered as subjective synonyms, DNA reassociation data (Xun et al., 1989) suggest that *M. bourgensis* and *M. olentangyi* are synonyms. *M. olentangyi* is the senior (older) synomym, so the species must be called *M. olentangyi*. However, this action removes *M. bourgensis*, the type species of *Methanoculleus*, from the taxonomy, so the genus should be conserved

Table 1.14 Characteristics of *Methanoculleus* species[a]

Property	Species		
	olentangyi	*marisnigri*	*thermophilicum*
Morphology	Irregular coccus	Irregular coccus	Irregular coccus
Diameter (μm)	1–2	1–2	0.7–1.8
Gram stain	–	–	NR
Motility[c]	–	NR[b]	(+)
Flagellation	None	Peritrichous	One
pH:optimum	6.7	7–8	6.7–7.2
range	5.5–8.0	6–8.7	6.2–8.0
T(°C):optimum	37	40	55–60
range	30–50	25–55	30–60
NaCl range(M)	<0.4	<1.0	<0.4
Substrates[d]	H_2, for, (iP, iB)	H_2, for, iP, iB	H_2, for
Autotrophy	–	–	–
Growth factors[e]	ac (YE, Tryp)	ac, peptone	ac
mol% G+C[f]	54–59 (Bd)	61–62 (Bd)	56–57 (Tm), 59–60 (Bd)
Type strain	RC/ER	JR1	CR-1
Culture collections[g]	DSM 2772, OCM 52	DSM 1498, OCM 56	DSM 2373

[a]References: Corder et al., 1983; Ferguson and Mah, 1983; Maestrojuán et al., 1990; Ollivier et al., 1986; Rivard and Smith, 1982; Zabel et al., 1985.
[b]NR = not reported.
[c]Motility: – = nonmotile, (+) = weakly motile.
[d]Substrates for methanogenesis: H_2 = hydrogen gas; for = formate; iP = isopropanol; iB = isobutanol.
[e]Growth factors: ac = acetate; YE = yeast extract; Tryp = Trypticase peptones.
[f]Bd = determined by buoyant density; Tm = determined by melting point, Lc = determined by liquid chromatography.
[g]Culture collections: DSM = Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany); OCM = Oregon Collection of Methanogens (Beaverton, OR, USA).

by an Opinion of the Judicial Commission; a request for such an Opinion has been submitted.

The other species of *Methanoculleus* (*M. marisnigri* and *M. thermophilicus*) are phylogenetically distinct from *M. olentangyi* (Xun et al., 1989).

1.3.4 Methanosarcinales

The family *Methanosarcinaceae* was recently proposed to be separated into a new order, *Methanosarcinales* (Rouvière et al., 1991). This proposal is taxonomically sound because the separation is consistent with the phylogenetic separation

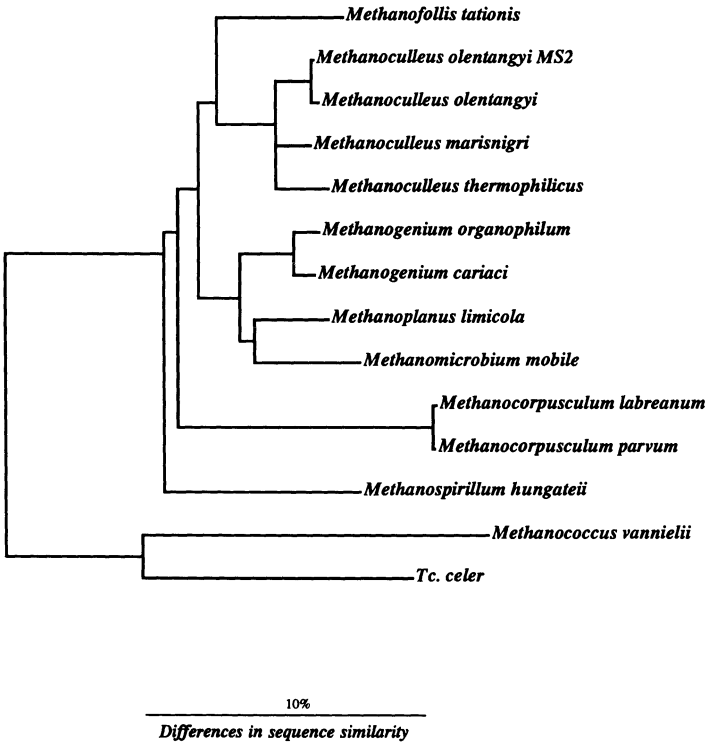

Figure 1.4. Phylogenetic relationships of *Methanomicrobiales* based on partial sequences of 16S rRNA.

of these organisms and consistent with conspicuous physiological distinction. The genera classified as *Methanosarcinales* include *Methanosarcina*, *Methanosaeta* (= *Methanothrix*), *Methanolobus*, *Methanococcoides*, *Methanohalophilus*, and *Methanohalobium*. The genus *Halomethanococcus* (Yu and Kawamara, 1987) could also be included; however, we excluded this genus because it is impossible to know whether it is distinct from other genera and species which have precedence. There are no known cultures of the type strain of the only species of the genus (*Halomethanococcus doii* IY-1) with which to do comparative studies, and the published description does not clearly differentiate this genus from *Methanohalophilus* or *Methanohalobium*, nor the species *Halomethanococcus doii* from *Methanohalophilus halophilus* or from *Methanohalobium evestigatum*.

The order *Methanosarcinales* comprises two families, *Methanosarcinaceae* and *Methanosaetaceae* fam. nov. Members of *Methanosarcinaceae* are all meth-

ylotrophic, that is, they grow by catabolizing methyl-group containing compounds such as methanol, methylamines, or methyl sulfides (Tables 1.15, 1.16, 1.17). All known strains can grow by dismutating trimethylamine (to ammonia, carbon dioxide, and methane) or methanol (to methane and carbon dioxide). Many can also grow by using H_2 to reduce CO_2 to methane or by splitting acetate (to methane and carbon dioxide). No other methanogens are methylotrophic, except *Methanosphaera* species, which reduce methanol to methane (in contrast to *Methanosarcinaceae*, *Methanosphaera* require H_2 as electron donor for methyl-group reduction). Although some *Methanosarcinaceae* can also grow on H_2 plus CO_2, their apparent affinity for H_2 suggests that they may not be competitive for H_2 in natural environments. None use formate as a catabolic substrate. *Methanosarcinaceae* are mesophilic or thermophilic, and habitats range from fresh water to saturated brines. Members of *Methanosarcinaceae* often have no requirements for organic nutrients (other than their catabolic substrates), except that some require vitamins (*p*-aminobenzoate, biotin, and thiamine). They have cell walls of protein and often an external wall of a heteropolysaccharide. *Methanosarcinaceae* are coccoid, occurring singly or in clusters of a few or many thousands, or pseudosarcinal (appearing somewhat like sarcinae, but with nonperpendicular planes of division and uneven daughter cells).

Methanosarcina contains nonhalophilic organisms which grow on methylotrophic substrates and sometimes acetate or $H_2 + CO_2$ (Table 1.15). *Methanosarcina barkeri* is the type species, which is phylogenetically distinct from the other nonhalophilic, mesophilic *Methanosarcina* species (Sowers et al., 1984b). It was first isolated by Schnellen (Schnellen, 1947), but the original type strain has been lost. Later, a strain isolated by Marvin P. Bryant (strain MS, known as "Marv's strain" [Bryant and Boone, 1987a]) was adopted as the neotype (Balch et al., 1979).

M. mazeii has several morphological forms, including coccoid. The first description of the species was based on enrichment cultures in which the coccoid forms were evident, so it was classified in a separate genus from *Methanosarcina* (*Methanococcus mazeii* [= *Methanococcus mazei*] was the first name of this species [Barker, 1936]). This species was later obtained in pure culture (Mah, 1980). Its similarities to *Methanosarcina barkeri* were recognized and this species was transferred to *Methanosarcina* as *Methanosarcina mazeii* (Mah and Kuhn, 1984).

M. acetivorans was isolated from a marine source and grows well in marine medium (Sowers et al., 1984a), although it is not halophilic (Maestrojuán and Boone, 1991).

M. vacuolata is phylogenetically distinct from other *Methanosarcina* species (Lysenko and Zhilina, 1985) and is the only type strain in the genus *Methanosarcina* that has gas vesicles (Zhilina and Zavarzin, 1987a). However, the possession of gas vesicles is not an exclusive characteristic of *M. vacuolata*, because at least one strain of *M. barkeri* has vesicles (Archer and King, 1983; Maestrojuán and Boone, 1991).

Table 1.15 Characteristics of *Methanosarcina* species[a]

Property	Species				
	barkeri	*mazeii*	*thermophila*	*acetivorans*	*vacuolata*
Morphology	Large and small aggregates	Coccus, macrocyst	Coccus, irregular aggregates	Coccus, macrocyst	Small aggregates
Gas vesicles	(+)	−	−	−	+
Gram stain	+	±	+	−	+
Motility	−	−	−	−	−
pH:optimum	5–7	6–7	6–7	6.5	6
range	4–8	5.5–8.5	5.5–8.0	5.5–8.0	4.5–7
T(°C):optimum	35–42	35–42	50	35–40	35–42
range	20–50	20–45	35–50	15–45	20–45
NaCl(M):optimum	<0.2	<0.2	<0.1, 0.6[b]	0.1–0.4	0.1
range	<0.9	<1.0	<1.2	<1.0	<0.6
Substrates[c]	H_2, Me, ac (H_2)	Me, ac (H_2)	Me, ac	Me, ac	(H_2), M, ac
Autotrophy	+	+	−	−	−
Growth factors[d]	(riboflavin)	(ac, YE, peptone)	PABA	none	none
mol% G+C[e]	39–44 (Bd)	42 (Bd)	42 (Tm)	41 (Tm)	36 (Tm)
Type strain	MS	S-6	TM-1	C2A	Z-761
Culture collections[f]	DSM 800, OCM 38	DSM 2053, OCM 26	DSM 1825, OCM 12	DSM 2834, OCM 95	DSM 1232, OCM 85

[a]References: Blotevogel and Fischer, 1989; Blotevogel et al., 1986; Bryant and Boone, 1987a, Liu et al., 1985; Mah, 1980; Mah and Kuhn, 1984; Murray and Zinder, 1985; Ollivier et al., 1984; Scherer and Sahm, 1981; Sowers and Gunsalus, 1988; Sowers et al., 1984a, 1984b; Touzel and Albagnac, 1983; Touzel et al., 1985; Zhilina, 1976; Zhilina and Zavarzin, 1979a, 1987a; Zinder and Mah, 1979; Zinder et al., 1985.

[b]Optimum NaCl concentration depends on growth conditions of the inoculum.

[c]Substrates for methanogenesis: H_2 = hydrogen gas; Me = methylated C_1 compounds such as methanol, methylamine, dimethylamine, trimethylamine, methanol + H_2; ac = acetate. (H_2) indicates poor growth or growth of only some strains.

[d]Growth factors: ac = acetate; YE = yeast extract; PABA = *p*-aminobenzoate. Parentheses indicate that factors are either stimulatory for growth or required by only some strains.

[e]Bd = determined by buoyant density; Tm = determined by melting point.

[f]Culture collections: DSM = Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany); OCM = Oregon Collection of Methanogens (Beaverton, OR, USA).

Table 1.16 Characteristics of slightly halophilic or marine, obligately methylotrophic species[a]

Property	*Methanolobus* spp.				*Methanococcoides*
	tindarius	*siciliae*	*volcani*	*oregonensis*	*methylutens*
Morphology	Irregular coccus	Irregular coccus	Irregular coccus	Irregular coccus	Irregular coccus
Diameter (μm)	0.8–1.3	NR[b]	NR	1.0–1.5	0.8–1.2
Gram stain	–	–	–	–	–
Motility	+	NR	NR	–	–
pH: optimum	6.5	6.5–6.8	NR	8.1–9.1	7.0–7.5
T(°C): optimum	37	37–40	37	35–37	30–35
range	10–40	20–48	15–45	20–40	15–35
NaCl (M): optimum	0.5	0.4–0.6	NR	<0.5	0.4
range	0.05–1.3	0.1–1.5	NR	0.1–1.5	0.2–1.0
Substrates[c]	Me	Me	Me, MeS	Me, MeS	Me
Growth factors[d]	none	(YE)	NR	thiamine, (YE)	Biotin
mol% G+C[e]	40 (Tm)	42–43 (Lc)	39	41 (Lc)	42 (Tm)
Type strain	Tindari 3[f]	T4/M	PL-12/M	WAL1	TMA-10
Culture collections[g]	DSM 2278, OCM 150	DSM 3028, OCM 156	DSM 3029, OCM 157	DSM 5435, OCM 99	DSM 2657, OCM 158

[a]References: König and Stetter, 1982; Ni and Boone, 1991; Sowers and Ferry, 1983, 1985; Stetter, 1989.
[b]NR = not reported.
[c]Substrates for methanogenesis: Me = C-1 compounds such as methanol, trimethylamine, dimethylamine, monomethylamine; MeS = dimethylsulfide or methanethiol.
[d](YE) = yeast extract is stimulatory for growth.
[e]Tm = determined by melting point; Lc = determined by liquid chromatography.
[f]Also called "strain T-3."
[g]Culture collections: DSM = Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany); OCM = Oregon Collection of Methanogens (Beaverton, OR, USA).

Table 1.17 Characteristics of moderately halophilic, obligately methyltrophic species[a]

Property	*Methanohalophilus*		*Methanohalobium*	*Methanosalsus*
	halophilus	*mahii*	*evestigatum*	*zhilinaeae*
Morphology	Irregular coccus	Irregular coccus	Flat polygons	Irregular coccus
Diameter (μm)	0.5–2.0	0.8–1.8	0.2–2.0	0.8–1.5
Gram stain	–	NR[b]	NR	NR
Motility	–	–	–	–
pH: optimum	6.5–7.4	7.5	7.0–7.5	9.2
range	6.3–8.0	6.5–8.5	6.5–8.3	8.2–10.3
T(°C): optimum	26–36	35	50	45
range	<18–40	NR	20–60	NR
NaCl (M): optimum	1.2–1.5	2.0	4.3	0.7
range	0.3–2.6	0.5–3.5	2.6–5.1	0.2–2.1
Substrates[c]	Me	Me	Me	Me, MeS
Growth factors	None	NR	B-vitamins	None
mol% G+C[d]	44 (Tm)	45–49 (Tm)	37 (Tm)	38–41 (Tm)
Type strain	Z-7982	SLP	Z-7303	WeN5
Culture collections[e]	DSM 3094, OCM 160	DSM 5219, OCM 68	DSM 3721, OCM 161	DSM 4017, OCM 62

[a]References: Liu et al., 1990; Lysenko and Zhilina, 1985; Mathrani and Boone, 1985; Mathrani et al., 1988; Paterek and Smith, 1985, 1988; Whilharm et al., 1991; Zhilina, 1983, 1986; Zhilina and Svetlichnaya, 1989, Zhilina and Zavarin, 1987b.

[b]NR = not reported.

[c]Substrates for methanogenesis: Me = C_1 compounds such as methanol, trimethylamine, dimethylamine, and monomethylamine; MeS = dimethylsulfide or methanethiol.

[d]Tm = determined by melting point.

[e]Culture collections: DSM = Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany); OCM = Oregon Collection of Methanogens (Beaverton, OR, USA).

M. thermophila is the thermophilic species in *Methanosarcina* (Zinder et al., 1985; Table 1.14).

The other genera of *Methanosarcinaceae* are halophilic. *Methanolobus* and *Methanococcoides* are slightly halophilic. These genera are physiologically and morphologically similar but with some differences (Table 1.16), and they are phylogenetically distinct (Sowers et al. 1984b). *Methanococcoides* contains one species, *Methanococcoides methylutens*. A second, more halophilic species, "*Methanococcoides euhalobium*" was proposed but has not been validated. The taxonomy of this latter species is not well defined (Obraztsova et al., 1987).

Methanolobus previously contained three species (*M. tindarius*, *M. siciliae*, and *M. vulcani* [Stetter, 1989]). Sequence comparisons of 16S rRNA indicated that *Methanohalophilus oregonensis* is more closely related to *Methanolobus* species than to *Methanohalophilus mahii*, so we propose to transfer *Methanoha-*

lophilus oregonensis to *Methanolobus* as *Methanolobus oregonensis* comb. nov. *Methanohalophilus* is a genus of moderately halophilic methanogens containing two similar species, *M. mahii* and *M. halophilus* (Table 1.17). DNA reassociation studies (Wilharm et al., 1991) suggest that these two species could be considered as subjective synonyms, but unpublished studies (Boone) indicate low sequence similarity between the species, suggesting that they are not synonymous. We believe that these species should not be considered synonymous unless additional data, such as ΔT_m, support their synonymy. If these species are considered to be synonyms, *M. halophilus* has priority and *M. mahii* would be eliminated from the taxonomy. Because *M. mahii* is the type species, the genus *Methanohalophilus* might be considered to lack a type and therefore be illegitimate (although it could be conserved by a published Opinion of the Judicial Commission).

Sequence analysis of 16S rRNA indicates that *Methanohalophilus zhilinaeae* should be separated into a new genus (Figure 1.5). Therefore, we propose to

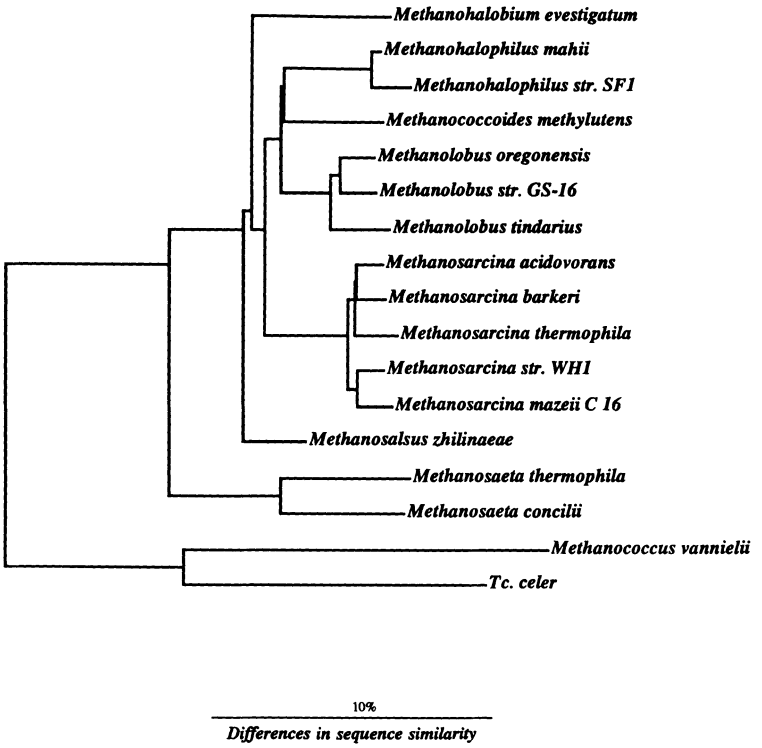

Figure 1.5. Phylogenetic relationships of *Methanosarcinales* based on partial sequences of 16S rRNA.

transfer this species to *Methanosalsus* gen. nov., as *Methanosalsus zhilinaeae* gen. nov., comb. nov.

The family *Methanosaetaceae* contains a single, aceticlastic genus, *Methanosaeta* (= *Methanothrix*). *Methanosaeta* all grow by the aceticlastic reaction, and no other substrates support growth (Table 1.18). These organisms grow within a sheath which confers a rod shape, often in chains which may be very long.

There is currently some controversy regarding the taxonomy of aceticlastic rods, whether they should be classified in the genus *Methanothrix* or *Methanosaeta*. The type species of these two competing names are *Methanothrix soehngenii* and *Methanosaeta concilii*, and there is little disagreement that these species are subjectively synonymous (Boone, 1991). *Methanothrix soehngenii* has priority, but many consider that the original description was based on an impure culture (Boone, 1991; Patel, 1992). The matter will be decided shortly

Table 1.18 Characteristics of *Methanosaeta (Methanothrix)* species[a]

	Species	
Property	*concilii* (*soehngenii*)	*thermophila*
Morphology	Rod	Rod
Dimensions (μm)	0.8×2.5–6.0	0.8–1.3×2–6
Gas vesicles	–	(+)
Filament length (μm)	>100	<100
Gram stain	–	–
Motility	–	–
pH:optimum	7.0–7.5	7
range	6–8	6.1–7.5
T(°C):optimum	35–40	55–60
range	10–45	>30–<70
NaCl(M):range	NR[b]	<0.2
Substrate[c]	ac	ac
Growth factors[d]	(biotin, thiamine, PABA)	(SF)
mol% G+C[e]	49–50 (Tm), 51 (Lc)	53–54 (Lc)
Type strain	GP6	P_T
Culture collections[f]	DSM 3671, OCM 69	DSM 6194

[a]References: Huser et al., 1982; Kamagata and Mikami, 1991; Kamagata et al., 1992; Nozhevnikova and Chudina, 1984; Nozhevnikova and Yagodina, 1982; Patel, 1984; Patel and Sprott, 1990; Touzel et al., 1988; Zehnder et al., 1980; Zinder et al., 1987.

[b]NR = not reported.

[c]Acetate (ac) is the only substrate for methanogenesis.

[d]Stimulatory growth factors indicated in parentheses. PABA = *p*-aminobenzoate; SF = sludge fluid.

[e]Tm = determined by melting point; Lc = determined by liquid chroma tography.

[f]Culture collections: DSM = Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany); OCM = Oregon Collection of Methanogens (Beaverton, OR, USA).

Table 1.19 Characteristics of *Methanopyrus* species[a]

Property	Species *kandleri*
Morphology	Rod
Cell width (μm)	0.5
Cell length (μm)	2–14
Motility	+
Flagella	Polar tufts
Gram stain	+
pH:optimum	6.5
range	5.5–7
T(°C):optimum	98
range	84–110
NaCl (M):optimum	0.3–0.4
range	<0.7
Substrate	H_2
Autotrophy	+
Growth factors	None
mol% G+C[b]	59 (Tm), 60 (Lc)
Type strain	AV19
Culture collection[c]	DSM 6324

[a]References: Burggraf et al., 1991b; Kurr et al., 1991.
[b]Tm = determined by melting point; Lc = determined by liquid chromatography.
[c]DSM = Deutsche Samlung von Mikroorganismen (Braunschweig, Germany).

by the Judicial Commission, but until then our taxonomy assumes that the description of *Methanothrix soehngenii* was based on an impure culture, and therefore this species and the genus *Methanothrix* are illegitimate.

In a related matter, *Methanothrix thermoacetophila* was proposed as a species of thermophilic *Methanothrix*, but the characterization was based on an impure culture and is illegitimate (Boone, 1991; Kamagata et al., 1992). Kamagata et al. (1992) proposed the name *Methanothrix thermophila* for thermophilic *Methanothrix*. That proposal for a species of a thermophilic, sheathed, aceticlastic methanogen is the first that was based on study of a pure culture, so this name is legitimate, regardless of the legitimacy of the genus *Methanothrix* (Rule 32b of Bacteriological Code [Sneath, 1992]). However, based on the assumption that *Methanothrix* is illegitimate, we propose to transfer this thermophilic species to *Methanosaeta* as *Methanosaeta thermophila* comb. nov.

1.3.5 Methanopyrales ord. nov.

The branch between *Methanopyrus kandleri* and other methanogens is deep enough to justify its placement in a new family (*Methanopyraceae*) and a new

order (*Methanopyrales*) (Figure 1.1). This order, family, and the genus *Methanopyrus* contain a single species, *M. kandleri*, so the description of the order and family is the same as that of the genus (Kurr et al., 1991): rod-shaped methanogens which grow near the boiling point of water and above (Table 1.19). Catabolic substrates for this order are limited to H_2 plus CO_2.

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2

Microscopy

G. Dennis Sprott and Terry J. Beveridge

2.1 Introduction

Based on molecular comparisons it is proposed that all living things should be subdivided into the three domains of Archaea, Bacteria, and Eucarya; presently referred to as the Archaeobacteria, Eubacteria and Eukaryotes (Woese et al., 1990). In a structural context, however, with the possible exceptions of ribosome shape, Archaea and Bacteria are cells that are difficult to distinguish from one another. Both bacterial domains consist of taxonomic representatives having a wide variety of shapes, sizes, and ultrastructural variations. In this chapter we review the structural data so far acquired on methanogens, much of which has appeared subsequent to other reviews (Aldrich et al., 1988; Whitman, 1985; Zeikus and Brown, 1975). Some data from our own laboratories have not yet been published.

Prokaryotic cells share the properties of a unicellular organization based on containment of cytoplasmic contents within a semipermeable cytoplasmic membrane, usually surrounded by a rigid cell wall. The archaeobacteria are equally small compared to eubacteria, presumably to provide high surface area to volume ratios and to satisfy their needs for maximum diffusion of molecules into or out of the cells (Beveridge, 1989). Each bacterium, whether eubacterial or archaeobacterial, must fabricate a design incorporating variations in size and shape which is a compromise between the need to be small and the provision of sufficient internal space to house cytoplasmic constituents essential for growth and survival (Beveridge, 1989). Perhaps all bacterial cells would appear similar except for other considerations such as the need for internal membranes (e.g., phototrophs and nitrifiers), cytoplasmic storage depots (e.g., polyhydroxybutyrate, glycogen

and polyphosphate granules), and specialized locomotion (e.g., flagella), adhesion (e.g., fimbriae and capsules), flotation (e.g., gas vesicles) or extension (e.g., prosthecae) devices. It is also possible that certain design differences are due to the materials used to manufacture or assemble a rigid envelope (e.g., S-layer protein versus pseudomurein versus methanochondroitin, etc.), and to the need to ensure division and separation of daughter cells.

2.2 Morphology

With notable exceptions much of the microscopy performed on the methanogens currently in pure culture (Part I, Chapter 1) are "first look" micrographs providing essential, but only partial data of newly described isolates. This is because most authors view descriptions of new isolates incomplete without at least some visual data to describe shape and size. Light microscopy using bright field (stained material) or phase contrast (live material) optics is excellent for this but frequently scanning electron microscopy (SEM) or transmission electron microscopy (TEM) are used to provide higher resolutions and to discern surface features, appendages, or internal detail (e.g., thin-sections).

Initially the genus name for many methanogens was descriptive of cell shape. Rod-shaped methanogens are illustrated by *Methanobacterium* spp. or *Methanopyrus kandleri* (Figure 2.1a). Very short rods of *Methanobrevibacter arboriphilicus* grown in liquid culture medium have a tendency to chain and to form longer filamentous cells when grown on agar medium (Zeikus and Henning, 1975). Isolations of coccoid methanogens are as numerous as the rod-forms. Many of these methanococci, including species of the genera *Methanogenium* (Figure 2.1c), *Methanococcus* (Figure 2.1h), *Methanocorpusculum* (Figure 2.1d), *Methanococcoides* (Figure 2.1e), *Methanolobus* (Figure 2.1f), *Methanohalophilus* (Liu et al., 1990), and *Methanoculleus* (Blotevogel et al., 1991) are irregularly shaped as a result of the pliability of the surface arrays which is a common feature to these organisms. Presumably, these S-layers are not so strongly knit together as other wall matrices (e.g., the methanochondroitin of *Methanosarcina*) and are more easily deformed. For example, integrity of the S-layer of *Methanococcoides euhalobius* requires Ca^{++} and the cells will round-up in its absence (Charak'yan et al., 1989). *Methanosphaera stadtmanae* (Figure 2.1j) appears as rounded-up cells (often in pairs, tetrads or clusters) but has a pseudomurein matrix rather than a S-layer surface (Miller and Wolin, 1985). An angular plate-shaped genus, *Methanoplanus* spp., has also been described (Figure 2.1g). Some methanogens have such a distinctive shape that they can be tentatively identified by light microscopy even in mixed cultures. These are *Methanospirillum* (long thin spirals, Figure 2.1b), *Methanosaeta* (*"Methanothrix"*) (Patel, 1992) (also long but thicker filaments, Figure 2.1i), and *Methanosarcina* (clusters of round cells, Figure 2.1, l–n).

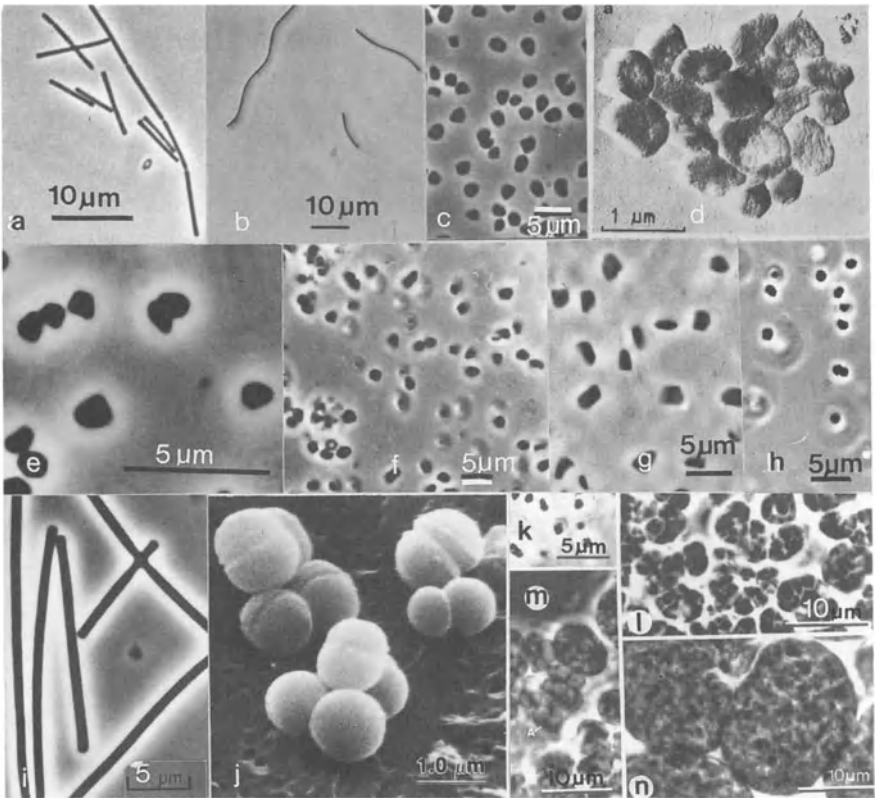


Figure 2.1. Morphology of methanogen cells. a, phase contrast micrograph of cells of *Methanopyrus kandleri* during the exponential growth phase; b, phase contrast micrograph of *Methanospirillum hungatei* GP1; c, phase contrast micrograph of *Methanogenium cariaci*; d, electron photomicrographs of plantinum-iridium-shadowed *Methanocorpusculum parvum*; e, phase contrast micrograph of *Methanococcoides methylutens* TMA-10; f, phase contrast micrograph of *Methanolobus tindarius*; g, phase contrast micrograph of *Methanoplanus limicola*; h, phase contrast micrograph of *Methanococcus thermolithotrophicus*; i, phase contrast micrograph of *Methanosaeta concilii* GP6; j, scanning electron micrograph illustrating the spherical cellular morphology and the cleavage furrow seen in rapidly dividing cells of *Methanosphaera stadtmanae*; k-n, phase contrast micrographs of *Methanosarcina acetivorans* strain C2A grown on acetate: k, single cells from an exponential phase culture; l, cell aggregates from a late exponential phase culture; m, aggregrates of large cells (A); n, communal cysts from a late exponential phase culture. Reproduced with permission. a, Kurr et al. (1991); b, Patel et al. (1976); c, Romesser et al. (1979); d, Zellner et al. (1987); e, Sowers and Ferry (1983); f, Konig and Stetter (1982); g, Wildgruber et al. (1982); h, Huber et al. (1982); i, Patel (1984); j, Miller and Wolin (1985); k-n, Sowers et al. (1984).

Even as early as 1936, Barker's photographs of *Methanosarcina mazei* revealed multiple forms (Barker, 1936). *M. acetivorans* grown on acetate (Figure 2.1, k–n) contain single spheroidal-shaped cells in exponential phase cultures which cluster into small and large cell aggregates of 2–12 cells (pseudosarcinae) during late exponential phase; sometimes the cells themselves are also enlarged. Stationary phase cultures form predominantly communal cysts composed of single cocci enclosed within a common envelope. Possible resting forms (microcysts) with thickened cell walls are found in more aged cultures (Barker, 1936; Robinson, 1986). Evidence presented by Maestrojuán & Boone (Meistrojuán and Boone, 1991) generally favors aggregation as a survival mechanism during unfavorable growth conditions. It is possible that cells clustered together generate a common microenvironment which is more suitable for survival. Some cells within the aggregate may even survive at the expense of others. Disaggregation to single cells is associated with the renewed onset of exponential growth and faster growth rates (Boone and Ma, 1987). *M. mazei* S-6 and LYC produce a regulated disaggregatase whose activity is responsible for conversion of aggregates to single cells (Xun et al., 1990). The purified enzyme has endopolysaccharide hydrolase activity against the methanochondroitin "matrix" that encompasses cells and binds them together. The matrix stains with ruthenium red which can be indicative of acidic mimicopolysaccharides.

An unshaken exponential growth of *M. mazei* S-6 in 40 mM trimethylamine medium containing 8.3 to 15.6 mM Ca^{++} or Mg^{++} forms visible, flat sheets of cells called "lamina" connected by intercellular connective material (Figure 2.2;

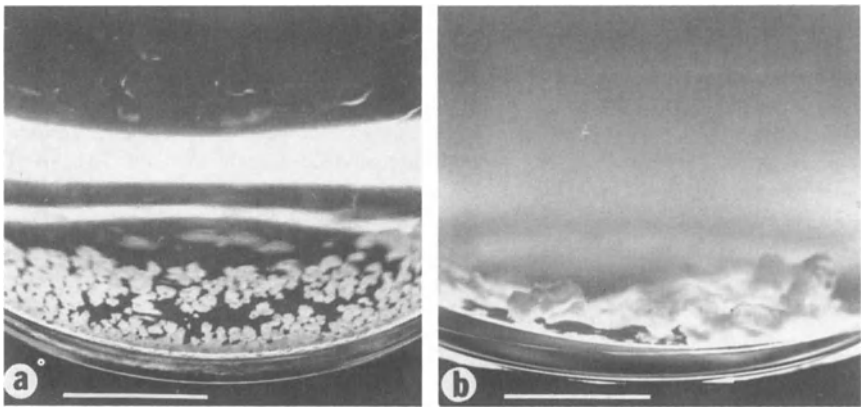


Figure 2.2. *Methanosarcina mazei* S6 forms in culture bottles (frontal view). a, packets; b, lamina, kept in the same medium for several days, at which time dissociated cells and very small packets made the supernatant turbid. bars = 1 cm. Reproduced with permission, Mayerhofer et al. (1992).

Mayerhofer et al., 1992). This formation was restricted to this strain and was associated with the expression of a cell surface antigen.

Methanogens are generally free-living but may, in some cases, be endosymbionts of eukaryotic cells. Microscopy has aided in identifying the endosymbiotic relationship found between *M. formicicum* and an anaerobic protozoon *Metopus striatus* (van Bruggen et al., 1984).

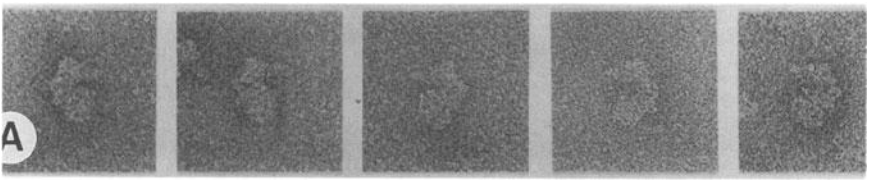
2.3 Ribosome Structure

Electron microscopy of small ribosomal units (Lake et al., 1982) has revealed three different shapes corresponding to the three lineages established by rRNA sequencing (Woese and Fox, 1977). Two distinctive features, namely, the presence of an archaeobacterial "bill" on the head of the small subunit and the absence of eukaryotic lobes at the base of this subunit established the distinctiveness of the archaeobacterial ribosome. The 30 S subunit of *M. thermoautotrophicum* is shown in different orientations in Figure 2.3A to illustrate this characteristic shape. The small subunit of the eukaryotic ribosome differs from that of archaeobacteria by exhibiting both the archaeobacterial bill and the eukaryotic lobes, whereas that of eubacteria lack both of these features. Generalized profiles of these three ribosomal small subunit shapes are shown (Figure 2.3B). Sulfur-dependent archaeobacteria may be distinguished from other archaeobacteria by the presence of both archaeobacterial bill and basal lobes on the small subunit and of a characteristic lobe, gap and bulge on the large subunit (Henderson et al., 1984).

2.4 Cell Envelopes

The cell envelopes of methanogens may be divided into at least five types (Kandler and Konig, 1985) if we exclude the report of the mycoplasma-like *Methanoplasma elizabethii* (Rose and Pirt, 1981) which is still of uncertain status and which is apparently unavailable for culture verification. The recognition that methanogens lack murein typical of eubacteria, and that they are a rich source of various S-layer arrangements, has increased attention to their cell envelopes. Reviews on archaeobacterial envelopes have previously appeared (Baumeister et al., 1989; Kandler and Konig, 1985; Konig, 1988; Konig and Stetter, 1986; Sleyter et al., 1986). Here we restrict ourselves not only to methanogens, but also mainly to the progress made on methanogen surface features through use of electron microscopy. A summary of the five envelope types presently authenticated is shown in Table 2.1.

In stained thin-sections pseudomurein appears as an amorphous electron dense



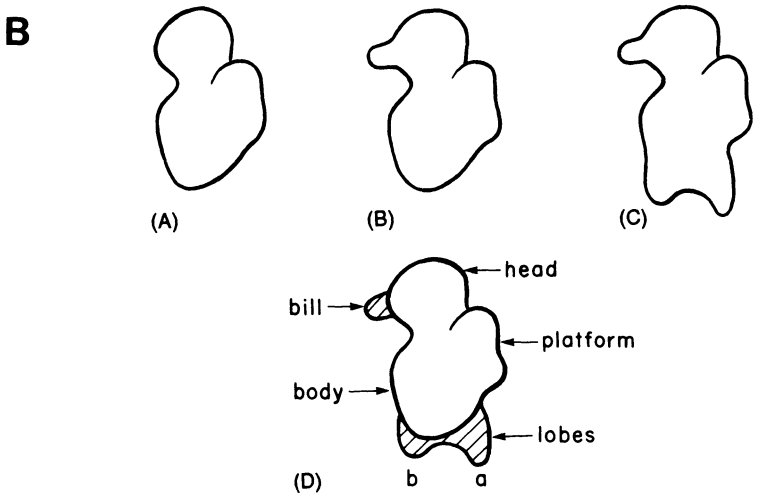


Figure 2.3. Electron micrographs and generalized profiles of small ribosomal subunits from archaeobacteria. In the field of A, small subunits from *Methanobacterium thermoautotrophicum* illustrate the asymmetric projection of small subunits. B, Generalized profiles of small ribosomal subunits, in the asymmetric profile. The eubacterial, archaeobacterial, and eukaryotic profiles are shown in A, B, and C, respectively. The common ribosomal regions are named in D, and the archaeobacterial eukaryotic lobes (a and b) and bill are shown in diagonal stripes. Reproduced with permission, Lake et al. (1982).

Table 2.1 Envelopes of methanogens

Envelope[a] Type	Methanogen	S-layer kDa-Monomer[b]		Lattice	Spacing (nm)	Reference[c]
1. RS-layer + CM	*Methanococcus voltae*	76	NG	H	10	1
	M. vannielii	60	NG	H	10.8	2
	M. thermolithotrophicus	82.5	NG	H	9.8	2
	M. jannaschii	90	NG	H	10.5	2
	M. aeolicus	—	—	H	12.3	3
	Methanocorpusculum sinense	92	G	H	16	4
	Methanoplanus limicola	143	G	H	14	5
	Methanolobus tindarius	156	G	H	12	6
	Methanococcoides methylutens	—	—	—	—	7
	Methanoculleus thermophilicus	130	G	H	—	8
	M. marisnigri	138	G	H	—	8,9
	Methanogenium liminatans	118	G	H	15.4	10
	M. tatii	120	G	H	—	11
	M. cariaci	117	NG	H	—	8,9
	Methanohalophilus oregonense	—	—	—	—	12
	Methanomicrobium mobile	—	—	—	—	13
	Methanosarcina acetivorans	—	—	—	—	14
2. MC + S-layer + CM	*Methanosarcina mazei*	—	—	—	—	15,16
3. RS-layer + P + CM	*Methanothermus fervidus*	76	G			17
	M. sociabilis	76	G			17
	Methanopyrus kandlerii	—	—	—	—	18
4. Sheath + S-layer + CM	*Methanospirillum hungatei* GP1	12	G	P2	2.8	19,20
	M. hungatei JF1	—	—	P1	2.5	21
	JF1	—	G	P2	2.8	22
	Methanothrix soehngenii FE		G	—	—	23
	Methanosaeta concilii GP6		G	P2	2.8	22
5. P + CM	*Methanobacterium*					
	Methanosphaera					
	Methanobrevibacter					

[a]RS, regularly structured; CM, cytoplasmic membrane; MC, methanochondroitin; P, psudomurein.
[b]NG, non-glycosylated; G, glycosylated.
[c]References: 1, Koval and Jarrell, 1987; 2, Nuber and Konig, 1987; 3, Sleytr et al., 1986; 4, Zellner et al., 1989; 5, Wildgruber et al., 1982; 6, Konig and Stetter, 1986; 7, Sowers and Ferry, 1983; 8, Zabel et al., 1985; 9, Romesser et al., 1979; 10, Zellner et al., 1990; 11, Zabel et al., 1984; 12, Liu et al., 1990; 13, H. Konig, personal communication to Sleytr et al., 1986; 14, Sowers et al., 1984; 15, Aldrich et al., 1986; 16, Kreisl and Kandler, 1986; 17, Brockl et al., 1991; 18, Kurr et al., 1991; 19, Sprott et al., 1986; 20, Stewart et al., 1985; 21, Shaw et al., 1985; 22, Patel et al., 1986; 23, Pellerin et al., 1990.

layer about 15–20 nm thick covering the cell and, therefore, resembles the wall profile of its eubacterial counterparts (e.g., *Bacillus subtilis*). These pseudomurein-containing walls require chemical analysis to distinguish them from the murein-containing ones of eubacteria. Fundamental chemical differences include the substitution of D- by L-amino acids in the peptide cross-linkages and replacement of *N*-acetylmuramic acid by *N*-acetyl-L-talosaminuronic acid in the glycan strands. Also, the linkage configuration (between *N*-acetyl-D-glucosamine and *N*-acetyl-L-talosaminuronic acid) in the glycan strands is $\beta(1,3)$ rather than $\beta(1,4)$ as in murein (Kandler and Konig, 1985; Konig, 1988).

Most of those methanogens which do *not* possess pseudomurein have at least one paracrystalline array, and these are usually hexagonal possessing p6 symmetry with center-to-center spacings varying among species from 10–16 nm (Table 2.1). *M. voltae*, which is a moderate halophile and which undergoes cell lysis without adequate protection by salts, has been especially difficult to stain and to demonstrate its hexagonal array (Koval and Jarrell, 1987). Since 2% NaCl is incompatible with heavy metal negative stains, prefixation with 2% glutaraldehyde was attempted prior to staining, but unsuccessfully. The array was eventually demonstrated in freeze-etch replicas of cells and envelope preparations (Koval and Jarrell, 1987).

Based on the periodic acid-Schiff staining reaction of the protein monomers following separation on SDS-PAGE, it is apparent that many S-layer proteins are glycosylated (Table 2.1) whereas others are not (e.g., *Methanococcus* spp.). The S-layer protein of *M. cariaci* is an exception for the *Methanogenium* genus, since it is also not glycosylated. Monomer molecular masses vary among strains from 60 to 156 kDa. The cell wall of *M. euhalobius* is unusual, since it consists of a protein S-layer over which lies a polysaccharide layer composed of glucose and mannose (Charakhch'yan et al., 1991). The external layer is more abundant on cells growing on trimethylamine than on methanol and is excreted from the cells to form an external film which the cells may use to attach to solid surfaces. *M. acetivorans* is included in this grouping because in thin-sections only a narrow 10 nm cell wall is seen. This layer appears to be proteinaceous based on lytic susceptibility to detergents such as Triton X-100 and sodium dodecyl sulfate (SDS) (Sowers et al., 1984).

The S-layer in *M. mazei* is a 12 nm thick layer removed by pronase (supporting its proteinaceous nature) and is connected to the cytoplasmic membrane by regular dense bridges (Aldrich et al., 1986; Robinson, 1986). Most *Methanosarcina* spp. are surrounded by a polymeric network, either loosely or more tightly knit, of methanochondroitin which is external to a single S-layer similar to that described for *M. acetivorans*. Because of the loose nature of the methanochondroitin fibrils in *M. mazei* the term "matrix" was proposed to replace its initial description as a wall (Aldrich et al., 1986). The thickness of this external matrix can be up to 200 nm in *M. barkeri* (Konig, 1988) or 60 nm in *M. mazei* S6 (Robinson, 1986).

Thin-sections of the isolated matrix devoid of intact cells showed that this is a rigid layer since it still retained the shape of the cells (Kreisl and Kandler, 1986). Chemical analysis revealed a non-sulfated polymer of *N*-acetylgalactosamine, D-glucuronic (or D-galacturonic) acid, minor amounts of D-glucose, and traces of D-mannose; this is chemically similar to the chondroitin of eukaryotes and hence its name "methanochondroitin" (Kreisl and Kandler, 1986). Methanochondroitin is responsible for cell-cell adhesion, since its loss mediates disaggregation and dispersal of cells (Xun et al., 1990).

Methanosarcina spp. can be induced to grow in marine medium (K. Sowers, personal communication), which results in growth as single cells. Thin-section electron micrographs reveal these single cells to be devoid of methanochondroitin.

Two species of *Methanothermus* have a pseudomurein layer encompassed by a glycoprotein S-layer (Table 2.1). Both genes encoding these glycoproteins have been cloned and sequenced (Brockl et al., 1991). Compared to others, this S-layer contains exceptionally high amounts of isoleucine, asparagine, and cysteine. The predicted secondary structure contains only 7% α-helix and has high β-sheet content (44%), which presumably adds intermolecular bonding to the layer which is necessary to withstand the hyperthermophilic environment of these methanogens. Carbohydrate comprises 17 mol% of the S-layer of *M. fervidus* and consists of mannose, 3-*O*-methylglucose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine (Hartmann and Konig, 1989).

Methanopyrus kandleri (gen. and sp. nov.) with an optimum growth temperature of 98°C has a protein layer external to a newly identified type of pseudomurein (Kurr et al., 1991). Ultrathin sections of cells treated with SDS revealed that the S-layer was removed by the detergent. Further chemical analysis has yet to be done.

Early studies employing thin-sections of *M. hungatei* JF1 (Zeikus and Bowen, 1975) and an enriched culture of *M. soehngenii* (Zehnder et al., 1980) revealed unique ultrastructures. Both methanogens consisted of chains of cells within a sheath (initially called the outer wall) of unusual striated surface appearance. The cells were spaced apart by multiple lamellae (spacer plugs) in *M. hungatei* and in the case of *M. soehngenii* by a plug composed of concentric rings.

The remarkable resilience of these sheath structures to chemical reagents was used to advantage in obtaining highly purified preparations for chemical and ultrastructural analysis (Beveridge et al., 1985; Patel et al., 1986; Sprott and McKellar, 1980). *M. concilii* cells were lysed by exposure to 5 M NaOH at room temperature followed by treatment with 1% SDS at 100°C (Patel et al., 1986). Plugs in the process of ingrowth are often an integral part of these sheaths (Figure 2.4). A milder alkali treatment of 0.1 M NaOH followed by SDS was adequate to isolate sheath tubes from *M. hungatei* (Patel et al., 1986; Sprott and McKellar, 1980). Sheath may also be isolated from *M. hungatei* following removal of spheroplasts from the sheath tubes with dithiothreitol at alkaline pH (Sprott et

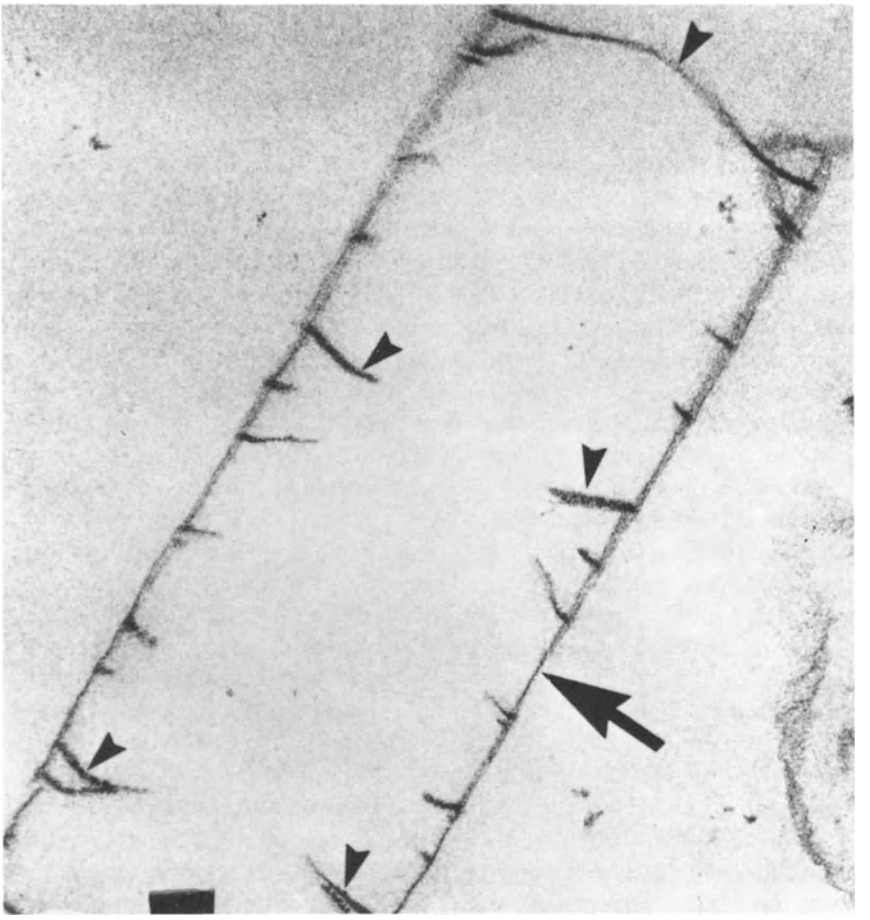


Figure 2.4. Thin section of an isolated and purified sheath from *Methanosaeta concilii* showing partial and complete spacer plugs still attached. The arrowheads point to representative spacer plugs. Large arrow points to the sheath. bar = 120 nm. Reproduced with permission, Patel et al. (1986).

al., 1979). In this case, the sheath is recovered from protoplast lysates by discontinuous sucrose gradient centrifugation, and spacer plugs removed from the isolated sheath by treatment with 0.1 M NaOH (Beveridge et al., 1985; Sprott and McKellar, 1980).

The striated nature of the isolated sheath surface and the sheath's tendency to split perpendicular to its long axis during negative staining for electron microscopy is illustrated in Figure 2.5A for *M. hungatei*. This suggested the sheath was composed of hoops stacked together rather than in coiled configuration. Analysis of occasional single hoops by negative stain showed that a surface array was present on a backing matrix (Figure 2.5C; Stewart et al., 1985). Positive proof for the concept of stacked hoops forming a hollow tube was obtained when it was discovered that the use of β-mercaptoethanol at 90°C dramatically disassembled the tubes into their free hoop form (Figure 2.5B). Other thiols tested, including dithiothreitol, were relatively ineffective. The strong attachment of hoops to each other and the release of short "glue peptides" during hoop disassembly by β-mercaptoethanol and heat, suggested a covalent linkage perhaps via a peptide with sensitive disufide bonds (Sprott et al., 1986).

One of the disadvantages of electron microscopy is the difficulty of obtaining high resolution topographic analyses of surfaces. Negative stains surround and penetrate biological structures such as the sheath (Figure 2.5A) and it is therefore difficult to decipher whether or not the hoop junctions are actual circumferential depressions on the outer face, inner face, or both, or whether they are loosely-knit regions capable of high stain absorption. Scanning tunneling microscopy (STM) is a newly developed technique that can produce atomic images of hard inanimate surfaces such as graphite and the technique is being adapted to provide submolecular views of biological structures. A new "hopping mode" STM was used to analyze both inner and outer faces of the *M. hungatei* sheath and showed, surprisingly, that the inner face had greater topography than the outer face (Beveridge et al., 1990) On the inner face deep grooves were found at hoop boundaries, and ridges corresponding to the surface 2.8 nm repeat were seen where the hoops resided. The hoop junctions on the outer face were not as deep, and the 2.8 nm lattice could just barely be discerned from the relatively smooth background (Beveridge et al., 1990). Presumably, the abrupt angular discontinuity between subunit particles and intersubunit depressions were too extreme for detection by STM; clearly, the 2.8 nm lattice exists at the outer face, since it is easily seen at the periphery of isolated hoops by electron microscopy (Figure 2.5B). This subunit periodicity can also be detected by selected area electron diffraction (SAED) as well as the cross-β folding of 0.46 nm within the particles themselves (Stewart et al., 1985). Presumably, most molecular folding within the subunit particles is cross-beta, and this is the prime reason for the sheath's high resiliency to physical, chemical and enzymatic perturbants (Beveridge et al., 1985).

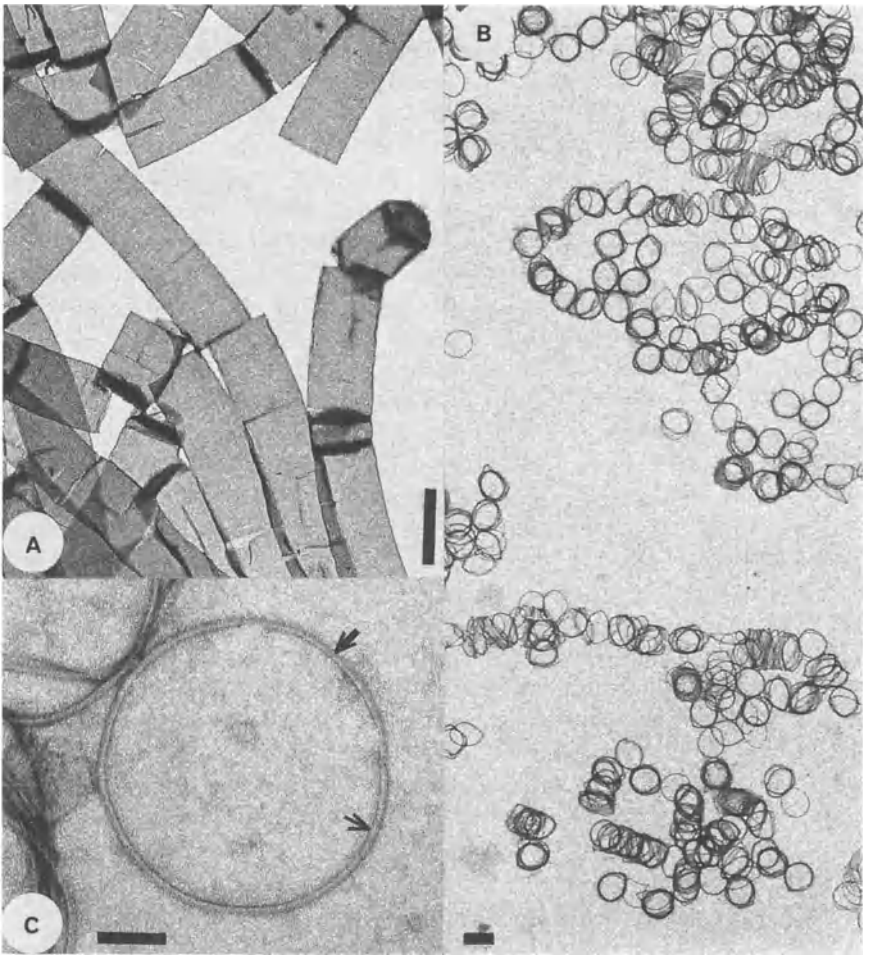


Figure 2.5. Electron micrographs of negatively stained preparations of the *Methanospirillum hungatei* GP1 sheath before (A) and after (B and C) treatment with 2% -mercaptoethanol (90°C, 30 min). A and B, bar = 500 nm; C, bar = 100 nm. Note the presence of the surface array and the circular nature of the hoops. Reproduced with permission, Sprott et al. (1986).

The surface array on the sheaths of isolated tubes and cells of *M. hungatei* strains GP1 and JF1 and *M. concilii* were compared by SAED and found to be indistinguishable from each other (Patel et al., 1986)(Figure 2.6). All sheath types had identical subunit arrangements with p2 symmetry (a = 5.6 nm, b = 2.8 nm, and γ= 86°) as previously found in *M. hungatei* GP1 (Stewart et al., 1985).

Purified sheath or hoops of *M. hungatei* GP1 and JF1 were solubilized rapidly at 90°C in arginine-KOH buffer, pH 12.6, or by β-mercaptoethanol plus SDS, pH 9.0 (Sprott et al., 1986). HPLC analysis of solubilized hoops revealed major polypeptides of 12, 24, and 45 kDa suggesting that two copies of the 24 kDa polypeptide form the two domains of the 5.6 × 2.8 nm unit cell as a dimer. The 2.8 nm particle may itself be a dimer of the 12 kDa protein. Compared to other S-layers the center-to-center spacing of the sheath array is very small (Table 2.1), suggesting a relatively impermeable structure (Shaw et al., 1985; Stewart et al., 1985). The pores through the sheath of *M. hungatei* JF1 were estimated to be no more than 2 nm.

Although the subunit arrangement of the sheaths of *M. hungatei* GP1 and JF1 and *M. concilii* are similar (Figure 2.6) each sheath differs in resistance to dissolution by β-mercaptoethanol-SDS, pH 9.0, at 90°C and by 0.1 M NaOH at 80°C. Marked chemical differences occur as well with respect to amino acid, sugar, and metal ion analyses (Patel et al., 1986). Sheath isolated from an enriched culture called *M. soehngenii* strain FE was found to be soluble in anhydrous hydrazine, suggesting that amide linkages between subunits may be responsible for the resilience of this structure (Pellerin et al., 1990). Oligosaccharides were released which were glycan chains of rhamnose, ribose, fucose, and hexoses with branches from rhamnosyl and ribosyl chains (Debeire et al., 1988). The glycans appeared to be linked to Asn-X-Ser glycosylation sites by asparaginyl-rhamnose linkages.

Variations in the envelope chemistry among methanogens has been exploited for the production of specific monoclonal antibody probes. A monoclonal antibody prepared against *M. hungatei* JF1 was specific for a determinant found in the sheath of strain JF1, and did not react with other methanogens including strain GP1 of the same species (Conway de Macario et al., 1986). Similar strain specificity was found for a monoclonal antibody prepared against *M. cariaci* JR1c. Monoclonals recognizing five antigenic determinants of the pseudomurein structure of *M. thermoautotrophicum* vary from recognizing a common antigen to having species and even strain specificities (Conway de Macario et al., 1983). A panel of monoclonals prepared against a wide variety of methanogen surface epitopes is now available for strain typing (Conway de Macario and Macario, 1986).

Recently, the chemical resiliency of *M. hungatei* GP1's sheath has been re-examined using a battery of chemical denaturants in combination with one another

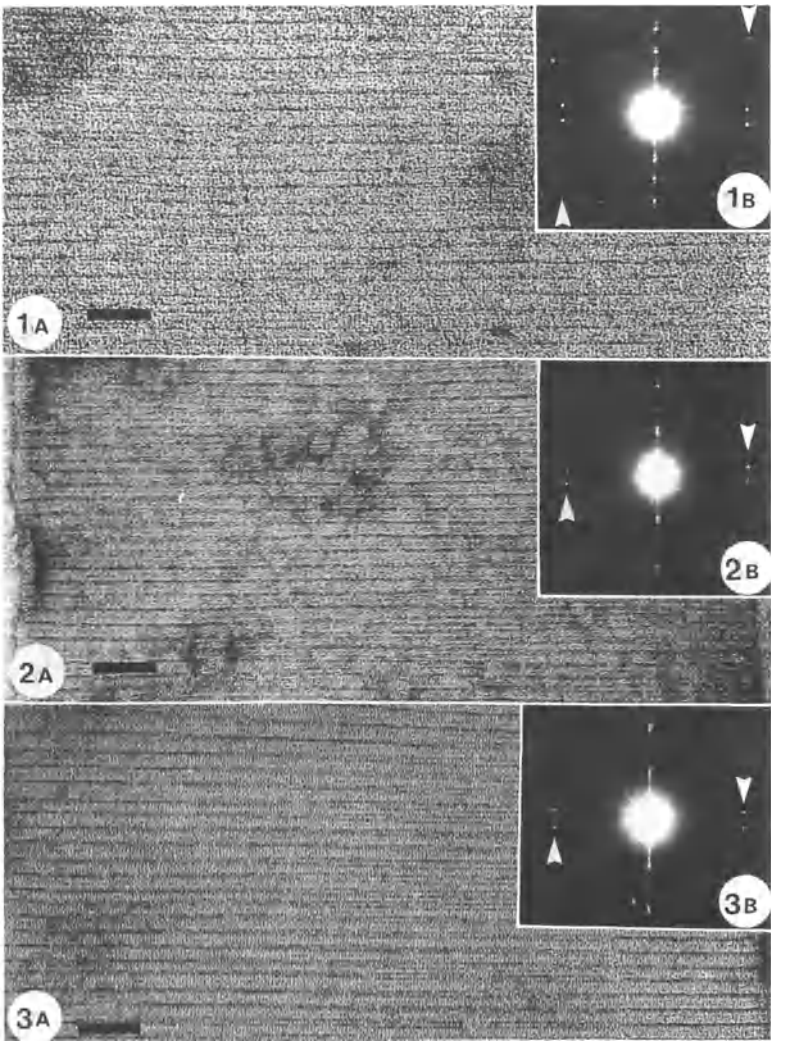


Figure 2.6. 1A, High magnification of a negatively stained *Methanosaeta concilii* sheath. The broad striations are the hoops from which the sheath is composed; the long axis of the sheath runs from top to bottom on the page. The fine pattern within each hoop is the result of the small subunits which make up the hoop and it is these that produce the 2.8 nm spacing seen in diffraction patterns. Since this image and the following images are of collapsed tubes of sheath, there are actually two layers of sheath which superimpose on one another to give a moiré. Bars = 50 nm; 1B, Electron diffraction pattern from a sheath similar to that seen in 1A. The 2.8 nm row line is pointed out; 2A, similar to 1A, but this is of the *Methanospirillum hungatei* GP1 sheath; 2B, electron diffraction pattern from GP1 sheath; 3A, similar to 1A, but this is of the *Methanospirillum hungatei* JF1 sheath; 3B, electron diffraction pattern from JF1 sheath. The spacings of all three diffraction patterns (1B, 2B, and 3B) are virtually identical, with a = 5.6 ± 0.1, b = 2.8 ± 0.1, and γ = 86 ± 2°. Reproduced with permission, Patel et al. (1986).

and at elevated temperatures. Three dissolution procedures were determined which produced a range of soluble products (Southam and Beveridge, 1991). Use of 0.05 M L-arginine buffer (pH 12.6) at 90°C for 10 min solubilized 74% by dry weight of the sheath; however, these solubilized polypeptides were extensively degraded. Two other methods were more successful. 2% β-mercaptoethanol in 2% SDS at 90°C in 0.005 M CHES buffer (pH 9.0) released a group of 30–40 kDa polypeptides which represented 42% (dry weight) of the sheath mass, whereas 5% β-mercaptoethanol plus 20 mM EDTA in 2% SDS at 100°C for 2 hours released 74% of the mass (dry weight) as 10–40 kDa polypeptides. None of the polypeptides released by these two regimens were degraded and none possessed glyco substituents. When polyclonal and monoclonal antibodies were raised against these extracted proteins and immunogold labeling for electron microscopy performed, distinct proteins were shown to reside on different faces of the sheath (Southam and Beveridge, 1991). Accordingly, these observations suggested that these constituent proteins were asymmetrically aligned across the thickness of the sheath, that presumably the ~45 kDa protein previously identified by Sprott et al. (1986) does not extend entirely across the thickness, and that there must be at least two molecular protein layers within the sheath.

Later research revealed the sheath's structure to be even more complicated. When suspended in 90% (wt/vol) aqueous phenol, another group of polypeptides representing 19% (dry weight) of the sheath was recovered which differed from those solubilized by the other regimens (Southam and Beveridge, 1992). Once these were extracted from the sheath, the sheath's rigidity was lost. Immunogold labeling of intact, unextracted sheath with antibodies specific for the phenol soluble proteins revealed these proteins to be below both the inner and outer faces of the sheath (Southam and Beveridge, 1992). Consequently, instead of a two molecular thick sheath as proposed initially by Southam and Beveridge (1991), now they propose a three molecular layered structure in which the phenol soluble products lie at the middle of the sheath to rigidize it. These products are asymmetrically overlaid, on each side, by the polypeptides which were extracted by the 1991 regimens.

Methanospirillum possesses other paracrystalline structures besides the sheath. One of these structures is the plugs of the cell spacers (Figure 2.7e,f) which consist of at least three paracrystalline plates. Treatment with 0.1 M NaOH releases the plugs from strain GP1 and they can be separated into two structural types designated the particulate and holey layers or plates (Beveridge et al., 1991). Two were particulate composed of 14 nm particles with a spacing of $a=b=18$ nm and the third was a holey layer with 12.5 nm holes sandwiched between the two particulate layers (Figure 2.8). Optical diffraction and computer reconstruction of aligned particulate and holey layers gave a center-to-center spacing of 18 nm, three to six times larger than that of the sheath. This larger spacing makes the combined permeability of the plates much greater than that of

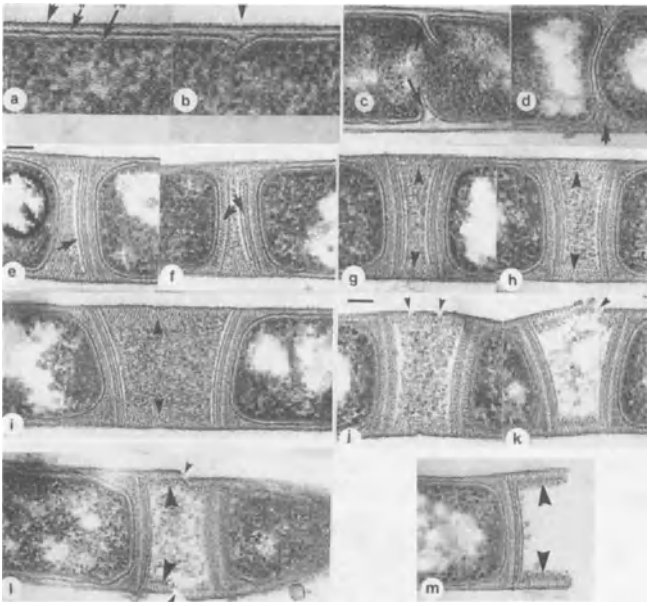


Figure 2.7. Thin sections of *Methanospirillum hungatei*. a, enveloping layers (S, sheath; W, wall; PM, plasma membrane) bar = 100 nm; b, The first recognizable stage of division is a slight indentation of the plasma membrane and wall (arrowhead). bar = 100 nm; c, a more advanced stage of septation. The arrows point to the ingrowing wall. Bar = 100 nm; d, the septum has split into two separate walls. The arrows point to the amorphous material filling the gaps between the two new walls. bar = 100 nm; e-i, outline of the growth of the cell spacer. After the amorphous material has completely filled in the gap between the two new polar walls produced by the septum, the gap gets larger as more and more material enters. At one pole, material rapidly organizes into periodic layers with an 18.0-nm repeat (e). Once complete (usually three electron-dense layers are formed), similar layers are organized at the adjacent pole (the arrows point to the layers at each pole in f). These periodic layers compose the plugs of the cell spacers. With time, the cell spacer grows larger owing to the incorporation of amorphous material into its central cavity and sheath extension. At the same time, an 18-nm network is laid down at the inner sheath surface (arrowheads in g, h, and i). Eventually, the spacer can obtain a length equal to the cell diameter (ca. 500 nm). In practice, spacer size is a function of the turbulence of the growth environment (high turbulence produces short chains of cells and, therefore, smaller cell spacers). g-i show the sequence of cell spacer expansion in a low turbulence culture. Figures e-i are the same magnification; bar in e = 100 nm. Figures j-m represent the sequence of events leading to filament splitting. Localized holes appear in one (or more) side of the sheath at a cell spacer midpoint (see small arrowheads in j). The central mass of amorphous material leaks out (see small arrowheads in k and l) and, eventually, a split occurs around the entire circumference of the sheath separating the two halves of the filament (l). Although the amorphous spacer material leaks out during the splitting, the 18-nm network on the inner sheath surface remains intact (see large arrowheads, l and m). Occasionally, filament ends are found in close proximity to each other as if they were once part of the same cell spacer. Figures j-m are the same magnification; bar in j = 100 nm. Reproduced with permission, Beveridge et al. (1986).

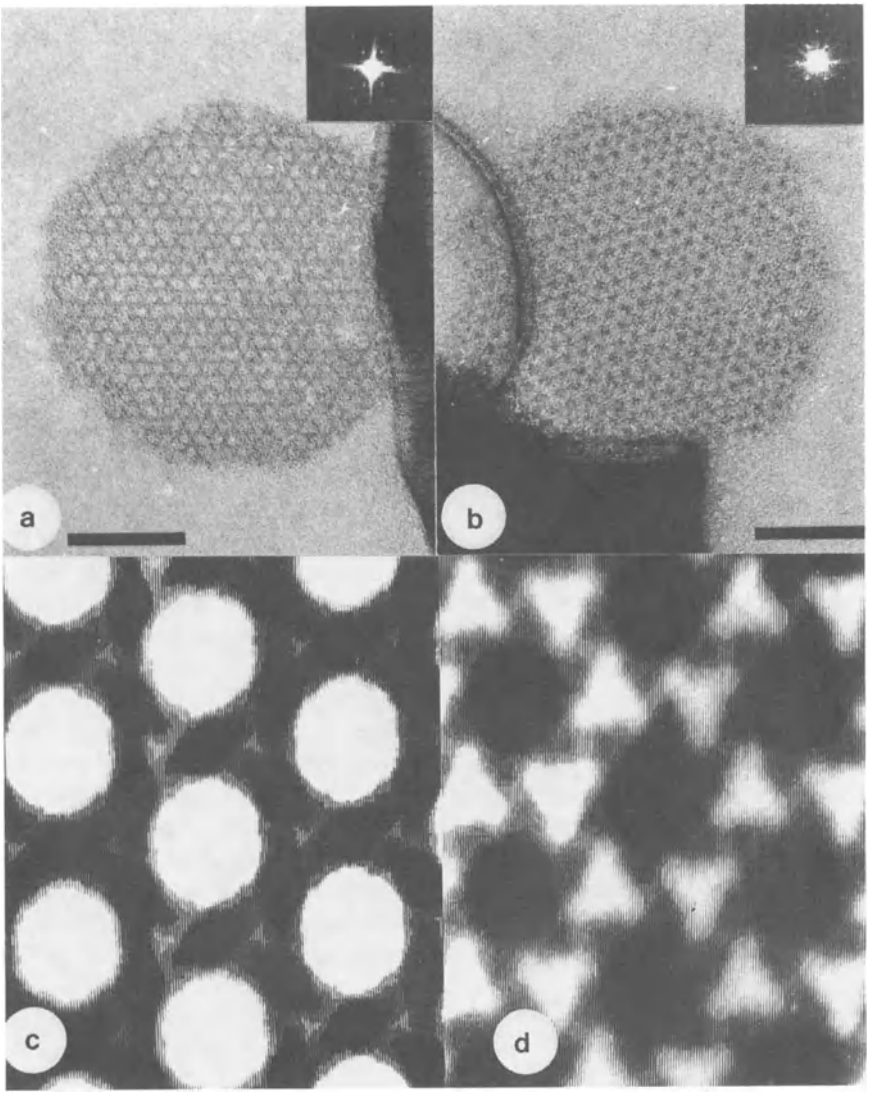


Figure 2.8. Two separated layers which form the plugs of *Methanospirillum hungatei*. a, high-resolution micrograph of the particulate layer and its optical transform. Bar = 100 nm. b, high-resolution micrograph of the holey layer and its optical transform. Bar = 100 nm. c, computer reconstruction of the particulate layer at high magnification. The subunit particles are white, and the thin arms extending from each particle are gray. The holes are black. Center-to-center spacing = 18.0 nm. d, computer reconstruction of the holey layer at high magnification. The subunit particles are delta-shaped units linked to one another by delicate gray linkers at each tip. The holes are black. Center-to-center spacing = 18.0 nm. Reproduced with permission, Beveridge et al. (1991).

the sheath. In fact, this permeability difference is emphasized by the way in which each filament of cells reacts to the Gram stain. Only the cells at each filament's termini stain gram-positive; the rest of the filament is gram-negative. This difference was confirmed by using a modified Gram reaction in which Gram's iodine is replaced by a platinum compound, so that the Gram stain can be followed by electron microscopy (Davies et al., 1983). The result was that precipitates of crystal violet-platinum formed only near the filament termini. Crystal violet and the platinum compound as individual reactants could not penetrate through the sheath to stain all cells in the filament because of the sheath's low permeability. They could only enter the terminal cells via the more permeable cell plugs at the filament termini, where they formed large purple precipitates which could not be washed out by ethanol decolorization (Beveridge et al., 1991). Carbol fuchsin, as a counterstain, complexes with the sheath and stains the rest of the filament red. Triton X-100 is also too large to penetrate the sheath, enters through the terminal plugs and causes cells to lyse within the filament beginning with the cells at the termini (Beveridge et al., 1991). These results indicate that all but very small molecules enter only through the end plugs and are restricted from the other cells within the filament by diffusion rates along the filament axis.

The cell wall (Figure 2.7a), which entirely encompasses each individual cell, resides underneath the sheath and has proven difficult to purify or analyze. Following spheroplast formation with dithiothreitol the wall is retained on deformed cells as a flexible layer (Beveridge et al., 1987). In filaments treated with β-mercaptoethanol, intact cells complete with wall can be extruded from the sheath and these retain the rod-shape of the cell; the cell wall on these cells is a paracrystalline array with p6 symmetry (T. Beveridge, G. Patel, and G. Sprott, unpublished data). This methanogen has the capacity, therefore, of forming envelope arrays of p2 and p6 symmetry plus at least two different types of paracrystalline plug layers.

2.5 Cell Division

2.5.1 M. hungatei

From the first observations of thin-sections it was apparent that the ultrastructure of *M. hungatei* was unique and that during the division process the wall and cytoplasmic membrane invaginated with either true septum formation as in gram-positive eubacteria or by constriction common among the gram-negative eubacteria (Zeikus and Bowen, 1975). Beveridge et al. (1987) confirmed and extended these observations by presenting a sequence of events in cell division which is novel amongst prokaryotes (Figure 2.7) and which showed septation as only a

part of the division process. Cells varying in length between 3.5 to 7.0 μm were seen randomly distributed along the filament. It appeared that the shortest cells were newly formed daughter cells, intermediate length cells were growing cells, and the longest cells were those undergoing division. Division required two separate events, septation and a complex process of filament splitting. Like septation in gram-positive eubacteria, the first event in time was the ingrowth of the wall at the middle of the longer cells within the filament. As the septum grew inward (Figure 2.7b) it gradually separated into two layers starting at the periphery of the cell (Figure 7c). Eventually, the two new daughter cells separated and two new cell poles were formed (Figure 7d). Amorphous material of unknown chemistry began to infiltrate the gap between the new cell poles (Figure 7d). The cells began to move apart and a set of three paracrystalline plates (a single plug) with 18 nm periodicity was laid down at one cell pole (Figure 7e) followed by plug assembly at the adjacent pole (Figure 7f). Since no intermediate steps in plug assembly were seen, the process was either very rapid or unstable until completed. Sheath extension followed to elongate the spacer region and was accompanied by the formation of an 18 nm periodic network parallel to the sheath (Figure 7g–i). When spacers extended to ~0.5 μm in length small breaks appeared in the sheath (Figure 7j). The amorphous material within the spacer region leaked out (Figure 7k), and the filament split apart at the spacer region (Figure 7l) forming two new filament termini (Figure 7m). Since the wall appeared to be a flexible layer retained on the rounded-up cells following spheroplast formation in alkaline buffer containing dithiothreitol and sucrose, it was difficult to rationalize how septation could occur without the input of a rigid wall (Beveridge et al., 1987). It now appears that the above spheroplasting conditions destroyed the normal periodicity and rigidity of the wall, thus solving the dilemma (T. Beveridge and G. Sprott, unpublished data). Although the wall can maintain cell shape following cell removal from sheath by β-mercaptoethanol treatment, it has yet to be successfully isolated for chemical analysis.

2.5.2 M. concilii

Cell division in *M. concilii* is also unusual and is a second example of the uniqueness of these division processes in methanogens (Beveridge, Harris, et al., 1986; Zehnder et al., 1980.). Thin-sections of *M. concilii* GP6 (Beveridge, Patel, et al., 1986) showed chains of rod-shaped cells aligned within a sheath; the cells were separated from one another and from the sheath by an amorphous, granular matrix of unknown chemistry or function (Figure 2.9a,b). The granular nature of the amorphous material was verified in negative stains, thin-sections and freeze-etchings. A 10 nm thick cytoplasmic membrane layer lying beneath the matrix had the unusual property of staining asymmetrically and exhibiting an extremely electron dense outer layer about 4nm deep (Figure 2.9b). This staining effect is

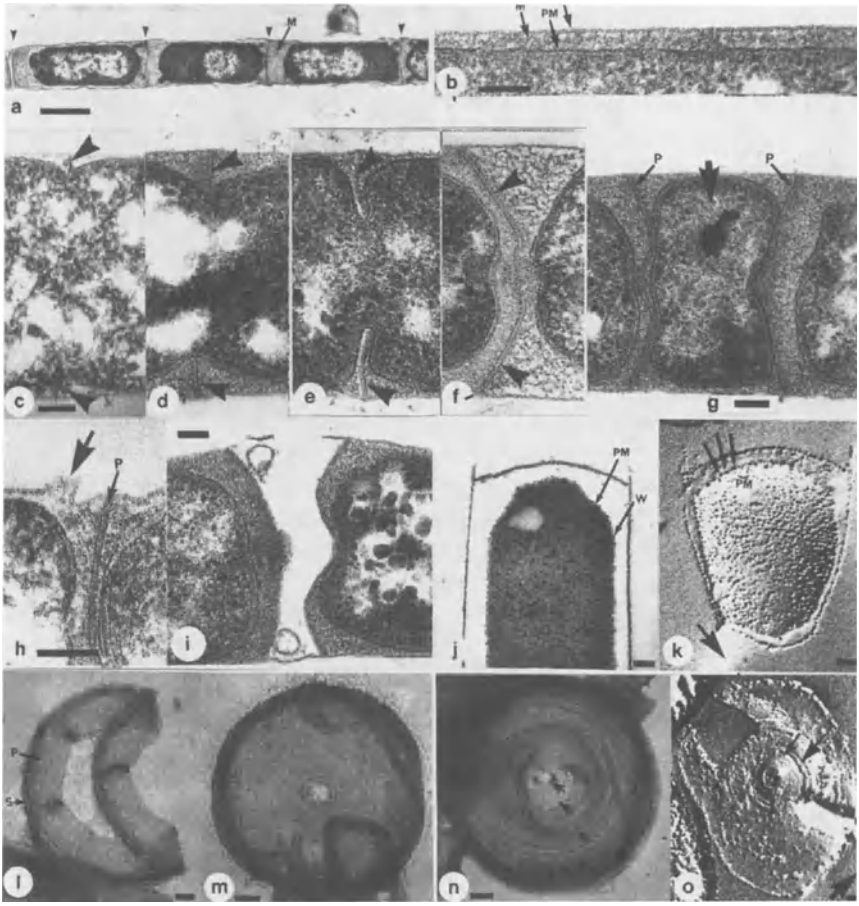


Figure 2.9. Ultrastructure, cell division sequence, and spacer plugs of *Methanosaeta concilii* GP6. a, thin section of three cells of *M. concilii* within a filament. The entire filament is bounded by a tubular sheath and each cell is separated from the next by a spacer plug (arrowheads). Surrounding each cell is an amorphous granular matrix (M). Bar = 1 μm. b, higher magnification showing the enveloping layers in thin section. The plasma membrane (PM) has an unusually thick and electron-dense surface leaflet. (S, sheath) Bar = 100 nm. c-f, a sequence of thin sections showing the cell division process in *M. concilii*. The large arrowheads point to the developing division annulus which is physically dividing the cell as it grows towards the cell centre. On completion of growth, the annulus becomes a spacer plug. All micrographs are the same magnification and the bar represents 100 nm. g-i, these images represent the process of filament splitting. g shows the production of a small protoplasmic element (arrow) which will be prone to filament fracture. Note that spacer plugs (P) which restrain the protoplasmic element are both conical and point in the same direction. h shows the sheath breaking down (arrow) above the plasma membrane

due to the close juxtaposition of a thin wall layer with the outer face of the cytoplasmic membrane, and is easily seen in sections of cells treated to remove the matrix (Figure 2.9j) and as particles in freeze-etch images (Figure 2.9k).

Ingrowth of a division annulus which is separate from the cell wall, and which begins from the sheath inner surface, initiated the division process and pushed the cell wall-cytoplasmic membrane inward (Figure 2.9, c–f). Isolated sheath tubes have multiple division annuli of different ingrowth states which apparently occur at random lengths along the inner surface and which, presumably, represent regions of cell division (Figure 2.4). The division process was shown in thin-sections (Figure 2.9, c–i), negative stains, and freeze-etchings of cells and division annuli (Figure 2.9, l–o), to involve ingrowth of a monolayer composed of concentric rings, followed by a second layer of wider concentric ribbons (Beveridge, Patel, et al., 1986). Completed division annuli (spacer plugs) were all aligned within the filament with their conical centers facing with the same polarity and the ribbon-like layer laid down with the same sidedness. Cell division is complete after the ingrowth and maturation of the division annulus (Figure 2.10, a–c), but this does not entirely explain the complicated process of multicellular filament splitting. Filament splitting occurs at a site placed usually towards the middle of a filament where a small fragment of protoplasm is partitioned by two spacer plugs (Figure 2.9g). After this small cytoplasmic fragment has been

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Figure 2.9. *Continued* residue within one of these small chambers. i shows the actual process of filament splitting. Bars = 100 nm. j, thin section of a plasmolyzed cell during protoplast extrusion in the initial stages of our sheath isolation and purification procedure. The matrix has been extruded leaving an empty space between the sheath, spacer plug, and cell. A thin granular layer (W) is still associated with the plasma membrane (PM) and completely encapsulates the protoplast; k, freeze-etch image of a cell similar to j. The concave hydrophobic surface of the plasma membrane (PM) is exposed and, at its periphery, small particles can be seen studding the external surface. Presumably these are the particles (W) seen in j. The shadow direction is denoted by the large arrow; l-o, these are individual division annuli which are negatively stained or freeze-etched. l is an immature annulus and entirely consists of fine concentric rings. P, developing plug; S, sheath remnant. m is an almost mature annulus of fine rings and this is infrequently seen. More frequently, the annulus looks like that in n; the outer arrow points to a series of concentric ribbons which lie on top of the layer of concentric rings. This layer is following the ingrowth of the underlying ring layer and has advanced to the area immediately beneath the second arrow. This second arrow points to the concentric ring layer; the third arrow points to the central hole left in this uncompleted annulus. o is a freeze-etching: the ring area is denoted by the small arrow, the ribbon region by the intermediate-sized arrow, and the shadow direction by the large arrow. Note the height (thickness) of the ribbon layer and that the annulus is not flat but, rather, conical. bars = 100 nm. Reproduced with permission. a,b,j,k from Beveridge, Patel, et al. (1986) and c-i plus l-o from Beveridge, Harris, et al. (1986).

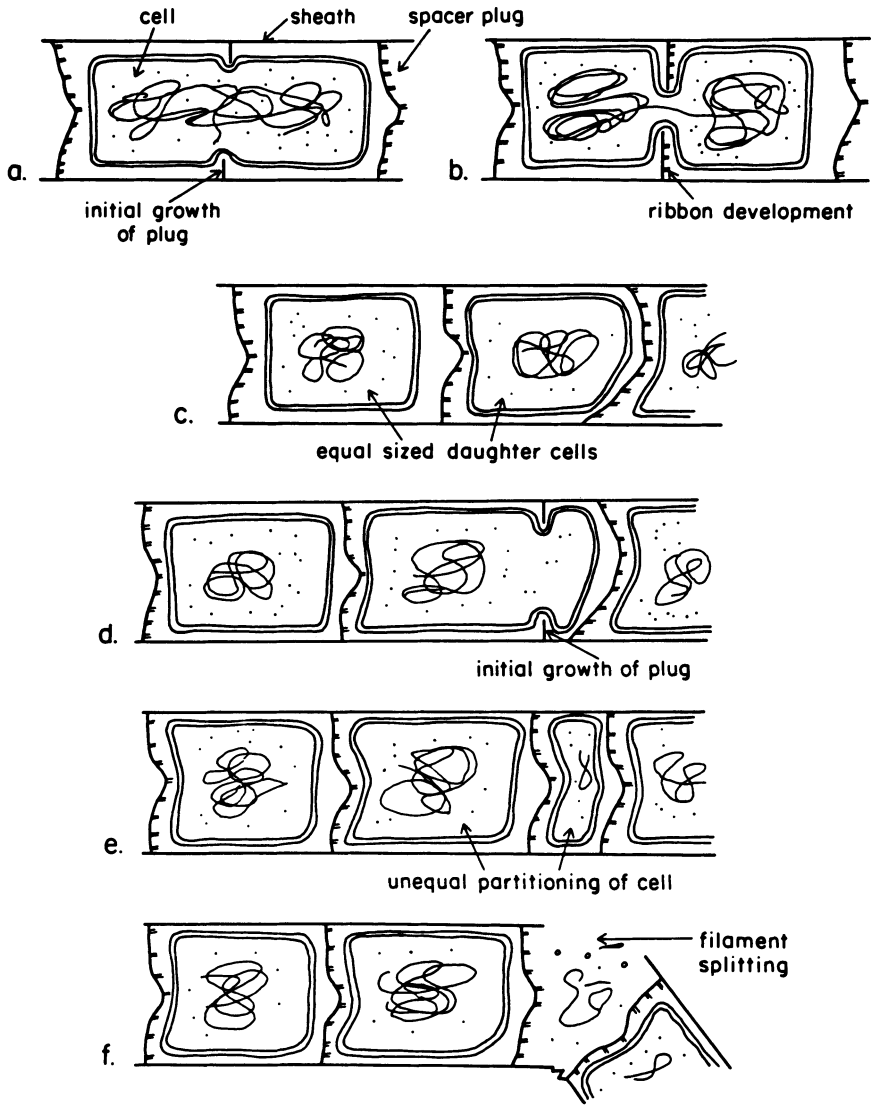

Figure 2.10. Diagrammatic representation of the series of structural events leading to cell division (a-c) and filament splitting (d-f) in *Methanosaeta concilii*. No attempt has been made to draw to scale. The cell wall and the particulate matrix, which lies between the wall and the sheath and spacer plugs, has been left out to simplify the diagrams. The spacer plugs consist of fine concentric rings denoted as a single transverse line and/or large concentric ribbons denoted by the short double lines. The chromosome is denoted by the tangled line at the center of each cell. Reproduced with permission, Beveridge, Harris, et al. (1986).

separated from adjacent cells by two division annuli, the sheath is broken (Figure 2.9h) and the two filament halves separate (Figure 2.9i). Presumably, the small cytoplasmic fragment is sacrificed during filament splitting (Beveridge, Harris, et al., 1986). A diagrammatic illustration of the complex division (Figure 2.10a–c) and filament splitting (Figure 2.10d–f) processes is presented.

2.5.3 Other methanogens

In *M. sinense* the envelope is composed of a single S-layer which confers the highly lobed shape to these cells (Pum et al., 1991). Evidence favours the insertion of new elements to the growing S-layer at lattice faults which align along the direction of septation. Small multiple invaginations on the cell surface appear to fuse together forming a deep invagination at more advanced stages of division (Figure 2.11a–f). Unlike more robust polymers, such as those that make up murein or pseudomurein which are joined together into an encompassing matrix by covalent bonding, S-layers depend on weaker bonding linkages (e.g., electrostatic, hydrophobic and H-bonding forces) and their formation is through the self-assembly of preformed subunits. Pum et al. (1991) give us the first explanation as to how these single S-layers, as the sole wall layer, can contribute to cell division.

Other reports of division in methanogens include thin-sections of a *Methanosarcina* sp. in the process of wall ingrowth (Figure 2.12) and of apparently true septum formation in a number of the *Methanobacteriaceae* (Figure 2.13 is representative).

2.6 Cytoplasmic or Plasma Membrane

Invaginations of the cytoplasmic membrane to form internal membrane systems have been reported in many methanogens viewed in thin-section (Zeikus and Bowen, 1975). These structures were given functional and organelle status when they were coined "methanochondria" to parallel the mitochondria of eukaryotes, and evidence was presented to show that they represented the site of methane and ATP synthesis (Kell et al., 1981; Sauer et al., 1981). This concept was disproven subsequently by ultrastructural (Aldrich, Beimborn, and Schonheit, 1987; Sprott et al., 1984) and biochemical (Kramer and Schonheit, 1987) data.

The frequency of "methanochondrial" sightings varied depending on the strain tested and on growth conditions, and were independent of the capacity of the culture to synthesize methane (Sprott et al., 1984). Some methanogens, such as *M. bryantii*, produced internal membranes of similar form in both thin-sections and freeze-fractures, but these were found only rarely (Figure 2.14a–c); most cells were devoid of such structures (Figure 2.14d). This indicated no obligatory,

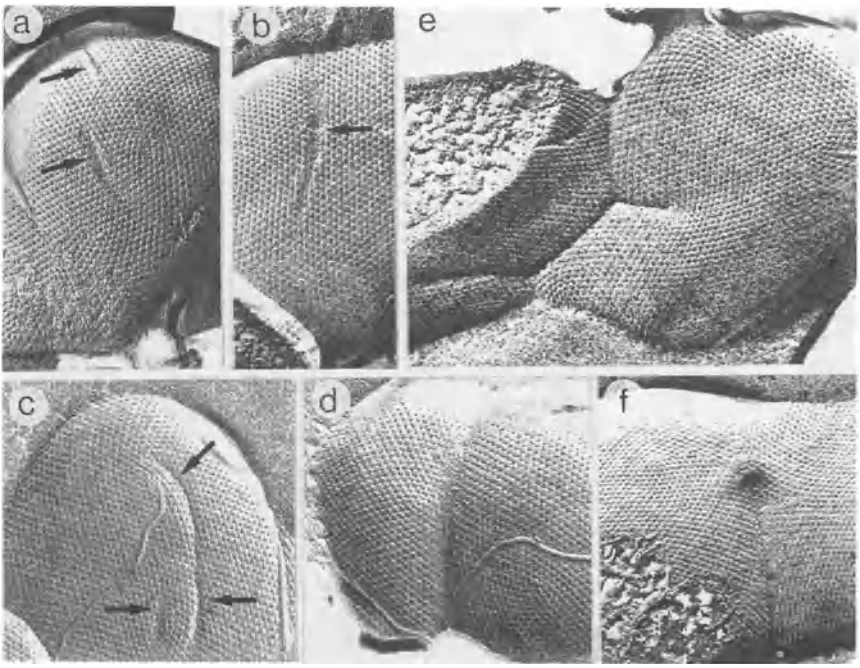

Figure 2.11. Freeze-etched preparations of intact cells of *Methanocorpusculum sinense* showing consecutive stages in the invagination of the cell wall and cell septation. Initially shallow invaginations are formed which become longer and deeper as new S-layer material is incorporated (a to f). Three discontinuous grooves of differing orientations are shown in panel a. The division of deeper invaginations show that they can also fuse or branch (e). A far advanced stage in the cell fission process is shown in panel f. The alignment of lattice faults in line with the septation direction indicates the direction of the progressing cell septation. Reproduced with permission, Pum et al. (1991).

bioenergetic role for "methanochondria". Thin-sections of *M. thermoautotrophicum* ΔH had numerous uni- and multilamellar membranous inclusions, which decreased dramatically in cells grown at depressed growth rates. Aldrich et al. (Aldrich, Beimborn, and Schonheit, 1987) re-examined the issue with this methanogen and identified a fixation protocol which, like the freeze-fracture technique, produced specimens devoid of internal membranes (Figure 2.13). Evidence favors the cytoplasmic membrane as the only energy transducing membrane in methanogens.

Until recently, cytoplasmic membranes had only been isolated and characterized in *M. hungatei* (Sprott et al., 1983), despite the development of spheroplast/protoplast formation methodologies for several methanogens (Sleytr et al., 1986;

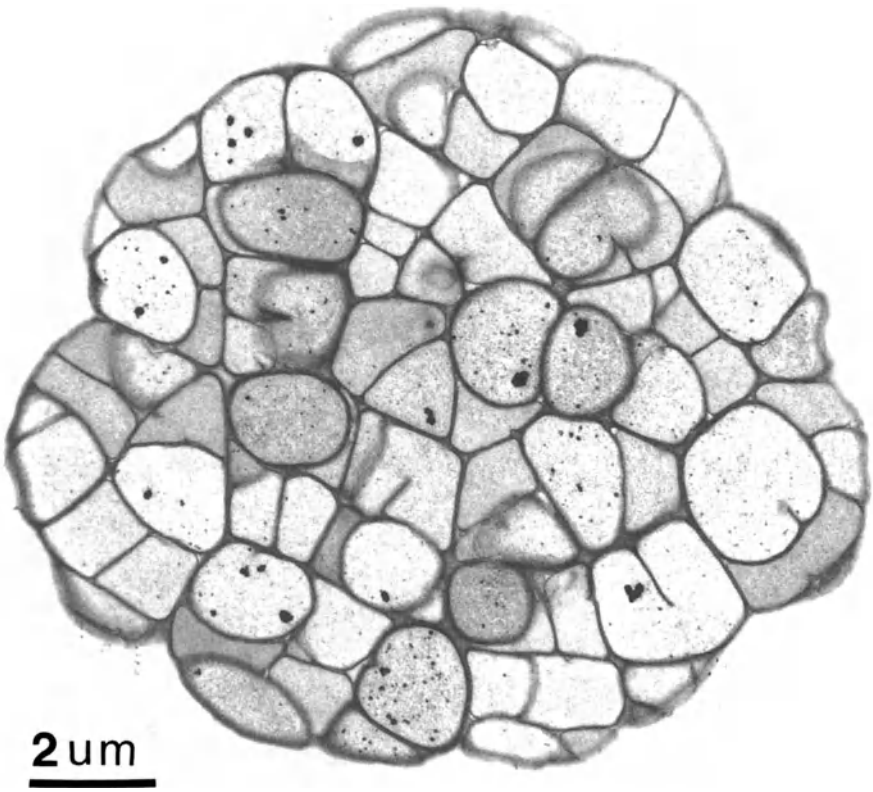

Figure 2.12. Thin-section of acetate grown *Methanosarcina* strain CHTI 55. Invaginations of the cell wall indicate active cell division. Reproduced with permission, Touzel et al. (1985).

Jarrell et al., 1982; Jussofie et al., 1986; Sprott et al., 1979). A method to form protoplasts of *M. voltae* by manipulation of cation concentrations (Patel et al., 1993) may well apply to many other irregularly-shaped coccoid methanogens which lyse in low ionic fluids such as water, and may prove useful in removing S-layers prior to cytoplasmic membrane purifications. Presumably, the absence of salts disrupts the bonding forces that hold these S-layers together. Membranes purified by sucrose gradient centrifugations of osmotically lysed spheroplasts of *M. hungatei* formed closed vesicles often with outward extensions (Figure 2.15b). Like the *Methanococcus* vesicles of Patel et al. (Patel et al., 1993), those of *M. hungatei* did not possess the S-layered cell wall and it may have been solubilized inadvertently during purification of the membranes. The isolated membrane consisted on a dry weight basis of numerous proteins (45–50%) separable on SDS-

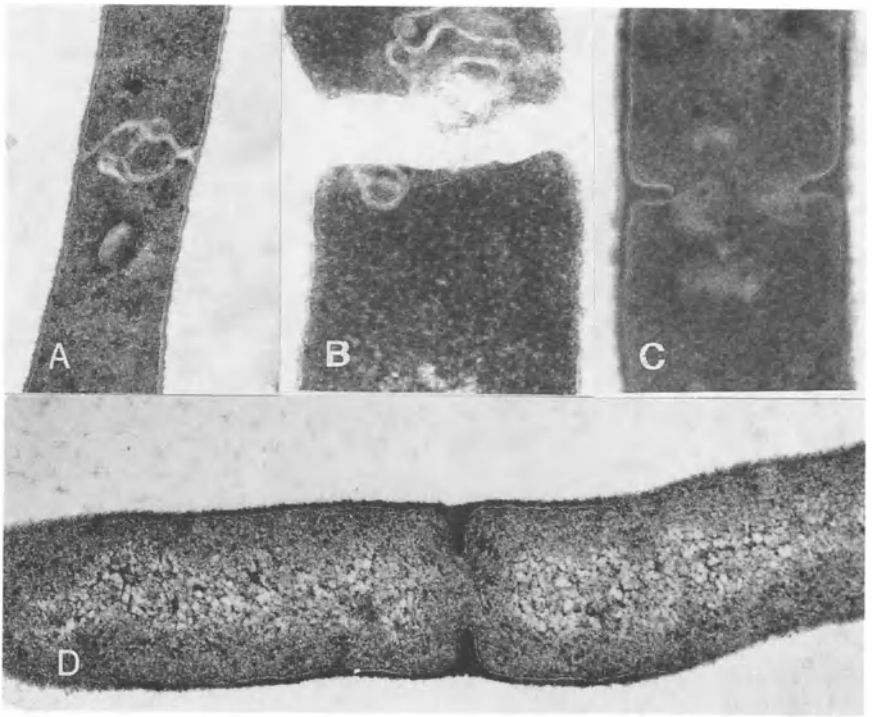

Figure 2.13. Formation of internal membranous structures in *Methanobacterium thermoautotrophicum* during some fixation protocols for thin-sectioning.
Panel A, Technikosome is visible at upper arrow. Septum (lower arrow) has convoluted membrane associated with it. Cell fixed in 0.5% glutaraldehyde–2.5% formaldehyde in cacodylate buffer, followed by 1% OsO_4. Panel B, Septum (s) has convoluted membrane associated with it. Cell fixed in 2.5% glutaraldehyde–2.5% formaldehyde (15 min, room temperature) in phosphate buffer, followed by 1% OsO_4. Panel C, Mesosomes (m) are visible on both sides of the septum (s). Cell fixed in 2.5% glutaraldehyde in phosphate buffer, followed by 1% OsO_4. Panel D, this is one of the best protocols involving chemical fixation. It compares favorably with freeze substitution. No internal membranes. Nucleoid (n) contains slightly coagulated fibrils of DNA. Cell fixed in 2.5% glutaraldehyde–2.5% formaldehyde (15 min, room temperature) in cacodylate buffer, postwashed for 15 min on ice and fixed with 1% OsO_4 for 30 min at room temperature. Reproduced with permission, Aldrich et al. (1987).

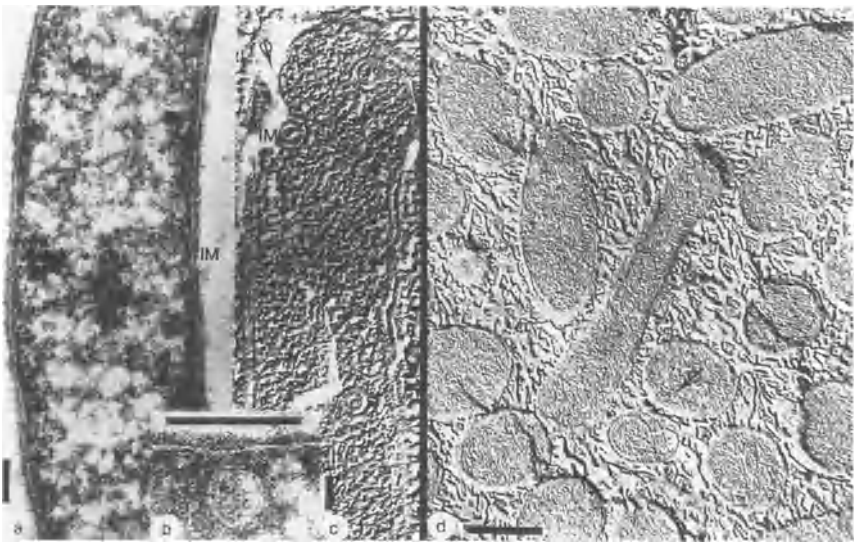

Figure 2.14. Comparison of the intracellular membranes of *Methanobacterium bryantii* in (a) thin section and (c) freeze-fracture. The few internal membranes (IM) that were seen resemble each other by the two techniques and occurred near the division site or the polar region. A high magnification of an IM in this section is seen in b. Bars = 0.1 μm. d, A Pt-C replica of a culture of *M. bryantii*. Cells were grown at a 150-rpm agitation rate and g = 12 h. The direction of shadowing is indicated by the arrow. Bar = 0.3 μm. Reproduced with permission, Sprott et al. (1984).

PAGE gels, lipid (35–37%), non-lipid carbohydrate (primarily as rhamnose, galactose and glucose) (5–7%), and ash (6–15%). About 94% of the lipid fraction was comprised of ether lipids divided fairly equally between tetraether and diether core structures; the remainder was neutral squalenes and hydrocarbons (Kushwaha et al., 1981). Complete structures are published for all ether lipids excluding several present in small amounts (Kushwaha et al. 1981, Ferrante et al. 1987).

Inside-out vesicles were made from protoplasts of *M. voltae* and strain Gö1 prepared by trypsin treatment (Hoppert and Meyer, 1990), and a membrane fraction from *M. thermoautotrophicum* was shown to catalyze methanogenesis (Sauer et al., 1980).

Freeze-fracturing has been carried out on several methanogens and the cytoplasmic membrane usually splits down its center to reveal exoplasmic (EF) and protoplasmic (PF) hydrophobic faces when using the nomenclature of Branton et al. (Branton et al., 1975). Freeze-etch replicas have been produced for *M. vannielii* (Jones et al., 1977), *M. voltae* (Koval and Jarrell, 1987), *M. concilii* (Beveridge, Harris, et al., 1986), and *M. mazei* (Aldrich et al., 1986), all of

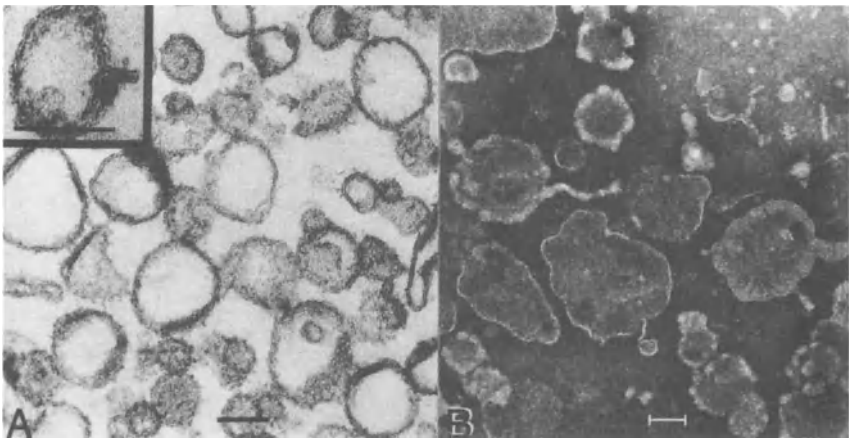

Figure 2.15. Electron micrographs of isolated cytoplasmic membrane (CM) vesicles from *Methanospirillum hungatei*. A, thin section of CM vesicles, the inset showing an evaginated segment of CM; B, isolated CM stained with phosphotungstic acid revealing projections of membrane from the vesicles. Reproduced with permission, Sprott et al. (1983).

which show typical PF fracture faces with numerous intramembranous particles and EF faces with depressions, particle-free areas, and relatively few particles (Figure 2.9k is representative). The conclusion that tetraether lipids with C_{40}-hydrocarbon chains of abundance in thermoacidophiles span the tetraether-rich cytoplasmic membranes with polar headgroups on either side (DeRosa et al., 1986) is partially based on the absence of intramembrane fracture planes (Langworthy, 1978). This suggestion is supported by the above results, since these methanogens are tetraether negative and contain diether and hydroxydiether polar lipids (Sprott et al., 1990). Contrary to this are results showing intramembrane fractures in the case of *M. thermoautotrophicum* which produce both diether and tetraether lipids in ratios depending on growth phase (Kramer and Sauer, 1991). In a thermodynamic sense, the cleavage of covalent bonds between tetraether headgroups by freeze-fracture is difficult to explain. Possibly, in *M. thermoautotrophicum* the tetraether lipids are segregated to distinct regions of the membrane which cannot fracture. Those areas which have fractured are the remaining membrane regions which contain diether lipids. Of note in this methanogen are the large 14 × 28 nm cylindrical particles of unknown function which were seen in the PF fracture face in addition to the common 10 nm particles.

To address the freeze-fracture issue of tetraether bilayers properly, it is necessary to present both the relative frequency of intramembrane fractures to cross fractures and the quantitative proportions of diether to tetraether lipids. *M. jan-*

naschii was chosen for such a study (Beveridge et al., 1993) because the proportion of diethers to tetraether lipids can be varied dramatically by changing growth temperature (Sprott et al., 1991). Ether lipids (about 90% of the total membrane lipid) were composed of 20% tetraether lipids after growth at 50°C and these increased to 45% at 70°C. The corresponding frequencies of cross fractures (Figure 2.16a) to intramembrane fractures (Figure 2.16b) were about 1:1 at 50°C and 9:1 at 70°C, indicating that tetraether lipids can result in a significant reduction of intramembrane fractures when at high concentration.

Liposomes can be prepared by detergent dialysis from the total polar lipids extracted from several methanogens (Chocquet et al., 1992). Because of their unusual chemically stable-lipid composition, these liposomes may be resistant to chemical and physical perturbants and have biotechnological value. Freeze-fractures of liposomes of *M. voltae* lipids (devoid of the proteins from the parent membranes) revealed hydrophobic fracture surfaces, both concave and convex, which were smooth (Figure 2.17). Multiple fracture faces were never seen, indicating that the liposomes were unilamellar.

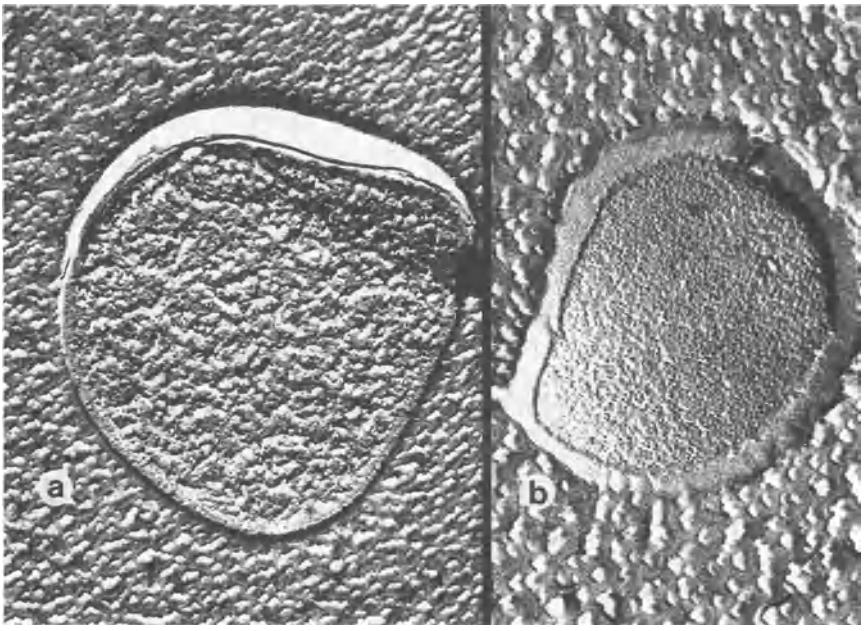

Figure 2.16. Freeze fracture micrographs of *Methanococcus jannaschii* cells grown at different temperatures. a, cross fracture of a cell grown at 70°C; b, protoplasmic fracture face from a cell grown at 50°C. Data of Beveridge and Sprott (unpublished).

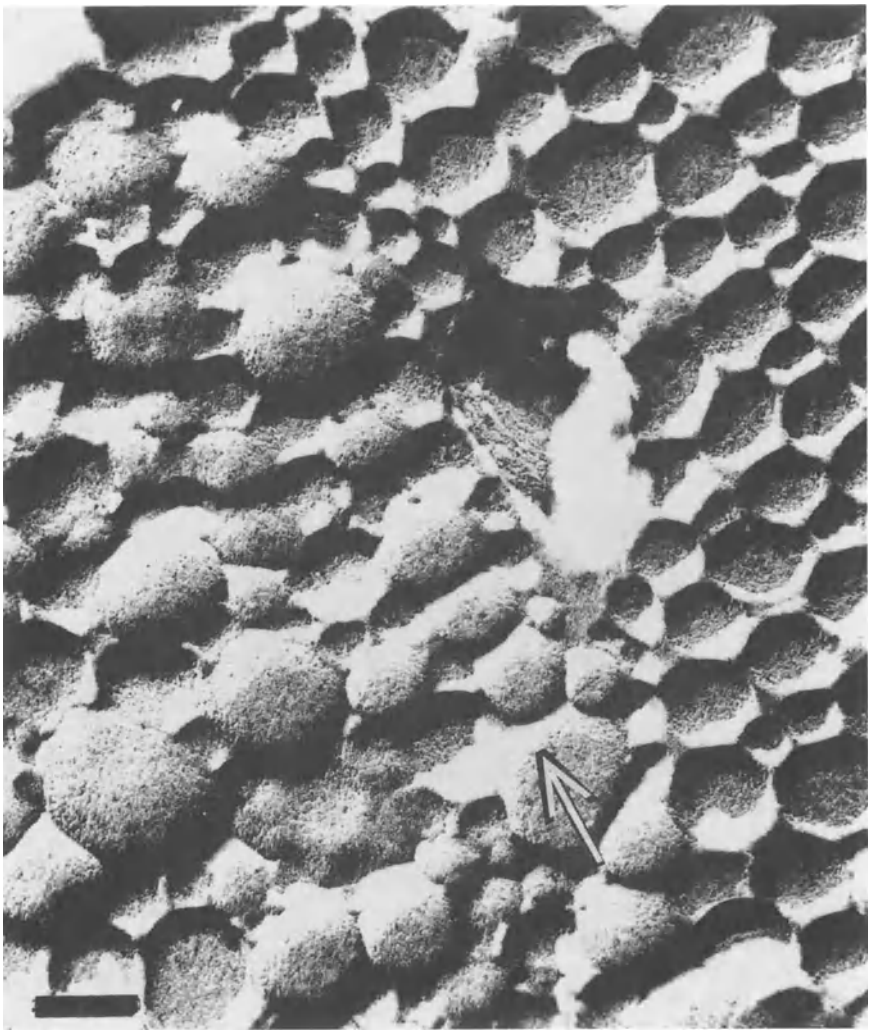

Figure 2.17. Freeze-fracture micrograph of liposomes from *Methanococcus voltae*, indicating their unilamellar nature. Bar = 100 nm. The arrow denotes shadow direction. Reproduced with permission, Choquet et al. (1992).

2.7 Particulate Enzymes

2.7.1 Localization

The F_{420}-reducing hydrogenases (FRH), and to a lesser extent the methyl coenzyme M reductases (MCR), of methanogens behave on hydrophobic column matrices as very hydrophobic enzymes, suggesting a functional association with the cytoplasmic membrane. Immunogold labeling of thin-sectioned cells revealed that FRH was localized predominantly in the cytoplasmic membrane (Baron et al., 1989; Lunsdorf et al., 1991; Muth, 1988). MCR labeling occurred throughout the cytoplasm of *M. mazei* (Thomas et al., 1987) and *M. thermoautotrophicum* (Hoppert and Meyer, 1990). However, under growth conditions which limited the amount of the F_{430} prosthetic group of MCR, localization on the cytoplasmic membrane was also demonstrated (Aldrich, Beimborn, Bokranz, et al., 1987; Ossmer et al., 1986).

2.7.2 FRH

Negative staining with uranyl acetate and shadow-casting with platinum indicated that FRH purified from *M. thermoautotrophicum* was a ring structure of dimensions 15.7 nm wide × 8.5 nm deep with an inner channel, or invagination, 4 nm in diameter (Wackett et al., 1987). The calculated molecular weight for this particle was 800 kDa. *M. hungatei* FRH showed similarly sized spherical particles (15.9 nm × 12.5 nm) with a central area being stained only in some cases (Figure 2.18a). The simplest interpretation favors that only one side of the flattened sphere has a central pocket or depression (Figure 2.18b–d), rather than a channel running completely through the particle which can become filled with stain from either side (Sprott et al., 1987). FRH is proposed to consist of two stacked rings. Each ring of the stack consists of four subunits and each of these subunits is an assembly of an α, β, and γ polypeptide (Wackett et al., 1987).

In the absence of cryoprotectants, freezing at −20°C and thawing destroyed the structural properties of FRH (Sprott et al., 1987).

2.7.3 MCR

Negative stains of MCR show an enzyme complex that is also spheroidal of dimensions 8.5 × 9.0 × 11.0 nm with a stain-filled central area (Figure 2.19). Concentrated enzyme suspensions formed crystals that upon disruption appeared as paracrystalline layers of MCR particles (Wackett et al., 1987).

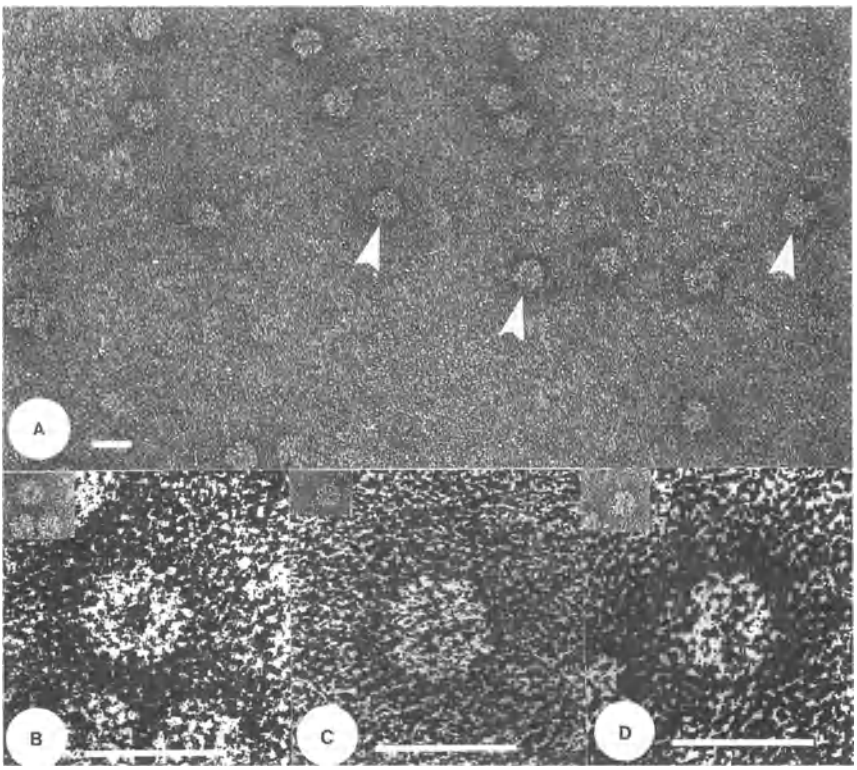

Figure 2.18. Electron micrographs of the native 15.9 nm F_{420}-reducing hydrogenase negatively stained with 1% uranyl acetate. No alterations to the particle profiles were observed when they were stained in ammonium molybdate. A, representation of a typical field of the enzyme. The arrowheads point to the three particle profiles which were observed; from left to right, flat subunit surface, surface with central depression, and oblate edge view; B, high magnification of the surface which has the central depression and of the particle seen in the upper left of the figure; C is of the flat surface and D is the edge view. The images at the upper left of each figure are lower magnifications of the same particles. Bar = 20 nm. Reproduced with permission, Sprott et al. (1987).

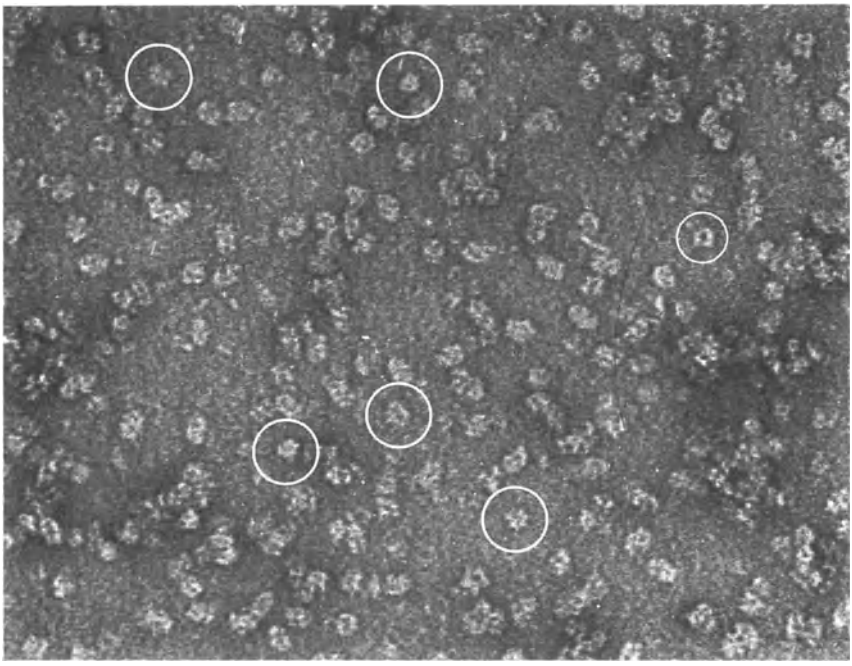

Figure 2.19. Electron micrographs of methyl reductase negatively stained with uranyl acetate (magnification, ×280,000). This micrograph shows structures determined to be the 3×10^5 kDa form of methyl reductase, as well as particles twice that size. Some representative images of the 3×10^5 kDa form are highlighted with circles. Reproduced with permission, Wackett et al. (1987).

2.7.4 ATPase

A mixture of inside-out vesicles, right-side out vesicles, and membrane fragments was prepared from protoplast lysates of *Methanosarcina* strain Gö1. Immunogold labeling with a polyclonal antiserum directed against the β-subunit of *Escherichia coli* F_0F_1-ATPase revealed a total of 5 ($\pm$ 1.4) gold particles per 1000 nm^2 of membrane surface (Mayer et al., 1987). Since background labeling was negligible, it can be concluded that a dense population of ATPase particles is anchored to the cytoplasmic membrane of this methanogen. Observation of negatively stained vesicles (lacking the immunogold to prevent masking of structural data) revealed a high number of stalked particles (38 $\pm$ 4.0 per 1000 nm^2) of subunit construction with a head-piece diameter of 10.0 $\pm$ 0.7 nm. This size and shape is similar to the F_0F_1-proton translocating ATPase found in eubacteria (Futai and Kanazawa, 1983).

2.7.5 *Methanoreductosomes*

Inside-out vesicle preparations from *Methanosarcina* strain Gö1 and *M. voltae* revealed a high molecular weight complex 19–36 nm in diameter attached via stalk-like connections with the same outer surface orientation as ATPase particles (Hoppert and Mayer, 1990; Mayer et al., 1987). Immunolocalization showed that the head-piece of the complex, called a methanoreductosome, contained MCR (Mayer et al., 1987, 1988). The known tendency of MCR to aggregate in concentrated solution (Hoppert and Mayer, 1990; Wackett et al., 1987) might be suspected as a source of artifact formation during the drying process of negative staining. Evidence against this is derived from freeze-etchings of cytoplasmic membranes of strain Gö1 showing numerous particles corresponding in size to the methanoreductosomes and the smaller F_1 portion of ATPase molecules (Mayer et al., 1988). A model of the methanoreductosome and its size relative to the ATPase is shown in Figure 2.20.

2.8 Appendages

Both flagella and fimbriae are produced by some species of methanogenic bacteria (Doddema et al., 1979; Jones et al., 1977). Only a few examples will be given here. *M. hungatei* is novel since mono- or bipolar flagella are inserted through the relatively permeable end plugs of filaments (Ferry et al., 1974; Southam et al., 1990). Surprisingly, filaments containing two or more cells are able to coordinate flagellar rotation so that propulsion is achieved; in this case, each tuft of flagella is contained by a separate terminal cell in the filament and, often, central cells separate these terminal cells. *M. voltae* has peritrichous flagella and a few thinner fimbriae (Koval and Jarrell, 1987). Three strains of *M. thermophilicum* possessed a single flagellum per cell; fimbriae were detected in only one strain (Zabel et al., 1985).

The flagellar filaments of methanogens are about 13 nm in width (Faguy et al., 1992; Kalmokoff et al., 1988; Southam et al., 1990; Zabel et al., 1985) which differs from the usual 20 nm thickness of eubacterial filaments (Kalmokoff et al., 1988). Intact flagella consisting of filament, hook and basal body may be purified by treating cell envelopes or spheroplasts with Triton X-114 (EDTA is required with gram-negative eubacteria), and subsequent partitioning into the upper aqueous phase by temperature shift (Kalmokoff et al., 1988). *M. vannielii* (Figure 2.21) and three other species of this genus (not shown) had flagella atypical of eubacteria with a basal structure represented by only a small knob, and a poorly differentiated hook appearing as only a slight thickening of the filament near the basal structure. The hook region and basal body rings are also difficult to differentiate in *M. hungatei* (Southam et al., 1990).

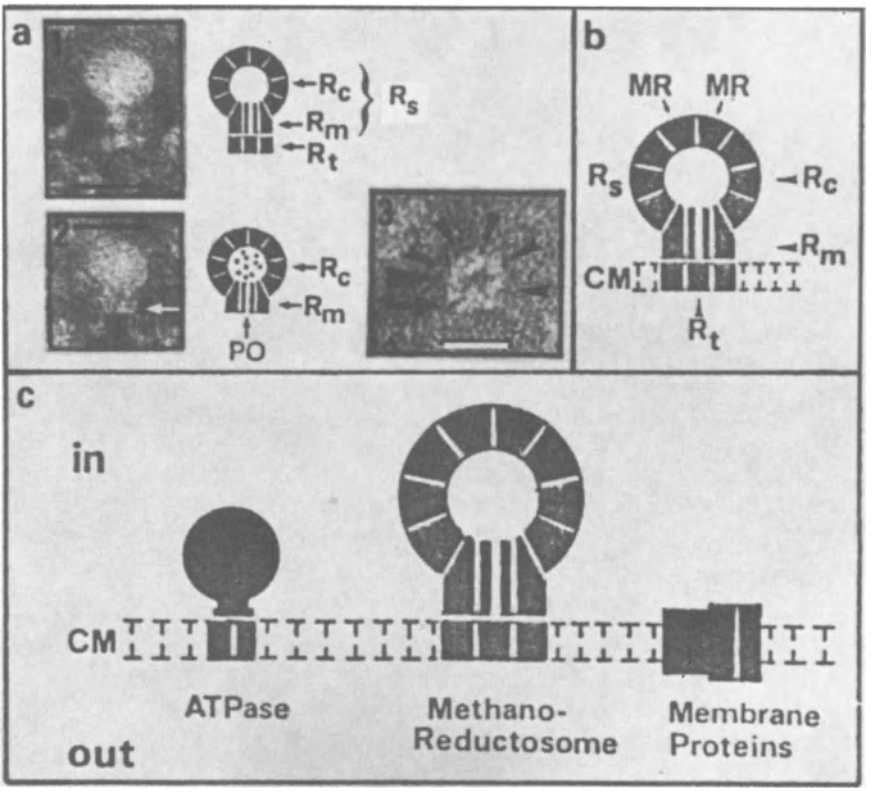

Figure 2.20. Ultrastructural aspects of the methanoreductosome. a1, an enzyme particle is shown along with a diagram exhibiting the composition of the enzyme complex out of a number of constituents. a2 and a3, free spherelike enzyme particles with an additional group of subunits (arrows) but devoid of the membrane-integrated part seen in panel 1. The particle is depicted with a diagram. The internal volume of R_c in this diagram is dotted: this indicates that the negative-staining solution can penetrate into R_c, presumably through the equivalent of a functional pore if the R_cR_m complex is not attached to R_t (see panel 1). Panels 1 and 2 were negatively stained with phosphotungstic acid after extensive air drying of the mounted sample; panel 3 was negatively stained with uranyl acetate without air drying before the application of the negative staining solution. b, diagrammatic view of the R_cR_m complex containing several copies of methyl-CoM methylreductase attached to R_t, the membrane-integrated part of the enzyme complex. c, the methanoreductosome associated with the cytoplasmic membrane, together with an F_0F_1-like ATPase particle and unspecified membrane proteins. The diagram is drawn to scale. Abbreviations: ATPase, F_0F_1-like ATPase particle; CM, cytoplasmic membrane; MR, copies of methyl-CoM methylreductase; PO, equivalent of a functional pore in R_m. Reproduced with permission, Mayer et al. (1988).

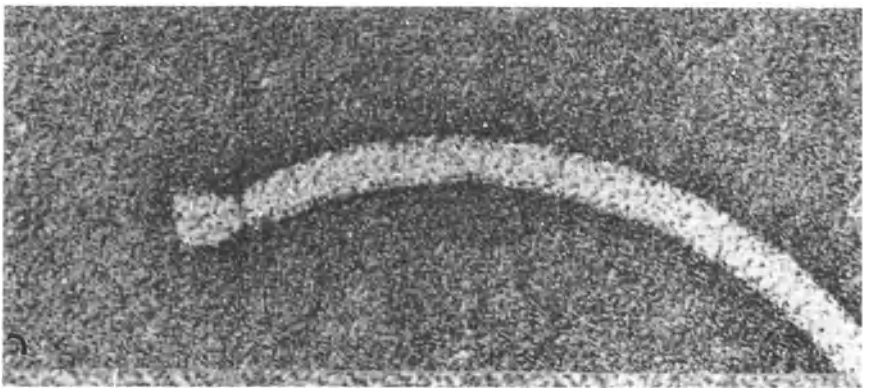

Figure 2.21. Intact flagellum of *Methanococcus vannielii*, prepared by Triton X-114 extraction of envelopes and negatively stained with 1% uranyl acetate. Bar = 50 nm. Reproduced with permission, Kalmokoff et al. (1988).

2.9 Intracellular Granules and Vesicles

2.9.1 Storage Granules

Twelve strains and species of the *Methanosarcinaceae*, especially when grown on methanol, were found to contain characteristic polyphosphate-like electron dense 0.15–0.25 μm particles in unstained thin-sections (Figure 2.22). Energy dispersive X-ray microanalysis established that the granules contained P, Ca, Fe and sometimes Mg, S, and Cl (Scherer and Bochem, 1983). Another species, *M. frisia*, when grown on methanol contained granules of similar appearance which could comprise up to 14% of the cell dry weight. By ^{31}P NMR analysis they were shown to consist of long chain polyphosphates (Rudnick et al., 1990). Electron dense granules were observed in thin-sections of *M. tindarius* and, when the granules were stained for light microscopy for polyphosphate, with toluidine blue they stained positive (Konig and Stetter, 1982).

Glycogen granules were seen in thin-sections of *Methanothrix* strain FE and these cytoplasmic granules when purified were found to contain glycogen with an average chain length of 13 glucose residues (Pellerin et al., 1987).

2.9.2 Crystals and tubules

Crystals with a lattice of 7.7–8.0 nm appear near the cell periphery in older cultures of *M. mazei* (Aldrich et al., 1986). Their origin is unknown and may relate to overproduction of proteins such as FRH and MCR which tend to self-aggregate.

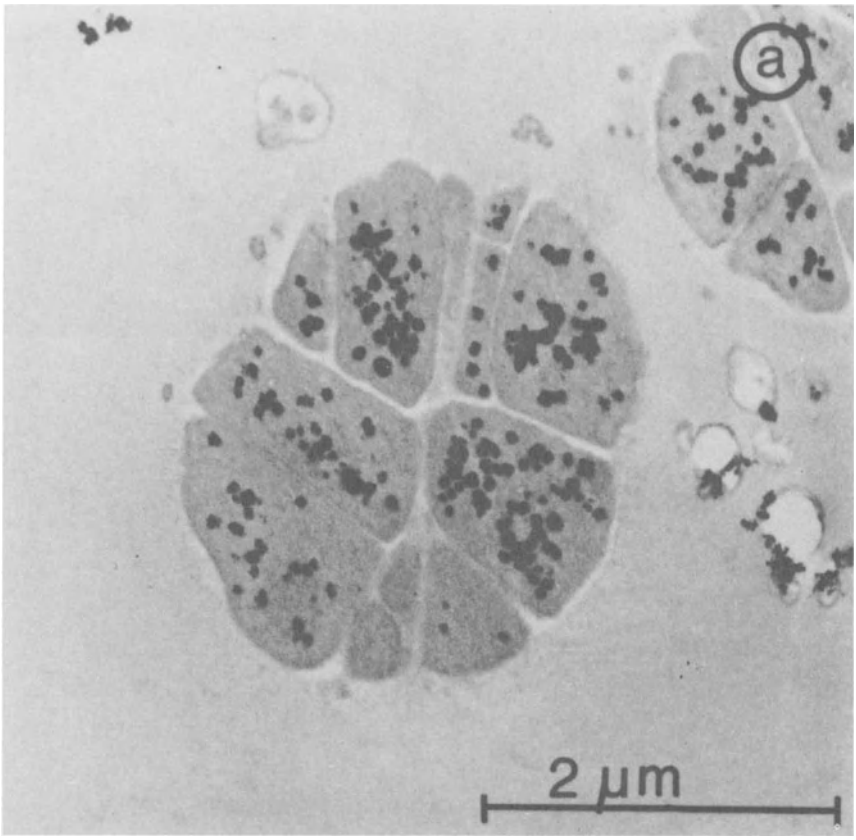

Figure 2.22. Electron micrographs of *Methanosarcina barkeri* Fusaro grown on acetate. Cells were fixed with glutaraldehyde and the embedded and sectioned material was poststained by lead citrate to demonstrate fibrils at the outside of the cells (hardly visible). Reproduced with permission, Scherer and Bochem (1983).

Tubules that appear to be intrusions of the cytoplasmic membrane have been observed in *M. mazei* (Aldrich et al., 1986) and *M. voltae* (Koval and Jarrell, 1987, 1989).

2.9.3 Gas Vesicles

Gas vesicles are found in *M. vacuolata* (Zhilina and Zavarzin, 1987), *M. barkeri* strain FR-1 (Archer and King, 1984), and *Methanothrix* sp. CALS-1 (Zinder et al., 1987). These cylindrically shaped vesicles (Figure 2.23) can occupy a large proportion of the cell volume. Purified gas vesicle membranes of FR-1 had a rib spacing of 4.8nm (Archer and King, 1984).

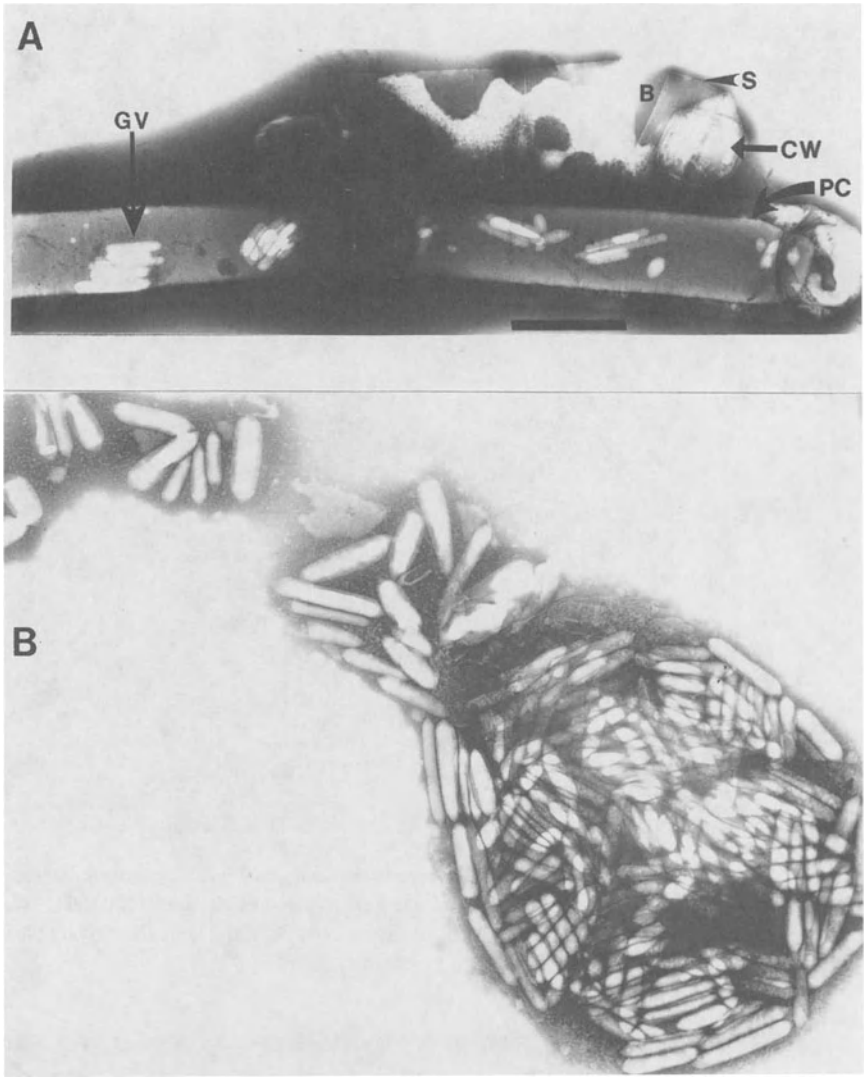

Figure 2.23. Gas vesicles. A, negative stain electron micrograph of a filament of *Methanothrix* sp. strain CALS-1 along side an empty sheath. Note striations (S) in the sheath, the break (B) in the empty sheath, the cross-wall (CW), the protoplasmic cylinder (PC) within the sheath, and gas vesicles (GV). Gas vesicles which have their long axis close to perpendicular to the plane of the photograph appear American football-shaped. Marker bar represents 1 μm; B, Release of gas vesicles from a vacuolate protoplast of *Methanosarcina barkeri* FR-1 stained with uranyl acetate. The marker bar represents 200 nm. Reproduced with permission: A, Zinder et al. (1987); B, Archer and King (1984).

2.10 Summary

In this chapter we have attempted to present much of the research conducted on methanogenic bacteria through the use of electron microscopy. This technique has clearly revealed methanogens to possess several structural traits that are unusual and unlike their eubacterial counterparts. This is especially true when enveloping layers are compared. Of all others, the cell wall of *Methanobacterium* spp. most closely resembles that of eubacteria (i.e., the gram-positive wall of rods like *B. subtilis*). So far, no methanogens have been discovered which are bounded by typical gram-negative eubacterial cell walls resembling an outer membrane and thin peptidoglycan layer like that possessed by *E. coli*. Many methanogens possess only a single S-layer as their wall (e.g., *Methanococcus* spp.), whereas others such as *Methanosarcina* spp. can be bounded by thick amorphous matrices of methanochondroitin depending on their environmental conditions. *M. hungatei*, *M. concilii* and other representatives of these genera are bounded by the most complex enveloping layers yet discovered; they possess cell walls, sheaths, and spacer plugs. These envelope differences which are found in methanogens ensure that their modes of cell division (Beveridge, Harris, et al., 1986; Beveridge et al., 1987) (with the possible exception of *Methanobacterium* spp; Aldrich, Beimborn, and Schonheit, 1987) must be subtly different from the typical constrictive or septal fissions of eubacteria (Beveridge, 1989). Indeed, these envelope differences found in methanogens and other archaeobacteria may even alter our understanding of how prokaryotes react to such fundamental microbiological criteria as the Gram stain (cf., Beveridge and Davies, 1983; Beveridge et al., 1991).

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3

Physiological Ecology of Methanogens

Stephen H. Zinder

3.1 Introduction

Biological methanogenesis plays a major role in the carbon cycle on Earth. Methanogenesis is the terminal step in carbon flow in many anaerobic habitats, including marine and freshwater sediments, marshes and swamps, flooded soils, bogs, geothermal habitats, and animal gastrointestinal tracts. CH_4 escaping from anaerobic habitats can serve as a carbon and energy source for aerobic methanotrophic bacteria, and can escape to the atmosphere, where it is a major participant in atmospheric chemical reactions and is an important greenhouse gas.

Methanogenesis also has many practical applications. Anaerobic treatment of organic wastes has been used in sewage treatment plants for nearly a century. There is now increased interest in treating various industrial and agricultural wastes using methanogenic mixed cultures, since methanogenic waste treatment systems can be energy efficient, or even energy producing. Methanogenic mixed cultures are being seriously considered for the treatment of certain toxic wastes, including halogenated and aromatic organic compounds. Another human-made methanogenic habitat is the sanitary landfill, and CH_4 is now being harvested from many landfill sites.

Because of their impact on the environment and their roles in waste treatment and energy conservation, there has been considerable study of methanogenic habitats and mixed cultures. The study of the ecology of methanogens is easier than for many other microbial groups because methanogens carry out well-defined reactions which often account for a significant fraction of the carbon flow in anaerobic habitats, and because methanogens have unique properties (e.g., resistance to antibiotics, presence of F_{420}) which facilitates their enumeration in natural

habitats. This chapter will discuss the ecology of methanogens in anaerobic habitats, with an emphasis on physiological characteristics of methanogens important to their ecology, and on how methanogen physiology may affect carbon and electron flow in those habitats. Recent monographs can be consulted for more detailed information concerning the biology of anaerobic microorganisms (Zehnder, 1988), and the processes leading to CH_4 in the atmosphere (Rogers and Whitman, 1991).

3.2 Substrate Range of Methanogens

Probably the most salient physiological feature of the methanogens is their extreme catabolic specialization. Despite the enormous phylogenetic diversity represented by the methanogens (Woese, 1987), as a group they can only use a small number of simple compounds, most of which contain one carbon (Table 3.1). Many methanogens use only one or two substrates, with the greatest versatility represented in some strains of *Methanosarcina,* which can use seven substrates. A major consequence of this specialization is that in most anaerobic habitats, methanogens are dependent on other organisms for their substrates. Therefore, a food web of interacting groups of anaerobes is required to convert most organic matter to methane, in contrast to aerobic ecosystems, where single organisms can usually effect the complete oxidation of a complex organic molecule to carbon dioxide.

It is not clear why methanogens cannot degrade more complex molecules, such as glucose, to CH_4 and CO_2. One view is that methanogenesis requires such complex and specialized metabolic machinery that methanogens are unable to compete with fermentative organisms more specialized for using complex substrates. A related view, expressed by McInerney and Beaty (1988), is that the free energy per electron is considerably higher for fermentative reactions than for complete reactions coupled to methanogenesis or sulfate reduction. For example, fermentation of glucose to acetate and H_2 has a $\Delta G^{\circ\prime}$ of -27 kJ per electron (and a $\Delta G'$ of -39 kJ per electron under anaerobic bioreactor conditions) while the complete dissimilation of glucose to CH_4 and CO_2 has a $\Delta G^{\circ\prime}$ of only -16.8 kJ per electron. Thus, a putative glucose utilizing methanogen would have to compete with a more efficient fermentative organism. However, a glucose utilizing methanogen which produces acetate and CH_4 from glucose could conserve -42.7 kJ per electron, and it is not clear why such an organism has not been found to exist. McInerney and Beaty (1988) also point out that anaerobic reactions are more entropic than enthalpic (heat producing), in contrast to aerobic and nitrate reducing reactions which are mainly enthalpic (e.g., burning wood, nitroglycerin). As a result, efflux of metabolic products may be important in driving anaerobic reactions, and may be coupled to development of a proton motive force.

Table 3.1 Methanogenic reactions

Reactants	*Products*	$\Delta G^{\circ\prime}$ *kJ/mol* CH_4	*Organisms*
$4H_2 + HCO_3^- + H^+$	$CH_4 + 3H_2O$	−135	Most methanogens
$4HCO_2^- + H^+ + H_2O$	$CH_4 + 3HCO_3^-$	−145	Many hydrogenotrophic methanogens
$4CO + 5H_2O$	$CH_4 + 3HCO_3^- + 3H^+$	−196	*Methanobacterium* and *Methanosarcina*
$2CH_3CH_2OH + HCO_3^-$	$2CH_3COO^- + H^+ + CH_4 + H_2O$	−116	Some hydrogenotrophic methanogens[a]
$CH_3COO^- + H_2O$	$CH_4 + HCO_3^-$	−31	*Methanosarcina* and *Methanothrix*
$4CH_3OH$	$3CH_4 + HCO_3^- + H_2O + H^+$	−105	*Methanosarcina* and other methylotrophic methanogens
$4(CH_3)_3{-}NH^+ + 9H_2O$[b]	$9CH_4 + 3HCO_3^- + 4NH_4^+ + 3H^+$	−76	*Methanosarcina* and other methylotrophic methanogens
$2(CH_3)_2{-}S + 3H_2O$[c]	$3CH_4 + HCO_3^- + 2H_2S + H^+$	−49	Some methylotrophic methanogens
$CH_3OH + H_2$	$CH_4 + H_2O$	−113	*Methanosphaera stadtmanii*, methylotrophic methanogens

[a]Other short chain alcohols are utilized including isopropanol.
[b]Other methylated amines utilized include dimethylamine and methylamine.
[c]Methyl mercaptan (methane thiol) is also used.
[d]$\Delta G^{\circ\prime}$ values from Thauer et al., 1977.

The most widespread catabolic reaction carried out by the methanogens is the reduction of CO_2 to CH_4 using H_2 as a reductant (Table 3.1). H_2 is a major fermentation product in many species of anaerobic bacteria, fungi, and protozoa. Most methanogens that can use $H_2{-}CO_2$ for methanogenesis (hydrogenotrophs), can also use formate as the electron donor for CO_2 reduction, using a formate dehydrogenase (Schauer and Ferry, 1980). Formate is a common fermentation product, especially in organisms which use a pyruvate-formate lyase in their fermentative metabolism, such as *Escherichia coli*. Formate is also a fermentation product of the plant metabolite oxalic acid (Allison et al., 1985).

Methanobacterium thermoautotrophicum (Daniels et al., 1978) and *Methanosarcina barkeri* (O'Brien et al., 1984) have been shown to grow on CO, apparently extracting electrons from CO using CO dehydrogenase, although H_2 may be an intermediate in the reaction. Growth on CO was very slow in both cases, and it

is not clear that CO is an important methanogenic precursor in anaerobic habitats. It has been more recently found that some hydrogenotrophic methanogens can also use short chain alcohols as electron donors, oxidizing secondary alcohols to ketones and primary alcohols to carboxylic acids (Widdel, 1986; Zellner and Winter, 1987). This finding overturned the conventional wisdom that alcohols other than methanol were not directly used by methanogens (Bryant et al., 1967), as will be described later.

Acetate is the ultimate endproduct of many fermentative pathways (see below) and can be an important CH_4 precursor in many habitats. Only two genera of methanogens are known to use acetate: *Methanosarcina* and *Methanothrix*. Of these two acetotrophic methanogens, *Methanosarcina* generally grows faster and to higher yield, and can use several different substrates, including methylated compounds and sometimes H_2-CO_2. *Methanothrix* is also called *Methanosaeta* (Patel and Sprott, 1990). A restricted subset of the methanogens, the methylotrophs, can use methanol and methylated amines, and some cultures can use methylated sulfides (Table 1.2). These include *Methanosarcina*, *Methanococcoides*, *Methanolobus*, and *Methanohalophilus*, all of which are members of the *Methanosarcinaceae* in the *Methanomicrobiales*. Methanol is probably not a major CH_4 precursor in anaerobic habitats, with small amounts arising from cleavage of methylated compounds such as pectin (Schink and Zeikus, 1980). Methanol may have been a more important methane precursor in early earth history since it has been found in high concentrations in comets, which may have made a significant contribution to the inventory of organic matter in the primordial earth (Bockelée-Morvan et al., 1991). *Methanosphaera stadtmanii*, which is a member of the *Methanobacteriales* is phylogenetically unrelated to the other methylotrophic methanogens, and can only grow on methanol and H_2 (Miller and Wolin, 1985).

Methylated amines, especially trimethylamine, are anaerobic breakdown products of methylated amino compounds such as choline and betaine. Choline is a constituent of the important lipid lecithin, and is readily broken down to trimethylamine. Betaine (glycine betaine) is a common osmoprotectant, accumulated to high concentrations inside the cells of many organisms growing in the presence of high salt (Csonka, 1989), and therefore considerable amounts of betaine can be expected to occur in saline and hypersaline environments with decomposing biomass. Trimethylamine N-oxide is also a common osmolyte in fish, and can be readily reduced to trimethylamine by anaerobes. Methylated sulfides are the breakdown products of the terminal methiol group of methionine (Zinder and Brock, 1978), and can be derived from sulfonium osmoprotectants such as dimethylpropiothetin (Kiene, 1990). They are generally minor methane precursors, and not all methylotrophic methanogens can use methylated sulfides (Oremland et al., 1989).

3.3 Physiological Adaptations of Methanogens to Their Environments

3.3.1 Salinity

Methanogens can be found in the complete range of salinities from freshwater to hypersaline. Freshwater methanogens typically require at least 1 mM Na^+ since an inwardly directed sodium motive force is involved in the bioenergetics of methanogenesis (Kaesler and Schönheit, 1989; Müller et al., 1987). While there is a great diversity of freshwater and marine methanogens, there are only a few known extremely halophilic methanogens, all of which are methylotrophs belonging to the *Methanosarcinaceae*. The high abundance of methylated osmoprotectants (e.g., betaine, dimethylpropiothetin) in halophiles has probably selected for methylotrophic methanogens in hypersaline environments in which anaerobic decomposition occurs. The most halophilic methanogens described are in the genus *Methanohalophilus* (Mathrani et al., 1988; Paterek and Smith, 1988; Zhilina and Zavarzin, 1990). *Methanohalophilus mahii* can grow well in salt concentrations up to 3 M. It is intriguing that 16S and 23S rRNA sequence comparisons show a specific relationship between the aerobic extremely halophilic archaeobacteria, such as *Halobacterium*, and the *Methanomicrobiales* (Burggraf et al., 1991), suggesting that the halobacteria and methylotrophic methanogens may have shared a common ancestor. Moreover, the *Methanosarcinaceae* are the only methanogens known to possess cytochromes (Kuhn et al., 1983), which are found in halobacteria.

An interesting adaptation to salt is found in the genus *Methanosarcina* (Sowers and Gunsalus, 1988). *M. thermophila*, considered to be a freshwater methanogen, was found to grow in marine medium after an adaptation period. Salt-adapted cells no longer had a thick "methanocondroitin" sacculus (Kreisl and Kandler, 1986) surrounding them, were sensitive to lysis by detergents, and grew as nearly single cells rather than as large clumps. Adaptation to marine medium caused a drop in the maximum growth temperature from 55°C to 45°C, consistent with the sacculus playing a role in cellular stabilization. Salt-adapted cells could be slowly adapted back to freshwater conditions. Similar results were obtained for several mesophilic *Methanosarcina* cultures, although not all lost their sacculi. These findings imply that the "methanocondroitin" sacculus in *Methanosarcina* is an adaptation to low salinity, acting as a corset to counter internal turgor pressure. That this sacculus is not found in the marine strain *Methanosarcina acetivorans* further supports this hypothesis.

Like other organisms, methanogens adapt to salinity by accumulating compatible solutes in their cytoplasm to equalize the external and internal osmolarity. In the marine methanogen *Methanococcus thermolithotrophicus*, β-glutamate was detected along with α-glutamate (Robertson et al., 1990). The novel metabolite N^{ε}-acetyl-β-lysine was detected in *Methanosarcina thermophila* and in *Methano-*

genium cariaci, *Methanohalophilus* sp., and *Methanococcus deltae*. At low osmolarity α-glutamate was the primary cytoplasmic solute, but at higher salinities, the proportion of N^{ϵ}-acetyl-β-lysine increased. When cells were grown in 1 M NaCl, the cytoplasmic concentration of N^{ϵ}-acetyl-β-lysine was close to 0.6 M in *M. thermophila*. It has also been found that methanogens can accumulate betaine if present in the growth medium (Robertson et al., 1990). Betaine was found in cells grown in medium containing yeast extract, which presumably provided betaine, or a precursor such as choline. More recently, both *N*,*N*,-dimethyl glycine and betaine were detected in *Methanohalophilus mahii* (Boone, 1992). Cells of *M. thermophila* or *M. cariaci* grown with betaine did not synthesize N^{ϵ}-acetyl-β-lysine when grown in medium containing betaine, indicating that genetic or biochemical mechanisms must exist to regulate its synthesis.

3.3.2 Temperature

Methanogens are found in a wide variety of thermal regimes, from marine sediments which are permanently at 2°C to geothermal areas above 100°C. There is a great diversity of both mesophilic and thermophilic methanogens. In general, thermophilic species grow more rapidly than corresponding mesophiles. For example, the doubling time on H_2-CO_2 at 37°C for *Methanococcus voltae* is near 2 h while that for *Methanococcus thermolithotrophicus* at 65°C is near 1 h, and the doubling time for *Methanococcus jannaschii* growing at 85°C in mineral medium is under 30 min (Jones et al., 1983), faster than the growth of *Escherichia coli* in glucose mineral salts medium. Growth rates of thermophilic acetotrophic methanogens are also higher than those of corresponding mesophiles (Zinder, 1990).

Of the methanogens described in a comprehensive review (Jones et al., 1987), only three species, *Methanolobus tindarius*, *Methanogenium cariaci*, and *Methanogenium marisnegri*, have growth optima below 30°C and none of them can grow below 10°C. There is evidence that temperatures below 15°C can greatly limit methanogenesis in freshwater habitats such as lake sediments and rice paddies (Conrad et al., 1987; Zeikus and Winfrey, 1976) and that the optimum for methanogenesis in these sediments is often near 35°C. Conrad et al. (Conrad et al., 1989) found that nearly all of the H_2 turnover in Lake Constance sediments incubated at 4°C was resistant to chloroform inhibition and was therefore presumably due to acetogens. H_2-CO_2 or methanol enrichments from sediments incubated at 4°C or 15°C only yielded acetogens. An acetogen isolated from Lake Constance sediments was capable of growth at 0°C, while a methanogen isolated from a rice paddy (none was isolated from Lake Constance) could not grow at temperatures below 18°C. It is well known in the anaerobic digestor literature that best results are obtained operating at temperatures near 35°C (McCarty, 1964), although operation of a manure digestor at 15°C has been reported (Zeeman

et al., 1988). Non-coastal marine sediments are often near 4°C, and it is clear that methanogenesis can occur in them (e.g., Warford et al., 1979), albeit slowly. Such sediments would be a good place to find methanogens capable of growing at temperatures less than 10°C.

Methanobacterium thermoautotrophicum, described in 1972 (Zeikus and Wolfe, 1972) was the first thermophilic methanogen ever isolated. This hydrogenotrophic methanogen grows optimally at 65°C and is cosmopolitan in its distribution, being found in hot springs (Zeikus et al., 1980) and thermophilic anaerobic digestors worldwide. Since that time, a considerable number of thermophilic methanogens have been described (Chapter 1). Notable among them are the extreme to hyperthermophiles isolated by K. O. Stetter and colleagues: *Methanococcus jannaschii* , a member of the *Methanococcales* isolated from an undersea spreading center with an temperature optimum (t_{opt}) of 85°C (Jones et al., 1983), *Methanothermus fervidus*, a member of the *Methanobacteriales* isolated from an Icelandic hot spring with a t_{opt} near 83°C (Stetter et al., 1981), and *Methanopyrus kandleri* (Kurr et al., 1991), which was isolated from shallow marine hydrothermal systems, has a t_{opt} near 100°C, and which does not belong to previously described methanogenic orders. It is of interest that, to this point, no extremely thermophilic methanogens belonging to the *Methanomicrobiales* have been isolated, especially since acetotrophic methanogens belong to this order (Zinder, 1990). The upper temperature limit for methanogenesis from acetate is presently 70°C, although one would expect higher temperature strains to exist.

It is not surprising that thermophily is common in methanogens since it is widespread in the archaeobacteria (Woese, 1987). To adapt to high temperature, an organism must ensure that its macromolecules (proteins, nucleic acids, and lipids) can maintain their structure and function at elevated temperatures (Sundaram, 1986). In general, proteins from thermophiles are stable at high temperature *in vitro* and this thermostability is reflected in subtle changes in their amino acid sequence (Sundaram, 1986). There is no evidence for novel amino acids occurring in proteins from extremely thermophilic methanogens.

An interesting phenomenon is the accumulation of high concentrations of the novel metabolite cyclic 2,3-diphosphoglycerate (cDPG) (see Part III, Chapters 10 and 11) in certain thermophilic methanogens. In the moderate thermophile *Methanobacterium thermoautotrophicum*, the cDPG concentration in the cytoplasm of non phosphate-limited cells is near 65 mM, while it is near 0.3 M (with K^+ as a counter-ion) in *Methanothermus fervidus* cells (Hensel and König, 1988), and is present at 1.1 M in *Methanopyrus kandleri* cells (Kurr et al., 1991). Glyceraldehyde-3-phosphate dehydrogenase and malate dehydrogenase from *M. fervidus* were stabilized against denaturation at 90°C by the presence of 0.3 M KcDPG (Hensel and König, 1988). This evidence is intriguing, but a cause and effect relationship between cDPG and thermostability has yet to be established.

There is also evidence that methanogens produce "heat shock proteins" in response to elevated temperature (Herbert et al., 1991), and that some hyperthermophilic archaeobacteria, including methanogens, may constitutively produce chaperonin-like proteins to maintain protein stability (Phipps et al., 1991).

High temperature denatures double stranded DNA into single strands, and higher G+C content increases the temperature of denaturation. Therefore, one might expect DNA from thermophiles to have a high G+C ratio, but this ratio for the DNA from *Methanothermus fervidus* is, in fact, near 33% (Stetter et al., 1981). While the high cytoplasmic potassium cDPG content in this organism stabilizes the DNA somewhat (Hensel and König, 1988), a histone-like protein appears to play a role in DNA stabilization in *M. fervidus*. This protein, called HMf, binds nonspecifically to DNA and has been shown to raise the melting temperature of linearized pUC18 in low ionic strength buffer by nearly 25°C (Sandman et al., 1990). Whether HMf-like proteins are more widespread in thermophilic methanogens is not known. A novel DNA topoisomerase, called reverse gyrase, has been found in hyperthermophilic archaeabacteria, including *M. fervidus* and *M. kandleri* (Bouthier de la Tour et al., 1990), as well as in hyperthermophilic eubacteria (Bouthier de la Tour et al., 1991). It is hypothesized that this enzyme, which produces positive supercoils in DNA, leads to greater stability of the more tightly coiled DNA.

Methanogens and other archaeobacteria have ether-linked isoprenoid lipids [see Part I, Chapter 3 and Jones et al. (1987)]. Since only archaeobacteria can grow at temperatures above 90°C, these lipids may be important in allowing membrane integrity at those temperatures. Many thermophilic methanogens have lipids in which the tail groups are covalently linked end-to-end, thereby forming a single membrane-spanning tetraether molecule spanning the membrane. It makes intuitive sense that membranes containing such lipids would be more stable. *Methanococcus jannaschii* had a higher ratio of tetraethers to diethers when grown at 75°C than at 45°C (Sprott et al., 1991), supporting this contention. However, it should be noted that *Methanopyrus kandleri*, which grows a temperatures over 100°C, does not have any tetraether lipids (Kurr et al., 1991).

3.3.3 pH

Most methanogens have pH optima near neutrality (Jones et al., 1987). There are examples of methanogens existing in extreme pH environments; for example, peat bogs can have pH values of 4.0 or below and can produce CH_4. Peat samples (initial pH = 3.9) still showed significant methanogenic activity when incubated at pH = 3.0, although the optimal pH value for methanogenesis was near 6.0 (Williams and Crawford, 1984). H_2-CO_2 addition stimulated methanogenesis while acetate addition inhibited it, most likely due to acetic acid/acetate accumulation inside cells (Russell, 1991). It has long been known that high concentrations

of fatty acids can enhance the inhibitory effect of low pH on methanogenesis in anaerobic bioreactors (McCarty, 1964). A hydrogenotrophic methanogen, most likely *Methanobacterium*, was isolated from peat bogs, and was found to be able to grow to pH values as low as 5 and to produce some methane down to pH = 3 (Williams and Crawford, 1985). A study of carbon flow in bog sediments (pH = 4.9) using radiotracers (Goodwin and Zeikus, 1987) demonstrated that both CO_2 reduction and methanogenesis from acetate could occur in those sediments at pH values as low as 4.0, although optimal methanogenesis occurred at pH 5–6.

There are also some moderately alkaliphilic methanogens, such as *Methanobacterium thermoalcaliphilum* (Blotevogel et al., 1985), which grows optimally near pH = 8 and can grow at pH = 9. Many hypersaline habitats are alkaline, and *Methanohalophilus zhilinae*, which has an optimum pH value of 9.2, was isolated from an alkaline hypersaline lake in Egypt (Mathrani et al., 1988).

3.3.4 Oxygen

Methanogens have the reputation of being the strictest of anaerobes. It is generally considered that methanogens need an oxidation/reduction potential in the growth medium more negative than −0.3 V (Hungate, 1967). Hungate (1967) calculated that the O_2 concentration at that potential is theoretically 10^{-56} mole per liter, so that it can safely be assumed that O_2 is not present in well reduced habitats.

Although methanogens will not grow or make CH_4 in the presence of oxygen, they can be fairly tolerant to oxygen exposure. The most comprehensive study of O_2 toxicity in methanogens was that of Kiener and Leisinger (Kiener and Leisinger, 1983). They took the important precaution of measuring survival in buffer lacking reducing agents, which can react with O_2, thereby forming peroxides and other toxic byproducts. They found that viability dropped about 100-fold within 10 h of exposure to air for *Methanococcus voltae* and *Methanococcus vanielii*. *Methanobrevibacter arboriphilus* and *Methanobacterium thermoautotrophicum* maintained viability for several hours before dying, and *Methanosarcina barkeri* maintained viability for over 24 h, which was attributed to its forming multicellular clumps. Thus, there is considerable variability in the sensitivity of methanogens to O_2. This suggests that methanogens can exist in some habitats in which there are anaerobic microenvironments or in which transient anaerobic conditions occur. For example, we have found (Chin and Zinder, 1982) viable counts of hydrogenotrophic and acetotrophic methanogens greater than 10^3 per gram dry weight in presumably aerobic soil under grass.

There is some evidence of adaptation to O_2 by methanogens. Low levels of superoxide dismutase have been detected in methanogens (Kirby et al., 1981). Another adaptation phenomenon was discovered in *Methanobacterium thermoau-*

totrophicum (Hausinger et al., 1985). When this methanogen was exposed to O_2, it forms an ester between the phosphate group of AMP or GMP and the 8-hydroxy group of the deazaflavin ring in the electron carrier coenzyme F_{420}. These modified cofactors had an absorbance maximum near 390 nm (and are therefore called F_{390} derivatives) and they no longer reacted with F_{420}-dependent hydrogenase. No F_{390} derivatives were detected in H_2 starved cells, and they were apparently converted back to F_{420} when anaerobiosis was restored (Kiener et al., 1988). This modification may therefore act by switching off reductive metabolism in the presence of O_2, and may also serve as a general alarmone for the presence of oxygen (Hausinger et al., 1985).

3.3.5 Genetic and Metabolic Regulation

An important aspect of the interaction between an organism and its environment is the regulation of the organism's activities in response to environmental stimuli. Short-term responses usually involve regulation of enzyme activity while long-term responses involve changes in gene expression (i.e., induction and repression). Only a few examples of this type of metabolic regulation have been found in methanogens. A more detailed description of the molecular mechanisms of gene regulation in methanogens can be found in Part IV, Chapter 12.

In terms of regulation of catabolic activity by substrate availability, many methanogens use only one or two substrates, so that one may not expect to see sophisticated regulatory networks. For example, *Methanobacterium formicicum* showed less than a twofold increase in formate dehydrogenase (FDH) activity when grown on formate versus growth on H_2-CO_2 (Schauer and Ferry, 1980), indicating that this enzyme is essentially constitutive in this organism. In contrast, *Methanococcus thermolithotrophicus* showed a 10-fold higher FDH activity when grown on formate alone than when grown on H_2-CO_2 or H_2-CO_2 plus formate (Sparling and Daniels, 1990), consistent with repression of FDH synthesis by H_2-CO_2. Methanogens that use alcohols longer than methanol as electron donors for CO_2 reduction will show alcohol dehydrogenase activity, even in the presence of H_2-CO_2 (Bleicher et al., 1989; Widdel and Wolfe, 1989). The presence of an alcohol (not always one which can be utilized) in the growth medium was necessary for this activity except in the case of *Methanogenium thermophilicum* which expressed this activity when H_2 was limiting even when no alcohol was present. Another response to H_2 limitation may be the synthesis of an autolytic enzyme by *Methanobacterium wolfei* (Kiener et al., 1987), although the exact purpose of this suicidal enzyme is unclear and it may even be related to induction of a defective bacteriophage (Meile et al., 1989).

Probably the best studied catabolic regulatory system in methanogens is the regulation of methanogenesis from acetate in *Methanosarcina*. Smith and Mah (Smith and Mah, 1978) first demonstrated that when *Methanosarcina* was pre-

sented with both methanol and acetate in the growth medium, it showed diauxie, using methanol preferentially. Similar results were obtained for H_2-CO_2 (Ferguson and Mah, 1983) or trimethylamine and acetate (Blaut and Gottschalk, 1982). The $\Delta G^{\circ\prime}$ per CH_4 for methanol or H_2-CO_2 is greater than for acetate (Table 3.1) and growth yields of *Methanosarcina* were greater on these substrates, so it is not surprising that they are preferred. In the case of *M. thermophila*, it was found that methanol-grown cells could use acetate at only 3% of the rate of acetate-grown cells, even when acetate was present in the medium (Zinder and Elias, 1985). Also, acetate-grown cells could only use methanol at 1% the rate that methanol-grown cells could. Cells in the acetate phase of growth on methanol-acetate medium could use either substrate alone or both together at rapid rates. The results were consistent with a regulatory model in which acetate is repressed by methanol, but methanol metabolism itself is inducible. There is now evidence for regulation of carbon monoxide dehydrogenase (Krzycki et al., 1982) and acetate kinase (Aceti and Ferry, 1988), two enzymes involved in methanogenesis from acetate in *Methanosarcina*. These two enzymes may represent the "tip of the iceberg" since out of 400 protein spots resolved in two-dimensional electrophoretograms from either acetate-grown or methanol-grown *M. thermophila* cells, there were ca. 140 spots present in one but not the other, although some of these changes may reflect changes in protein charge rather than synthesis (Jablonski et al., 1990).

In terms of regulation of biosynthetic pathways, it appears that they are constitutive in many methanogens. For example, most methanogens studied apparently do not take up exogenous amino acids and therefore synthesize them *de novo* with the exception of some *Methanococcus* spp. (Ekiel et al., 1985; Whitman et al., 1987). Acetate can be taken up and used as a major precursor of cell carbon in many methanogens (see Part III, Chapter 10). It was found that *M. thermoautotrophicum* strain THF had approximately sixfold higher activity of carbon monoxide dehydrogenase, an enzyme involved in acetyl-CoA synthesis in this organism, than did cells grown in the presence of acetate (Lee and Zinder, 1988), suggesting regulation of the acetyl-CoA biosynthetic pathway.

Nitrogen fixation is an energetically costly process to a cell, and free living eubacterial nitrogen fixing bacteria will regulate both biosynthesis and activity of nitrogenase in the presence of a preferred nitrogen source such as ammonia (Postgate, 1982). Nitrogen-fixing methanogens grown in the presence of ammonia have been shown not to reduce acetylene to ethylene (Belay et al., 1984; Lobo and Zinder, 1990), and Western immunoblot analysis showed that the dinitrogenase reductase was not present in ammonia grown cells (Lobo and Zinder., 1990; Magot et al., 1986), consistent with genetic regulation. Nitrogen fixing cells of *Methanosarcina* exposed to ammonia concentrations as low as 10 μM showed a transient cessation of acetylene reduction (Lobo and Zinder, 1990), a phenomenon

called switch-off. Thus nitrogen fixation can be a highly regulated process in methanogens with regulation occurring at both the genetic and metabolic levels.

The above examples show that methanogens can respond at the genetic and metabolic level to some environmental stimuli. As we understand the biology of methanogens better, other examples will arise, and perhaps they, in turn, will allow us to understand better their role in the ecology of methanogens.

3.3.6 Motility and Gas Vesicles

Motility is found in the Methanococcales, and in the *Methanomicrobiales* in the genera *Methanospirillum, Methanogenium, Methanolobus*, and *Methanomicrobium* (Jones et al., 1987). The architecture of the flagella of *Methanococcus voltae* and *Methanospirillum hungatei* closely resembles that eubacterial flagella, consisting of a helical filament, a hook region, and a disc-like basal body motor (Kalmokoff et al., 1988). The only convincing rationale for motility in bacteria is tactic response toward or away from environmental stimuli. *Methanococcus voltae* requires acetate, leucine, and isoleucine, and all three were shown to be attractants using an Adler capillary assay (Sment and Konisky, 1989). Histidine was not an attractant. *Methanospirillum hungatei* strain GP1 showed chemotaxis towards acetate (Migas et al., 1988), which it requires for growth.

It is likely that methanogenic substrates, such as H_2 or formate, are chemoattractants, but this has not been tested for methanogens, perhaps due to the formidable technical task of establishing anaerobic gradients of these substrates, which are also energy sources. Certainly, one can imagine that there would be a gradient of H_2 emanating from a cellulose particle being decomposed by H_2 -producing anaerobes, and that a methanogen which could follow that trail may have a selective advantage, although other fermentation products, such as acetate, may work as well. The advantage of chemotaxis would be negated in a mechanically mixed system such as a stirred bioreactor.

Another mechanism that microorganisms can use to adjust their positions in habitats is gas vesicles for flotation. Gas vesicles have only been detected in some mesophilic *Methanosarcina* strains (Mah et al., 1977; Zhilina and Zavarzin, 1987) and three thermophilic *Methanothrix* strains (Kamagata and Mikami, 1991; Nozhevnikova and Chudina, 1985; Zinder et al., 1987). The function of gas vesicles in these organisms is not known. It has been noted that *Methanothrix* sp. strain CALS-1 cells have fewer gas vesicles during earlier stages of growth, and have more gas vesicles when entering stationary phase (Zinder et al., 1987). I have occasionally noted bands of cells floating at the top of *Methanothrix* sp. strain CALS-1 cultures that have exhausted their substrate. It is possible that this is a mechanism to vacate a habitat poor in acetate. It should be mentioned that the gas vesiculated *Methanothrix* sp. strain CALS-1 and *Methanosarcina barkeri*

strain W (Mah et al., 1977) were both isolated from continuously mixed anaerobic bioreactors where flotation would not be an advantage. *Methanothrix*-like cells in a continuously mixed thermophilic bioreactor contained gas vesicles (Zinder et al., 1984), suggesting that their presence was either vestigial or that they were serving a function other than flotation.

3.3.7 Reserve Materials

Organisms require endogenous sources of energy and nutrients to survive times when there is no exogenous source, and evidence has accrued that this holds true for methanogens. For example, motile hydrogenotrophic methanogens often maintain motility in wet mounts for microscopy long after the small amount of H_2 present in the medium would have been exhausted. Often, these reserve materials are polymers that can be stored as reserve sources of energy or nutrients during times in which there is an excess of the nutrient they store. The reserve polymers glycogen and polyphosphate have been detected in methanogens.

Glycogen was detected in *Methanosarcina* (Murray and Zinder, 1987), *Methanothrix* (Pellerin et al., 1987), *Methanolobus* (König et al., 1985), and *Methanococcus* (König et al., 1985). The percentage of cell dry weight varied from about 1% up to about 13% in the case of *Methanococcus thermolithotrophicus*. Nitrogen limitation and carbon/energy excess, the classic stimulus for glycogen storage in other organisms, led to glycogen accumulation in *Methanosarcina thermophila* and *Methanolobus tindarius* (König et al., 1985; Murray and Zinder, 1987). Evidence for degradation of glycogen under energy starvation conditions was obtained in both *M. thermophila* and *M. tindarius* (König et al., 1985; Murray and Zinder, 1987), with complete mobilization occurring within 24 h in the case of *Methanosarcina*. One mole of CH_4 was detected per mole of glycogen degraded by *M. tindarius*. Evidence was obtained that glycogen-containing cells of *M. thermophila* maintained higher ATP levels upon starvation than those lacking glycogen and could switch more readily from acetate to methanol as a methanogenic substrate (Murray and Zinder, 1987). These studies, while not clearly showing cause and effect, suggest that glycogen can serve as a short-term energy reserve in methanogens. It is curious that glycogen, an internal carbohydrate, can be used by methanogens while utilization of exogenous carbohydrates has never been detected. This may reflect the inability of methanogens to compete with present-day fermentative bacteria for such substrates (McInerney and Beaty, 1988), as previously discussed.

Polyphosphate has also been detected in *Methanosarcina* (Rudnick et al., 1990; Scherer and Bochem, 1983). It was shown that the amount of polyphosphate stored by *Methanosarcina frisia* was dependent on the phosphate concentration in the growth medium, with up to 0.26 g polyphosphate stored per gram of protein in cells grown in medium with 1 mM phosphate (Rudnick et al., 1990). No

physiological role has been investigated for polyphosphate in methanogens. Indeed, while there is evidence polyphosphate can serve to phosphorylate sugars and AMP and can serve as a phosphate reserve, the physiological roles of polyphosphate in eubacteria are still not clear (Wood and Clark, 1988).

As previously mentioned, members of the *Methanobacteriales* and *Methanopyrus* (Kurr et al., 1991) contain cyclic 2,3-diphosphoglycerate (cDPG). *Methanosarcina frisia* also has low cDPG levels (Rudnick et al., 1990). Because of the high-energy ester bonds in this molecule, it is reasonable to assume that this compound can serve as an energy reserve, and it was originally called "methanophosphogen" (Kanodia and Roberts, 1983), although direct evidence that it serves such a role is lacking. *Methanobacterium thermoautotrophicum* was found to store cDPG only when phosphate and H_2 were available (Seely and Fahrney, 1984). Evidence has also been obtained that cDPG may serve as a biosynthetic intermediate (Evans et al., 1985). Recently it was found that extracts of *M. thermoautotrophicum* contained high levels of 2,3-diphosphoglycerate, which could be converted to ATP via the formation of phosphoenolpyruvate (van Alebeek et al., 1991). The authors speculated that the 2,3-diphosphoglycerate could be derived from cDPG, and this reaction was recently detected in *M. thermoautotrophicum* extracts (Sastry et al., 1992). Thus, a variety of roles for cDPG have been proposed (phosphogen, phosphate storage compound, biosynthetic intermediate, protein stabilizer, osmolyte) and it may fulfill one or more of these in different methanogens.

3.4 Microbial Interactions

3.4.1 Competition for Methanogenic Substrates: General Considerations

Methanogens generally compete with three other major anaerobic metabolic groups for their substrates in natural habitats: sulfate reducing bacteria, acetogens (Table 3.2), and ferric iron (Fe^{3+}) reducers. Most sulfate reducing bacteria are clustered in the delta branch of the Gram negative proteobacteria (Woese, 1987). The genus *Desulfotomaculum* is in the Gram positive branch of the eubacteria, and the extreme thermophile *Archaeoglobus* is an archaebacterium (Woese, 1987). They can use sulfate, or other oxidized forms of sulfur such as thiosulfate, sulfite, and elemental sulfur, as electron acceptors producing sulfide as the major reduced product (Widdel, 1988). As a group, they can use a much greater diversity of electron donors than do methanogens, including organic acids, alcohols, amino acids, and aromatic compounds. Acetogens (sometimes called H_2-consuming acetogens, or homoacetogens) are in the Gram positive branch of the eubacteria (Woese, 1987), and, as a group, can use an even greater variety of substrates, including sugars, purines, and methoxyl groups of methoxylated aromatic com-

Table 3.2 H_2 and acetate utilization by Fe^{3+} reducing bacteria, sulfate reducing bacteria, methanogens, and acetogens

Reactants	*Products*	$\Delta G^{\circ\prime}$ *kJ/rxn*
$4H_2 + 8Fe^{3+}$	$8H^+ + 8Fe^{2+}$	−914
$4H_2 + SO_4^{2-} + H^+$	$HS^- + 4H_2O$	−152
$4H_2 + HCO_3^- + H^+$	$CH_4 + 3H_2O$	−135
$4H_2 + 2HCO_3^- + H^+$	$CH_3COO^- + 4H_2O$	−105
$CH_3COO^- + 8Fe^{3+} + 4H_2O$	$2HCO_3^- + 8Fe^{2+} + 9H^+$	−809
$CH_3COO^- + SO_4^{2-}$	$2HCO_3^- + HS-$	−47
$CH_3COO^- + H_2O$	$CH_4 + HCO_3^-$	−31

[a] $\Delta G^{\circ\prime}$ values from (Thauer et al., 1977).

pounds (Ljungdahl, 1986). Fe^{3+} reducers have only recently been described. One Fe^{3+} reducer, called GS-15, can use acetate or aromatic compounds as electron donors (Lovley and Lonergan, 1990; Lovley et al., 1987), while *Shewanella putrifaciens* can use H_2, formate, or organic compounds as electron donors for ferric iron reduction (Lovley et al., 1989).

From ecological data described below, it is found that in habitats in which organic substrate (electron donor) is limiting, there is a hierarchy for competition for electron donor in which Fe^{3+} reducers can outcompete other organisms if their electron acceptor is present, followed by sulfate reducing bacteria, methanogens, and acetogens. For example, addition of sulfate to freshwater sediments, where it is usually present in limiting amounts, often greatly inhibits methanogenesis (Abram and Nedwell, 1978; Lovley et al., 1982; Winfrey and Zeikus, 1977). While this is in agreement with the $\Delta G^{\circ\prime}$ values for the reactions (Table 3.2), the differences between the values are modest, with a 12.5% more favorable $\Delta G^{\circ\prime}$ for sulfate reduction versus methanogenesis using H_2 as the electron donor. Yet methanogenesis is often nearly completely inhibited in habitats high in sulfate, such as marine sediments. However, these $\Delta G^{\circ\prime}$ values are for substrates and products at molar concentrations for solutes and 1 atm partial pressures for gases, and these differences in reaction thermodynamics become more significant under conditions found in natural habitats. First, competition for H_2 will be discussed, followed by competition for acetate and other substrates.

3.4.2 Competition for H_2

H_2 in methanogenic habitats is simultaneously produced and consumed and its steady-state concentration is often extremely low in habitats receiving limiting

amounts of organic matter, such as in lake sediments (Table 3.3). The development of the reduction gas detector to detect H_2 in sub part-per-million partial pressures has greatly increased our understanding of H_2 dynamics in such systems. The partial pressure of H_2 in headspaces in equilibrium with methanogenic lake sediments, for example, is typically only a few pascals (1 Pa $\approx 10^{-5}$ atm or 10 parts-per-million), and this represents a dissolved H_2 concentration in the nanomolar range. H_2 partial pressures can be higher in systems with a higher organic loading rate or shorter retention time, i.e., anaerobic digestors and the animal rumen (Table 3.3).

The first approach to understanding competition between H_2 -consuming anaerobes was to examine apparent K_m values for H_2 utilization. Robinson and Tiedje (1982) pointed out the importance, when measuring the progress of H_2 utilization, of ensuring that the H_2-consuming reaction was occurring slowly enough so that the reaction is not limited by H_2 transfer from the gas phase to the liquid phase. Such phase transfer limitation can cause overestimation of apparent K_m, a problem in some previous studies. Results for pure cultures and some natural systems are shown in Table 3.4. Apparent K_m values for H_2 uptake by methanogens and methanogenic habitats were 4–8 μM H_2 (550–1100 Pa), while values were lower for sulfate reducing bacteria, about 2 μM, and *Sporomusa termitida*, an acetogen, had a K_m value of 6 μM. V_{max} values were more variable, with methanogens and sulfate reducing bacteria having similar values (Robinson and Tiedje, 1984). While K_m values did correlate with the ability of sulfate reducing bacteria to outcompete methanogens, they are considerably higher than the H_2 concentrations found in natural habitats (Table 3.3). These high K_m values probably represent intrinsic limitations on on the ability of hydrogenases to use H_2 at low partial pressures.

An alternative to the Michaelis-Menton model to describe the interactions of anaerobic hydrogenotrophs is one involving minimum thresholds for substrate

Table 3.3 Representative H_2 concentrations in methanogenic habitats

Habitat	*H_2(Pa)*[a]	*H_2(nM)*	*Reference*[b]
Lake Mendota sediments	4.8	36	(1)
Knaak Lake sediments	3.7	28	(1)
Rice paddy	2.7	28	(2)
Sewage sludge	27.	203	(1)
Rumen fluid (basal level)	187	1,400	(3)
Rumen fluid (post-feeding)	2,000	15,000	(3)

[a](Pascals, 1 atm = 101.1 × 10^5 Pa.

[b]References: 1, Conrad et al., 1986; 2, Conrad et al., 1987; 3, Smolenski and Robinson, 1988.

utilization. Lovley et al. (1982) showed that addition of sulfate to Wintergreen Lake sediments corresponded with a decrease in the H_2 partial pressure from ca. 1 to 0.2 Pa concurrent with inhibition of methanogenesis and suggested that the latter H_2 partial pressure was below the point at which methanogens could use H_2. Subsequently, Lovley (1985), showed that resting cells of several mesophilic hydrogenotrophic methanogens could not use H_2 at partial pressures below 6 Pa. The the pure cultures were assayed at 39°C, and as will be discussed presently, temperature can affect the thermodynamics of the reactions (Conrad and Wetter, 1990; Lee and Zinder, 1988) with higher thresholds occurring at higher temperatures.

A wide range of hydrogenotrophic anaerobes were examined for H_2 thresholds (Cord-Ruwisch et al., 1988), and there was an inverse correlation between the free energy available for the reaction and the threshold (Table 3.5). The threshold for methanogens was considerably below that for an acetogen, while that for a sulfate reducer was lower still. Thus, sulfate reducers can reduce the partial pressure of H_2 to a level low enough that methanogens cannot use it. *Acetobacterium woodii* could use H_2 at much lower partial pressures when it used caffeate as an electron acceptor, showing that thresholds were more reaction-specific than organism-specific.

Thresholds can be readily explained by the thermodynamic effect of H_2 partial pressure on H_2 consuming reactions. This can be estimated by the free energy form of the Nernst Equation. For a chemical reaction occurring at 25C: aA + bB $\rightarrow$ cC + dD, we can estimate the $\Delta G'$ (pH = 7) in kJ as:

Table 3.4 Apparent K_m values for H_2 uptake by pure cultures and methanogenic habitats

	Apparent K_m		
Organism or habitat	*μM*	*Pa*	*Reference*[a]
Methanospirillum hungatei	5	670	1
Methanosarcina barkeri	13	1,700	1
Methanobacterium thermoautotrophicum	8	1,100	2
Methanobacterium formicicum	6	800	2
Desulfovibrio vulgaris	2	250	1
Desulfovibrio desulfuricans	2	270	2
Sporomusa termitida	6	800	3
Rumen fluid	4–9	~860	4
Sewage sludge	4–7	~740	4

[a]References: 1, Robinson and Tiedje, 1984; 2, Kristijansson et al., 1982; 3, Breznak et al., 1988; 4, Robinson and Tiedje, 1982.

$$\Delta G' = \Delta G^{\circ\prime} + RT \ln \frac{(C)^c(D)^d}{(A)^a(B)^b} = \Delta G^{\circ\prime} + 5.7 \log \frac{(C)^c(D)^d}{(A)^a(B)^b}$$

where (A) denotes the molar concentration of A if it is a solute, or its partial pressure in atm if it is a gas, R is the ideal gas constant and T is the absolute temperature in degrees kelvin. H_2O or H^+ are not included in the quotient since their concentrations are constant at 55 M and 10^{-7} M (i.e., pH = 7), respectively, under standard conditions. For methanogenesis from H_2–CO_2, with HCO_3^- at 10 mM and CH_4 at 0.5 atm, one can calculate the dependence of the $\Delta G'$ on H_2 partial pressure as follows:

$$\Delta G' = -131 + 5.7 \log \frac{(CH_4)}{(HCO_3^-)} - 5.7 \log (H_2)^4 = -123 - 22.8 \log (H_2)$$

Thus, plotting the log of H_2 partial pressure versus $\Delta G'$, a straight line with slope 22.8 kJ/10-fold increase in H_2 partial pressure is generated (Figure 3.1). Lines of identical slope are generated for acetogenesis and sulfate reduction using H_2 since they also use four moles of H_2 per reaction. However, they intersect $\Delta G' = 0$ at different H_2 partial pressures.

Table 3.5 Thresholds for hydrogenotrophic anaerobes

Organism	*Electron Accepting Rxn*	$\Delta G^{\circ\prime}$ *(kJ/molH_2)*	*H_2 Threshold (Pa)*	*(nM)*
Acetobacterium woodii	$CO_2 \rightarrow$ acetate	−26.1	52.	390.
Methanospirillum hungatei	$CO_2 \rightarrow CH_4$	−33.9	3.0	23.
Methanobrevibacter smithii	$CO_2 \rightarrow CH_4$	−33.9	10.	75.
Desulfovibrio desulfuricans	$SO_4^= \rightarrow H_2S$	−38.9	0.9	6.8
Acetobacterium woodii	caffeate → hydrocaffeate	−85.0	0.3	2.3
Wolinella succinogenes	fumarate → succinate	−86.0	0.002	0.015
Wolinella succinogenes	$NO_3^- \rightarrow NH_4^+$	−149.	0.002	0.015

[a]Data from Cord-Ruwisch et al. (1988).

Also represented on Figure 3.1 are the minimum thresholds for the three groups from Table 3.5. There is approximately 25 kJ/rxn of energy available for energy for methanogens and acetogens at their thresholds, clearly not enough to conserve an ATP (30–45 kJ/mol, Thauer et al., 1977), while sulfate reducing bacteria can conserve close to 45 kJ/rxn under the specified conditions. These values are considerably less than the $\Delta G^{\circ\prime}$ values, which better approximate the energy potentially available to pure cultures incubated under several atm of H_2. Thus, these organisms are energetically "geared" to use H_2 at the low partial pressures found in natural habitats.

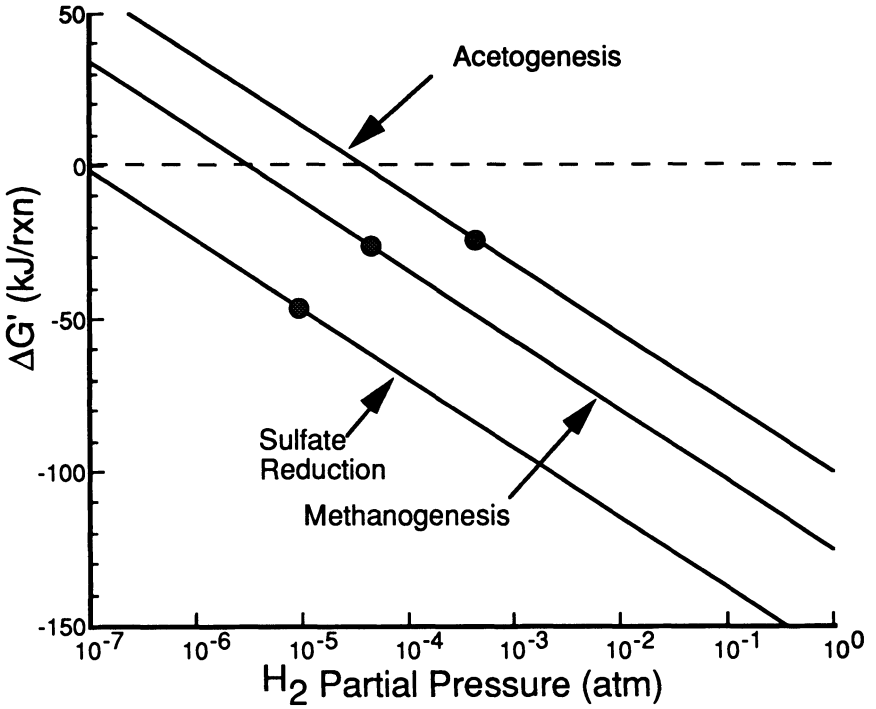

Figure 3.1. Effect of H_2 partial pressure on the free energy of methanogenesis, sulfate reduction, or acetogenesis using H_2 . The equations for the reactions are in Table 3.2. The dots show typical H_2 thresholds for the various microbial groups shown in Table 3.5. The concentrations for the products and reactants other than H_2 are: HCO_3^- and SO_4^{2-}, 20 mM; CH_4, 0.5 atm; HS^- = 1 mM; acetate, 10 mM.

At 10^{-4} atm (10 Pa) H_2, a methanogen could still be active, while an acetogen would not be able to use H_2. Similarly, a H_2 partial pressure of 10^{-5} atm would allow a sulfate reducer to use H_2, but not a methanogen. Thus, the threshold model provides a good explanation for the competitive hierarchy seen in natural habitats. It should also be mentioned that at 10^{-4} atm, the $E^{\circ\prime}$ value for the H_2/H^+ couple is only -296 mV, not the standard $E^{\circ\prime}$ value of -414 mV for 1 atm H_2 at pH = 7.

Fe^{3+} is often supplied to anaerobic habitats, such as sediments, by diffusion from aerobic zones, where Fe^{+2} is chemically or biologically oxidized by O_2. The energetics of Fe^{3+} reduction are extremely favorable (Table 3.2), although the limited solubility of Fe^{3+} probably makes the actual free energies considerably lower. Both methanogenesis and sulfate reduction in Potomac river sediments

were inhibited by addition of Fe^{3+} (Lovley and Phillips, 1987). The H_2 partial pressures were 0.82 Pa, 0.17 Pa, and 0.03 Pa in methanogenic, sulfate amended, and Fe^{3+} amended sediments, respectively (Lovley and Phillips, 1987), consistent with threshold models. Amorphous ferric hydroxide was the most effective form of Fe^{+3}, apparently because of the greater availability of Fe^{3+} as compared to more highly crystalline forms.

3.4.3 Competition for Acetate

While little is known about the physiological properties that will favor one hydrogenotrophic methanogen species over another, there is considerable evidence that high acetate concentrations favor the faster growing and more versatile *Methanosarcina*, while the slow growing specialist *Methanothrix* is usually favored by low acetate concentrations. For example, in our own studies on populations in a thermophilic anaerobic bioreactor (Zinder et al., 1984), a displacement of *Methanosarcina* by *Methanothrix* as the dominant acetotrophic methanogen was correlated with acetate concentrations falling below 1 mM, and similar results have been obtained in another thermophilic bioreactor (Wiegant and de Man, 1986). As in the case of hydrogenotrophic methanogens, both Michaelis-Menton and threshold models have been used to describe this competition.

Values for apparent K_m for acetate utilization by *Methanosarcina* typically are 3–5 mM, while those for *Methanothrix* cultures are typically less than 1 mM (Table 3.6). The K_m values presented in Table 3.6 were measured in different

Table 3.6 Apparent K_m values and minimum thresholds for acetate catabolism by acetotrophic anaerobic cultures.

Organism	*Apparent* K_m	*Reference*[a]	*Threshold*	*Reference*[a]
Methanosarcina barkeri Fusaro	3.0[b]	1	0.62[b]	2
Methanosarcina barkeri 227	4.5	3	1.2	3
Methanothrix sp.	—	—	0.069	3
Methanothrix soehngenii Opfikon	0.8	4	0.005	5
Methanothrix sp. CALS-1	>0.1	6	0.012	6
Methanothrix soehngenii GP 1	0.86	7	—	—
Methanothrix soehngenii MT-1	0.49	7	—	—
TAM organism	0.8 mM	8	0.075	8
Acetate-oxidizing syntrophic culture	—	—	>0.2 mM	9
Desulfobacter postgatei	0.23	1	—	—

[a]References: 1, Schönheit et al., 1982; 2, Fukazaki et al., 1990; 3, Westermann et al., 1989; 4, Huser et al., 1982; 5, Jetten et al., 1990; 6, Min and Zinder, 1989; 7, Ohtsubo et al., 1992; 8, Ahring and Westermann, 1987; 9, Zinder and Koch, 1984.

[b]All values are millimoles per liter.

ways: some were from progress curves for acetate utilization (Ahring and Westermann, 1987; Jetten et al., 1990; Min and Zinder, 1989; Schönheit et al., 1982), some were from initial rates at different acetate concentrations (Ohtsubo et al., 1992; Westermann et al., 1989), and others were based on the effect of acetate on doubling time for methanogenesis (Huser et al., 1982). Thus, it is not surprising that different results can be obtained, sometimes even for the same strain. For example, *Methanothrix soehngenii* Opfikon had a K_m value, based on the doubling time for methanogenesis from acetate, near 0.8 mM (Huser et al., 1982), while progress curves for acetate utilization showed no diminution of rate down to concentrations below 0.1 mM (Jetten et al., 1990). Temperature can also affect K_m values for acetate (Westermann et al., 1989). In any event, it is clear that K_m values are lower for *Methanothrix* than *Methanosarcina*. This can be attributed to their respective mechanisms of acetate activation. There is strong evidence that *Methanosarcina* uses acetate kinase/phosphotransacetylase to form acetyl-CoA (Aceti and Ferry, 1988), while *Methanothrix* uses an acetyl-CoA synthetase (acetate thiokinase) (Jetten et al., 1990), which have K_m values for acetate close to those determined for the organism in which they are found (for a more detailed description see Part II, Chapter 6).

Minimum thresholds for acetate utilization have also been found for acetotrophic methanogens. Those for *Methanosarcina* are typically 0.5 mM and higher, while those for *Methanothrix* are in the micromolar range (Table 3.6). An interesting contribution was made by Fukazaki et al. (1990), who showed that the acetate threshold for *M. barkeri* Fusaro was 0.26 mM at pH = 6.3, and 2.17 mM at pH = 7.4. Interestingly, the amount of undissociated acetic acid at these pH values was 6.7 and 4.4 μM, respectively, suggesting that acetic acid is the actual substrate responsible for the threshold. This pH effect may be a partial explanation for different results obtained for acetate thresholds and apparent K_m values in various studies. Figure 3.2 shows that there is roughly twice as much free energy available to *Methanosarcina* at its threshold near 1 mM acetate than there would be to *Methanothrix* at 5 μM acetate. Indeed, yield coefficients for growth on acetate are typically lower for *Methanothrix* than for *Methanosarcina* (Huser et al., 1982; Smith and Mah, 1978; Zinder et al., 1987), suggesting that *Methanothrix* conserves less energy than does *Methanosarcina* from methanogenesis from acetate, and that this *lower* efficiency of energy conservation allows it to use acetate at lower concentrations.

Methanothrix can be considered a specialist that only uses acetate, uses it slowly and with low yield, but can use it even at very low concentrations. On the other hand, *Methanosarcina* is more of a generalist, being able to grow on several substrates, use them rapidly with higher cell yield [cell yields of *Methanosarcina* on H_2-CO_2 are also generally higher than strictly hydrogenotrophic methanogens (Daniels et al., 1984)], but can only use acetate at relatively high concentrations. All other things being equal, *Methanosarcina* would be

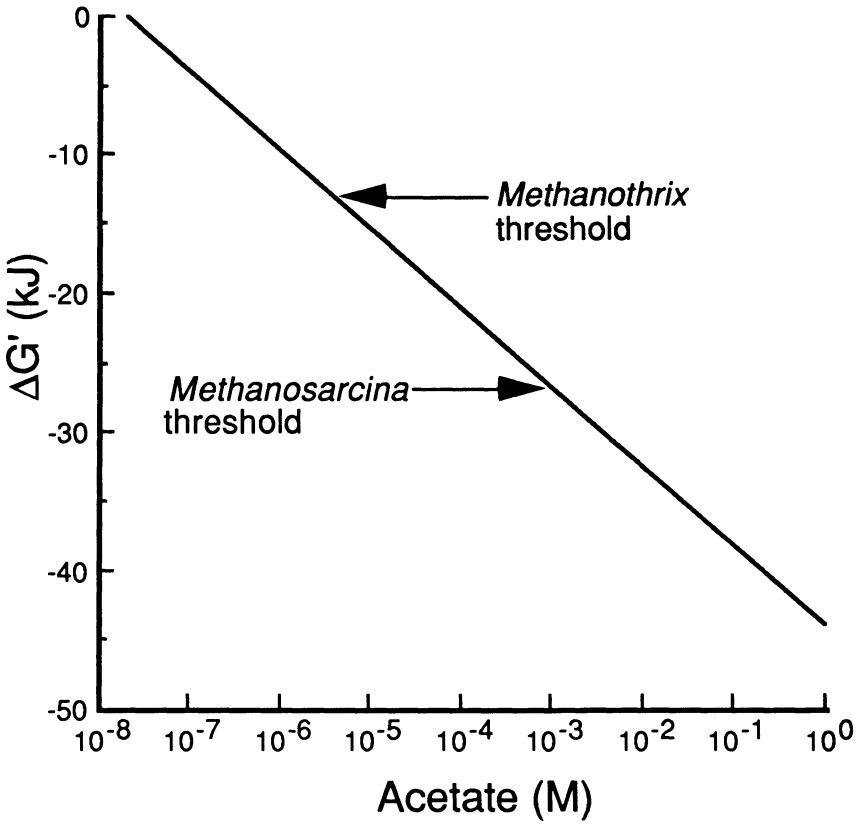

Figure 3.2. Effect of acetate concentration on the free energy available from methanogenesis from acetate (Table 3.1) by mesophilic acetotrophic methanogens. It is assumed that the thresholds for acetate are 1 mM for *Methanosarcina* (Fukazaki et al., 1990) and 5 μM for *Methanothrix* (Jetten et al., 1990), and that the HCO_3^- concentration is 10 mM and the CH_4 partial pressure is 0.5 atm.

favored under conditions in which a high input of organic matter leads to rapid accumulation of acetate, such as a starting up anaerobic bioreactor or in decomposing fecal matter, while *Methanothrix* should be favored in much more stable habitats, such as a bioreactor operating efficiently under long term stable conditions or lake sediments. This interaction between the two genera has been compared to *r* versus *K* selection in animals (Zinder et al., 1984). It should be remembered that this is only one facet of competition between two organisms, and many other factors will influence their competition.

The TAM organism is a thermophilic acetotrophic methanogen that can use

H_2-CO_2 and formate in addition to acetate (Ahring and Westermann, 1985). It has a doubling time on acetate of four days, considerably longer than typical doubling times for thermophilic *Methanosarcina* (ca. 0.5 d) or *Methanothrix* (ca. 1 d). Its kinetic constants are closer to those of *Methanothrix* (Table 3.6). An acetate oxidizing coculture (see below) was not specifically examined for threshold values, but a recent analysis by the author of data on acetate consumption by this culture (Zinder and Koch, 1984) indicates that it could use acetate down to concentrations at least as low as 0.2 mM. This is of interest in light of the recent finding of acetate oxidation at acetate concentrations below 1 mM in a thermophilic anaerobic bioreactor in which *Methanosarcina* was present, but not *Methanothrix* (Petersen and Ahring, 1991).

Other acetotrophic anaerobes include sulfate reducers and Fe^{3+} reducers. As with H_2, high sulfate or Fe^{3+} typically inhibits methanogenesis from acetate in sediments. The acetate concentration in methanogenic Potomac river sediments was near 5 μM (Lovley and Phillips, 1987), identical to the minimum threshold of *Methanothrix soehngenii* (Jetten et al., 1990). Addition of sulfate to those sediments lowered the acetate concentration to near 2 μM, and addition of Fe^{3+} lowered it to 0.5 μM.

3.4.4 Competition for Other Methanogenic Substrates

Low rates of methanogenesis are found in sulfate dominated marine and estuarine sediments. Sulfate inhibited methanogenesis from added H_2/CO_2 and acetate in San Francisco Bay sediments, but not methanogenesis from methanol, trimethylamine, or from methionine, which can be converted to methane thiol and dimethyl sulfide (Oremland and Polcin, 1982). Furthermore, addition of the methanogenic inhibitor bromoethane sulfonic acid caused buildup of methanol concentrations in the sediments, and ^{14}C-methanol was converted to methane in these sediments (Oremland and Polcin, 1982). It was postulated that these methyl compounds were "noncompetitive" substrates that sulfate reducers utilized poorly if at all. However, evidence for methanol oxidation by sulfate reducing bacteria in marine sediments has been obtained (King, 1984), as well as for some oxidation of methylamines, and it is not clear which environmental conditions favor methanogenesis versus sulfate reduction using methylated substrates (Kiene, 1991).

3.4.5 Obligate Interspecies H_2/Formate Transfer

Methanogens were originally believed to be able to grow on propionate and butyrate and alcohols longer than methanol. One such culture was *Methanobacillus omelianskii*, which was believed to oxidize ethanol to acetate and use the electrons to reduce CO_2 to CH_4. This culture was resolved by M. P. Bryant and collaborators (Bryant et al., 1967) into two organisms: the S (symbiotic) organ-

ism, which oxidizes ethanol, and *Methanobacterium* MoH (methanogen oxidizing hydrogen, now *Methanobacterium bryantii*). This finding was a major conceptual breakthrough in our understanding about anaerobes and their interactions. It was realized that two organisms, an H_2 producer and an H_2 consumer, could couple together to break down a single substrate, an example of syntrophism (Gk.-eating together).

The equations describing the reactions carried out by the partners in the *M. omelianskii* culture are shown in Table 3.7. In essence, the H_2 consumer pulls the reaction of the H_2 producer by removing H_2, keeping its partial pressure low. The S organism grows poorly on ethanol, because the H_2 partial pressure rapidly builds up to levels that make ethanol oxidation thermodynamically unfeasible. The methanogen cannot grow alone on ethanol, which it is unable to use as an electron donor. Thus, this is a true mutualistic symbiosis in which each organism requires the other in order to grow. After this discovery, methanogens were not considered to be able to grow on ethanol and longer alcohols, but Widdel and coworkers (Widdel, 1986) demonstrated that some hydrogenotrophic methanogens indeed can use alcohols.

The effects of H_2 partial pressure on the thermodynamics of ethanol oxidation and methanogenesis are shown in Figure 3.3. Lower H_2 partial pressures make ethanol oxidation more favorable and methanogenesis less favorable. The H_2 partial pressure must be poised at a point at which both organisms can conserve energy. As discussed previously, methanogens cannot use H_2 at partial pressures below about 6×10^{-5} atm at 35°C. A study of energy partitioning in syntrophic cocultures of the ethanol oxidizer *Pelobacter acetylenicus* (Seitz et al., 1990), it was found that ethanol oxidation did not occur if H_2 partial pressures caused the $\Delta G'$ to be less favorable than -16 kJ/mol ethanol, which would correspond to an H_2 partial pressure near 10^{-2} atm in Figure 3.3. Therefore, for both organisms to conserve energy, the H_2 partial pressure must be poised within the "window" of 6×10^{-5} to 10^{-2} atm.

Fatty acid oxidizing syntrophic cocultures are more tightly coupled (Table 3.7), and their "co-isolation" had to await the development of the ingenious technique of isolating colonies of the H_2 producer on a lawn of H_2 consumers (McInerney et al., 1979), thereby ensuring close proximity. Using this technique, cocultures were co-isolated of the butyrate-oxidizing *Syntrophomonas wolfei* (McInerney et al., 1981), the propionate-oxidizing *Syntrophobacter wolinii* (Boone and Bryant, 1980), the benzoate-oxidizing *Syntrophus buswellii* (Mountfort et al., 1984) and the thermophilic acetate-oxidizing AOR (acetate-oxidizing rod) (Zinder and Koch, 1984), among others. *Syntrophomonas* cultures, which grow on long chain fatty acids (e.g., stearate), have also been isolated (Roy et al., 1986). *Syntrophomonas* and *Syntrophus* have been cultured axenically on crotonate (Beaty and McInerney, 1987; Hopkins and McInerney, 1991), the AOR on ethylene glycol or pyruvate (Lee and Zinder, 1988), while *Syntrophobacter* has

Table 3.7 Properties of obligate methanogenic syntrophic cultures.

Substrate	*Organism*	*Reaction*	$\Delta G^{\circ\prime}$ *(kJ/rxn)*	$\Delta G^{\circ\prime}$ *(kJ/mol CH_4)*	T_d^a *(h)*
Ethanol	"S organism"	$2Ethanol + 2H_2O \rightarrow 2Acetate^- + 2H^+ + 4H_2$	+19.3		
	Methanogen	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	−135.6		
	Sum	$2Ethanol + HCO_3^- \rightarrow 2Acetate^- + H^+ + CH_4 + H_2O$	−116.3	−116.3	<24
Butyrate	*Syntrophomonas wolfei*	$2Butyrate^- + 4H_2O \rightarrow 4Acetate^- + H^+ + 4H_2$	+96.2		
	Methanogen	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	−135.6		
	Sum	$2Butyrate^- + HCO_3^- + H_2O \rightarrow 4Acetate^- + CH_4 + H^+$	−39.4	−39.4	84
Propionate	*Syntrophobacter wolinii*	$4Propionate^- + 12\ H_2O \rightarrow 4Acetate^- + 4HCO_3^- + 4H^+ + 12H_2$	+304.6		
	Methanogen	$12H_2 + 3HCO_3^- + 3H^+ \rightarrow 3CH_4 + 9H_2O$	−406.6		
	Sum	$4\ Propionate^- + 3H_2O \rightarrow 4Acetate^- + HCO_3^- + H^+ + 3CH_4$	−102.0	−34	168
Benzoate	*Syntrophus buswelii*	$4Benzoate^- + 28H_2O \rightarrow 12Acetate^- + 4HCO_3^- + 12H^+ + 12\ H_2$	+359.0		
	Methanogen	$12H_2 + 3HCO_3^- + 3H^+ \rightarrow 3CH_4 + 9H_2O$	−406.6		
	Sum	$4\ Benzoate^- + 19H_2O \rightarrow 12Acetate^- + HCO_3^- + 9H^+ + 3CH_4$	−47.6	−15.8	168
Acetate	AOR[b]	$Acetate^- + 4H_2O \rightarrow 2HCO_3^- + 4H_2 + H^+$	+104.6		
	Methanogen	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	−135.6		
	Sum	$Acetate^- + H_2O \rightarrow HCO_3^- + CH_4$	−31.0	−31.0	36

[a]Doubling time in hours.

[b]AOR = Thermophilic acetate oxidizing rod. The relatively rapid growth of this culture is most likely due to its being a thermophile.

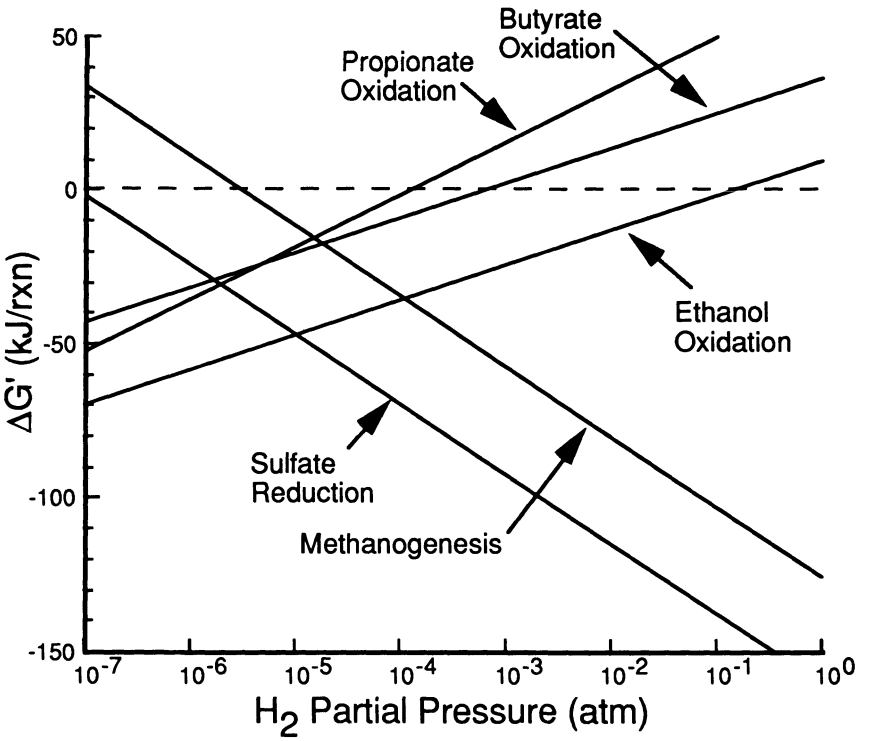

Figure 3.3. Effect of H_2 partial pressure on reactions involved in interspecies H_2 transfer during oxidation of ethanol, propionate, or butyrate coupled to methanogenesis or sulfate reduction. The equations for the oxidations are in Table 3.7. The concentrations of ethanol, propionate, butyrate, and acetate are all 10 mM, and the concentrations of the rest of the products and reactants are as in Fig 3.1.

eluded axenic culture. Cultures of these organisms coupled to hydrogenotrophic sulfate reducing bacteria tend to grow faster than methanogenic cocultures, but such cultures are probably artificial, since sulfate reducing bacteria exist that use these substrates directly (Widdel, 1988).

As shown in Figure 3.3, the windows for butyrate and propionate oxidations coupled to methanogenesis are much narrower than for ethanol oxidation. This is especially true for propionate oxidation, and it is not surprising that propionate oxidation is considered to be very easily perturbed in anaerobic bioreactors (McCarty, 1964). It has been suggested that physical juxtaposition between hydrogen consumers and producers facilitates hydrogen transfer (Conrad et al., 1985; Thiele et al., 1988). A related observation is that of Dubourgier et al. (1988)

that microbial flocs from an anaerobic bioreactor containing *Syntrophobacter*, identified by immunoelectron microscopy, had methanogens interspersed between *Syntrophobacter* cells, reflecting the stringent requirement of propionate oxidation for low H_2 partial pressures, while flocs containing *Syntrophomonas* often had *Syntrophomonas* cells clumped together with methanogens grouped outside them, reflecting a more relaxed requirement for H_2 consumption in butyrate degrading cultures.

Thiele and Zeikus (Thiele and Zeikus, 1988) have proposed that formate rather than H_2 is the electron carrier in many cases of interspecies electron transfer on the basis of isotope studies of an ethanol degrading syntrophic coculture. Formate and H_2 can be interconverted by formate-hydrogen lyase enzyme systems following the equation:

$$HCOO^- + H_2O \rightarrow HCO_3^- + H_2, \Delta G^{\circ\prime} = 1.3 \text{ kJ/rxn}$$

Formate-hydrogen lyase systems are common in anaerobes, and it would be expected that formate and H_2 would be in equilibrium with each other in most anaerobic habitats. Boone et al. (1989) examined a methanogenic butyrate-oxidizing coculture and found that the dissolved H_2 concentrations were near 63 nM during active metabolism, dropping to a minimum threshold of 35 nM near the end of growth. A formate concentration of 16.4 μM was thermodynamically equivalent to 63 nM H_2. Using a diffusion model with these concentrations, it was calculated that even though the diffusion coefficient of formate is fivefold lower than for H_2, formate could be expected to transfer electrons 98-fold more rapidly than H_2. It was calculated that H_2 diffusion could not account for rates of CO_2 reduction to CH_4 found in anaerobic bioreactors, but that calculation was based on a methanogenic population size of 10^7/ml, when actual counts are typically more than 30-fold higher (Mackie and Bryant, 1981; Zinder et al., 1984). In a more recent study, H_2 diffusion within microbial flocs degrading lactate was great enough to account for the methanogenesis detected (Goodwin et al., 1991).

Thiele and Zeikus (1988) proposed that an advantage of formate is that its excretion and uptake could be electrogenic, thereby leading to a contribution to the proton motive force in both syntrophic partners. For example, *Oxalobacter formigenes* can conserve energy from formate excretion (Allison et al., 1985), and *Methanobacterium formicicum* showed a 1.4 fold higher Y_{CH_4} on formate than on H_2-CO_2 (Schauer and Ferry, 1980), in agreement with this proposal.

The relative roles of formate versus H_2 in interspecies electron transfer need to be assessed under a variety of conditions, since factors such as the bicarbonate concentration and pH can affect the H_2/formate equilibrium. It is difficult to determine the turnover of either H_2 or formate because both are susceptible to isotopic exchange, and because their turnovers are so rapid, about 72 turnovers

per second calculated for H_2 in one instance (Boone et al., 1989). One approach is to examine syntrophic cocultures in which the "electron consumer" cannot use formate or H_2. *Syntrophomonas wolfei* did not grow as rapidly when coupled to *Methanobacterium bryantii*, which does not use formate, as to *Methanospirillum hungatei*, which does (McInerney et al., 1981). However, there may be other physiological differences between the two genera that accounted for the difference. A thermophilic acetate oxidizer (Lee and Zinder, 1988) and a thermophilic propionate oxidizer (Stams et al., 1992) could both couple as well to *Methanobacterium thermoautotrophicum* ΔH as to a thermophilic *Methanobacterium* sp. which could use formate. H_2 partial pressures in syntrophic cultures at high temperatures are higher (see below), and there is greater diffusion at high temperature, so that formate may not be as important in interspecies electron transfer under thermophilic conditions. Conversely, an amino acid oxidizing anaerobe, *Eubacterium acidaminophilum*, was able to grow syntrophically coupled to *Desulfovibrio baarsii*, which can use formate but not H_2 (Zindel et al., 1988). Ideally, one would want to compare the coupling capabilities of isogenic methanogen mutants, which have lost the ability to use formate or H_2 to test the relative contributions of each electron carrier.

Finally, it should be mentioned that temperature can have a significant effect on partial pressures of H_2 in syntrophic cocultures. This is due to the temperature term in the free energy form of the Nernst Equation, as well as in the equation:

$$\Delta G = \Delta H - T\Delta S$$

in which ΔH is the change in enthalpy and ΔS is the change in entropy. In the cases of equations that lead to H_2 formation and consumption, the upper and lower boundaries for H_2 partial pressure can vary 250 fold over the range 0–80°C (Lee and Zinder, 1988; Zinder, 1990), with higher H_2 partial pressures at higher temperatures. It has been experimentally demonstrated that the minimum H_2 threshold for methanogenesis and acetogenesis by several pure cultures increased with temperature in a manner consistent with this relationship (Conrad and Wetter, 1990), and that H_2 partial pressures in thermophilic syntrophic cocultures were considerably higher than predicted for mesophilic cocultures (Lee and Zinder, 1988; Stams et al., 1992). The higher H_2 partial pressures in thermophilic systems, along with the greater diffusion at high temperature, allow syntrophic reactions in thermophilic cultures to proceed with less diffusion limitation than in mesophilic systems.

3.4.6 Facultative Interspecies H_2/Formate Transfer

While the above examples involve an obligate requirement for a H_2 consuming partner, at least for the reactions described, some organisms couple to methano-

gens when it is advantageous, but the interaction is not required. For example, many carbohydrate-utilizing fermentative anaerobes produce H_2 as a product. H_2 production from pyruvate oxidation to acetyl-CoA is quite favorable and cultures carrying out this reaction can produce H_2 in excess of one atmosphere. In contrast, H_2 production from NADH ($E^{\circ\prime} = -320$ mV) is not favorable unless the H_2 partial pressure is less than 10^{-3} atm (100 Pa) (Wolin and Miller, 1982). Thus, a fermentative organism growing in axenic culture must recycle NADH to form reduced products such as ethanol, lactate, or fatty acids. However, in natural habitats, fermentative anaerobes are usually growing in the presence of hydrogenotrophs such as methanogens which can maintain the H_2 partial pressure at a level low enough to allow H_2 production from NADH. Thus freed from the restraint of recycling NADH, hydrogenase-containing fermentative anaerobes can produce more acetate and other fatty acids. This allows greater energy conservation by the fermentative organism, since the acyl-CoA intermediates in these pathways can be conserved as ATP.

An example of this coupling between a fermentative organism and a methanogen is shown in Figure 3.4, which is based on the results of Weimer and Zeikus (Weimer and Zeikus, 1977). *Clostridium thermocellum* in pure culture ferments glucose units derived from cellulose mainly to ethanol and acetate (some lactate is also formed) with three ATP's conserved via substrate-level phosphorylation. However, when *Methanobacterium thermoautotrophicum* is present, interspecies H_2 transfer allows *C. thermocellum* to produce more acetate, thereby conserving an extra mole of ATP. When *C. thermocellum* was grown on cellobiose instead of cellulose, it grew much faster, and *M. thermoautotrophicum* could not keep pace; however, the conditions in a batch culture with a high concentration of a sugar are not relevant to natural situations. Similar couplings with methanogens and a variety of fermentative anaerobes have been observed (Wolin and Miller, 1982). An interesting coupling occurred between an acetogen growing on a carbohydrate and a hydrogenotrophic methanogen. In the presence of the methanogen, the homoacetogen produced two moles of acetate rather than three, indicating that the methanogen can outcompete an acetogen for its own electrons (Cord-Ruwisch et al., 1988; Winter and Wolfe, 1980).

These results are congruent with those in methanogenic habitats, which indicate that carbon flow from carbohydrates is mainly to acetate and other fatty acids (Hungate, 1975; King and Klug, 1982; Lovley and Klug, 1982; Zinder and Elias, 1985). An exception may be lactose, which is often metabolized by lactic acid bacteria, which lack hydrogenase and are not able to couple to methanogens (Chartrain and Zeikus, 1986).

Many amino acids can be fermented singly or in pairs by the Stickland reaction. It has also been found that alanine can be oxidized to acetate (Zindel et al., 1988) and isoleucine to 2-methyl butyrate (Wildenaurer and Winter, 1986) by syntrophic methanogenic cocultures essentially carrying out the oxidation half of the

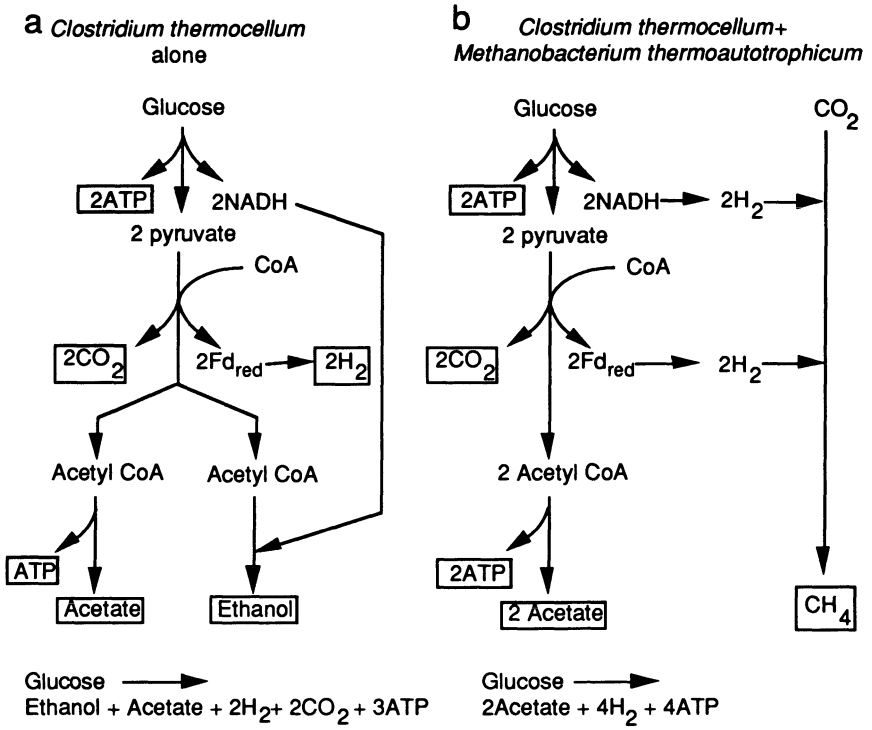

Figure 3.4. Fermentation products of *Clostridium thermocellum* growing axenically on cellulose (a) or together with *Methanobacterium thermoautotrophicum* (b). Some lactate is also produced by the axenic *C. thermocellum* culture.

Stickland reaction. Thus, interspecies electron transfer can lead to fatty acid formation from many amino acids.

As described previously, sulfate reducers are generally considered to be in competition with methanogens. However, it has been shown that some *Desulfovibrio* strains can grow on lactate or ethanol in the absence of sulfate when syntrophically coupled to a methanogen (Bryant et al., 1977). When sulfate was added to these cultures during syntrophic growth, further methanogenesis was greatly diminished. Under conditions of sulfate depletion, a competitor therefore becomes a syntrophic partner, in analogy to transient political alliances.

3.4.7 Symbiosis with Protozoa

While most known protozoa are aerobic and contain mitochondria, there are several species of flagellate and ciliate and ameboid protozoa which lack mito-

chondria, and cannot carry out true respiration, although some can reduce oxygen using peroxysomes. These protozoa often contain membrane bound organelles, called hydrogenosomes, for conversion of pyruvate to acetate and H_2 (Müller, 1988). Such protozoa are commonly found in anaerobic sediments and animal gastrointestinal tracts.

It was first noted in 1980, on the basis of autofluorescence of F_{420}-containing methanogenic cells (see Section 3.5.2), that methanogenic bacteria were associated with the surfaces of rumen ciliate protozoa (Vogels et al., 1980). Since then, there have been numerous observations of methanogens either on the surface or inside protozoa from anaerobic sediments (van Bruggen et al., 1983), landfill sediments (Finlay and Fenchel, 1991), termite guts (Lee et al., 1987), and cockroach guts (Guzen et al., 1991). Not surprisingly, the methanogenic bacteria are often associated with hydrogenosomes (Guzen et al., 1991). In a few of these cases, hydrogenotrophic methanogens, such as *Methanobacterium formicicum* and *Methanoplanus endosymbiosus* (van Bruggen et al., 1986) have been isolated from protozoal cells.

The advantage of this symbiosis to the endosymbiotic methanogens is clear, and it is reasonable to assume that removal of H_2 would allow more acetate production by the host protozoan. This hypothesis was tested recently for the anaerobic ciliate *Trymyema compressum* by Holler and Pfennig (Holler and Pfennig, 1991). When grown rapidly, this organism "outgrew" its methanogenic symbiont; however, it could be reinfected by *Methanobacterium formicicum*, which was not lysed upon ingestion into food vacuoles, presumably because of the resistance of pseudomurein to lysozyme (Wagener et al., 1990). When the reinfected protozoa were grown on *Bacteroides* as a food source, there was essentially no effect on acetate production from the carbon source or on host cell yield as compared to protozoa without methanogens. Little or no effect was seen on host cell yield in a similar study of three anaerobic ciliates cured of methanogens using bromoethane sulfonate (Fenchel and Finlay, 1991), although fermentation products were not measured in that study. Thus, the symbiosis between the methanogens and anaerobic protozoa may be commensal, with the protozoa not producing more H_2 in the presence of the methanogen than it does in its absence. There may be no biochemical mechanism to convert NADH to H_2 (i.e., NADH/ferredoxin oxidoreductase) in the protozoa.

It was recently demonstrated that a *Methanobacterium formicicum* culture isolated from a protozoan could fix nitrogen (Magingo and Stumm, 1991), introducing the possibility that the methanogen could provide the protozoan with fixed nitrogen.

3.4.7 Interspecies Acetate Transfer

It has long been known that acetotrophic methanogens play an important role in pH homeostasis in anaerobic environments by preventing acetic acid buildup.

There is increasing appreciation that acetate removal is important to syntrophic reactions. Since acetate is a product of most syntrophic reactions, its concentration can affect the thermodynamics of the reaction, and its removal should make thermodynamically marginal reactions more favorable. For example, two moles of both acetate and H_2 are produced per mole of butyrate degraded by syntrophic cultures (Table 3.7), and therefore, a 10-fold change in acetate concentration would have the same effect as H_2 on reaction thermodynamics. Addition of *Methanosarcina barkeri* to a *Syntrophomonas wolfei-Methanospirillum hungatei* coculture increased the extent of butyrate degradation and the number of *S. wolfei* cells per mole of butyrate degraded (Beaty and McInerney, 1989). Addition of an acetotrophic methanogen resembling the TAM organism stimulated propionate degradation by a purified thermophilic propionate degrading syntrophic culture (Stams et al., 1992). Since acetate is usually present in concentrations several orders of magnitude higher than dissolved H_2 in anaerobic environments, acetate turnover is probably not as readily perturbed as H_2 turnover, which is extremely dynamic.

A potential example of obligate interspecies acetate transfer is the acetone-degrading methanogenic enrichment described by Platen and Schink (Platen and Schink, 1987). This culture, which received acetone as the sole carbon and energy source, was dominated by a short rod, presumably the acetone degrader, and a long filamentous rod resembling *Methanothrix*. Isotopic labeling experiments indicated that acetone was carboxylated to acetoacetate (presumably the CoA derivative), which was split into two moles of acetate, and then was converted to CH_4. Addition of acetate to this culture inhibited acetone utilization until the acetate was consumed, and inhibitors of methanogenesis (bromoethane sulfonate or acetylene) greatly inhibited acetone degradation and led to accumulation of low levels of acetate. This evidence suggests that even though the $\Delta G^{\circ\prime}$ for conversion of acetone to acetate was -34.2 kJ/rxn, the acetone degrader was dependent on acetate degradation, perhaps because it required enough energy to conserve an ATP by substrate level phosphorylation.

3.5 Methods to Study Methanogens in Natural Habitats

3.5.1 Cultural Methods

Cultural methods are still valuable tools in understanding methanogen ecology, and provide information that complements other more recently developed methods. Simple enrichment cultures from natural samples have yielded interesting and important new cultures of methanogens. However, enrichments from low dilutions of sample do not necessarily contain the numerically dominant species present in the habitat, since the organism selected by such procedures can be a

fast growing "weed." Thus, it is important to perform appropriate dilutions of samples in order to show that the methanogen in question is present in significant numbers. For example, an undiluted sample inoculated into acetate medium might yield *Methanosarcina* while a 10^{-6} dilution might yield the slower growing *Methanothrix*.

Viable counting of methanogens in natural habitats has been used with some success, but this technique suffers from many inherent drawbacks that typically cause it to underestimate microbial numbers. For example, the growth medium must be able to support the microorganisms in which you are interested. An important concept in this regard originally articulated by Hungate (Hungate, 1967) is habitat simulation. The conditions in the growth medium (pH, E_h, ionic strength, etc.) should match those in the environment as closely as possible. Also, addition of rumen fluid or anaerobic digestor sludge supernatant may provide growth factors present in the microbial habitat but lacking in conventional media. Another drawback is that a viable count unit may represent more than one organism due to attachment of cells to particles, or aggregate or microcolony formation. For example, a nearly spherical clump of *Methanosarcina* 10 μm in diameter has approximately one thousand times the biomass of a single cell 1 μm in diameter, yet represents a single viable unit, as would be a *Methanothrix* filament one hundred cells long. Mechanical disruption or treatment with chemical dispersants (e.g., detergents or pyrophosphate) may help disperse microorganisms, but can often lead to loss of viability, and such processes should be carefully monitored and optimized. Problems with viable counting procedures should be suspected when numbers of methanogens are not high enough to account for the methanogenic activity detected.

There are also difficulties in identifying some methanogens in viable counts down to the species level. For example, it is difficult to differentiate between the mesophilic autotrophic methanococci *Methanococcus vannielii, M. deltae,* and *M. maripaludis*, although one dimensional SDS gel electrophoresis of cell proteins has promise in this regard (Franklin et al., 1988), as do the immunological and molecular biological techniques described below.

The viable counting method most likely to give the highest numbers of methanogens, since it involves the least perturbation of the organisms, is the most probable number (MPN) technique. This technique has been used to enumerate methanogens in anaerobic sediments (Franklin et al., 1988) and bioreactors (Chartrain and Zeikus, 1986; Grotenhuis et al., 1991; Mackie and Bryant, 1981; Zinder et al., 1984). The MPN method is of low precision, giving essentially an order of magnitude estimate (de Man, 1975). This method also provides you with an enrichment culture of an organism present in high numbers in your original sample, which you can use for subsequent isolation.

Hungate developed the anaerobic roll tube technique (Hungate, 1967) for the enumeration and isolation of anaerobic microorganisms, although the culture

tubes he originally described have been replaced by tubes with crimp-top stoppers. Other improvements have been made in the methodology, including the use of anaerobic glove-boxes (Balch et al., 1979). In this technique, which is essentially a variation of the pour plate technique, a suitable dilution of the sample is placed in a tube of molten agar medium, and the tube is rapidly rotated in ice water so that the agar forms a thin film coating the inside of the tube. After incubation, colonies form in this agar layer, which can be counted using a dissecting microscope, and can be picked with a sterile Pasteur pipette. Methanogen colonies can be identified by microscopic examination of the colonies using a phase-contrast or epifluorescence microscope, and the colonies themselves may show F_{420} autofluorescence (see below). Antibiotics which inhibit growth of eubacteria, such as penicillin and other β-lactam antibiotics (Zinder and Mah, 1979), D-cycloserine (Zinder and Mah, 1979), or vancomycin (Huser et al., 1982) can be useful in preventing growth of eubacterial contaminants after it has been ascertained that they are not inhibitory to the methanogens in question. For example, penicillin derivatives are inhibitory to *Methanothrix soehngenii* (Huser et al., 1982). Organisms enumerated using this technique must be able to withstand exposure to molten agar and the rapid temperature drop involved in agar solidification. Also, not all organisms may form colonies in agar. For example, it has been noted that *Methanothrix* forms colonies in agar only when highly contaminated, if at all (Huser et al., 1982; Zinder et al., 1987).

Methods for culturing methanogens in petri dishes have been developed. Typically, petri dishes are inoculated inside an anaerobic glove-box, but are incubated in airtight anaerobic chambers (Balch et al., 1979). Methanogen cells on the agar surface in spread plates are extremely sensitive to exposure to even trace levels of oxygen present in the glove box (Jones et al., 1983) so that great care must be taken. Soft agar overlays probably protect the organisms better than spread plates.

3.5.2 Microscopical Methods

Some methanogens, most notably *Methanosarcina*, *Methanothrix*, and *Methanospirillum*, have distinct enough morphologies that they may be identified tentatively in natural samples using a phase contrast microscope, although such identification should always be corroborated using other methods. Epifluorescence microscopy is a useful method in studying methanogen ecology. Many methanogens contain high concentrations of the electron carrier F_{420} (Gorris and van der Drift, 1986) so that their cells autofluoresce blue-green when illuminated with light near 420 nm (blue violet) (Doddema and Vogels, 1978). This cofactor has been detected in eubacteria, but not in high enough concentrations to autofluoresce. However, F_{420} levels vary widely in methanogens and growth conditions may affect those levels (Gorris and van der Drift, 1986). *Methanothrix* usually does not have high enough concentrations to autofluoresce (Huser et al., 1982;

Zinder et al., 1987), so that nonfluorescent cells in samples may also be methanogens. This technique has aided in qualitative identification of methanogens in natural samples (Lee et al., 1987; Vogels et al., 1980; Zinder et al., 1984). Interference by fluorescent material in natural samples can cause problems in detecting autofluorescent cells. Extracting natural samples and measuring F_{420} concentrations has been suggested as a method for estimating methanogenic biomass, but the great variation in F_{420} content in different methanogens (Gorris and van der Drift, 1986) makes this method untenable.

Scanning and transmission electron microscopy can also be used to tentatively identify methanogens with distinctive ultrastructural elements. The sheath and crosswall structures in *Methanothrix* and *Methanospirillum* make them relatively easy to identify by transmission electron microscopy in microbial assemblages, and cause *Methanothrix* filaments to resemble bamboo when viewed with the scanning electron microscope (Dolfing et al., 1985; Zinder et al., 1984). The thick outer polysaccharide and colonial organization of *Methanosarcina* makes it relatively easy to identify with electron microscopy (Robinson and Erdos, 1985). Pseudomurein-containing methanogens show a typical Gram positive outer layer and are not readily identifiable under the electron microscope. Immunological methods are an important adjunct to microscopical methods (see below).

3.5.3 Immunological Methods

The first use of immunofluorescence microscopy to identify a methanogen in a natural sample was by Strayer and Tiedje (1978) who used fluorescent antibody to detect *Methanobacterium formicicum* in Wintergreen Lake sediments. The more recent use of antibodies to study methanogen diversity and ecology has been spearheaded by E. Conway de Macario and colleagues, who developed a panel of antibodies against a wide variety of methanogens and determined their crossreactivities (Conway de Macario et al., 1982; Macario and Conway de Macario, 1983). In general, antibody specificity correlates with phylogenetic relationship. More recently, they have used these antibodies to directly identify and enumerate methanogenic populations in anaerobic bioreactors using fluorescent immunomicroscopy (Macario and Conway de Macario, 1988; Macario et al., 1989; Visser et al., 1991). Using this method, they showed that a considerable diversity of methanogens was present in mesophilic bioreactors, and less diversity was evident in thermophilic bioreactors (Visser et al., 1991), as might be expected. Immunogold labeling of electron microscope specimens has also been used to identify methanogens and other anaerobes in mixed microbial populations (Dubourgier et al., 1988; Robinson and Erdos, 1985).

The limitations on the use of antibodies to study methanogen ecology center around their specificity, which can be too low and crossreact with organisms other than the one desired, or too high and essentially give information about the

ecology of serotypes of the same species. An example of low specificity is an instance where antibodies against *Methanosarcina thermophila* TM-1 cross-reacted with *Methanothrix*-like cells in one bioreactor sample (Macario et al., 1989). For this reason, bulk immunological methods, such as enzyme-linked immunoassay (ELISA) should not be used to study natural habitats, since microscopy will at least provide information about the morphology of the reacting organisms. Another potential pitfall is that it is possible that methanogens may present different antigens on their surface when they are in a bioreactor than when in pure culture. Finally, this method will not identify organisms which are not reactive to the panel of antibody probes, so that novel undescribed methanogens might be missed. Despite these limitations, immunological analysis is a valuable tool to study methanogen ecology. It would be useful to have systematic studies in which the results of immunological and cultural techniques are compared.

3.5.4 Molecular Biological Methods

The techniques of molecular biology are beginning to be applied to the study of microbial ecology, and their sensitivity and specificity makes them a potentially useful addition to the arsenal of techniques used to study the numbers and diversity of microbial species in a habitat. Most of the techniques that can be applied to methanogens involve 16S rRNA, or its corresponding rDNA, for which there is now a rich phylogenetic sequence database (Woese, 1987). Many methods involve extraction of nucleic acids from a sample and then analyzing them by hybridization to organism-specific probes in "dot blots" (Stahl et al., 1988), or by sequencing of the rRNA either using reverse transcriptase and chain terminators, cloning a cDNA copy made by reverse transcriptase, or using polymerase chain reaction to amplify copies of rDNA (Steffan and Atlas, 1991). For example, Ward et al. (Ward et al., 1990) extracted rRNA from the Octopus Spring (Yellowstone National Park) cyanobacterial mat, and determined the sequence of fourteen individual clones. These clones fell into eight similarity groups and not one clone showed significant identity with sequences of several thermophilic bacteria that have been isolated from that or similar cyanobacterial mats. *Methanobacterium thermoautotrophicum* is numerous in lower anaerobic layers of the Octopus spring mat (Sandbeck and Ward, 1982; Zeikus et al., 1980), but all of the clones were eubacterial. These results show that many of the organisms present in that community have yet to be cultured. A major concern with these methods is the efficiency of extraction of nucleic acids from different species, since the resulting nucleic acid pool may not be representative of the community. This is especially true for methanogens, which have unusual outer layers which make extraction of nucleic acids difficult, especially pseudomurein in *Methanobacteriales* and the polysaccharide sacculus of *Methanosarcina* (Balch et al., 1979).

A more autecological method with great promise for *in situ* identification of

microorganisms was devised by N. Pace and colleagues (Giovannoni et al., 1988). Fluorescently labeled oligonucleotide probes complementary to rRNA are used to label individual cells which can then be examined and counted using a fluorescence microscope, or by fluorescence flow cytometry (Amman et al., 1990). The power of these methods is that since 16S rRNA has both highly variable and highly conserved regions (Woese, 1987), probes can be designed to detect individual species or entire phylogenetic domains (Giovannoni et al., 1988) depending on the question being asked. Both fluorescent probe and analysis of nucleic acid extracts dot blots were used in a preliminary study of the interactions between sulfate reducing bacteria and methanogens in anaerobic biofilms (Kane et al., 1991). There are many technical problems associated with these methods, including selective permeabilization of cells to allow the probe in while retaining rRNA in all cell types, background fluorescence in samples, possible low rRNA content of some cells, etc. However, these single cell labeling techniques have considerable potential in improving our understanding of methanogen ecology.

The use of molecular biological techniques in microbial ecology has just begun, and there has been little application to methanogens, but it is clear that these techniques will play a major role in future ecological studies. It is likely that probes for specific methanogen genes such as methylreductase or carbon monoxide dehydrogenase may be used in ecological studies. These techniques will be most valuable when coupled with conventional cultural, microscopical, and activity measurement techniques so that a more complete understanding of methanogen abundance and function in the environment can be obtained.

3.5.5 Activity Measurements

CH_4 is the primary catabolic product of methanogens, and is readily quantified by headspace analysis using gas chromatography. Rates of methanogenesis by samples, usually incubated in serum vials, can be readily measured, and effects of various treatments on those rates can be assessed. The potential of the methanogenic community in question to degrade added methanogenic substrates or their precursors can be determined (Shelton and Tiedje, 1984). Incubation in serum vials involves perturbation of some habitats, such as stratified aquatic sediments, and this should be taken into account before drawing conclusions.

Information on the contribution of potential precursors of CH_4 in a given habitat is best obtained by measuring the turnover of radiolabeled precursors to CH_4. Typically, ^{14}C-labeled precursors are used, such as $^{14}CO_2$ or $^{14}CH_3COO^-$, although ^3H-labeled methyl compounds such as C^3H_3OH or $C^3H_3COO^-$, can also be used. If an intermediate is at a constant steady state concentration, its turnover can be measured by the exponential disappearance of label (Hungate, 1975; Smith and Mah, 1966). If the concentration is not constant, initial rates can be measured for a short period of time (Zinder et al., 1984). When performing quantitative

studies on turnover rates, care should be taken that addition of the label does not significantly increase the concentration of the compound in the sample, since a higher concentration can change the kinetics of turnover. Radiolabeled CH_4 can be detected rapidly and with high specificity but low sensitivity with a gas chromatograph-gas proportional counter (Nelson and Zeikus, 1974), or with greater sensitivity but less specificity using a scintillation vial technique (Zehnder et al., 1979). Aqueous radiolabeled compounds can be fractionated using a high pressure liquid chromatograph. The BioRad HPX87H organic acid analysis column (Bio Rad Laboratories, Richmond, CA) is especially well suited to fractionate organic acids, alcohols, and sugars, and has been used to examine carbon flow from ^{14}C-carbohydrate to CH_4 in methanogenic habitats (Chartrain and Zeikus, 1986; King and Klug, 1982; Zinder, 1986).

Metabolic inhibitors have proven useful in study of methanogens in natural habitats. Chloroform and carbon tetrachloride were used as inhibitors of methanogens in early studies (Oremland, 1988), but they have been supplanted by bromoethane sulfonate (BrES), an inhibitory analogue of coenzyme M (Gunsalus et al., 1978), which has the advantages of being water soluble and more specific than chlorinated methanes. While methyl reductase is inhibited by micromolar concentrations of BrES, whole cells are usually less sensitive, with millimolar quantities often required, presumably due to lack of BrES uptake. Different methanogenic populations in the same habitat may have different sensitivities to BrES (Zinder et al., 1984). Acetylene also inhibits methanogens, but it is less specific and can be biodegraded in natural habitats (Oremland, 1988). Molybdate inhibits sulfate reduction (Taylor and Oremland, 1979) and is useful in studies of competition between methanogens and sulfate reducing bacteria in natural habitats.

3.5.6 Stable Isotope Fractionation

The stable isotope ^{13}C represents about 1.1% of the total carbon on earth, with the remainder being ^{12}C. Many biological reactions discriminate between these two isotopes, usually favoring the lighter ^{12}C, thereby producing isotopically light products. The ratio of these isotopes in CH_4 can be measured using a high-resolution mass spectrometer. CO_2-reducing methanogens have fractionation values ($\delta^{13}C$) between $-25‰$ and $-50‰$ (per mil) (Oremland et al., 1982), while the discrimination for methanol has been measured as $-70‰$ (Krzycki et al., 1987; Oremland, 1988) and that for acetate as $-21‰$ (Krzycki et al., 1987). Geochemists have used $\delta^{13}C$ values to infer the source of terrestrial and atmospheric CH_4. CH_4 from clearly biological sources (sediments, ruminants, etc.) typically has $\delta^{13}C$ values of $-60‰$ to $-90‰$ when compared to a carbonate standard. Natural gas, which is considered to be produced by thermal breakdown of organic matter, typically has $\delta^{13}C$ values of $-30‰$ to $-50‰$ (Oremland, 1988; Tyler, 1991). The isotopically light values for biogenic CH_4 are due to

isotope discrimination by methanogens as well as by the phototrophs which originally fixed the carbon, and perhaps also by other organisms involved in the breakdown of the organic matter. Indeed, CH_4 from ruminants eating plants using the C−4 shunt to fix CO_2 is not as isotopically light as CH_4 from ruminants eating plants using the usual C-3 pathway (Oremland, 1988; Tyler, 1991). Researchers have also used H/D ratios in the CH_4 to determine the percentage of methane derived from acetate, since transfer of an intact methyl group in methanogenesis from acetate leads to a higher H/D ratio (Tyler, 1991). These isotope methods give indirect evidence as to sources of CH_4 detected in the environment.

3.6 Methanogenic Habitats

3.6.1 The Anaerobic Digestor

The anaerobic digestor is a human-made habitat designed for the conversion of organic wastes to methane. It is instructive to discuss this habitat first since it is the best studied and most easily manipulated of methanogenic habitats and is representative of methanogenic habitats in which degraded organic matter is completely converted to CH_4. More recent developments in the design and use of anaerobic bioreactors will be discussed in Section 3.8.1.

Anaerobic digestors are found in nearly all sewage treatment plants and are used to treat the particulate fraction (sludge) generated by primary settling of raw sewage and secondary settling of activated sludge. This waste is usually high strength (~5% w/v organic matter) and is mainly polymeric, consisting of undigested plant polymers in feces, toilet paper, and microbial cells. The readily biodegradable components of the waste are converted to CH_4 and CO_2 (biogas) leaving a stabilized organic waste. The biogas can be burned by cogenerators to provide heat for the process and usually also generate electricity which can be used for other functions in the sewage treatment plant. The classical anaerobic digestor is essentially a large fermentation vat which is heated to near 35°C, and is fed substrate continuously or semicontinuously so that it can be considered analogous to a chemostat. It has long been known that the retention time (= volume/flow rate) for such anaerobic digestors must be greater than 10 days or there can be substantial buildup of fatty acids (Gujer and Zehnder, 1983; McCarty, 1964).

Figure 3.5a shows carbon flow to CH_4 in an anaerobic bioreactor. Polymers are broken to soluble monomers and oligomers by hydrolytic enzymes (cellulases, proteases, etc.) which, owing to interspecies H_2/formate transfer (Sections 3.4.5 and 3.4.6), are then fermented mainly to fatty acids and H_2. Fatty acids longer than acetate, including long-chain fatty acids derived from lipids, are oxidized to acetate, H_2, and CO_2 (in the case of propionate). These reactions result in the

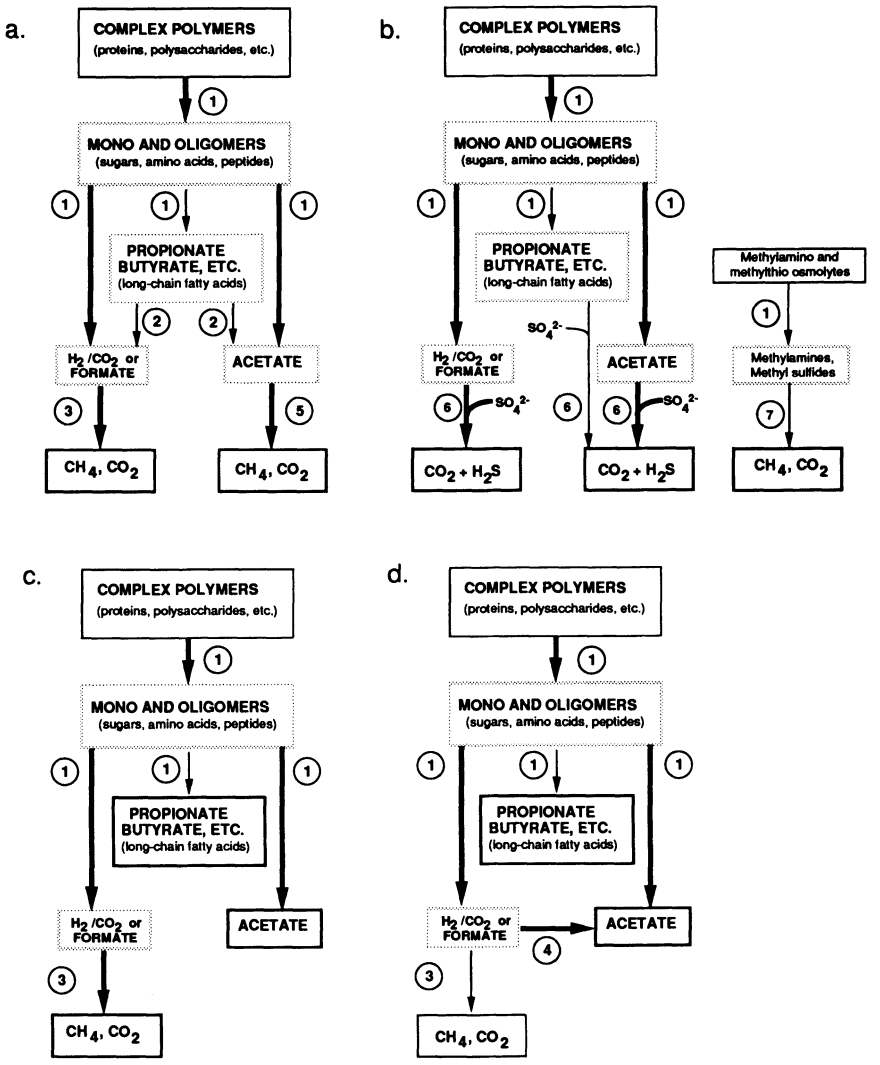

Figure 3.5. Carbon flow to CH_4 in anaerobic habitats: (a) anaerobic digestor or freshwater sediments; (b) marine sediments containing sulfate; (c) animal rumen; (d) gut of lower termites; Microbial groups involved: (1) fermentative anaerobes; (2) H_2 producing acetogens; (3) H_2 consuming methanogens; (4) H_2 consuming acetogens; (5) acetotrophic methanogens; (6) sulfate reducing bacteria; (7) methylotrophic methanogens. Intermediates are shown in dashed boxes, and major endproducts are in heavy boxes. Major carbon flows shown with heavy arrows, minor carbon flows shown with light arrows.

conversion of the original substrates to the primary substrates for methanogenesis: H_2-CO_2/formate and acetate.

Studies of turnover of labeled acetate have shown that acetate is the precursor of about two thirds of the methane produced in anaerobic digestors (Boone, 1982; Mountfort and Asher, 1978; Smith and Mah, 1966; Zinder et al., 1984), with CO_2 reduction using H_2 or formate accounting for nearly all of the remainder. Depending on the nature of the substrate, other CH_4 precursors include methylamines (derived mainly from choline and betaine), methyl sulfides (derived from methionine and sulfonium compounds), or methanol (derived from methoxy groups). The two to one stoichiometry of acetate versus CO_2 as a CH_4 precursor is in accordance with predicted stoichiometries from known biodegradative pathways in which acetate is a central intermediate. For example, for the methanogenic degradation of glucose, it would be ultimately be converted to acetate, H_2, and CO_2 due to interspecies electron transfer and the following equations would describe its conversion to CH_4:

1	$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 4H_2 + 4H^+$
2	$4H_2 + HCO_3^- + H^+ \rightarrow \mathbf{CH_4} + 3H_2O$
3	$2CH_3COO^- + 2H_2O \rightarrow \mathbf{2CH_4} + 2HCO_3^-$
Sum	$C_6H_{12}O_6 + 3H_2O \rightarrow \mathbf{3CH_4} + 3HCO_3^- + 3H^+$

From these equations two out of the three moles of CH_4 from glucose are predicted to be derived from acetate. Owing to similar considerations, the predicted proportion of CH_4 derived from acetate for valine, palmitate, or benzoate would be 67%, 70%, and 80%, respectively. Thus, acetate is the major methane precursor in anaerobic digestors and other habitats that completely convert biodegraded substrate to CH_4.

One factor limiting the conversion of organic matter to CH_4 is the slow growth of the two groups responsible for consuming acids: the syntrophic fatty acid degraders, and the acetotrophic methanogens. As shown in Table 3.6, methanogenic syntrophic associations using propionate or butyrate have doubling times of several days, and *Methanothrix*, which is often the dominant acetotrophic methanogen in anaerobic bioreactors, has a doubling time in excess of 3.5 days, while *Methanosarcina* has a doubling time of 1–2 days. An important consequence of the slow growth of these organisms is that the retention time of the digestor must be long enough so that they are far from washout, in accord with engineering studies that the retention time must be greater than ten days for efficient and stable operation.

In general, there must be balanced interactions among the microbial groups in the anaerobic digestor for efficient operation. Failure of acid consumers to keep pace with acid production leads to pH drop and toxicity of un-ionized fatty acids. Since H_2 (or formate) turnover is so dynamic (Boone et al., 1989), even slight

perturbations in the rate of hydrogenotrophic methanogens leads to partial pressures too high to allow syntrophic fatty acid oxidation, causing fatty acid buildup.

A wide variety of hydrogenotrophic methanogens can be cultured from anaerobic digestors or detected using antibodies, including the genera *Methanobrevibacter, Methanobacterium, Methanospirillum,* and *Methanogenium* (Conway de Macario et al., 1982; Macario and Conway de Macario, 1988; Macario et al., 1989; Visser et al., 1991). Both *Methanosarcina* and *Methanothrix* can be detected or cultivated from anaerobic digestors, with *Methanothrix* often being the predominant morphotype detected when anaerobic digestor sludge is viewed microscopically (Gujer and Zehnder, 1983).

3.6.2 Freshwater Sediments and Soils

Carbon flow in most anaerobic freshwater sediments essentially resembles that in anaerobic bioreactors (Lovley and Klug, 1982) except that the organic loading rate (from settling plant material, algal cells, detritis, etc.) is considerably lower, and there is usually no organism washout. Thus it is not surprising that the H_2 concentration is near its minimum threshold (Table 3.3) and acetate is present in micromolar concentrations (Lovley and Klug, 1982; Winfrey and Zeikus, 1979) rather than the millimolar concentrations typically found in anaerobic digestors. The contribution of acetate to methanogenesis in Wintergreen Lake sediments was near 60% (Lovley and Klug, 1982), similar to anaerobic bioreactors. The ratio of organic matter to sulfate reaching the sediments determines the proportion of methanogenesis versus sulfate reduction occurring in them, since sulfate reducers can outcompete methanogens for substrate and are limited by the amount of sulfate diffusing into the sediments (see above). Methanogenesis can be limited by temperatures below 15°C in aquatic sediments (Conrad et al., 1989; Conrad et al., 1987; Zeikus and Winfrey, 1976).

Soils have numerous microhabitats and even ostensibly aerobic soil can have anaerobic microhabitats (Chin and Zinder,). Rice paddies and other flooded soils receiving large amounts of organic matter rapidly turn anaerobic upon flooding and produce CH_4 (Conrad et al., 1987). Rice paddies are often limited for nitrogen, and nitrogen fixation can occur in overlying waters by the *Azolla-Anabaena* plant-cyanobacterium symbiosis as well as in the sediments. It is of interest that two out of three methanogens isolated from rice paddy soils were capable of nitrogen fixation (Rajagopal et al., 1988).

3.6.3 Marine Habitats

Electron flow in anaerobic marine habitats is usually dominated by sulfate reducing bacteria (Figure 3.5b) owing to the approximately 27 mM sulfate in seawater. Thus, in most marine aquatic sediments, little or no methane is produced

in sediment layers in contact with seawater, and most of that which is produced is from methyl compounds (King, 1984; Oremland et al., 1982). In certain circumstances in which there are non-limiting amounts of readily degradable organic matter, i.e., a large amount of decaying plant material, sulfate reduction and methanogenesis can occur simultaneously. Methanogenesis can also occur in deeper layers of marine sediments that receive a high enough input of organic matter so that sulfate is depleted. Considerable amounts of CH_4 can accumulate in such sulfate-depleted zones, and under high hydrostatic pressures can actually form CH_4-water clathrates (Tyler, 1991). CH_4 diffusing up into the sulfate reducing zone is usually consumed before it reaches aerobic zones. The nature of this anaerobic CH_4 oxidation has eluded understanding (Kiene, 1991; Oremland, 1988). Since aerobic CH_4 oxidation requires O_2 as a reactant, anaerobic CH_4 oxidation must use a very different mechanism.

There is evidence that carbon flow to CH_4 in some sulfate-depleted methanogenic marine sediments does not resemble flow in anaerobic digestors or freshwater sediments. $^{14}CH_3COO^-$ added to such sediments is often mainly oxidized to $^{14}CO_2$ (Sansone and Martens, 1981; Warford et al., 1979) rather than converted to $^{14}CH_4$, as would be expected for acetotrophic methanogens. This indicates that methanogenesis from acetate is due to a syntrophic association between acetate oxidizing hydrogen producers and hydrogenotrophic methanogens, similar to the association described for a thermophilic acetate oxidizing syntrophic coculture (Lee and Zinder, 1988; Zinder and Koch, 1984). Stable isotope patterns, in which the δ^{13} C values for CH_4 and CO_2 paralleled each other with depth in Santa Barbara Basin sediments (Claypool and Kaplan, 1974) indicate that methanogenesis CO_2 was the major CH_4 precursor in those sediments. The identity of the acetate oxidizers in these sediments is not known. It should be pointed out that evidence has been obtained that the presence of hydrogenotrophic sulfate reducers can pull *Methanosarcina* toward acetate oxidation rather than splitting (Phelps et al., 1985). $^{14}CO_2$ production from $^{14}CH_3COO^-$ is generally considered evidence for sulfate reduction in freshwater aquatic sediments (Lovley and Phillips, 1987; Winfrey and Zeikus, 1977), but it is possible that syntrophic acetate oxidation occurs in freshwater sediments also.

Hydrogenotrophic methanogens isolated from marine environments are mainly members of the genus *Methanococcus*, while *Methanolobus* and *Methanococcoides* are the marine methylotrophic genera (Jones et al., 1987). *Methanosarcina acetivorans* was isolated from a marine habitat (Sowers et al., 1984), but, as previously mentioned, most *Methanosarcina* strains can grow in marine salts (Sowers and Gunsalus, 1988).

CH_4 is detected in oxygenated ocean waters in concentrations considerably greater than would be expected if it were simply in equilibrium with the atmosphere (Kiene, 1991). It has been postulated that this CH_4 is derived from anoxic microhabitats associated with fecal pellets, decaying organic matter, etc. (Kiene,

1991; Oremland, 1988). One problem is that one would expect sulfate reducers to outcompete methanogens, but it is possible that organic matter is not limiting in the anaerobic microhabitats, or that "noncompetitive" methylated substrates are used (Kiene, 1991).

3.6.4 Animal Gastrointestinal Tracts

Many animal gastrointestinal tracts have one or more chambers in which food is held for a long enough period of time and under suitable conditions to allow microbial growth. Since the amount of organic matter is high and the amount of oxygen diffusion is low, the microbial processes in these chambers are anaerobic. These chambers can occur before the stomach (foregut, e.g., the rumen), or after (hindgut, e.g., the human large intestine). In general, such fermentations can augment the digestive capabilities of the animal, often allowing the breakdown of cellulose, which is digested poorly, if at all, by animals in the absence of symbiotic microorganisms.

The animal rumen is a foregut fermentation vessel and is one of the best studied microbial habitats extant, largely due to the pioneering work of R. Hungate and colleagues (Hungate, 1966; Hungate, 1975). Ruminants include cows, sheep, goats, deer, elk, bison, and gazelles. The rumen microbial population ferments otherwise undigestible cellulosic plant material to fatty acids, which can be absorbed by the ruminant. The rumen contains approximately 10^{10} bacteria and 10^6 ciliate protozoa per ml, as well as cellulolytic anaerobic fungi (Hobson, 1988), and these microbial cells pass through the remaining digestive system to be used as a source of protein and other nutrients.

Carbon flow in the rumen is depicted in Figure 3.5c. The salient feature in the rumen is that fatty acids are not further metabolized by microbial populations, in contrast to an anaerobic digestor. Rather, they accumulate to high concentrations (ca. 60 mM acetate, 20 mM propionate, and 10 mM butyrate), making them available to the animal host (Miller, 1991). A major contributing factor to the absence of acid consuming organisms is that sufficient water and saliva (which also provides bicarbonate as a pH buffer) are swallowed such that the rumen retention time is less than one day. Thus, the slow-growing fatty acid oxidizers and acetotrophic methanogens are washed out of the system. In fact, it could be argued that *Methanosarcina*, with its doubling time on acetate of one to two days, is an enemy of ruminants, causing them to forego more efficient fiber digestion in order to keep the hydraulic retention time short enough to prevent methanogenesis from acetate. *Methanosarcina* can be present in the rumen in low numbers (Patterson and Hespell, 1979) presumably growing on methylamines or methanol, which allow rapid enough growth to keep pace with dilution.

Hydrogenotrophic methanogens are present in the rumen and are responsible for the major part of rumen methanogenesis. Their growth rates are sufficient to

allow them to keep pace with dilution; however, basal H_2 partial pressures are about tenfold higher than in an anaerobic digestor (Table 3.3), too high to allow syntrophic fatty acid oxidation. The basal H_2 partial pressure allows NADH oxidation. Consequently, Hungate (1975) has shown, using radioisotope turnover, that lactate and ethanol are unimportant intermediates in a well-functioning rumen. He also concluded, on the basis of K_m measurements, that H_2 was a more important intermediate than formate, although this issue might be readdressed in light of more recent determinations of these values (Lovley et al., 1984). The major hydrogenotrophic methanogenic genus in the rumen, and in gastrointestinal environments in general, is *Methanobrevibacter* (Miller, 1991), some of which are associated with protozoa (Vogels et al., 1980). Thus, the rumen can be considered a truncated ecosystem when compared to an anaerobic digestor, owing to the lack of fatty acid oxidizers and acetotrophic methanogens. Although considerably less CH_4 is produced per unit carbon digested compared to the anaerobic digestor, because of the large amounts of plant material ingested, the rumen fermentation is vigorous, leading to eructation (belching) of 400 liters of CH_4 per day form the bovine rumen (Tyler, 1991).

Other examples of animals with foregut fermentations are marsupials, colobid and langur monkeys, hippopotamus, camels, and llamas (Hobson, 1988), and the hoatzin or stinkbird (Grajal et al., 1989).

Prominent among the xylophagous (wood eating) insects are the termites. Termites have a hindgut pouch which contains cellulose digesting microbiota, protozoa and bacteria in termites classified as "lower," and only bacteria in "higher" termites (Breznak, 1982). These microbial communities were considered to be miniature rumens until Breznak and Switzer (Breznak and Switzer, 1986) showed that $^{14}CO_2$ added to gut contents of five termite genera was converted mainly to ^{14}C-acetate rather than $^{14}CH_4$. This observation held true for both lower and higher termites (Breznak and Kane, 1990). There is presently no explanation as to why acetogens can outcompete methanogens in the termite gut. An acetogen isolated from a termite gut had a K_m value for H_2 (6 μM) similar to those from methanogens and a minimum threshold for H_2 (466 Pa) considerably higher than those of methanogens (Table 3.5). It is possible that there is some condition in the gut inhibiting growth of methanogens such as high O_2, low pH, or an inhibitory metabolite. O_2 is a likely candidate because methanogenesis can be abolished in the termite gut by exposing whole termites to hyperbaric O_2, or stimulated by adding H_2 to termite atmospheres (Odelson and Breznak, 1983), indicating significant diffusion of gas into the termite gut. In the termite *Zootermopsis*, nearly all of the methanogens detected by F_{420} autofluorescence were found inside small flagellate protozoa (Lee et al., 1987) suggesting that the protozoa were providing refuge from an inhibitory factor. It has also been proposed that mixotrophy, the ability to use both organic substrates or H_2, gives the acetogens a

competitive advantage (Breznak and Kane, 1990), but it is not clear why mixotrophy would not be an advantage in other habitats.

One consequence of CO_2 reduction by acetogens instead of methanogens is that more of the substrate carbon and reducing power is made available to the animal host rather than being lost as CH_4. It has been estimated that about 12% of feed energy is lost as CH_4 in ruminants (Russell and Strobel, 1989). This predominance of acetogenesis also means that the amount of CH_4 emitted to the atmosphere per unit substrate consumed is considerably lower. It would be advantageous in terms of animal nutrition if the rumen fermentation could be shifted toward greater acetogenesis, and this would also lead to lower CH_4 emission to the atmosphere (see below). Feeding cattle with ionophore antibiotics, especially monensin, can decrease CH_4 emissions by 30%, leading to greater concentrations of reduced products, especially propionate (Russell and Strobel, 1989). Gram positive organisms, including pseudomurein-containing methanogens, tend to be more sensitive to these antibiotics, and this apparently leads to fermentation shifts (Russell and Strobel, 1989).

Most animals have hindgut fermentation chambers, including humans. The human large intestine receives undigestible food, mainly plant fiber, as well as sloughed off intestinal mucosa, etc. These are fermented mainly to fatty acids as in the rumen. Between 10% and 30% of humans produce considerable amounts of CH_4, with the remainder producing H_2 and CO_2, (Miller, 1991). The CH_4 production rate for an individual corroborates with fecal numbers of *Methanobrevibacter*, which can vary between 10^1 and 10^{10} per g dry weight. Reduction of $^{13}CO_2$ to ^{13}C-acetate was detected using ^{13}C-nuclear magnetic resonance in human and rat feces, with higher rates of acetogenesis in individuals producing little CH_4 (Lajoie et al., 1988). The reasons why some individuals are more methanogenic than others are unclear. It is also of interest that 5–10% of our diet energy can be derived from fatty acids absorbed from our large intestine (Miller, 1991). A methanogen growing on H_2 and CH_3OH, *Methanosphaera stadtmanii* has also been isolated from human feces, but the importance of this reaction to overall methanogenesis is probably not great. Finally, another anaerobic microenvironment that is part of the gastrointestinal system that can harbor methanogens is dental plaque (Belay et al., 1988; Kemp et al., 1983).

3.6.5 Geothermal Habitats

Terrestrial geothermal areas are widespread, and microbial populations in hot springs ranging in temperature from 40 to 100°C have been studied microbiologically in Yellowstone National Park in Wyoming, and in California, Iceland, Italy, and New Zealand. H_2 and CO_2 are among the gases flushing terrestrial hot springs, and it is not surprising that *Methanobacterium thermoautotrophicum* (t_{opt} = 65°C)

(Zeikus et al., 1980) and *Methanothermus fervidus* (t_{opt} = 83°C) (Stetter et al., 1981) have been isolated from terrestrial hot spring waters. Another habitat associated terrestrial hot springs is the cyanobacterial-bacterial mat, which is found in neutral to alkaline spring outflows with temperatures below 73°C. *M. thermoautotrophicum* is found in anaerobic layers underlying the upper photosynthetic layer (Sandbeck and Ward, 1982; Zeikus et al., 1980). Acetotrophic methanogens are less frequently found in hot springs, although a thermophilic *Methanothrix* was enriched from "decaying algal ooze" from hot springs in Kamchatka, Russia (Nozhevnikova and Chudina, 1985).

Marine hot springs can be found near shore and range up to 100°C. *Methanococcus thermolithotrophicus* (t_{opt} = 65°C) was isolated from sediments off the coast of Italy. More spectacular are the undersea hydrothermal vents found in spreading centers (Jannasch and Taylor, 1984) such as the Galapagos Rift site. Because of the high hydrostatic pressure at these sites, the water temperature can reach as high as 350°C. The hydrothermal fluid contains reduced compounds, including H_2, CO_2 and H_2S and the ability of these compounds to support a well-developed aerobic chemosynthetic community in the surrounding waters is well-documented (Jannasch and Taylor, 1984). The springs themselves are also an ideal place to determine the upper temperature limit of life, and many novel hyperthermophilic organisms have been cultured from these sites along the thermal gradient emanating from these springs, especially by K. Stetter and colleagues (Stetter et al., 1990). *Methanococcus jannaschii* (t_{opt} = 85°C) (Jones et al., 1983), *Methanococcus igneus* (t_{opt} = 88°C) (Burggraf et al., 1990), and *Methanopyrus kandleri* (t_{opt} = 105°C) (Kurr et al., 1991) have been isolated from these sites. These are all hydrogenotrophic methanogens, and hyperthermophilic methanogens growing on substrates other than H_2, even formate, have not been described.

3.6.6 Other Habitats

Methanogens can be found in many other anaerobic habitats. Landfills receiving refuse can rapidly turn anaerobic because of the large amount of organic matter being degraded, and produce CH_4. This CH_4 can be hazardous to housing built over the site. In some landfills, CH_4 is now being "harvested" by underground pipes for energy generation. Initial aerobic oxidation in landfills can heat them to 70°C, so that thermophilic anaerobes may be important once anaerobiosis ensues. *Methanobacterium*, *Methanosarcina*, and *Methanothrix* have been cultured from landfills (Archer and Peck, 1989).

The heartwood of trees can become water saturated (wetwood) and support a variety of anaerobic bacteria including hydrogenotrophic methanogens such as *Methanobrevibacter arboriphilus* (Zeikus, 1977).

Iron pipe exposed to anaerobic conditions is subject to corrosion. While corrosion is mainly attributed to sulfate reducing bacteria, it has also been shown that

methanogens can play a role in iron biocorrosion (Daniels et al., 1987). Elemental iron undergoes a process in which a thin film of H_2 is formed on the surface in thermodynamic equilibrium with elemental iron:

$$Fe^\circ + 2H^+ \rightarrow Fe^{2+} + H_2 \, (\Delta G^{\circ\prime} = +1 \text{ kJ/rxn})$$

The H_2 inhibits its further production, but the presence of a hydrogenotrophic methanogen can "pull" the reaction by consuming H_2 in a process analogous to interspecies H_2 transfer.

3.7 The Global CH_4 Cycle

Methanogenesis represents a significant portion of the carbon flow in the anaerobic habitats described above. In many of those habitats, CH_4 diffusing away reaches an anaerobic/aerobic interface, which is the habitat for aerobic CH_4 oxidizing bacteria (methanotrophs) (Kiene, 1991). Thus, much of the CH_4 produced in soils, sediments, and lake bottoms never reaches the atmosphere, since it is consumed by methanotrophs. It was recently shown that methyl fluoride at ca. 1% v/v in the headspace gas inhibited CH_4 oxidation, but was only slightly inhibitory to methanogenesis (Oremland and Culbertson, 1992). Addition of methyl fluoride to sand along a lake shoreline, creek sediments, or compost, led to a 3- to 400-fold increase in CH_4 released into chambers covering those sites, indicating that a significant fraction of CH_4 produced was consumed by methanotrophs in these habitats. There was no stimulation of CH_4 emission by methyl fluoride in San Francisco Bay saltmarsh sediments, indicating that methanotrophs played a minor role in those sediments.

A considerable amount of biogenic CH_4 is not consumed by methanotrophs in some habitats and reaches the atmosphere. This includes CH_4 produced in shallow sediments such as marshes and rice paddies. CH_4 can diffuse out of the sediments and can reach the atmosphere before oxidation, or be transported directly to the atmosphere as bubbles. Another mechanism for CH_4 to leave aquatic anaerobic systems is via the vascular system of plants as a conduit (Dacey and Klug, 1979), and rice plants may be especially important conduits (Tyler, 1991). CH_4 can also diffuse out of landfill sites. CH_4 produced in foregut or hindgut fermentations can also be released to the atmosphere.

Atmospheric CH_4 eventually reaches the stratosphere, where it reacts with free radicals, mainly hydroxyl radical, forming $CH_3\cdot$, which can then participate in a number of complex reactions leading to both ozone production and destruction (Tyler, 1991). CH_4 can react with chlorine radical, and deplete this ozone destroying species. CH_4 oxidation in the stratosphere may also be an important source of water which forms ice clouds at a height of 85 km (Thomas et al., 1989). It

is estimated that the average lifetime for CH_4 in the atmosphere is 7–11 years (Pearman and Fraser, 1988).

CH_4 is an important greenhouse gas. Although its current atmospheric concentration, 1.7 ppm (by volume) is considerably lower than that of CO_2 (ca. 345 ppm) it has a higher infrared absorbance and, per molecule, is considered to be thirty times as effective a greenhouse as CO_2 (Tyler, 1991). CH_4 is deemed to be the second most important greenhouse gas after CO_2, making roughly 25% of the contribution to greenhouse warming that CO_2 does (Tyler, 1991).

It has been determined that the CH_4 in our atmosphere is presently increasing by 1% per year. CH_4 can be measured in air bubbles trapped in polar ice at different depths, and those samples can also be accurately dated. Using such techniques, it has been determined that CH_4 was relatively constant at 0.8 ppm from 1600 until 1850, but that since then there has been a steady increase to the present value of 1.7 ppm (Pearman and Fraser, 1988). CH_4 has been measured in ice samples of up to 160,000 years old (Chappellaz et al., 1990), and it was found that CH_4 varied between 0.35 and 0.65 ppm until the recent time period. Higher values corresponded with periods of higher temperature and deglaciation, but it is not clear whether the higher CH_4 was a cause or an effect of higher temperature.

Thus, atmospheric CH_4 is presently at a concentration unprecedented in the past 160,000 years. The increase in the past one hundred and fifty years strongly suggests an impact by human activity. Attempts have been made to determine the magnitude of various natural and anthropogenic contributions to CH_4 in the atmosphere. A typical ledger for CH_4 emission to the atmosphere is shown in Table 3.8. There is considerable uncertainty about the actual values, since they usually involve extrapolation from a small scale to a global scale. Khalil and Rasmussen (1990) discuss CH_4 budgets proposed by various investigators and describe constraints on them.

Despite uncertainties concerning the precise contributions of various CH_4 sources, it can be concluded that many of the methanogenic habitats discussed previously make a major contribution to atmospheric CH_4, especially ruminants, wetlands, rice paddies, and landfills. The contribution of termites to CH_4 emissions has been controversial. A large contribution was initially proposed (Zimmermann et al., 1982), but most researchers favor a lower value (Tyler, 1991) consistent with results that show that termites convert only a small fraction of the carbon they digest to CH_4 (Figure 3.5d).

Human activity (industrial processes, livestock, rice paddies, biomass burning, etc.) has had a major impact on biogenic and abiogenic CH_4 emission. Agriculture accounts for a large fraction of the biogenic portion of anthropogenic CH_4 emissions (Table 3.8). As previously stated it would be desirable from an animal nutrition standpoint to lower CH_4 emissions from livestock, and modest decreases have been obtained with ionophore antibiotics. It is possible that adopting certain

Table 3.8 Estimates of CH_4 released into the atmosphere, based on estimates of Tyler (1991).

Source	*CH_4 Emission (10^{12} g/yr)*
Biogenic	
Livestock	80–100
Termites	25–150
Rice paddy fields	70–120
Natural wetlands	120–200
Landfills	5–70
Oceans	1–20
Tundra	1–5
Nonbiogenic	
Coal mining	10–35
Natural gas flaring and venting	10–30
Industrial and pipeline losses	15–45
Biomass burning	10–40
Methane hydrates	2–4
Volcanoes	0.5
Automobiles	0.5
Total	355–870
Total biogenic	302–715
Total anthropogenic	201–441

practices in rice paddy management might reduce their CH_4 emissions. However, complex scientific, economic, and political issues come into play when discussing the magnitude of global climate change, the role of CH_4 in global climate change, and which steps might be taken to reduce CH_4 emissions, issues that are beyond the scope of this chapter.

3.8 Biotechnological Uses for Mixed Methanogenic Cultures

3.8.1 Novel Substrates and Anaerobic Bioreactor Configurations

Anaerobic digestion is now considered an attractive method for energy-efficient treatment of a variety of high strength wastes other than sewage sludge, including animal manures, crop wastes, food processing wastes, distillery wastes, and municipal wastes (for a review, see Hall and Hobson, 1988). In general, there has been increasing appreciation for the abilities of anaerobes to degrade a wide variety of organic compounds, including aromatic compounds (Schink, 1988), and halogenated organics (see below). Most of the carbon in the waste is converted

to CH_4, while many of the nutrients are retained, making the treated sludge an excellent fertilizer if there are no toxic compounds or pathogens present. Anaerobic digestion has also been considered to be a method to turn biomass, usually lignocellulose or kelp, to energy. However, such schemes must presently be considered economically unfeasible when compared to the cost of natural gas because of the costs associated with converting biomass to CH_4, including the biomass feedstock, the pretreatment of that feedstock, process costs, and costs, in some cases, to remove CO_2, H_2S and other impurities to make pipeline-quality gas. Thus, anaerobic digestion must be considered a method of energy efficient waste treatment rather than a method for net energy generation at the present time.

Long retention time makes the conventional anaerobic digestor unsuitable for the treatment of many dilute soluble wastes that are generated. New bioreactor designs incorporate methods of retaining biomass, so that the organism retention times are decoupled from the hydraulic retention times, thereby preventing washout of the slow growing acid consuming organisms. Another advantage of bioreactors in which biomass is retained is that their high biomass content can lead to extremely high rates of methanogenesis, and hydraulic retention times as short as a few hours. Two such bioreactor configurations, fixed biofilm reactors and sludge blanket reactors, will be discussed.

In a fixed biofilm reactor, a surface is provided on which microorganisms can colonize and form a biofilm. The first fixed biofilm anaerobic reactor developed was the anaerobic filter (Young and McCarty, 1969), analogous to the aerobic trickling filter, in which macroscopic supports are provided for colonization, and the waste flows by the support. The first support used was rocks, but more recently glass beads, ceramic particles, or a plastic matrix, have been used (van den Berg, 1984). Anaerobic filters have been used successfully to treat a variety of wastes, but are prone to clogging (van den Berg, 1984).

In expanded/fluidized bed reactors (Fig 3.6a), the colonization substrate consists of small (< 1 mm) particles such as sand, alumina, or diatomaceous earth (Switzenbaum, 1983; van den Berg, 1984). The waste must be pumped fast enough to expand or fluidize (depending on the degree of bed expansion) the bed of particles so that the liquid can flow past them without any clogging caused by particulates in the waste or by reactor biomass. Since the hydraulic flow rate of the feed is usually not sufficient for bed expansion, a recycle pump is used to increase the flow rate. The major advantage of expanded/fluidized bed reactors is the extremely high surface area available for colonization, allowing high concentrations of biomass in the reactor. Because of their high biomass content, expanded/fluidized reactors can efficiently treat wastes with hydraulic retention times as short as 1 h (Jewell et al., 1981; Schraa and Jewell, 1984). In one microscopic study of an anaerobic biofilm treating swine wastes (Robinson et al., 1984), several different morphotypes of autofluorescent methanogens were

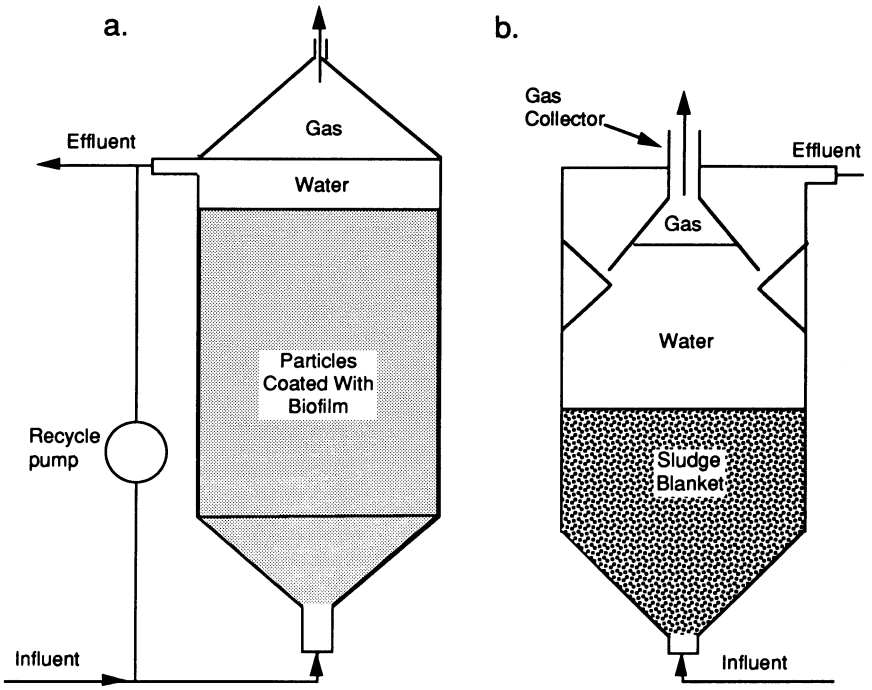

Figure 3.6. Two novel bioreactor configurations: a, the expanded/fluidized bed reactor; b, the upflow anaerobic sludge blanket (UASB) reactor.

detected. *Methanothrix*-like filaments were on the film surface, while *Methanosarcina*-like clumps were embedded within the lower regions of the film, and similar methanogen diversity was detected in a butyrate degrading biofilm (Zellner et al., 1991).

Limiting the use of fixed biofilm reactors is the long startup period, often months, before sufficient biomass has colonized the surfaces to perform efficiently (Switzenbaum, 1983; van den Berg, 1984). However, once started up, such reactors are extremely robust. In one study (Gorris et al., 1989), biofilm formation was found to consist of three periods: a lag phase in which the substrate was initially colonized, followed by a biofilm production phase, after which a steady state phase in which biomass is sloughed off at roughly the same rate as it forms. The colonization of a variety of plastic supports by methanogens was studied, and it was found that *Methanothrix soehngenii* favored hydrophobic polymers, *Methanospirillum hungatei* JF-1 favored hydrophilic polymers, and *Methanobrevibacter arboriphilus* AZ adhered to all supports (Verrier et al., 1988). On the other hand, *Methanosarcina mazei* MC3 did not adhere to any of the supports,

and probably can only colonize after other organisms have initiated the biofilm. More recently, the hydrophobicities and charge on methanogens was determined, and *Methanothrix soehngenii* was very hydrophobic with little charge, while *Methanosarcina barkeri* had high negative charge on its surface (Grotenhuis et al., 1992), which is not surprising since its sacculus is very acidic (Kreisl and Kandler, 1986). This high negative charge and the resulting repulsion between cells is consistent with my personal observation that it is difficult to obtain tight pellets of *Methanosarcina* when centrifuging cultures. Understanding the interaction between anaerobic bacteria and various surfaces should help in the optimization of startup of fixed film reactors.

The upflow anaerobic sludge blanket (UASB) reactor was developed in Holland by Lettinga and colleagues (Lettinga et al., 1980) and is depicted in Figure 3.6b. The key to this reactor is the development of microbial "granules" a few mm across that are retained by the reactor, so that the sludge blanket is nearly solid microbial biomass, with bacterial densities in excess of 10^{12} per ml (Grotenhuis et al., 1991). Granule retention is facilitated by the gas collecting apparatus shown in Figure 3.6b, in which a tortuous path to the effluent outlet prevents loss of most of the granules. This selects for organisms which are able to remain in the reactor as part of granular consortia. The UASB reactor has been used to treat a wide range of municipal and industrial wastes (Switzenbaum, 1983; van den Berg, 1984), and high loading rates and short retention times are possible. A thermophilic UASB reactor (Wiegant and de Man, 1986) produced more than 50 times its volume in CH_4 daily!

As in fixed biofilm reactors, the start-up of UASB reactors leading to granule formation is critical and considerable effort has gone into studying the microbiology of the granules and their formation. The microbial composition of the granules can vary significantly with the substrate, since some substrates, such as sugars, will select for more complex communities than other substrates, such as fatty acids, which will lack fermentative bacteria (Figure 3.5a). The density of the granules is 1.00–1.05, barely more dense than water, so that according to Stoke's law, fairly large size is needed to obtain a reasonable settling velocity (Dolfing et al., 1985). Chemical analysis of granules treating sugar plant wastes indicated that large amounts of extracellular polysaccharide were not present in the granules (Dolfing et al., 1985; Grotenhuis et al., 1991). Analysis of methanogenic and nonmethanogenic organisms isolated from granules, including *Methanothrix,* indicates that they were all quite hydrophobic and carry little electrical charge (Grotenhuis et al., 1992) suggesting an important role for hydrophobic interactions in associations leading to granule formation. Transmission electron microscopy using ruthenium red staining for polysaccharides (Grotenhuis et al., 1991) showed little extracellular polysaccharide surrounding *Methanothrix* cells, while *Methanosarcina* aggregates had a strongly staining layer surrounding it, perhaps to overcome the high negative charge of its sacculus.

When viewed by scanning or transmission microscopy, short or long *Methanothrix*-like filaments are often visible and can account for 30% of the cells in granules treating sugar plant wastes (Dolfing et al., 1985), and because of its hydrophobicity (Grotenhuis et al., 1992) and its ability to form a filamentous matrix, *Methanothrix* is considered to play an important structural role in forming compact efficient granules (Dolfing et al., 1985). In one study of acetate-fed thermophilic UASB reactors (Wiegant and de Man, 1986), granules consisting mainly of *Methanosarcina* were produced by keeping the feeding rate sufficiently high so that the effluent acetate concentration was higher than 10 mM. They found that reactors with the *Methanosarcina*-rich granules could only reach one tenth the volumetric CH_4 production rate of reactors with *Methanothrix* granules, and that the *Methanosarcina* granules were loose and easily washed out. However, *Methanosarcina* clumps can be found embedded in granules containing more complex microbial communities (Grotenhuis et al., 1991; Visser et al., 1991).

Evidence for macroscopic structure in some granules has been obtained. In an electron microscopic study of granules grown on sucrose, three layers were discerned (MacLeod et al., 1990). The outer layer, about 20 μm thick, consisted of various morphotypes in relatively loose association, and the underlying layer consisted of rod shaped bacteria in much closer association reminiscent of the fatty acid degrading syntrophic associations described using immunoelectron microscopy (Dubourgier et al., 1988). The inner layer of the granules was mainly organisms clearly resembling *Methanothrix*, and also contained cavities which were presumed to be gas pockets. On the basis of purely morphological criteria, it was proposed that the three layers contained different trophic groups, with fermentative bacteria and hydrogenotrophic methanogens in the outer layer, H_2-producing acetogens and hydrogenotrophic methanogens in the middle layer, and acetotrophic methanogens in the inner layer. Another study of granule structure, in which histological sections of mesophilic and thermophilic granules using fatty acids were viewed by light microscopy, found a two-layered structure (Macario et al., 1991; Visser et al., 1991). Organisms reacting with antibodies to *Methanosarcina thermophila* TM-1 were found in both the interior and exterior layer of granules, while organisms reacting with *Methanothrix soehngenii* antibodies were found only in the exterior layer. Organisms reacting to antiserum to *Methanobacterium thermoautotrophicum* were found as large aggregated colonies on the exterior of thermophilic granules, and organisms reacting with *Methanobrevibacter arboriphilus* antibody were evenly distributed throughout the granule exterior layer.

In contrast, another study failed to find a layered structure in propionate or ethanol utilizing granules (Grotenhuis et al., 1991). Interestingly, using cultural and immunoelectron microscopy, it was found that *Methanothrix* and *Methanobrevibacter arboriphilus* were the predominant methanogens in propionate utiliz-

ing granules, while *Methanosarcina* and *Methanospirillum* predominated in ethanol utilizing granules All of these organisms were found in granules treating sugar plant waste.

Anaerobic biofilms and the granules in UASB reactors are fascinating miniature ecosystems and warrant continued further study. There is still much to learn about factors which lead to their formation, and in community structure in already-formed biofilms and granules.

Another anaerobic bioreactor configuration is the two-phase reactor. In this system, two bioreactors are run in series. The first phase is acidogenic, and is operated at short retention time, so that the waste is converted to organic acids in a manner resembling the rumen. The second phase is the methanogenic or acid consuming phase, and can be run at long retention time or as a fixed film reactor to favor slow-growing acid consuming organisms. In theory, each phase can be optimized for its respective process (van den Berg, 1984). A two-phase system treating cellulose, in which the first phase uses microbial populations derived from a sheep rumen while the second methanogenic phase is an UASB reactor, has been described (Gijzen et al., 1988). An interesting variation of the two stage reactor was described (Thiele and Zeikus, 1987) in which anion exchange resin was used to shuttle fatty acids from an acidogenic reactor treating cheese whey to an upflow anaerobic filter methanogenic reactor. One notable aspect of the study was that the methanogenic reactor, upon receiving a shock load of substrate, produced the equivalent of 100–200 times its own volume in CH_4 per day for a period of a few hours. The shuttle cannot transfer neutral products such as ethanol, and it remains to be seen whether the process is practical.

One final topic in anaerobic digestion which deserves mention is microbial nutrition. Methanogenic bacteria require a several trace metal nutrients in order to grow, including nickel, cobalt, molybdenum, and large amounts of iron (Jarrell and Kalmokoff, 1988). It is conceivable that these elements can limit methanogenesis in anaerobic bioreactors or in other habitats, either because they are not present in high enough amounts, or because they are not available due to formation of metal sulfide precipitates. Many metals have a solubility in the nanomolar range when in the presence of sulfide. Studies have shown that nickel can stimulate methanogenesis in anaerobic bioreactors treating a defined medium containing acetate (Speece et al., 1983), or bean blanching waste (Murray and van den Berg, 1981). In the latter study, cobalt was also stimulatory. It is not clear whether similar nutritional deficiencies occur when more complex wastes are treated. Whether naturally present complexing agents help make metals more available, or whether the methanogens themselves have mechanisms to make the metals more available is presently unknown, but could be of great importance in regulating their activity in anaerobic bioreactors and in natural habitats.

In conclusion, new anaerobic bioreactor configurations have been devised

which allow the treatment of a wide variety of wastes besides sewage sludge. Some of these configurations, especially the UASB, have been commercialized and show promise in the the energy-efficient treatment of organic wastes. Further understanding of the physiology and biochemistry of methanogens should improve process efficiency and stability.

3.8.2 Thermophilic Anaerobic Digestion

It has been known since the 1930s that anaerobic conversion of wastes can occur in the temperature range 50–60°C, and a large number of laboratory, pilot, and full-scale thermophilic anaerobic bioreactors have been described (Buhr and Andrews, 1977; Zinder, 1986). Thermophilic versions of the expanded bed (Schraa and Jewell, 1984) and UASB (Wiegant and de Man, 1986) reactors have been successfully operated. Carbon flow to CH_4 in thermophilic bioreactors essentially resembles that in mesophilic reactors (Mackie and Bryant, 1981; Winter and Zellner, 1990; Zinder et al., 1984), although there appears to be less tendency to form propionate from carbohydrates (Winter and Zellner, 1990). Operation at temperatures over 60°C often causes drastic drops in efficiency in thermophilic anaerobic bioreactors (Pfeffer, 1974; Varel et al., 1980). As previously stated, no acetate utilizing methanogens with optima above 60–65°C are presently known (Zinder, 1990).

Among the advantages of thermophilic anaerobic digestion over the mesophilic process are increased reaction rates and lower retention times, destruction of pathogens (pasteurization), and lower viscosity, which can make sludge dewatering easier. Among the considered disadvantages are increased heat requirement, and reports of poor process stability (Buhr and Andrews, 1977). While increased heat requirement can be an important drawback, many thermophilic bioreactors produce more than enough CH_4 for their own heating. Some wastes, such as distillery wastes, are already heated, and many industries have heat as a waste product of other processes. Reports of poor process stability is probably due to poor temperature control. A thermophilic bioreactor operated in our laboratory for several years (Zinder et al., 1984) showed excellent stability.

The primary advantage of the thermophilic process is that lower retention times are possible. It was shown that a laboratory-scale thermophilic cattle waste digestor could be operated at retention times as short as three days (Varel et al., 1977). An advantage of shorter retention time is that a smaller bioreactor is needed. For example, shifting a full scale anaerobic digestor at a sewage treatment plant to thermophilic operation allowed the decrease of its retention time from 14 to 7 days, thereby doubling its capacity and saving the capital cost of building a new digestor (Rimkus et al., 1982). This decrease in retention time can be directly attributed to the more rapid growth of thermophilic acetotrophic methano-

gens and other acid consuming organisms [i.e., fatty acid oxidizing syntrophs (Ahring and Westermann, 1987)] when compared to mesophiles.

Thermophilic anaerobes capable of growth at 50–60°C appear to be common in mesophilic environments. A 60°C cattle waste digestor could be started up using mesophilic digestor sludge or rumen fluid with little lag (Varel et al., 1977). A UASB reactor shifted from 38°C to 55°C produced CH_4 within a week, and was treating waste with 80% efficiency within three weeks. The rapid appearance of several thermophilic methanogen serotypes in the granules after the temperature increase was documented using fluorescent antibody techniques (Visser et al., 1991). Several thermophilic methanogenic and nonmethanogenic anaerobes which can play a role in thermophilic anaerobic digestion have been described (Winter and Zellner, 1990; Zinder, 1986). The most common methanogens reported to be present in thermophilic anaerobic bioreactors are *Methanobacterium thermoautotrophicum* (Visser et al., 1991; Zinder et al., 1984), *Methanogenium thermophilicum* (Widdel, 1986; Zabel et al., 1985), *Methanosarcina thermophila* and thermophilic *Methanothrix* (Visser et al., 1991; Wiegant and de Man, 1986; Zinder et al., 1984), and the TAM organism (Ahring and Westermann, 1985; Stams et al., 1992). A thermophilic acetate oxidizing syntrophic coculture has been described (Zinder and Koch, 1984), and recently, evidence has been obtained that acetate oxidation rather than acetate splitting, may play a role in some thermophilic bioreactors when acetate concentrations are low (Petersen and Ahring, 1991).

3.7.3 Anaerobic Dehalogenation

Halogenated organic compounds are major environmental pollutants. While there has been extensive study of their degradation by aerobic microorganisms, it was not until 1982 that the potential of anaerobes in the biodegradation of halogenated organics began to be appreciated. In that year, Suflita et al. (1982) showed that several halogenated benzoates were degraded by anaerobic sewage digestor sludge. Since then, the dehalogenation of a wide variety of halogenated organic compounds has been demonstrated (Table 3.9). The mechanism in most of these dehalogenations is reductive, which can be represented by the reaction:

$$R—X + 2[H] \rightarrow R—H + HX$$

in which R represents an organic molecule, X a halogen, and [H] an electron (plus proton). Reductive dehalogenation is therefore the equivalent of the addition of H_2 across the carbon-halogen bond.

Reductive dehalogenation is energetically favorable compared to many anaerobic reactions. For example, the $E^{\circ\prime}$ for reductive dehalogenation of chlorinated ethylenes ranges from +0.37 to +0.58 V (Vogel et al., 1987), similar to nitrate

reduction to nitrite (+0.43 V). Therefore, one may predict that organisms could evolve to use halogenated compounds as terminal electron acceptors. Presently, there are only two organisms shown to couple growth to using a halogenated organic as an electron acceptor. One is *Desulfomonile tiedjei*, a sulfate reducer which can grown on H_2 or formate and several halogenated benzoates (DeWeerd et al., 1990). The other is the recently described *Dehalobacter restrictus* (Holliger, 1992) which can grow on H_2 or formate and reduces tetrachloroethylene to dichloroethylene.

Both methanogenic habitats and cultures have been shown to carry out reductive dehalogenation of a wide spectrum of halogenated organic compounds (Table 3.9). Notable among these are some highly chlorinated compounds which are resistant to aerobic degradation, and are considered major pollutants, such as tetrachloroethylene, chloroform, and polychlorinated biphenyls. In the case of tetrachloroethylene (perchloroethylene or PCE), which is completely resistant to

Table 3.9 Selected examples of dehalogenation of halogenated organic compounds by methanogenic habitats or pure cultures (many other examples exist)

Compound	*Products*	*System*	*Reference*[a]
Chlorobenzoates	Benzoate	Digestor sludge	1
Pentachlorophenol	Phenol, benzoate	Anaerobic sediments	2
Hexachlorobenzene	Tri- and dichloro-benzenes	Digestor sludge	3
Tetrachlorobiphenyls	Dichlorobiphenyls	Contaminated sediments	4
Tetrachloroethylene	Vinyl chloride and CO_2	Acetate-utilizing anaerobic biofilm	5
Tetrachloroethylene	Ethylene	Anaerobic enrichment	6
Tetrachloroethylene	Ethane	Anaerobic enrichment	7
Tetrachloroethylene	Trichloroethylene	*Methanosarcina barkeri*	8
1,2-Dichloroethane	Chloroethane, ethylene	Various methanogens	9
1,2-Dibromoethane	Ethylene	Various methanogens	10
Carbon tetrachloride	Chloroform, CO_2	Acetate-utilizing anaerobic biofilm	11
Chloroform	Dichloromethane	*Methanobacterium thermoautotrophicum*	12
Chloroform	Dichloromethane	*Methanosarcina barkeri*	13
Dichloromethane	Acetate, CO_2	Methanogenic enrichment	14
CCl_3F	$CHFCl_2$, CH_2FCl, CO	*Methanosarcina barkeri*	15

[a]References: 1, Suflita et al., 1982; 2, Mikesell and Boyd, 1986; 3, Fathepure et al., 1988; 4, Quensen III et al., 1990; 5, Vogel and McCarty, 1985; 6, DiStefano et al., 1991; 7, de Bruin et al., 1992 ; 8, Fathepure and Boyd, 1988; 9, Holliger, 1992; 10, Belay and Daniels, 1987; 11, Bouwer and McCarty, 1983; 12, Egli et al., 1987; 13, Mikesell and Boyd, 1990; 14, Freedman and Gossett, 1991; 15, Krone and Thauer, 1992.

aerobic degradation, some habitats effect only partial dechlorination to dichloroethylene (Gibson and Sewell, 1992) or vinyl chloride (Vogel and McCarty, 1985), which is considered more toxic than tetrachloroethylene, while others can completely dechlorinate PCE to ethylene (DiStefano et al., 1991; Freedman and Gossett, 1989) or even ethane (de Bruin et al., 1992). The reasons for these differences are unclear.

Pure cultures of methanogens listed in Table 3.9 generally carry out reductive dehalogenation at rates usually lower than dehalogenating habitats, and dehalogenation represents only a small fraction of their methanogenic metabolism. It has been found that cobalamins and cofactor F_{430}, both of which are plentiful in methanogens, can catalyze reductive dehalogenation of one and two-carbon halogenated organic compounds when provided with a strong reducing agent, such as Ti^{3+} (Gantzer and Wackett, 1991; Holliger, 1992; Krone et al., 1989; Krone et al., 1989). It is therefore likely that dehalogenation by methanogens is a non-specific reaction catalyzed by reduced transition metal complexes in them. Apparently, there are other organisms in methanogenic habitats which carry out more rapid and specific dehalogenation. If these organisms use H_2 /formate as their reductant, as *Desulfomonile tiedjei* and *Dehalobacter restrictus* do, they should be competing with methanogens for their substrate, and should be expected to outcompete methanogens because of the superior energetics of reductive dehalogenation versus CO_2 reduction to CH_4.

Even if their role in dehalogenation in methanogenic habitats is minor, methanogens still play important roles in carbon flow in those systems, as in the complete conversion of 3-chlorobenzoate to CH_4 and CO_2 by a microbial consortium (Dolfing and Tiedje, 1986). In this consortium, 3-chlorobenzoate was converted to benzoate by *Desulfomonile tiedjei* and benzoate was oxidized to acetate and H_2 by an organism resembling *Syntrophus buswellii*. Some of the H_2 produced from benzoate oxidation was used for reductive dechlorination, while acetate and the remaining H_2 were consumed by methanogens. Proper functioning by the methanogens in removing the products of benzoate oxidation is important to efficient functioning of the culture.

There is considerable potential for the use of mixed methanogenic cultures in the bioremediation of sites contaminated with halogenated organics (Bhatnagar and Fathepure, 1991). Contaminated wastes can be treated either by pumping them through a treatment facility, or by stimulating *in situ* activity of organisms, especially since many waste sites are already anaerobic. Compounds with several halogens are often dehalogenated more rapidly by anaerobes than when only one or a few halogens are present, which is the opposite trend from that in aerobic biodegradation (Vogel et al., 1987). This has led to the idea of treatment using sequential anaerobic/aerobic bioreactors (Fathepure and Vogel, 1991), although complete dechlorination by anaerobes is possible in many cases. Understanding the role of methanogens in anaerobic microbial communities involved in bio-

remediation schemes will be essential for efficient and reliable removal of halogenated organics and other pollutants.

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II

BIOCHEMISTRY

4

Reactions and Enzymes Involved in Methanogenesis from CO_2 and H_2

Rudolf K. Thauer, Reiner Hedderich, and Reinhard Fischer

4. Introduction

This chapter concentrates on the reactions and enzymes involved in methanogenesis from CO_2 and H_2. The coenzymes and electron carriers involved are described only as far as necessary for the understanding of their functions; they are dealt with extensively in other chapters. The bioenergetics of CO_2 reduction to CH_4 are only briefly discussed. For details the reader is referred to Chapter 8. We have tried to cover the complete literature dealing with the enzymology of methanogenesis from CO_2 and H_2. However, mainly work is cited which was performed with purified or at least partially purified preparations following the famous dictum of Efraim Racker: don't waste clean thinking on dirty enzymes.

4.1.1 Hydrogenotrophic Methanogens and Energetic Aspects

Most methanogenic bacteria can grow on molecular hydrogen and CO_2 as sole energy source (Balch et al., 1979; Jones et al., 1987). Exceptions are, e.g., *Methanothrix* spp., which metabolize only acetate (Huser et al., 1982); *Methanosphaera stadtmaniae*, which reduces methanol with H_2 (Miller and Wolin, 1985); and *Methanolobus tindarius*, which uses only methylamine and methanol (König and Stetter, 1982):

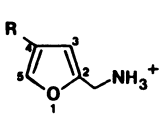
$$4\,H_2 + CO_2 \rightarrow CH_4 + 2\,H_2O,\ \Delta G^{\circ\prime} = -131\ kJ/mol \qquad (4.1)$$

In methanogenic ecosystems the partial pressure of H_2 is generally between 1 Pa and 10 Pa. At these low H_2 concentrations the free energy change $\Delta G'$ associated

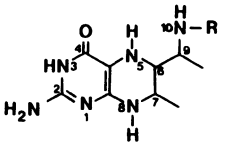
with methanogenesis from CO_2 and H_2 is between -20 kJ/mol and -40 kJ/mol. *In vivo* at least 50 kJ/mol are required to drive the synthesis of ATP from ADP and inorganic phosphate (Thauer et al., 1977; Thauer and Morris, 1984). Thus, under physiological growth conditions, less than 1 mol ATP per mol CH_4 can be generated. Evidence is available that the exergonic formation of CH_4 and the endergonic phosphorylation of ADP are coupled via a chemiosmotic mechanism (Blaut et al., 1990; Gottschalk and Blaut, 1990; Kaesler and Schönheit, 1989a and b) (see Part II, Chapter 8).

4.1.2 C_1-Unit Carriers and Electron Carriers

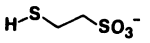
The reduction of CO_2 to CH_4 proceeds via coenzyme bound C_1-intermediates. Methanofuran (MFR), tetrahydromethanopterin (H_4MPT), and coenzyme M (H-S-CoM) are the three C_1-unit carriers found in all methanogens analyzed in this respect (DiMarco et al., 1990a; Keltjens et al., 1990; Wolfe, 1991).



MFR



H_4MPT



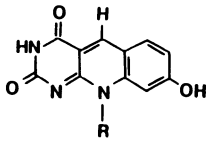
H-S-CoM

Methanofuran (Leigh et al., 1984 and 1985) is a C_4-substituted furfurylamine found in all methanogenic bacteria and in *Archaeoglobus fulgidus* (White, 1988; Möller-Zinkhan et al., 1989). There exists at least five chemically different methanofuran-derivatives which differ in the substituent R (White, 1988) (for structures see Part III, Chapter 9). Tetrahydromethanopterin (van Beelen et al., 1984a) is also found in non-methanogenic archaebacteria (Möller-Zinkhan et al., 1989). It is very similar to tetrahydrofolate, from which it differs by the two methyl groups in positions 7 and 9, and by the nature of the substituent R (for structures see chapter 9). Tetrahydrosarcinapterin (H_4SPT) is found in *Methanosarcina* spp. that differs from H_4MPT only by an additional glutamyl moiety in the substituent R (van Beelen et al., 1984b). Coenzyme M (Taylor and Wolfe, 1974) is the simplest coenzyme known to date. It is exclusively found in methanogenic bacteria (Balch and Wolfe, 1979).

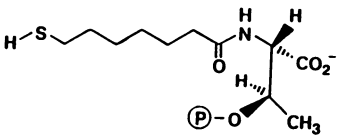
The functional groups of these three coenzymes, to which the C_1-intermediates are bound, are the amino group of methanofuran, the nitrogens 5 and 10 of tetrahydromethanopterin, and the mercapto group of coenzyme M.

The reduction of CO_2 to methane with H_2 also involves several electron carriers

(see Part II, chapter 7). Coenzyme F_{420} (Eirich et al., 1978) and *N*-7-mercaptoheptanoyl-*O*-phospho-L-threonine (H-S-HTP) (Noll et al., 1986; Kobelt et al., 1987) are two coenzymes for which such a function has been ascertained.



F_{420} (oxidized)



H-S-HTP

The substituent R (see Part III, chapter 9) in coenzyme F_{420} may contain two glutamyl moieties ($F_{420}-2$), three glutamyl moieties ($F_{420}-3$), four glutamyl moieties ($F_{420}-4$), or five glutamyl moieties ($F_{420}-5$) (Eirich et al., 1979; Peck and Archer, 1987).

The methanogens also contain ferredoxin (Hatchikian et al., 1982; Moura et al., 1982; Terlesky and Ferry, 1988), a polyferredoxin with 12 [4Fe–4S] clusters (Hedderich et al., 1992) and other iron-sulfur proteins (Rogers et al., 1988) of still unknown function. Methanogens capable of oxidizing methyl-groups (of acetate, methanol, or methylamine) to CO_2 additionally contain cytochromes (Kühn et al., 1983; Kühn and Gottschalk, 1983; Jussofie and Gottschalk, 1986; Kemner et al., 1987). Whether these are active when methylotrophic methanogens grow on H_2 and CO_2 has not yet been elucidated.

4.1.3 C_1-Intermediates and Partial Reactions

The following coenzyme-bound C_1-intermediates in CO_2 reduction to methane have been identified: N-formyl-MFR (CHO—MFR), N^5-formyl-H_4MPT (CHO—H_4MPT), N^5, N^{10}-methenyl-H_4MPT (CH≡H_4MPT$^+$), N^5, N^{10}-methylene-H_4MPT (CH_2=H_4MPT), N^5-methyl-H_4MPT (CH_3—H_4MPT), and methylcoenzyme M (CH_3—S—CoM) (Keltjens et al., 1990; DiMarco et al., 1990a; Thauer, 1990). Seven partial reactions can be formulated:

$$CO_2 + MFR + H_2 \rightarrow CHO\text{—}MFR + H_2O + H^+, \quad \Delta G^{\circ\prime} = +16 \text{ kJ/mol} \quad (4.2a)$$

$$CHO\text{—}MFR + H_4MPT \rightarrow CHO\text{—}H_4MPT + MFR, \quad \Delta G^{\circ\prime} = -4.4 \text{ kJ/mol} \quad (4.2b)$$

$$CHO\text{—}H_4MPT + H^+ \rightarrow CH{\equiv}H_4MPT^+ + H_2O, \quad \Delta G^{\circ\prime} = -4.6 \text{ kJ/mol} \quad (4.2c)$$

$$CH{\equiv}H_4MPT^+ + H_2 \rightarrow CH_2{=}H_4MPT + H^+, \quad \Delta G^{\circ\prime} = -5.5 \text{ kJ/mol} \quad (4.2d)$$

$$CH_2{=}H_4MPT + H_2 \rightarrow CH_3{-}H_4MPT, \quad \Delta G^{\circ\prime} = -17.2 \text{ kJ/mol} \quad (4.2e)$$

$$CH_3{-}H_4MPT + H{-}S{-}CoM \rightarrow CH_3{-}S{-}CoM + H_4MPT, \quad \Delta G^{\circ\prime} = -29.7 \text{ kJ/mol} \quad (4.2f)$$

$$CH_3{-}S{-}CoM + H_2 \rightarrow CH_4 + H{-}S{-}CoM, \quad \Delta G^{\circ\prime} = -85 \text{ kJ/mol} \quad (4.2g)$$

The free energy changes given for reactions 4.2a–g have been calculated from experimentally determined equilibrium constants or have been estimated from analogous reactions (Keltjens and van der Drift, 1986; Keltjens and Vogels, 1988). They account for a total free energy change of −130.4 kJ/mol. This value differs by only 0.6 kJ/mol from the free energy change calculated for the reduction of CO_2 with H_2 to CH_4 from the standard free energies of formation from the elements (Thauer et al., 1977; the thermodynamic data are given for CO_2, H_2, and CH_4 in the gaseous state). Evidence is available that the exergonic reactions 4.2f and 4.2g are coupled with energy conservation by a chemiosmotic mechanism (Blaut et al., 1990; Gottschalk and Blaut, 1990) and that the endergonic reaction 4.2a is energy driven by reversed electron transport (Kaesler and Schönheit, 1989a and b) (see Chapter 8). An overview of the metabolic pathway is given in figure 4.1.

4.1.4 Methanogenic Bacteria Investigated in Detail

The enzymes involved in the catalysis of reactions 4.2a–g have mainly been studied in three organisms which belong to three of the four known taxonomic orders of methanogenic bacteria (Balch et al., 1979; Woese, 1987); *Methanobacterium thermoautotrophicum* strain ΔH and strain Marburg (Brandis et al., 1981) with optimal growth at a temperature of 65°C which belongs to the order *Methanobacteriales; Methanosarcina barkeri* strain Fusaro and strain MS (Kandler and Hippe, 1977; Bryant and Boone, 1987) with a growth temperature optimum at 37°C which belong to the order of *Methanomicrobiales;* and *Methanopyrus kandleri* (Kurr et al., 1991) with a growth temperature optimum at 98°C, which is the sole member of its group so far (Burggraf et al., 1991). Some of the enzymes are also found in *Archaeoglobus fulgidus* (Achenbach-Richter et al., 1987), which is a sulfate-reducing archaeon phylogenetically closely related to the *Methanomicrobiales* (Woese et al., 1991). Where information is available, their properties are included in the comparison.

4.1.5 Transition Metals Required for Growth on H_2 and CO_2

Methanogens are dependent on iron, cobalt, nickel, and molybdenum (Schönheit et al., 1979) and/or tungsten for growth on H_2 and CO_2 (Winter et al., 1984;

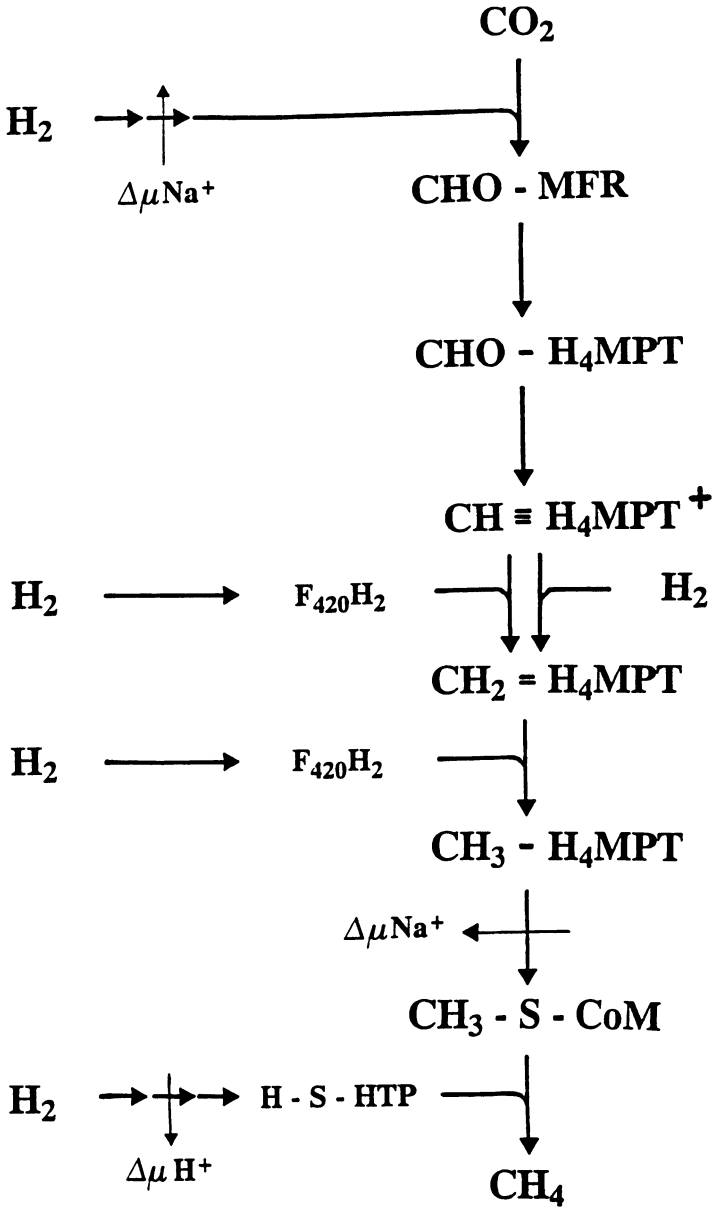


Figure 4.1. metabolic pathway of methanogenesis from CO_2 and H_2. CHO-MFR, N-formylmethanofuran; CHO-H_4MPT, N^5-formyltetrahydromethanopterin; CH≡H_4MPT$^+$, N^5, N^{10}-methenyltetrahydromethanopterin; CH_2=H_4MPT, N^5, N^{10}-methylenetetrahydromethanopterin; CH_3-H_4MPT$^+$, N^5-methyltetrahydromethanopterin; CH_3-S-CoM, methyl-coenzyme M; H-S-HTP, *N*-7-mercaptoheptanoyl-*O*-phospho-L-threonine; $F_{420}H_2$, reduced coenzyme F_{420}.

van Bruggen et al., 1986; Widdel, 1986). For each of these transition metals at least one metallo-enzyme is known which is essential for methanogenesis from CO_2 and H_2. The role of the transition metals in the catalysis of reactions 4.2a–g will be emphasized.

4.1.6 Outline

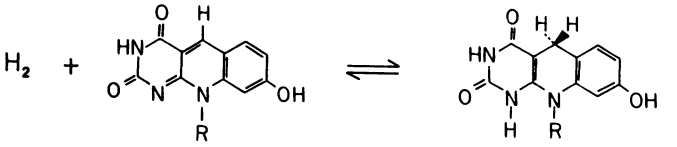
Molecular hydrogen is a substrate in four of the seven partial reactions described above. For the activation of H_2 the organisms contain several hydrogenases. The properties of these proteins will be discussed first, followed by a description of the other enzymes involved in the catalysis of reactions 4.2a–g.

4.2 Activation of Molecular Hydrogen

Methanogenic bacteria growing on H_2 and CO_2 generally contain two (NiFe) hydrogenases (Grraf and Thauer, 1981): a coenzyme F_{420}-reducing hydrogenase, and a coenzyme F_{420}-non-reducing hydrogenase (Jacobson et al., 1982). The physiological electron acceptor of the latter enzyme is still not known. In the literature this enzyme is also referred to as methylviologen-reducing hydrogenase, which is, however, misleading since both (NiFe) hydrogenases can catalyze the reduction of viologen dyes as artificial one-electron acceptors. In addition to the two (NiFe) hydrogenases, most methanogens contain a third very active hydrogenase, designated H_2-forming methylenetetrahydromethanopterin dehydrogenase, which differs from all other hydrogenases known to date in that it does not contain nickel and/or iron-sulfur clusters (Zirngibl et al., 1990; Schwörer and Thauer, 1991).

4.2.1 Coenzyme F_{420}-reducing (NiFe) hydrogenase

This (NiFe) enzyme catalyzes the reversible reduction of coenzyme F_{420} with H_2. Coenzyme F_{420} is a two-electron hydride acceptor with an $E^{\circ\prime}$ of -360 mV (Walsh, 1986; Gloss and Hausinger, 1987). The reduction of F_{420} is stereospecific (Teshima et al., 1985; Yamazaki et al., 1985).



(4.3)

The free energy change ($\Delta G^{\circ\prime}$) associated with this reaction is -11 kJ/mol. Reduced coenzyme F_{420} generated in this reaction is required for the reduction

of $CH{\equiv}H_4MPT^+$ to $CH_2{=}H_4MPT$ in reaction 4.2d and for the reduction of $CH_2{=}H_4MPT$ to $CH_3{-}H_4MPT$ in reaction 4.2e as evidenced by the finding that all H_2/CO_2 grown methanogens investigated in this respect contain a very active coenzyme F_{420}-dependent methylene-H_4MPT dehydrogenase and a coenzyme F_{420}-dependent methylene-H_4MPT reductase (Schwörer and Thauer, 1991).

The F_{420}-reducing hydrogenase appears to be a peripheral membrane protein localized at the inner surface of the cytoplasmic membrane as revealed by immunogold labeling techniques (Muth, 1988; Baron et al., 1989; Lünsdorf et al., 1991). However, upon cell breakage most of the activity is recovered in the soluble cell fraction. The enzyme, which is reversibly inactivated by O_2, has been purified and characterized from *Methanobacterium thermoautotrophicum* (Fox et al., 1987), *Methanobacterium formicicum* (Baron and Ferry, 1989), *Methanosarcina barkeri* (Fusaro) (Fiebig and Friedrich, 1989), *Methanococcus voltae* (Muth et al., 1987), *Methanococcus vannielii* (Yamazaki, 1982), and *Methanospirillum hungatei* (Sprott et al., 1987; Choquet and Sprott, 1991).

The enzyme purified from *M. thermoautotrophicum* consists of three different subunits designated α (47 kDa), β (31 kDa), and γ (26 kDa), and contains per mol $\alpha_1\beta_1\gamma_1$ trimer approximately 1 mol nickel, 1 mol FAD, and 13–14 mol non-heme iron and acid-labile sulfur (Fox et al., 1987). The iron-sulfur clusters appear to be organized in [4Fe-4S]-clusters. The 47 kDa α-subunit most probably harbors the nickel as evidenced by characteristic nickel-binding motifs in the amino terminus (Arg-X-Cys-X_2-Cys-X_3-His) and the carboxy terminus (Asp-Pro-Cys-X_2-Cys-X_2-His) (Alex et al., 1990). Its sequence is homologous (30%) to that of the large subunit of the F_{420}-non-reducing hydrogenase from *M. thermoautotrophicum* and of other nickel containing hydrogenases (Alex et al., 1990). The 26 kDa γ-subunit is an iron-sulfur protein as evidenced by the presence of a bacterial ferredoxin-like iron-sulfur cluster motif (Cys-X_2-Cys-X_2-Cys-X_3-Cys-X_3-Arg-X_{14-16}-Cys-X_2-Cys-X_2-Cys-X_3-Cys) (Meyer, 1988). It does not display significant direct homology to the classical small subunit of other (NiFe) hydrogenases. The 31 kDa β-subunit is probably an iron-sulfur flavoprotein and the site of coenzyme F_{420} reduction as evidenced by the finding that the $\alpha_1\gamma_1$ dimer is still capable of H_2-activation but lacks FAD and the ability to reduce F_{420}. The amino acid sequence of the β-subunit exhibits 26% direct homology to the β-subunit of the F_{420}-reducing formate dehydrogenase from *Methanobacterium formicicum*.

The structures of the F_{420}-reducing hydrogenase from the other methanogens are similar but not identical to that of the enzyme from *M. thermoautotrophicum*. For example, the enzyme from *M. voltae* is composed of three subunits α (45 kDa), β (37 kDa), and (27 kDa) (Muth et al., 1987). It contains, per mol $\alpha_1\beta_1\gamma_1$ trimer, 1 mol of FAD, 1 mol selenium (see below), and 4–5 mol non-heme iron and acid-labile sulfur.

The genes for the F_{420}-reducing hydrogenase have been cloned and sequenced from *M. thermoautotrophicum* (Alex et al., 1990; Reeve and Beckler, 1990) and from *M. voltae* (Halboth and Klein, 1992; see also chapter 12). The putative

operon in both organisms is composed of four genes encoding four subunits, indicating that one subunit must have been lost during purification of the enzyme. However, the loss appears to have no effect on the catalytic properties (Fox et al., 1987). It has therefore been suggested that via the fourth subunit the hydrogenase could be anchored to the inner surface of the cytoplasmic membrane (Przybyla et al., 1992).

As mentioned above, the F_{420}-reducing hydrogenase also catalyzes the reduction of viologen dyes with H_2, indicating that one-electron redox steps are involved in the oxidation of H_2. This is also suggested by the presence of iron-sulfur clusters and explains why the enzyme contains a flavin, which is required as a one-electron/two-electron switch for the two-electron reduction of coenzyme F_{420} (Walsh, 1979).

4.2.2 Coenzyme F_{420}-Non-reducing (NiFe) Hydrogenase

The physiological electron acceptor of the (NiFe) hydrogenase is not yet known. It probably accepts one electron at a time since the enzyme catalyzes the reduction of viologen dyes and contains iron-sulfur clusters, but lacks a flavin and thus a one-electron/two-electron switch.

$$H_2 + 2X \rightarrow 2X^- + 2H^+ \qquad (4.3)$$

The $E^{\circ\prime}$ of the H^+/H_2 couple is -414 mV. At the H_2 partial pressures prevailing in the natural habitat of the methanogens the E' of the H^+/H_2 couple is between -270 mV and -300 mV. The electron acceptor X thus should have an $E^{\circ\prime}$ near -300 mV.

The F_{420}-non-reducing hydrogenase is probably involved in the catalysis of reactions (4.2a) and (4.2g). This is mainly deduced from the finding that *in vivo* the reduction of CO_2 to formyl-MFR and of methyl-coenzyme M to CH_4 with H_2 are not dependent on coenzyme F_{420}. The localization of the enzyme in the intact cells has not yet been determined via immuno-gold labeling. Upon cell breakage most of the activity is recovered in the soluble cell fraction.

The enzyme, which is reversibly inactivated by O_2, has been purified and characterized from *M. thermoautotrophicum* (strain ΔH and strain Marburg (Coremans et al., 1989; Hedderich et al., 1990; Kolodziej et al., 1992) and *Methanobacterium formicicum* (Jin et al., 1983). The enzyme from *M. thermoautotrophicum* is composed of three subunits, α, β, and γ of apparent molecular mass of 52 kDa, 41 kDa, and 17 kDa, respectively, and contains per mol trimer approximately 1 mol nickel and up to 19 mol non-heme iron. The 52 kDa α-subunit is homologous (30%) to the 47 kDa α-subunit of the F_{420}-reducing hydrogenase and contains the characteristic nickel binding motifs at the amino terminus (Arg-X-Cys-Gly-X-Cys-X_3-His) and carboxy terminus (Asp-Pro-Cys-

X_2-Cys-X_2-His) (Reeve et al., 1989; Reeve and Beckler, 1990). The 41 kDa β-subunit is probably an iron-sulfur protein. It is homologous to the small subunit of the nickel-containing hydrogenases from sulfate-reducing bacteria and the (NiFe) hydrogenase-1 from *Escherichia coli* (Przybyla et al., 1992). Comparison of its amino acid sequence with that of other (NiFe) hydrogenases indicates the presence of 10 conserved cysteinyl residues. In contrast to the small subunit genes for the (NiFe) hydrogenases from eubacteria, the gene encoding the β-subunit does not encode an N-terminal signal peptide, consistent with the presence of the hydrogenase in the soluble fraction of methanogenic bacteria. The function of the 17 kDa γ-subunit is not known.

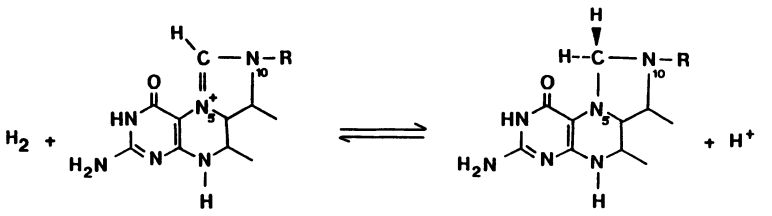
The genes for the F_{420}-non-reducing hydrogenase have been cloned and sequenced from *M. thermoautotrophicum* (Reeve et al., 1989), from *Methanothermus fervidus* (Steigerwald et al., 1990), and from *M. voltae* (Halboth and Klein, 1992) (see chapter 12). The putative operon is composed of four genes coding for the three subunits of the enzyme and a polyferredoxin containing 12 × [4Fe-4S] clusters. This polyferredoxin has recently been isolated. It is only slowly reduced by H_2 in the presence of the F_{420}-non-reducing hydrogenase, making it questionable whether this iron-sulfur protein is the physiological electron acceptor of the (NiFe) hydrogenase (Hedderich et al., 1992).

4.2.3 The Selenocysteine-Containing Hydrogenases

The F_{420}-reducing hydrogenase (Muth et al., 1987) and the F_{420}-non-reducing hydrogenase from *Methanococcus voltae* contain a selenocysteine instead of a cysteine in the carboxy terminal nickel binding site of the α-subunit (Asp-Pro-selenocysteine-X_2-Cys-X_2-His) (Halboth and Klein, 1992). Interestingly, the organism additionally contains the genes for the two (NiFe) hydrogenases without selenocysteine (Halboth and Klein, 1992). Whether and when these are expressed is still not known. The (NiFe) hydrogenase from *M. vannielii* has also been shown to contain selenium (Yamazaki, 1982).

4.2.4 H_2-Forming Methylenetetrahydromethanopterin Dehydrogenase

This hydrogenase catalyzes the reversible reduction of $CH{\equiv}H_4MPT^+$ with H_2 to $CH_2{=}H_4MPT$ (reaction 4.2d).



(4.2d)

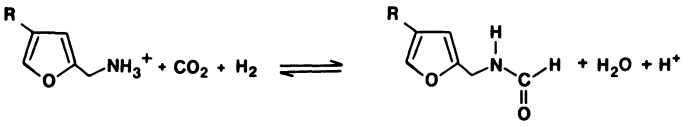
The enzyme neither catalyzes the reduction of viologen dyes nor an H_2H^+ exchange, both of which are characteristic properties of the two (NiFe) hydrogenases. H_2-forming methylene-H_4MPT dehydrogenase is found in all methanogens of the orders *Methanobacteriales*, *Methanococcales*, and also in *Methanopyrus kandleri*, but appears to be absent in the order *Methanomicrobiales* (Schwörer and Thauer, 1991).

A very active soluble hydrogenase, which is rapidly inactivated in the presence of air, has been purified from *Methanobacterium thermoautotrophicum* (Zirngibl et al., 1990), *Methanobacterium wolfei* (Zirngibl et al., 1992), and *Methanopyrus kandleri* (Ma et al., 1991a). It is a homodimer of a polypeptide of apparent molecular mass of 43 kDa. The purified enzyme does not appear to contain nickel or iron-sulfur clusters. The only transition metal found in significant amounts was zinc and the enzyme also lacks a chromophoric prosthetic group (Zirngibl et al., 1992). The high activity soluble hydrogenase genes have been cloned and sequenced from *M. thermoautotrophicum* (von Bünau et al., 1991) and *M. kandleri* (Zirngibl et al., 1992). The primary structure lacks sequence motifs characteristic of the nickel-binding site of (NiFe) hydrogenases and of iron-sulfur clusters of bacterial ferredoxins.

The catalytic mechanism of the enzyme has been studied in detail. Available evidence indicates that the H_2-forming dehydrogenase catalyzes a stereospecific hydride transfer from H_2 to $CH{\equiv}H_4MPT^+$ via a ternary complex mechanism. How this is achieved in the absence of a redox active transition metal is not yet known.

4.3 CO_2 Reduction to N-Formylmethanofuran

Free formate is not an intermediate in CO_2 reduction to CH_4. Those methanogens capable of growing on formate first oxidize this compound to CO_2, which is subsequently reduced (Sparling and Daniels, 1986). The direct CO_2 reduction product is N-formylmethanofuran which is an N-substituted formamide (Leigh et al., 1985).

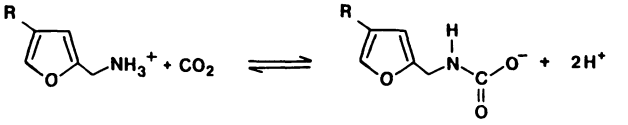


(4.4)

The free energy change associated with reaction 4.4 is estimated to be +16 kJ/mol (Keltjens and van der Drift, 1986). This estimate is based on a $\Delta G^{\circ\prime}$ value

of +3.45 kJ/mol for the reduction of CO_2 with H_2 (both in the gaseous state) to formate and a $\Delta G^{\circ\prime}$ value of +12.5 kJ/mol for the formation of formylmethanofuran from formate and methanofuran, the latter assumed to be equal to the formation of N-formyl-glutamate from formate and glutamate (Robinson, 1971). The reduction of CO_2 to N-formylmethanofuran is thus an endergonic reaction, in particular with respect to the low H_2 partial pressures of 1–10 Pa prevailing in the natural habitats of the methanogens. Under these conditions the free energy change is between +34 and +40 kJ/mol.

The reduction of CO_2 to formylmethanofuran most probably proceeds via the carbamate which is formed from CO_2 and methanofuran in a very rapid spontaneous reaction (see Ewing et al., 1980).



(4.5)

The free energy change associated with reaction 4.5 is estimated to be +6.4 kJ/mol, which is calculated from $\Delta G^{\circ\prime}$ = + 1.6 kJ/mol for $HCO_3^- + NH_3 \rightarrow NH_2COO^- + H_2O$ (Raijman and Jones, 1973) and $\Delta G^{\circ\prime}$ = +4.8 kJ/mol for CO_2 (gaseous) + $H_2O \rightarrow HCO_3^- + H^+$ (Thauer et al., 1977).

4.3.1. Three Enzyme Complexes Involved in CO_2 Reduction to N-Formylmethanofuran with H_2

Probably three enzyme complexes are involved in the catalysis of N-formylmethanofuran formation from methanofuran, CO_2, and H_2 (partial reaction 4.2a): (i) the F_{420}-non-reducing (NiFe) hydrogenase, which catalyzes the reduction of a still unknown electron acceptor X (see Section 4.2.2), (ii) a formylmethanofuran dehydrogenase, which catalyzes the reversible reduction of CO_2 + MFR to CHO—MFR with a still unknown one-electron donor Y, and (iii) an oxidoreductase complex, which catalyzes the reversed electron transport from reduced X to oxidized Y:

$$H_2 + 2\,X \rightarrow 2\,X^- + 2\,H^+ \qquad (4.6a)$$

$$CO_2 + MFR + 2\,Y^- + H^+ \rightarrow CHO\text{—}MFR + H_2O + 2\,Y \qquad (4.6b)$$

$$2\,X^- + 2\,Y \rightarrow 2\,X + 2Y^- \qquad (4.6c)$$

The redox potential $E^{\circ\prime}$ of X has been estimated to be near −300 mV (see section 4.2.2). The redox potential of Y must be near −500 mV since the $E^{\circ\prime}$ of the CO_2/

CHO—MFR couple is −497 mV (calculated from $\Delta G^{\circ\prime} = +16$ kJ/mol of partial reaction 4.2a and $E^{\circ\prime} = -414$ mV of the H^+/H_2 couple). The reduction of Y by X^- is thus an endergonic reaction which can only proceed if driven by energy. Evidence is available that the driving force is the sodium motive force (Kaesler and Schönheit, 1989a and b) (see chapter 8).

The properties of the F_{420}-non-reducing (NiFe) hydrogenase, which catalyzes reaction 4.6a, have been described in Section 4.2.2. The proposed reduced X:oxidized Y oxidoreductase complex, which catalyzes reaction 4.6c, has not yet been identified because of the lack of knowledge about the chemical nature of X and Y. Because the mechanism of reversed electron transport involves ion gradients as a driving force, it is proposed that this complex is an integral part of the cytoplasmic membrane. The formylmethanofuran dehydrogenase, which catalyzes reaction 4.6b, has been purified and characterized.

Formylmethanofuran dehydrogenase catalyzes the reversible reduction of artificial one-electron acceptors such as viologen dyes with N-formylmethanofuran (Börner et al., 1989). The enzyme does not contain a flavin indicating that, because of the lack of a one-electron/two-electron switch, the physiological electron acceptor Y is most probably a one- (or $n \times$ one) electron carrier. The enzyme is found in all H_2/CO_2-grown methanogens investigated (Schwörer and Thauer, 1991). It appears to be associated with the soluble cell fraction because after cell breakage more than 90% of the enzyme activity is recovered in the 100,000 × *g* supernatant. Formylmethanofuran dehydrogenase, which is rapidly inactivated in the presence of air, has been purified from *Methanobacterium thermoautotrophicum* (Börner et al., 1991), from *Methanosarcina barkeri* (Karrasch et al., 1989), and from *Methanobacterium wolfei* (Schmitz et al., 1992) and partially purified from *Archaeoglobus fulgidus* (Schmitz et al., 1991). The enzyme contains either molybdenum or tungsten.

4.3.2. Molybdenum-Containing Formylmethanofuran Dehydrogenases

The enzyme from *M. thermoautotrophicum* is composed of two different subunits of apparent molecular mass of 60 kDa and 45 kDa, respectively, and contains, per mol $\alpha_1\beta_1$ dimer, approximately 1 mol molybdenum, 1 mol molybdopterin dinucleotide, and 4 mol non-heme iron and acid-labile sulfur. The pterin dinucleotide was found to be a mixture of molybdopterin guanine dinucleotide (Figure 4.2), molybdopterin adenine dinucleotide, and molybdopterin hypoxanthine dinucleotide (Börner et al., 1991).

The formylmethanofuran dehydrogenase from *M. barkeri* is composed of six different subunits of apparent molecular mass of 65 kDa, 50 kDa, 37 kDa, 34 kDa, 29 kDa, and 17 kDa, and contains per mol hexamer approximately 1 mol molybdenum, 1 mol molybdopterin guanine dinucleotide (Figure 4.1), 28 mol non-heme iron, and 28 mol acid-labile sulfur (Karrasch et al., 1989; Karrasch et

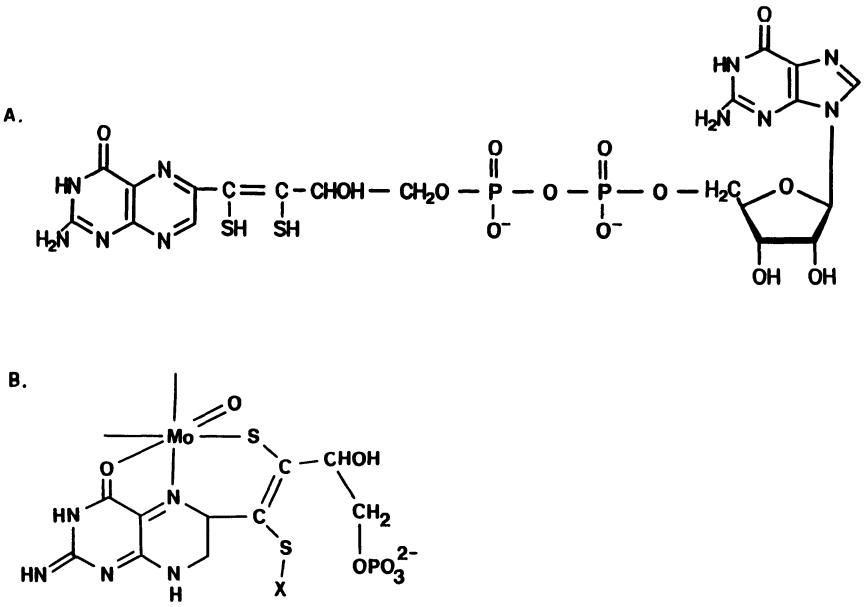


Figure 4.2. (A) Structure of molybdopterin guanine dinucleotide in the oxidized form (Johnson et al., 1990) and (B) proposed structure of the complex with molybdenum in the active site of formylmethanofuran dehydrogenase (Fischer and Strähle, 1991).

al., 1990a and b). This enzyme also catalyzes the oxidation of N-furfurylformamide (Breitung et al., 1990), N-methylformamide, formamide, and formate albeit with much lower catalytic efficiency (V_{max}/K_m) than the oxidation of formylmethanofuran (Thauer et al., unpublished results).

4.3.3 A Tungsten-Containing Formylmethanofuran Dehydrogenase in M. wolfei

M. wolfei contains two formylmethanofuran dehydrogenases which can be separated by anion exchange chromatography. Enzyme I is a molybdenum protein and is preferentially formed when the growth medium contains molybdenum. Enzyme II is a tungsten protein, which is only formed when the growth medium contains tungstate. The molybdenum enzyme is composed of three different subunits of apparent molecular mass of 63 kDa, 51 kDa, and 31 kDa, and contains per mol $\alpha_1\beta_1\gamma_1$ trimer approximately 0.3 mol molybdenum, 0.3 mol molybdopterin guanine dinucleotide, and 4–6 mol non-heme iron and acid-labile sulfur. The tungsten enzyme II is also composed of three subunits of apparent

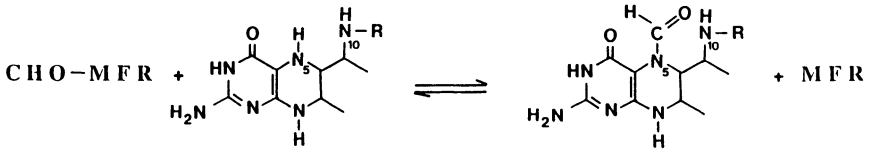
molecular mass of 64 kDa, 51 kDa, and 35 kDa and contains per mol $\alpha_1\beta_1\gamma_1$ trimer 0.4 mol of tungsten, 0.4 mol molybdopterin guanine dinucleotide, and 4–6 mol non-heme iron and acid-labile sulfur. The molybdenum enzyme and the tungsten enzyme differ significantly in their catalytic properties. Besides N-formylmethanofuran the molybdenum enzyme can also use N-furfurylformamide and formate as substrates, whereas the tungsten enzyme cannot do so (Schmitz et al., 1992).

4.3.4 Comparison with Xanthine Oxidase

Formylmethanofuran dehydrogenase catalyzes a reaction that is analogous to that catalyzed by milk xanthine oxidase (Hille and Massey, 1985), which is also a molybdenum pterin enzyme (Wootton et al., 1991): formylmethanofuran and xanthine are both formamide derivatives. This is most convincingly demonstrated by the finding that both xanthine oxidase (Morpeth et al., 1984) and formylmethanofuran dehydrogenase (Thauer et al., unpublished results) can catalyze the oxidation of formamide. Formylmethanofuran dehydrogenase and xanthine oxidases may therefore be considered to be related enzymes.

4.4 Formyl Group Transfer from Formylmethanofuran to H_4MPT

Formylmethanofuran:H_4MPT formyltransferase catalyzes the formation of N^5-formyl-H_4MPT from formylmethanofuran and H_4MPT (Donnelly and Wolfe, 1986). The formyltransferase can also use N-furfurylformamide as substrate, but with much lower catalytic efficiency (V_{max}/K_m) (Breitung et al., 1990).



Reports that in some methanogens N^{10}-formyl-H_4MPT rather than N^5-formyl-H_4MPT is formed have turned out to be incorrect (Breitung and Thauer, 1990). The generation of the N^5-isomer is of special interest because in eubacteria and in eucaryotes N^{10}-formyltetrahydrofolate is the intermediate, which is formed from free formate and tetrahydrofolate in an ATP-dependent reaction rather than in a formyltransferase reaction (Whitehead et al., 1988).

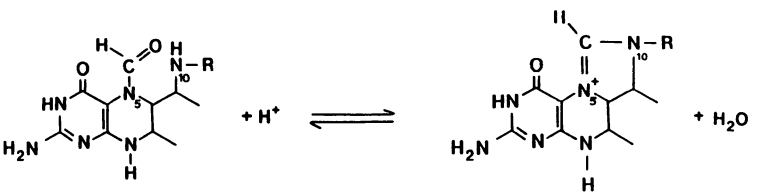
The formyltransferase, which is a soluble enzyme stable in air, has been

purified from *Methanobacterium thermoautotrophicum* (Donnelly and Wolfe, 1986), from *Methanosarcina barkeri* (Breitung and Thauer, 1990), from *Methanopyrus kandleri* (Brietung et al., 1992), and from *Archaeoglobus fulgidus* (Klein et al., 1992). It is a monomer or a tetramer of a polypeptide of apparent molecular mass 32–41 kDa. The enzyme is devoid of a chromophoric prosthetic group. The gene encoding the enzyme from *M. thermoautotrophicum* has been cloned, sequenced, and expressed in *Escherichia coli* in an active form (DiMarco et al., 1990b). The N-terminal amino acid sequences of the enzyme from the three other organisms have been determined. Interestingly, the first 16 N-terminal amino acids of the four formyltransferases investigated were found to be identical. This is amazing since the enzymes exhibit considerably different physical and chemical properties and are of different phylogenetic origin (Breitung et al., 1992).

The catalytic properties of the four enzymes have been studied in detail. The kinetic mechanism was determined to be of the ternary complex type (Breitung et al., 1992). Salts were found to specifically affect the catalytic efficiency (V_{max}/K_m) and thermostability of the enzymes (Breitung et al., 1992).

4.5 Conversion of N^5-formyl-H_4MPT to N^5,N^{10}-Methenyl-H_4MPT

N^5,N^{10}-Methenyl-H_4MPT cyclohydrolase catalyzes the reversible hydrolysis of N^5,N^{10}-methenyl-H_4MPT to N^5-formyl-H_4MPT (Donnelly et al., 1985):



(4.8)

N^5,N^{10}-methenyl-H_4MPT spontaneously hydrolyzes to N^{10}-formyl-H_4MPT under alkaline conditions. The rate of hydrolysis for reaction 4.8 is enhanced in the presence of anions (Breitung et al., 1991). This was not realized by those workers who provided evidence that the N^{10}-formyl isomer is formed from N^5,N^{10}-methenyl-H_4MPT by the cyclohydrolase from *Methanosarcina barkeri* (te Brömmelstroet et al., 1990a). It may be of interest to note that the N^5,N^{10}-methenyltetrahydrofolate cyclohydrolase from eubacteria and eucaryotes mediates the formation of the N^{10}-formyl isomer from N^5,N^{10}-methenyltetrahydrofolate (e.g., Ljungdahl and Clark, 1986).

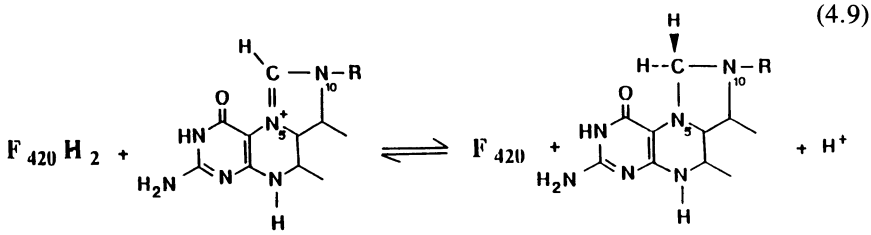
The cyclohydrolase, which is a soluble oxygen insensitive enzyme, has been

purified from *Methanobacterium thermoautotrophicum* (DiMarco et al., 1986), from *Methanosarcina barkeri* (te Brömmelstroet et al., 1990a), from *Methanopyrus kandleri* (Breitung et al., 1991), and from *Archaeoglobus fulgidus* (Schwörer et al., 1993). It is a monomer or a dimer of a polypeptide with an apparent molecular mass of 40 kDa and lacks a chromophoric prosthetic group. A comparison of the N-terminal amino acid sequences revealed a high degree of homology. The catalytic properties have been analyzed and compared. The catalytic efficiency and thermostability of the enzymes were found to be specifically affected by salts (Breitung et al., 1991).

4.6 Reduction of N^5, N^{10}-Methenyl-H_4MPT to N^5,N^{10}-Methylene-H_4MPT

All H_2/CO_2-grown methanogenic microbes analyzed in this respect contain a coenzyme F_{420}-dependent methylene-H_4MPT dehydrogenase, which catalyzes the reversible reduction of N^5,N^{10}-methenyl-H_4MPT with reduced coenzyme F_{420} to N^5,N^{10}-methylene-H_4MPT (Schwörer and Thauer, 1991). Reduction proceeds via hydride transfer and is probably stereospecific.

(4.9)



Together with the coenzyme F_{420}-reducing (NiFe) hydrogenase (see Section 4.2.1) the two enzymes mediate the reduction of N^5,N^{10}-methenyl-H_4MPT with H_2:

$$H_2 + F_{420} \rightarrow F_{420}H_2, \quad \Delta G^{\circ\prime} = -11 \text{ kJ/mol} \tag{4.10a}$$

$$\underset{F_{420}H_2}{CH{\equiv}H_4MPT^+} + \rightarrow \underset{F_{420} + H_+,}{CH_2{=}H_4MPT} + \quad \Delta G^{\circ\prime} = +5.5 \text{ kJ/mol} \tag{4.10b}$$

The two reactions are also catalyzed in one step by the H_2-forming methylene-H_4MPT dehydrogenase, which is present in most methanogenic bacteria, except those belonging to the order *Methanomicrobiales* (see Section 4.1.4):

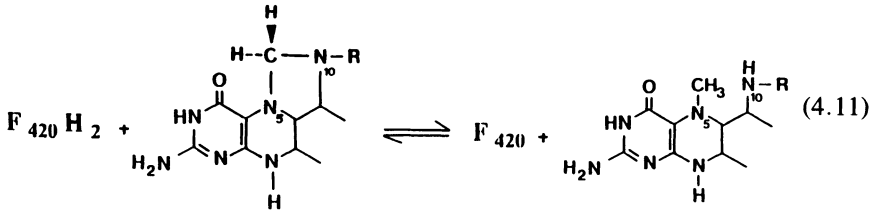
The coenzyme F_{420}-dependent methylene-H_4MPT dehydrogenase from *Methanobacterium thermoautotrophicum* (Mukhopadhyay and Daniels, 1989; te Brömmelstroet et al., 1991a), from *Methanosarcina barkeri* (Enssle et al., 1991; te Brömmelstroet et al., 1991b), and from *Archaeoglobus fulgidus* (Schwörer and

Thauer, 1993) has been purified and characterized. It is a soluble enzyme stable in air. The dehydrogenase is a homopolymer of a polypeptide of apparent molecular mass of 32 kDa. The dehydrogenase lacks a chromophoric prosthetic group, just as the N^5,N^{10}-methylenetetrahydrofolate dehydrogenase from eubacteria and eucaryotes (Wohlfarth et al., 1991).

The presence of two enzyme systems mediating the reduction with H_2 of methenyl-H_2MPT to methylene-H_4MPT (reaction 4.2d) in methanogenic bacteria is not understood. The H_2-forming methylene-H_4MPT dehydrogenase generally has a lower apparent affinity for H_2 but a higher specific activity in cell extracts than the F_{420}-reducing hydrogenase (Thauer et al., unpublished results). This suggests that the H_2-forming dehydrogenase is operative mainly when the H_2 partial pressure is high, and the F_{420}-dependent system when the H_2 partial pressure is low.

4.7 Reduction of N^5,N^{10}-Methylene-H_4MPT to N^5-Methyl-H_4MPT

A coenzyme F_{420}-dependent methylene-H_4MPT reductase has been found in every H_2/CO_2-grown methanogen investigated in this respect. The enzyme catalyzes the reduction of N^5,N^{10}-methylene-H_4MPT to N^5-methyl-H_4MPT with reduced coenzyme F_{420} as electron donor. Reduction is via hydride transfer and probably proceeds stereospecifically.



(4.11)

Together with the F_{420}-reducing (NiFe) hydrogenase (see Section 4.2.1) the enzyme catalyzes the reduction of methylene-H_4MPT with H_2 to N^5-methyl-H_4MPT (reaction 4.2e).

$$H_2 + F_{420} \rightarrow F_{420}H_2, \quad \Delta G^{\circ\prime} = -11\ \text{kJ/mol} \quad (4.12a)$$

$$CH_2{=}H_4\text{MPT} + F_{420}H_2 \rightarrow CH_3{-}H_4\text{MPT} + F_{420}, \quad \Delta G^{\circ\prime} = -6.2\ \text{kJ/mol} \quad (4.12b)$$

The F_{420}-dependent reductase, which is a soluble enzyme relatively stable in air, has been purified from *Methanobacterium thermoautotrophicum* strain ΔH (te Brömmelstroet et al., 1990b) and strain Marburg (Ma and Thauer, 1990a and b), from *Methanosarcina barkeri* (Ma and Thauer, 1990c; te Brömmelstroet et al.,

1991b), from *Methanopyrus kandleri* (Ma et al., 1991b), and from *Archaeoglobus fulgidus* (Schmitz et al., 1991). The enzyme is a homotetramer or homohexamer of a polypeptide of apparent molecular mass of 35 kDa. The N-terminal amino acid sequences of the four purified enzymes show a high degree of similarity. The enzyme does not contain FAD or FMN and thus differs from the respective N^5,N^{10}-methylenetetrahydrofolate reductase of eubacteria and eucaryotes, which are flavoproteins (Wohlfarth et al., 1990).

It has been postulated that the reduction of methylene-H_4MPT to methyl-H_4MPT with H_2 is coupled with energy conservation. This hypothesis was abandoned after it became clear that the methylene-H_4MPT reductase is a completely soluble homomeric enzyme without a prosthetic group. Also, at physiological H_2 partial pressures (pH_2 = 1 Pa − 10 Pa) the free energy change associated with partial reaction 5 is too small to drive the phosphorylation of ADP.

4.8 Methyltransfer from N^5-Methyl-H_4MPT to Coenzyme M

Until recently, the formation of methyl-coenzyme M from N^5-methyl-H_4MPT and coenzyme M has only been studied in cell extracts (Sauer, 1986; Sauer et al., 1986; van der Wyngaard et al., 1991). The reaction was found to be dependent on ATP and on strong reducing conditions. In the absence of coenzyme M a methylated corrinoid accumulated which was demethylated upon the addition of coenzyme H (Poirot et al., 1987; Kengen et al., 1988, 1990, 1992). The corrinoid was identified as 5-hydroxybenzimidazolyl cobamide (Pol et al., 1982; Kräutler et al., 1987) (Figure 4.3). From these studies it was postulated that methyltransfer from methyl-H_4MPT to coenzyme M proceeds in two steps: in the first step the methyl group of CH_3-H_4MPT is transferred to a corrinoid protein in the reduced form [Co(I)]; and in the second step this methyl group is further transferred to coenzyme M:

$$CH_3\text{—}H_4MPT + [Co(I)] \rightarrow CH_3\text{—}[Co(III)] + H_4MPT \quad (4.13a)$$

$$CH_3\text{—}[Co(III)] + H\text{-}S\text{-}CoM \rightarrow CH_3\text{-}S\text{-}CoM + [Co(I)] \quad (4.13b)$$

The requirement for reducing conditions and ATP for activity was explained by assuming that in the presence of traces of O_2 the corrinoid protein is oxidized to the Co(II) form, which can be reduced back to the Co(I) form in an ATP dependent reaction (Fischer et al., 1992). The role of ATP is not yet understood.

$$[Co(I)] + O_2 \rightarrow [Co(II)] + O_2^- \quad (4.14a)$$

$$[Co(II)] + e^- \xrightarrow[ATP]{} [Co(I)] \quad (4.14b)$$

Recently, fractionation studies revealed that for methyltransfer from methyl-H_4MPT to coenzyme M only the membrane fraction is required and that the polypeptides involved are integral membrane proteins (Fischer et al., 1992). An enzyme complex catalyzing the overall reaction was isolated from the membrane fraction of *Methanobacterium thermoautotrophicum* (strain Marburg) (Gärtner et al., 1992). It is composed of seven different subunits of apparent molecular mass of 12.5 kDa, 13.5 kDa, 21 kDa, 23 kDa, 24 kDa, 28 kDa, and 34 kDa. The 23 kDa polypeptide is probably the corrinoid-binding polypeptide (G. Fuchs, personal communication). The complex (156,000 kDa) contains per mol 1.6 mol 5-hydroxybenzimidazolyl cobamide (Fig. 4.3), 8 mol non-heme iron, and 8 mol acid-labile sulfur.

Recently Kengen et al. (1992) described the purification of the methyltransferase from *M. thermoautotrophicum* strain ΔH. The 100 kDa protein is composed of three subunits of apparent molecular mass 35 kDa, 33 kDa, and 31 kDa, and contains 0.2 mol 5-hydroxybenzimidazolyl cobamide.

Immunological evidence exists (Stupperich et al., 1990) that the methyltransferase could be identical with the membrane-associated corrinoid protein first described by Schulz and Fuchs (1986) (see also Dangel et al., 1987; Schulz et al., 1988).

The free energy change associated with the methyltransfer reaction is assumed to be identical to that of methionine synthesis from CH_3-tetrahydrofolate and homocysteine, which is −29.7 kJ/mol (Rüdiger and Jaenecke, 1969). Based on indirect evidence it has been postulated that the free energy change associated with methyl-coenzyme M formation from methyl-H_4MPT is conserved via a chemiosmotic mechanism (see chapter 8). The finding that the methyl-H_4MPT:coenzyme M methyltransferase is an integral membrane protein complex supports this proposal. Recently preliminary experiments have been published indicating that inverted membrane vesicles of *Methanosarcina barkeri* strain Gö1 catalyze the formation of methyl-coenzyme M from methyl-H_4MPT and coenzyme M. Evidence was obtained that the reaction was coupled with an electrogenic transport of sodium ions (Becher et al., 1992). Thus the methyltransferase appears to be a site of energy conservation (see chapter 8).

4.9 Methyl-Coenzyme M Reduction to Methane

The reduction of CO_2 with H_2 to methyl-coenzyme M proceeds through steps for which analogous reactions in non-methanogenic bacteria are known. In contrast, the reduction of methyl-coenzyme M to methane is a reaction unique to methanogenic microbes. The reaction involves three novel coenzymes (H-S-CoM, H-S-HTP, and coenzyme F_{430}) and four enzyme complexes. Methyl-coenzyme M reductase and heterodisulfide reductase are discussed in greater detail.

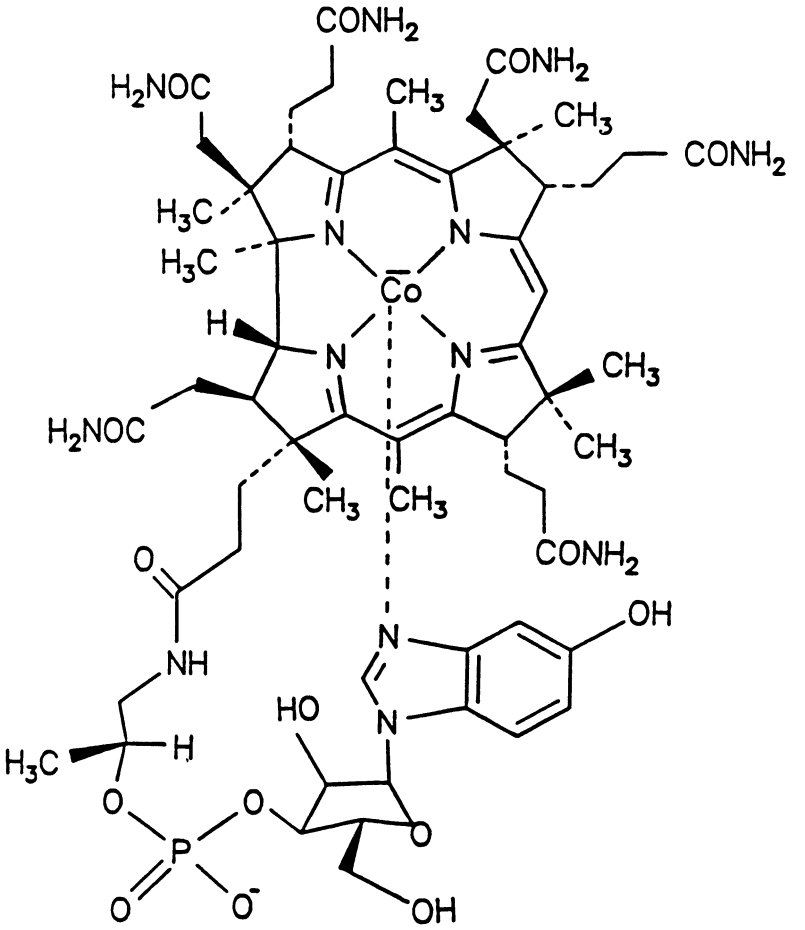


Figure 4.3. Structure of 5-hydroxybenzimidazolyl cobamide, the corrinoid found in methanogenic bacteria (Pol et al., 1982; Kräutler et al., 1987).

4.9.1 Four Enzyme Complexes Involved in Methyl-Coenzyme M Reduction with H_2 to CH_4

The last step in methanogenesis, the reduction of methyl coenzyme M to methane with H_2 (reaction 4.2g), involves four enzymes or enzyme complexes which catalyze the following reactions.

$$\begin{array}{lll} CH_3\text{-S-CoM} + \rightarrow CH_4 + \text{CoM-S-S-HTP} & \Delta G^{\circ\prime} = -45 \text{ kJ/mol} & (4.15a) \\ \quad \text{H-S-HTP} & & \\ H_2 + 2X \rightarrow 2X^- + 2H^+ & & (4.15b) \\ 2X^- + 2Z \rightarrow 2X + 2Z^- & \Delta G^{\circ\prime} = -40 \text{ kJ/mol} & (4.15c) \\ 2Z^- + 2H^+ + \rightarrow \text{H-S-CoM} + \text{H-S-HTP} + 2Z & & \\ \text{CoM-S-S-HTP} & & (4.15d) \end{array}$$

CoM-S-S-HTP is the heterodisulfide of coenzyme M (H-S-CoM) and *N*-7-mercaptoheptanoyl-*O*-phospho-L-threonine (H-S-HTP). X is the still unknown electron acceptor of the coenzyme F_{420}-non-reducing (NiFe) hydrogenase. Its redox potential has been proposed to be near −300 mV (see section 4.2.2). Z is the still unknown electron donor of the heterodisulfide reductase. Its redox potential, $E^{\circ\prime}$, must be near −200 mV since $E^{\circ\prime}$ of the CoM-S-S-HTP/H-S-CoM + H-S-HTP couple is −210 mV (Hedderich and Thauer, 1988). The reduction of Z (−200 mV) by X^- (−300 mV) is thus an exergonic reaction. Evidence is available that the reduction of CoM-S-S-HTP with H_2 is coupled with ADP phosphorylation via a chemiosmotic mechanism (Peinemann et al., 1989 and 1990; Deppenmeier et al., 1991). The electron transport chain between X and Z appears to be the most probable site of energy conservation (see chapter 8).

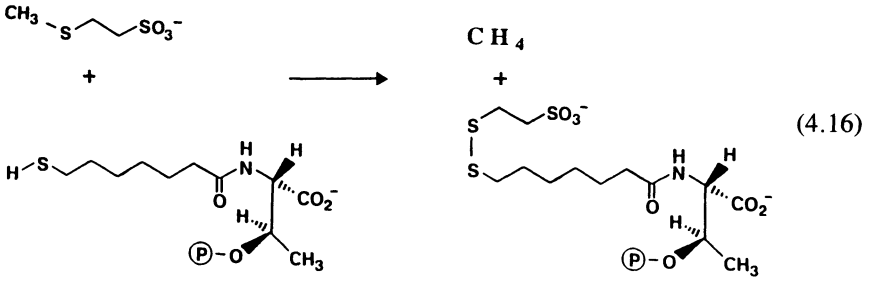
The properties of the coenzyme F_{420} non-reducing hydrogenase, which catalyzes reaction 4.15b, have been described (see Section 4.2.2). The proposed reduced X:oxidized Z oxidoreductase complex, which catalyzes reaction 4.15c, has not yet been identified because of the lack of knowledge of the chemical nature of X and Z. Because the mechanism of electron transport phosphorylation involves vectorial reactions it has been postulated that this enzyme complex is an integral part of the cytoplasmic membrane. The methyl-coenzyme M reductase and the heterodisulfide reductase, which catalyze reactions 4.15a and 4.15d, respectively, have been purified and characterized.

4.9.2 Methyl-Coenzyme M Reductase

(a) Reaction Catalyzed by Methyl-Coenzyme M Reductase

Until recently it was believed that methyl-coenzyme M reductase catalyzes the reduction of methyl-coenzyme M to methane and free coenzyme M. The

physiological electron donor was not known. The role of H-S-HTP, which was absolutely required for enzyme activity, was unclear (Ankel-Fuchs et al., 1987). In 1987 R. Wolfe and coworkers provided evidence that in cell extracts of *Methanobacterium thermoautotrophicum* H-S-HTP was converted to CoM-S-S-HTP when it was added to methyl-coenzyzme M in substrate amounts (Bobik et al., 1987). Ellermann et al. (1998) showed that purified methyl-coenzyme M reductase catalyzes the reduction of methyl-coenzyme M with H-S-HTP to methane and CoM-S-S-HTP in a 1:1:1:1 stoichiometry (reaction 4.15a).



(4.16)

(b) Substrate Specificity and Inhibitors

The methyl-coenzyme M reductase is highly specific for both methyl-coenzyme M and H-S-HTP. From the methyl-coenzyme M analogues tested only ethyl-coenzyme M, selenomethyl-coenzyme M, monofluoromethyl-coenzyme M, and difluoromethyl-coenzyme M are slowly reduced (Wackett et al., 1987), *N*-6-Mercaptohexanoyl-*O*-phospho-L-threonine and *N*-8-mercaptooctanoyl-*O*-phospho-L-threonine cannot substitute for H-S-HTP as electron donor; rather, both compounds act as inhibitors (Ellermann et al., 1988; see also Olson et al., 1992). Dephospho-H-S-HTP is neither active nor inhibitory (unpublished results). The same is true for H-S-HTP synthesized from D-threonine phosphate (Kobelt et al., 1987). Methyl-coenzyme M reductase is effectively inhibited by 2-bromoethanesulfonate ($K_I = 4\ \mu M$), 2-azidoethane sulfonate ($K_I = 1\mu M$), and by 3-bromopropanesulfonate ($K_I = 50$ nM) (Ellermann et al., 1989).

(c) Is H-S-HTP the Naturally Occurring Coenzyme?

Despite the fact that H-S-HTP is a highly specific and effective electron donor for methyl-coenzyme M reduction, evidence became available that the physiological electron donor of methyl-coenzyme M reductase could possibly be a larger molecule, which contains H-S-HTP covalently bound through a mixed

anhydride linkage to a sugar moiety (Sauer et al., 1987; Sauer et al., 1990). This compound, the structure of which is depicted in Figure 4.4, has been isolated from *M. thermoautotrophicum* and has been shown to substitute for H-S-HTP in the methyl-coenzyme M reductase assay. However, it was not significantly more active than H-S-HTP. The specific activity of the cell extracts tested were very low. Because the rate limiting step in the assay used is not known, it is difficult to interpret the results. Therefore, final evidence awaits the determination of the activity with purified active enzyme and the demonstration that the compound is not hydrolyzed to H-S-HTP under the assay conditions by showing the formation of the respective heterodisulfide.

(d) Localization within the Cell

Upon cell breakage the majority of methyl-coenzyme M reductase is recovered in the soluble cell fraction. Immunogold labeling studies revealed that in intact cells the enzyme is preferentially localized at the inner aspect of the cytoplasmic membrane, although this is not evident in all preparations (Ossmer et al., 1986; Aldrich et al., 1987). In *Methanosarcina barkeri* (strain Gö1) the methyl-coenzyme M reductase appears to be part of a larger protein complex associated with the cytoplasmic membrane. The complex has been designated methanoreductosome (Mayer et al., 1988; Hoppert and Mayer, 1990).

(e) Possible Site of Energy Conservation

As already mentioned, the reduction of methyl-coenzyme M with H_2 (reaction 4.2g) *in vivo* is coupled with the phosphorylation of ADP via a chemiosmotic mechanism. At first it was thought that the reduction of methyl-coenzyme M with H-S-HTP ($\Delta G^{\circ\prime} = -45$ kJ/mol) is the site of energy conservation. This idea was abandoned when evidence became available that the reduction of CoM-S-S-HTP with H_2 ($\Delta G^{\circ\prime} = -40$ kJ/mol) is coupled with ADP phosphorylation (Peinemann

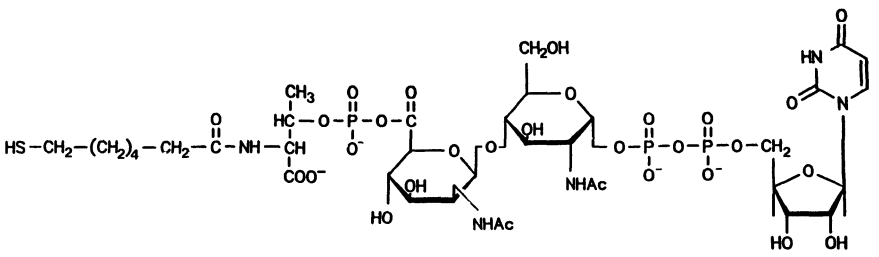


Figure 4.4. Structure of the physiological electron donor for methyl-coenzyme M reductase as proposed by Sauer et al. (1987 and 1990).

et al., 1990; Deppenmeyer et al., 1991). It could be, however, that there are two coupling sites in the conversion of methyl-coenzyme M with H_2 to CH_4 ($\Delta G^{\circ\prime} = -85$ kJ/mol).

(f) Molecular Properties of the Purified Enzyme

Methyl-coenzyme M reductase has been purified from *M. thermoautotrophicum* strain ΔH (Ellefson and Wolfe, 1981; Hartzell and Wolfe, 1986) and strain Marburg (Ankel-Fuchs and Thauer, 1986; Ellermann et al., 1988 and 1989), from *Methanosarcina barkeri* (Moura et al., 1983; Hoppert and Mayer, 1990), *Methanosarcina thermophilia* (Jablonski and Ferry, 1991), *Methanothrix soehngenii* (Jetten et al., 1990), *Methanopyrus kandleri* (Rospert et al., 1991a), and *Methanococcus voltae* (Konheiser et al., 1984). The native enzyme has an apparent molecular mass of approximately 300 kDa. It is composed of three different subunits, α, β, and γ, in an $\alpha_2\beta_2\gamma_2$ arrangement. The enzyme contains 2 mol of tightly but not covalently bound coenzyme F_{430} per mol (Ellefson and Wolfe, 1981), which is a nickel porphinoid. Its structure is shown in Figure 4.5 (for reviews see Friedmann et al., 1990 and 1991). The enzyme also appears to

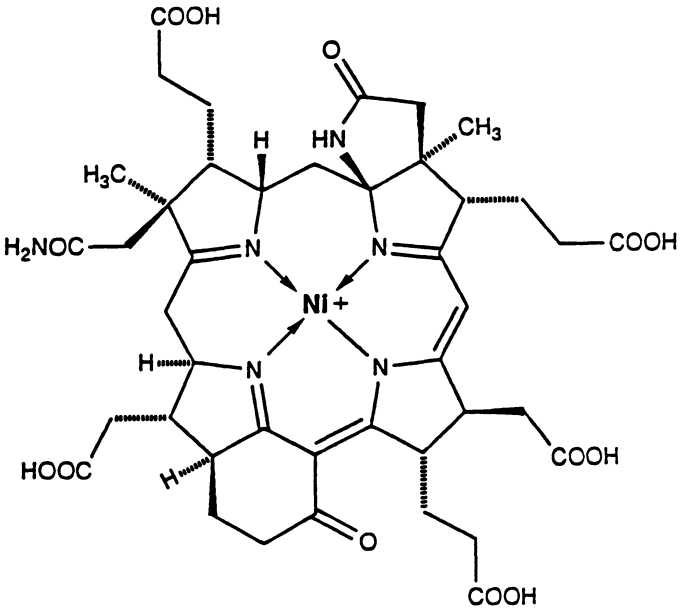


Figure 4.5. Structure of coenzyme F_{430} (Pfaltz et al., 1982; Livingston et al., 1984; Pfaltz et al., 1985; Fässler et al., 1985; Färber et al., 1991).

contain 2 mol of H-S-CoM (Hartzell et al., 1987) and 2 mol H-S-HTP (Noll and Wolfe, 1986).

(g) Enzyme Activity

In cell extracts the methyl-coenzyme M reductase is generally more or less inactive. The enzyme can be activated to a few percent of the *in vivo* activity in an H_2- and ATP-dependent enzyme catalyzed reaction. The enzymes involved in the activation have been partially purified. The mechanism of activation is not yet understood (see Rouvière et al., 1988; Rouvière and Wolfe, 1989). Indirect evidence suggests that activation occurs by reduction of the protein-bound coenzyme F_{430} to the Ni(I) state (Albracht et al., 1986 and 1988; Olson et al., 1991).

Rospert et al. (1991b) recently obtained cell extracts containing a high specific activity methyl-coenzyme M reductase, and partially purified the enzyme in the active form. The enzyme exhibited a strong EPR signal assigned to Ni(I)F_{430} by comparison with the EPR spectrum of non-enzyme-bound Ni(I)F_{430}. It was shown that the specific activity of methyl-coenzyme M reductase correlated with this EPR signal: after the addition of chloroform, which is known to be a potent inhibitor of methanogenesis and to rapidly oxidize the Ni(I)F_{430} to the Ni(II)-form (Krone et al., 1989), the activity of methyl-coenzyme M reductase and the nickel EPR signal decreased in parallel. These findings strongly suggest that active methyl-coenzyme M reductase contains coenzyme F_{430} in the Ni(I) oxidation state.

(h) The Catalytic Mechanism

In order to understand the role of coenzyme F_{430} in the catalytic mechanism, it is probably important that coenzyme F_{430} in the reduced Ni(I) form can be reductively methylated yielding methyl-Ni(II)F_{430} (Lin and Jaun, 1991). This metalloorganic compound is subject to rapid protonolysis to methane and Ni(II)F_{430} (Jaun and Pfaltz, 1988; Lin and Jaun, 1991).

$$\text{Ni(II)}F_{430} + CH_3\text{-X} + 2e^- \rightarrow [CH_3\text{—Ni(II)}F_{430}]^- + X^- \qquad (4.17a)$$

$$[CH_3\text{—Ni(II)}F_{430}]^- + H^+ \rightarrow CH_4 + \text{Ni(II)}F_{430} \qquad (4.17b)$$

Recently it was found that the enzyme-catalyzed reduction of methyl-coenzyme M with H-S-HTP proceeds via inversion of stereoconfiguration (Ahn et al., 1991). This finding is consistent with a methyl-Ni(II)F_{430} as an enzyme-bound intermediate; methylation of Ni(I)F_{430} with CH_3-S-CoM is thought to proceed by means of inversion and subsequent protonolysis with retention of stereoconfiguration.

The redox potential, $E^{\circ\prime}$, of the Ni(II)F_{430}/Ni(I)F_{430} couple is more negative

than −500 mV (Jaun and Pfaltz, 1986; Jaun, 1990). This could explain why it is difficult to activate the enzyme once the coenzyme F_{430} is in the oxidized form.

It has been suggested that H-S-HTP could reduce the enzyme bound coenzyme F_{430} in a one-electron redox reaction. Based on this proposal a catalytic mechanism from methyl-coenzyme M reductase has been proposed which is depicted in Figure 4.6 (Berkessel, 1991; Jaun, 1990 and 1993).

(i) Molecular Biology

The genes encoding the three subunits of methyl-coenzyme M reductase have been cloned and sequenced from *M. thermoautotrophicum* (strain Marburg),

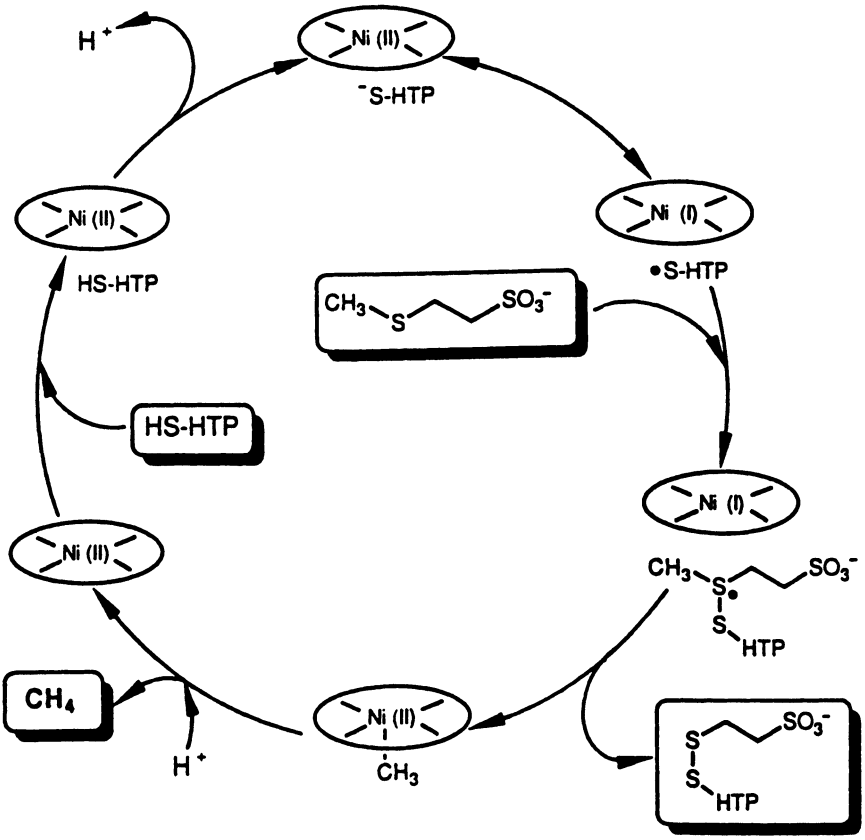


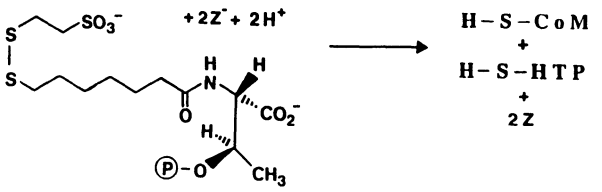
Figure 4.6. Catalytic mechanism of methyl-coenzyme M reductase as proposed by Berkessel (1991). The figure was kindly provided by A. Berkessel.

Methanococcus vannielii, M. voltae, Methanothermus fervidus, and *M. barkeri* (Allmansberger et al., 1989; Weil et al., 1989) (see chapter 12). In all of these organisms the three genes were found to comprise a single transcriptional unit, which additionally contains two open reading frames encoding two small polypeptides of molecular masses below 20 kDa. The two polypeptides, which can be detected in cell extracts (Sherf and Reeve, 1990), appear to be absent in purified active methyl-coenzyme M reductase preparations (Ellermann et al., 1989; Rospert et al., 1991b). This indicates that they have no apparent function in the catalytic cycle.

M. thermoautotrophicum and *Methanobacterium wolfei* have been shown to contain two genetically distinct isozymes of methyl-coenzyme M reductase (Rospert et al., 1990). The expression of the two isozymes is differentially regulated by the growth conditions (Bonacker et al., 1992).

4.9.3 Heterodisulfide Reductase

Heterodisulfide reductase catalyzes the reduction of CoM-S-S-HTP with reduced viologen dyes as electron donor, and the oxidation of H-S-HTP and H-S-CoM to CoM-S-S-HTP with methylene blue as electron acceptor (Hedderich et al., 1989). The physiological electron donor Z is not known:



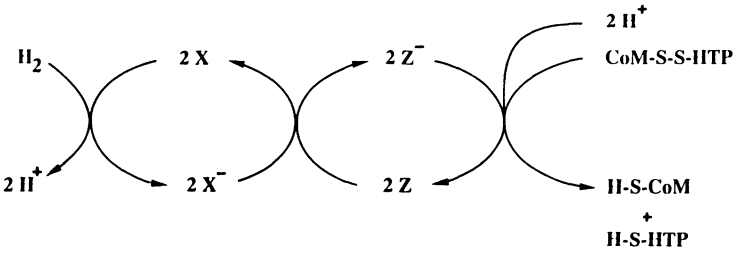
(4.18)

Heterodisulfide reductase is associated with the membrane fraction. In *Methanobacterium thermoautotrophicum* the enzyme is less hydrophobic than in *Methanosarcina barkeri*. It has been purified from these two organisms. Heterodisulfide reductase from *M. thermoautotrophicum* in the native form has an apparent molecular mass of 550 kDa and is composed of three different subunits of apparent molecular mass of 80 kDa, 36 kDa, and 21 kDa, respectively. The enzyme contains 4 mol FAD and 72 mol non-heme iron and the same amount of acid-labile sulfur per $\alpha_4\beta_4\gamma_4$ polymer (Hedderich et al., 1990).

The as-isolated enzyme from *M. barkeri* is composed of three subunits of apparent molecular mass of 45 kDa, 26 kDa, and 24 kDa, respectively. The preparation also contains cytochrome b in addition to FAD and iron-sulfur clusters (Thauer et al., unpublished results). It thus appears that the electron transport

chain from H_2 to CoM-S-S-HTP in *M. barkeri* differs from that in *M. thermoautotrophicum*, which, like all methanogens growing solely on H_2 and CO_2, lacks cytochromes (Kühn et al., 1983).

As outlined above, the electron transport chain from H_2 to CoM-S-S-HTP theoretically involves three partial reactions: (i) the reduction of X with H_2, (ii) the reduction of Z with reduced X, and (iii) the reduction of CoM-S-S-HTP with reduced Z.



For *M. thermoautotrophicum* it has been shown that heterodisulfide reductase and F_{420}-non-reducing hydrogenase form a tightly bound complex (Hedderich et al., 1990; Kolodziej et al., 1992). The two enzymes are each composed of three subunits, one or two of which are most likely iron-sulfur proteins. One of the subunits of heterodisulfide reductase from *M. barkeri* is most likely a cytochrome b. It is therefore postulated that X and Z are not separate entities, but subunits of the hydrogenase and heterodisulfide reductase, respectively.

4.10 Coupling of CO_2 Reduction with H_2 and of CoM-S-S-HTP Reduction with H_2: The RPG Effect

Cell extracts of *Methanobacterium thermoautotrophicum* (strain ΔH) have been shown to catalyze the reduction of CO_2 with H_2 to formylmethanofuran only in the presence of CoM-S-S-HTP, which is simultaneously reduced to H-S-CoM and H-S-HTP. Almost all of the methanofuran added to the extract is converted to formylmethanofuran despite the fact that the reduction of CO_2 to formyl-MFR with H_2 is an endergonic reaction at the H_2 and CO_2 concentrations employed (Bobik and Wolfe, 1988 and 1989a). This finding indicates that the endergonic reduction of CO_2 with H_2 to formyl-MFR and the exergonic reduction of CoM-S-S-HTP with H_2 in the cell extracts are energetically coupled.

$$H_2 + CO_2 + MFR \rightarrow CHO\text{-}MFR + H_2O, \quad \Delta G^{\circ\prime} = +16 \text{ kJ/mol} \quad (4.2a)$$
$$H_2 + CoM\text{-}S\text{-}S\text{-}HTP \rightarrow H\text{-}S\text{-}CoM + H\text{-}S\text{-}HTP, \quad \Delta G^{\circ\prime} = -40 \text{ kJ/mol} \quad (4.15d)$$

The interpretation of these results also provides an explanation for the so called RPG effect. In 1977 *R. P. G*unsalus observed that cell extracts of *M. thermoautotrophicum* catalyzed the reduction of CO_2 to methane only when catalytic amounts of CH_3-S-CoM or other compounds were added which were later shown to regenerate CoM-S-S-HTP (Gunsalus and Wolfe, 1977; Romesser and Wolfe, 1982; Bobik and Wolfe, 1989b).

Direct experimental evidence for an energetic coupling of the first and the last step of methanogenesis from CO_2 and H_2 in cell extracts is not available. In order to invoke chemiosmotic coupling, extracts exhibiting the RPG effect would have to contain inverted membrane vesicles. This is not unlikely since the cell extracts were only subjected to centrifugation at 30,000 × *g* which, at the high protein concentrations employed (32 mg/ml), is not sufficient to sediment the membrane fraction. Because the rates of methanogenesis from CO_2 and H_2 obtained in cell extracts were only 1% of the *in vivo* rates, only a few vesicles would have been sufficient to allow a chemiosmotic coupling.

Acknowledgments

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5

Conversion of Methanol and Methylamines to Methane and Carbon Dioxide

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5.1 Introduction

5.1.1 Methylotrophic Methanogenic Bacteria

The first report on methane formation from a methylated one-carbon compound, notably methanol, goes back to 1920 (Groenewegen, 1920). In the thirties, methylotrophic methanogens were systematically studied in the laboratory of Kluyver and Van Niel (1936). Here, Barker (1936) enriched an organism, then called *Methanococcus mazei,* which was capable of growth not only on methanol, but also on butanol and acetone. The organism was not pure and the original cultures were lost. Only about 40 years later, the methanogen that met the original description was reisolated and renamed *Methanosarcina mazei* (Mah, 1980; Mah and Kuhn, 1984). The first methylotroph obtained in axenic culture, and in fact one of the first pure methanogenic species, was isolated by Schnellen (1936), a student of Kluyver. Again, the original cultures of the organism, *Methanosarcina barkeri,* were lost. *M. barkeri* has been reisolated as a number of distinct strains from a variety of sources. The type strain, MS, was obtained by Bryant in 1966 (Bryant, 1966; Bryant and Boone, 1987). Biochemically, *M. barkeri* is the best studied methylotrophic methanogen and most of the work reviewed in this chapter refers to it.

Methylotrophy, defined as the capability to grow on methylated compounds, is displayed by about 20 species of methanogens (see for recent taxonomic overviews: Jain et al., 1988; Whitman, 1985; Garcia, 1990; Boone et al., this volume). A survey of the methylotrophs and some of their characteristics is presented in Table 5.1. They include a vacuolated methanogen, *Methanosarcina vacuolata* (Zhilina and Zavarzin, 1987a), and two thermophiles, *Methanosarcina*

Table 5.1. Methylotrophic methanogenic bacteria

Organism (type strain)	*Source*	*[NaCl] (optimum) (M)*
Family *Methanobacteriaceae*		
Methanosphaera cuniculi (1R7)	Rabbit rectum	
Methanosphaera stadtmanae (MCB-3)	Human feces	
Family *Methanococcaceae*		
Methanococcus halophilus (Z-7982)	Cyanobacterial mats	0.26–2.6(1.2)
Family *Methanosarcinaceae*		
Methanosarcina acetivorans (C2A)	Marine sediment	0.2
Methanosarcina alcaliphilum		
Methanosarcina barkeri (MS)	Freshwater, sewage digestors, marine mud, lagoons, rumen	
Methanosarcina frisia (DSM 3316)	Marine sediment	0.3–0.7
Methanosarcina mazei (S-6)	Sludge, soil	
Methanosarcina thermophila (TM-1)	Anaerobic digestor	
Methanosarcina vacuolata (Z-761)	Sewage digestor, soil	
Methanolobus siciliae (T4/M)	Marine sediment, oil well	0.4–0.6
Methanolobus tindarius (T3)	Marine mud	0.06–1.27 (0.5)
Methanolobus vulcani (PL-12/M)	Marine mud, oil well	
Methanococcoides euhalobius (283)	Oil deposit	0.18–2.4 (1.0)
Methanococcoides methylutens (TMA-10)	Marine sediment	0.2–0.6 (0.4)
Methanohalophilus mahii (SLP)	Great Salt Lake	1.0–2.5 (2.0)
Methanohalophilus oregonense (WAL1)	Saline aquifer	0.1–1.4 (0.35)
Methanohalophilus zhilinae (WeN5)	Saline lake	0.2–2.1 (0.7)
Methanohalobium evestigatus (Z-7303)	Salt lagoon	2.6–5.2 (4.3)
Halomethanococcus alcaliphilum		
Halomethanococcus doii (IY-1)	Solar salt pond	1.8–3.6 (3.0)

Continued

Table 5.1 *Continued*

	Substrate use[a]					
Organism	*MeOH*	*MA*	*DMS*	H_2/CO_2	Ac^-	*Literature*
M. cuniculi	+ (+H_2)	−		−	−	Biavati et al., 1988
M. stadtmanae	+ (+H_2)	−		−	−	Miller and Wolin, 1985
M. halophilus	+	+		−	(+)	Zhilina, 1983
M. acetivorans	+	+		−	+	Sowers, Johnson, et al., 1984
M. alcaliphilum						Nakatsugawa and Horikoshi, 1989a
M. barkeri	+	+	−	+	+	Bryant and Boone, 1987
M. frisia	+	+		+	−	Blotevogel and Fischer, 1989, Blotevogel and Macario, 1989
M. mazei	+	+	−	+/−	+/−	Mah and Kuhn, 1984 Liu et al.,1985
M. thermophila	+	+	−	+/−	+	Zinder and Elias, 1985
M. vacuolata	+	+		+	+	Zhilina and Zavarzin, 1987a
M. siciliae	+	+	+	−	−	Stetter, 1989; Ni and Boone, 1991
M. tindarius	+	+		−	−	König and Stetter, 1982
M. vulcani	+	+		−	−	Stetter, 1989
M. euhalobium	+	+		−	−	Obraztsova et al., 1987
M. methylutens	+	+		−	−	Sowers and Ferry, 1983
M. mahii	+	+		−	−	Paterek and Smith, 1985 1988
M. oregonense	+	+	+	−	−	Liu et al., 1990
M. zhilinae	+	+	+	−	−	Mathrani et al., 1988
M. evestigatus	+	+		−	−	Zhilina and Zavarzin, 1987b
H. alcaliphilum						Nakatsugawa and Horikoshi,1989b
H. doii	+	+		−	−	Yu and Kawamura, 1987

[a]Abbreviations: MeOH, methanol; MA, methylamines; DMS, dimethylsulfide; Ac^-, acetate; +/−, strain-dependent.

thermophila (Zinder et al., 1985) and *Methanohalobium evestigatus* (Zhilina and Zavarzin, 1987b). A substantial number of the more recent isolates have been obtained from marine sediments and salt lakes; they are halotolerant or moderately to extremely halophilic (Table 5.1). These habitats usually contain high sulfate concentrations, and here hydrogenotrophic methanogens are outcompeted by sulfate-reducing bacteria. Owing to their ability to grow on methanol and methylamines derived from decaying plants, algal mats, and fish, the methylotrophs have gained their ecological niche (King, 1984).

Methylotrophic methanogens comprise eight different genera, all of which, with two exceptions, belong to the family of the *Methanosarcinaceae* (Balch et al., 1979; Sowers et al., 1984a). The exceptions concern *Methanosphaera,* represented by *M. stadtmanae* (Miller and Wolin, 1983, 1985) and *M. cuniculi* (Biavati et al., 1988), and *Methanococcus halophilus* (Zhilina, 1983). *Methanosphaera* is a member of the *Methanobacteriaceae* and is otherwise exceptional since it is only capable of growth on methanol with hydrogen as electron donor. The taxonomic position of *M. halophilus* in the *Methanococcaceae* is not clear (Jain et al., 1988) and it may need transfer to either *Methanosarcina* (Blotevogel and Fischer, 1989) or *Methanohalobium* (Garcia, 1990). In addition, the moderately halophilic genera *Methanohalobium* and *Halomethanococcus* could be subjective synonyms of *Methanohalophilus* (Liu et al., 1990).

5.1.2 Substrates Used by Methylotrophic Methanogenic Microbes

All methylotrophs are capable of deriving their energy for growth from the conversion of methanol into methane and CO_2 (Tables 5.1 and 5.2, Equation 1.1). Hippe et al. (1977) were the first to isolate a methanogen, *M. barkeri* strain Fusaro, that used mono-, di-, and trimethylamine (Equations 3–5, in Table 5.2). The *N*-methyl compounds were subsequently shown to be common substrates (Tables 5.1). Conversion of acetate, a methylated two-carbon compound (Equation 10, in Table 5.2), is restricted to the few *Methanosarcina* species listed in Table 5.1, and a number of poorly classified thermophilic strains that may be closely related to *M. thermophila* (Touzel and Albagnac, 1984; Touzel et al., 1985; Zinder and Mah, 1984; Ollivier et al, 1984). The *Methanosarcina* species tend to grow faster on acetate than specialists like *Methanothrix* (*Methanosaeta*). Obligate acetotrophs, however, show a high affinity for their substrate and can metabolize acetate to levels that are 10- to 100-fold lower than *Methanosarcina* can metabolize (Min and Zinder, 1989; Zinder, 1990; Clarens and Moletta, 1990; Jetten et al., 1990).

Growth on hydrogen and CO_2 (Equation 9, in Table 5.2) is limited to some *Methanosarcina* species, in which the potential to use CO_2 as the electron acceptor may vary among different strains of the same species (Table 5.1). *Methano-*

Table 5.2. Energy-yielding reactions utilized by methylotrophic methanogens

Reaction	$\Delta G^{\circ\prime}$ *(kJ/mol CH_4)*
1. $4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$	−106
2. $CH_3OH + H_2 \rightarrow CH_4 + H_2O$	−112.5
3. $4\ CH_3NH_2 + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 4\ NH_3$	−76.7
4. $2\ (CH_3)_2NH + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 2\ NH_3$	−74.8
5. $4\ (CH_3)_3N + 6\ H_2O \rightarrow 9\ CH_4 + 3\ CO_2 + 4\ NH_3$	−75.8
6. $2\ (CH_3)_2S + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 2\ H_2S$	−52.1
7. $4\ (CH_3)SH + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 4\ H_2S$	−51
8. $(CH_3)SH + H_2 \rightarrow CH_4 + H_2S$	−69.3
9. $4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$	−130.4
10. $CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$	−36.0
11. $4\ CO + 2\ H_2O \rightarrow CH_4 + 3\ CO_2$	−211

sphaera, however, is obligately hydrogenotrophic: hydrogen is strictly required to reduce methanol to methane (Equation 2 in Table 5.2). CO_2 and acetate are provided in the medium as a source of cell carbon (Miller and Wolin, 1985). In fact, *M. barkeri* is also able to use H_2 and methanol substrates for growth (Kenealy and Zeikus, 1982; Müller et al., 1986). Since methanol is exclusively reduced to methane, growth only occurs when acetate is present as a cell carbon source. Typically, none of the methylotrophic methanogens listed in Table 5.1 are capable of growth on formate, a substrate used by about 40% of the methanogenic species. Methylotrophs apparently lack active formate dehydrogenase. *M. barkeri* may grow, albeit very slowly, with carbon monoxide as the sole carbon and energy source (Equation 11 in Table 5.2), or mixotrophically with CO and methanol (Zeikus, 1983; O'Brien et al., 1984). CO presumably does not represent a physiological substrate and is metabolized as an alternate substrate of acetyl-CoA synthetase (CO dehydrogenase). Carbon monoxide is converted into CO_2 and hydrogen, which are intermediates that accumulate in the gas phase.

Dimethyl sulfide (DMS) was only recently recognized as an energy and growth substrate for methylotrophs (Kiene et al., 1986; Oremland et al., 1989) (Equation 6 in Table 5.2). Apart from the coccoid strain GS-16 isolated by the authors, DMS is "noncompetitively" metabolized by *Methanohalophilus zhilinae, Methanohalophilus oregonense,* and *Methanolobus siciliae* (Mathrani et al., 1988; Liu et al., 1990; Ni and Boone, 1991). A number of *Methanosarcina* species tested were incapable of using the compound (Oremland et al., 1989) (Table 5.1). Hence, DMS conversion seems to be restricted to halophilic obligate methylotrophs. DMS-metabolizing cells produced methanethiol as a transient intermediate (Kiene et al., 1986; Oremland et al., 1989; Ni and Boone, 1991). Apparently,

methanethiol is also used by the organisms. However, growth supported by methane formation from methanethiol alone or in combination with hydrogen (Equations 7 and 8 in Table 5.2), has not been demonstrated.

5.1.3 Methanogenesis from methylated one-carbon compounds: an outline.

Methylotrophic methanogens use methanol as the common substrate. A schematic view, which incorporates our present biochemical knowledge of the process, is shown in Figure 5.1. Methanogenesis from methylamines and methylsulfides has hardly been investigated at a biochemical level. Available evidence, which will be surveyed in Section 5.3, suggests that the substrates are metabolized by some modification of the methanol pathway.

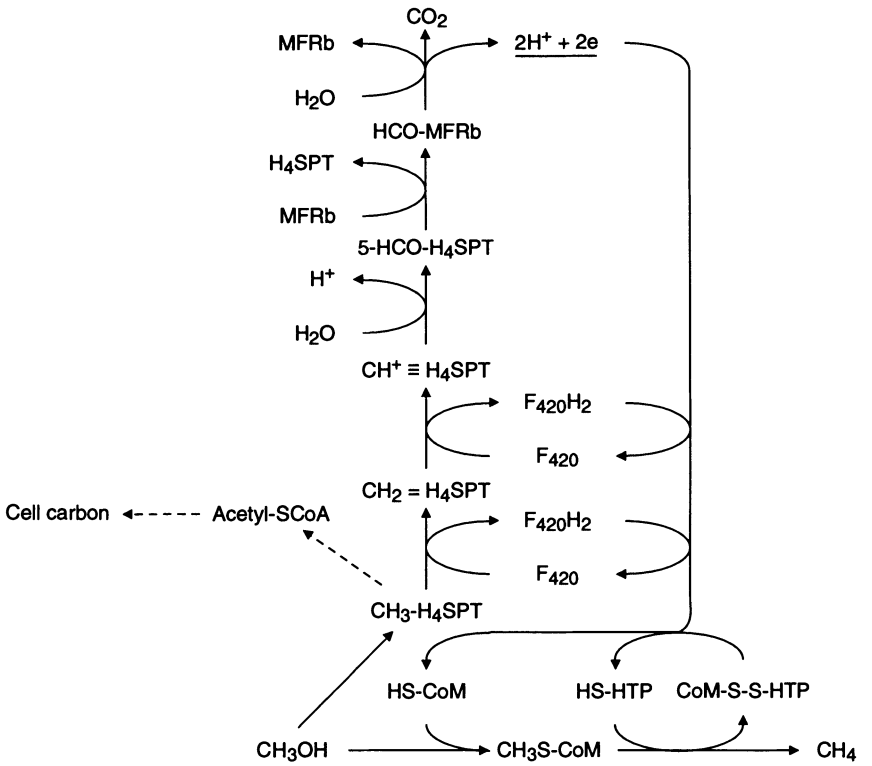

Figure 5.1. The pathway of methanol conversion to methane and CO_2. One-carbon carriers: HS-CoM, coenzyme M; H_4SPT, 5,6,7,8-tetrahydrosarcinapterin; MFRb, methanofuran-b. Electron carriers: F_{420}, coenzyme F_{420}; HS-HTP, 7-mercatoheptanoyl-L-threonine phosphate.

Methanol conversion involves two different routes (Figure 5.1): the reduction of the substrate to methane and concomitant methanol oxidation to CO_2 according to the following overall reactions:

$$CH_3OH + 6\,[H] \rightarrow 3\,CH_4 + 3\,H_2O \qquad (5.1a)$$

$$CH_3OH + H_2O \rightarrow CO_2 + 6\,[H] \qquad (5.1b)$$

$$\text{net: } 4\,CH_3OH \rightarrow 3\,CH_4 + CO_2 + 2\,H_2O \qquad (5.1)$$

As described in detail in Section 5.2.1, methanol reduction to methane (reaction 5.1a) occurs by the transfer of the methyl group to coenzyme M (HS-CoM). Reductive demethylation of methylcoenzyme M (CH_3S-CoM) with 7-mercaptoheptanoyl-L-threonine phosphate (HS-HTP) as the reductant produces methane and the heterodisulfide (CoM-S-S-HTP) of HS-CoM and HS-HTP. In order to replenish the latter compounds, CoM-S-S-HTP has to be reduced with electrons derived from methanol oxidation (reaction 5.1b). Methanol oxidation, which is the subject of Section 5.2.2, generally proceeds by the same sequence of reactions as CO_2 reduction (see Chapter 4), albeit in the reverse order and with the participation of slightly modified one-carbon and electron carriers. The oxidative pathway is, in more than one respect, of crucial importance in methylotrophic metabolism; it provides the cell with 5-methyl-tetrahydrosarcinapterin (5-methyl-H_4SPT) and CO_2 required for the synthesis of acetyl-CoA, the starting point for autotrophic cell carbon synthesis (see Part III, Chapter 10, this volume). Next, reducing equivalents are produced for anabolic reduction reactions and for the reduction of CoM-S-S-HTP to HS-CoM and HS-HTP described in Section 5.2.3. The latter reaction is chemiosmotically coupled to proton extrusion, which drives the phosphorylation of ADP by means of a proton-translocating ATPase (for reviews see Blaut and Gottschalk, 1985; Blaut et al., 1990; Thauer, 1990; Chapter 8, this volume). Finally, methanol oxidation is connected with electrogenic events, notably sodium transport. In whole cells the initial step in methanol oxidation is driven by a sodium-motive force [Section 5.2.2(a)]. The sodium-motive force is created either by secondary Na^+ export connected to the methane-forming reactions or by primary sodium export coupled to some reaction in the terminal steps of methanol oxidation (Kaesler and Schönheit, 1989 a,b). A more detailed treatment of the chemiosmotic processes is given by Müller et al. (Chapter 8, this volume).

Methylotrophs like *M. barkeri* are capable of methane formation from a variety of substrates or combinations of substrates. Substrate conversion proceeds by closely interconnected pathways. It is to be expected that substrate use is subject to some metabolic regulation and we shall return to this aspect in our last section (5.4).

5.2 Methanogenesis from Methanol

5.2.1 The Route of Methanol Reduction to Methane

The first biochemical studies on methanol conversion to methane were conducted in the sixties by Blaylock and Stadtman (Blaylock, 1968; Blaylock and Stadtman, 1963, 1964, 1966). These authors demonstrated that cell extracts of *M. barkeri* could reductively cleave methylcobalamin to methane. Methylcobalamin was formed in the extracts from methanol and electrochemically prepared Cob(I)alamin. The role of corrinoids in methane-forming processes, however, was clouded by the discovery of HS-CoM as the methyl carrier in the terminal step of methanogenesis (Taylor and Wolfe, 1974). Moreover, the observed lack of inhibition by propyliodide during methanogenesis from methanol seemed to disqualify a role for B_{12} derivatives in the reactions (Shapiro, 1982). Presently, the role of corrinoids in methanol conversion is fully appreciated, as shown in the next section. Section 5.2.1(b) then deals with CH_3S-CoM reduction in methylotrophs.

(a) The Conversion of Methanol to CH_3S-CoM

As mentioned above, Blaylock and Stadtman were able to methylate free cobalamin with methanol. The reaction is complex and requires, in addition to ATP and hydrogen, four components: a high-molecular-weight corrinoid protein, a protein of unknown function, ferredoxin, and a heat- and acid-stable dialyzable cofactor. CH_3S-CoM, rather than (free) methylcobalamin, was later found to be an intermediate in methanol conversion (Shapiro and Wolfe, 1980; Hutten et al., 1981). The enzyme system which catalyzes the methylation of HS-CoM by methanol has been investigated by Van der Meijden et al. (1983a,b,c; 1984 a,b); these results are schematically represented in Figure 5.2. CH_3S-CoM synthesis proceeds in two subsequent enzymic reactions. In the first reaction, catalyzed by methanol:5-hydroxybenzimidazolyl methyltransferase (MT1), the methyl group from methanol is bound to the corrinoid prosthetic groups of MT1. Next, methylcobalamin:HS-CoM methyltransferase (MT2) transfers the methyl group to HS-CoM. The two-step reaction implies that CH_3S-CoM formation from methanol should occur with a net retention of methyl group configuration and without significant racemization. This was, indeed, found to be the case (Zydowski et al., 1987).

MT1 has been purified from *M. barkeri* by a factor of 5.7, as determined from the increase of the specific corrinoid content (Van der Meijden et al., 1984a). Nevertheless, the enzyme preparation was over 90% pure, which indicates MT1 comprises about 15% of the soluble protein in the organism. Purification required strict anoxic conditions and, even then, major loss in activity occurred. Native

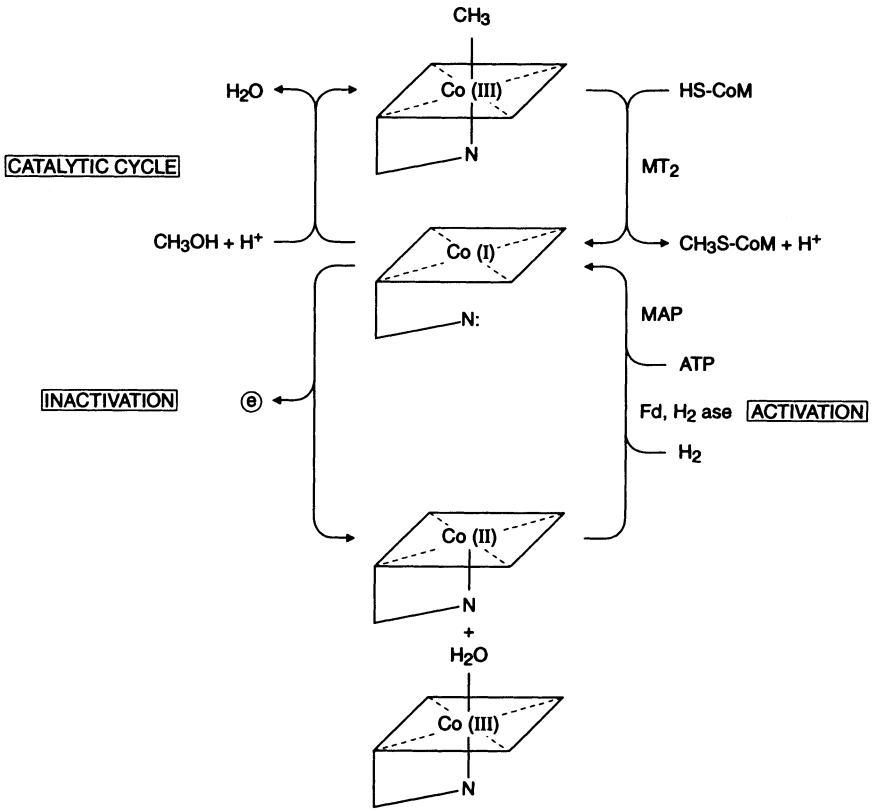

Figure 5.2. The methanol:HS-CoM methyltransferase system. Methanol: 5-hydroxybenzimidazolylcobamide methyltransferase (MT1) is schematically represented by its corrinoid prosthetic group. Other abbreviations: N, 5-hydroxybenzimidazol; MT_2, methylcobamide: HS-CoM methyltransferase; MAP, methyltransferase activating protein; Fd, ferredoxin; H_2ase, hydrogenase; e, electron.

MT1 showed an apparent molecular mass of 122 kDa and contained two different subunits of 34 kDa and 53 kDa, respectively, consistent with an $\alpha_2\beta$ configuration. As isolated, 3.4 mol corrinoid were present per 122 kDa trimer, conferring an orange-brown color to the enzyme. The corrinoid was identified as Factor III (B_{12}HBI), a cobamide with the nitrogeneous base 5-hydroxybenzimidazole as the lower (α) ligand (Figure 5.3); B_{12}HBI is the characteristic B_{12} derivative found in most methanogenic microbes (Pol et al., 1982; Stupperich and Kräutler, 1988). The prosthetic group is tightly, but *not* covalently, bound to the holoenzyme, and the component becomes dissociated after treatment with SDS and 2-mercaptoetha-

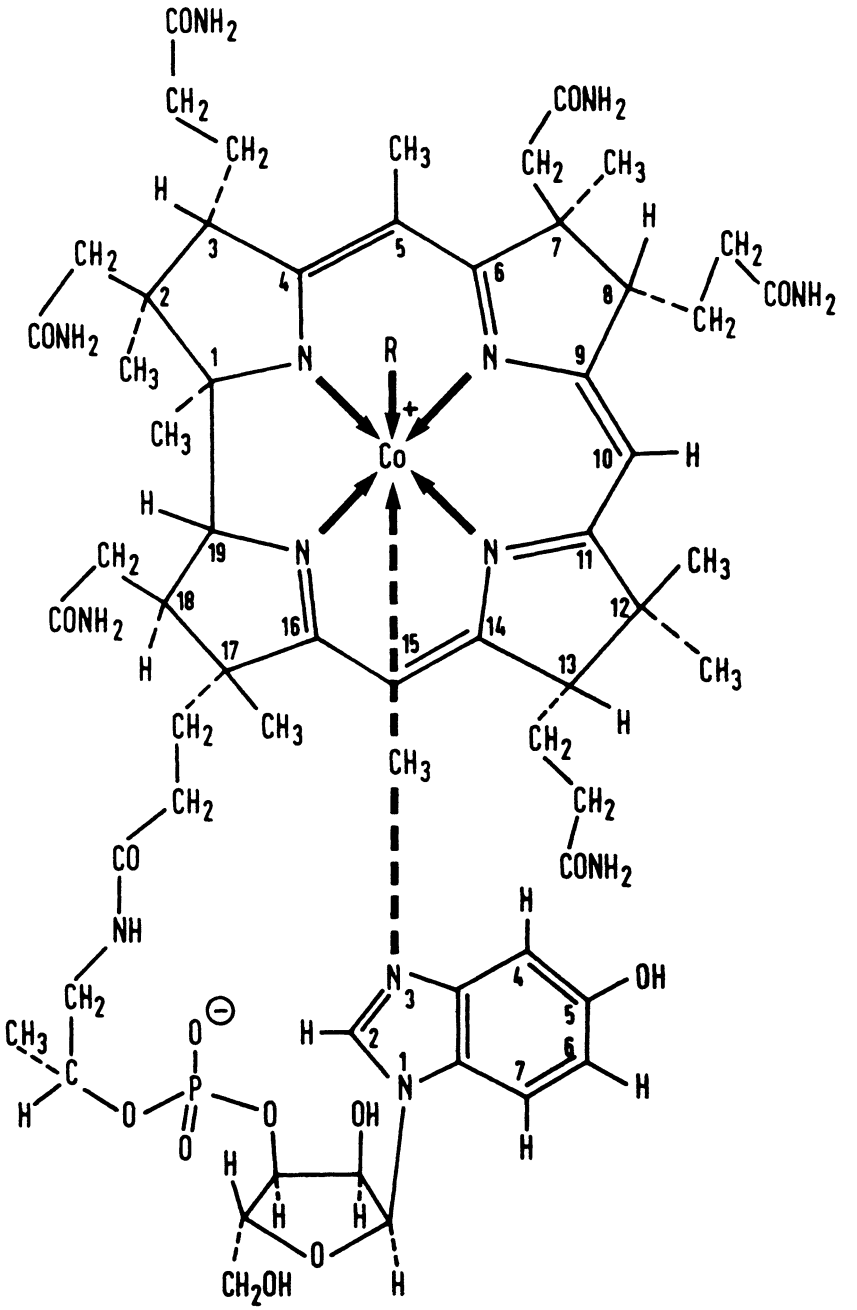

Figure 5.3. Structure of 5-hydroxybenzimidazolylcobamide (B_{12}HBI).

nol. Therefore, it was not possible to establish by means of SDS gel electrophoresis to which subunit B_{12}HBI was bound (Van der Meijden et al., 1984a).

MT2 has been purified 86-fold to apparent homogeneity (Van der Meijden et al., 1983a). Gel chromatography of the native protein and denaturing SDS electrophoresis indicated that MT2 was composed of a single polypeptide of about 40 kDa. Though free methylcobamide is not an intermediate in the CH_3S-CoM synthesis, MT2 uses methylcobalamin with high specific activity as an artificial substrate. By this property the enzyme may be assayed independently from the other components of the enzyme system. In fact, *M. barkeri* contains two different MT2 isozymes of essentially the same size (34 kDa) (Grahame, 1989). The isozymes can be distinguished on the basis of slightly different electrophoretic mobilities, retention during hydroxyl appatite chromatography, and cross-reactivity with specific antibodies. Interestingly, one type of MT2 was predominantly (89% of total activity) present after growth of *M. barkeri* on methanol, whereas the other type constituted 60 to 80% of the overall MT2 activity after growth on acetate (Grahame, 1989). This would suggest that both isozymes take part in two different reactions.

In a resolved system, MT1 and MT2 alone are not sufficient to bring about methyl group transfer from methanol to HS-CoM. This has to do with the specific properties of the catalytic center of MT1, B_{12}HBI. Corrinoids are exquisitely designed to mediate transmethylation with high velocity (Kräutler, 1987, 1990; Matthews et al., 1990). This, however, holds only when the central cobalt atom is kept in the highly-reduced Co(I) state. In this oxidation state the cobamide exists as a "base-off" (distorted) square planar complex (Figure 5.2). Cobalt then has the chemical properties of a "supernucleophile" capable of accepting a CH_3^+ from the methylated substrate. The methyl-Co bond is stabilized by nucleotide coordination resulting in an oxygen-stable, though light-sensitive, hexacoordinate ("base-on") complex with cobalt in the formal Co(III) state. Spectral changes that occurred during reaction of MT1 with methanol, indeed, were in agreement with "base-on" methyl-B_{12}HBI formation (Van der Meijden et al., 1983c, 1984b). When extracted under the appropriate conditions, up to 80% of the total cobamide in *M. barkeri* consists of methyl-B_{12}HBI (Höllriegl et al., 1983).

In the presence of mild oxidants (traces of oxygen, dithiols, oxidized flavins or viologen dyes) Co(I) will be oxidized to pentacoordinate Co(II)-corrin and hexacoordinate Co(III)-corrin, and MT1 becomes catalytically incompetent. This at least partly explains inactivation of the enzyme during its purification. MT1, however, may be reductively reactivated by an enzyme system present in *M. barkeri* (Van der Meijden et al., 1983b,c;1984a,b). Electrons required for the activation are provided by hydrogen, hydrogenase, and ferredoxin (Figure 5.2); both the F_{420}-dependent and the viologen-reducing hydrogenase may serve to oxidize hydrogen (P. Daas, unpublished results). At pH 7 the reduction potential of the Co(I)/Co(II) couple in free cobamide is -640 mV (Lexa and Saveant,

1983), which is far below that of the H_2/H^+ couple ($E^{\circ\prime} = -414$ mV). Though the redox potential of enzyme-bound B_{12}HBI may be more positive, Van der Meijden et al. (1983c, 1984b) found that the prosthetic group of inactivated MT1 was not reduced below the Co(II) level with hydrogen as electron donor, and methyl-B_{12}HBI was not formed after addition of methanol. In crude cell extracts, and in the methanol:methyltransferase system composed of partly purified enzymic components, catalytic amounts of ATP are absolutely required for full activity (Shapiro and Wolfe, 1980; Van der Meijden et al., 1983b,d). In crude extracts ATP may be removed after an activation phase without an appreciable affect on the methyltransferase activity. In the resolved system, however, the rate of CH_3S-CoM synthesis progressively decreased after removal of ATP, but subsequent addition of ATP restored the activity. When MT1 activation in the presence of hydrogen was followed spectrophotometrically, a sharp absorption peak arose at 390 nm only when ATP was added (Van der Meijden et al., 1983c). This peak is characteristic of Co(I) cobamide (Matthews et al., 1990). With even small amounts of methanol the corrin was then trapped as methyl-B_{12}HBI. Enhanced resolution of the methyltransferase system resulted in a complete lack of activity, even in the presence of ATP, hydrogen, hydrogenase and ferredoxin (Van der Meijden et al., 1948a; P. Daas, unpublished results). Activity now became strictly dependent on the addition of a heat-labile, oxygen-sensitive enzyme, which separated from the other components. In Figure 5.2 the enzyme is termed methyltransferase-activating protein (MAP).

The requirement for ATP and the MAP protein in the reductive activation of MT1 is evident, but the way in which these components participate in the process is unclear. In general, two modes of action may be envisaged: (1) The reduction potential of the electrons derived from hydrogen is lowered, at the expense of ATP hydrolysis, to a level which permits Co(II) to Co(I) reduction. This mechanism might resemble the role of ATP in the dinitrogenase reaction (Mortenson and Thornley, 1979). An ATP-driven reversed electron flow appears unlikely, since the enzyme system is composed of soluble proteins. (2) ATP modifies the structure of MT1 or its B_{12}HBI prosthetic groups in a process in which hydrogen reduction of Co(II) to Co(I) becomes feasible. Further studies are needed to shed more light on the activation phenomenon and the role of ATP and MAP.

(b) The Reduction of CH_3S-CoM

The most common methane-forming reaction is the reductive demethylation of CH_3S-CoM according to reaction 5.2 with HS-HTP as the reductant (for review see Rouvière and Wolfe, 1988; Rouvière et al., 1990; Friedmann et al., 1990).

$$CH_3S\text{-CoM} + \text{HS-HTP} \rightarrow CH_4 + \text{CoM-S-S-HTP} \qquad (5.2)$$

Since CH_3S-CoM reduction and the enzyme which catalyzes the reaction, methyl-coenzyme M reductase (MCR), have been described in relation to methanogenesis from hydrogen and CO_2 (see Chapter 4, this volume). We will restrict this section to some specific findings on methylotrophic methanogens.

MCR has been purified and characterized from *M. barkeri* strain 227 (Hartzell and Wolfe, 1986), *M. mazei* (Thomas et al., 1987), *M. thermophila* (Jablonski and Ferry, 1991) and from the methylotrophic strain Göl (Hoppert and Mayer, 1990). MCR from methylotrophs is usually composed of three subunits in an $\alpha_2\beta_2\gamma_2$ configuration, and the enzyme contains F_{430} as a prosthetic group. However, the apparent molecular masses of the native enzymes from *M. thermophila* and strain Göl were reported to be 132–141 kDa and 182 kDa, respectively, suggesting that they were isolated as $\alpha_1\beta_1\gamma_1$ proteins. The gene from *M. barkeri* strain Fusaro has been sequenced (Bokranz and Klein, 1987) and the amino acid sequence shows strong homology with those of other methanogens (Klein et al., 1988; Weil et al., 1989). Moreover, at least part of the CH_3S-CoM reductase activity could be recovered when subunits prepared from *M. barkeri* MCR were reconstituted with subunits from other methanogens, suggesting a common structural motif (Hartzell and Wolfe, 1986); still there are differences. With an average molecular mass of about 30 kDa, the γ subunit of MCR from *Methanomicrobiaceae*, to which most methylotrophs belong, is significantly lower than that of other methanogens (Rouvière and Wolfe, 1987). The subunit pattern of the enzyme from *M. stadtmanae*, however, is in agreement with its taxonomic status in the *Methanobacteriaceae*. Antibodies raised against MCR's from *Methanosarcina* species showed strong cross-reactivity with enzymes from other representatives of the genus, but failed to react with MCR's prepared from other methanogens (Hartzell and Wolfe, 1986; Thomas et al., 1987; Mayer et al., 1988).

As found for other methanogens, MCR from methylotrophs occurs predominantly as a soluble protein (Thomas et al., 1987; Jablonski and Ferry, 1991; Hoppert and Mayer, 1990). Upon gentle enzymic lysis of strain Göl some enzyme remains associated with the membrane, which is easily removed by washing with buffer (Deppenmeier et al., 1988). In the latter organism MCR present in the cytoplasmic fraction is often clustered into hollow sphere-like entities with one small gap (Mayer et al., 1988). Part of the clusters with an estimated M_r of 4×10^6 seems to be connected at the open end with a proteinacous, channeled stalk, which in turn is attached to a membrane-integrated protein complex with an M_r between 5×10^5 and 1×10^6. The function of the toadstool-like construction, termed methanoreductosome, was discussed in relation to proton transport across the cell membrane (Mayer et al., 1988). However, heterodisulfide reduction, rather than CH_3S-CoM reduction, is found to be coupled with proton translocation. Since the nature of the stalk and membrane proteins has not been established, a role for methanoreductosomes in chemiosmotic processes cannot be ruled

out completely. Alternatively, structural organization of MCR into sphere-like multicopy complexes might be a prerequisite for optimal enzymic activity. Usually, cell–free extracts are prepared by passage through a French pressure cell. This step is accompanied by a dramatic loss in MCR activity. In contrast, crude inside-out vesicles from strain Gö1 obtained by lytic procedures retained some 67% of the *in vivo* MCR activity (Deppenmeier et al., 1988). Thus, disintegration of the MCR complexes as the result of harsh cell rupture could be one reason for the loss in activity.

As mentioned above, F_{430} is the prosthetic group of MCR. Though the precise function has not been elucidated, its chemical and structural properties make F_{430} the most likely candidate to act as the catalytic center. The coenzyme could accept the methyl group from CH_3S-CoM via ligation to the central nickel atom. Subsequent protonolysis of the methyl group would then result in the formation of methane, with net inversion of the original methyl configuration (Ahn et al., 1991). Using (*S*)−[1−$^2H_1$,^3H] ethylcoenzyme M as an artificial substrate for MCR from *M. barkeri,* these authors were in fact able to demonstrate methyl group inversion.

F_{430} is presumed to be catalytically active with nickel in the reduced Ni(I) state [$E^{\circ\prime}$(Ni(I)/Ni(II) = −504 mV]. While Ni(I)−F_{430} shows characteristic EPR signals (Albracht et al., 1988) and exhibits an absorption maximum around 380 nm, Ni(II)−F_{430} is EPR-silent and the absorption maximum is at 430 nm (for a review see Friedmann et al., 1990). Highly active MCR (30 μmol·min^{-1}·mg^{-1}) isolated from *M. thermoautotrophicum* showed the expected spectral characteristics of Ni(I)−F_{430} (Rospert et al., 1991). Pertinent EPR signals were observed in intact cells of *M. barkeri* growing on acetate (Krzycki and Prince, 1990). MCR purified from *M. barkeri* (Hartzell and Wolfe, 1986), *M. mazei* (Thomas et al., 1987) and *M. thermophila* (Jablonski and Ferry, 1991) showed the UV-visible light spectral features of Ni(II)−F_{430}, and the enzyme preparations showed only low, if any, activity. Oxidative inactivation of MCR may be another reason for loss of activity. Reductive activation of purified *M. thermophila* MCR with Ti(III) citrate ($E^{\circ\prime}$ = −480 mV), or enzymatically with carbon monoxide and CO dehydrogenase ($E^{\circ\prime}$ = −524 mM) (Jablonski and Ferry, 1991), supports this mechanism. With the latter reducing system, ferredoxin was specifically required as an electron carrier. Reactivation occurred in the absence of ATP, though a 1.7-fold stimulation was found in its presence. One should keep in mind that after reactivation, CH_3S-CoM reduction with HS-HTP as electron donor proceeded at a rate of about 35 nmol·min^{-1}·mg^{-1}, which may be only 1–2% of the *in vivo* activity. Relatively high MCR activities were measured with vesicles from Gö1 (Deppenmeier et al., 1988). It is not known whether F_{430} is present in the Ni(I) state. ATP was not required in the H_2-dependent reduction of CH_3S-CoM by the vesicle system, but it was indispensable when the same reaction was followed with conventionally prepared cell extracts of strain Gö1 and *M. barkeri*. Evidently,

inactivation of MCR can result from both disintegration of MCR complexes and oxidation of the F_{430} prosthetic group. The mechanism by which inactive MCR may be reactivated and the putative role of ATP in this process requires further investigation.

5.2.2 The Route of Methanol Oxidation to CO_2

In order to generate reducing equivalents for the reduction of CoM-S-S-HTP, one-fourth of the methanol has to be oxidized to CO_2. Methanol oxidation proceeds largely by the reversed CO_2 reduction route (5.2.2(b)). The point at which methanol enters this pathway is not yet resolved, and is discussed in more detail below.

(a) The Initial Step in Methanol Oxidation

Detailed studies performed in the laboratory of Gottschalk with resting cells of *M. barkeri* and strain Göl provided conclusive evidence that the initial step in methanol oxidation is driven by a sodium-motive force (Blaut et al., 1985; Müller et al., 1987b, 1988a,b, 1990; Winner and Gottschalk, 1989; Gottschalk and Blaut, 1990; Müller et al., Chapter 8, this volume). This conclusion was based on the following observations: (1) Disproportionation of methanol into methane and CO_2 is dependent on the presence of sodium. The cation, however, is not required when formaldehyde is present. In this case formaldehyde is oxidized to CO_2, whereas methanol is specifically reduced to methane. This implies that sodium is involved in some reaction between methanol and the formaldehyde oxidation level of the 5,10-methylene-H_4MPT one-carbon unit. (2) Methanogenesis from methanol was impaired by an artificial sodium gradient ($Na^+_{in} > Na^+_{out}$). Dissipation of the sodium-motive force caused the inhibition of methanogenesis, even if the proton-motive force was high. (3) Methanol reduction under hydrogen to methane generates a proton-motive force, which then drives a Na^+/H^+ antiporter. Inhibition of the antiporter by harmaline and amiloride inhibited methanogenesis from methanol. From the latter observations it was concluded that the sodium-motive force is the result of secondary events (Blaut et al. 1990). Alternatively, evidence has been presented that the sodium-motive force is generated by primary Na^+ extrusion coupled to a reaction in formaldehyde oxidation to CO_2 (Kaesler and Schönheit, 1988, 1989).

By a similar line of experimentation the groups of Gottschalk and Schönheit showed that reduction of formaldehyde to methanol, i.e., CH_3S-CoM, is coupled with primary Na^+ export (Blaut et al., 1990; Kaesker and Schönheit, 1989a). Conversion of formaldehyde to CH_3S-CoM involves: (i) nonenzymatic binding of the substrate to H_4MPT, or rather H_4SPT (see below), resulting in 5,10-methylene-H_4SPT, (ii) reduction of the latter to 5-methyl-H_4SPT, and (iii) methyl

group transfer to HS-CoM (Figure 5.4). Since methylene-H_4SPT reduction to methyl-H_4SPT is a sodium-independent reaction (see below), the exergonic transfer of the methyl group must be the site where primary sodium export takes place. This agrees with the finding that methanogenesis from acetate in resting cells, which includes the methyl-H_4SPT:HS-CoM methyltransfer step, is sodium-dependent (Peinemann et al., 1988; Fischer and Thauer, 1988). The presence of the methyltransferase with the characteristics of a corrinoid enzyme (Kengen et al., 1992) has been demonstrated in cell extracts of *M. barkeri* grown on methanol and acetate (Van de Wijngaard et al. 1988, 1991b).

The necessity of a sodium-motive force in methanol oxidation to formaldehyde (reaction 5.3) has been explained in terms of the thermodynamics of the reaction (Blaut and Gottschalk, 1985; Blaut et al., 1985; Müller et al., 1988b). Under standard conditions, with protons as the electron acceptor, the reaction (5.3) is strongly endergonic.

$$\begin{gathered} CH_3OH + H_2O \rightarrow HCHO + 2H^+ + 2e^-, \\ \Delta G^{\circ\prime} = +44.8\ \text{kJ/mol};\ E^{\circ\prime} = -185\text{mV} \end{gathered} \qquad (5.3)$$

It was proposed (Blaut and Gottschalk, 1984) that methanol oxidation proceeded by use of a methanol dehydrogenase and a one-carbon carrier (X) (Figure 5.4). The generated electrons ($E^{\circ\prime} = -185$ mV) have to be transported uphill by sodium-driven reversed electron transport to allow reduction of a methanogenic low-potential redox carrier, such as F_{420} ($E^{\circ\prime} = -350$ mV). In this case the methanol would enter the oxidation pathway at the level of 5,10-methylene-H_4SPT. However, the presence of methanol dehydrogenase has never been demonstrated. Moreover, methanol disproportionation is also catalyzed, albeit with low velocity, by crude cell-free extracts of *M. barkeri* (Hutten et al., 1981; J. Keltjens, unpublished results). With the present biochemical knowledge in mind, some alternatives may be formulated (Figure 5.4): (1) Methanol is first converted to CH_3S-CoM by the MT1/MT2 system described above, followed by methyl group transfer to H_4SPT. This reaction is endergonic ($\Delta G^{\circ\prime} = +29.7$ kJ/mol) (Keltjens and Van der Drift, 1986) and may depend on the sodium gradient. (2) The methyl group of methanol is bound to the corrinoid prosthetic group of MT1 and subsequently transferred to H_4SPT. (3) Methyl-H_4SPT is formed directly from methanol according to reaction 5.4.

$$CH_3OH + H_4SPT \rightarrow 5\text{-methyl-}H_4SPT + H_2O,\ \Delta G^{\circ\prime} = 2.2\ \text{kJ/mol} \qquad (5.4)$$

The latter reaction is only slightly endergonic under standard conditions. Under physiological conditions, however, the $\Delta G'$ may be substantially more positive (Keltjens and Van der Drift, 1986).

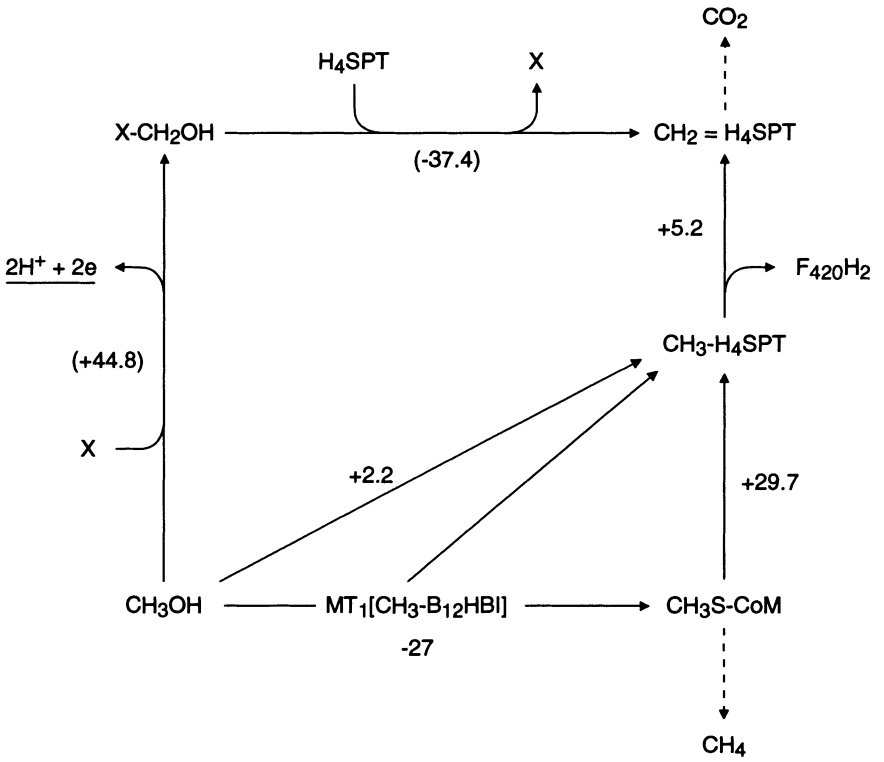

Figure 5.4. Alternative pathways for the methanol methyl group oxidation. Numbers next to arrows represent free energy changes of reactions in the indicated direction under standard conditions. X, unknown one-carbon carrier. ( ), reactions proposed by Blaut and Gottschalk, 1984.

We recently found that cell-free extracts of *M. barkeri* that had been thoroughly washed to remove low-molecular weight compounds mediated methyl group transfer from methanol to H_4MPT (J. Keltjens and R. Dijkerman, unpublished results). The product of the reaction, 5-methyl-H_4MPT, was identified on the basis of the HPLC retention and the UV spectrum, using the authentic compound as control. Methyltransfer was independent of ATP and HS-CoM, but was stimulated more than 25-fold by Ti(III)citrate. These observations rule out the possibility that 5-methyl-H_4SPT synthesis occurs via MT1, which is strictly dependent on ATP or CH_3S-CoM, and suggests a direct transfer of the methyl group to H_4SPT. It is not known whether the reaction requires a separate enzyme, or whether the corrinoid enzyme involved in the methyl-H_4SPT:HS-CoM methyl transferase reaction also serves as a methyl group acceptor from methanol. The

corrinoid enzyme is activated by Ti(III)citrate (Van de Wijngaard, 1991b) and a role for the enzyme in sodium transport was implied.

(b) Methyl Group Oxidation to CO_2

As stated above, 5-methyl-H_4SPT is the product of the initial step in methanol oxidation. The methyl group is then oxidized in the sequence of reactions listed in Table 5.3 (see also Figure 5.1). This reaction sequence is identical to the CO_2 reduction pathway described by Thauer et al. (Chapter 4, this volume), but it proceeds in the reverse direction and with the participation of somewhat different coenzymes (Figure 5.5). Methylotrophs characteristically contain a methanopterin derivative, called sarcinapterin (SPT) (Van Beelen et al., 1984), with an additional glutamyl group in the sidechain (Figure 5.5A). In *M. barkeri* a methanofuran, termed methanofuran-b (MFRb), is present with a diglutamyl bound to the 4-[*N*-(γ-L-glutamyl-γ-L-glutamyl)-p-(β-aminoethyl)phenoxymethyl]-2-(aminomethyl)furan core (Figure 5.5B)(Bobik et al., 1987; White, 1988). Methylotrophic methanogens also contain F_{420} derivatives with three to five glutamate groups, and different F_{420} species may be isolated from the same organism (Fig. 5.5C; Table 5.4). During batchwise growth the total cellular concentration of F_{420} remains fairly constant, but the relative proportions of the analogs markedly change (Heine-Dobbernack et al., 1988; Peck, 1989). The reason for this is not completely understood. Activity of F_{420}-dependent methylene dehydrogenase and reductase, for instance, is strongly affected by the length of the F_{420} side chain (te Brömmelstroet et al., 1991). Hence variation of the levels of F_{420} analogs could be a means to regulate the metabolic flux.

In agreement with the central metabolic role of SPT, or rather tetrahydrosarcinapterin (H_4SPT), and F_{420}, the concentrations of these coenzymes in methylotrophic organisms are high, and easily compare to those of hydrogenotrophic organisms (Table 5.4). This also holds for the activities of the enzymes involved in methyl group oxidation (Table 5.5). The exception is *M. stadtmanae,* which is incapable of methanol oxidation. The modest amounts of the coenzymes and enzymes present in this organism may serve anabolic reactions (Schwörer and Thauer, 1991; Van de Wijngaard et al., 1991a). From Tables 5.4 and 5.5 it can be seen that enzyme and coenzyme contents in the methylotrophs vary with the growth substrate. Notably, after growth on acetate activities of most enzymes are prominently lower. This makes sense, since during growth on this substrate methyl group oxidation is only required to produce reducing equivalents for anabolic reactions such as pyruvate synthesis. The SPT content, however, is strongly enhanced: H_4SPT is crucially important as the methyl group acceptor during acetyl-CoA cleavage (Fischer and Thauer, 1989). Apparently, versatile methylotrophs like *M. barkeri* are capable of regulating their metabolism by enzyme and coenzyme synthesis.

Table 5.3. Enzymic reactions involved in methyl group oxidation to CO_2

Enzyme	*Reaction*		$\Delta G^{\circ\prime}$ *(kJ/mol)*[a]	$E^{\circ\prime}$ *(mV)*
1. Methylene–H_4SPT reductase	5-Methyl–H_4SPT + F_{420}	$\rightleftharpoons$ 5,10-methylene–H_4SPT + $F_{420}H_2$	+5.2	−323
2. Methylene–H_4SPT dehydrogenase	5,10-Methylene–H_4SPT + H^+ + F_{420}	$\rightleftharpoons$ 5,10-methenyl–H_4SPT + $F_{420}H_2$	−2.3	−362
3. Methenyl–H_4SPT cyclohydrolase	5,10-Methenyl–H_4SPT + H_2O	$\rightleftharpoons$ 5-formyl–H_4SPT + H^+	+4.65	
4. Formylmethanofuran:H_4SPT formyltransferase	5-Formyl–H_4SPT + MFRb	$\rightleftharpoons$ formyl–MFRb + H_4SPT	+3.68	
5. Formylmethanofuran dehydrogenase	Formyl–MFRb + H_2O	$\rightarrow$ CO_2 + MFRb + 2 H^+ + 2e	−16	−497

[a]Free energy changes were taken from Keltjens and Van der Drift (1986) or were calculated from reported equilibrium constants (Keltjens et al., 1990, 1992; te Brömmelstroet et al., 1991).

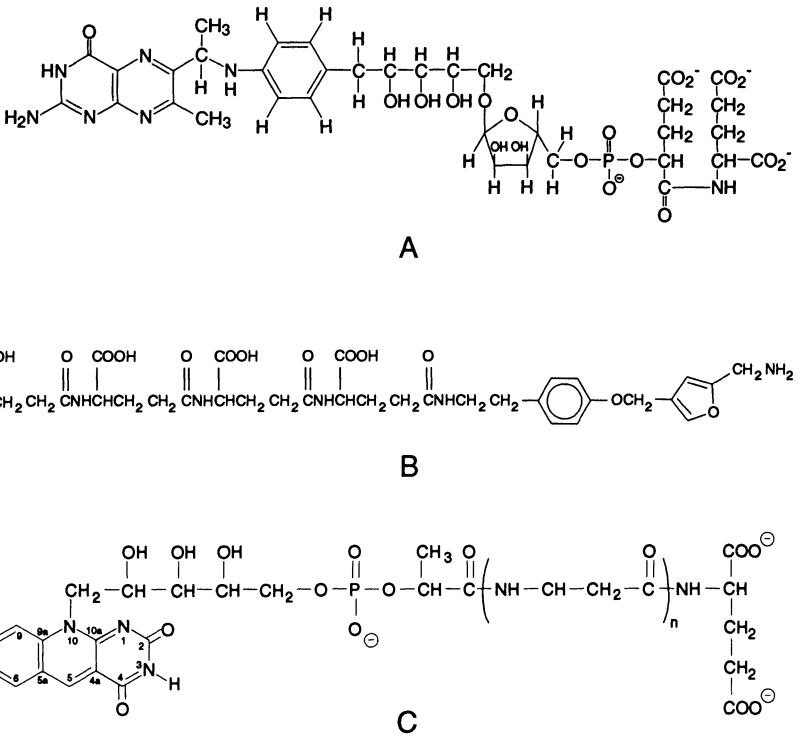

Figure 5.5. Structures of (A) sarcinapterin, (B) methanofuran and (C) F_{420} derivatives. In methylotrophic methanogens F_{420}^{-3} (n=2), F_{420}^{-4} (n=3) and F_{420}^{-5} (n=4) can be found.

The enzymes involved in methyl group oxidation have all been purified from *M. barkeri*. Properties of the H_4SPT-dependent enzymes isolated from strain MS and strain Fusaro of the organism are given in Table 5.6. All are cytoplasmic proteins and none of them harbor distinct prosthetic groups. The various bisubstrate reactions that are catalyzed by the enzymes proceed by ternary (sequential) complex mechanisms, except for formylmethanofuran:H_4SPT formyltransferase from strain Fusaro, for which a ping-pong mechanism was reported (Breitung and Thauer, 1990). It should be mentioned that the kinetic constants given in Table 5.6 were all determined with H_4MPT and MFR derivatives and with F_{420}^{-2}, rather than with the coenzymes present in *M. barkeri*. Consequently, the actual kinetic constants may differ to some extent. 5-Methyl- to 5,10-methylene-H_4SPT oxidation catalyzed by methylene-H_4SPT reductase (Equation 1 in Table 5.3) is kinetically unfavorable, and *in vitro* the presence of methylene-H_4SPT dehydrogenase (Equation 2 in Table 5.3) is required to drive the reaction (te Brömmelstroet

Table 5.4. Contents of methanopterin and F_{420} derivatives of methyltrophs and other methanogens[a]

Organism (strain)	*Energy substrate*	*Cofactor content (μmol/g protein)*[b]						
		MPT	*SPT*	*F_{420} (total)*	*F_{420}^{-2}*	*F_{420}^{-3}*	*F_{420}^{-4}*	*F_{420}^{-5}*
Methylotroph								
Methanosarcina barkeri (MS)	methanol	—[d]	47.3	1.41	0.01	0.04	0.18	1.17
	methylamine	—	8.4					
	H_2+CO_2	—	40.1	0.65	0.07	0.02	0.24	0.32
	acetate	—	186.9	3.14	0.10	0.04	1.16	1.84
Methanosarcina barkeri[c]	methanol			0.65–1.42				
(Fusaro)	acetate	—	204.5	1.26	0.05	0.07	0.67	0.47
Methanosarcina mazei	methanol	—	76.9	1.30	0.13	0.74	0.29	0.14
Methanolobus tindarius	methanol	—	32.4	1.55	1.20	0.33	0.01	0.01
Methanosphaera stadtmanae	methanol+H_2	—	—	0.14	0.01	0.01	0.10	0.02
Hydrogenotroph								
Methanobacterium thermo-autotrophicum (ΔH)	H_2+CO_2	117.2	—	2.27	2.01	0.26	—	—
Methanobacterium bryantii	H_2+CO_2	28.6	—	0.67	0.67	—	—	—
Acetotroph								
Methanosaeta soehngenii	acetate	—	2.72	0.02	0.00	—	0.01	0.01

[a]Data were taken from Gorris and Van der Drift (1986).
[b]MPT, methanopterin; SPT, sarcinapterin.
[c]Heine-Dobbernack et al.,1988.
[d]—, not detectable.

Table 5.5. Specific activities of enzymes involved in methyl oxidation and CO_2 reduction present in cell extract

Organism (strain)	*Energy substrate*	*Enzyme activity*[a] *(μmol·min^{-1}·mg protein^{-1})*					*Reference*
		MTR	*MDH*	*CH*	*FT*	*FDH*	
Methylotroph							
Methanosarcina barkeri (Fusaro)	Methanol	5.4	2.8		1.25	1.3	Schwörer and Thauer, 1991; Breitung and Thauer, 1990
	H_2+CO_2	6.9	2.5			1.3	Schwörer and Thauer, 1991
	Acetate	2.2	0.14			0.27	Schwörer and Thauer, 1991
Methanosarcina barkeri (MS)	Methanol	4.6	7.8	1.5	0.33		te Brömmelstroet et al., 1990, 1991; Keltjens et al., 1992
	Acetate	0.72	0.14			0.3	Schwörer and Thauer, 1991
Methanosarcina thermophila	Methanol		0.05	0.09	1.0	1.5	Jablonski et al., 1990
	Acetate		0.02	0.03	0.7	0.08	
Methanosphaera stadtmanae	Methanol+H_2	0.02	0.01			0.04	Schwörer and Thauer, 1991
Hydrogenotroph							
Methanobacterium thermo-autotrophicum	H_2+CO_2	4.6	3.1–7	1.2	2.1	1.1	Schwörer and Thauer, 1991 Jablonski et al., 1990
Methanobacterium wolfei	H_2+CO_2	5.26	2.3			1.7	Schwörer and Thauer, 1991
Acetotroph							
Methanosaeta soehngenii	Acetate	0.01	0.02			0.15	Schwörer and Thauer, 1991

[a]Activities do not represent V_{max} values and may depend on assay conditions used by the authors.

Abbreviations: MTR, methylene–H_4MPT reductase; MDH, methylene–H_4MPT dehydrogenase; CH, methenyl–H_4MPT cyclohydrolase; FT, formylmethanofuran:H_4MPT formyltransferase; FDH, formylmethanofuran dehydrogenase.

Table 5.6. Structural and kinetic properties of H_4SPT-dependent enzymes

Enzyme (subunit size in kDa; quaternary structure)	*Kinetic constants*[a]	Organism (strain)	Reference
Methylene reductase (38; α_4)	K_m (CH_3-) = 250; $K_m(F_{420})$ = 40 V_{max} = 200; k_{cat} = 127	*M. barkeri* (MS)	te Brömmelstroet et al., 1991
Methylene reductase (36; α_4)	K_m ($CH_2=$) = 15; $K_m(F_{420}H_2)$ = 12 V_{max} = 2200; k_{cat} = 1360	*M. barkeri* (Fusaro)	Ma and Thauer, 1990
Methylene dehydrogenase (35; α_6)	K_m ($CH_2=$) = 6; $K_m(F_{420})$ = 18 V_{max} = 3000; k_{cat} = 1650	*M. barkeri* (MS)	te Brömmelstroet et al., 1991
Methylene dehydrogenase (31; α_8)	K_m ($CH_2=$) = 6; $K_m(F_{420})$ = 40 V_{max} = 4000; k_{cat} = 2066	*M. barkeri* (Fusaro)	Enssle et al., 1991
Cyclohydrolase (41; α_2)	K_m ($CH^+=$) = 570	*M. barkeri* (MS)	te Brömmelstroet et al., 1990
Formyltransferase (32; α_8)	K_m (H_4MPT) = 230; K_m(fMFR) = 47 V_{max} = 2330; k_{cat} = 1227	*M. barkeri* (MS)	Keltjens et al., 1992
Formyltransferase (32; α_1)	K_m (H_4MPT) = 400; K_m(fMFR) = 400 V_{max} = 3700; k_{cat} = 1973	*M. barkeri* (Fusaro)	Breitung and Thauer, 1990

[a] K_m values are given in μM; V_{max} is $\mu mol \cdot min^{-1} \cdot mg^{-1}$; k_{cat} is s^{-1}. Abbreviations: (CH_3-), 5-methyl$-H_4MPT$; ($CH_2=$), 5,10-methylene$-H_4MPT$; ($CH^+=$), 5,10-methenyl$-H_4MPT$; (fMFR), formylmethanofuran.

et al., 1991). The latter enzyme shows a very low K_m of 6 μM for 5,10-methylene-H_4SPT. Cellular activities of both enzymes are more than sufficient to accommodate methanol oxidation in a non-rate-limiting way (te Brömmelstroet et al., 1991). Sodium is not required in the reaction. Both methylene-H_4SPT reductase and dehydrogenase use F_{420} as the sole electron acceptor: the proton-reducing dehydrogenase found in obligately hydrogenotrophic methanogens is absent in the methylotrophs (Schwörer and Thauer, 1991). The product of the dehydrogenase reaction, 5,10-methenyl-H_4SPT, then called YFC ("Yellow Fluorescent Component"), was originally detected by Daniels and Zeikus (1978) as a short-term labeling product of $^{14}CH_3OH$.

5,10-Methenyl-H_4SPT is subsequently hydrolyzed by methenyl-H_4SPT cyclohydrolase (Equation 3 in Table 5.3). In a previous report we suggested that 10-formyl-H_4SPT was formed in the reaction (te Brömmelstroet et al., 1990). This is incorrect, and by a variety of criteria the 5-formyl species is the established product of methenyl-H_4SPT cyclohydrolase (Breitung and Thauer, 1990; Keltjens et al., 1992). Following the formyl group transfer according to Equation 4 in Table 5.3, formyl MFRb, the substrate of the terminal oxidation step, is produced. Formyl-MFRb:H_4SPT formyltransferase has been purified from *M. barkeri* strain Fusaro as a 32 kDa protein (Breitung and Thauer, 1990) and from strain MS of the organism as an octamer of 32 kDa subunits (Keltjens et al., 1992). *M. barkeri* formyltransferase may use *N*-furfurylformamide as a pseudo-substrate for formyl-MFR (Breitung et al., 1990). However, the apparent K_m of the artificial substrate is 900-fold higher and the V_{max} is 72-fold lower, which stresses the considerable importance of the composition of the side chain in the kinetic and energetics of coenzyme binding.

Whereas the free energy changes of the H_4SPT-dependent reactions are small (Table 5.3), formyl-MFR oxidation according to Equation 5 in the table is quite exergonic. Formylmethanofuran dehydrogenase from *M. barkeri* has been purified 100-fold to apparent homogeneity (Karrasch et al., 1989b, 1990a). The enzyme oxidized formyl-MFR (K_m = 20 μM) with methyl viologen (K_m = 20 μM) or metronidazole as artificial electron acceptors with a specific activity of 175 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ (k_{cat} = 640 s^{-1}) The mechanism of catalysis could not be established. *N*-Furfurylformamide also acted as a pseudosubstrate for the dehydrogenase, albeit with a high K_m of 200 mM (Breitung et al., 1990). The dehydrogenase from *M. barkeri* is structurally complex. The native 220 kDa protein is composed of five different subunits present in equal amounts and showing apparent molecular masses of 65, 50, 37, 34, 29 and 17 kDa. In addition, 28 mol non-heme iron, 28 mol acid-labile sulfur, 0.5–0.8 mol molybdenum, and 0.6–0.9 mol of a pterin cofactor were present per 220 kDa protein (Karrasch et al., 1990a). The pterin cofactor was identified as molybdopterin guanine dinucleotide (Figure 5.6) (Karrasch et al., 1990b). As in other molybdoenzymes,

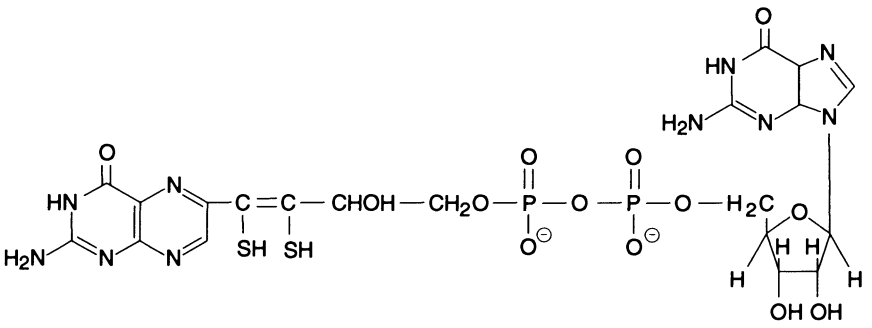

Figure 5.6. Structure of molybdopterin guanine dinucleotide.

cyanide is an inhibitor of the dehydrogenase: addition of 0.1 mM cyanide results in an almost instantaneous inhibition of the reaction (Börner et al., 1989). Formyl-MFR dehydrogenase from *M. thermoautotrophicum* has a simpler structure. This 110 kDa molybdopterin protein is composed of two subunits with an apparent molecular mass of 60 kDa and 45 kDa, respectively, and only 4 mol acid-labile sulfur and 4 mol non-heme iron are present per mol of protein (Börner et al., 1991).

When cell extracts of *M. barkeri* were subjected to centrifugation at 160,000 x *g* for 2h, approximately 50% of the dehydrogenase activity was recovered from the pellet fraction (Börner et al., 1989). Under these conditions H_4SPT-dependent enzymes involved in methanol oxidation and MCR were present only in the supernatant. This might imply that the dehydrogenase is a membrane protein and that some of the subunits play a role in membrane-binding or even membrane-poring. As mentioned above, experiments performed by Kaesler and Schönheit (1989a,b) suggested formaldehyde (methylene-H_4MPT) oxidation to be coupled with primary Na^+ extrusion. It is now tempting to speculate that the exergonic formyl-MFR dehydrogenase reaction is the site where Na^+ export takes place.

In crude cell-free extracts of *M. barkeri*, F_{420} (K_m = 25 μM) could act as the electron acceptor of the formyl-MFR dehydrogenase reaction (Börner et al., 1989); however, with the purified enzyme, F_{420} is inactive (Karrasch et al., 1989b). Using washed membrane preparations of the organism, we also did not detect formyl-MFR oxidation coupled to F_{420} reduction, although this activity was present in crude cell extracts and in the soluble enzyme fraction (Keltjens and Brugman, unpublished results). However, the membrane pellet fraction could catalyze formylmethanofuran oxidation and F_{420} reduction at the same rate as cell extracts, when purified ferredoxin was added. Moreover, ferredoxin was strictly required to couple formyl-MFR, and also $F_{420}H_2$, oxidation to CoM-S-S-HTP

reduction with the pellet fraction. These unpublished findings suggest that ferredoxin is a physiological electron acceptor of formyl-methanofuran dehydrogenase in *M. barkeri*.

5.2.3 The Reduction of CoM-S-S-HTP

(a) CoM-S-S-HTP Reduction and Proton Translocation

The oxidation of one mol methanol leads to the formation of 2 mol $F_{420}H_2$ and an additional 2 mol electrons (reduced ferredoxin). The reducing equivalents are used for CoM-S-S-HTP reduction. This redox process is of primordial importance in the energy metabolism (see Chapter 8). Deppenmeier et al. (1990b) found that in everted vesicle systems of strain Gö1, oxidation of $F_{420}H_2$ and CoM-S-S-HTP reduction (reaction 5.5) is coupled with the transport of 2 mol H^+ per mol $F_{420}H_2$ oxidized. This proton translocation drives (in the system used) the synthesis of 0.4 mol ATP/$F_{420}H_2$. Gö1 contained a second

$$F_{420}H_2 + \text{CoM-S-S-HTP} \rightarrow F_{420} + \text{HS-CoM} + \text{HS-HTP}, \quad \Delta G^{\circ\prime} = -30.3 \text{ kJ/mol} \tag{5.5}$$

$$H_2 + \text{CoM-S-S-HTP} \rightarrow \text{HS-CoM} + \text{HS-HTP}, \quad \Delta G^{\circ\prime} = -42.5 \text{ kJ/mol} \tag{5.6}$$

energy-conserving system which is independent of F_{420} (Deppenmeier et al., 1991) and which used hydrogen as the electron donor (reaction 5.6). Two mol of H^+ were translocated per mol of CoM-S-S-HTP reduced. The findings suggest that electron transport takes place with the participation of at least one H^+-translocating, membrane-spanning protein. Vesicle systems of Gö1 and membrane preparations of *M. tindarius*, but not the soluble protein fractions, catalyzed the overall reaction (5.6) (Deppenmeier et al., 1990a,b). In contrast, it has been found by many authors (see, for example: Baresi, 1984; Nelson and Ferry, 1984; Krzycki and Zeikus, 1984; Krzycki et al., 1985; Deppenmeier et al., 1989; Fischer and Thauer, 1990b) that the reduction of CH_3S-CoM under hydrogen, which includes reaction 5.6, is catalyzed by soluble protein fractions. Apparently, under the conditions of extract preparation, which usually involves passage of whole cells through a French pressure cell, the process is independent of membrane proteins. However, CH_3S-CoM reduction under hydrogen catalyzed by the soluble protein fraction of Gö1 obtained after mild cell lysis was stimulated up to 10-fold by the addition of membrane preparations of various methanogenic species (Deppenmeier et al., 1989). In the absence of membranes, oxidation of one mol formaldehyde coupled to the reduction of one mol CH_3S-CoM, whereas an additional mol of hydrogen was formed. In their presence 2 mol of CH_3S-CoM were reduced at the expense of 1 mol of formaldehyde. Electron transport

in methylotrophs obviously requires the availability of suitable cell-free systems, in which coupling between soluble and membrane-bound redox carriers remains intact. Presently, such system seems to be available only for Gö1.

Hereafter, we will describe several of the components of the methylotrophic electron transport chain, notably membrane-bound redox carriers (5.2.3(b)), hydrogenase (5.2.3(c)), and ferredoxin (5.2.3(d)). In 5.2.3(e) a model will be presented which incorporates the present knowledge of the redox processes.

(b) Membrane-Bound Redox Carriers in Methylotrophic Methanogens

EPR analysis of membrane preparations of *M. barkeri* showed the presence of multiple iron-sulfur centers of the [4Fe-4S] type, as well as high-spin heme-containing species (Kemner et al., 1987). The signals could not be attributed to putative membrane-bound iron-sulfur proteins like hydrogenase, formylmethanofuran dehydrogenase or heterodisulfide reductase. Though the latter enzyme has not been purified from a methylotrophic methanogen, the protein from *M. thermoautotrophicum* contains large amounts of iron and acid-labile sulfur (Hedderich et al., 1990). To date, two classes of membrane-bound proteins have been identified in the methylotroph *M. tindarius*: cytochromes and $F_{420}H_2$ dehydrogenase.

In striking contrast with other methanogenic bacteria, organisms capable of growth on methylated one-carbon compounds or acetate contain physiologically significant levels of cytochromes in their membranes (Table 5.7). *M. stadtmanae* is the notable exception (Miller and Wolin. 1985). *Methanosarcina* species are characterized by the presence of two types of cytochrome *b* in about equal amounts showing extremely low redox potentials of −325 mV and −183 mV, respectively (Kühn et al., 1983; Kühn and Gottschalk, 1983). Interestingly, a third cytochrome *b* ($E^{\circ\prime} = -250$ mV) can be detected after growth on acetate, while the total amount of cytochrome is also higher than in cells obtained on other substrates. Apart from the cytochromes *b*, some cytochrome *c* is present. The latter type is the major representative in obligately methylotrophic marine organisms like *M. methylutens* and *M. tindarius* (Table 5.7); the organisms may contain more than one cytochrome *c* species (Jussofie, 1984; Jussofie and Gottschalk, 1986). Cytochrome *b* present in the membranes of *M. barkeri* and *M. thermophila* could be reduced with hydrogen or CO, and were oxidized with CH_3S-CoM, which clearly suggests a role in electron transport (Kemner et al., 1987; Terlesky and Ferry, 1988a).

$F_{420}H_2$ serves as an electron donor in CoM-S-S-HTP reduction (reaction 5.5). An enzyme which catalyzed $F_{420}H_2$ ($K_m = 5.4\ \mu M$) oxidation coupled to methyl viologen and metronidazole reduction has recently been purified from *M. tindarius* (Haase et al., 1992). The enzyme, $F_{420}H_2$ dehydrogenase, showed an apparent molecular mass of 120 kDa and was composed of equal amounts of 45, 40, 22,

Table 5.7. Cytochrome contents of methylotrophic methanogenic bacteria[a]

Organism (strain)	Growth substrate	Cytochrome content[b] (μmol/g membrane protein)	
		cytochrome *b*	cytochrome *c*
Methanosarcina barkeri (Fusaro)	Methanol	0.30	0.024
	Monomethylamine	0.38	0.075
	Dimethylamine	0.27	0.019
	Trimethylamine	0.38	0.016
	Acetate	0.50	n.d.
	H_2+CO_2	0.42	n.d.
Methanosarcina vacuolata	Methanol	+++	+
Methanosarcina thermophila	Methanol	+++	+
Methanosarcina mazei	Methanol	0.27	n.d.
Methanococcoides methylutens	Trimethylamine	0.007	0.306
Methanolobus tindarius	Methanol	0.016	0.189

[a]Data were taken from Kühn and Gottschalk (1983), Kühn et al. (1983), Jussofie (1984) and Jussofie and Gottschalk (1986).
[b]+++, major species; +, minor species; n.d., not determined.

18 and 17 kDa subunits. The native enzyme had to be solubilized from the membrane by mild treatment with detergent. Since under these conditions cytochromes remained bound, it appears $F_{420}H_2$ dehydrogenase is membrane-associated rather than membrane-bound, and some of the subunits might serve as membrane anchors. Per 120 kDa protein, 16 mol non-heme iron and 16 mol acid-labile sulfur were present; hemes or flavins were absent. The latter observation is remarkable, since the enzyme catalyzes a two-electron/one-electron switch, which is usually brought about by flavin groups. The physiological electron acceptor has not been established. It is unknown whether the enzyme is a specific constituent of obligate methylotrophs.

(c) Hydrogenase and Methylotrophic Electron Transport

During methanogenesis from methylated one-carbon compounds, substrate oxidation and reduction largely proceeds in a balanced way according to the reaction stoichiometries listed in Table 5.2. Both the versatile and obligate methylotrophs, however, produce hydrogen during growth (Lovley and Ferry, 1985; Boone et al., 1987; Krzycki et al. 1987; Ahring et al., 1991). Apparently, part of the reducing equivalents are consumed in proton reduction. The rate of hydrogen formation is low, about 1% of the rate of methanogenesis. Since in closed systems the gas is taken up again, production and consumption result in

net steady-state partial hydrogen pressures ranging from 8 to 160 Pa (55 to 1100 nM). Reducing equivalents generated during substrate oxidation are required for anabolic and catabolic reactions. Thus, intermediary hydrogen formation could be a means of fine-tuning the processes. The presence of hydrogen could also play a role in metabolic regulation. Boone et al. (1987), for instance, found that the presence of the gas was a prerequisite for *M. barkeri* to shift from one type of metabolism to another (see also Section 5.4).

Intermediary hydrogen formation presumes the presence of hydrogenase (H_2ase). Indeed, all methylotrophs appear to contain this enzyme. Cellular activities are especially high in organisms that are capable of growth on hydrogen and CO_2, whereas only modest activities are found in obligate methylotrophs like *M. tindarius* (Deppenmeier et al., 1991). In addition, activities may vary with respect to the growth substrate, indicating that enzyme synthesis is subject to regulation (Nelson and Ferry, 1984; Bhatnagar et al., 1987; see, however, Baresi and Wolfe, 1981).

When crude cell extracts of *Methanosarcina* species are separated by non-denaturing gel electrophoresis a number of H_2ase bands can be detected (Bhosale et al., 1989, 1990; Fiebig and Friedrich, 1989). The bands may represent charge isomers and multimers of two types of H_2ase that are generally encountered in methanogens (see also Chapter 7): a viologen-reducing species and F_{420}-reducing H_2ase. The former has not been purified from a methylotroph. The latter was isolated from *M. barkeri* strain MS (Fauque et al., 1984) and strain Fusaro (Fiebig and Friedrich, 1989). In these organisms the enzyme represented about 0.2% and 1.5%, respectively, of the cellular proteins. Though after extract preparation activity is mainly recovered in the soluble fraction, part of it remains associated with particulate material. Purified F_{420}-reducing H_2ase from *M. barkeri* strain MS evolved hydrogen at a rate of 270 μmol·min^{-1}·mg protein^{-1}. The 800 kDa protein was composed of 60 kDa subunits (Fauque et al., 1984). One mol FMN, 8 to 10 mol iron and 0.6 to 0.8 mol nickel were present per 60 kDa subunit. The enzyme from strain Fusaro occurred in two forms showing apparent molecular weights of 845 kDa and 198 kDa, respectively; both species were composed of 48 kDa (α), 33 kDa (β) and 30 kDa (γ) subunits in an $\alpha_2\beta_2\gamma$ configuration. The enzyme reduced F_{420} (K_m = 25 μM) and methyl viologen (K_m = 3.3 mM) with V_{max} values of 25 μmol·min^{-1}·mg^{-1} (K_{cat} = 353 s^{-1}) and 655 μmol·min^{-1}·mg^{-1} (K_{cat} = 9226 s^{-1}), respectively. Interestingly, ferredoxin from *M. barkeri* (Fauque et al., 1984) and *Clostridium* (Bhosale et al., 1989) was also used as an electron acceptor. Moreover, the *M. barkeri* enzyme catalyzed $F_{420}H_2$ oxidation coupled to benzyl viologen reduction at high rate (Keltjens, unpublished results). This reaction equals the one performed by $F_{420}H_2$ dehydrogenase from *M. tindarius*. In fact, we always found that F_{420}, H_2ase, and dehydrogenase activities co-purify, and we did not detect a distinct $F_{420}H_2$ dehydrogenase.

The structural and catalytic properties of the *M. barkeri* F_{420}-reducing H_2ase

are in agreement with the presence of the three functional domains as proposed for the enzyme from *M. thermoautotrophicum* (Fox et al., 1987; Livingston et al., 1987) (Figure 5.7): (1) a nickel center involved in hydrogen activation and proton reduction, (2) Fe-S clusters, which mediate a one-electron transfer to viologen dyes or ferredoxin, and (3) the flavin prosthetic group, which performs the 1-electron/2-electron switch to F_{420}. In *M. barkeri* growing on methanol the function of F_{420}-dependent H_2ase could be one of a "three-way valve": $F_{420}H_2$ is oxidized by the enzyme with ferredoxin as the electron acceptor and in the presence of **excess** $F_{420}H_2$, proton reduction and hydrogen formation takes place.

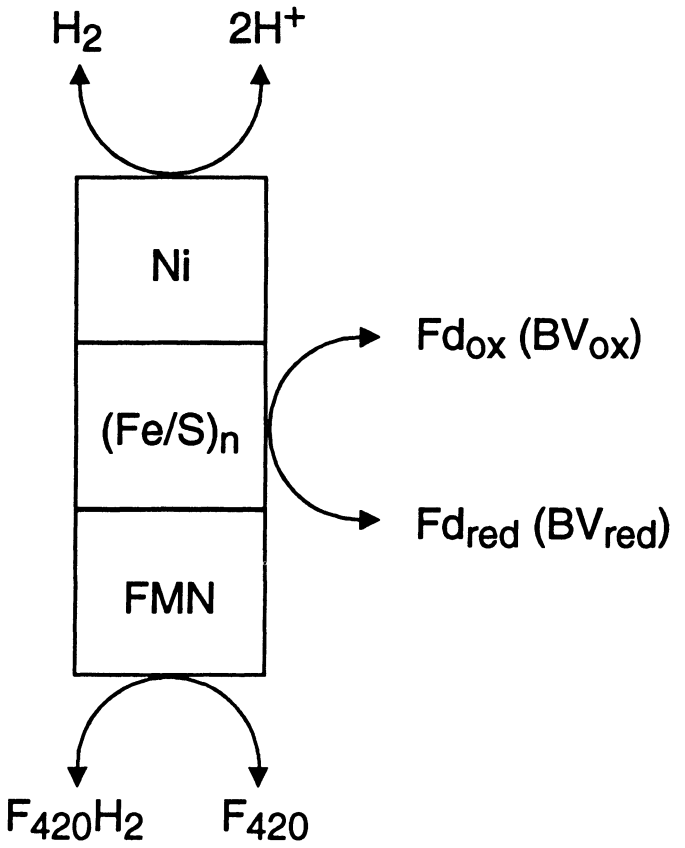

Figure 5.7. Schematic representation of the functional domains of F_{420}-reducing hydrogenase and partial reactions catalyzed by the enzyme. Abbreviations: Fd, ferredoxin; BV, (benzyl/methyl) viologen.; $(FeS)_n$, iron-sulfur centers; ox/red, oxidized/reduced.

(d) Ferredoxin, A Central Electron Carrier in Methylotrophic Metabolism

Methanosarcina species characteristically contain copious amounts of ferredoxin. The acidic soluble iron-sulfur protein has been purified from *M. barkeri* strain MS (Moura et al., 1982; Hausinger et al., 1982; Van der Meijden et al., 1984b), from strain Fusaro of the same species (Hatchikian et al., 1982) and from *M. thermophila* (Terlesky and Ferry, 1988b) (see also Chapter 7).

Ferredoxin is the substrate in a series of reactions shown in Figure 5.8. It is the electron acceptor in the acetyl-CoA cleavage reaction (Terlesky and Ferry, 1988a) and thereby mediates hydrogen formation in cell-free systems (Fischer and Thauer, 1990b). The iron-sulfur protein is an electron acceptor of F_{420}-dependent hydrogenase (Fauque et al., 1984; Bhosale et al., 1989); a role as such (as suggested in Figure 5.8) for viologen-reducing H_2ase remains to be verified.

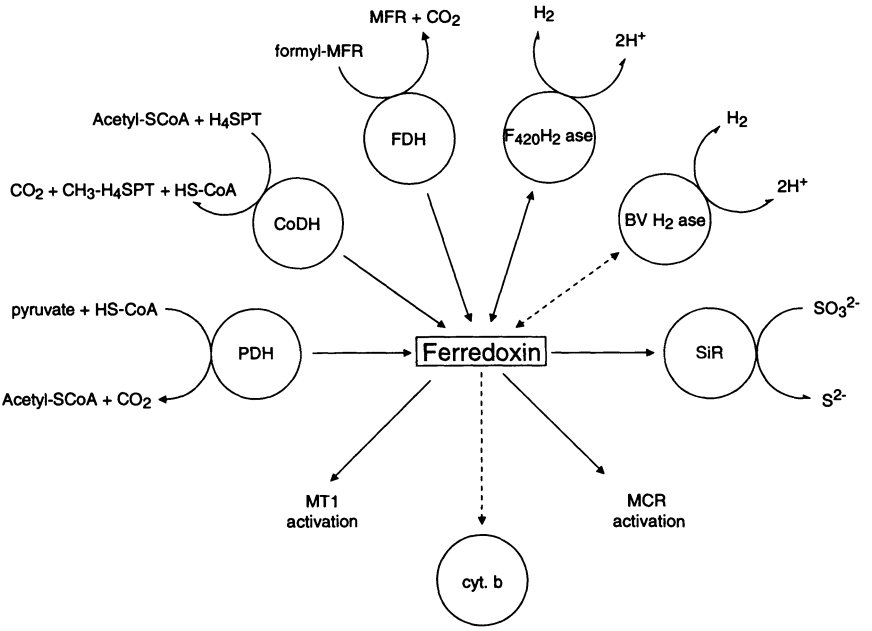

Figure 5.8. Role of ferredoxin as a redox carrier in methylotrophic methanogens. Enzymes: PDH, pyruvate dehydrogenase; CODH, acetyl-CoA synthetase (carbon monoxide dehydrogenase); FDH, formylmethanofuran dehydrogenase; F_{420} H_2ase, F_{420}-reducing hydrogenase; BV H_2ase, viologen-reducing hydrogenase; SiR, sulfite reductase; MCR, methylcoenzyme reductase; MT1, methanol:B_{12}HBI methyltransferase; cyt b, cytochrome b. Reactions given by dashed lines have not been established.

With a washed membrane fraction, ferredoxin was required to couple formylmethanofuran and $F_{420}H_2$ oxidation to CoM-S-S-HTP reduction. In addition, pyruvate conversion to acetyl-CoA and hydrogen (Hatchikian et al., 1982) and the reduction by hydrogen of sulfite to sulfide (Fauque et al., 1984) is strictly dependent on ferredoxin. Finally, the protein functions as an electron donor in the reductive activation of MT1 (5.2.1.a) and MCR (Jablonski and Ferry, 1991). Obviously, the enzyme takes a central position in methylotrophic electron transport. A function as electron donor in cytochrome *b* reduction is yet to be proven: circumstantial evidence follows from the observation that hydrogen and carbon monoxide are capable of reducing the cytochrome (Jussofie, 1984; Kemner et al., 1987; Terlesky and Ferry, 1988a) and ferredoxin is the electron acceptor of CO dehydrogenase.

(e) Electron Transport in CoM-S-S-HTP Reduction

Though many elements are still unknown, a minimal model for electron transport which incorporates the above findings and the midpoint potentials of the relevant redox couples is shown in Figure 5.9. It is proposed that electrons derived from $F_{420}H_2$ ($E^{\circ\prime} = -350$ mV) and formyl-MFR ($E^{\circ\prime} = -497$ mV) oxidation, or acetyl-CoA cleavage ($E^{\circ\prime} = -200$ to -400 mV; Thauer, 1990), are collected by ferredoxin and transferred to a membrane-bound oxidoreductase. Hydrogen ($E^{\circ\prime} = -414$ mV) formed as a by-product of methanol disproportionation can be reoxidized, and here the viologen-reducing H_2ase might play a role. Since the physiological electron acceptor is unknown it is uncertain whether the enzyme donates the electrons to ferredoxin or to a membrane-bound redox carrier. Via both types of cytochrome b, electrons are transported to heterodisulfide reductase. The $E^{\circ\prime}$ value of -193 mV for CoM-S-S-HTP reduction was presumed to be equal to that measured for the couple HS-CoM/$(S\text{-CoM})_2$ (Kell and Morris, 1979).

Energy released by electron transport is conserved by proton translocation. The site(s) where H^+ transport takes place is unknown. Heterodisulfide reductase could be one. The finding, however, that upon extract preparation enzyme activities are about equally distributed between soluble and particulate fractions (Schwörer and Thauer, 1991) suggests that the enzyme is tightly membrane-associated rather than membrane-integrated.

It should be stressed that the model (Figure 5.9) is only a minimal one. The actual situation will be more complex and may vary among different methylotrophic methanogens, and may alter with respect to the growth substrate. For example, in the obligate methylotroph *M. tindarius,* membrane-bound $F_{420}H_2$ dehydrogenase will be involved (5.2.3(b)). Acetate-cultured *M. barkeri* also contains an additional cytochrome *b* ($E^{\circ\prime} = -250$ mV) (Kühn and Gottschalk, 1983). When grown on the substrate cells are capable of coupling the exergonic

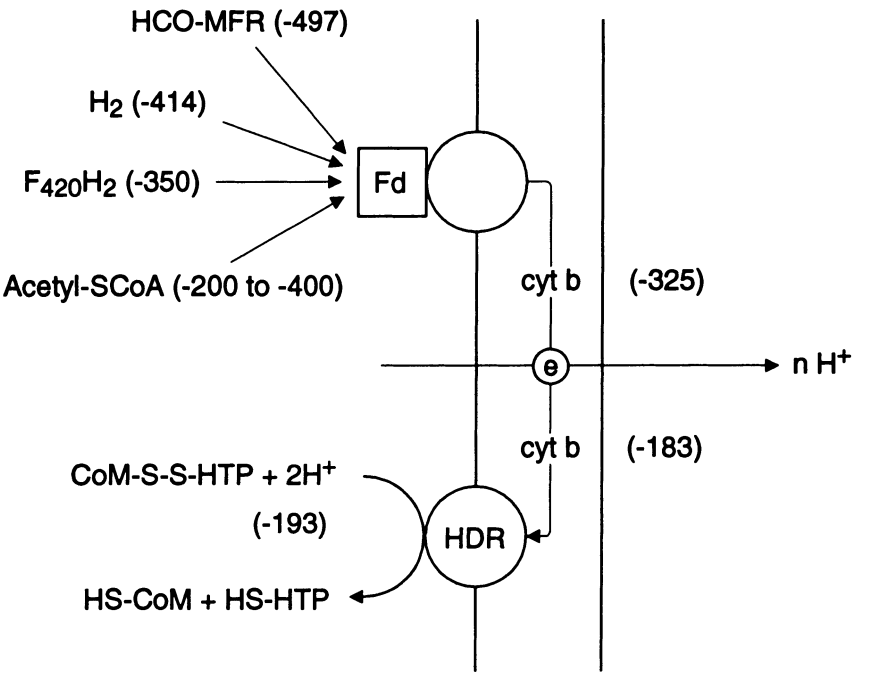

Figure 5.9. Model for electron and proton transport in CoM-S-S-HTP reduction. The values in parentheses represent the standard midpoint potentials of the redox couples. Abbreviations: HCO-MFR, formylmethanofuran; Fd, ferredoxin; Cyt b, cytochrome b; HDR, CoM-S-S-HTP (heterodisulfide) reductase; e, electron.

carbon monoxide oxidation (reaction 5.7) with proton extrusion (Bott et al., 1986; Bott and Thauer, 1989).

$$CO + H_2O \rightarrow CO_2 + H_2,\ \Delta G^{\circ\prime} = -20\text{kJ/mol} \qquad (5.7)$$

While keeping the elements of Figure 5.9, this may be explained by assuming that the reducing equivalents from CO oxidation are transferred via ferredoxin and cytochrome *b* ($E^{\circ\prime} = -325$ mV) to a hydrogenase, which accepts the electrons from the $E^{\circ\prime} = -183$ mV cytochrome.

5.3 Methanogenesis from Methylamines and Methylsulfides

At the biochemical level, methane formation from mono-, di- and trimethylamine is only poorly investigated. Available evidence suggests that the N-methyl

compounds are metabolized analogously to methanol. Experimental reaction stoichiometries were in agreement with the theoretical ones given in Table 5.2 (Hippe et al., 1977). This implies that three-quarters of the methyl groups are reduced to methane and the remainder is oxidized to CO_2. CH_3S-CoM is the intermediate in the reduction route (Naumann et al., 1984). In whole cells reduction of trimethylamine to methane by hydrogen is coupled with the generation of a proton-motive force (Müller et al., 1987c). When grown with the substrate, *M. barkeri* contains a trimethylamine:HS-CoM methyltransferase, which catalyzes reaction 1 in Table 5.8. The reaction requires an ATP-dependent reductive activation as found in the MT1/MT2 system (5.2.1(a)), which supports a role for a corrinoid enzyme (Naumann et al., 1984). The activity is absent in methanol-cultured organisms, and is detectable only after growth on di- or monomethylamine. With the latter two compounds as substrates CH_3S-CoM is produced by reactions 2 and 3 in Table 5.8. Again, these activities are absent after growth on methanol. Di- and monomethylamine, products of reaction 1 and 2 are observed as transient intermediates in trimethylamine-metabolizing cultures (Hippe et al., 1977) and during conversion of the compound by cell extracts (Naumann et al., 1984). The above findings exclude the possibility that the N-methyl methyltransfer reactions are performed by the methanol:HS-CoM methyltransferase, but rather are catalyzed by distinct inducible enzymes. Like in methanol disproportionation, the initial step in N-methyl oxidation is driven by chemiosmotic processes (Müller et al., 1987a). Whether the sodium-motive force is the driving factor has not been established. Analogous to methanol oxidation, the initial step could be a methyl group transfer to H_4SPT (Equations 6a–6c in Table 5.8). N-methyl transfer is considerably more endergonic than with methanol.

Table 5.8. Methyl group transfer in methylamine and methylsulfide conversion

	Reaction	*$\Delta G^{\circ\prime}$ (kJ/mol)*[a]
1	$(CH_3)_3N + HS-CoM \rightarrow CH_3S-CoM + (CH_3)_2NH$	−6.1
2	$(CH_3)_2NH + HS-CoM \rightarrow CH_3S-CoM + (CH_3)NH_2$	−2.6
3	$(CH_3)NH_2 + HS-CoM \rightarrow CH_3S-CoM + NH_3$	−5.0
4	$(CH_3)_2S + HS-CoM \rightarrow CH_3S-CoM + (CH_3)SH$	+12.2
5	$(CH_3)SH + HS-CoM \rightarrow CH_3S-CoM + H_2S$	+14.1
6a	$(CH_3)_3N + H_4SPT \rightarrow$ 5-methyl-$H_4SPT + (CH_3)_2N$	+24.0
6b	$(CH_3)_2NH + H_4SPT \rightarrow$ 5-methyl-$H_4SPT + (CH_3)NH_2$	+27.2
6c	$(CH_3)NH_2 + H_4SPT \rightarrow$ 5-methyl-$H_4SPT + NH_3$	+25.1
7a	$(CH_3)_2S + H_4SPT \rightarrow$ 5-methyl$-H_4SPT + (CH_3)SH$	+42.3
7b	$(CH_3)SH + H_4SPT \rightarrow$ 5-methyl-$H_4SPT + H_2S$	+44.2

[a]Free energy changes were derived from overall reactions using the thermodynamic properties of the reactants (Wagman et al., 1983) and partial reactions given by Keltjens and van der Drift (1986). H_4SPT, tetrahydrosarscinapterin.

A selected number of halophilic obligate methylotrophs are capable of growth on dimethyl sulfide. Methanethiol is transiently produced (Kiene et al., 1986; Oremland et al., 1989; Ni and Boone, 1991). Though this has to be experimentally verified, dimethyl sulfide conversion might represent another variation on the theme of methanol conversion. By the participation of some specific methyltransfer reactions (Equations 4, 5 and 7a–7b in Table 5.8), CH_3S-CoM and 5-methyl-H_4SPT are formed, that subsequently are metabolized according to established routes. Calculation shows that the S-methyl transfer reactions are very endergonic, even under standard conditions. Due to the toxicity of the substrates or H_2S, a reaction product, methyl sulfides, can be administered only in low concentrations (5–10 mM), which make the *in vivo* reactions thermodynamically even more unfavorable. Trimethylamine-grown cells appeared to be incapable of dimethyl sulfide conversion, whereas organisms cultured with the latter substrate used both methylamines and methanol (Oremland et al., 1989). This suggests that dimethyl sulfide metabolism is dependent on distinct inducible enzymes.

5.4 Metabolic Regulation in Methylotrophic Metabolism

Methanosarcina species are the most metabolically versatile methanogens (Table 5.1). This enables the organisms to adapt to changes in substrate supply in their natural habitats as opposed to obligate hydrogenotrophs or acetotrophs. It is to be expected that these organisms respond, within the limits of their metabolic potential, to changes in the environment. Regulation could be on the level of enzyme synthesis, enzyme activity, or both. In the following we will restrict the discussion to regulation during growth on hydrogen and CO_2, methanol, acetate and combinations of those substrates. To facilitate the discussion, a comprehensive scheme of methanogenesis from the three substrates is shown in Figure 5.10.

Methanol is the common substrate for all methylotrophs (Table 5.1) and, in general, the enzymes involved in methanol utilization are constitutive: the compound is converted by whole cells and cell extracts irrespective of the conditions of pre-growth (Smith and Ma, 1978; Ferguson and Mah, 1983; Krzycki et al., 1983; Eikmanss and Thauer, 1984; see also Section 5.3 and references therein). A notable exception is *M. thermophila,* in which methanol catabolism appears to be inducible (Zinder and Elias, 1985). The potential for growth on hydrogen and CO_2 is limited to some *Methanosarcina* species (Table 5.1) and is often strain-dependent. Organisms like *M. acetivorans, M. mazei* or *M. thermophhila* characteristically contain low hydrogen uptake activities (Nelson and Ferry, 1984; Boone et al., 1987; Ahring et al., 1991). During mixotrophic growth on methanol and hydrogen plus CO_2, both substrates are simultaneously consumed (Ferguson and Mah, 1983; Kenealy and Zeikus, 1982, Müller et al., 1986).

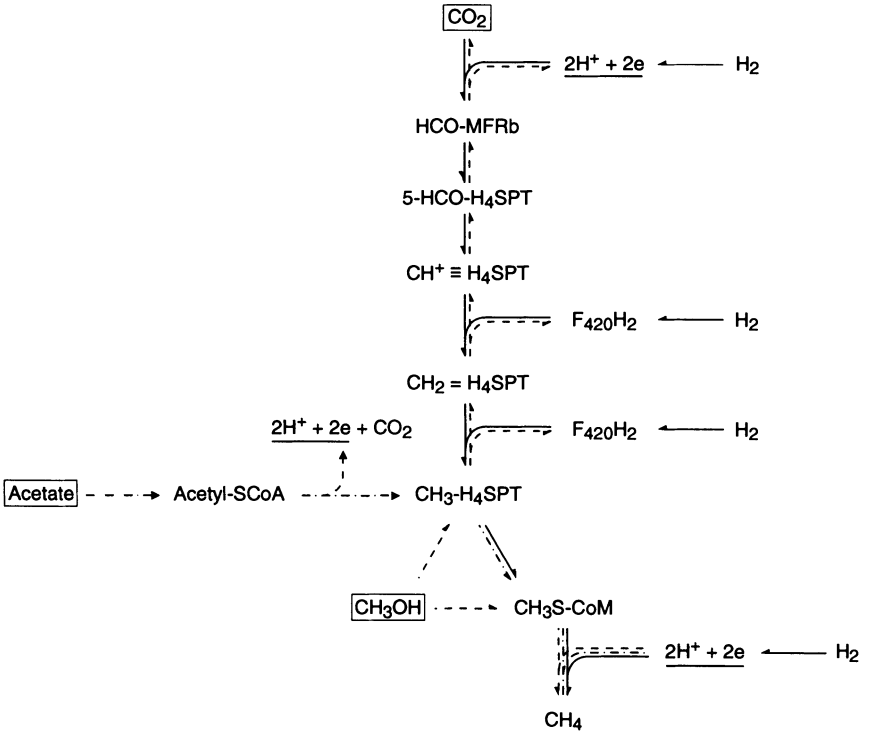

Figure 5.10. Interconnection of the routes of methanogenesis from hydrogen and CO_2 (——), methanol (— — —) and acetate (— · —). Abbreviations: MFRb, methanofuran-b; H_4SPT, tetrahydrosarcinapterin.

However, methanol is preferentially reduced to methane with hydrogen as the electron donor. In contrast, methanol oxidation and CO_2 reduction, which require energetically unfavorable initial reactions, are inhibited. Here, regulation of the metabolic flow takes place at the level of enzyme activity.

With acetate as the co-substrate growth is usually biphasic. This has been documented for growth on H_2 plus CO_2 and acetate (Ferguson and Ma, 1983), methanol and acetate (Smith and Mah, 1978; Mah et al., 1983; Zinder and Elias, 1985) and trimethylamine and acetate (Blaut and Gottschalk, 1982). In these cases acetate is only utilized when the other substrates are consumed, suggesting that the aceticlastic machinery is subject to repression and derepression. Indeed, enzyme patterns of extracts of acetate- and methanol-grown *M. thermophila* show multiple differences (Jablonski et al., 1990). The levels of the enzymes required in acetate activation, acetate kinase and phosphotransacetylase, are substantially higher in acetate-cultured organisms (Blaut and Gottschalk, 1982; Aceti and

Ferry, 1988; Lundie and Ferry, 1989). The amount of acetyl-CoA synthetase is specifically enhanced (Terlesky et al., 1986). This also holds for partial reactions catalyzed by the enzyme, notably CO oxidation (Krzycki et al., 1982; Nelson and Ferry, 1984) and isotope exchange between CO_2 and the carbonyl group of acetate (Eikmanns and Thauer, 1984). After growth on methanol the latter reaction could not be detected: the organism then was even incapable of acetate degradation (Eikmanns and Thauer, 1984). Furthermore, the cellular concentration of H_4SPT, the acceptor of the methyl group in acetyl cleavage, is increased (Table 5.4). MT2 present in acetate-cultured *M. barkeri* differs from the one in methanol-grown cells (Grahame, 1989). The former isozyme may be involved in the 5-methyl-H_4SPT:HS-CoM methyltransferase reaction, whereas the latter is active in the methanol:HS-CoM methyltransfer step. Hydrogenase activities and patterns may be different after growth on acetate (Nelson and Ferry, 1984; Bhatnagar et al., 1987). Finally, acetate-grown *Methanosarcina* specifically contains carbonic anhydrase (Karrasch et al., 1989a) and an additional cytochrome *b* (Kühn and Gottschalk, 1983). Thus, a whole series of enzyme levels are increased; the enzymes required for methyl-H_4SPT oxidation, however, seem to be suppressed (Table 5.5). Clearly, regulation takes place at a genetic level. It has frequently been observed that organisms require long periods of adaptation on acetate before this substrate is utilized. Conversely, cultures grown in the absence of the compound often lose the ability to use acetate. The mechanism underlying the genetic regulation is unknown.

Yet regulation by enzyme synthesis in mixotrophic growth is not the only factor involved. As mentioned, growth on hydrogen plus CO_2 and acetate is biphasic. Here regulation may also take place at the level of enzyme activity. Hydrogen is a potent inhibitor of acetate catabolism, also in acetate-adapted organisms (Ferguson and Mah, 1983; Eikmanns and Thauer, 1984; Boone et al., 1987; Fischer and Thauer, 1990a; Ahring et al., 1991). The site of inhibition is likely to be the acetyl-CoA cleavage reaction (8).

$$\text{Acetyl-CoA} + H_4\text{SPT} \rightarrow \text{HS-CoA} + \text{5-methyl-}H_4\text{SPT} + CO_2 + 2\,H^+ + 2e,\ \Delta G^{\circ\prime} = +41\ \text{kJ/mol};\ E^{\circ\prime} = -200\ \text{mV} \qquad (8)$$

Under standard conditions the reaction is very endergonic and shows a high $E^{\circ\prime}$ of -200 mV (Thauer, 1990). Since four products are formed from two substrates, the midpoint potential is highly dependent on the concentrations of the reactants. During acetate conversion, organisms maintain a steady state partial pressure of approximately 30 Pa H_2 (Lovley and Ferry, 1985; Boone et al., 1987; Ahring et al., 1991) which equals an $E' = -310$ mV. Assuming the hydrogen pressure to be in equilibrium with the redox state within the cell, the latter value also may hold for reaction 5.8. Thus, by tediously balancing the ratios of the substrates

and products, acetyl-CoA cleavage would become feasible. It will be clear that addition of hydrogen has a dramatic negative impact on the reaction. Usually, acetate consumption starts when hydrogen is depleted.

Diauxy observed during growth on methanol and acetate will likely be the combined result of the regulation of enzyme synthesis and enzyme activities. Non-acetate-adapted cells only started to convert acetate when methanol was consumed (Smith and Mah, 1978; Mah et al., 1982). In acetate-adapted cells, in which acetoclastic apparatus is active, acetate and methanol are co-metabolized (Zinder and Mah, 1979; Krzycki et al., 1982; Zinder and Elias, 1985). In this case the substrates have to compete for common one-carbon (HS-CoM and H_4SPT) and electron (ferredoxin) carriers (Fig. 5.10). The substrate flow in mixotrophic catabolism shows a marked change compared to unitrophic growth with either of both compounds. Methanol reduction to methane is enhanced and the extra reducing equivalents required are derived from oxidation of the methyl group of acetate to CO_2. Hereby, competition for common substrates may be circumvented.

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6

Fermentation of Acetate

James G. Ferry

6.1 Introduction

It was 100 years after the discovery of "combustible air" by Alessandro Volta in 1776 that acetate was first implicated as a substrate for methanogenesis. In 1876, Hoppe-Seyler demonstrated that sewage sludge produced methane when amended with acetate. Later, Hoppe-Seyler (1887) showed that enrichment cultures converted the substrate to equimolar amounts of methane and carbon dioxide. Nearly 40 years later, Söehngen (1906) described Gram-negative sarcina and a filamentous rod-shaped microorganism in acetate-utilizing enrichment cultures. However, nearly another 40 years elapsed before Schnellen (1947) described the first pure cultures (of *Methanosarcina barkeri*) which grew on acetate. Growth was slow and subsequent isolates also produced methane from acetate at rates which were considered too low to account for methanogenesis in the environment. As a result, it was hypothesized that cocultures were required for the rapid conversion of acetate to methane. During the 1960s, it became evident that most of the methane in freshwater environments is derived from acetate (Jeris and McCarty, 1965; Smith and Mah, 1966), a development which created a renewed interest in methanogenic acetotrophs. In the late 1970s and early 1980s, it was shown that pure cultures of *Methanosarcina* converted acetate to methane and carbon dioxide in defined media and at rates much greater than previously reported (Mah et al., 1978; Weimer and Zeikus, 1978; Smith and Mah, 1980). These important milestones showed that significant rates of acetate conversion in nature do not require cocultures or cosubstrates, and sparked studies on the microbiology, physiology and biochemistry of acetate utilization during the 1980s and into the present. The current understanding of the pathway provides impressions of

nature's solutions to the problems of methyl transfer, electron transport, energy conservation, and gene regulation which are unique to the methanogenic conversion of acetate. The pathway is now at a level of understanding that will permit productive investigations of these and other interesting questions.

6.2 Microbiology

6.2.1 Ecology

The methanogenic decomposition of organic matter in most anaerobic freshwater habitats requires microbial consortia composed of at least three interacting metabolic groups. The first group (fermentative bacteria) degrades polymers to H_2, CO_2, formate, acetate, and higher volatile fatty acids. The second group (the acetogenic bacteria) oxidizes the higher acids to acetate and H_2 or formate. The third group (methane-producers) employs two independent pathways for the utilization of H_2, formate, or acetate as substrates for growth. One pathway involves the reduction of CO_2 with electrons derived from the oxidation of H_2 or formate (CO_2-reducers). The other pathway involves a fermentation of acetate by reduction of the methyl group to methane and oxidation of the carbonyl group to CO_2 (acetotrophs).

Most of the methane produced in nature originates from acetate; however, the relative amounts of methane produced from the methyl group of acetate or reduction of CO_2 can vary depending on the presence of other metabolic groups of anaerobes and the environment. Homoacetogenic microorganisms oxidize H_2 or formate and reduce CO_2 to acetate, a process which increases the amount of methane derived from acetate and the importance of methanogenic acetotrophs. A non-methanogenic microorganism called AOR (*a*cetate *o*xidizing *r*od) has been described which oxidizes acetate to H_2 and CO_2 (Zinder and Koch, 1984). The extent to which microorganisms like the AOR occur in anaerobic environments is unknown; however, their presence would diminish the relative importance of acetotrophic methane producers. In marine environments, the acetotrophic sulfate-reducing bacteria dominate (see Part I, Chapter 3); thus, when sulfate is present, the reduction of CO_2 and conversion of methylamines is the major route of methanogenesis. Finally, acetotrophs grow more slowly than CO_2-reducers and, therefore, methane from acetate is not likely to predominate where the residence time for organic matter is short.

Although acetate is a major precursor for methane formation, only two genera of methanogenic acetotrophs (*Methanosarcina* and *Methanothrix*) and a few species have been described. The genera cluster in one branch of the *Methanomicrobiales* (Stackebrandt et al., 1982; Macario and de Macario, 1987) along with other methane-producing methylotrophs. It has been proposed to separate this

branch from the *Methanomicrobiales* to form a new order, the *Methanosarcinales* (Part I, Chapter 1). The *Methanosarcina* are the most versatile of all methanogenic species and have the capability for growth on H_2-CO_2, methanol, methylamines, and acetate. Some *Methanosarcina* species grow weakly on H_2-CO_2 or may require a period of adaptation (Sowers et al., 1984; Zinder et al., 1985; Mukhopadhyay et al., 1991); *Methanothrix*, however, is able to utilize only acetate. The *Methanosarcina* grow on acetate more rapidly than *Methanothrix* species, but the K_m and minimum threshold concentrations for growth are much greater than for the *Methanothrix* (Min and Zinder, 1989; Westermann et al., 1989b; Westermann et al., 1989c; Ohtsubo et al., 1992); as a result, *Methanosarcina* species thrive in environments where acetate concentrations are high and *Methanothrix* species predominate where acetate is low (Zinder et al., 1984).

6.2.2 Growth and Physiology

(a) *Methanosarcina*

Generally, the *Methanosarcina* grow in large aggregates, a condition which may facilitate survival and (or) recovery of H_2 produced during growth on acetate (see below). Several *Methanosarcina* species exhibit life cycles in which aggregates or cysts dissociate into single cells. The dissociation may be in response to changes in environmental conditions in order to provide a means of dispersal (Mah, 1980; Sowers et al., 1984; Robinson, 1986). Cells cultured in high salt loose the outer heteropolysaccharide layer and grow as single cells instead of in large aggregates, a condition which will undoubtedly facilitate development of genetics in this genus (Sowers and Gunsalus, 1988). At 30 mM Mg^{2+} or less, acetate-grown strain TM-1 appears as aggregates; however, above 100 mM Mg^{2+} the organism grows as single cells, a result which implicates a role for Mg^{2+} and not Na^+ in the production and stability of single cells grown on acetate (Ahring, Alatriste-Mondragon, et al., 1991).

M. thermophila strain TM-1 (Zinder et al., 1985) has a temperature optimum for growth at 50°C and is unable to grow at 60°C (Zinder and Mah, 1979). The organism is capable of growth on acetate in a defined mineral salts medium which contains *p*-aminobenzoic acid (Murray and Zinder, 1985). Sulfanilamide inhibits growth, a result consistent with a requirement for *p*-aminobenzoic acid in the synthesis of folate or sarcinapterin. Acetate-grown batch cultures of *M. thermophila* strain TM-1 can be limited for nitrogen at NH_4^+ concentrations below 2.0 mM. Strains of *M. barkeri* also grow on acetate in defined mineral salts media (Weimer and Zeikus, 1978; Scherer and Sahm, 1980; Smith and Mah, 1980). The lower growth yields and longer generation times in mineral *versus* complex media is likely the result of the availability of assimilable carbon precursors (Hutten et al., 1980). Growth in complex media also has a positive effect on the

ability of H_2-CO_2-grown *M. barkeri* strain 227 to adapt to growth on acetate (Ferguson and Mah, 1983), possibly by supplying growth factors or compounds for the synthesis of cofactors specifically required in the pathway. Acetate-grown *Methanosarcina* can be cultured on a large scale in a pH auxostat (Sowers et al., 1984). This method provides a constant pH and substrate concentration and rapidly produces high yields of acetate-grown cells, a development which has facilitated biochemical studies.

Inhibition of methanogenesis from acetate has detrimental effects on the rate of conversion of organic matter to methane and carbon dioxide in anaerobic digestors (see Part I, Chapter 3). Both H_2 and formate have been reported to inhibit methanogenesis from acetate, either in pure cultures or enrichments. Since formate and H_2-CO_2 are interconvertable by a diversity of anaerobes, specific inhibition by either of these compounds in enrichment cultures is uninterpretable (Pretorius, 1972; Baresi et al., 1978; Guyot and Ramirez, 1989). Growth and methanogenesis by cultures of *M. barkeri* strain MS (Hutten et al., 1980) are not inhibited by formate. However, formate (10 mM) inhibits methanogenesis from acetate in pure cultures of *M. barkeri* strain 227 and *M. thermophila* strain TM-1 (Guyot, 1986); H_2 was not evolved, a result which is consistent with a specific inhibition by formate. Another study with strain TM-1 reported H_2 evolution from formate (Zinder and Anguish, 1992). Low concentrations of H_2 (approximately 250 to 5000 Pa) inhibit methanogenesis from acetate in pure cultures (Hutten et al., 1980; McInerney and Bryant, 1981; Ferguson and Mah, 1983; Ahring et al., 1991b; Zinder and Anguish, 1992); however, not all strains are inhibited at the same concentrations (Boone et al., 1987; Krzycki et al., 1987).

The amount of free energy available for the conversion of H_2-CO_2, methanol, or trimethylamine to methane is considerably larger than that for the conversion of acetate to methane (Part II, Chapter 5). Thus, it is not surprising that growth on a mixture of acetate and another substrate is biphasic (Smith and Mah, 1978; Zinder and Mah, 1979; Scherer and Sahm, 1980; Hutten et al., 1980; Blaut and Gottschalk, 1982; Ferguson and Mah, 1983). Two-dimensional gel electrophoresis of cell extract proteins from *M. thermophila* reveal more than 100 mutually exclusive peptides in acetate- and methanol-grown cells (Jablonski et al., 1990). This regulation of enzyme synthesis, in response to the growth substrate, presents an opportunity for investigations into the molecular basis for transcriptional regulation in the "*Archaea*." Whole cell studies with *M. thermophila* grown on mixtures of substrates are consistent with a model for regulation in which methanol represses the synthesis of enzymes specific for methanogenesis from acetate (Zinder and Elias, 1985). It has also been proposed that differences in the specific growth rates on methanol and acetate, together with changes of pH, may influence diauxic growth (Hutten et al., 1980). An understanding of regulation will require a molecular understanding of transcription. Transcription in the "*Archaea*" domain appears to be fundamentally different from the "*Bacteria*". The DNA-dependent

RNA polymerases from the "*Archaea*" have a subunit composition more like the eucaryal nuclear polymerases than the polymerases from the "*Bacteria*". These differences are reflected in the different consensus promoter sequences between the domains (Hausner et al., 1991).

(b) *Methanothrix*

The proposal that the name *Methanothrix* be changed to *Methanosaeta* (Patel and Sprott, 1990; Boone, 1991; Patel, 1992) is still under consideration; therefore, the name *Methanothrix* is used in this chapter only because it is most familiar in the existing literature.

Unlike the more generalist *Methanosarcina*, the *Methanothrix* utilize only acetate as a substrate for growth and methanogenesis. Although *Methanothrix* sp. CALS-1 contains formate dehydrogenase, this organism does not utilize formate and formate-hydrogen lyase activity is not present (Zinder and Anguish, 1992). Earlier reports that formate is converted to H_2 and CO_2 by *M. soehngenii* (Zehnder et al., 1980; Huser et al., 1982) have not been confirmed. However, an organism with a cellular morphology similar to *Methanothrix* has been described called the TAM (*t*hermophilic *a*cetate-utilizing *m*ethanogenic) organism which utilizes H_2-CO_2 and formate as substrates (Ahring and Westermann, 1984; Ahring and Westermann, 1985). The optimum temperatures for the TAM organism and thermophilic strains of *Methanothrix* are near 70°C, which is higher than for the *Methanosarcina*; thus, it appears that the optimum temperature for methanogenesis from acetate is near 70°C (Zinder, 1988; Zinder, 1990). The first description of *M. soehngenii* reported that the organism grows in a mineral salts medium (Zehnder et al., 1980; Huser et al., 1982) and most strains which have been described since then are able to grow in chemically defined media. *M. soehngenii* strain CALS-1 requires biotin (Zinder et al., 1987) and growth of *M. concilii* is stimulated by *p*-aminobenzoic acid, biotin and thiamine (Patel et al., 1988). Methanogenesis from acetate by *M. soehngenii* strain VNBF is stimulated by the addition of Ni, Co, and Mo (Fathepure, 1987); however, methanogenesis is inhibited at concentrations greater than 5 μM of these metals. Interestingly, formate (5 mM) inhibits methanogenesis from acetate by *M. soehngenii* strain VNBF (Fathepure, 1983). Neither formate (10 mM) or H_2 (40 kPa) inhibits methanogenesis from acetate by strain CALS-1 (Zinder and Anguish, 1992).

6.3 Biochemistry

6.3.1 History and Perspective

Several hypotheses for the general mechanism of acetate conversion to methane were introduced by early investigators. In 1936, the evidence indicated that CO_2

was reduced to CH_4 with $4H_2$ generated by the complete oxidation of acetate to two molecules of CO_2 (Barker, 1936). Later, using ^{14}C-labeled acetate, it was shown that most of the CH_4 was derived from the methyl group and little from the carboxyl carbon, a result which ruled out the CO_2 reduction theory (Buswell and Sollo, 1948; Stadtman and Barker, 1949; Stadtman and Barker, 1951). These studies were extended to show that the hydrogen (deuterium) atoms of the methyl group are transferred intact to CH_4 (Pine and Barker, 1956). Additional studies led to the conclusion that the ultimate step in methanogenesis from all substrates (reduction of CO_2 or conversion of the methyl group of other substrates) is the reductive demethylation of a common precursor X-CH_3 (Stadtman and Barker, 1951; Pine and Vishniac, 1957). Later, another theory was advanced in which acetate is reduced to $2CH_4$ with exogenously supplied H_2 (Zeikus et al., 1975); however, this mechanism was ruled out by the finding that acetate is a sole source for growth and methanogenesis by pure cultures.

Most acetate-utilizing anaerobes from the "*Bacteria*" domain cleave acetyl-CoA followed by oxidation of the methyl and carbonyl groups to CO_2 and reduction of an exogenous electron acceptor (Thauer et al., 1989). The acetotrophic methane-producing "*Archaea*" also cleave acetate; however, the methyl group is reduced to methane with electrons derived from oxidation of the carbonyl group to CO_2 (reaction 13, Table 6.1). Thus, the conversion of acetate to CH_4 and CO_2 is a fermentation. The phylogenetic extremes between the two domains raises questions regarding the comparative biochemical mechanisms of acetate activation and cleavage. Recently, two short reviews have summarized the biochemistry and energetics of the fermentation of acetate (Ferry, 1992; Jetten and Stams et al., 1992).

6.3.2 General Features of the Pathway

Figure 6.1 depicts the pathway as it is currently understood in the *Methanosarcina* and, with only minor modifications, in *Methanothrix*. In the pathway, acetate is activated to acetyl-CoA followed by cleavage of the carbon-carbon and carbon-sulfur bonds (decarbonylation) catalyzed by the nickel/iron-sulfur component of the CODH (CO dehydrogenase) enzyme complex. The nickel/iron-sulfur component oxidizes the carbonyl group to CO_2 and reduces a ferredoxin. The methyl group is transferred to the corrinoid/iron-sulfur component within the complex and finally to coenzyme M (HS-CoM) catalyzed by at least two methyltransferases. The CH_3-S-CoM is reductively demethylated to methane with electrons derived from the sulfur atoms of CH_3-S-CoM and HS-HTP which results in formation of the heterodisulfide CoM-S-S-HTP. Reduction of the heterodisulfide to the corresponding sulfhydryl forms of the cofactors is accomplished with electrons derived from reduced ferredoxin; however, the electron transport chain from the ferredoxin to the heterodisulfide is unknown.

Table 6.1 Reactions involved in the fermentation of acetate by *Methanosarcina* and *Methanothrix*

Reaction	$\Delta G^{\circ\prime}$	$\Sigma\Delta G^{\circ\prime(a)}$	$\Sigma\Delta G^{\circ\prime(b)}$
(1) $CH_3COO^- + ATP \rightarrow CH_3CO_2PO_3^{2-} + ADP^{(c)}$	+13	+13	—
(2) $CH_3CO_2PO_3^{2-} + CoA \rightarrow CH_3COSCoA + Pi^{(c)}$	−9	+4	—
(3) $CH_3COO^- + CoA + ATP \rightarrow CH_3COSCoA + AMP + PPi^{(c)}$	—	—	−6
(4) $CH_3CO-S-CoA + H_4SPT \rightarrow CO + CH_3-H_4SPT + CoA^{(c)}$	+62	+66	+56
(5) $CH_3-H_4SPT + HS-CoM \rightarrow CH_3-S-CoM + H_4SPT^{(c)}$	−29	+37	+27
(6) $CH_3-S-CoM + HS-HTP \rightarrow CH_4 + CoM-S-S-HTP^{(c)}$	−43	−6	−16
(7) $CO + H_2O \rightarrow CO_2 + H_2^{(c)}$	−20	−26	−36
(8) $CoM-S-S-HTP + H_2 \rightarrow HS-CoM + HS-HTP^{(c)}$	−42	−68	−78
(9) $ADP + Pi \rightarrow ATP^{(c)}$	+32	−36	—
(10) $AMP + ATP \rightarrow 2ADP^{(d)}$	0	—	−78
(11) $PPi \rightarrow 2Pi^{(d)}$	−22	—	−100
(12) $2ADP + 2Pi \rightarrow 2ATP^{(d)}$	+64	—	−36
(13) $CH_3COO^- + H^+ \rightarrow CH_4 + CO_2^{(c)}$	−36	—	—

[a]Sum of reactions for *Methanosarcina*.
[b]Sum of reactions for *Methanothrix*.
[c]$\Delta G^{\circ\prime}$ values (kJ/mol) were taken from Section II, Chapter 5.
[d]$\Delta G^{\circ\prime}$ values (kJ/mol) were taken from Jetten and Stams et al. (1992).

6.3.3 Activation of Acetate to Acetyl-CoA

As early as 1967, a requirement for "active acetate" prior to cleavage was proposed (Stadtman, 1967). Methane formation from acetyl phosphate and acetyl-CoA by cell extracts of *M. barkeri* implicated these compounds as potential activated forms (Grahame and Stadtman, 1987b; Fischer and Thauer, 1988). *M. thermophila* synthesizes acetate kinase and phosphotransacetylase only when grown on acetate (Aceti and Ferry, 1988; Lundie and Ferry, 1989), a result which is consistent with acetate activation by reactions 1 and 2 (Table 6.1). In *M. soehngenii*, acetyl-CoA synthetase (Kohler and Zehnder, 1984) catalyzes the activation of acetate to acetyl-CoA (reaction 3, Table 6.1). Half-maximal rates for the synthetase were obtained at 0.86 mM acetate (Jetten et al., 1989a), a value that is considerably lower than the 22 mM obtained for the acetate kinase from *M. thermophila* and consistent with the ability of *Methanothrix* to outcompete the *Methanosarcina* for low concentrations of acetate in the environment (Jetten et al., 1990a). One ATP equivalent is expended for each acetate metabo-

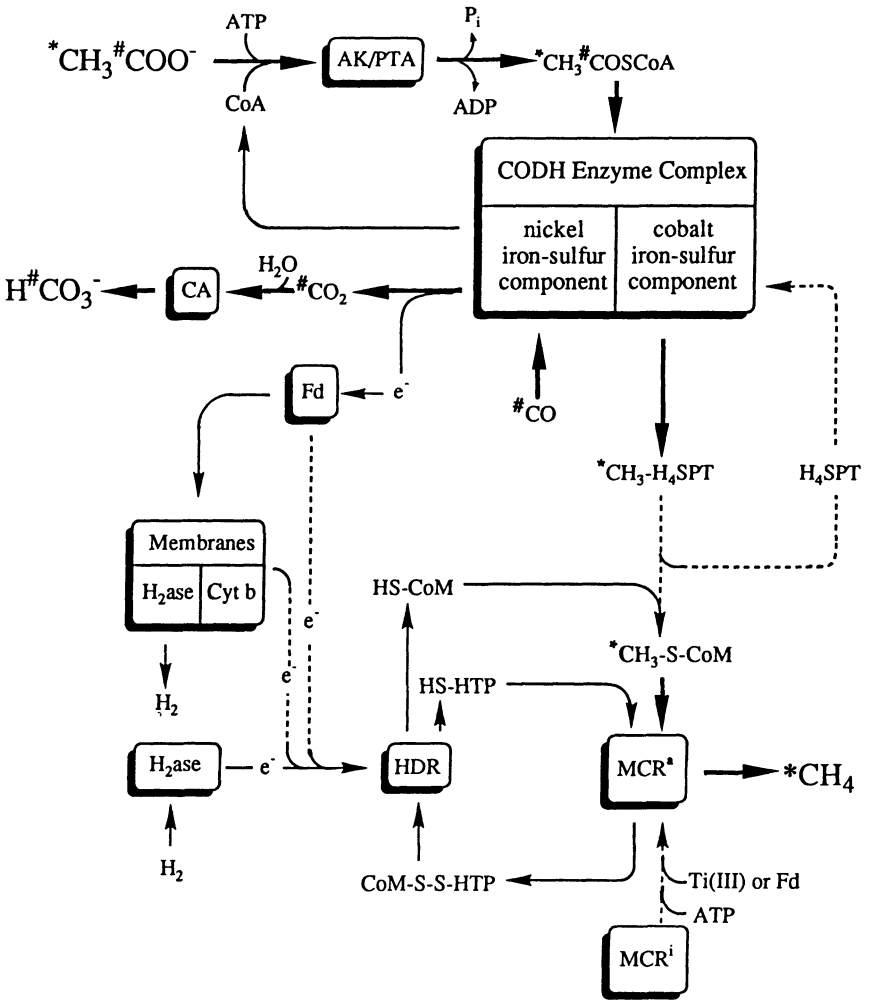

Figure 6.1. Proposed pathway for the conversion of acetate to CO_2 and CH_4 in *Methanosarcina*. AK, acetate kinase; PTA, phosphotransacetylase; H_4SPT, tetrahydrosarcinapterin; CA, carbonic anhydrase; MCR^i, inactive methylreductase; MCR^a, active methylreductase; HDR, heterodisulfide (CoM-S-S-HTP) reductase; Fd, ferredoxin; CODH, carbon monoxide dehydrogenase; cyt b, cytochrome b; H_2ase, hydrogenase. Dashed lines represent gaps in understanding of the pathway. The carbon atoms of acetate are marked with a (*) and a (#) symbol to distinguish between the carboxyl and methyl groups. By permission from Ferry (1992).

lized, a considerable investment for both *Methanosarcina* and *Methanothrix* when considering the small amount of energy available for ATP synthesis (reaction 13, Table 6.1).

Although *M. thermophila* is a strict anaerobe, the acetate kinase is stable in air and the enzyme is purified from the soluble fraction as an α_2 homodimer with a subunit M_r of 53,000 (Aceti and Ferry, 1988). The K_m for ATP is 2.8 mM. The nucleotide triphosphates TTP, ITP, UTP, and GTP substitute for ATP with retention of greater than 80% of the activity. The air-stable monomeric (M_r = 42,000) phosphotransacetylase is purified from the soluble fraction of *M. thermophila* (Lundie and Ferry, 1989). The activity of this enzyme is stimulated over seven-fold by the presence of 50 mM potassium or ammonium ions; however, phosphate, arsenate, and sulfate are inhibitory. The K_m for CoA is 91 μM and the K_m for acetyl-PO_3^{2-} is 165 μM. The acetyl-CoA synthetase from *M. soehngenii* is also an air-stable enzyme and is isolated from the soluble fraction as an α_2 homodimer with a subunit molecular mass of 73,000 Da (Jetten et al., 1989a). The K_m for CoA is 48 μM. Based on the amino acid sequence deduced from the gene, the synthetase appears to have two ATP-binding domains similar to other ATP-binding proteins (Eggen, Geerling, Boshoven, et al., 1991).

6.3.4 Carbon-Carbon and Carbon-Sulfur Bond Cleavage of Acetyl-CoA

The central enzyme in the pathway of methanogenesis from acetate is CO dehydrogenase (CODH), which catalyzes the cleavage of acetyl-CoA. CODHs are wide spread among anaerobes and serve a variety of functions. Several anaerobes from the "*Bacteria*" domain oxidize acetate completely to CO_2 and reduce various electron acceptors (Thauer et al., 1989); most contain CODH which cleaves acetyl-CoA yielding the methyl and carbonyl groups, both of which are oxidized to CO_2. The homoacetogenic bacteria employ CODH (acetyl-CoA synthase) in the energy-yielding Ljungdahl-Wood pathway to catalyze the synthesis of acetyl-CoA. CODH is also utilized by the methanogenic "*Archaea*" for cell carbon synthesis from CO_2 (Ladapo and Whitman, 1990). Microorganisms from the "*Archaea*" and "*Bacteria*" domains are at the extremes of bacterial evolution (Woese et al., 1990) and comparative studies of CODHs from these widely divergent organisms are likely to enhance an understanding of the mechanism and molecular evolution of this key enzyme.

Much of what is known regarding the mechanism of acetate cleavage is based on the prior understanding of acetyl-CoA synthesis from two molecules of CO_2 in the Ljungdahl-Wood pathway (Ragsdale, 1991). In the pathway, one CO_2 is reduced to the methyl level in the form of CH_3-THF (methyltetrahydrofolate) and the other CO_2 is reduced to the redox level of CO which is bound to CODH (acetyl-CoA synthase). The methyl group of CH_3-THF is transferred to a corrinoid/iron-sulfur (corrinoid/Fe-S) protein which then transfers the methyl group to the

CODH. The CODH catalyzes acetyl-CoA synthesis from the CODH-bound methyl, CO, and CoA moieties. The first indication of an involvement of CODH in methanogenesis from acetate came from the observation that CO-oxidizing activity was elevated in acetate-grown cells of *M. barkeri* strain MS (Krzycki et al., 1982). Later, it was shown that antibodies to CODH inhibited methane production from acetate in cell extracts of *M. barkeri* (Krzycki et al., 1985). Furthermore, CODHs purified from acetate-grown *Methanosarcina* and *Methanothrix* constitute a major portion of total cell protein (Krzycki and Zeikus, 1984b; Terlesky et al., 1986; Grahame and Stadtman, 1987a; Jetten et al., 1989b). Finally, biochemical evidence for an acetyl-CoA cleavage function was obtained by the demonstration of an exchange of the carbonyl group of acetyl-CoA with CO (Jetten and Hagen et al., 1991; Raybuck et al., 1991).

(a) The CODH Enzyme Complex from *M. Thermophila*

In *M. thermophila*, CO-oxidizing activity is present in an enzyme complex which catalyzes the synthesis of acetyl-CoA (Abbanat and Ferry, 1991) and cleavage of the C-C and C-S bonds of acetyl-CoA (Raybuck et al., 1991). Because the enzyme complex oxidizes CO to CO_2, it is commonly referred to as a CODH enzyme complex; however, the primary function is cleavage of acetyl-CoA during growth on acetate. The five-subunit *M. thermophila* CODH complex can be resolved into two enzyme components: a 200,000 Da CO-oxidizing nickel/iron-sulfur (Ni/Fe-S) component which contains 89,000 Da and 19,000 Da subunits, and a 100,000 Da corrinoid/iron-sulfur (Co/Fe-S) component which contains 60,000 Da and 58,000 Da subunits (Abbanat and Ferry, 1991). The fifth subunit (71,000 Da) of the complex has not been characterized. The CO-reduced enzyme complex has a spin-coupled Ni-Fe-C center with an EPR spectrum indistinguishable from the Ni-Fe-C center of the CO-reduced CODH (acetyl-CoA synthase) from the homoacetogenic organism *Clostridium thermoaceticum* (Terlesky et al., 1987). A structure is proposed for the center with the composition $[NiXFe_{3\text{-}4}S_4]$-C≡O where X bridges the Ni and Fe components (Kumar and Ragsdale, 1992). The reduced Ni-Fe-C center in the *C. thermoaceticum* enzyme is the proposed site for synthesis of the acetyl moiety of acetyl-CoA from CO and a methyl group donated by the methylated corrinoid/Fe-S protein (Lu et al., 1990; Lu and Ragsdale, 1991); thus, the Ni-Fe-C center in the Ni/Fe-S component from *M. thermophila* is the proposed site for cleavage of the acetyl moiety of acetyl-CoA (Abbanat and Ferry, 1991). *M. barkeri* strains MS and 227 and *M. mazei* strain S-6 produce small amounts of acetate when grown on H_2-CO_2, methanol, or trimethylamine (Westermann et al., 1989a); however, it is unknown whether acetate synthesis is catalyzed by the same enzymes involved in acetate cleavage.

The involvement of a corrinoid cofactor in methanogenesis from acetate had

been suspected for several years (Stadtman, 1967). Corrinoids are abundant in acetate-grown cells of *Methanosarcina* (Scherer and Sahm, 1980; Stupperich and Krautler, 1988; Silveira et al., 1991) and *Methanothrix* (Kohler, 1988). Evidence has accumulated for an involvement of corrinoids in methyl transfer reactions from studies with whole cells and cell extracts (Eikmanns and Thauer, 1985; van de Wijingaard et al., 1988; Patel and Sprott, 1990); particularly as the immediate methyl acceptor (or donor) for acetyl-CoA cleavage (or synthesis) (Laufer et al., 1987). Recent studies with the CODH enzyme complex from *M. thermophila* have provided direct biochemical evidence for involvement of factor III (Coα-[α-(5-hydroxybenzimidazolyl)]-cobamide) in the initial methyl transfer reactions leading to CH_3-S-CoM (Abbanat and Ferry, 1991). The Co/Fe-S component of the CODH enzyme complex contains factor III, the cobalt atom of which is reduced to the Co^{1+} state with electrons donated directly by the Ni/Fe-S component. In this redox state, Co^{1+} is a supernucleophile which is able to accept a methyl group.

(b) Mechanism of Acetate Cleavage in *M. Thermophila*

The component enzymes in the CODH complex from *M. thermophila* have properties in common with the CODH (acetyl-CoA synthase) and corrinoid/iron-sulfur protein from *C. thermoaceticum*. The CODHs from both organisms are two-subunit, nickel-containing, iron-sulfur proteins with CO-oxidizing activity that reduce ferredoxin. Each of the CODHs are associated with a unique two-subunit corrinoid/iron-sulfur protein. Both CODHs transfer electrons to the respective corrinoid/iron-sulfur proteins in the absence of additional electron carriers. The acetyl-CoA synthase from *C. thermoaceticum* catalyzes the synthesis of acetyl-CoA from CH_3I, CO, and CoA with an absolute requirement for the corrinoid/iron-sulfur protein, which is methylated with CH_3I followed by transfer of the methyl group to the synthase (Ragsdale, 1991). The five-subunit enzyme complex from *M. thermophila* also catalyzes the synthesis of acetyl-CoA from CH_3I, CO (or CO_2 + $2e^-$) and CoA (Abbanat and Ferry, 1990).

The composition and properties of the enzyme components from the CODH complex of *M. thermophila* are consistent with a proposed acetyl-CoA cleavage mechanism (Figure 6.2) which is analogous to a reversal of the mechanism proposed for acetyl-CoA synthesis in *C. thermoaceticum*. In the proposed mechanism (Figure 6.2), the Ni/Fe-S component cleaves the C-C and C-S bonds of acetyl-CoA at the Ni-Fe site. After cleavage, the methyl group is transferred to the Co^{1+} atom of the Co/Fe-S component. The *M. thermophila* enzyme complex catalyzes an exchange of CoA with acetyl-CoA at rates five-fold greater than the *C. thermoaceticum* acetyl-CoA synthase (Raybuck et al., 1991). The difference in rates may reflect the acetyl-CoA cleavage function for the *M. thermophila* enzyme, as opposed to the *C. thermoaceticum* enzyme which functions in the

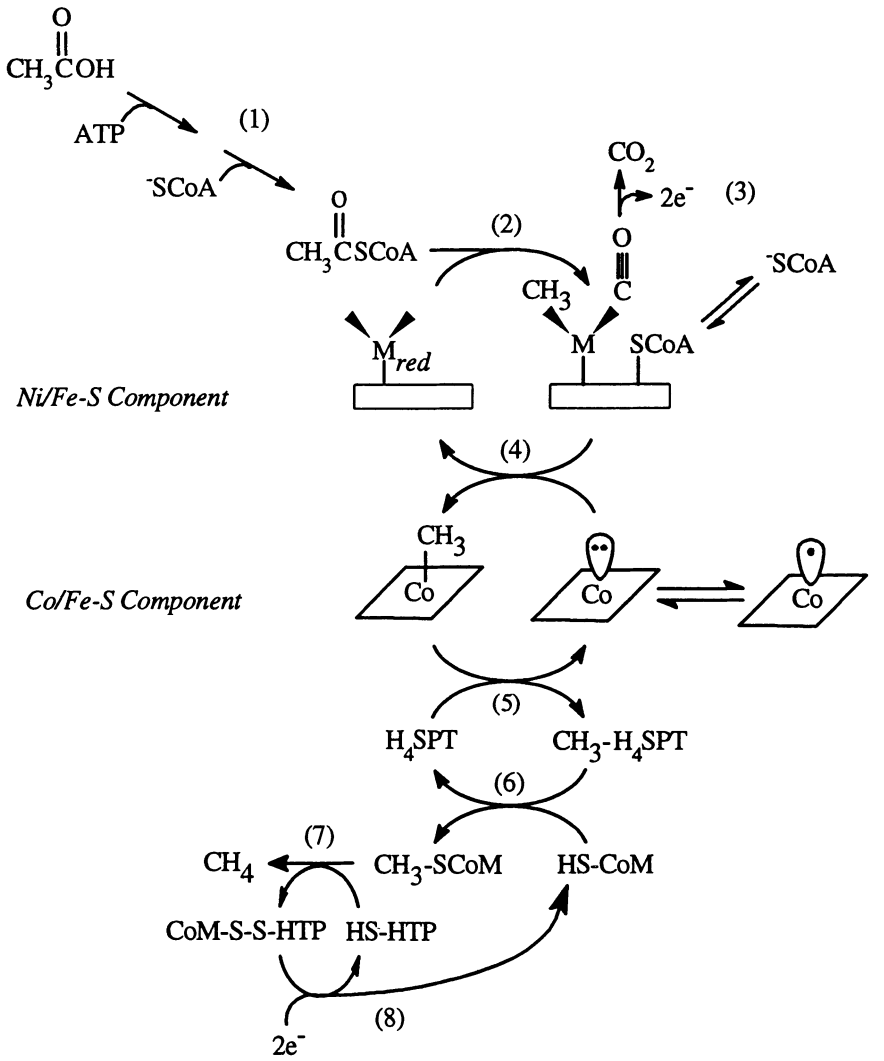

Figure 6.2. Proposed mechanism for acetyl-CoA cleavage by the CO dehydrogenase complex from *M. thermophila*. The M above the rectangle represents the active site metal center of the Ni/Fe-S component; the dots in the orbitals above the rhombus represent the Co^{2+} and Co^{1+} states of the cobamide center of the Co/Fe-S component. The numbering refers to: (1) activation of acetate, (2) cleavage by the Ni/Fe-S component, (3) oxidation of the carbonyl group, (4) methyl transfer to the Co/Fe-S component, (5) methyltransfer to tetrahydrosarcinapterin, (6) methyl transfer to coenzyme M, (7) reductive demethylation to methane, and (8) reductive regeneration of cofactors to the cooresponding sulfhydryl forms. Adapted from Jablonski et al. (1993).

biosynthesis of acetyl-CoA. The complex also catalyzes an exchange of CO with the carbonyl group of acetyl-CoA (Raybuck et al., 1991); however, the rate of exchange is an order of magnitude less than the *C. thermoaceticum* enzyme. This low rate of exchange is consistent with a low rate of exchange of CO with the carbonyl group during methanogenesis from acetate (Nelson et al., 1987) and no detectable accummulation or consumption of CO during growth on acetate (Zinder and Anguish, 1992). In addition to the C-C and C-S bond cleavage activity, it is proposed that the Ni-Fe center binds the carbonyl group and oxidizes it to CO_2. This proposed function is supported by the ability of the Ni/Fe-S component to oxidize CO and reduce a ferredoxin purified from *M. thermophila* (Abbanat and Ferry, 1991). In addition to the synthesis of acetyl-CoA from CoA, CO, and CH_3I, the complex is also able to reduce CO_2 to the carbonyl precursor with electrons from titanium (III) citrate; however, CO is preferentially incorporated into acetyl-CoA without prior oxidation to free CO_2 (Abbanat and Ferry, 1990). CO is not directly incorporated during the synthesis of acetate from CH_3I, CO_2 and CO in cell extracts or whole cell suspensions of *M. barkeri*; rather, CO is a source of electrons for reduction of CO_2 to the carbonyl precursor (Eikmanns and Thauer, 1984; Laufer et al., 1987; Fischer and Thauer, 1990b). This apparent discrepancy between *M. thermophila* and *M. barkeri* is unexplained but could be a result of rapid oxidation of CO to CO_2 in whole cells of *M. barkeri* before any significant acetate synthesis.

(c) Electron Paramagnetic Resonance (EPR) Spectroscopy of CODH

The component enzymes from the *M. thermophila* complex have been characterized in greater detail by EPR spectroscopy (Lu, Jablonski, Ferry, and Ragsdale, unpublished results; Jablonski et al., 1993). The CO-oxidizing Ni/Fe-S component contains three species of Fe-S clusters with EPR properties similar to those reported for the *C. thermoaceticum* acetyl-CoA synthase (Lindahl et. al., 1990a; Lindahl et. al., 1990b) and other CODHs from methanogenic acetotrophs (Table 2). Two of the Fe-S centers in the *M. thermophila* Ni/Fe-S component have EPR spectra typical of bacterial 4Fe-4S centers (g_{av} = 1.95 and 1.97); however, the third Fe-S center (g_{av} = 1.87) has an EPR spectrum which is atypical of 4Fe-4S centers. Presently, it is not known if the g_{av} = 1.95 and 1.97 signals derive from the same or different 4Fe-4S centers. The function of these 4Fe-4S centers is unknown but they are likely to be involved in the transfer of electrons to ferredoxin or the Co/Fe-S component. The CO-reduced Ni/Fe-S component also exhibits the spin-coupled Ni-Fe-C EPR spectrum (Lu, Jablonski, Ferry and Ragsdale; unpublished results), a finding consistent with the proposed mechanism for acetyl-CoA cleavage (Figure 6.2); however, it is not known which, if any, of the Fe atoms from the iron-sulfur centers participate in formation of the Ni-Fe center. EPR spectroscopy of the as-isolated Co/Fe-S component indicates a low-spin

Co^{2+} (Jablonski et al., 1993). There is no superhyperfine splitting from the nitrogen nucleus (I = 1/2) of the 5-hydroxybenzimadazole base in factor III, a result which indicates the absence of a lower axial ligand to the cobalt atom (base-off configuration). Redox titration of the $Co^{2+/1+}$ couple reveals a midpoint potential of −486 mV (Jablonski et al., 1993) similar to that reported for the *C. thermoaceticum* corrinoid/Fe-S protein in which the corrinoid is also in the base-off configuration (Harder et al., 1989). The base-off configuration of corrinoids changes the midpoint potential of the $Co^{2+/1+}$ couple from approximately −600 mV (base-on) to a less negative value which allows reduction to the methyl-accepting Co^{1+} redox state by physiological electron donors which have midpoint potentials in the range of −500 mV. Indeed, ATP is not required for reduction to the Co^{1+} redox state with electrons donated by the Ni/Fe-S component. The Co/Fe-S component of the *M. thermophila* enzyme complex contains a 4Fe-4S center with a midpoint potential of −502 mV which is nearly isopotential with the $Co^{2+/1+}$ couple and is probably involved in electron transfer from the Ni/Fe-S component to the cobalt atom (Jablonski et al., 1993). These properties are similar to the 4Fe-4S center present in the *C. thermoaceticum* corrinoid/Fe-S protein. The genes encoding the two subunits of the Co/Fe-S component have been cloned and sequenced (Maupin and Ferry, unpublished results); the deduced amino acid sequences share a high degree of homology with the sequence deduced from the genes encoding the subunits of the corrinoid/Fe-S protein from *C. thermoaceticum* (S. W. Ragsdale, personal communication). In summary, the biochemical properties of the enzyme components from the *M. thermophila* enzyme complex have a striking resemblance to properties of the *C. thermoaceticum* acetyl-CoA synthase and corrinoid/Fe-S protein, a finding which further supports the proposed mechanism for acetyl CoA cleavage (Figure 6.2).

The CODH characterized from *Methanosarcina barkeri* is purified as an $\alpha_2\beta_2$ oligomer with subunit M_rs of approximately 90,000 and 19,000 (Grahame and Stadtman, 1987a; Krzycki et al., 1989), similar to the CO-oxidizing Ni/Fe-S component of the *M. thermophila* complex. Recently, it was reported that the *M. barkeri* $\alpha_2\beta_2$ CODH can be purified in an enzyme complex which also contains a corrinoid protein (Grahame, 1991). The complex catalyzes cleavage of acetyl-CoA and transfer of the methyl group to tetrahydrosarcinapterin (H_4SPT), a result consistent with studies which implicate tetrahydromethanopterin (H_4MPT) as an intermediate in methanogenesis from acetate in *M. barkeri* (Fischer and Thauer, 1989). H_4SPT and H_4MPT are one-carbon carriers found exclusively in the "*Archaea*" (Part II, Chapter 1). The *M. barkeri* $\alpha_2\beta_2$ CODH component contains Ni and Fe; however, no EPR signals attributable to Ni have been reported. Six [4Fe-4S] clusters per $\alpha_2\beta_2$ tetramer are indicated by core extrusion experiments (Krzycki et al., 1989). Low temperature EPR spectroscopy has identified a 4Fe-4S cluster (Table 6.2). A second low temperature EPR signal (g_{av} = 1.89) is obtained from the reduced enzyme, which is atypical of bacterial 4Fe-4S centers

Table 6.2 Comparison of the iron-sulfur clusters in the Ni/Fe-S component from *Methanosarcina thermophila* and the CO dehydrogenases from other methanogenic *Archaea* and *Clostridium thermoaceticum*

Source of CODH	g_{av}	g values	E_m (mV)	g_{av}	g values	E_m (mV)	g_{av}	g values	E_m (mV)
Methanosarcina thermophila (Lu, Jablonski, Ferry, and Ragsdale; unpublished results)	1.87	2.02,1.87,1.72	154	1.95	2.04,1.93,1.89	−444	1.97	2.05,1.95,1.90	−540
Clostridium thermoaceticum (Ragsdale, 1991)	1.82	2.01,1.81.1.65	−220	1.94	2.04,1.94,1.90	−440	1.86	1.97,1.87,1.75	−530
Methanosarcina barkeri (Krzycki *et al.*, 1989)	1.89	2.005,1.91,1.76	−35	1.96	2.05,1.94,1.90	−390	NS[a]	NS	NS
Methanothrix soehngenii (Jetten and Hagen *et al.*, 1991)	1.87	2.005,1.894,1.733	−230	1.95	2.05,1.93,1.865	−410	NS	NS	NS

[a]NS = not studied

and is similar to the atypical iron-sulfur signal in all other CODHs from acetotrophic and acetogenic anaerobes (Table 6.2). The spectrum is perturbed on incubation with CO and is observed in whole cells of *M. barkeri* during methanogenesis, a result which indicates that the cleavage of acetate yields a moiety that CODH recognizes as CO (Krzycki and Prince, 1990).

Unlike the enzymes from *Methanosarcina*, the CODH from *M. soehngenii* can be purified in the presence of air but requires strictly anaerobic conditions for CO-oxidizing activity (Jetten et al., 1989b). The native enzyme is an $\alpha_2\beta_2$ oligomer composed of subunits with molecular masses of 79,400 and 19,400 Da, and contains Ni and Fe. Thus, the *M. soehngenii* enzyme is similar to the enzymes from the *Methanosarcina* except that it has not been purified in association with a corrinoid-containing protein. The *M. soehngenii* enzyme catalyzes the exchange of CO with the carbonyl group of acetyl-CoA, demonstrating C-C and C-S cleavage activity (Jetten, Hagen, et al., 1991). The gene encoding the α subunit has a deduced amino acid sequence which has some homology with acyl-CoA oxidases; however, no consensus sequences involved in acetyl-CoA or CoA binding are evident (Eggen, Geerling, Jetten, et al., 1991). The deduced amino acid sequence of the largest subunit also contains eight cysteine residues with spacings that could accommodate Fe-S centers; accordingly, the anaerobically purified (reduced) enzyme exhibits two low-temperature EPR signals (Jetten, Hagen, et al., 1991; Jetten, Pierik, et al., 1991) (Table 6.2). One of these signals is attributable to a 4Fe-4S center and the other, as in the *Methanosarcina*, is atypical of bacterial 4Fe-4S centers with a $g_{av} = 1.87$. The atypical signal is substoichiometric in intensity and partially disappears when the enzyme is incubated with CO. It is postulated that this signal may arise from a 6Fe-6S prismane-like center. No EPR signals attributable to a Ni center have been reported for the *M. soehngenii* enzyme.

In summary, all CODHs characterized from acetate-grown methanogenic organisms have a similar subunit composition, contain 4Fe-4S centers and Ni, and have a Fe-S center with $g_{av} < 1.9$ (Table 6.2). A Ni-Fe center is the proposed site for cleavage of acetyl-CoA in the *M thermophila* CODH; however, it is not yet known if the $g_{av} < 1.9$ signal is associated with the Ni-Fe center.

6.3.5 Methyl Transfer to HS-CoM and Reductive Demethylation of CH_3-S-CoM to Methane

After cleavage of acetyl-CoA, the methyl group is ultimately transferred to HS-CoM (2-mercaptoethanesulfonic acid). *M. thermophila* grown with CD_3COO^- contained CD_3-S-CoM (83%) and CD_2H-S-CoM (17%) in a proportion similar to that of the deuterated methane (CD_3H and CD_2H_2), a result which demonstrates that CH_3-S-CoM is an intermediate in the pathway (Lovley et al., 1984). Methyl-H_4SPT is an intermediate (reactions 4 and 5, Table 6.1) in transfer of the methyl

group to HS-CoM, which implies a requirement for enzymes in transfer of the methyl group from the Co/Fe-S component of the CODH enzyme complex to HS-CoM. An enzyme (M_r = 34,000) with methylcobalamin:HS-CoM methyltransferase activity from acetate-grown *M. barkeri* has been described (Grahame, 1989); however, it is unknown if CH_3-H_4SPT can replace methylcobalamin and recent evidence suggests this enzyme is not involved in transfer of the methyl group from CH_3-H_4SPT in acetate-grown cells (Fischer et al., 1992). Two corrinoid-containing proteins (480,000 Da and 29,000 Da) present in the cytoplasmic fraction of *M. barkeri* have been described (Cao and Krzycki, 1991). The 480,000-Da protein is methylated at the onset of methanogenesis in whole cells and demethylated when methanogenesis stops, a result which indicates an involvement in methyltransfer. The 29,000 Da protein is methylated only when reductive demethylation of CH_3-S-CoM is inhibited. Thus, several potential cytoplasmic methyltransfer proteins have been identified but their function in the pathway is unknown. Recently, CH_3-H_4SPT:HS-CoM methyltransferase activity was demonstrated in a particulate fraction from acetate-grown *M. barkeri* (Fischer et al., 1992). The activity could be solubilized with detergents, suggesting it is integral to the membrane.

The final step in the utilization of all methanogenic substrates is the reductive demethylation of CH_3-S-CoM catalyzed by CH_3-S-CoM methylreductase (reaction 6, Table 6.1). The two electrons required for the reduction derive from the sulfur atoms of CH_3-S-CoM and HS-HTP (7-mercaptoheptanoylthreonine phosphate) (Part II, Chapter 1) yielding the heterodisulfide CoM-S-S-HTP. In the pathway for methanogenesis from acetate, the mixed disulfide is reduced to the corresponding sulfhydryl forms of the cofactors with electrons originating from oxidation of the carbonyl group of acetyl-CoA (Hedderich, 1989; Schwörer and Thauer, 1991). The thermodynamics of both heterodisulfide formation and reduction (reactions 6 and 8, Table 6.1) suggests a potential for electron transport coupled to ATP synthesis.

All methylreductases studied thus far contain factor F_{430}, which is a yellow, Ni-containing, structural hybrid of the corrin and porphyrin ring systems (Part II, Chapter 2). The function of F_{430} is unknown, but it is postulated that a Ni(I)-S-HTP species reacts with CH_3-S-CoM to form Ni(II)-CH_3 which returns to the nonligated Ni(I) species yielding CoM-S-S-HTP and CH_4 (Albracht et al., 1988; Jaun and Pfaltz, 1988; Krzycki and Prince, 1990; Lin and Jaun, 1991).

The methylreductase purified from *M. thermophila* has a subunit composition of $\alpha_1\beta_1\gamma_1$ with M_r's of 69,000, 42,000, and 33,000 (Jablonski and Ferry, 1991). The native enzyme (M_r = 141,000) contains 1 mol of F_{430} and utilizes HS-HTP as the electron donor. The as-isolated enzyme requires a reductive reactivation which can be accomplished *in vitro* with a ferredoxin purified from *M. thermophila*; ATP is not required but stimulates the reactivation. Ti(III)citrate can substitute for ferredoxin in a non-enzymatic reaction. It is proposed that the

methylreductase is isolated in two forms: a ready form that can be reactivated with reduced ferredoxin and an unready form unable to be reactivated unless converted to the ready form by an unknown mechanism that requires ATP (Figure 6.1).

The *Methanosarcina mazei* methylreductase contains F_{430} and has a native molecular mass of 283,400 Da with subunits of 68,000 Da, 43,215 Da, and 30,500 Da in a $\alpha_2\beta_2\gamma_2$ configuration (Thomas et al., 1987). The CH_3-S-CoM methylreductase from *M. soehngenii* has the same subunit composition and utilizes HS-HTP as the electron donor (Jetten et al., 1990b); a requirement for reductive activation of this enzyme was not investigated. Immunogold labeling of several acetate-grown *Methanosarcina* species and *M. soehngenii* indicates that the methylreductase of these acetotrophic organisms is primarily located in the cytoplasm (Thomas et al., 1987); however, the cells were grown with abundant nickel in the growth medium, conditions which may have influenced the amount of cytoplasmic methylreductase relative to membrane-associated enzyme (Aldrich et al., 1987).

6.3.6 Electron Transport and Bioenergetics

A relatively small amount of energy is available from the conversion of acetate to CH_4 and CO_2 (reaction 13, Table 6.1) and an equivalent of one ATP is already expended in the activation of acetate. Thus, these organisms must have evolved a very efficient mechanism for energy conservation. There are no obvious reactions leading to a substrate-level phosphorylation; however, several lines of evidence support a chemiosmotic mechanism for ATP synthesis. Acetate-degrading whole cells of *M. barkeri* generate a proton-motive-force of -120 mV (Peinemann et al., 1988). It is likely that the transport of electrons from the carbonyl of acetyl-CoA to CoM-S-S-HTP is dependent on membrane-bound carriers involved in generation of the proton-motive-force. Three *b*-type cytochromes with low midpoint potentials ranging from -330 to -182mV have been reported in acetate-grown cells of *Methanosarcina,* and cytochrome *b* is also present in *M. soehngenii* (Kühn and Gottschalk, 1983; Kühn et al., 1983). In addition, over 50% of the heterodisulfide reductase activity in acetate-grown *M. barkeri* is associated with the membrane fraction (Schwörer and Thauer, 1991; Fischer et al., 1992). CO-dependent methylreductase activity is stimulated by the addition of membranes to the soluble fraction, a result which further supports the involvement of a membrane-bound electron transport chain (Kemner et al., 1987). Although coenzyme F_{420} (a 5-deazaflavin) (Part II, Chapter 1) is an important electron carrier in the CO_2-reducing pathway, it is not required for conversion of acetyl-CoA to methane in cell extracts of *M. barkeri* (Fischer and Thauer, 1989). In addition, F_{420} is not an electron acceptor for CODH (Kohler and Zehnder, 1984; Terlesky and Ferry, 1988a). However, F_{420} may be involved in oxidation of the methyl

group of acetate to CO_2 to provide electrons for reductive biosynthesis (see below). As in CO_2-reducing methanogenic species, acetate-grown *M. barkeri* has an enzyme system to convert F_{420} to F_{390} in an ATP-dependent reaction (van de Wijngaard et al., 1991); however, the logic for the conversion is unknown (see Part I, Chapter 3).

Recent results strongly implicate an involvement of ferredoxin in electron transport. Ferredoxin is required for methanogenesis from acetate in extracts of *M. barkeri* (Fischer and Thauer, 1990a) and is a direct electron acceptor for the Ni/Fe-S component of *M. thermophila* (Abbanat and Ferry, 1991); however, the carriers mediating electron flow from ferredoxin to the heterodisulfide reductase are unknown. The gene encoding the ferredoxin from *M. thermophila* has a deduced amino acid sequence which contains eight cysteines in a spacing characteristic of 2[4Fe-4S] ferredoxins from the "*Bacteria*" domain (Clements and Ferry, 1992). The gene is transcribed in cells grown on either acetate, trimethylamine or methanol, a result which suggests a requirement for the ferredoxin in the metabolism of each substrate.

The production and consumption of H_2 during the growth of *Methanosarcina* species on acetate led to the hypothesis that H_2 could be an obligatory intermediate in electron transport during methanogenesis from acetate (Lovly and Ferry, 1985). A ferredoxin-dependent evolution of CO_2 and H_2 from acetyl-CoA has been demonstrated in extracts of *M. barkeri* (Fischer and Thauer, 1990a), and a CO-oxidizing:H_2-evolving system (reaction 7, Table 6.1) from *M. thermophila* has been reconstituted with the CODH complex, ferredoxin and purified membranes which contain an hydrogenase linked to cytochrome *b* (Terlesky and Ferry, 1988b). In addition, H_2 is a source of electrons for the reductive demethylation of CH_3-S-CoM in the soluble fraction of *M. thermophila* (Nelson and Ferry, 1984) and in extracts of *M. barkeri* strain 227 (Baresi and Wolfe, 1981). Although acetate-grown *M. thermophila* is unable to utilize H_2-CO_2 for methanogenesis, these cells contain a H_2-dependent heterodisulfide reductase activity with a rate which is similar to H_2-CO_2-utilizing species (Clements et al., 1993). Thus, it is reasonable to postulate that oxidation of the carbonyl group of acetate could be coupled to H_2 evolution by membrane-bound electron carriers and that H_2 is subsequently oxidized to provide electrons for the reduction of CoM-S-S-HTP. This hypothesis implies a potential for ATP synthesis by a chemiosmotic mechanism; indeed, proton translocation is coupled to the oxidation of CO to CO_2 and H_2 in cell suspensions of acetate-grown *M. barkeri* (Bott et al., 1986; Bott and Thauer, 1989). However, a membrane-bound electron transport chain without the participation of H_2 as an intermediate cannot be excluded. The dependence on H_2 for production of methane from acetate by a soluble enzyme preparation from *M. barkeri* led to the proposal that a membrane-bound electron transport system was operative in whole cells and that H_2 was only necessary to circumvent electron flow in the absence of membranes (Krzycki and Zeikus, 1984a). This

proposal is consistent with the ability of a particulate fraction from *M. barkeri* to produce methane in the absence of H_2 (Baresi, 1984). Oxidation of the methyl group of acetate to CO_2 could be the sole source of H_2 produced during growth on acetate, in which case H_2 would not play a major role as an intermediate in electron transport from ferredoxin to the heterodisulfide reductase.

Methane formation from acetate is dependent on sodium and is accompanied by the generation of a secondary sodium ion gradient in *M. barkeri* (Peinemann et al., 1988; Patel and Sprott, 1991). The membrane-bound CH_3-H_4MSPT:HS-CoM methyltransferase recently reported for acetate-grown *M. barkeri* (Fischer et al., 1992) catalyzes an exergonic reaction (reaction 5, Table 6.1). This reaction is coupled to a primary electrogenic sodium extrusion in the CO_2-reducing and methanol-utilization pathways (Part II, Chapter 5); however, it is unknown if the reaction is coupled to sodium extrusion during methanogenesis from acetate. The generation of a sodium gradient during methanogenesis from acetate is not understood. Reactions that may be driven by a sodium ion potential are (i) ATP synthesis, (ii) the uptake of acetate, and (iii) the endergonic cleavage of acetyl-CoA (Table 6.1).

The bioenergetics of *Methanothrix* requires special consideration in that the activation of acetate yields inorganic pyrophosphate (Table 6.1). Thus, it appears that *Methanothrix* is at a thermodynamic disadvantage compared to *Methanosarcina* unless the energy in pyrophosphate can be conserved (Table 6.1). A pyrophosphatase was isolated from *Methanothrix* (Jetten, Fluit, et al., 1992) with a subunit composition and other properties different from other pyrophosphatases studied. It is not yet known if this enzyme is involved in the recovery of energy from pyrophosphate formed in the activation of acetate. The enzyme is isolated from the soluble fraction; however, there is some indication it may be a peripheral membrane protein. Thus, it has been postulated that the pyrophosphatase from *Methanothrix* may have a proton translocation function. The maximum energy charge for *Methanothrix* is low compared to other cells, and it has been suggested that this condition offers an advantage since the free energy required for phosphorylation of ADP would be significantly lower (Jetten, Stams, et al., 1991).

6.3.7 Other Enzyme Activities

The methyl group of acetate is primarily converted to methane; however, a small proportion of methyl groups are oxidized to CO_2 to provide electrons for reductive biosynthesis. Acetate-grown *Methanosarcina* species contain low levels of enzymes which are utilized in the CO_2-reduction pathway for methanogenesis; thus, it is proposed that the methyl group of acetate is oxidized by a reversal of the pathway for CO_2 reduction to methane (Jablonski et al., 1990). Acetate-grown *M. barkeri* contains formyl-methanofuran dehydrogenase and 5,10-methylene-H_4MPT(tetrahydromethanopterin) dehydrogenase (Schwörer and Thauer, 1991)

and acetate-grown *M. thermophila* contains formyl-methanofuran:H_4MPT formyltransferase, 5,10-methenyl-H_4MPT$^+$ cyclohydrolase, and F_{420}-dependent 5,10-methylene-H_4MPT dehydrogenase (Jablonski et al., 1990). The low levels of these enzymes suggest they are not involved in conversion of the methyl group of acetate to methane; rather, oxidation of the methyl group of acetate to CO_2. The route for methyl transfer from acetate is not known; however, the cell must have evolved a mechanism to distinguish between methyl group oxidation or reduction to methane.

Growth of *M. barkeri* and *M. thermophila* on acetate induces carbonic anhydrase activity, but the function of this enzyme in the conversion of acetate to methane is only speculative (Karrasch et al., 1989; Jablonski and Ferry, 1991). It is proposed that the formation of carbonic acid may be required in an antiport mechanism for transport of the acetate anion into the cell. The enzyme from *M. thermophila* has been purified and characterized; it is a homodimer with a subunit molecular weight of 23,000 Da and is sensitive to inhibitors of all carbonic anhydrases studied to date (Alber and Ferry, unpublished results). The amino acid sequence deduced from the gene sequence indicates a potential leader sequence suggesting the enzyme could be transported outside of the cell.

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7

Redox Enzymes of Methanogens: Physicochemical Properties of Selected, Purified Oxidoreductases

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7.1 Introduction

The numerous oxidation/reduction reactions involved in the metabolism of methanogens require the participation of equal or greater numbers of different oxidoreductase enzymes. In this chapter only a selected group of these enzymes are considered. A number of additional important redox enzymes including hydrogenase, methylcoenzyme M methylreductase, CoM-S-S-HTP heterodisulfide reductase, dehydrogenases involved in tetrahydromethanopterin-dependent reactions, and formylmethanofuran dehydrogenase are described in other chapters. The purpose of the present chapter is to provide a review with some perspective of the present state of knowledge concerning the structural, catalytic, and physiological functions of a few important oxidoreductase enzymes in methanogens. The examples chosen have been characterized as well-purified preparations and are now among the more extensively studied methanogen oxidoreductase enzymes.

The early Dutch microbiologists in Delft observed that mixed cultures of methanogens could utilize molecular hydrogen as electron donor for the reduction of carbon dioxide to methane (Söhngen, 1906, 1910). Since then, it has been established that the ability to utilize hydrogen as reductant is a common property of methanogens that have been studied in pure culture. Initially, it was thought that a variety of simple alcohols and lower fatty acids could be used directly as reductants, and a number of observations were made that appeared to support Van Niel's unifying concept that carbon dioxide is the ultimate oxidant and thus the unique source of methane in nature (Barker, 1936a,b). However, the ethanol oxidizing organism, *Methanobacterium omelianskii* (Barker, 1939-40), later was

shown to be a consortium of two bacterial species, one that oxidized ethanol to acetate and molecular hydrogen and the other, *Methanobacterium* M.o.H. (*Methanobacterium bryantii*), that utilized the hydrogen for reduction of carbon dioxide to methane (Bryant et al., 1967; Whitman and Wolfe, 1983). A purified culture termed *Methanobacterium suboxydans* (Stadtman and Barker, 1951a) capable of coupling carbon dioxide reduction to the oxidation of even-numbered carbon atom fatty acids, C4-C8, to acetate and odd-numbered carbon atom fatty acids, C5 and C7, to acetate and propionate presumably consisted of a similar consortium (McInerney et al., 1979). In contrast it is now clear that acetate (see Part II, Chapter 6), formate and methanol are exceptions and these substrates are fermented directly by several species of methane-producers. Acetate and formate, in particular, which are produced in anaerobic environments as end products of fermentations of more complex substrates, finally are converted to carbon dioxide and methane. Methanol derived from methyl esters of numerous abundant natural products can serve either as electron donor or acceptor for several species. Black mud in swamps and estuaries, sewage sludge, and the rumen contents of the digestive tract of herbivores are rich sources of these types of organisms. Although these developments suggested that, except for the simplest members of the series, alcohols and fatty acids probably are not used directly by methanogens as reductants for CO_2 conversion to CH_4, strains recently have been described that can utilize a limited number of primary, secondary, and cyclic alcohols. The properties of the dehydrogenases that metabolize these alcohols will be discussed later in this chapter.

7.2 Formate Dehydrogenases

Formate dehydrogenases are widely distributed in nature and the enzymes differ considerably in composition, properties, and types of electron acceptors utilized. *Methanococcus vannielii*, an organism that grows in a mineral salts medium on formate as sole carbon and energy source, was isolated from San Francisco bay mud in 1948 and shown to ferment formate to carbon dioxide and methane according to Equation 7.1 (Stadtman and Barker, 1951b). In poorly buffered media containing sodium formate as substrate, growth and methane production ceased when, due to sodium carbonate accumulation, the pH increased to 9 or above. Continued oxidation of formate under these conditions gave exclusively hydrogen and CO_2.

$$4\,HCOOH \rightarrow 3\,CO_2 + CH_4 + 2\,H_2O \qquad (7.1)$$

Many years later, two formate dehydrogenases were isolated from *M. vannielii* (Jones and Stadtman, 1981). One of these, present in bacteria cultivated in

selenium supplemented media, is a large enzyme complex made up of approximately 100 kDa selenium-containing subunits and 105 kDa subunits containing molybdenum and Fe/S centers. Cells, which grew very slowly in selenium-deficient media (Jones and Stadtman, 1977), contained only the molybdenum/iron-sulfur enzyme species. Essentially homogeneous preparations of this non-selenium enzyme contained varying amounts of molybdenum and Fe/S. However, based on the correlation of enzymic activity and metal content, particularly molybdenum, observed for several different preparations, it was deduced that fully active enzyme contains 1 equivalent of Mo and 10 of Fe and acid-labile sulfide per mole of enzyme. This 105 kDa formate dehydrogenase later was found to consist of two dissimilar subunits that migrated in SDS-PAGE gels as 60 kDa and 33 kDa species (S. Yamazaki and T.C. Stadtman, unpublished). The large-molecular-weight selenium-dependent formate dehydrogenase contains selenium in the form of selenocysteine residues (Jones et al., 1979). Marked stimulation of growth of *M. vannielii* by added selenium is correlated with the simultaneous appearance in the cells of this high-molecular-weight enzyme complex, which is comprised of the molybdopterin-iron-sulfur protein and selenocysteine-containing protein subunits. Additional supplementation with tungsten further stimulated growth and, in cells from these cultures, the large selenoenzyme complex was the major formate dehydrogenase present. Enzyme isolated from cells grown in the presence of ^{185}W contained radioactive tungsten and a lowered molybdenum content indicating partial replacement of molybdenum with tungsten. Although highly purified fully active forms of the selenium-dependent and selenium-independent formate dehydrogenases of *M. vannielii* have not been compared in detail, it appears that the selenoenzyme complex may be the more active catalyst. Unfortunately, the marked oxygen sensitivity of both enzymes and their tendency to lose variable amounts of metal components during isolation have prevented accurate comparison of fully active forms of the seleno- and non-selenoenzyme species.

Methanobacterium formicicum, a formate-fermenting organism isolated from sewage sludge (Schauer and Ferry, 1980), produces a formate dehydrogenase that is about 3% of the total soluble protein of the cell (Schauer and Ferry, 1982; Schauer and Ferry, 1986). This enzyme consists of two dissimilar subunits (α = 85 kDa and β = 53 kDa) in an $\alpha_1\beta_1$ structure of 177 kDa. Compositional analysis of the homogeneous protein showed the presence of 1 g atom of molybdenum, 2 g atoms of zinc, 21–24 g atoms of iron, and 25–29 g atoms of inorganic sulfur per mol of enzyme. The enzyme also contained 1 mol of bound FAD. Spectral analysis of denatured enzyme preparations showed that, in addition to FAD, a fluorescent compound with excitation and emission spectra typical of molybdopterin cofactors had been released from the protein. Further characterization of this pterin derivative indicated a close similarity to the molybdopterin cofactor present in xanthine oxidase (May et al., 1986; Kramer et al., 1987).

The *M. formicicum* formate dehydrogenase, like the 105 kDa enzyme from *M.vannielii*, lacks selenocysteine. Sequence analysis of the gene encoding the *M.formicicum* enzyme revealed the absence of a TGA codon, thus explaining the failure to detect selenium in the enzyme (Shuber et al., 1986). As deduced from the gene sequence, the α subunit size is 75,725 Da rather than the estimated 85,000 Da and the β subunit is 43,927 Da rather than 53,000 Da. It is of interest that there is a high degree of sequence homology in the portion of the *M. formicicum* gene corresponding to the TGA codon flanking region of the *Escherichia coli* formate dehydrogenase fdhF gene (Zinoni et al., 1986) with the exception that a cysteine codon, TGC, occurs in place of TGA. However, mutation of the TGC codon in the *M. formicicum* gene to TGA was not sufficient to allow readthrough of the cloned message in *E. coli* and insertion of selenocysteine at the UGA codon. For efficient incorporation of selenocysteine in the *E. coli* expression system it was necessary to generate a sequence in the *M. formicicum* message at the 3′ side of the UGA codon identical to that shown to be essential in the *E. coli* fdhF message (Zinoni et al., 1990; Heider and Bock, 1992). It will be interesting to compare the catalytic activities of the isolated naturally occurring sulfur enzyme with the mutated selenocysteine-containing species produced in *E. coli*. The latter may be a much more efficient catalyst in view of the fact that in the case of the *E. coli* formate dehydrogenase (fdhF), mutation of the TGA to TGC resulted in the production of a cysteine-containing formate dehydrogenase of greatly reduced catalytic activity (Axley et al., 1991).

The naturally occurring deazaflavin, an 8-hydroxy-5-deazaflavin derivative (F_{420}), serves as the physiological electron acceptor for the *M. formicicum* formate dehydrogenase and the selenium-dependent and selenium-independent enzymes of *M. vannielii*. All of these enzymes also reduce FMN, FAD, and methyl viologen, but not NAD^+ or $NADP^+$. The *M. vannielii* enzymes also reduce benzyl viologen and tetrazolium dyes. Reduction of the deazaflavin by the *M. formicicum* formate dehydrogenase is dependent on the presence of enzyme-bound FAD, whereas with methyl viologen as electron acceptor FAD-depleted enzyme is fully active. This enzyme is markedly sensitive to cyanide (K_i = 6 μM), is inhibited by azide (K_i = 39 μM), α,α'-dipyridyl, and 1,10-phenanthroline, and is rapidly inactivated by oxygen. Both *M. vannielii* enzymes were inhibited by cyanide (10 mM) in crude extracts; the purified enzymes were rapidly inactivated by oxygen or by treatment with 1 mM iodoacetamide and were inhibited by metal-chelating agents such as EDTA, 1,10-phenanthroline, and "Tiron." The selenium-dependent enzyme appeared to be more sensitive to metal binding agents than the 105 kDa selenium-independent enzyme. Whereas growth of *M. vannielii* in tungsten-containing media resulted in incorporation of W in the selenoenzyme complex and retention of catalytic activity, growth of *M. formicicum* in the presence of 1 mM tungstate abolished formate dehydrogenase activity. Inhibition of formate

dehydrogenase activity by growth in the presence of tungsten has been observed in *E. coli* (Pinset, 1954; Enoch and Lester, 1972).

The pH optimum for oxidation of formate by *M. formicicum* formate dehydrogenase, with either methyl viologen or 8-hydroxy-5-deazaflavin as substrate, is about 7.9 and the optimum temperature is 55°C. Both *M. vannielii* enzymes exhibited pH and temperature optima of 9.2 and 55-60°C, respectively, with 2,3,5-triphenyltetrazolium chloride as electron acceptor.

Electron paramagnetic resonance (EPR) spectroscopic studies (Barber et al., 1983) conducted with the *M. formicicum* formate dehydrogenase revealed resonances that could be assigned to molybdenum(V) and reduced Fe/S centers. These resonances were not present in EPR spectra of oxidized enzyme. The nearly isotropic signal ($g_{av} \geq 2.0$) observed at 193K that was postulated to be due to Mo(V) was dramatically split in spectra of enzyme enriched in ^{95}Mo ($I=5/2$), confirming that this signal indeed was a property of a molybdenum center. Although the Mo(V) EPR signal was present in spectra of dithionite-treated whole cells of *M. formicicum* that contained active formate dehydrogenase, it was missing from spectra of dithionite-treated cells lacking active formate dehydrogenase due to growth in the presence of tungstate. Addition of cyanide, an inhibitor of formate dehydrogenase, to active cells altered the Mo(V) signal. By poising extracts of *M. formicicum* at a series of potentials at pH 7.7 and measuring the resulting Mo(V) EPR signal intensity, it was calculated that the redox midpoint potential for the Mo(VI)/Mo(V) couple is −330 mV, and that for the Mo(V)/Mo(IV) couple is −470 mV. When EPR spectra of reduced, purified formate dehydrogenase were recorded at 20K, a paramagnetic species of rhombic symmetry typical of a reduced Fe/S cluster was observed. Based on computer simulations, values of $g_1 = 2.047$, $g_2 = 1.948$, and $g_3 = 1.911$ were calculated. Deviation in the g_2 region from the simulated spectrum was due to overlap with the Mo(V) signal. The fact that addition of formate as reducing agent elicited the same EPR signals as dithionite indicates that the molybdopterin and Fe/S both function as redox centers during formate oxidation by the *M. formicicum* formate dehydrogenase. By analogy with the wild type and mutant *E. coli* fdhF formate dehydrogenases (Axley et al., 1991), the cysteine residue corresponding in position to selenocysteine or cysteine, respectively, should also serve as a redox center. In the *M. vannielii* formate dehydrogenases selenocysteine or cysteine, molybdopterin, and Fe/S centers presumably function in a similar electron transport chain.

7.3 Hydrogenase

The fact that most species of methanogens studied to date can use molecular hydrogen as electron donor for methane synthesis indicates that the necessary

enzyme, hydrogenase, is ubiquitous. For a further discussion of hydrogenases that have been characterized from methanogens, the reader is referred to Part II, Chapters 5 and 6. Like the formate dehydrogenases from this strictly anaerobic group of microorganisms, the hydrogenases also are complex enzymes containing a variety of redox centers. Instead of molybdenum, the hydrogenases contain nickel. Iron-sulfur centers are present, and selenocysteine and FAD are components of some of these enzymes. The physiological electron acceptor for many is the abundant cofactor, F_{420}, a 8-hydroxy-5-deazaflavin derivative.

The first hydrogenase shown to be a selenoenzyme is that present as a soluble enzyme in *M. vannielii* (Yamazaki, 1982). This enzyme was isolated in near homogeneous form and shown to contain Ni (^{63}Ni labeled enzyme from bacteria grown with ^{63}Ni), selenocysteine, Fe/S, and FAD (Yamazaki, 1984). The 340 kDa enzyme, having a subunit structure of $\alpha_2\beta_4\gamma_2$ (α = 56 kDa, β = 43 kDa, and γ = 35 kDa), contains 4 g atoms of selenium in the form of selenocysteine residues located in the four β subunits, 2 g atoms of nickel, 18–20 g atoms of iron, and 2 mol FAD per mol. Upon heating with reducing agent and SDS for 2–3 times longer than usual for SDS PAGE analysis, the α subunit of the enzyme dissociates into two identical 27 kDa subunits and thus is actually a dimer. A hydrogenase of similar subunit composition and metal and cofactor content was purified from *Methanococcus voltae* (Muth et al., 1987). Reduction of the deazaflavin cofactor by these hydrogenases serves to couple molecular hydrogen oxidation to the terminal methylreductase system for methane synthesis.

Deazaflavin-Linked $NADP^+$ Reductase

An electron transport system that includes an 8-hydroxy-5-deazaflavin-dependent $NADP^+$ reductase as an essential component to link formate oxidation or molecular hydrogen oxidation to NADPH-dependent synthesis of cell material is functional in a number of species of methane bacteria. Extracts of *Methanobacterium ruminantium* were shown to reduce $NADP^+$ to NADPH with formate as electron donor (Tzeng et al., 1975a) or with molecular hydrogen (Tzeng et al., 1975b). The enzyme catalyzing this reaction was purified from *M. vannielii* and shown to reconstitute a formate-$NADP^+$ oxidoreductase system when added to purified formate dehydrogenase (Jones and Stadtman, 1980). Homogeneous preparations of the deazaflavin-dependent $NADP^+$ reductase were isolated (Yamazaki and Tsai, 1980) using a rapid and highly sensitive fluorometric assay in which the reduced deazaflavin was used as substrate for $NADP^+$ reduction (Equation 2). The differences in the fluorescence spectra of the reduced and oxidized forms of the deazaflavin (excitation λ_{max} = 328 nm and emission λ_{max} = 390 nm versus excitation λ_{max} = 425 nm and emission λ_{max} = 475 nm, respectively)

allowed ready measurement of the product by monitoring the change in fluorescence intensity at 475 nm during irradiation of samples at 425 nm.

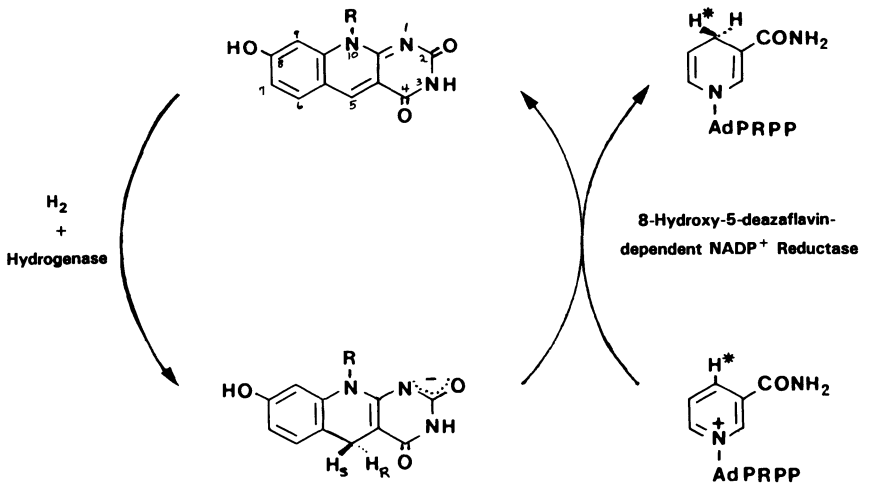
$$\text{1,5-dihydro-8-hydroxy-5-deazaflavin} + \text{NADP}^+ \rightleftharpoons \text{8-hydroxy-5-deazaflavin} + \text{NADPH} + \text{H}^+ \quad (7.2)$$

The pH maxima for the forward and reverse reactions are 7.9 and 4.8, respectively. At pH7, the k_{cat} value of the forward reaction is 24 times greater than that of the reverse reaction, showing that the production of NADPH is the favored process. The standard reduction potentials of the 8-hydroxy-5-deazaflavin cofactor ($E_o' = -0.37$ V) (Eirich et al., 1978) and $NADP^+$/NADPH ($E_o' = -0.32$ V) also indicate the forward reaction is favored. The purified $NADP^+$ reductase does not use NAD^+ or NADH as substrates, and it does not reduce FAD, FMN, or riboflavin with NADPH. Studies with a number of substrate analogues of 8-hydroxy-5-deazaflavin (Yamazaki et al., 1982) revealed that the basic 5-deazaflavin heterocyclic system of the cofactor is essential since neither riboflavin nor 1,5-dideazariboflavin was reduced. The N-10 side chain of the natural cofactor was not essential for activity, but introduction of a methyl group at the 8-position (8-methoxy derivative) prevented reduction by the enzyme. The importance of C-5 as the reduction site was indicated by the finding that 5-methyl-deazariboflavin is not reduced but instead is an inhibitor of the reduction of 5-deazariboflavin.

The native 85 kDa 5-deazaflavin-dependent $NADP^+$ reductase is a dimer of two apparently identical 43 kDa subunits. Inhibition of catalytic activity by treatment with alkylating agents or organic mercurials indicated the presence of one or more essential sulfhydryl groups in the enzyme. Unlike the oxygen-sensitive formate dehydrogenase of *M.vannielii*, the $NADP^+$ reductase could be isolated in the presence of air using buffers containing 1 mM DTT. Inclusion of 20% ethylene glycol in the buffers prevented loss of activity of dilute enzyme preparations. Homogeneous preparations were shown to contain 2g atoms of zinc per mole of enzyme (Yamazaki, S., L. Tsai, and T. C. Stadtman, unpublished). One-half of the zinc content could be readily removed from the enzyme by dialysis with concomitant loss of 50% of enzymic activity.

Stereochemical studies of the 8-hydroxy-5-deazaflavin-dependent $NADP^+$ reductase using tritium-labeled substrates showed the oxidation-reduction reaction between deazaflavin and pyridine nucleotide to be a direct hydride transfer process (Yamazaki et al., 1980). With (5R,S)-[5-^{3}H]8-hydroxy-5-deazaflavin-H_2 and excess $NADP^+$ as substrates, the enzyme catalyzed the transfer of 49% of the ^{3}H to NADPH at pH 7.6 and the remainder (48%) remained with the deazaflavin. The release of only 2% of the tritium to solvent showed that the tritium transfer was direct. Oxidation of the [4-^{3}H]NADPH product with either a 4-S or 4-R-stereospecific enzyme indicated the product to be (4S)-[4-^{3}H]NADPH. This was

confirmed by showing that in the reverse direction only the (4S)-[4-^{3}H]NADPH was oxidized by the enzyme, whereas with the (4R)-[4-^{3}H]NADPH as substrate no tritium was transferred to the deazaflavin. By comparing the specificity of the hydride transfer process catalyzed by the $NADP^+$ reductase and a hydrogenase from *Methanobacterium thermoautotrophicum*, it was shown that, with respect to the prochiral center at C-5, both enzymes act at the same face of the deazaflavin cofactor. To determine the absolute stereochemical position of the hydrogen introduced by the enzymes at the C-5 center, a labeled dihydro-deazaflavin synthetic analogue of the natural cofactor, produced by reduction of 7,8-didemethyl-8-hydroxy-[5-^{2}H]-5-deazariboflavin with the homogeneous selenium-dependent hydrogenase from *M. vannielii*, was subjected to a chemical degradation procedure that preserved the resulting chirality of the C-5 center intact (Yamazaki et al., 1985). Comparison of the ORD spectra obtained for the degradation product with those of authentic samples of known absolute configurations established that reduction of the deazaflavin cofactor occurs on the *si* face of the molecule and oxidation proceeds by removal of the pro-S hydrogen at C-5. Thus, the selenium-containing hydrogenase and the 8-hydroxy-5-deazaflavin $NADP^+$ reductase from *M. vannielii* are *si* face-specific enzymes. These reactions occur as shown in Scheme 7.1.



Scheme 7.1

7.5 Carbon Monoxide Dehydrogenase

The German and Dutch microbiologists in the early 1900s recognized that bacteria were capable of oxidizing carbon monoxide and concluded that carbon monoxide

would serve as *Nährstoff* in the growth of bacteria (Kaserer, 1906). It was found later that anaerobic microorganisms present in sludge converted carbon monoxide into a mixture of carbon dioxide and methane (Fischer et al., 1931, 1932). Yet it was not until after the second World War that Kluyver and Schnellen employed *Methanosarcina barkeri* and *M. formicicum* in the first studies of pure cultures which produced methane from carbon monoxide and from carbon monoxide plus hydrogen (Kluyver and Schnellen, 1947). Extensive, quantitative measurements of substrates and products were conducted, establishing the fundamental stoichiometries for fermentations of carbon monoxide to methane. In 1958, Tatsuhiko Yagi published the first conclusive evidence that oxidation of carbon monoxide was catalyzed by a specific enzyme (Yagi, 1958). The evidence was provided from work on the non-methanogenic organism *Desulfovibrio desulfuricans*, in which it was later demonstrated that carbon monoxide oxidoreductase is an enzyme distinct from hydrogenase and/or formate dehydrogenase (Yagi, 1959).

In 1977, during the course of studies of growth on carbon monoxide, Lacy Daniels and others reported on the properties of crude carbon monoxide dehydrogenase present in cell-free extracts of methanogens (Daniels et al., 1977). Partial purification of the enzyme was accomplished later from *Methanobrevibacter arboriphilicus* (Hammel et al., 1984). However, it was not until the account of Krzycki and Zeikus in 1984 that the enzyme, now commonly termed carbon monoxide dehydrogenase (or CODH), was first obtained in pure form from a methanogen. The growing inventory of carbon monoxide dehydrogenase enzymes which have been purified and characterized from various methanogens now consists of enzymes from: *Methanosarcina thermophila* (Abbanat and Ferry, 1991), *Methanothrix soehngenii* (Jetten et al., 1989, 1991), *M. vannielii* (DeMoll et al., 1987), and two different isolates from *M. barkeri* (Krzycki and Zeikus, 1984; Krzycki et al., 1989; Grahame and Stadtman, 1987). It is now established that these enzymes are involved in physiologically important reactions of acetyl-CoA synthesis and decomposition. The ability of the enzyme to reversibly oxidize the carbonyl moiety of acetyl-CoA is central to its function in acetyl-CoA synthesis and decomposition. Oxidoreductase activity, studied with carbon monoxide as substrate, serves as a paradigm for carbonyl redox reactions of acetyl-CoA. A thorough discussion of carbon monoxide dehydrogenase, as it is involved in decomposition of acetyl-CoA ultimately to produce methane and carbon dioxide, is set forth in Part II Chapter 6. The present section is concerned with structural and catalytic properties of the methanogen enzymes with regard to their redox activity toward carbon monoxide.

7.5.1 Structural properties

(a) Oligomeric Structure

In all of the methanogenic organisms tested so far, CODH is found as an enzyme of roughly 220 kDa. Its structure consists of two different subunits

with molecular masses of approximately 90,000 Da and 20,000 Da (SDS gel electrophoresis), present in an $\alpha_2\beta_2$ configuration. The genes from *M. soehngenii* encoding both subunits have been cloned, sequenced, and expressed (albeit lacking enzymic activity) in both *Desulfovibrio vulgaris* and *E. coli*. The molecular masses calculated from the deduced amino acid sequences of the two genes are 89,461 and 21,008, in agreement with data from SDS gel electrophoresis (Eggen etal., 1991; Jetten et al., 1989). As yet, the $\alpha_2\beta_2$ type of structure of CODH that occurs in methanogens has not been found in other organisms.

(b) Metal Content

Nickel and high amounts of non-heme iron, in the form of Fe/S clusters, are present in all known methanogen CODH enzymes. An early indication that the Fe/S centers are located in the large subunit came from amino acid analyses of the individual subunits of the enzyme from *M. barkeri*. The results showed that the large subunit contains 23 cysteine residues, whereas only one cysteine was found in the small subunit of this enzyme (Grahame and Stadtman, 1987). Later, the proposal that Fe/S clusters are held in the large subunit was confirmed by nucleotide sequence information for the *Chd* genes from *M. soehngenii*. From the deduced amino acid sequences, there is no cysteine in the small subunit and 32 cysteine residues are in the large subunit of this enzyme (Eggen et al., 1991).

The metal contents of five pure $\alpha_2\beta_2$ enzymes from different sources are presented in Table 7.1. It is found that the enzymes generally contain approximately 2 g atoms of nickel and an amount of iron which may vary from 15–30 g atoms. In the case of *M. thermophila*, it was necessary to isolate the enzyme by dissociating it from other proteins present in a corrinoid-containing complex. The unusually low level of nickel observed in the resolved CODH component

Table 7.1 Principal metal components of $\alpha_2\beta_2$ CO oxidoreductase in methanogens

Organism	*Ni*	*Fe*	*Zn*	*other*	*Ref.*
M. barkeri	1.3 ± 0.3	15.6 ± 5.6	1.1	NR[1]	*a*
M. barkeri MS	1.5 ± 0.3	29.7 ± 5.7	0.85 ± 0.1	Cu, 0.94	*b*
M. vannielii	2.0 ± 0.1	16 ± 7	0.4	NR	*c*
M. thermophila[2]	0.42 ± 0.04	15.4 ± 2.6	5.4 ± 0.2	NR	*d*
M. soehngenii	2.0 ± 0.1	18 ± 2	NR	NR	*e*

References: *a*, Grahame and Stadtman, 1987; *b*, Krzycki et al., 1989; *c*, DeMoll et al., 1987; *d*, Abbanat and Ferry, 1991; *e*, Jetten et al., 1991.

[1]Not reported.

[2]Values were reported per mol of subunit of the CODH component resolved by detergent treatment of the CODH-corrinoid complex, and were doubled for inclusion in the table.

may, therefore, have resulted from loss during the procedures for disruption of the complex. Large quantities of zinc are detected in the enzymes as isolated. However, extensive removal of zinc is readily accomplished, without effect on activity, by treatment with chelators such as EDTA. Once adventitiously bound zinc has been eliminated, the residual levels are substoichiometric with respect to the basic $\alpha_1\beta_1$ heterodimeric unit. Thus zinc is unlikely to participate in redox reactions involving carbon monoxide.

When prepared in the oxidized form, the enzyme is deep brown in color with strong absorption in the region around 400 nm which extends out to 800 nm and beyond. Characteristics of the absorption band are typical of those due to Fe←S charge-transfer interactions found in ferredoxin-like Fe/S centers. The absorption is markedly diminished upon addition of reducing agents such as dithionite or the substrate carbon monoxide. Joseph Krzycki and others have investigated the nature of the Fe/S centers by core-extrusion studies using thiophenol/hexamethylphosphoramide (Krzycki et al., 1989). Most of the iron present in the enzyme is extrudable in the form of 6.1 ± 0.78 [4Fe-4S] clusters. However, in this type of analysis, reorganization of cluster types is quite possible.

(c) EPR Spectroscopy

Low temperature, X-band EPR studies have been conducted on the purified enzymes from *M. soehngenii* (Jetten et al., 1991) and *M. barkeri* (Krzycki et al., 1989). The results show that these enzymes possess various features in common with one another and also with the more extensively studied enzyme from *Clostridium thermoaceticum* (Lindahl etal., 1990a,b). Upon reduction, both methanogen enzymes exhibit complex EPR spectra, in which two different, overlapping rhombic signals can be discerned. The signal of greatest intensity was characterized, in the case of *M. soehngenii*, by *g*-values of 2.05, 1.93, and 1.865, corresponding to 0.9 mol of an $S=1/2$ spin per $\alpha\beta$ unit. In the case of *M. barkeri*, the predominant signal exhibited *g*-values of 2.05, 1.94, and 1.90, with double integration of the entire spectrum yielding approximately 1.1 mol of $S=1/2$ spin per $\alpha\beta$ heterodimer. Redox potentiometric titrations, performed on both enzymes, indicated that the predominant rhombic component arises from a center with midpoint potential of around −400 mV. Since $E^{\circ\prime}$ of the CO/CO_2 couple is −520 mV, reaction of the enzyme with carbon monoxide at pH7 results in extensive reduction of this center. On the whole, the EPR and redox properties of this center are quite analogous to an isolated bacterial ferredoxin cubane-like $[4Fe,4S]^{2+/1+}$ cluster. Moreover, in a region of the large subunit (residues 466–576) which contains 8 cysteine residues and bears homology to the archael-type ferredoxins, the existence of one or two [4Fe,4S] clusters seems likely (Eggen et al., 1991).

The second, low-intensity component exhibits *g*-values of 1.997, 1.886, and 1.725 in *M. soehngenii* (Jetten et al, 1991), and *g*-values of 2.005, 1.91, and

1.76 in the enzyme from *M. barkeri* (Krzycki et al., 1989). The *g*-values of this center, being rather low, are not typical of bacterial ferredoxin [4Fe,4S] centers. The temperature dependence of the weak signal indicates a structure with higher spin relaxation rates than found in the major [4Fe,4S] component (Krzycki et al., 1989). In part, this may help to explain the low observed spin quantitation of only 0.1 mol $S=1/2$ spin per mol $\alpha\beta$ heterodimer. Moreover, the possibility of metal centers with $S=3/2$ has been raised for the enzyme from *C. thermoaceticum* (Lindahl et al., 1990a). Compared with the major signal, the low-intensity component persists at much higher potentials and vanishes with an *apparent* midpoint potential of -230 mV in *M. soehngenii*. In *M. barkeri* the signal disappears within a region somewhat more positive than -100 mV. Despite such high apparent midpoint potentials, it is this low-intensity component, and not the signal ascribed to a [4Fe,4S] structure, which shows detectable perturbation when carbon monoxide is added to the reduced enzyme. It has been speculated that the structure of this center may resemble a [6Fe,6S] prismane-like cluster (Jetten et al., 1991). Incorporation of nickel into one of the Fe sites in such a structure would be permissible, perhaps forming a portion of the active site of the enzyme. An amino-terminal region of the large subunit consisting of a patch of four cysteine residues (numbers 55, 58, 63, and 73), in combination with cysteine residues from other parts of the molecule, has been proposed as the site for fixation of the putative metallo-prismane structure (Eggen et al., 1991).

Finally, it should be noted that, under certain conditions, exposure to carbon monoxide evokes a strong EPR signal of nearly axial symmetry with apparent values of $g_{\parallel}$ and $g_{\perp}$ around 2.03 and 2.07, respectively. The signal is readily observed under temperature conditions ($\approx$100 K), in which paramagnetic relaxation in Fe/S centers is too rapid to permit detection of the latter. The CO-induced signal was originally discovered and later characterized in the purified CO oxidoreductase from *C. thermoaceticum* (Ragsdale et al. 1982; Ragsdale et al., 1985; Fan et al., 1991). The complexes of very high molecular mass, which contain corrinoid and CO-dehydrogenase components and are present in *M.thermophila* (Terlesky et al., 1986, 1987) and in *M. barkeri* (Grahame, 1992), also exhibit a CO-dependent EPR spectrum with characteristics nearly identical to the Clostridial enzyme. In contrast, this type of EPR signal has not yet been reported for the purified methanogen $\alpha_2\beta_2$ CO oxidoreductase component once removed from its association with other proteins in the complex. In Part II Chapter 6, characteristics of this CO-induced EPR signal are described, as related to the action of CODH-corrinoid complexes in methane formation from acetate.

(d) Thermal Stability and Inactivation by CO

Heat-inactivation studies have been performed on the enzyme from *M. soehngenii* (Jetten et al., 1989) and *M. barkeri* (Grahame and Stadtman, 1987). Al-

though the *M. barkeri* enzyme appears to be slightly more resistant toward thermal inactivation, test conditions, such as buffer composition, used in the different studies were not equivalent. However, in general, a relatively high degree of thermostability is exhibited. Upon incubation at 60°C for 10 min, neither enzyme is appreciably inactivated. Nevertheless, incubation of the enzyme from *M. barkeri* under an atmosphere of 100% CO for 10 min at 24°C causes significant inactivation of the enzyme. Furthermore, at higher temperatures, losses of activity in the presence of carbon monoxide are greater still. It is unlikely that the conformation of the reduced enzyme is intrinsically less stable than the enzyme in the oxidized state. Alternatively, as a normal consequence of the catalytic mechanism, one may envision that in the presence of CO a reactive, high-energy intermediate is formed or stabilized at the active site. The lifetime of such an intermediate would be expected to be short under conditions wherein an electron acceptor is available to allow the reaction to proceed on to products. However, in the absence of an electron acceptor, the reactive group would persist for a longer time, increasing the probability of its entry into detrimental side-reactions. Enzyme-bound radical species such as $CO\cdot^{+}$ and $CO_2\cdot^{-}$ may be considered as one-electron oxidized forms of carbon monoxide, perhaps formed at the enzyme active site. If so, the steady-state concentration of the radical must be sufficiently low to have escaped detection in EPR experiments.

7.5.2 Catalytic properties

(a) Kinetic Mechanism

There have been no reports of a thorough study of the kinetic mechanism of CO dehydrogenase from methanogens. However, by analogy with the enzyme from *C. thermoaceticum* (Diekert and Thauer, 1978), and since the enzyme can exist in both reduced and oxidized forms, a *ping-pong* mechanism is assumed. Apparent K_m values for carbon monoxide have been measured under conditions wherein the electron acceptor (second substrate) is present at near-saturating levels. In contrast to the enzyme from *Clostridia*, none of the methanogen CO oxidoreductases are fully saturated with carbon monoxide at partial pressure of 1 atm. With methyl viologen as second substrate, apparent K_m values for carbon monoxide have been estimated at 0.7 mM in *M. soehngenii* (Jetten et al., 1989) and 5 mM in *M. barkeri* (Krzycki and Zeikus, 1984).

(b) Electron Acceptor Substrates

In the acetotrophic methanogens the physiological electron acceptor substrate is likely to be ferredoxin (Fisher and Thauer, 1990; Terlesky and Ferry, 1988) whereas, *in vitro*, CO dehydrogenase also reduces various other low-potential

electron acceptors. Reduction of viologen derivatives with midpoint potentials as low as −650 mV has been observed (DeMoll et al., 1987), which attests to the capacity of the enzyme to store reducing equivalents in centers of very low redox potential.

Variability exists among the enzymes from different methanogens in specificity for electron acceptor substrates. Albeit, certain characteristics are held in common, including the ability to act on viologen substrates and the inability to reduce pyridine nucleotides such as NAD^+ and $NADP^+$. The enzymes from *M. barkeri* and *M. vannielii* reduce flavin derivatives FAD and FMN readily, but are totally inactive toward the 5-deazaflavin derivative coenzyme F_{420} (Grahame and Stadtman, 1987; DeMoll et al., 1987). Similar results are found for the enzyme from *M. thermophila*, as it exists as a component of a corrinoid-containing complex (Terlesky et al., 1986). In contrast, reactivity toward coenzyme F_{420} was detected (at low levels) for the enzyme from *M. soehngenii*, yet FAD and FMN were reduced more slowly than F_{420}. Under a gas phase of 100% carbon monoxide, the enzymes purified from different strains of *M. barkeri* exhibit comparable values of $K_{m(app)}$ for methyl viologen of 5.0 mM (Krzycki and Zeikus, 1984) and 7.1 mM (Grahame and Stadtman, 1987), whereas 3.0 mM and 12 mM are the values found for the enzymes from *C. thermoaceticum* and *Acetobacterium woodii*, respectively (Ragsdale et al., 1983a,b). Despite the fact that only apparent K_m values are available for comparison, in *M. soehngenii* a remarkably low $K_{m(app)}$ value for methyl viologen of 65 μM (Jetten et al. 1989) further illustrates the unique electron acceptor substrate specificity of the enzyme in this organism. In general, the low reactivity toward two-electron, low-potential acceptor substrates is consistent with export of electrons directly from Fe/S centers on the enzyme via an $n=1$ redox process.

(c) pH Optimum and Efficiency

Carbon monoxide dehydrogenase enzymes from methanogenic bacteria exhibit reasonably high catalytic efficiencies. The turnover numbers for the CO-dependent reduction of methyl viologen are presented in Table 7.2. The values in Table 7.2 are obtained from directly measurable specific activities of the purified enzymes observed in the presence of 1 atm CO. However, because carbon monoxide is not present at saturating levels, these values may be rather conservative. For example, V_{max} of the enzyme from *M. barkeri* strain MS was calculated as 1,300 U/mg (Krzycki and Zeikus, 1984). This corresponds to a turnover number of 143,000 min^{-1}, whereas the value obtained at 1 atm CO is 23,800 min^{-1}. Within an order of magnitude, catalytic efficiency of the methanogen enzymes is comparable with CO dehydrogenases from other anaerobic organisms such as *C. thermoaceticum* (Ragsdale et al., 1983a; Diekert and Ritter, 1983) and *A. woodii* (Ragsdale et al., 1983b) and from aerobic phototrophs such as

Table 7.2 Catalytic activities of CO oxidoreductase enzymes from methanogens

Organism	pH optimum	Turnover number[1] (CO oxidized min^{-1})	Conditions: pH	Conditions: Temperature (°C)	Refs.
M. barkeri	7–9	14,600	7.0	25	*a*
M. barkeri MS	NR[2]	23,800	7.0	37	*b*
M. vannielii	10.5	20,900	7.7	23	*c*
M. thermophila[3]	8.4	7,810 (10,650)	8.4	23	*d,e*
M. soehngenii	9.0	12,870	8.9	35	*f*

References: *a*, Grahame and Stadtman, 1987; *b*, Krzycki & Zeikus, 1984; *c*, DeMoll et al., 1987; *d*, Abbanat & Ferry, 1991; *e*, Terlesky et al., 1986; *f*, Jetten et al., 1991.
[1]Calculated from specific activity of the final preparation per mol 110 kDa heterodimer.
[2]Not reported.
[3]CODH component resolved by detergent treatment of the CODH-corrinoid complex. Value in parenthesis as measured in the intact complex.

Rhodospirillum rubrum (Bonam and Ludden, 1987). Aerobic organisms, such as *Pseudomonas carboxydoflava* (Krüger and Meyer, 1986), possess a CO dehydrogenase with properties so unlike the methanogen enzymes that few direct comparisons are possible.

CO dehydrogenase enzymes from various anaerobic bacteria exhibit optimal pH values considerably above pH 7. The methanogenic organisms offer no exceptions, as can be seen from the values of optimal pH listed in Table 7.2. The reason that these enzymes exhibit an alkaline pH optimum is understandable based upon thermodynamic considerations of the reaction catalyzed, written as follows:

$$CO + H_2O \rightleftharpoons CO_2 + 2e^- + 2H^+ \qquad (7.3)$$

As one proceeds toward alkaline pH, the overall free energy change becomes steadily more favorable; gaining −2.73 kcal/mol per unit pH at room temperature. Furthermore, as the redox potential of the acceptor substrate decreases, the sensitivity to pH is predicted to increase. This is borne out by the observation that the reaction catalyzed by the enzyme from *M. vannielii* continues to increase in rate as a function of pH until a very high optimum pH of 10.5 is reached. Above this value, decline in activity may be due to factors such as changes in the conformation of the enzyme. Similar behavior was found for CO dehydrogenase in crude extracts of *Clostridium pasteurianum*. Remarkably, no maximum peak of activity could be reached. Instead, the rate persisted to climb up to the highest value of pH tested, pH 12 (Thauer et al., 1974). Nevertheless, it is clear

that not all CO dehydrogenases behave as unregulated catalysts such as this. At least, in the case of the enzymes from *Methanosarcina*, pH dependence of the reaction rate is presumably controlled by factors other than enzyme denaturation and overall thermodynamics of the reaction.

(d) Inhibitors

For many years sensitivity to oxygen and inhibition by cyanide, a substrate analogue isoelectronic with CO, had been found for all CO dehydrogenase enzymes examined from anaerobic bacteria. Thus, inhibition by O_2 and CN^- was considered to be a general property of these enzymes. A rigorous kinetic study of cyanide inhibition was reported for the enzyme from *R. rubrum* (Ensign et al., 1989). The observed pattern of inhibition indicates that cyanide is a slow-binding, active-site directed inhibitor, which does not combine with [4Fe,4S] centers, yet exhibits specificity for nickel contained in the enzyme. Cyanide combines directly to produce an inactive complex, which can be reactivated upon exposure to carbon monoxide (Ensign et al., 1989). As mentioned earlier, carbon monoxide itself is capable of causing inactivation of the *M. barkeri* enzyme; however, the kinetic pattern has not been established.

Studies on the *R. rubrum* enzyme have also provided insight into the mechanism of action of several inhibitors which are analogues of CO_2, the most thoroughly studied being carbonyl sulfide (COS). COS displays rapid-equilibrium, competitive inhibition with respect to carbon monoxide (Hyman et al., 1989). Like carbon monoxide, COS was effective in displacing cyanide from the CN^--inhibited enzyme. Inhibition by COS was uncompetitive versus the electron acceptor methyl viologen; a finding consistent with the previously suggested ping-pong mechanism. Unfortunately, detailed investigations such as these have not been carried out on the enzymes isolated from methanogenic organisms.

Qualitatively, the effects of cyanide on CO dehydrogenase enzymes from methanogenic bacteria are consistent, in general, with the detailed kinetic picture provided from the studies on the enzyme from *R. rubrum*. However, the enzyme purified from *M. soehngenii* serves as a remarkable exception to this rule. CO dehydrogenase from *M. soehngenii* is extraordinary, since neither oxygen nor cyanide causes marked inhibition (Jetten et al., 1989). Whether purified under anaerobic or aerobic conditions, the enzyme from *M. soehngenii* displays high levels of activity with a virtually unchanged content of metal ions (Jetten et al., 1991). However, EPR studies clearly show that purification carried out in the presence of oxygen causes alteration of the structure of certain metal centers (Jetten et al., 1991). Ultimately, an understanding of the structural basis for resistance to oxygen and cyanide may also shed light on the unusual electron acceptor substrate specificity of this enzyme.

7.6 Alcohol Dehydrogenase

In 1967, Bryant et al. demonstrated that *M. omelianskii* was not a single organism but instead consisted of a mixed culture in which an ethanol-oxidizing bacterium, the "S-organism", provided hydrogen for growth on CO_2 of a methanogenic organism now known as *M. bryantii* (Bryant et al., 1967). As mentioned earlier in this chapter, following this discovery greater restrictions were required for the range of carbon substrates considered possible for use by methanogenic organisms in pure culture. Thus, in the middle 1980s, when enrichment cultures of methanogens were obtained which utilized ethanol, 2-propanol, and/or 2-butanol, the presence of H_2-producing, perhaps obligately syntrophic microorganisms was suspected (Widdel, 1986). However, after the application of several techniques to assess microbiological purity, each of which now are sufficiently sophisticated to have revealed the impure nature of "*M. omelianskii*", Friedrich Widdel first reported on the isolation of pure cultures of methanogens which grow using alcohol substrates higher than methanol as electron donors (Widdel, 1986).

Numerous species, from several different orders of methanogenic organisms, have now been identified which are capable of growth on 2-propanol/CO_2 and/or 2-butanol/CO_2 (Zellner and Winter, 1987; Zellner et al., 1987; Widdel et al., 1988; Zellner, Bleicher, et al., 1989; Zellner, Stackebrandt, etl al., 1989; Widdel and Wolfe, 1989; and Zellner et al., 1990). A few organisms also have been discovered that can exist on cyclopentanol/CO_2 (Bleicher et al., 1989). As a source of energy, secondary alcohol utilization is accompanied by conversion to the corresponding ketone derivative. Furthermore, the ketones, acetone and 2-butanone, can be reduced back to 2-propanol and 2-butanol, respectively, upon readmittance of hydrogen to the pure cultures. Carbon atoms from the carbon-skeleton of alcohol substrates other than methanol are not converted to methane in significant quantities. Rather, the apparent role for secondary alcohol substrates is to serve, in the absence of hydrogen, as a source of reducing equivalents for CO_2 reduction to methane. Although organisms exist which can utilize the primary alcohol ethanol in a similar capacity, there are fewer such examples. The best known case is *Methanogenium organophilum* in which acetaldehyde, formed by oxidation of ethanol, undergoes subsequent dismutation to yield ethanol plus acetate. Dismutation of aldehydes was observed in dilute suspensions of *Methanogenium thermophilum* (Widdel and Wolfe, 1989) and was demonstrated also in cell-free extracts of *M. organophilum* (Frimmer and Widdel, 1989), thereby offering a possible explanation for the accumulation of high levels of acetate during growth and only low amounts of acetaldehyde.

Recently, substantial progress has been made on the purification and characterization of novel alcohol dehydrogenase (ADH) enzymes from methanogens capable of secondary alcohol oxidation. In general, low yields of total cell mass occur when alcohol substrates are supplied as sole source of energy for growth. In

contrast, when grown on H_2/CO_2, cell mass is high, but little or no ADH is detectable. However, when cells of *Methanogenium liminatans* and *Methanobacterium palustre* were grown on H_2/CO_2 in the presence of 0.5% 2-propanol, ADH was expressed at levels of 3–5% of total cellular protein (Bleicher and Winter, 1991). The ability of various alcohols to induce or derepress ADH has been tested in four different species of methanogenic organisms (Widdel and Wolfe, 1989). In all of the species tested, addition of either 2-propanol or 2-butanol brought about the expression of ADH. Under conditions of hydrogen limitation, expression of ADH also was elicited by acetone in all of the species tested. In contrast, *M. organophilum* was the only organism for which expression of ADH was elicited by ethanol. In the sections which follow, characteristics of alcohol dehydrogenase structure and function are discussed as determined from studies on the purified enzymes from *M. liminatans*, *M. thermophilum*, *M. palustre*, and *Methanocorpusculum parvum*.

7.6.1 Structure and function

(a) Coenzyme Specificity

Two types of ADH are distinguishable in methanogenic organisms based upon their coenzyme specificity. One type employs $NADP^+$, whereas factor F_{420} serves as the required coenzyme in the other class. No ability to utilize NAD^+, FAD, or FMN has been observed for ADH enzymes purified from methanogens. Coenzyme F_{420}-dependent enzymes have been purified from both *M. liminatans* (Bleicher and Winter, 1991), and *M. thermophilum* (Widdel and Wolfe, 1989). The purified enzymes from *M. palustre* and *M. parvum* require $NADP^+$ (Bleicher and Winter, 1991). The ethanol-oxidizing enzyme present in crude extracts of *M. organophilum* also appears to be specific for $NADP^+$, since F_{420} was not reduced, and F_{420}:$NADP^+$ oxidoreductase, although present in the extract, was inactive at the apparent pH optimum of ADH, pH 10.0 (Frimmer and Widdel, 1989).

(b) Alcohol Substrate Specificity

The ADH enzymes from methanogenic organisms exhibit rather restricted specificities for alcohol substrates. A comparative kinetic study of the enzymes from *M. liminatans* and *M. palustre* showed that activity is limited mostly to oxidation of short chain secondary alcohols, 2-propanol and 2-butanol, with only marginal rates of 2-pentanol oxidation (Bleicher and Winter, 1991). Both enzymes show weak abilities to oxidize cyclopentanol, whereas cyclohexanol is oxidized only by the enzyme from *M. palustre*. The range of allowed substrates is slightly greater for *M. palustre* ADH, than for the *M. liminatans* enzyme. Both

enzymes catalyze the reduction of the corresponding ketone derivatives, of which acetone is reduced most efficiently. Neither enzyme catalyzes oxidation of primary alcohols (including ethanol), diols, polyols, benzyl alcohol, or secondary alcohols with more than five carbon atoms (Bleicher and Winter, 1991). However, with the enzyme purified from *M. thermophilum*, oxidation of ethanol and 1-propanol has been observed, albeit at only 0.1% of the rate of 2-propanol oxidation (Frimmer and Widdel, 1989). Furthermore, dismutation of acetaldehyde, measurable in crude extracts of this organism, is found also using the purified enzyme (Frimmer and Widdel, 1989). When eventually purified, the enzyme from *M. organophilum* would be especially interesting to study, since this organism is much more active in oxidation of ethanol than *M. thermophilum*.

(c) Additional Structural and Catalytic Properties

At first glance, secondary ADHs from methanogenic organisms appear similar in oligomeric structure to certain other recognized examples of bacterial and eukaryotic ADH enzymes. On SDS gel electrophoresis the methanogen enzymes all exhibit a single band corresponding to a subunit of molecular mass around 40,000 Da. Native molecular mass measurements indicate canonical dimeric or tetrameric structures, in which all subunits are presumed to be identical (Bleicher and Winter, 1991). Polyclonal antibodies against the $NADP^+$-dependent enzyme from *M. parvum* cross-react with the $NADP^+$-dependent *M. palustre* enzyme, but also react with the $NADP^+$-dependent ADH from *Thermoanaerobium brockii*, an organism not related to the methanogens. Nevertheless, no immunological similarity was found between methanogen ADHs and the ADH enzymes from equine liver or yeast. Furthermore, the F_{420}-dependent ADH from *M. liminatans* shows no immunological similarity with any of the other ADH enzymes tested, including those from other methanogens (Bleicher and Winter, 1991). Homology exists in the amino-terminal sequences of the $NADP^+$-requiring ADH enzymes from *M. palustre* and *M. parvum*; however, no resemblance is evident in the N-terminal region of the F_{420}-dependent ADH from *M. liminatans* (Bleicher and Winter, 1991).

The apparent absence of zinc is another feature which sets the F_{420}-dependent enzyme of *M. liminatans* apart from the $NADP^+$-requiring enzyme from *M. palustre*. The $NADP^+$-dependent enzyme contains approximately 1–2 g atoms of Zn per subunit (but, no other metals above control levels), whereas only 0.07 g atom of zinc was found per mol subunit in the *M. liminatans* enzyme (Bleicher and Winter, 1991). Furthermore, activity of the F_{420}-dependent enzyme was not stimulated by addition of zinc. Iron was detected at levels above background in this enzyme, although similar amounts were found in both native and denatured states. No other metals were found in the F_{420}-dependent enzyme (Bleicher and Winter, 1991). Iron-sulfur centers were excluded, since the enzyme preparations

are colorless. However, the *M. liminatans* F_{420}-dependent enzyme is inhibited by 1,10-phenanthroline and α,α'-dipyridyl. Notably, these chelators are without effect on the $NADP^+$-requiring enzyme from *M. palustre*. In contrast, the sulfhydryl-reactive reagent iodoacetate (2 mM) had no effect on the F_{420}-dependent enzyme of *M. liminatans*, but completely inactivated the $NADP^+$-dependent enzyme from *M. palustre* (Bleicher and Winter, 1991).

The effects of temperature and salt concentration on the stability of the F_{420}-dependent enzymes also distinguish these enzymes from the $NADP^+$-utilizing ADH enzymes in methanogens. Furthermore, the F_{420}-dependent ADH enzymes exhibit pH optima which are well below the values found for $NADP^+$-dependent enzymes. Optimum activity for alcohol oxidation by enzymes which utilize $NADP^+$ was observed at pH 8 for the pure ADH from *M. palustre* (Bleicher and Winter, 1991) and at pH 10.0 in crude extracts of *M.organophilum* (Frimmer and Widdel, 1989). In comparison, alcohol oxidation by coenzyme F_{420}-dependent enzymes exhibited pH optima at pH 6 for the *M. liminatans* enzyme (Bleicher and Winter, 1991) and at pH 4.2 for the enzyme from *M. thermophilum* (Widdel and Wolfe, 1989). The dissimilar pH optima of the two classes of methanogen ADH enzymes might be explained, at least in part, by the difference in the number of protons taken up in reduction of their obligate cofactors. The unusual features of substrate and coenzyme specificity provide much incentive for further studies on the alcohol dehydrogenase enzymes of methanogens.

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8

Bioenergetics of Methanogenesis

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8.1 Introduction

The bioenergetics of methanogens could be expected to have special features, given that they are members of the *Archaea* and utilize a number of unique reactions and coenzymes in the pathways of methanogenesis. This turns out to be so; however, it was not until 1980 that the elucidation of the biochemistry of methanogenesis attained a stage where the bioenergetics could be studied. Since then, progress has been made, reactions involved in energy conservation have been identified, and it became clear that at least some of these reactions proceed in or at the cytoplasmic membrane. For example, it now makes sense that only methylotrophic methanogens contain cytochromes that were first discovered in methanogens in 1979; likewise, the general sodium ion dependence of methanogenesis is now understood. Indeed, the bioenergetics of methanogenesis has unique features: ATP synthesis takes advantage of proton as well as of sodium gradients, both of which are generated by primary pumps.

8.2 Thermodynamic Aspects of Methanogenesis

8.2.1 Methanogenesis from H_2 + CO_2

The most common substrate of methanogens is $H_2 + CO_2$, which can be utilized by almost all representatives of this group with the exception of a few obligate methylotrophic and acetotrophic species. Carbon dioxide reduction with

H_2 to CH_4 is a highly exergonic process under standard conditions (Table 8.1, reaction 1). However, it has to be kept in mind that the H_2 partial pressure in methanogenic ecosystems is only 10^{-5} to 10^{-4} atm, resulting in a decrease of the free energy change associated with this fermentation to about 30 kJ/mol (Thauer, 1990). Taking into account that about 50 kJ/mol is required to synthesize ATP from ADP and P_i under cellular conditions, less than one mol of ATP can be synthesized per mol of CH_4 formed.

Looking at the individual steps of the CO_2 reduction pathway it becomes

Table 8.1 Standard free energy changes of methanogenic fermentations and reactions involved in methanogenesis from various methanogenic substrates.

Reaction	$\Delta G^{\circ\prime}$
1 $CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$	−130 kJ/mol CH_4
2 $4HCOOH \rightarrow 3\ CO_2 + CH_4 + 2\ H_2O$	−119 kJ/mol CH_4
3 $4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$	−106 kJ/mol CH_4
4 $4\ (CH_3)_3NH^+ + 6\ H_2O \rightarrow 9\ CH_4 + 3\ CO_2 + 4\ NH_4^+$	−76 kJ/mol CH_4
5 $CH_3COOH \rightarrow CH_4 + CO_2$	−36 kJ/mol CH_4
6 $\mathbf{CO_2} + H_2 + MF \rightarrow \mathbf{HCO}—MF + H_2O$	+16 kJ/mol
7 $\mathbf{HCO}—MF + H_4MPT \rightarrow \mathbf{HCO}—H_4MPT + MF$	−5 kJ/mol
8 $\mathbf{HCO}—H_4MPT + H^+ \rightarrow \mathbf{CH}{\equiv}H_4MPT^+ + H_2O$	−2 kJ/mol
9 $\mathbf{CH}{\equiv}H_4MPT^+ + F_{420}H_2 \rightarrow \mathbf{CH_2}{=}H_4MPT + F_{420} + H^+$	+6.5 kJ/mol
10 $\mathbf{CH_2}{=}H_4MPT + F_{420}H_2 \rightarrow \mathbf{CH_3}—H_4MPT + F_{420}$	−5 kJ/mol
11 $\mathbf{CH_3}—H_4MPT + HS{-}CoM \rightarrow \mathbf{CH_3}—S{-}CoM + H_4MPT$	−29 kJ/mol
12 $H_2 + F_{420} \rightarrow F_{420}H_2$	−13.5 kJ/mol
13 $\mathbf{CH_3}—S{-}CoM + H_2 \rightarrow CH_4 + HS{-}CoM$	−85 kJ/mol
14 $\mathbf{CH_3}—S{-}CoM + HS{-}HTP \rightarrow \mathbf{CH_4} + CoM{-}S{-}S{-}HTP$	−43 kJ/mol
15 $CoM{-}S{-}S{-}HTP + H_2 \rightarrow HS{-}CoM + HS{-}HTP$	−42 kJ/mol
16 $HCOOH + F_{420} \rightarrow CO_2 + F_{420}H_2$	−16 kJ/mol
17 $\mathbf{CH_3OH} + HS{-}CoM \rightarrow \mathbf{CH_3}—S{-}CoM + H_2O$	−27.5 kJ/mol
18 $CoM{-}S{-}S{-}HTP + \mathbf{HCO}—MF \rightarrow HS{-}CoM + HS{-}HTP + \mathbf{CO_2} + MF$	−58 kJ/mol
19 $CoM{-}S{-}S{-}HTP + F_{420}H_2 \rightarrow HS{-}CoM + HS{-}HTP + F_{420}$	−29 kJ/mol
20 $CH_3{-}COOH + ATP \rightarrow CH_3{-}CO{-}P + ADP$	+13 kJ/mol
21 $CH_3{-}CO{-}P + CoA{-}SH \rightarrow CH_3{-}CO{-}S{-}CoA + P_i$	−9 kJ/mol
22 $CH_3{-}COOH + ATP + CoA{-}SH \rightarrow CH_3{-}CO{-}S{-}CoA + AMP + PP_i$	−6 kJ/mol
23 $ADP + P_i \rightarrow ATP$	+32 kJ/mol
24 $CH_3{-}CO{-}S{-}CoA + H_4MPT \rightarrow CH_3H_4MPT + CO + HS{-}CoA$	+62 kJ/mol
25 $CH_3{-}H_4MPT + CO + H_2O \rightarrow CH_4 + CO_2 + H_4MPT$	−134 kJ/mol
26 $CO + H_2O \rightarrow CO_2 + H_2$	−20 kJ/mol

evident that the formation of formyl-methanofuran (CHO-MF) from methanofuran (MF), CO_2, and H_2 is endergonic under standard conditions (Table 8.1, reaction 6). At a hydrogen partial pressure of 10^{-5} to 10^{-4} atm, $\Delta G'$ increases to about +40 kJ/mol. This highly endergonic reaction necessitates the input of energy in a yet unknown form; this reaction is discussed in more detail in Section 8.4.1(b) and 8.4.3(b).

The reactions involved in the conversion of formyl-MF to methyltetrahydromethanopterin (CH_3-H_4MPT) are catalyzed by the cytoplasmic enzymes formyl-MF:H_4MPT formyltransferase, methenyl-H_4MPT cyclohydrolase, methylene-H_4MPT dehydrogenase, and methylene-H_4MPT reductase (Table 8.1, reactions 7-10). The $\Delta G^{\circ\prime}$ values associated with these reactions are small under standard conditions and they can be considered as being fully reversible. Reduced coenzyme F_{420} ($F_{420}H_2$) serves as the electron donor in the methylene-H_4MPT dehydrogenase and the methylene-H_4MPT reductase reactions. The $F_{420}H_2$ oxidized in these reactions is regenerated by the F_{420}-reducing hydrogenase which appears to be present in all hydrogenotrophic methanogens. The H_2-dependent F_{420} reduction is exergonic under standard conditions (Table 8.1, reaction 12). The F_{420}-dependent hydrogenases which were purified from methanogens such as *Methanobacterium thermoautotrophicum*, *Methanosarcina barkeri*, and *Methanococcus voltae* are found in the cytoplasmic fraction after cell breakage (Fauque et al., 1984; Fox et al., 1987; Muth et al., 1987). However, a membrane association could be demonstrated in three of these organisms with the immunogold labeling technique (Baron et al., 1989; Lünsdorf et al., 1991; Muth, 1988). Electron transfer from H_2 via the F_{420}-dependent hydrogenase to methenyl-H_4 MPT or methylene-H_4MPT is not associated with energy conservation, but a role of the F_{420}-dependent hydrogenase in energy transduction in other reactions is still a matter of debate.

Methyl group transfer from CH_3-H_4MPT to coenzyme M (HS-CoM) gives rise to methyl-CoM and is exergonic (Table 8.1, reaction 11). In *M. thermoautotrophicum* more than 80% of the methyl-H_4MPT:CoM methyltransferase which catalyzes this reaction is bound to the cytoplasmic membrane (Kengen et al., 1992). Moreover, recent findings in *Methanosarcina* strain Gö1 indicate a novel type of energy transduction linked to this methyl transfer reaction (see Section 8.4.1(a)).

Methane formation from methyl-CoM with H_2 is the energetically most favorable reaction of the CO_2 reduction pathway (Table 8.1, reaction 13). That this reaction is sufficiently exergonic to support a methanogenic cell's energy demand can be concluded from the fact that *Methanosphaera stadtmanae* grows exclusively on methanol $+H_2$ as an energy source (Miller and Wolin, 1985). The H_2-dependent reductive demethylation of methyl-CoM consists of two partial reactions (Bobik et al., 1987; Ellermann et al., 1989) both of which are associated with a large free energy change (Table 8.1, reactions 14 and 15). Interestingly,

as we will see further below, it is the H_2-dependent heterodisulfide reduction which is directly linked to energy generation (Table 8.1, reaction 15).

Many hydrogenotrophic methanogens are also capable of utilizing formate as a substrate. Methanogenesis from formate is about as exergonic as that from H_2 + CO_2 (Table 8.1, reaction 2). Formate is oxidized to CO_2 which in turn enters the carbon dioxide reduction pathway. Reducing equivalents generated by the oxidation of formate enter the pathway at least partly by way of $F_{420}H_2$ (Table 8.1, reactions 9 and 10). There are no indications for an energetic coupling associated with the formate-dependent reduction of F_{420} (Table 8.1, reaction 16) as catalyzed by F_{420}-dependent formate dehydrogenase (Barber et al., 1983; Jones and Stadtman, 1981). It is not yet clear whether $F_{420}H_2$ serves also as electron carrier in the first CO_2 reduction step and in the heterodisulfide reduction during growth on formate, or whether a F_{420}-nonreactive formate dehydrogenase is capable of transferring the electrons directly to the respective redox reaction. Although produced in small quantities, H_2 does not appear to be an obligate intermediate in methanogenesis from formate (Schauer and Ferry, 1980).

8.2.2 Methanol

Methanol utilization in the absence of an external electron donor such as H_2 is restricted to the Methanosarcinaceae. The methanol utilization pathway consists of an oxidative and of a reductive branch. The overall process is highly exergonic (Table 8.1, reaction 3). The $\Delta G^{\circ\prime}$ associated with methanogenesis from methanol is theoretically sufficient for the synthesis of two ATP from ADP and P_i per CH_4 formed (assuming a $\Delta G'$ of 50 kJ/mol required under cellular conditions for ATP synthesis).

Methanol utilization is initiated by the exergonic methyl group transfer to coenzyme M in a reaction that involves corrinoids (Table 8.1, reaction 17) (Van der Meijden, Heythuysen, et al., 1983; Van der Meijden, Jansen, et al., 1983). The methanol:HS-CoM methyltransferase is a soluble enzyme and there are no indications for an energetic coupling associated with this reaction. The utilization of methylamines occurs in an analogous fashion, with the exception that specific methylamine:HS-CoM methyltransferases are expressed. A trimethylamine:HS-CoM methyltransferase was demonstrated in *M. barkeri* (Naumann et al., 1984). The reduction of methyl-CoM occurs in principal in the same way as described above for the CO_2 reduction pathway with the exception that the electrons for methyl-CoM reduction are not derived from H_2 but from methyl group oxidation.

It is still a matter of debate whether methyl-CoM is an obligate intermediate for the methyl groups undergoing oxidation to CO_2. Direct methyl group transfer to H_4MPT has been suggested (Keltjens and van der Drift, 1986). Methyl group oxidation involves reactions 6 to 11 of Table 8.1, but in the reverse direction.

The electron acceptor in formyl-MF oxidation (Table 8.1, reaction 6) is not yet known (Fischer and Thauer, 1989; Mahlmann et al., 1989). Assuming that the methyl group oxidation occurs by way of methyl-CoM, the endergonic methyl group transfer to H_4MPT (Table 8.1, reaction 11) can be expected to require an energetic coupling. It is reasonable to assume that the dependence of methanol oxidation on the transmembrane electrochemical gradient of sodium ions ($\Delta\tilde{\mu}_{Na^+}$) in *M. barkeri* (Müller, and Blaut et al., 1988) reflects such an energetic coupling. The reaction is expected to be catalyzed by the membrane-bound methyl-H_4MPT:CoM methyltransferase mentioned above in connection with the CO_2 reduction pathway (for a detailed discussion see Section 8.4.3(a)). The reactions that follow and bring about the conversion of methyl-H_4MPT to formyl-MF occur in the cytoplasm and lead to the reduction of two F_{420}. The $\Delta G^{\circ\prime}$ values of these reactions are small and the reactions are fully reversible under standard conditions. Since the physiological electron acceptor employed in the oxidation of formyl-MF to CO_2 is unknown, the $\Delta G^{\circ\prime}$ of this reaction cannot be calculated. However, the midpoint potential at pH 7 ($E_{m,7}$) of the CO_2/CHO-MF couple (−500 mV) indicates that a low potential electron carrier can be reduced. There are indications that the formyl-MF oxidation is accompanied by the generation of a $\Delta\tilde{\mu}_{Na^+}$ across the membrane (see Section 8.4.1(b)). The CHO-MF-dependent heterodisulfide reduction is associated with a large $\Delta G^{\circ\prime}$ (Table 8.1, reaction 18). In contrast, $\Delta G^{\circ\prime}$ of the electron transfer from $F_{420}H_2$ to CoM-S-S-HTP is considerably smaller (Table 8.1, reaction 19). Both the CHO-MF-dependent and the $F_{420}H_2$-dependent heterodisulfide reduction are membrane processes associated with energy transduction.

8.2.3 Acetate

Methanogenesis from acetate has the smallest $\Delta G^{\circ\prime}$ value of all methanogenic substrates (Table 8.1, reaction 5). Therefore, less than one ATP can be synthesized per mol of methane formed. The conversion of acetate starts with an activation to acetyl-CoA. Whereas *Methanosarcina* species activate acetate by the combined action of acetate kinase and phosphotransacetylase (Table 8.1, reactions 20, 21) acetate activation in *Methanothrix* species is catalyzed by acetate synthetase (Table 8.1, reaction 22). Since in *Methanosarcina* one ATP is invested for acetate activation it has to be postulated that methanogenesis from acetyl-CoA delivers more than one ATP per CH_4 formed (Table 8.1, reaction 23). The cleavage of acetyl-CoA as catalyzed by carbon monoxide dehydrogenase yields enzyme-bound CO and a bound methyl group (Fischer and Thauer, 1989; Laufer et al., 1987). The latter is transferred via a corrinoid protein to H_4MPT; the subsequent transfer of the methyl group to CoM is expected to be catalyzed by the sodium-motive methyl-H_4MPT:HS-CoM methyltransferase. The reactions leading from methyl-H_4MPT to methane are the same ones that were already

discussed above for the CO_2 reduction pathway (Table 8.1, reactions 11, 14, 15) with the exception that CO but not H_2 serves as electron donor in reaction 15. Considering enzyme-bound CO as being equivalent to free CO, the acetyl-CoA cleavage is highly endergonic (Table 8.1, reaction 24) and must therefore be assumed to be coupled with the exergonic CO-dependent reduction of methyl-H_4MPT (Table 8.1, reaction 25) which occurs in several steps. In one step, enzyme-bound CO undergoes a ferredoxin-dependent oxidation to carbon dioxide as catalyzed by carbon monoxide dehydrogenase (Fischer and Thauer, 1990a; Terlesky and Ferry, 1988a). Evidence has been presented that the conversion of CO to CO_2 and H_2 by resting cells of *M. barkeri* (Table 8.1, reaction 26) is coupled with the synthesis of ATP (Bott et al., 1986). This suggests that the ferredoxin-dependent CO oxidation is also associated with energy transduction.

8.3 The Mechanism of ATP Synthesis in Methanogens

Chemotrophic bacteria such as methanogens derive their metabolic energy from the oxidation of organic or inorganic electron donors. The free energy changes ($\Delta G^{\circ\prime}$) associated with these reactions are transformed into a biologically useful form of energy which is ATP. Two principal mechanisms of ATP synthesis can be distinguished in chemotrophic organisms: substrate level phosphorylation (SLP) and electron transport phosphorylation (ETP).

8.3.1 Substrate Level Phosphorylation (SLP) versus Electron Transport Phosphorylation (ETP)

Whether methanogenic bacteria gain energy by ETP or SLP was a matter of controversy when investigators first addressed this question. From the beginning, however, it was clear that methanogens are not principally different from other organisms with respect to the utilization of ATP as the universal form of cellular energy. This was evident from an increase in the energy charge as observed during methanogenesis from H_2-CO_2 in *Methanobacterium* (Roberton and Wolfe, 1970). We will briefly discuss the differences between the two modes of energy generation in the context of methanogenesis.

ETP means a process in which the oxidation of a substrate involves electron transport along membrane-bound electron carriers with increasing redox potentials ($E_{m,7}$) to a terminal acceptor molecule. The electron carriers are arranged in such a way that the flow of electrons through this electron transport chain is accompanied by the extrusion of protons from the inside of the bacterial cell to its outside. This electrochemical potential difference (inside electrically negative and alkaline), the protonmotive force (Δp), is composed of an electrical potential difference ($\Delta\Psi$) and a chemical potential difference ($\Delta pH = pH_{in} - pH_{out}$):

$$\Delta p = \frac{\Delta\tilde{\mu}_{H^+}}{F} = \Delta\Psi - 2.303\frac{RT}{F}\Delta\text{pH} \qquad \text{(volts)}$$

or

$$\Delta\tilde{\mu}_{H^+} = F\Delta\Psi - 2.303\,RT\,\Delta\text{pH} \qquad (\text{J mol}^{-1})$$

where R is the gas constant (8.3 kJ $\text{mol}^{-1}\,\text{K}^{-1}$), T is the temperature in K, and F is Faraday's constant (96 500 J V^{-1} mol^{-1}). The energy stored in this transmembrane proton gradient is competent in driving a number of processes such as the synthesis of ATP from ADP and P_i (*via* a membrane-bound ATP synthase), active transport, and flagellar movement. The synthesis of ATP is accompanied by the influx of protons into the bacterial cell along their potential difference. The number of protons (n) needed for ATP synthesis and $\Delta\tilde{\mu}_{H^+}$ correlate with the phosphorylation potential (ΔG_p):

$$n(-\Delta p) = \frac{\Delta G_p}{F} \qquad \text{(volts)}$$

or:

$$n(-\Delta\tilde{\mu}_{H^+}) = \Delta G_p \qquad (\text{J mol}^{-1})$$

ΔG_p corresponds to the actual free energy change for the synthesis of ATP from ADP and P_i:

$$\Delta G_p = \Delta G^{\circ\prime} + 2.303\,RT\log\frac{[\text{ATP}]}{[\text{ADP}]\,[P_i]}$$

Typically, the synthesis of ATP, but not electron transport, is inhibited by protonophores which, because of their mode of action, are also called uncouplers. These agents render the membrane permeable to protons, thereby dissipating $\Delta\tilde{\mu}_{H^+}$. Other inhibitors such as *N,N'*-dicyclohexylcarbodiimide (DCCD) inhibit ATP synthesis by blocking the flow of protons through the ATP synthase and, at the same time, cause a decrease in electron transport because an increased $\Delta\tilde{\mu}_{H^+}$ slows down the electron transport-coupled H^+ ejection.

SLP refers to a process in which the free energy change ($\Delta G^{\circ\prime}$) of a reaction is conserved through the formation of a phosphorylated intermediate. The activated phosphoryl group of this intermediate is subsequently transferred to ADP, giving rise to ATP. In contrast to ETP, SLP does not depend on membranes. Organisms that gain ATP exclusively by SLP have to hydrolyze ATP in order to generate $\Delta\tilde{\mu}_{H^+}$ for processes that depend on this form of energy (Harold, 1972). In these organisms, the membrane-bound ATP synthase mentioned above works in the reverse direction from an ATPase, meaning that the hydrolysis of ATP to ADP and P_i drives the extrusion of H^+. In this situation, protonophores do not influence the ATP-generating reactions. However, since ATP is used to generate $\Delta\tilde{\mu}_{H^+}$,

protonophores may lead to a drop in the energy charge when the cells increase the ATPase activity to overcome the effect of the protonophore. In organisms that employ SLP as the sole mode of energy generation, ATPase inhibitors cause an increase rather than a decline of the energy charge because less ATP is hydrolyzed for $\Delta\tilde{\mu}_{H^+}$ generation when the ATPase is blocked.

There are several simple observations that cannot be reconciled with SLP-coupled methanogenesis: (1) If it is assumed that methanogens synthesize ATP via SLP, a phosphorylated intermediate (X~P) must be formed which serves as phosphoryl donor for ADP. Looking at the biochemistry of methanogenesis there is no indication for the formation of a phosphorylated intermediate. (2) SLP implies a 1:1 stoichiometry between the formation of X~P and the formation of ATP. The $\Delta G^{\circ\prime}$ associated with methanogenesis from acetate is not sufficient to synthesize one mol of ATP per one mol of acetate. (3) SLP does not depend on the presence of membranes, and can be observed *in vitro* in the cytoplasmic fraction. In the case of methanogens, however, a 1:1 stoichiometric formation of ATP coupled to any reaction of the methanogenic process could never be observed *in vitro*.

On the other hand, there are two studies that could not be reconciled with ETP as the mode of ATP generation in methanogens. ATP formation independent from $\Delta\tilde{\mu}_{H^+}$ was reported for *M. voltae* and *M. thermoautotrophicum* using H_2-CO_2 or formate as methanogenic substrate (Crider et al., 1985; Schönheit and Beimborn, 1985). Whereas the experiments with *M. voltae* were taken to suggest a theoretical model for ATP synthesis by SLP (Lancaster, 1986) the studies with *M. thermoautotrophicum* were extended, leading to results that explained many contradictory observations (Kaesler and Schönheit, 1988; Mountfort et al., 1986). It was shown that this organism generates a $\Delta\tilde{\mu}_{H^+}$ of -200 mV during methane formation, and that protonophores, in the concentrations used, were unable to decrease the $\Delta\tilde{\mu}_{H^+}$ below -100 mV. Interestingly, this potential could still drive ATP synthesis and the first step of methanogenesis. However, a gradual decrease of $\Delta\Psi$ from -100 to -10 mV diminishes both the cellular ATP content and methanogenesis. It was furthermore shown that some protonophores do not dissipate $\Delta\tilde{\mu}_{H^+}$ effectively, even when administered at high concentrations.

Inhibition of methanogenesis from $H_2 + CO_2$ by protonophores as observed in a number of studies (Blaut and Gottschalk, 1984b; Butsch and Bachofen, 1984; Jarrell and Sprott, 1983; Roberton and Wolfe, 1970) can be understood if it is assumed that the inhibition is unspecific or, if it is specific, that a coupling exists between a $\Delta\tilde{\mu}_{H^+}$-generating reaction and a $\Delta\tilde{\mu}_{H^+}$-utilizing reaction. Indeed, many observations support the idea of a coupling between the most exergonic reactions of the CO_2 reduction pathway and the endergonic formation of formyl-MF from CO_2, H_2, and MF (see above). Whether the coupling occurs via $\Delta\tilde{\mu}_{H^+}$ or some other kind of transmembrane ion gradient will be discussed further below. Due

to the inhibitory effect of protonophores on methanogenesis from $H_2 + CO_2$ the experiments done with this substrate are neither clearly in favor nor against ETP as the mode of ATP generation in methanogens.

A study with resting cells of *M. barkeri*, however, presented strong evidence for ATP synthesis by ETP in this organism (Blaut and Gottschalk, 1984a). Methanogenesis from H_2 + methanol needs only the transfer of the methyl group from methanol to coenzyme M and subsequent methyl-CoM reduction. Using H_2 + methanol as the methanogenic substrate it was possible to circumvent reactions that might need the input of energy in order to proceed and to study the relevant energetic parameters of the electron transport from H_2 to methyl-CoM in whole cells. Under such conditions, protonophores dissipate $\Delta\tilde{\mu}_{H^+}$ and lead to a rapid decline of the intracellular ATP content, but not to a decrease in the rate of methanogenesis. This indicates that ATP can only be formed in the presence of $\Delta\tilde{\mu}_{H^+}$. However, it is theoretically still possible to explain the low ATP content with a highly active H^+-translocating ATPase which rapidly hydrolyzes ATP in order to reestablish the $\Delta\tilde{\mu}_{H^+}$ dissipated by the protonophore. The latter possibility was excluded by showing that the ATPase inhibitor DCCD does not decrease $\Delta\tilde{\mu}_{H^+}$, which would be expected if $\Delta\tilde{\mu}_{H^+}$ was generated by ATP hydrolysis *via* an ATPase. Hence, it could be concluded that ETP is the mode of energy transduction during growth on methanol + H_2. This was corroborated by the direct demonstration of the extrusion of three to four protons per methane formed from methanol + H_2 (Blaut et al., 1987).

8.3.2 Electron Transport Reactions Linked to Energy Transduction

It was now of importance to find out which of the individual reactions involved in methanogenesis from H_2 + methanol is associated with the generation of a $\Delta\tilde{\mu}_{H^+}$. For this purpose it was necessary to establish a cell-free system capable of ATP synthesis. The methyl group transfer from methanol to coenzyme M as catalyzed by cytoplasmic enzymes (Van der Meijden, Heythuysen, et al., 1983; Van der Meijden, Jansen, et al., 1983) was excluded from being involved in energy transduction in response to methanogenesis from H_2 + methyl-CoM (Blaut and Gottschalk, 1984a). Therefore, only methyl-CoM reduction with H_2 could be the site coupled to proton extrusion from the cells. Since methyl-CoM does not simply cross the cytoplasmic membrane, a cell-free system had to be established in which the cytoplasmic side of the cytoplasmic membrane faces the outside. Such a system became available after the discovery that the protein cell wall surrounding *Methanosarcina* strain Gö1 could be easily digested by applying a time-dependent pronase treatment leading to the formation of protoplasts (Jussofie et al., 1986) which upon passage through a French pressure cell at a reduced pressure give rise to crude vesicles, 80% of which show an inside-out orientation. Such a vesicle preparation of *Methanosarcina* strain Gö1 catalyzes ATP synthesis

in response to H_2-dependent methane formation from methyl-CoM (Peinemann et al., 1989). In this system, ATP synthesis, but not methanogenesis, is inhibitable by protonophores. The main disadvantage of this vesicle system was its poor coupling between methanogenesis and ATP synthesis (1 mol ATP synthesized per 100 mol CH_4 produced). Moreover, this vesicle preparation still contained cytoplasmic components.

(a) The H_2-Dependent Heterodisulfide Reductase System

When it became evident that methyl-CoM reduction to methane consists of two partial reactions (Table 8.1, reactions 14 and 15) the question arose whether the HS-HTP-dependent methyl-CoM reduction or the H_2-dependent reduction of CoM-S-S-HTP is associated with the generation of $\Delta\tilde{\mu}_{H^+}$. The synthesis of ATP from ADP and P_i by the crude vesicle system in response to H_2-dependent CoM-S-S-HTP reduction suggested that only the electron transport from H_2 to the heterodisulfide, but not methanogenesis from HS-HTP and methyl-CoM, is competent in generating a $\Delta\tilde{\mu}_{H^+}$ which drives ATP synthesis (Peinemann et al., 1990). This result led to the conclusion that a membrane location of the methylreductase is not functionally necessary, although a membrane association of this protein could be demonstrated by immunoelectron microscopy in *Methanococcus voltae* and *M. thermoautotrophicum,* but not in *M. barkeri* (Aldrich et al., 1987; Ossmer et al., 1986). Electron microscopic studies in *Methanosarcina* strain Gö1 suggested the organization of the methylreductase in a huge membrane-associated complex referred to as methanoreductosome (Mayer et al., 1988). Nevertheless, in most methanogens the majority of the methylreductase activity is found in the cytoplasmic fraction after cell fractionation. This is also true for *Methanosarcina* strain Gö1, in which the fractionation of the crude inverted vesicles leads to inverted vesicles free of cytoplasmic components referred to as washed vesicles, and to a soluble fraction which contains the majority of the methylreductase activity (Deppenmeier et al., 1989).

The washed inverted vesicles of this organism catalyze a H_2-dependent reduction of CoM-S-S-HTP which is accompanied by H^+ translocation into the lumen of the vesicles. The $\Delta\tilde{\mu}_{H^+}$ thereby generated drives the synthesis of ATP from ADP and P_i. In contrast to the crude vesicle system, which still contains cytoplasmic components and shows a poor coupling, the washed inverted vesicles exhibit a stringent coupling between CoM-S-S-HTP reduction and ATP synthesis with maximal stoichiometries of 1 H^+ translocated/e^- and 1 ATP synthesized/4 e^- (Deppenmeier et al., 1991). Protonophores stimulate electron transport (heterodisulfide reduction) but inhibit proton translocation and ATP synthesis.

(b) Components Involved in H_2-dependent Heterodisulfide Reduction

(i) Hydrogenase. It is evident that the H_2-dependent heterodisulfide reductase system depends on the presence of a hydrogenase in order to oxidize H_2. Two

types of hydrogenase have been isolated from methanogens; they differ with respect to the electron acceptor utilized. The F_{420}-dependent hydrogenase reacts with F_{420} and viologen dyes, whereas the F_{420}-nonreactive hydrogenase cannot reduce F_{420} and reacts only with viologen dyes. The latter enzyme is therefore often referred to as methyl viologen (MV)-reducing hydrogenase. Factor F_{420}-dependent hydrogenases were purified from a number of methanogens such as *M. thermoautotrophicum*, *Methanobacterium formicicum*, *M. barkeri*, and *M. voltae* (Fauque et al., 1984; Fox et al., 1987; Jin et al., 1983; Muth et al., 1987; Baron and Ferry, 1989).

The question arises which of the two types of hydrogenase plays a role in the H_2-dependent heterodisulfide reduction. It has been suggested that the MV-reducing hydrogenase is employed in this reaction, whereas the F_{420}-dependent hydrogenase is used to supply $F_{420}H_2$ for the non-$F_{420}H_2$-dependent reactions in the carbon dioxide reduction pathway (Deppenmeier et al., 1991). Evidence in favor of this notion comes from the observation that after repeated washing of membranes from *Methanosarcina* strain Gö1, only the MV-dependent enzyme remains membrane-bound, whereas the F_{420}-dependent hydrogenase is washed off. These membranes display a rapid H_2-dependent heterodisulfide reduction. The MV-reducing hydrogenase is therefore thought to be part of this electron transport system. The enzyme was recently purified from membranes of *Methanosarcina* strain Gö1 after solubilization with the detergent CHAPS (Deppenmeier et al., 1992). It is composed of two subunits with molecular masses of 40 and 60 kDa, and it contains Ni, Fe, and acid-labile S. Flavins, however, were not detected. The F_{420}-nonreactive hydrogenases purified from *M. formicicum* and *M. thermoautotrophicum* exhibit similar features, but there are no indications of a membrane location (Jin et al., 1983; Kojima et al., 1983). Interestingly, the N-termini of the polypeptides of the hydrogenase from *Methanosarcina* strain Gö1 show a higher degree of similarity to the H_2 uptake hydrogenase of some eubacteria than to the F_{420}-dependent enzyme from *M. formicicum* and *M. thermoautotrophicum* (Deppenmeier et al., 1992). The genes encoding the large and small subunit of the F_{420}-nonreactive hydrogenase from *M. thermoautotrophicum* and *Methanothermus fervidus* were sequenced and revealed a high degree of similarity with eubacterial hydrogenases, indicating a common evolutionary ancestry (Reeve et al., 1989; Steigerwald et al., 1990). The genes are organized in an operon with two additional genes, one of which codes for a polyferredoxin, while the function of the other gene is still unclear. It is possible that the products of these genes may act as membrane anchors for the two catalytic subunits and participate in electron transport.

(ii) Heterodisulfide Reductase. The last part of the H_2-dependent heterodisulfide reductase system is the heterodisulfide reductase itself. This enzyme is assayed by following the CoM-S-S-HTP-dependent oxidation of reduced benzyl-viologen, or the oxidation of HS-CoM and HS-HTP with methylene blue as an

oxidant. The heterodisulfide reductase was purified from the soluble fraction of *M. thermoautotrophicum* after cell breakage (Hedderich et al., 1990). In contrast, in *Methanosarcina* strains the enzyme is membrane-bound and detergents have to be employed for their solubilization (U. Deppenmeier, unpublished results). Unfortunately, the purification of this enzyme from a methylotrophic methanogen has not yet been accomplished. The physiological electron donor for CoM-S-S-HTP reduction is not yet known. However, it is reasonable to assume that an additional electron carrier mediates the electron transfer from the hydrogenase to the heterodisulfide reductase, since otherwise it is difficult to see how the electron transport can be coupled to proton translocation. So far there is no hint as to the nature of such a postulated electron carrier. Cytochromes were suggested to be involved in electron transport from H_2 to methyl-CoM in *M. barkeri* (Kemner et al., 1987), but more recent experiments indicate that the H_2 -dependent cytochrome reduction observed may be nonphysiological (Kamlage and Blaut, 1992). Since cytochromes were only detected in the Methanosarcinaceae, the majority of the methanogens must rely on different electron carriers. It is conceivable that the aforementioned polyferredoxin is involved in this process.

(c) The $F_{420}H_2$-Dependent Heterodisulfide Reductase System

Washed inverted vesicles of *Methanosarcina* strain Gö1 and membranes from *Methanolobus tindarius* contain another energy-transducing heterodisulfide reductase system which does not depend on H_2, but on $F_{420}H_2$, as the reductant (Deppenmeier et al., 1990a; Deppenmeier et al., 1990b). The $F_{420}H_2$-dependent heterodisulfide reduction was also shown to be competent in driving proton translocation into the lumen of inverted vesicles of *Methanosarcina* strain Gö1, resulting in the generation of a $\Delta\tilde{\mu}_{H^+}$. Protonophores stimulate the heterodisulfide reduction, but prevent the establishment of $\Delta\tilde{\mu}_{H^+}$ and ATP synthesis. The ATP synthase inhibitor DCCD decreases the rate of $F_{420}H_2$-dependent heterodisulfide reduction. The reversal of this DCCD-mediated inhibition by protonophores and the stimulation of the $F_{420}H_2$-dependent heterodisulfide reduction by ADP indicate a stringent coupling between electron transport and and ATP synthesis. The $F_{420}H_2$-dependent heterodisulfide reductase system displays stoichiometries of 1 H^+ translocated/e^- and 0.8 ATP synthesized/4e^-.

(d) Components Involved in $F_{420}H_2$-Dependent Heterodisulfide Reduction

(i) The $F_{420}H_2$ Dehydrogenase. The $F_{420}H_2$ dehydrogenase is the entry point for the electrons derived from $F_{420}H_2$ oxidation to an electron transport chain that leads to the heterodisulfide reductase. The enzyme can be assayed *in vitro* by the artificial electron acceptor metronidazole in the presence of MV as mediator:

$$F_{420}H_2 + \text{metronidazole}_{\text{oxidized}} \rightarrow F_{420} + \text{metronidazole}_{\text{reduced}}$$

The $F_{420}H_2$ dehydrogenase was isolated from *M. tindarius* after solubilization of membranes with the zwitterionic detergent CHAPS (Haase et al., 1992). The apparent molecular mass of the native enzyme is 120 kDa, consisting of five different subunits of 45, 40, 22, 18, and 17 kDa. It contains 16 mol Fe and 16 mol acid-labile S per mol of enzyme, but flavin was not detectable. The absence of flavin is interesting with respect to the question of how the enzyme manages the two-electron/one-electron transition from the hydrogen carrier $F_{420}H_2$ to the one-electron carrier MV which serves as mediator in this system.

(ii) Electron Carriers Mediating the Electron Transport between $F_{420}H_2$ Dehydrogenase and Heterodisulfide Reductase. The question arises whether there are possible electron carriers that mediate the electron transport from the $F_{420}H_2$-dehydrogenase to the heterodisulfide reductase and couple this electron transport to the translocation of protons. The solubilization behavior of the $F_{420}H_2$-dehydrogenase in the purification process argues against an intrinsic membrane protein (Haase et al., 1992). In addition, little is known about how tightly the heterodisulfide reductase in *Methanosarcina* species binds to the membranes. The accessibility of the enzyme to CoM-S-S-HTP in inverted vesicles argues in favor of its location on the cytoplasmic side of the membrane. This and the presence of the heterodisulfide reductase in the soluble fraction of *M. thermoautotrophicum* argue against an intrinsic membrane location. As a consequence, the presence of an electron carrier in the membrane which is engaged in electron transport and proton translocation must be postulated (Deppenmeier et al., 1990b). Since quinones have not been detected in methanogenic bacteria, other components must be involved in proton translocation. Recent experiments strongly suggest the participation of one or several cytochromes in electron transport from $F_{420}H_2$ to CoM-S-S-HTP (Kamlage and Blaut, 1992). Membranes of *Methanosarcina* strain Gö1 contain two *b*- and two *c*-type cytochromes with midpoint potentials of −135 mV, −240 mV (*b*-type cytochromes) and −140 mV, −230 mV (*c*-type cytochromes). The cytochromes are reduced by $F_{420}H_2$ and oxidized by CoM-S-S-HTP at high rates. Addition of CoM-S-S-HTP to reduced cytochromes and subsequent low temperature spectroscopy showed the oxidation of cytochrome b_{564}. The presence of cytochromes appears to be restricted to the Methansosarcinaceae (Kühn et al., 1983), the same group of organisms that is assumed to have an $F_{420}H_2$-dependent heterodisulfide reductase system.

(e) The Physiological Role of the Two Heterodisulfide Reductase systems

In light of the finding that membranes from the obligate methylotroph *M. tindarius* are devoid of the H_2-dependent heterodisulfide reductase system, it is

reasonable to suggest that methanogens not capable of hydrogenotrophic growth contain only the $F_{420}H_2$-dependent heterodisulfide reductase. In accordance with this assumption, *Methanosarcina* strain Gö1, which may grow either methylotrophically or hydrogenotrophically, contains both the H_2-dependent and the $F_{420}H_2$-dependent system (Deppenmeier et al., 1991). However, the experimental evidence in the obligate hydrogenotroph *Methanococcus thermolithotrophicus* is not unambiguous: membranes from this organism are essentially devoid of benzylviologen-dependent heterodisulfide reductase. This activity is almost completely recovered from the soluble fraction, as was reported for *M. thermoautotrophicum* (Hedderich and Thauer, 1988). Since the membranes from *M. thermolithotrophicus* contain relatively high F_{420}-dependent hydrogenase activity, in contrast to those from *Methanosarcina* strain Gö1, it is uncertain whether the observed metronidazole/MV-dependent $F_{420}H_2$ oxidation is due to $F_{420}H_2$ dehydrogenase or F_{420}-dependent hydrogenase activity. It will be necessary to test more organisms for the presence of the two heterodisulfide reductase systems.

From the physiological point of view it appears reasonable to assume that the $F_{420}H_2$ dehydrogenase plays a prominent role during methylotrophic growth because at least two out of three redox reactions involved in methyl group oxidation lead to the formation of $F_{420}H_2$ (Table 8.1, reactions 9 and 10 in reverse direction). The $F_{420}H_2$-dependent heterodisulfide reductase then serves to regenerate F_{420} and to channel the electrons to the heterodisulfide (Figure 8.1). During hydrogenotrophic growth the situation is different. Carbon dioxide reduction to the level of methyl-H_4MPT involves in at least two out of three redox reactions with $F_{420}H_2$ as electron donor (Table 8.1, reactions 9 and 10). $F_{420}H_2$ is supplied by the F_{420}-dependent oxidation of H_2 as catalyzed by the F_{420}-reactive hydrogenase. It is theoretically possible that the latter enzyme also plays a role in the reduction of the heterodisulfide. However, as pointed out previously, the H_2-dependent heterodisulfide reductase system in *Methanosarcina* strain Gö1 is independent of F_{420}, and the inside out vesicles produced from this organism are devoid of F_{420}-dependent hydrogenase. On the basis of these facts it appears very likely that only the F_{420}-nonreactive hydrogenase is involved in H_2-dependent heterodisulfide reduction. The different roles of the two hydrogenases in the hydrogentrophic metabolism are summarized in Figure 8.2.

Which heterodisulfide reductase is involved in methanogenesis from formate is not yet clear. In light of the fact that all formate dehydrogenases isolated so far from methanogenic microbes utilize F_{420} as electron acceptor (Barber et al., 1983; Jones and Stadtman, 1981), it appears reasonable to assume that the $F_{420}H_2$-dependent heterodisulfide reductase is involved. However, cytochromes have not been found in (formate-utilizing) hydrogenotrophic methanogens. If the H_2-dependent reductase played a role, then H_2 should be an obligate intermediate, which is not in accordance with the experimental findings (Schauer and Ferry,

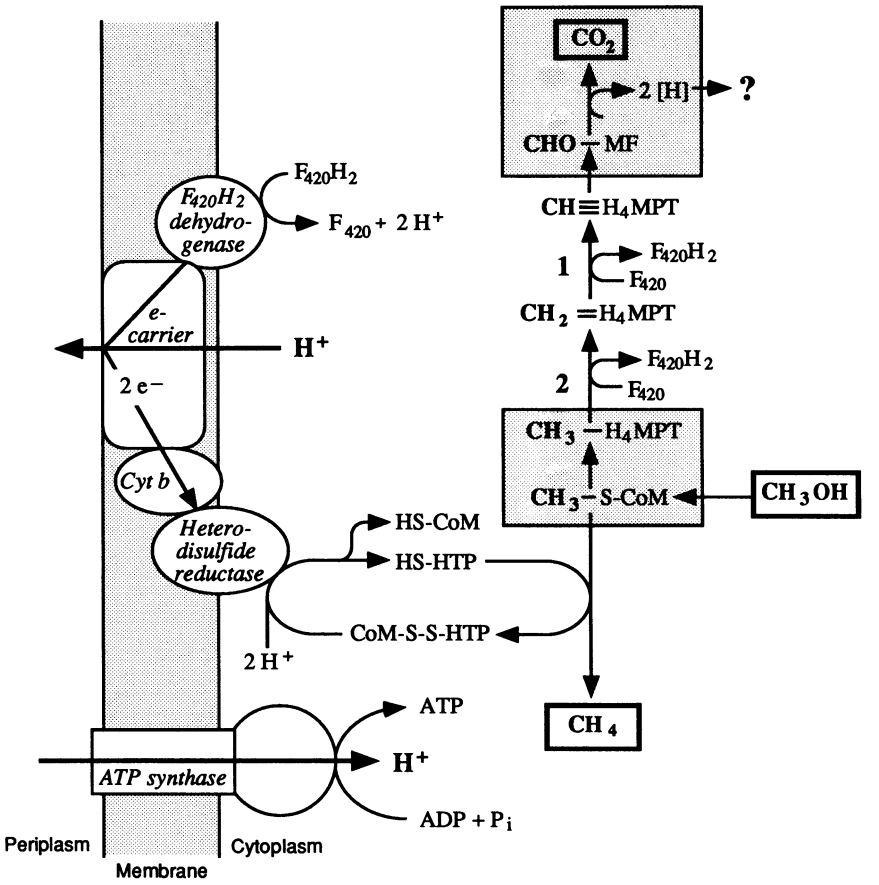

Figure 8.1. Hypothetical scheme showing the role of the $F_{420}H_2$-dependent heterodisulfide reductase system in energy transduction during methanogenesis from methanol. The stoichiometry of methanol conversion is not taken into consideration. Grey boxes indicate reactions involved in membrane-dependent energy transduction (formyl-MF dehydrogenase and methyl-H_4MPT:coenzyme M methyltransferase). The reactions generating reduced F_{420} are numbered: 1, methylene:H_4MPT dehydrogenase; 2, methylene:H_4MPT reductase. The unknown physiological electron acceptor in the formyl-MF dehydrogenase reaction is indicated by (?).

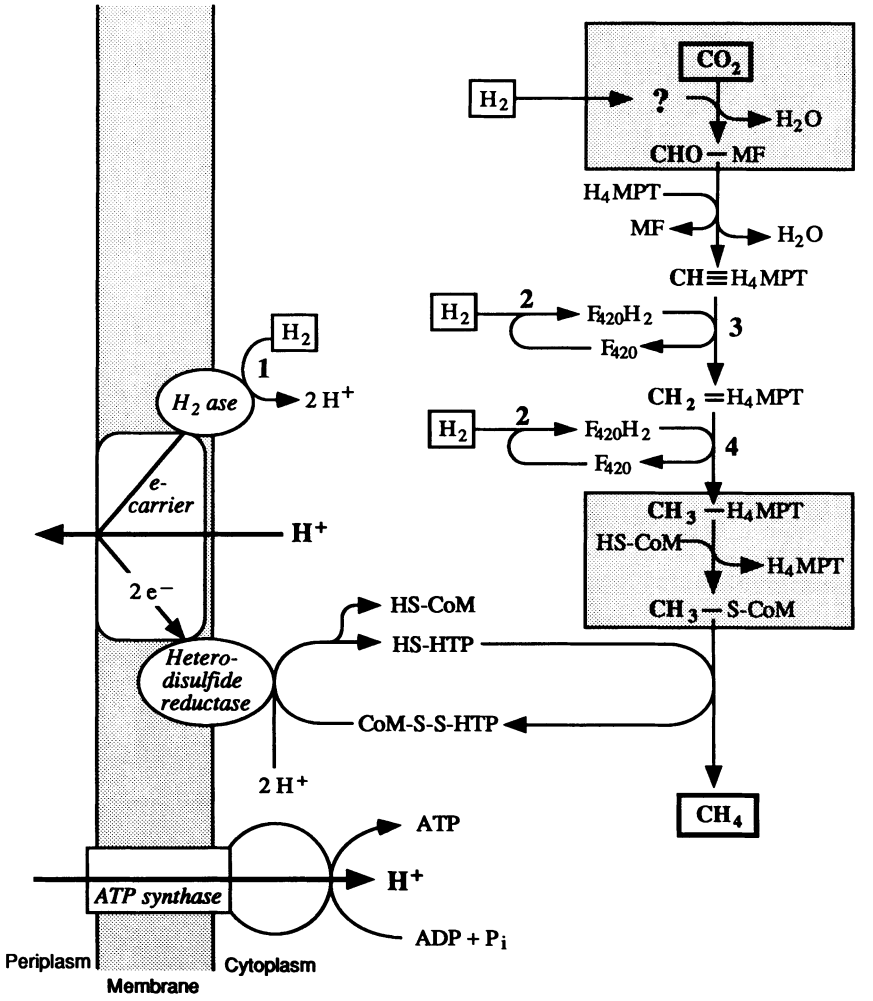

Figure 8.2. Hypothetical scheme showing the role of the H_2-dependent heterodisulfide reductase system in energy transduction during methanogenesis from H_2 + CO_2. Grey boxes indicate reactions involved in membrane-dependent energy transduction (formyl-MF dehydrogenase and methyl-H_4MPT:coenzyme M methyltransferase). The numbers indicate the following reactions: 1, F_{420}-non-dependent hydrogenase; 2, F_{420}-dependent hydrogenase; 3, methylene:H_4MPT dehydrogenase; 4, methylene:H_4MPT reductase. The unknown physiological electron donor in the formyl-MF dehydrogenase reaction is indicated by (?).

1980). Thus, how formate oxidation is coupled with energy conservation is still an open question.

(f) Possible Mechanisms of $\Delta\tilde{\mu}_{H^+}$ Formation Coupled to H_2- or $F_{420}H_2$-Dependent Heterodisulfide Reduction

If it is assumed that the MV-dependent hydrogenase is located on the periplasmic side and the heterodisulfide reductase on the cytoplasmic side of the membrane, the generation of $\Delta\tilde{\mu}_{H^+}$ can be easily envisaged, for the uptake of H_2 and transfer of electrons to an electron acceptor would lead to the liberation of scalar protons on the outside of the cytoplasmic membrane (Daniels et al., 1984). Such a mechanism of $\Delta\tilde{\mu}_{H^+}$ generation was reported for sulfate-reducing bacteria (Odom and Peck, 1981). Although such a role for hydrogenase cannot be ruled out completely, several findings argue against it: (1) There is no evidence for a periplasmic location of any of the hydrogenases isolated so far from methanogens. (2) Isotope fractionation studies performed with *M. thermoautotrophicum* indicated the release of protons in the cytoplasm upon H_2 oxidation (Daniels et al., 1980). (3) The H^+/CH_4 ratio of 3 to 4 determined in whole cells of *M. barkeri* for proton translocation in response to methanogenesis from methanol $+H_2$ cannot be accounted for by this mechanism (Blaut et al., 1987).

For the $F_{420}H_2$-dependent heterodisulfide reductase it is even more difficult to formulate a reasonable concept of how proton translocation is brought about. In light of the experiments with inverted vesicles of *Methanosarcina* strain Gö1 it is certain that both the $F_{420}H_2$-dehydrogenase and the heterodisulfide reductase are located on the cytoplasmic side of the cytoplasmic membrane, since both substrates can reach the respective enzyme only in inside-out, but not in right-side-out, vesicles. Therefore, scalar proton production on the periplasmic side can be excluded. The presence of additional hydrogen and electron carriers on the periplasmic side would allow formulation of a model of proton translocation. However, quinones appear to be absent from membranes of methanogens, while other proteins, besides the ones described above, have not yet been identified. In the case of the $F_{420}H_2$-dependent heterodisulfide reductase, a direct involvement of cytochromes in proton translocation is conceivable.

At present it appears most likely that proton translocation is brought about in both systems by a redox-driven proton pump. However, a final answer awaits the identification and functional reconstitution of all components involved.

8.3.3 Energy Transduction in Acetotrophic Methanogens

Very little is known about the energetic coupling in acetate degradation by methanogenic microbes. As pointed out previously, the free energy change associated with acetate activation, and the subsequent cleavage of acetyl-CoA to methyl-

H_4MPT and enzyme-bound CO, can be expected to be endergonic. This is true under both standard conditions and under cellular steady state conditions. The inhibtion of methanogenesis from acetate by ionophores in whole cells of *M.barkeri,* and the dependence on sodium ions (Peinemann et al., 1988; Perski et al., 1982), are in agreement with the idea of a coupling between the endergonic acetyl-CoA cleavage and the exergonic CO-dependent methyl-H_4MPT reduction to methane via a transmembrane ion gradient. On the other hand, the finding that the 100,000 x *g* supernatant of cell extracts of acetate-grown *M. barkeri* catalyzes the formation of CH_4 and CO_2 from acetyl-CoA, and that this activity is not affected by various ionophores (Fischer and Thauer, 1988; Krzycki and Zeikus, 1984), is difficult to reconcile with a coupling *via* transmembrane ion gradients. However, the considerable differences between the *in vivo* and the *in vitro* system must also be considered. The acetyl-CoA cleavage to CO_2 and CH_4 by cell extracts is not associated with ATP synthesis, and needs the presence of H_2 to proceed (Krzycki and Zeikus, 1984), whereas methane formation from acetate in whole cells is inhibited by H_2 (Eikmanns and Thauer, 1984). Note also that the low potential electron donor Ti(III)citrate could replace H_2 as an activator in the formation of H_2, CO_2, and methyl-H_4MPT from acetyl-CoA (Fischer and Thauer, 1990b). This might indicate that a low potential electron donor is also needed under *in vivo* conditions, and that transmembane ion gradients participate in the generation of a low potential electron donor by some sort of reverse electron transfer.

It is questionable whether H_2 is an obligate intermediate in the *in vivo* methanogenesis from acetate. In intact cells electron transport probably proceeds by way of membrane-bound electron carriers such as cytochromes *b* and *c* (Kemner et al., 1987; Terlesky and Ferry, 1988a) which are present in all organisms capable of methanogenesis from acetate (Kühn et al., 1983; Kühn and Gottschalk, 1983).

In view of the observation that CO oxidation to CO_2 and H_2 by resting cells of *M. barkeri* generates a $\Delta\tilde{\mu}_{H^+}$ (Bott and Thauer, 1989), it is reasonable to assume that there are two additional sites in CO-dependent methanogenesis associated with ion translocation. First, the heterodisulfide reduction is expected to be associated with $\Delta\tilde{\mu}_{H^+}$ generation and, second, the exergonic methyl transfer from methyl-H_4MPT to coenzyme M proceeds with $\Delta\tilde{\mu}_{Na^+}$ generation (see below). There are indications that reduced ferredoxin, which was shown in cell extracts of *M. barkeri* and *M. thermophila* to be formed during CO oxidation, serves as physiological electron donor in methyl-CoM reduction (Fischer and Thauer, 1990a; Terlesky and Ferry, 1988b). It is an open question how the electrons from reduced ferredoxin are channeled to the heterodisulfide. Are there any indications that the H_2-dependent or the $F_{420}H_2$-dependent heterodisulfide reductase plays a role in this electron transfer? It has already been pointed out that H_2 is most likely not an obligate intermediate in methanogenesis from acetate, and $F_{420}H_2$ does not appear to play a role either. This view is supported by the fact that the *in*

vitro methanogenesis from acetate in *M. barkeri* is not dependent on F_{420} (Fischer and Thauer, 1989) and that the F_{420} level is lower in acetate-grown cells than in cells grown on other substrates (Heine-Dobbernack et al., 1988). It is therefore conceivable that a third heterodisulfide reductase system exists in methanogenic bacteria which depends on reduced ferredoxin and is expressed during acetotrophic growth. Such a ferredoxin-dependent heterodisulfide reductase system would be engaged in proton-translocating electron transport. All of these ideas are summarized in a hypothetical scheme (Figure 8.3). More efforts are needed to elucidate the mode of energy transduction associated with methanogenesis from acetate.

8.4 Sodium Energetics in Methanogenic Bacteria

During the last decade it has become evident that sodium ions play a vital role in the bioenergetics of various organisms (Dimroth, 1991). This includes not only marine and alkalitolerant organisms, where an important function of this ion can be anticipated, but also freshwater isolates. Furthermore, a sodium-dependence of growth and product formation is found in both aerobic and anaerobic bacteria growing at neutral as well as alkaline external pHs (Skulachev, 1985; Müller et al., 1990). Recently the classical "proton" organism *Escherichia coli* was also reported to induce a respiration-linked primary sodium pump under conditions of a low $\Delta\tilde{\mu}_{H^+}$ (Avetisyan et al., 1989).

A number of studies revealed that sodium ions can substitute for protons in all types of membrane-linked reactions: osmotic (solute transport) (Maloy, 1990), chemical (ATP synthesis) (Hoffmann et al., 1990) and mechanical work (flagellar rotation) (Imae and Atsumi, 1989) can be driven by an electrochemical sodium ion gradient ($\Delta\tilde{\mu}_{Na^+}$) across the membrane. The $\Delta\tilde{\mu}_{Na^+}$ can be produced by different devices, including respiratory-linked reactions such as NADH-quinone reductases (Tokuda and Unemoto, 1985) and a terminal oxidase (Semeykina et al., 1989), by certain decarboxylases (Dimroth, 1987), as well as by ATPases (Hoffmann et al., 1990). Furthermore, there is at least one well studied organism, *Propionigenium modestum*, which does not have a proton energetics but relies solely on $\Delta\tilde{\mu}_{Na^+}$ as an "energy-rich" intermediate. In this example, the $\Delta\tilde{\mu}_{Na^+}$ is produced by the methylmalonyl-CoA decarboxylase and is in turn used to drive ATP synthesis via a sodium-translocating ATP synthase (Hilpert et al., 1984; Laubinger and Dimroth, 1988).

In 1981 it was reported that growth as well as methane formation from $H_2 + CO_2$ as carried out by *M. thermoautotrophicum* is strictly dependent on Na^+ (Perski et al., 1981). This is also true for methane formation from $H_2 + CO_2$, CH_3OH, or CH_3COOH, as carried out by resting cells of other methanogens (Perski et al., 1982). In analogy to the function of Na^+ in non-methanogenic

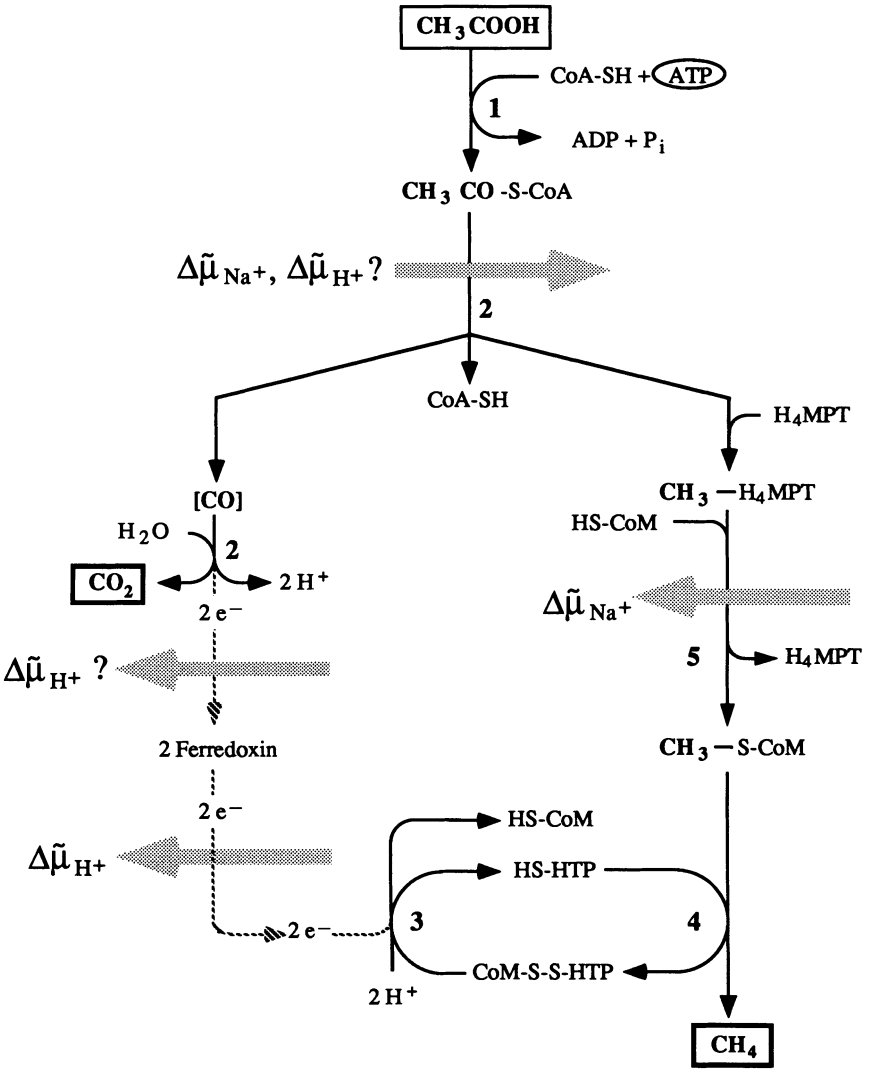

Figure 8.3. Hypothetical scheme of carbon and electron flow during methanogenesis from acetate. Horizontal grey arrows pointing to the left indicate reactions thought to be involved in the generation of transmembrane electrochemical gradients of H^+ ($\Delta\tilde{\mu}_{H^+}$) or Na^+($\Delta\tilde{\mu}_{Na^+}$). The horizontal grey arrow pointing to the right indicates that the highly endergonic acetyl-CoA cleavage reaction needs the input of energy, possibly in the form of $\Delta\tilde{\mu}_{H^+}$ or $\Delta\tilde{\mu}_{Na^+}$. The dashed lines and arrows indicate the flow of electrons. The numbers indicate the following reactions: 1, acetate kinase and phosphotransacetylase; 2, CO dehydrogenase; 3, heterodisulfide reductase; 4, methyl-CoM reductase; 5, methyl-H_4MPT:coenzyme M methyltransferase.

organisms, a role of this cation in methanogenic bacteria in intracellular enzyme catalysis, pH regulation, or ATP synthesis was envisaged. Since Na^+ is also required for methane formation by resting cells, it is evident that it plays a role in the energy-generating pathway of methanogenesis. Further studies led to the discovery of a novel, primary mechanism of sodium ion translocation not previously observed in living cells. In addition, under certain conditions methanogens also generate secondary sodium ion gradients by a Na^+/H^+ antiporter. The $\Delta\tilde{\mu}_{Na^+}$ serves as driving force for various energy-dependent reactions, including a novel function in driving a chemical reaction other than ATP synthesis. It is the purpose of the following section to describe the reactions and the mechanisms by which methanogenic bacteria create electrochemical sodium ion gradients, as well as to describe the reactions known so far which are driven by $\Delta\tilde{\mu}_{Na^+}$.

8.4.1 Primary Sodium Ion Pumps

In general, living cells tend to expel sodium ions from their cytoplasm (Lanyi, 1979); this is achieved by either primary or secondary mechanisms. Primary mechanisms can be linked to respiration, ATP hydrolysis, or decarboxylation of certain acids (Unemoto et al., 1990). As we will see below, methanogenic microbes add to this list a novel mechanism of primary Na^+ translocation.

(a) The methyl-H_4 MPT:HS-CoM Methyltransferase Is a Primary Sodium Ion Pump

The methyl-H_4MPT:HS-CoM methyltransferase was recently identified as the $\Delta\tilde{\mu}_{Na^+}$-generating reaction during methanogenesis from $H_2 + CO_2$. To elucidate the sodium-dependent reaction in the pathway of methanogenesis from $H_2 + CO_2$, the effect of Na^+ on methanogenesis from different methanogenic substrates was first compared. The substrates formaldehyde and methanol enter the central pathway at different redox levels, i.e., the level of methylene-H_4MPT and methyl-CoM, respectively; under an atmosphere of H_2 these substrates are exclusively reduced to methane.

By the use of these substrate combinations, the sodium dependence of the overall reaction from $H_2 + CO_2$ was, at least to some extent, localized (Müller, Blaut, et al., 1988). Methanogenesis from $H_2 + CO_2$, H_2 + HCHO, but not from $H_2 + CH_3OH$, was dependent on Na^+, indicating that the conversion of methylene-H_4MPT to the methyl-level (methyl-CoM or methyl-H_4MPT) is a sodium-dependent reaction during methanogenesis from $H_2 + CO_2$. This reaction has an apparent K_m for Na^+ of 0.8 ± 0.2 mM. It is accompanied by a free energy change of -34 kJ/mol and could therefore be involved in energy transduction, i.e., ion translocation. To elucidate the role of sodium ions during this reaction sequence, experiments with $^{22}Na^+$ were performed. Upon addition of H_2 + HCHO to cell suspensions of *M. barkeri*, sodium ions are actively extruded from the

cytoplasm resulting in the generation of a transmembrane Na^+ gradient of −60 mV. The mechanism of sodium ion translocation, i.e., primary versus secondary, was determined using various inhibitors. Na^+ translocation is not inhibited by protonophores or inhibitors of the Na^+/H^+ antiporter, indicating a primary mechanism. Na^+ translocation results in the generation of a protonophore-resistant membrane potential of −60 mV and, correspondingly, a protonophore-mediated reversed ΔpH (inside acidic) of the same magnitude as the $\Delta\Psi$ which is formed. Sodium ion extrusion is not inhibited by inhibitors of the ATP synthase present in this organism (Müller, Winner, et al., 1988). These results clearly demonstrate a primary and electrogenic mechanism of sodium ion translocation coupled to methanogenesis from formaldehyde $+H_2$. Experiments with *M. thermoautotrophicum* gave the same result, and a Na^+/HCHO stoichiometry of 3-4 was determined (Kaesler and Schönheit, 1989b).

The experiments described so far have shown that the conversion of formaldehyde to the formal redox level of methanol leads to a sodium transport across the membrane; however, this reaction sequence is complex and involves at least two enzymes, methylene-H_4MPT reductase and methyl-H_4MPT:HS-CoM methyltransferase (Table 8.1, reactions 10 and 11). Therefore, the approach described above leaves us with the question of which reaction is responsible for the ion translocation observed. The solution to this question came very recently from a study with inverted membrane vesicles using the intermediates methylene- and methyl-H_4MPT as substrates. Only 16% of the methylene-H_4MPT reductase in *Methanosarcina* strain Gö1 was found in the membrane fraction, which argues against a function in energy conservation (Becher et al., 1992b). The methyl-H_4MPT:HS-CoM methyltransferase activity was determined by measuring the methyl-H_4MPT or methyl-H_4F-dependent disappearance of HS-CoM. Interestingly, more than 88% of the activity was found in the membrane fraction, indicating that this enzyme is membrane-bound. Furthermore, the membrane-bound enzyme activity is strictly dependent on the presence of Na^+, indicating that the methyl-H_4MPT:HS-CoM methyltransferase is the actual sodium pump (Becher et al., 1992b).

This assumption was confirmed using $^{22}Na^+$ in transport studies. The overall reaction, the conversion of methylene-H_4MPT to methyl-CoM as catalyzed by washed inverted vesicles, is accompanied by a sodium ion transport into the lumen of the vesicles (Becher et al., 1992a). Na^+ transport was strictly dependent on HS-CoM, and inhibitor studies revealed that the sodium transport was primary and electrogenic. Of the partial reactions, the membrane-bound methylene-H_4MPT reductase activity is not coupled to sodium ion transport. On the other hand, the conversion of methyl-H_4MPT to methyl-CoM is coupled to a primary electrogenic sodium ion translocation. Interestingly, methyl-H_4MPT can be partly replaced by methyl-H_4F. That the reaction involves a corrinoid is demonstrated by the fact that methyl-CoM formation as well as sodium transport is inhibited

by propyliodide; upon illumination, methyl-CoM formation and, simultaneously, Na^+ transport are restored (Becher et al., 1992b). These experiments provide convincing evidence that the methyl-H_4MPT:CoM methyltransferase is a membrane-bound, corrinoid-containing enzyme that functions as a primary sodium ion pump. This is, to the best of our knowledge, the first demonstration of a methyltransferase functioning as an ion pump.

In view of a membrane-bound, corrinoid-containing methyl-H_4MPT:HS-CoM methyltransferase in *Methanosarcina* strain Gö1, the presence of a redox-active, membrane-bound corrinoid isolated from *M. thermoautotrophicum* is interesting (Schulz and Fuchs, 1986). Triton X-100 concentrations of 10 mg/mg protein are necessary to solubilize effectively the protein. It has an apparent molecular mass of 500 kDa and consists of subunits of 33, 28, 26 and 23 kDa, whereby the 33 kDa subunit carries the corrinoid (Schulz et al., 1988). Although a function in the central pathway could not be assigned to this protein, antibodies against it cross-react with a methyl-H_4MPT:HS-CoM methyltransferase isolated from *M. thermoautotrophicum* (Stupperich et al., 1990). These experiments provide further indications that the methyltransferase is a membrane-bound corrinoid protein. Interestingly, membrane-bound corrinoids are found in all methanogens tested so far (Dangel et al., 1987).

Recently, a membrane-bound methyl-H_4MPT:CoM methyltransferase has been purified from *M. thermoautotrophicum* strain ΔH (Kengen et al., 1992). The enzyme consists of three subunits in an $\alpha\beta\gamma$ configuration with apparent molecular masses of 35, 33 and 31 kDa. The enzyme contains 2 nmol of 5-hydroxybenzimidazolyl cobamide (B_{12}HBI) per mg of protein, and B_{12}HBI copurifies with the enzyme. Furthermore, the methyltransferase can be methylated by methyl-H_4MPT, indicating that it contains a corrinoid as a cofactor. The 33 kDa subunit might be identical to the 33 kDa corrinoid protein of unknown function isolated from membranes of *M. thermoautotrophicum*. This is substantiated by the finding that antibodies against the membrane-bound corrinoid protein inhibit the formation of methyl-CoM from formaldehyde $+H_2$ + HS-CoM (Kengen et al., 1992).

Several lines of evidence suggest that the membrane-bound, corrinoid-containing methyl-H_4MPT:HS-CoM methyltransferase is identical to the corrinoid protein found in membranes of different methanogenic bacteria. But how can the reaction be coupled to a sodium ion transport? Based on an analogous reaction, the transfer of a methyl group from methyl-H_4F to homocysteine (Banerjee and Matthews, 1990), the following working hypothesis is developed (Figure 8.4). The methyl-H_4MPT:HS-CoM methyltransferase is a membrane-integral protein with its corrinoid-containing subunit located in the membrane; the other two subunits do not necessarily have to be membrane-integral, but they must come into contact with the corrinoid as well as with methyl-H_4MPT and HS-CoM. Therefore, a membrane-peripheral location could account for such a function. Methyl-H_4MPT is bound to the enzyme and the methyl group then undergoes a

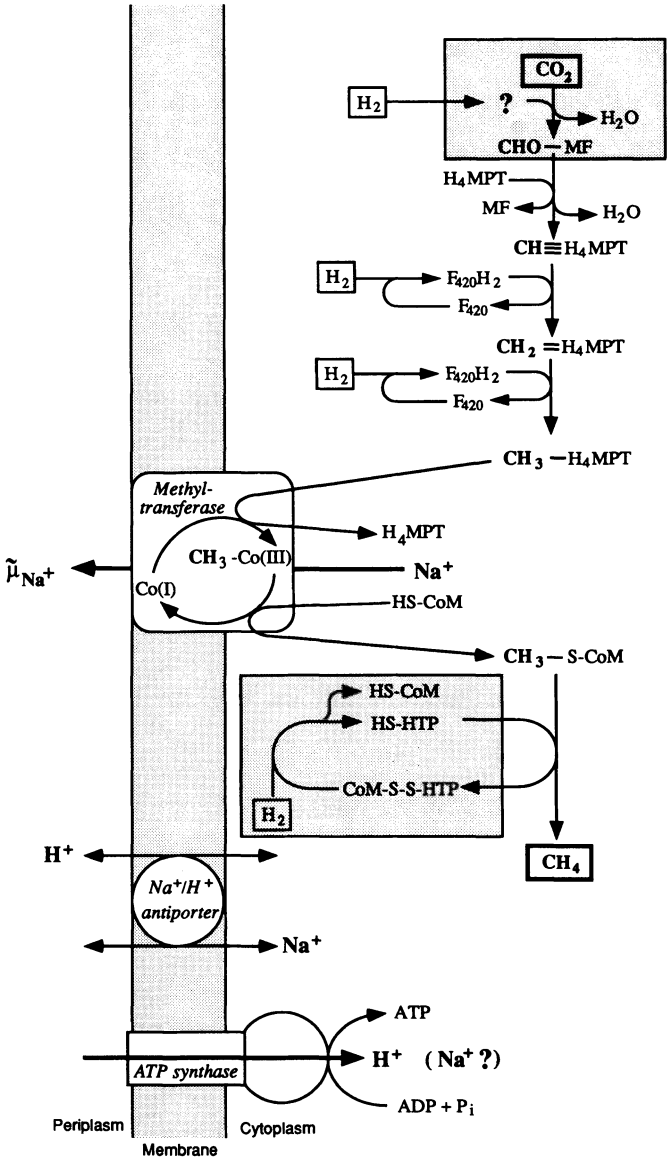

Figure 8.4. Hypothetical scheme showing the sodium-motive methyltransferase involved in methanogenesis fom H_2 + CO_2. Grey boxes indicate reactions involved in membrane-dependent energy transduction (formyl-MF dehydrogenase and H_2-dependent heterodisulfide reductase). The unknown physiological electron donor in the formyl-MF dehydrogenase reaction is indicated by (?). Also indicated is an Na^+/H^+ antiporter and a H^+-ATP synthase (for a possible involvement of Na^+ in ATP synthesis, see Section 8.5). Co(I) and methyl-Co(III) indicate the demethylated and methylated forms, respectively, of the methyl-H_4MPT:coenzyme M methyltransferase.

displacement by a nucleophilic attack of a Co(I) species; this reaction gives rise to H_4MPT, which is released from the enzyme, and an enzyme-bound methyl-Co(III) intermediate. The latter is then the substrate for a nucleophilic attack by the thiolate anion of HS-CoM; this reaction yields methyl-CoM and regenerates the Co(I) species. Future experiments will be designed to show how the sodium ion transport is linked to the methyl transfer reaction.

This model does not explain how the methyl-H_4MPT is activated prior to the nucleophilic displacement. A S_{N2} reaction mechanism at the α-carbon of a tertiary amine is without precedence. An activation could be achieved by a two electron oxidation giving rise to a quarternized N^5 in the molecule. However, since the methyl-H_4MPT:HS-CoM methyltransferase is assayed with washed membranes, the formation of a methyl-dihydromethanopterin seems unlikely unless the corresponding dehydrogenase is also membrane-bound. Another possibility is a one electron oxidation giving rise to an amine radical cation. Unless the methyltransferase itself is able to create and regenerate this radical, this mechanism is also unlikely. A likely explanation is an activation by protonation.

The demonstration of this primary sodium pump has important implications for the bioenergetics of methanogenesis from different substrates. Besides the proton-motive heterodisulfide reductase it represents a second site of energy transduction during growth and methanogenesis from all substrates which are converted via methyl-H_4MPT and methyl-CoM; these are: $H_2 + CO_2$, formate, H_2 + HCHO, and probably also acetate. As outlined above, the methyl group of acetate is transferred to H_4MPT (and not directly to HS-CoM) (Fischer and Thauer, 1989; Grahame, 1991) and the acetate-specific methyl-H_4MPT:HS-CoM methyltransferase is also expected to be sodium-motive. This is substantiated by a study on the bioenergetics of methanogenesis from acetate (Peinemann et al., 1988). As seen with $H_2 + CO_2$ as a substrate, the direction of ΔpH was reversed during methanogenesis from acetate (−20 mV, inside acidic). Most interesting was the fact that protonophores were unable to completely dissipate the electrical potential. A complete dissipation was only observed upon the combined action of protonophores and sodium ionophores, indicative of the presence of two primary transmembrane ion gradients, i.e., $\Delta\tilde{\mu}_{Na^+}$ as well as a $\Delta\tilde{\mu}_{H^+}$. However, the presence of a sodium motive methyl-H_4MPT:HS-CoM methyltransferase during methanogenesis from acetate still has to be proven experimentally.

(b) The Formylmethanofuran Dehydrogenase Reaction as Possible Site for Na^+-Translocation

As outlined above, the oxidation of formylmethanofuran to $CO_2 + H_2$ + methanofuran is part of the methanol oxidation pathway (see Section 8.2.2). Although the electron acceptor for this reaction is not known, the $\Delta G^{\circ\prime}$ as calculated with protons as oxidants is exergonic ($\Delta G^{\circ\prime} = -16$ kJ/mol; Table 8.1,

reversal of reaction 6). Kaesler and Schönheit (1989a) demonstrated that the energy is stored in the form of an electrochemical sodium ion gradient across the membrane. Cell suspensions of *M. barkeri* incubated in the presence of bromoethanesulfonate, which inhibits the reductive demethylation of methyl-CoM, oxidize formaldehyde in stoichiometric amounts to CO_2 and $2H_2$; therefore, this reaction sequence is, to some extent, suitable to address the question of the energetic coupling of the formylmethanofuran dehydrogenase reaction in whole cells. This reaction sequence is Na^+-dependent and accompanied by $^{22}Na^+$ translocation. Inhibitor studies revealed that Na^+ transport is primary and electrogenic, and results in the generation of a $\Delta\tilde{\mu}_{Na^+}$ of -118 mV. From the rate of Na^+ extrusion and HCHO oxidation a Na^+/HCHO stoichiometry of 3 was calculated. Essentially identical results were obtained with *M. thermoautotrophicum*. These results are in accordance with the presence of a sodium-motive enzyme involved in the oxidation of formaldehyde to CO_2, which is probably the formylmethanofuran dehydrogenase reaction. On the other hand, experiments performed in the laboratory of the authors do not reveal a sodium-dependence of this reaction sequence as analyzed in *M. barkeri* (Blaut et al., 1985; Winner and Gottschalk, 1989), and a re-investigation of the inhibitor studies of Kaesler and Schönheit could not be reproduced. This led to results which favor a proton-motive formylmethanofuran dehydrogenase reaction. More experiments with a purified system are needed to determine if a proton motive force drives the reaction catalyzed by the formylmethanofuran dehydrogenase.

Interestingly, more than 60% of the formylmethanofuran dehydrogenase is found in the membrane fraction after separation of a crude cell-free extract of *M. thermoautotrophicum* (Börner et al., 1989). The enzyme has been purified from *M. thermoautotrophicum* and *M. barkeri* (Karrasch et al., 1989; Karrasch et al., 1990a). In the latter case it has a molecular mass of 220 kDa and is composed of six non-identical subunits. The enzyme contains non-heme iron and acid-labile sulfur as well as a molybdopterin cofactor (Karrasch et al., 1990b) which occurs as a guanine, adenine and hypoxanthine dinucleotide derivative in a 1:0.4:0.1 ratio (Börner et al., 1991).

How the reaction can be coupled to ion transport remains in question. It is conceivable that the electrons derived from formylmethanofuran ($E_{m,7} = -500$ mV) are chanelled via a membrane-integral electron transport chain to the actual acceptor; the electron carriers involved are ion-motive and generate the ion gradient across the membrane. A hypothetical scheme is presented in Figure 8.5.

8.4.2 Secondary Sodium Ion Pumps

Whereas primary sodium ion pumps are only found in some bacteria, secondary sodium ion translocating systems are widespread amongst prokaryotes (Krulwich, 1983). These systems use the energy of the $\Delta\tilde{\mu}_{H^+}$ to extrude Na^+ from the

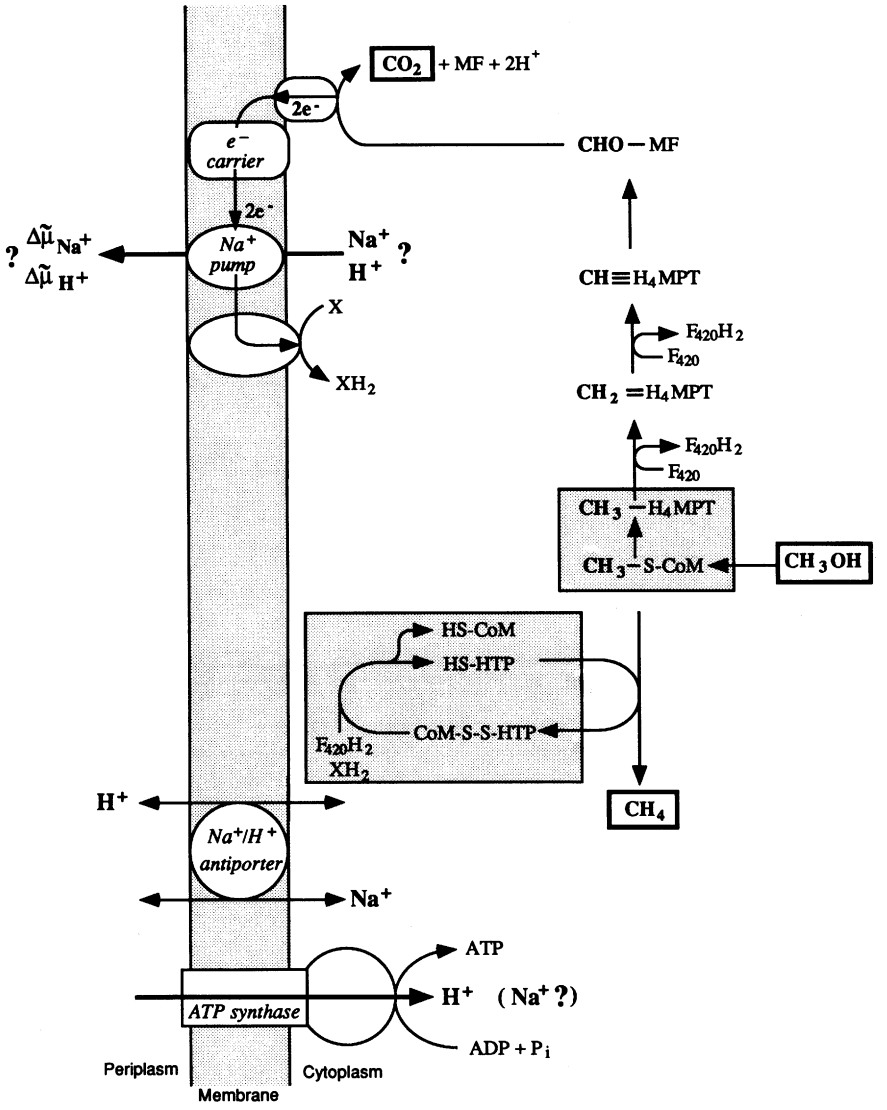

Figure 8.5. Hypothetical scheme showing the ion-motive formyl-MF dehydrogenase involved in methanogenesis from methanol. The ion transported may be Na^+ or H^+ (Section 8.4.1(b). Grey boxes indicate reactions involved in membrane-dependent energy transduction (methyl-H_4 MPT:coenzyme M methyltransferase and $F_{420}H_2$-dependent heterodisulfide reductase). X indicates the unknown electron acceptor of the formyl-MF dehydrogenase reaction. Also indicated is an Na^+/H^+ antiporter and a H^+-ATP synthase (for a possible involvement of Na^+ in ATP synthesis, see Section 8.5).

cytoplasm; this exchange results in the generation of both a ΔpNa and the influx of protons, resulting in regulation of the intracellular pH (Schuldiner an d Padan, 1992). This important dual function explains the ubiquitous presence of Na^+/H^+ antiporters .

(a) Na_+/H_+ Antiporter in Methanogenic Bacteria

Na^+/H^+ antiporters are found in *M. thermoautotrophicum* (Schönheit and Beimborn, 1985), *M. barkeri* (Müller et al., 1987a) and *Methanosarcina* strain Göl (V. Müller, unpublished results). They were determined by measuring the ΔpNa-dependent acidification of a weakly buffered cell suspension. Addition of NaCl to such suspensions results in a sudden drop of the extracellullar pH, indicating (but not proving) an exchange of Na^+ versus H^+. This exchange reaction is also observed with Li^+, but not with K^+. Furthermore, the acidification was inhibited by amiloride and harmaline, compounds known to inhibit the eukaryotic antiporter. This finding is further evidence for the presence of a Na^+/H^+ antiporter in methanogenic bacteria.

Although little is known about the kinetics and the stoichiometry of the exchange, the antiporter was shown to catalyze both, the generation of a ΔpNa (ΔpH-driven, during methane formation from methanol) (Müller et al., 1987a) as well as a ΔpH (ΔpNa-driven, during methanogenesis from $H_2 + CO_2$) (Kaesler and Schönheit, 1989a,b). If these exchanges are mediated by the same protein, we would have to postulate an electroneutral exchange ($H^+/Na^+ = 1$); in the case of the postulated $\Delta\tilde{\mu}_{Na^+}$-dependent ΔpH formation an electrogenic exchange ($H^+/Na^+ > 1$) would lead to the generation of a membrane potential which would, via a thermodynamic backup pressure, inhibit further proton extrusion. Interestingly, eukaryotic Na^+/H^+ antiporters are electroneutral whereas prokaryotic exchangers are electrogenic (Krulwich, 1983).

Most of the methanogenic microbes grow optimaly in neutral to slightly acidic media, but the external pH changes are due to the consumption or production of CO_2. In a number of studies the question of whether methanogenic microbes perform pH homeostasis was addressed, and the results obtained clearly showed the capacity of methanogenic microbes to regulate their intracellular pH (Jarrell and Sprott, 1981; Jarrell and Sprott, 1985; Müller et al., 1987a; Schönheit and Beimborn, 1985; Sprott et al., 1985). The Na^+/H^+ antiporter of *M. barkeri* was shown to be involved in pH regulation at alkaline but not acidic pH values (Müller et al., 1987a). Under alkaline conditions the protons re-enter the cytoplasm by exchange for Na^+, thus keeping the intracellular pH more acidic than the exterior. For *M. thermoautotrophicum* an involvement of Na^+ in pH regulation under alkaline conditions was not observed. On the other hand, from the impaired ΔpH formation at pH 5 in the absence of Na^+, an involvement of Na^+ in ΔpH formation was postulated (Schönheit and Beimborn, 1985). However, the meth-

ane formation rate in the absence of Na^+ was only 5% of the control and, therefore, the impaired ΔpH formation might also result from the low rate of electron transport-driven proton extrusion coupled to methanogenesis; normally, primary proton translocation is responsible for the alkalization under acidic conditions (Booth, 1985).

Besides its function in pH regulation, the antiporter is also essential for the generation of a ΔpNa under certain growth conditions. During the conversion of methanol, a $\Delta\tilde{\mu}_{H^+}$ is produced by the heterodisulfide reductase (see Section 8.2.2). At neutral to slightly acidic pH values (6.6–6.8) this primary proton gradient is converted to a secondary Na^+ gradient via a Na^+/H^+ antiporter; this was demonstrated by measuring proton as well as sodium ion movements across the membrane (Müller et al., 1987a). The ΔpNa formed is in turn used as driving force for various energy dependent-reactions.

Owing to the simultaneous presence of the two primary ion gradients, $\Delta\tilde{\mu}_{Na^+}$ and $\Delta\tilde{\mu}_{H^+}$, the antiporter plays a vital role in connecting the two primary ion gradients, and is, therefore, an interesting subject for further studies.

8.4.3 Functions of the Electrochemical Sodium Ion Gradient

In general, the $\Delta\tilde{\mu}_{Na^+}$ can be used for all three types of work, i.e., mechanical, osmotic and chemical. Whereas an involvement of Na^+ in flagellar rotation has not been investigated in methanogens, Na^+ was shown to be involved in the two latter types of work, whereby a new type of energy transduction with Na^+ as the coupling ion was observed.

(a) $\Delta\tilde{\mu}_{Na^+}$-Driven Conversion of Methanol to CO_2

The first clue for a function of Na^+ in methanogens came from a study of the methanol oxidation pathway in *M. barkeri*. As outlined above (see Section 8.2.2), methanol is disproportionated to CO_2 and CH_4. Sodium ions are not involved in the reductive branch but in the oxidative reaction sequence, specifically, in the conversion of methanol to the formal redox level of formaldehyde (i.e., methylene-H_4MPT). This reaction sequence is endergonic (reversal of reactions 11 and 10, Table 8.1); it depends on an energized membrane, i.e., it is not catalyzed by cell free extracts, and cell suspensions do not oxidize methanol in the presence of protonophores (Blaut et al., 1985). The same was observed with the substrate trimethylamine (Müller et al., 1987b).

Using different techniques this reaction was further analyzed. By measuring the energetically relevant parameters ΔpNa, $\Delta\Psi$ and ΔpH during methanogenesis from methanol it was shown that this endergonic reaction is driven by $\Delta\tilde{\mu}_{Na^+}$ (Müller, Blaut, et al., 1988). Furthermore, an electrogenic sodium influx accompanying this reaction was demonstrated. It was later shown that the oxidation of

methanol is impaired by the absence of a membrane potential, but can be restored by an artificial ΔpNa. However, this reaction sequence involves the methyl-H_4MPT:HS-CoM methyltransferase as well as the methylene-H_4MPT reductase and, using this approach, the question of which sodium-driven reaction is actually used could not be answered. The solution to this problem came from an investigation of the reverse reaction where it was shown that the methyl-H_4MPT:HS-CoM methyltransferase is the actual sodium pump (see Section 8.4.1(a)).

The finding of a sodium-motive methyltransferase has important implications for the pathway of the methyl group in the oxidation reactions. It was and is still a matter of debate whether the methyl group enters the central pathway at the level of HS-CoM or H_4MPT. From the Na^+-dependence of methanol oxidation, and the recent finding that the methyl transfer from methyl-H_4MPT to HS-CoM is the actual sodium-driven reaction, it can be deduced that methanol enters the pathway at the level of methyl-CoM, which is then converted to methyl-H_4MPT in a $\Delta\tilde{\mu}_{Na^+}$-driven reaction (Figure 8.6).

(b) $\Delta\tilde{\mu}_{Na^+}$-Driven Formation of Formyl Methanofuran from H_2 + CO_2

As outlined above, the formation of formylmethanofuran from methanofuran + H_2 + CO_2 is an endergonic reaction and necessitates the input of energy. Energy may be provided by a direct coupling with an exergonic reaction such as the hydrolysis of ATP, or by an electrochemical gradient of protons ($\Delta\tilde{\mu}_{H^+}$) or sodium ions ($\Delta\tilde{\mu}_{Na^+}$) to drive the unfavorable transport of electrons from an electron carrier with a more positive midpoint potential to one with a more negative midpoint potential (reverse electron transport). Whereas acetogenic bacteria couple the endergonic reduction of CO_2 to formyl-tetrahydrofolate in the formyl-tetrahydrofolate synthetase reaction (formyl-H_4F) with the hydrolysis of ATP (Ljungdahl, 1986), there is no indication for a role of ATP in the formation of CHO-MF or CHO-H_4MPT in methanogens.

The necessity for an energetic coupling in the direction of CHO-MF synthesis is indicated by several observations. First, R. P. Gunsalus observed in extracts of *M. thermoautotrophicum* that methyl-CoM greatly stimulates methanogenesis from H_2 + CO_2 (Gunsalus and Wolfe, 1977). It was later found that the stimulation of CHO-MF synthesis is due to the activation of an unknown electron carrier by CoM-S-S-HTP (Bobik and Wolfe, 1989). The low potential electron donor titanium(III) was shown to effectively activate CHO-MF synthesis in the absence of CoM-S-S-HTP, indicating that Ti(III) can replace the physiological low potential electron donor in this reaction. It is unclear in which way CoM-S-S-HTP leads to an activation of the first step in CO_2 reduction, but a coupling *via* transmembrane electrochemical gradients of ions (H^+ or Na^+) is conceivable. This means electron transport from H_2 to CoM-S-S-HTP would generate a $\Delta\tilde{\mu}_{H^+}$ or a $\Delta\tilde{\mu}_{Na^+}$, which drives the H_2-dependent reduction of an electron carrier with

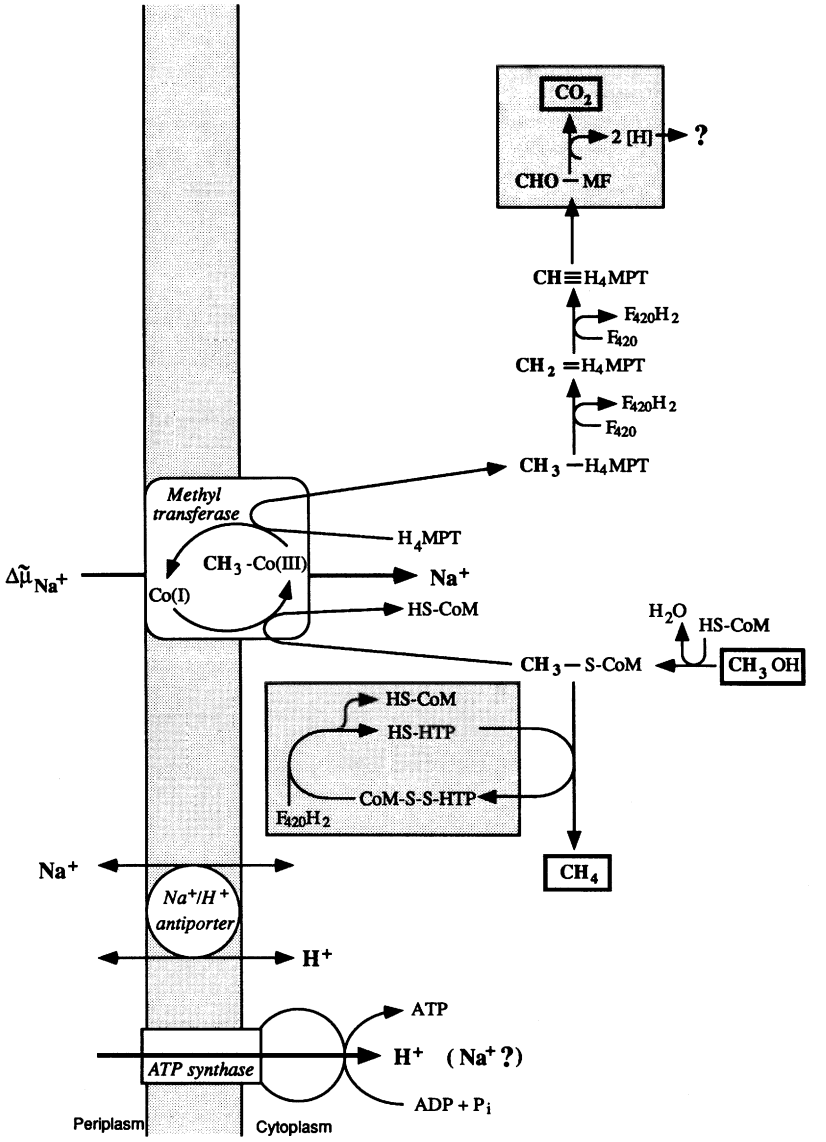

Figure 8.6. Hypothetical scheme of the involvement of the sodium-driven methyl-H_4MPT:coenzyme M methyltransferase in methanogenesis from methanol. Grey boxes indicate reactions involved in membrane-dependent energy transduction (formyl-MF dehydrogenase and $F_{420}H_2$-dependent heterodisulfide reductase). The unknown physiological electron acceptor in the formyl-MF dehydrogenase reaction is indicated by (?). Also indicated is an Na^+/H^+ antiporter and a H^+-ATP synthase (for a possible involvement of Na^+ in ATP synthesis, see Section 8.5). Co(I) and methyl-Co(III) indicate the demethylated and methylated forms, respectively, of the methyl-H_4MPT:coenzyme M methyltransferase.

a midpoint potential at pH 7 ($E_{m,7}$) sufficiently negative to serve as electron donor in CO_2 reduction (Figure 8.7). The $E_{m,7}$ of such an electron carrier should be in the range of that of the CO_2/CHO-MF couple (−500mv). The inhibition of methanogenesis from H_2 + CO_2 by agents affecting the $\Delta\Psi$, as observed in many laboratories (Butsch and Bachofen, 1984; Jarrell and Sprott, 1983; Roberton and Wolfe, 1970), favors such a notion. This is also substantiated by the recent finding that 60% of the formyl-MF dehydrogenase is found in the membrane fraction of *M. thermoautotrophicum* (Börner et al., 1989) (see Section 8.4.1(b)).

After the discovery of primary electrochemical sodium ion gradients in methanogens, the idea arose that the formation of formylmethanofuran might be driven by $\Delta\tilde{\mu}_{Na^+}$ (Müller, Blaut, et al., 1988). Indications for this assumption were obtained by Kaesler and Schönheit (1989b). As stated above, CO_2 reduction is sensitive to protonophores; however when the Na^+/H^+ antiporter of *M. barkeri* is inhibited by 5-(N-ethyl-N-isopropyl) amiloride (EIPA), CO_2 reduction to methane as carried out by resting cells becomes insensitive to protonophores. Under these conditions, a $\Delta\tilde{\mu}_{H^+}$ was not detected, but the $\Delta\tilde{\mu}_{Na^+}$ remained high at −120 mV. This experiment indicates a Na^+ cycle during methanogenesis from H_2 + CO_2 in the presence of protonophores and EIPA; the inhibition by protonophores in the absence of EIPA was explained by the activity of the Na^+/H^+ antiporter which dissipated $\Delta\tilde{\mu}_{Na^+}$ in the presence of protonophores. However, in earlier experiments the same group demonstrated that the inhibition of methanogenesis from H_2 +CO_2 by protonophores is relieved by addition of an artificial Na^+/H^+ antiporter, monensin, which is not in accordance with the results described above (Schönheit and Beimborn, 1986). Kaesler and Schönheit (1989b) demonstrated that CO_2 reduction rather than formaldehyde reduction is sensitive towards sodium ionophores, but since the energetic relevant parameters were not measured, this does not argue for $\Delta\tilde{\mu}_{Na^+}$ as a driving force. The most convincing experiment is the determination of the Na^+/substrate stoichiometries, which revealed a higher value for formaldehyde than for CO_2, which was then attributed to the $\Delta\tilde{\mu}_{Na^+}$-driven CO_2 reduction (Kaesler and Schönheit, 1988). However, it should be noted that experiments performed in our laboratory do not support $\Delta\tilde{\mu}_{Na^+}$ as driving force but favour $\Delta\tilde{\mu}_{H^+}$. Although the inhibitors are active in our system, their effect on various parameters tested is different from the finding of Schönheit and collaborators. Furthermore, if the aforementioned ion gradient, whose generation is coupled to the heterodisulfide reduction, is the actual RPG effector, than $\Delta\tilde{\mu}_{H^+}$ as driving force becomes very likely. Since all of these studies were done with resting cells and rely on inhibitor studies, experiments with purified systems are necessary in order to clarify this question.

(c) Na^+-Solute Symport and Other Transport Processes

Active transport in methanogenic bacteria has been described for only a few compounds. *Methanobacterium ruminantium* strain M1 takes up HS-CoM and

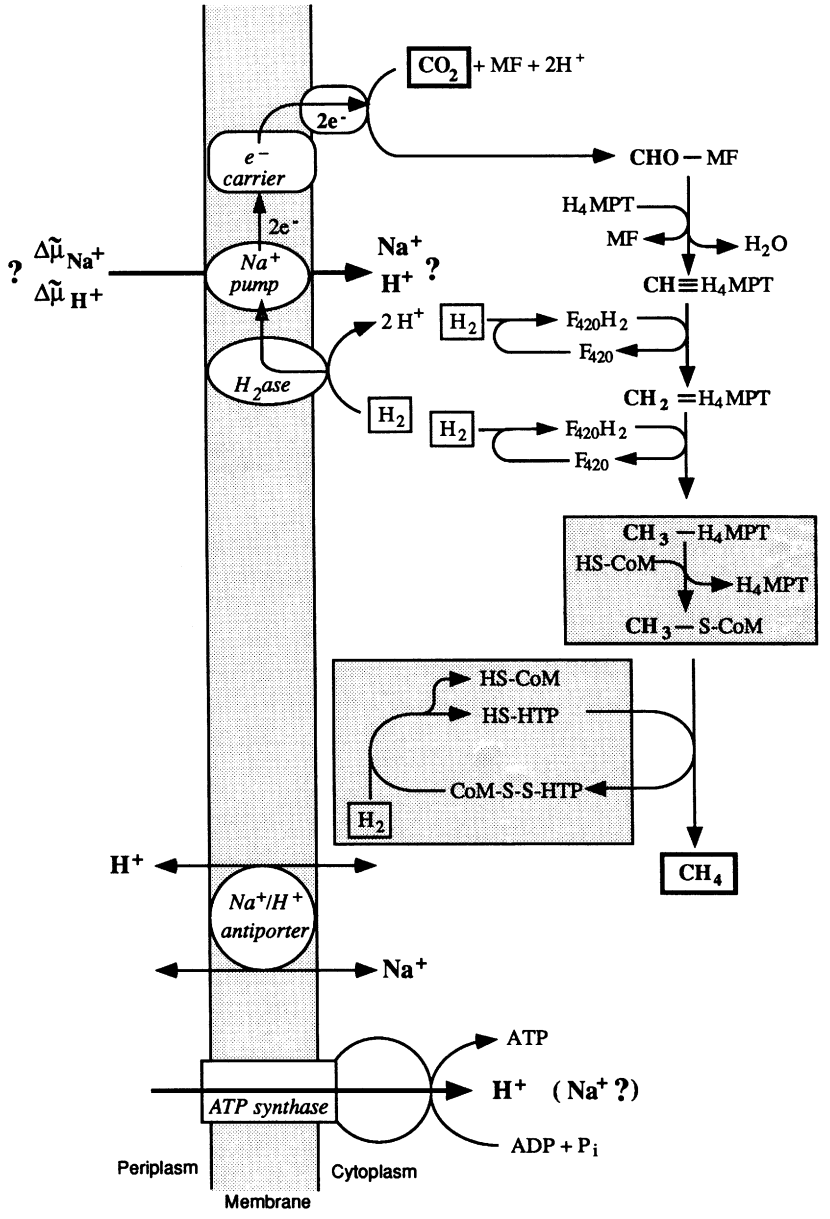

Figure 8.7. Hypothetical scheme of the ion gradient-driven formyl-MF dehydrogenase reaction involved in methanogenesis from H_2+ CO_2. Grey boxes indicate reactions involved in membrane-dependent energy transduction (methyl-H_4MPT:coenzyme M methyltransferase and H_2-dependent heterodisulfide reductase). Also indicated is an Na^+/H^+ antiporter and a H^+-ATP synthase (for a possible involvement of Na^+ in ATP synthesis, see Section 8.5).

methyl-CoM in an energy-dependent fashion (Balch and Wolfe, 1979). Air, bromoethanesulfonate (BES), and uncouplers inhibit the transport, but DCCD does not, indicating a secondary transport process. The K_m is 73 nM for HS-CoM and 50 nM for methyl-CoM and the V_{max} values are 312 and 320 pmol/min · mg dry weight for HS-CoM and methyl-CoM, respectively. Similar systems having only 30% and 10% of the *M. ruminantium* uptake rates were found in *Methanospirillum hungatei* and *Methanobacterium mobile*, respectively. The existence of a CoM transport system in *M. voltae* was suggested by a genetic study in which it was shown that a BES-resistant mutant is impaired in the transport of BES (Santoro and Konisky, 1987). Furthermore, BES transport is inhibited by HS-CoM and methyl-CoM in the wild type. In an extended study the transport systems were investigated in more detail (Dybas and Konisky, 1989). This led to the description of a HS-CoM-specific, high V_{max} system (960 pmol/ min · mg protein; K_m = 61 μM), and of a second, more general system that transports HS-CoM, methyl-CoM and BES with a K_m of 53 μM and has a V_{max} of 88 pmol/min · mg protein. The transport observed is carrier-mediated and the intracellular accumulation of HS-CoM and methyl-CoM is energy-dependent.

Active transport has also been demonstrated for Ni^{2+} (Jarrell and Sprott, 1982), which is needed for hydrogenase, and factor F_{430}. *M. bryantii* accumulates Ni^{2+} with an apparent K_m of 3.1 μM. From inhibitor studies, and from the effect of artificial driving forces, it was concluded that Ni^{2+} transport is coupled to proton movements.

Evidence for a Na^+/solute symport was obtained with *M. voltae*. The organism has a requirement for leucine and isoleucine; isoleucine transport is inhibited by protonophores or *N*-ethylmaleimide and activated by sodium ions (Ekiel et al., 1985; Jarrell et al., 1984). If the sodium ion concentration is increased from 2 to 25 mM the K_m for isoleucine decreased from 330 μM to 22 μM, while the V_{max} remained constant at 2.4 nmol isoleucine/min · mg dry weight (Jarrell and Sprott, 1985). In addition, *M. voltae* seems to possess constitutive transport systems for a variety of amino acids which are activated by sodium ions (Ekiel et al., 1985).

8.5 ATPases

As outlined above, the reduction of the heterodisulfide is coupled to ATP synthesis by electron transport phosphorylation, which necessitates the presence of an ATP synthase. A first indication of the presence of an ATP synthase in membranes of methanogenic bacteria came from a study of Mountfort, who demonstrated ATP synthesis driven by an artificial ΔpH in cell suspensions of *M. barkeri* (Mountfort, 1978). A membrane-bound ATPase was found in *M. thermoautotrophicum*, and ATP synthesis was also observed in whole cells of this organism when a ΔpH or an electrical potential was generated by a potassium

diffusion potenial (Doddema et al., 1978; Schönheit and Perski, 1983). Later on, the aforementioned DCCD-sensitive electron transport phosphorylation was demonstrated, as carried out during methanogenesis from H_2 + CH_3OH by *M. barkeri* (Blaut and Gottschalk, 1984a).

Membranes of *M. barkeri* were found to have a DCCD-sensitive ATPase consisting of a and b subunits which could be dissociated from the membrane by methods also used for the dissociation of F_1 from *E. coli* membranes (Inatomi, 1986). The properties of this ATPase were similar to those of the F_1-part of the F_1F_O-ATPase. When the enzyme is solubilized from the membranes with detergents it contains at least four additional subunits of 40, 27, 23 and 6 kDa (Inatomi, Maeda, et al., 1989). The 6 kDa subunit was shown to bind DCCD; the analogous DCCD-binding protein in F_1F_O-ATPases is about 7 kDa while the one in vacuolar ATPases is usually about 15–19 kDa (Pedersen and Carafoli, 1987). In addition, a 5.5 kDa DCCD-binding protein is found in membranes of *M. tindarius* (Scheel and Schäfer, 1990). A DCCD-sensitive ATPase is not restricted to methylotrophic methanogens but is is also found in hydrogenotrophic organisms such as *M. thermoautotrophicum* (Doddema et al., 1978; Smigan et al., 1988) and *Methanohalophilus halophilum* (Smigan et al., 1992). This is in accordance with the view that electron transport-driven ATP synthesis is ubiquitous in methanogens.

The genes coding for the α and β subunits of the ATPase from *M. barkeri* have been sequenced. 52% of the residues of the α subunit were identical to those of the large subunit of the vacuolar ATPase from carrot or *Neurospora crassa* and 59% of the residues from the β subunit were identical to the small subunit from *N. crassa* (Inatomi, Eya, et al., 1989). On the other hand, there is less but significant (22–24% identity) homology to the α and β subunits of the F_1F_O-ATPase from *E. coli*. Membrane-associated ATPases of other archaebacteria such as *Halobacterium salinarium (halobium)*, *H. saccharovorum*, *Sulfolobus acidocaldarius* and *M. thermolithotrophicus* have been analyzed (Ihara and Mukohata, 1991; Stan-Lotter et al., 1991; Denda et al., 1988a,b; Lübben et al., 1987; Gogarten, Rausch, et al., 1989). These data revealed that the archeaobacterial ATPases are closely related to one another, and that they can be separated from the V-type and F_1F_O-ATPases; they are classified as A-(archaeobacterial)-type ATPases. A-type ATPases are closely related to V-type ATPases, but distantly related to F_1F_O-type ATPases. It is believed that all types of ATPases were derived from a common ancestral protein (Gogarten, Kibak, et al., 1989).

V- and P- (E_1E_2-) type ATPases do not synthesize ATP, and it had long been believed that the F_1F_O-ATPase is the only type that operates as a H^+-ATP synthase in living cells. With the discovery of the ATP synthesizing A-type ATPase this assumption is no longer valid. Although the catalytic parts of the A-type ATPase are highly homologous to the V-type ATPase, the membrane intrinsic part may be considered as a functional homologue to the F_O-part. This is in accordance

with the finding that the proteolipid from *S. acidocaldarius* is closer related to the proteolipid from F_1F_O-ATPases than V-ATPases as deduced from the amino acid sequence of the cloned genes (Denda et al., 1989; Mandel et al., 1988). In summary, the presence of a membrane-bound ATPase is in accordance with the finding of electron transport phosphorylation in methanogenic bacteria. From the data available there is no doubt that the enzyme transports protons. Does it transport sodium ions in addition? Evidence against the presence of a Na^+-ATP synthase in methanogenic bacteria is the following: first, a specific ΔpNa-driven ATP synthesis in whole cells could not be observed (Müller, Winner, et al., 1988). Second, there was no effect of Na^+ on ATP synthesis as coupled to the heterodisulfide reduction (Blaut et al., 1985); however, since this reaction is definitely proton-motive, a participation of Na^+ is not expected. With the discovery of a primary sodium ion pump connected to the methyltransferase in the pathway of methanogenesis (Müller, Winner, et al., 1988) and the discovery of an ATPase in *P. modestum* able to translocate both, H^+ as well as Na^+ (Laubinger and Dimroth, 1988, 1989), the possibility of an Na^+-ATP synthase which couples the $\Delta\tilde{\mu}_{Na^+}$ produced in the methyltransferase reaction with the synthesis of ATP is not excluded. However, further experiments are necessary in order to provide a definite answer.

As stated above, a proton-motive ATP synthase of the A-type involved in electron transport-driven ATP synthesis is found in *M. barkeri*. Although the presence of such an enzyme is not excluded in the marine organism *M. voltae*, a different type of enzyme was found in its membranes. The enzyme is not inhibited by DCCD but by vanadate, a feature observed for P-type ATPases (Dharmavaram and Konisky, 1987). It can be removed from the membranes by salt extraction, a property not observed for integral membrane proteins. The enzyme was purified; it consists of one subunit of 74 kDa and it forms an acyl phosphate intermediate during ATP hydrolysis (Dharmavaram and Konisky, 1989). The gene has been cloned, but the deduced amino acid sequence of the protein does not show significant homologies to known ATPases. However, one region of the protein sequence shows homology to the conserved region of E_1E_2-ATPases centered around the aspartate residue, which becomes phosphorylated during ATP hydrolysis (Dharmavaram et al., 1991).

As to the function of this particular ATPase, studies with *M. voltae* indicated that, although it is not involved in ATP synthesis, it is involved in ion homoeostasis (Crider et al., 1985). The ion transported was shown to be sodium (Carper and Lancaster, 1986), which is of importance in marine environments. The mechanism of ATP synthesis in this organism is still a matter of debate (see Section 8.3.1) but if one assumes electron transport phosphorylation to be present, one would also expect an A-type ATP synthase to be present. This postulate is experimentally substantiated by the finding that both an artificial ΔpH and a ΔpNa can serve as driving force for ATP synthesis in *M. voltae* (Crider et

al., 1985). However, more experiments are needed to describe the mechanism by which the marine organism *M. voltae* produces ATP.

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III

BIOSYNTHESIS

9

Biosynthesis of the Coenzymes in Methanogens

Robert H. White and Dan Zhou

9.1 Introduction

At present six groups of new coenzymes involved in the biochemistry of methane formation have been structurally characterized. Structures representative of these groups are shown in Figure 9.1.

With the exception of coenzyme F_{420}, which has also been found to occur in several nonmethanogenic microorganisms, these coenzymes appear to occur only in methanogens (DiMarco et al., 1990). Work on the biosynthesis of these cofactors has proceeded at a very slow pace because of the limited number of scientists working in this research area. People trained and interested in coenzyme biosynthesis left the field in the late 1960s when the definitive work on the biosynthesis of many of the standard coenzymes was concluded.

Much of the present work on the biosynthesis of these coenzymes was established using the experimental method referred to by the authors as the in vivo stable isotope metabolic labeling technique. In this method, a microorganism is allowed to grow on an enriched, stable isotope-labeled substrate that represents a major part of its total carbon source. During growth, the isotope in this substrate is incorporated unevenly into different cellular components, including the coenzymes, according to the biosynthetic pathway of each coenzyme. After growth, a combination of chemical procedures, biochemical insight, and mass spectrometry is used to establish, as far as possible, both the extent and the position at which the isotope is incorporated into the coenzymes, as well as in other cellular molecules produced by the cells. With this information, conclusions can be reached as to the pathways and possible precursors used by the cells, in vivo, for the biosynthesis of any given coenzyme. The proposed pathway can then be

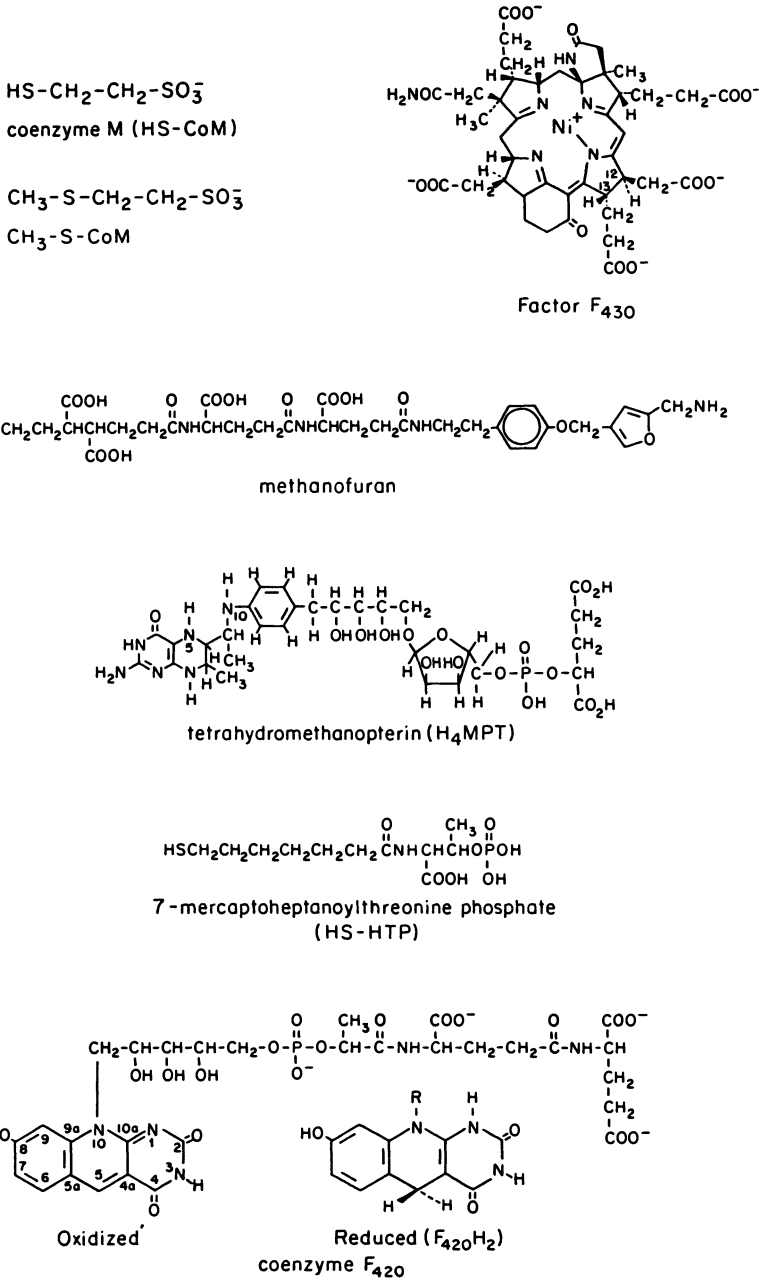

Figure 9.1. Structures of the coenzymes involved in methanogenesis (from Rouviere and Wolfe, 1988).

tested by measuring the incorporation of isotope-labeled intermediates into the coenzyme either by growing cells in their presence or by incubating the labeled intermediates with cell extracts of methanogens. The intermediates in the biosynthesis of the coenzyme can also be directly identified in the cells by means of trace organic identification methods that rely heavily on the use of gas chromatography-mass spectrometry (GC-MS) analysis.

This approach was used to outline the biosynthetic pathway of thiamine (White, 1978), lipoic acid (White, 1980a), and fatty acids (White, 1980b) in *Escherichia coli*. A similar approach that used ^{3}H- and ^{14}C-labeled compounds was developed and used by Csonka (1977) and Csonka and Fraenkel (1977) to determine, in vivo, pathways for the biosynthesis of amino acids and NADPH in *E. coli*. Ekiel et al. (1983, 1984) used the in vivo stable isotope metabolic labeling technique to determine biosynthetic pathways in methanogens, using ^{13}C NMR instead of mass spectrometry to determine the extent and position of the label incorporated into the biosynthetic products. (The use of NMR to determine biosynthetic pathways is a very powerful method, but is restricted in its application to coenzyme biosynthesis because coenzymes generally occur in such low amounts in cells that it is difficult to measure isotopic incorporations by this method. The primary strength in the use of mass spectrometry to study biosynthetic pathways is that this method can measure small isotope incorporations in much smaller amounts of samples than are required for NMR analysis.)

With this introduction, the present state of knowledge of the biosynthesis of the coenzymes present in methanogens is presented.

9.2 Methanofuran

Methanofuran was first identified by Romesser and Wolfe (1982) as a low molecular weight cofactor required for methanogenesis from CO_2 in extracts of *Methanobacterium thermoautotrophicum* that had been depleted of low molecular weight compounds. Based on these observations the coenzyme was named the carbon dioxide reduction factor (CRF). This name remained in place until its structure was established in 1984 by Leigh et al., at which time it was given the trival name of methanofuran because of the presence of the furan ring in its structure. Subsequently, studies of the methanofurans present in different methanogens revealed a structural diversity (Bobik et al., 1987; White, 1988a) arising from variations in the structure of the side chain attached to a core structure of 4-[*N*-γ-L-glutamyl-γ-L-glutamyl)-*p*-(β-aminoethyl) phenoxymethyl]-2-(aminomethyl)furan, which has been found to be common to all of the methanofurans characterized so far. When this side chain is 1,3,4,6-hexanetetracarboxylic acid, the methanofuran has the originally described structure and is designated as methanofuran. Attachment of the core to two γ-linked glutamates produces

methanofuran b, and attachment of the core to 1-hydroxy-1,3,4,6-hexanetetracarboxylic acid generates methanofuran c.

Several of the subunits of these methanofurans released by acid hydrolysis have structural features that are of particular interest from a biosynthetic point of view. These include 2-(aminomethyl)-4-(hydroxymethyl)furan (F1), 1,3,4,6-hexanetetracarboxylic acid (TCA), and 1-hydroxy-1,3,4,6-hexanetetracarboxylic acid (1-hydroxyTCA). Natural products containing a furan ring have been isolated and characterized from a large number of different organisms and can presently be classified into three major structural types: the furanoterpenoids, the furanocoumarins, and the fatty acid-derived furans, which are generally 2,5-disubstituted furans. On the basis of the structures of these furans and a limited number of labeling experiments by Hikino and Konno (1976), it can be concluded that the first two classes of these furans are terpene-derived and the latter is fatty acid-derived. None of these structures, however, contains a 2,4-disubstituted furan like that found in methanofuran, and as such, it is likely that the furan in methanofuran is biosynthesized by a new pathway. The second interesting structural elements in these methanofurans are the TCA present in methanofuran and the 1-hydroxyTCA present in methanofuran c. These chemical structures have never before been found in natural products, although many very similar compounds have been reported in fungal natural products (Turner, 1971).

As with several of the coenzymes discussed in this chapter, methanofuran is too large a molecule for the direct determination by mass spectrometry of the specific positions at which stable isotopes are incorporated. This coenzyme, however, can be easily degraded by acid hydrolysis into smaller fragments that can then be derivatized and examined individually by mass spectrometry. For the experiments to be discussed here, two of the hydrolytic fragments, TCA and F1, were analyzed by mass spectrometry as the tetramethyl ester and diacetyl derivatives, respectively.

By growing rumen isolate 10-16B with [2,2,2–$^{2}H_3$]acetate, deuterium was found to be incorporated into only two sites of the TCA molecule, with one site on each half of this symmetrical molecule. One site was found to be labeled 37% with deuterium, the same as the glutamic acid present in the cells; the other site was labeled 77% with deuterium, the same as malonate-derived compounds produced by the cells (White, 1987). This unequal distribution of deuterium clearly eliminates the possibility that TCA is biosynthesized by the condensation of two groups of similar biochemical origin such as two α-ketoglutarates or an α-ketoglutarate plus a 2-hydroxyglutarate derived from α-ketoglutarate. The correspondence between the extent of deuterium labeling of each of these positions with the extent of labeling of specific cellular compounds would indicate that the TCA molecule could, however, be derived from one α-ketoglutarate plus at least one malonate. This idea could be tested experimentally by growing cells with [1,2-$^{13}C_2$]acetate and measuring the incorporation of $^{13}C_2$ units into the TCA

molecule. However, because of the symmetrical nature of the TCA molecule, an experiment designed to measure the specific positions of incorporation of [1,2-$^{13}C_2$]acetate by mass spectrometry was impossible. Thus the measurement of the position of [1,2-$^{13}C_2$]acetate incorporated into the nonsymmetrical analog of TCA, 1-hydroxyTCA found in methanofuran c, was performed. In these experiments $^{13}C_2$ units from [1,2-$^{13}C_2$]acetate were found to be incorporated into TCA at three different positions of the 1-hydroxyTCA (White, 1987). One of the acetate $^{13}C_2$ units was incorporated into the nonhydroxyl-containing side of the molecule (carbons 4, 5, and 6, and the C-6 carboxylic acid group), and two acetate $^{13}C_2$ units were incorporated into the hydroxyl-containing side of the molecule (carbons 1, 2, and 3, and the C-1 carboxylic acid group). A pattern of incorporation of these labeled acetates into TCA and 1-hydroxyTCA consistent with these results is shown in Figure 9.2.

Based on these labeling experiments, as well as the detection of Compound **II** (Figure 9.2) in extracts of methanogens by GC-MS, the pathway for the biosynthe-

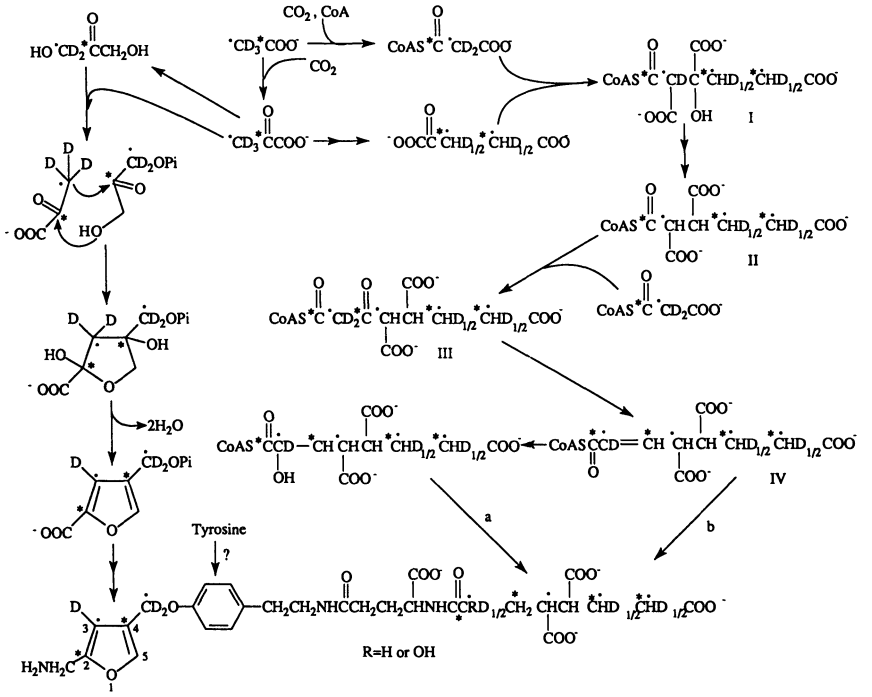

Figure 9.2. Biosynthetic pathway and pattern of label acetate incorporation into methanofuran and methanofuran c. Pathway (a) leads to methanofuran c containing hydroxyTCA, and pathway (b) leads to methanofuran containing TCA.

sis of TCA shown in Figure 9.2 was proposed. In this pathway the biosynthesis of TCA begins with the condensation of α-ketoglutarate with malonyl-CoA to form Compound **I**, which after elimination of water and reduction of the double bond, is converted into the CoA derivative of 1,1,2,4-butanetetracarboxylic acid (Compound **II**, Figure 9.2). This is further condensed with a second molecule of malonyl-CoA, in a series of reactions analogous to those observed during fatty acid biosynthesis, to form Compound **IV** (Figure 9.2). This molecule can then be reduced to the CoA derivative of TCA or alternatively, undergo a reverse addition of water to form the CoA derivative of 1-hydroxyTCA. As discussed below, the thioesters present in these intermediates could be used for the formation of the peptide bond that binds them into their respective methanofurans.

As with TCA, both $^{2}H_3$- and $^{13}C_2$-labeled acetates were found to be incorporated into the F1 fragment of methanofuran (White, 1988b). [1,2-$^{13}C_2$]Acetate was found to be incorporated as an intact unit into two separate positions of this molecule. The extent of incorporation of the $^{13}C_2$ unit into each of these positions was the same as that observed for the incorporation of acetate into the alanine and proline produced by the cells, indicating that pyruvate or molecules derived from pyruvate served as precursors for the biosynthesis of the furan ring. From [2,2,2-$^{2}H_3$]acetate, deuterium was incorporated into two separate sites of the F1 molecule, one containing from zero to two deuteriums and the other only one. On the basis of the fragmentation pattern of the F1 diacetyl derivative, it was determined that two of the deuteriums were incorporated into the hydroxymethyl group at C-4 and one was incorporated at C-3 of the furan ring. The extent and distribution of the incorporated deuterium at the C-4 methylene were the same as that observed for C-6 of the glucose produced by the cells. The extent of the incorporated deuterium at C-3 of the furan ring was the same as that calculated for the methyl group of pyruvate after exchange of the deuterium with solvent protons was taken into consideration. From this information it was concluded that F1 is generated by the condensation of dihydroxyacetone phosphate and pyruvate, as outlined in Figure 9.2. The first product of this condensation, a dihydroxy-substituted tetrahydrofuran, after elimination of two moles of water, would produce the phosphate ester of 2-carboxy-4-(hydroxymethyl)furan. Reduction of the carboxylic acid to an aldehyde followed by transamination would produce the phosphate ester of F1.

With the exception of the identification of α-ketoglutarate and Compound **II** shown in Figure 9.2 in cell extracts, no work has been reported confirming any of the proposed intermediates in the pathways leading to either TCA or F1. The confirmation of these pathways will be complete only after the proposed intermediates are identified in cell extracts and shown to be enzymatically converted into either the intact methanofuran molecule or its hydrolytic fragments.

The order and mechanism of the incorporation of the individual subunits into the final methanofuran structure are also not known. Some insight, however, into

how this assembly may occur can be obtained from the proposed biochemical structure of the intermediates leading to the individual subunits. Thus the proposed generation of F1 as the phosphate ester during its biosynthesis would produce an intermediate activated for the condensation with the phenol of the tyramine subunit by displacement of the phosphate. That this monophosphate ester could serve in such a capacity is consistent with the known susceptibility of 2- and 3-halomethylenefurans to substitution reactions (Cariou, 1978; Divald et al., 1976; Dunlap and Peters, 1953). The specific structure of the phenol at which this coupling could occur is unknown but could be either free tyrosine, tyramine, or conjugates of either of these molecules, attached to any number of the remaining components of the methanofuran.

In addition to generating F1 as an activated precursor molecule, the TCA molecule is also proposed to be biosynthesized as an activated ester, that is, the CoA ester. This thioester would be preactivated for condensation with an amino group of glutamic acid. This amino group could be from either free glutamic acid or glutamic acid attached to any number of the remaining components of the methanofuran structure. Since the three presently characterized methanofurans each consist of the same core structure attached through the glutamic acid to either TCA or other similar groups, the most likely condensation would be with this core structure. No information as to the order in which these reactions do occur is presently known. Determination of the exact pathways will require the isolation and characterization of each of the coenzyme intermediates involved.

9.3 Methanopterin

The chemical characterization of methanopterin (MPT) began in 1978 with the identification, by Gunsalus and Wolfe (1978), of a compound present in extracts of methanogens that showed an absorbance maximum at 342 nm. The structure of MPT was established after the identification of several of its degradation products (Keltjens et al., 1983; 1984), and the evaluation of two-dimensional NMR experiments conducted on the intact oxidized cofactor (van Beelen, Stassen, et al., 1984).

Because methanopterin is a relatively large molecule, successful application of the in vivo stable isotope metabolic labeling technique to the study of its biosynthesis required the degradation of the molecule to smaller subunits for subsequent mass spectral analysis. This was accomplished by means of two different procedures. The first procedure consisted of the oxidative cleavage of the reduced cellular form of MPT, 5,6,7,8-tetrahydromethanopterin (H_4MPT), which occurs when cell extracts of methanogenic bacteria are exposed to air. This oxidative cleavage, which has been extensively studied for 5,6,7,8-tetrahydrofo-

lates (Reed and Archer, 1980), results in the separation of the pterin from the arylamine side chain (White, 1985a). Among the products generated during the oxidative cleavage of H_4MPT are 7-methylpterin and methaniline, both of which can be isolated from the oxidized cell extracts for subsequent mass spectral analysis. The second procedure consisted of the reductive cleavage of the isolated methanopterin to 6-ethyl-7-methylpterin and methaniline (Van Beelen, Labro, et al., 1984; White, 1990). The advantage of this second procedure is that the isolated pterin fragment, 6-ethyl-7-methylpterin, contains both of the methyl groups of the methanopterin. Mild acid hydrolysis of the methaniline produced by either procedure can be used to isolate the 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane subunit of methanopterin.

The first experiments on the biosynthesis of MPT measured the incorporation of ^{15}N-glycine and $^{2}H_3$C-methionine into 7-methylpterin, an oxidative fragment of H_4MPT (White, 1986a). The labeled H_4MPT used in these experiments was produced by growing cells of rumen isolate 10-16B in the presence of the indicated labeled molecules. The amount of label incorporated into the 7-methylpterin was established by GC-MS analysis of its bistrimethylsilyl derivative. The results of this analysis showed that cells grown with ^{15}N-glycine readily incorporated one ^{15}N into the 7-methylpterin, and that cells grown with $^{2}H_3$C-methionine readily incorporated up to three deuteriums into the methyl group of the 7-methylpterin. The results of these experiments were consistent with the biosynthesis of the pterin portion of MPT originating from GTP, and its 7-methyl group being derived from the methyl group of methionine. The idea that the pterin was derived from GTP was also supported by the experimental results of Keller et al. (1986), who measured, using ^{13}C NMR, the incorporation of ^{13}C from [1-^{13}C]acetate into MPT by growing cells of *Methanobacterium thermoautotrophicum*.

Later work by White (1990) established that both of the methyl groups of MPT were derived from the methyl group of methionine. This was accomplished by measuring the incorporation of up to six deuteriums from $^{2}H_3$C-methionine into 6-ethyl-7-methylpterin, a reductive cleavage product of MPT that contains both of MPTs methyl groups. A diagram outlining the observed incorporation of the above labeled molecules into the pterin portion of MPT is shown in Figure 9.3.

Experiments measuring the incorporation of [3,3,3-$^{2}H_3$]acetate into the 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane moiety of MPT, established that the aromatic portion of this molecule was derived from an intermediate in the shikimic acid pathway (White, 1985b). The observation of ready incorporation of [*aromatic*-$^{2}H_2$]*p*-aminobenzoic acid, a component of folic acid, into the 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane moiety of MPT, showed that this intermediate is chorismic acid, the known metabolic precursor to *p*-aminobenzoic acid (White, 1985b). These observations were also consistent with experiments reported by Keller et al. (1986) that showed that ^{13}C from [1-^{13}C]acetate was incorporated into the aniline ring of MPT to the same extent and with the same labeling pattern

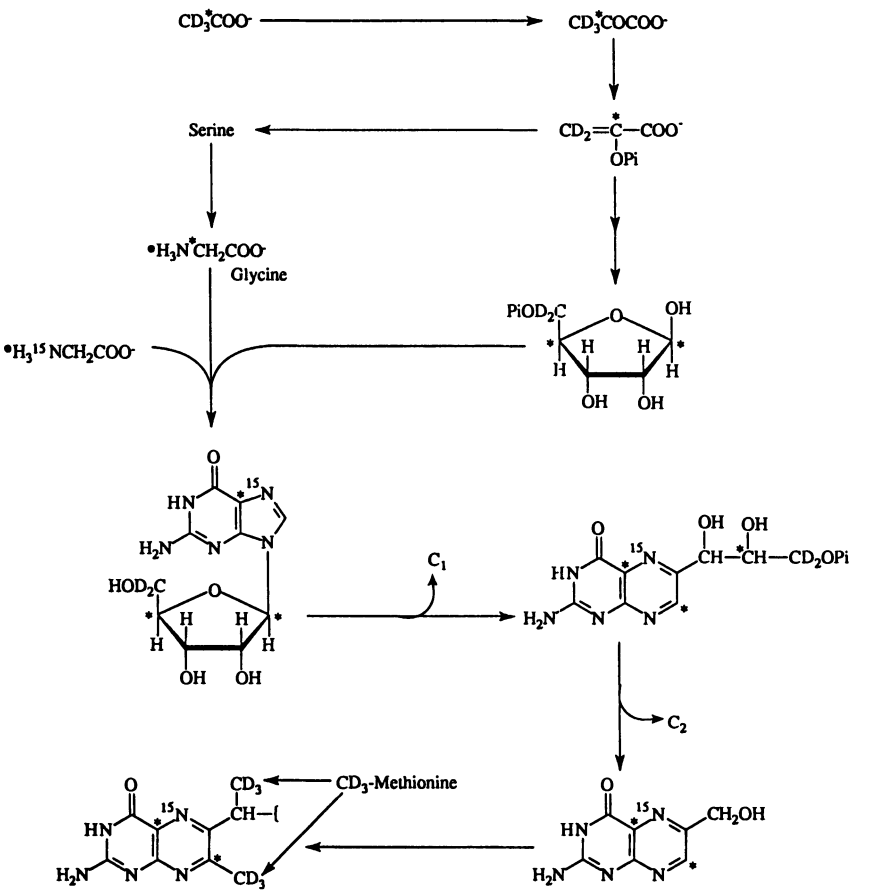

Figure 9.3. Labeling pattern observed for the incorporation of labeled precursors into the pterin portion of MPT.

as found in the cellular tyrosine, another shikimic acid-derived metabolite. The experiments by Keller et al. (1986) using cells of *M. thermoautotrophicum*, also clearly showed that the methylene group of the 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane was not derived from either the carboxyl group of shikimic acid or the C-3 of pyruvate, via chorismate. This followed from the observation that this methylene was labeled from [1-^{13}C]-acetate, whereas the carboxyl group of shikimic acid or the C-3 of pyruvate was found to be unlabeled. The labeling pattern of the pentyl side chain from [1-^{13}C]acetate was in fact in full agreement with the five-carbon side chain of the 5-(*p*-aminophenyl)-1,2,3,4-tetrahydroxypentane arising from a pentose. Based on the stereochemistry of the 5-(*p*-

aminophenyl)-1,2,3,4-tetrahydroxypentane, it was concluded that this pentose was actually ribose (White, 1986b). The hydroxyglutarate moiety of the MPT in these experiments was found to be labeled with the same pattern as the α-ketoglutarate present in *M. thermoautotrophicum*, suggesting its formation by the reduction of α-ketoglutarate. The observed labeling patterns for these experiments are diagrammed in Figure 9.4.

The data described above strongly suggested a close connection between the biosynthesis of folate and methanopterin. This connection was further strengthened by the observation that extracts of *Methanococcus volta* readily cleave 7,8-H_2-L-neopterin to 7,8-H_2-6-hydroxymethylpterin, a key reaction involved in the biosynthesis of folate (Brown and Williamson, 1987). The involvement of the product of this reaction, 7,8-H_2-6-hydroxymethylpterin, in the biosynthesis of

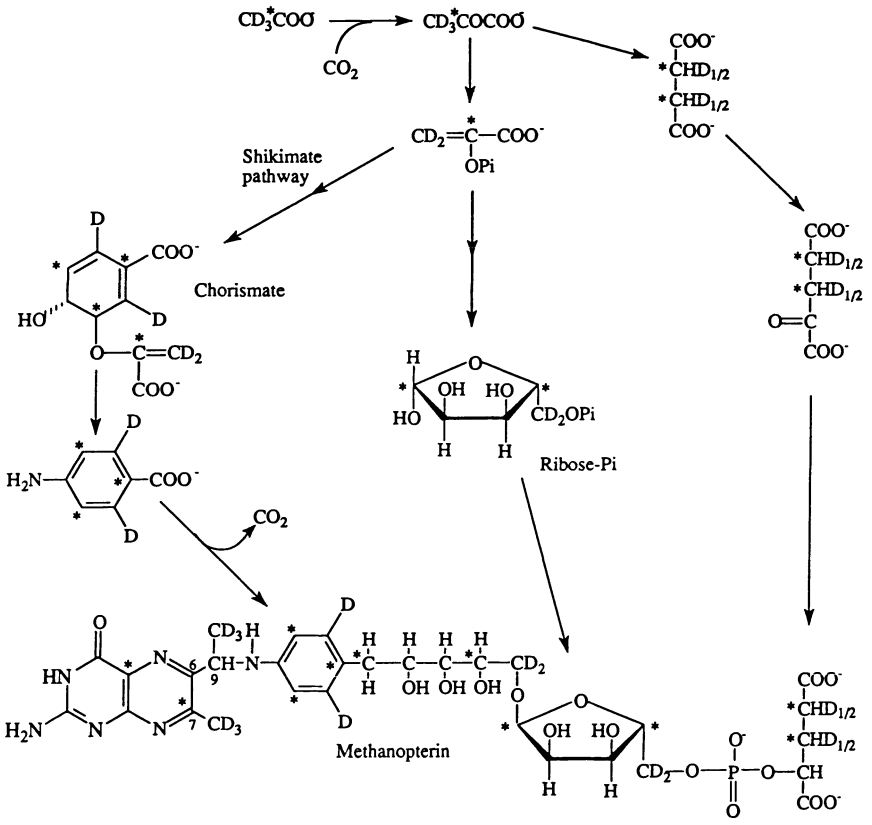

Figure 9.4. Observed labeling pattern of MPT from labeled acetates.

MPT was further confirmed by the incorporation of [*methylene*-^{2}H]-7,8-H_2-6-hydroxymethylpterin, generated in vivo from [*methylene*-^{2}H]-6-hydroxymethylpterin, to an extent of 30% into MPT, by growing cells of *M. volta*. Thus 7,8-H_2-6-hydroxymethylpterin appears to be a common intermediate in the biosynthesis of both folate and methanopterin.

The determination of 7,8-H_2-6-hydroxymethylpterin as an intermediate in the biosynthesis of MPT indicated that the introduction of the methyl groups into MPT must occur after the formation of this compound. In order to establish the positions at which the pterin was methylated, *M. volta* cells were grown with each of the following labeled pterins: [*methylene*-^{2}H]-6-hydroxymethylpterin, [*ethyl*-$^2H_4$]-6-[1(RS)-hydroxyethyl]pterin, [*methyl*-$^2H_3$]-6-hydroxymethyl-7-methylpterin, [*ethyl*-$^2H_4$-*methyl*-$^2H_3$]-6-[1(RS)-hydroxyethyl]-7-methylpterin, and [1-*ethyl*-^{3}H]-6-[1(RS)-hydroxyethyl]-7-methylpterin; and their incorporation into MPT was measured. The results of these experiments showed that only the non-7-methylated pterins were incorporated into methanopterin. These results indicated that the C-7 methyl group was probably added at a later stage of the biosynthetic pathway and that the C-9 methyl group was introduced either at the 7,8-H_2-6-hydroxymethylpterin stage of the pathway, or at a later stage of the biosynthetic pathway. If the C-9 methyl group was introduced at the 7,8-H_2-6-hydroxymethylpterin stage of the pathway, then both the 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase and the enzyme analogous to the 7,8-dihydropteroate synthase in folate acid biosynthesis would have to function with 7,8-H_2-6-(1-hydroxyethyl)pterin.

The position at which the methylation occurred was resolved by the demonstration that cell extracts of *M. formicicum* readily condense [*methylene*-^{3}H]-7,8-H_2-6-hydroxymethylpterin-PP with methaniline to generate desmethylated MPT, and that the desmethylated MPT is then subsequently dimethylated to MPT by the cell extract of *M. volta* in the presence of *S*-adenosylmethionine (White, 1990). These observations indicate that the pterin portion of methanopterin is biosynthetically derived from 7,8-H_2-6-hydroxymethylpterin-PP, which is coupled to methaniline in a reaction analogous to that involved in the biosynthesis of folic acid. This reaction as well as the other reactions involved in the biosynthesis of MPT are outlined in Figure 9.5.

From the above discussion it is clear that all of the early steps in the biosynthesis of MPT are the same as those found in folate biosynthesis, and that the differences between MPT and folate biosynthesis represent biochemical transformations that occur at the later stages in the biosynthesis of methanopterin. This finding would thus indicate that folate is the more primitive of these two coenzymes and that MPT represents only a biochemical modification to the folate structure. Since folate and MPT function as coenzymes carrying out the same basic biochemical transformations but, at the same time, show absolute specificity for the enzymes

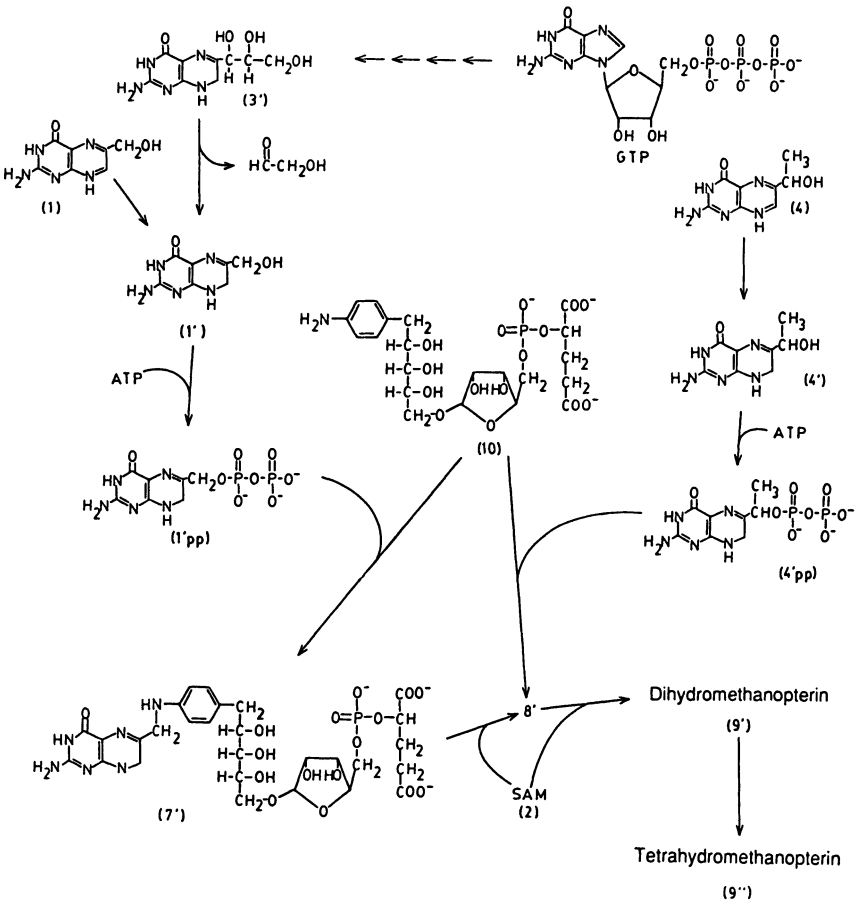

Figure 9.5. Proposed pathway for the biosynthesis of MPT (From White, 1990).

isolated from their host organisms (Rouvière and Wolfe, 1988), the pathway for the biosynthesis of MPT represents the first known example where a coenzyme is converted into another coenzyme by the transformation of its structure.

9.4 Factor F_{420} and Riboflavin

Factor 420 (F_{420}) was first isolated from methanogens by Cheeseman et al. in 1972, and was later identified as the *N*-(*N*-L-lactyl-γ-L-glutamyl)-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin 5′-phosphate by

Eirich et al. in 1978 (Figure 9.1). Although the cofactor is not unique to the methanogens (Lin and White, 1986) it has become an important indicator for the identification of the methanogens because of its high abundance in these cells and its intense fluorescence. The structural resemblance between the chromophore of riboflavin and the deazaflavin of F_{420} is the first clue that these molecules may be biosynthesized by similar pathways. As a result, information on the biosynthesis of riboflavin, which is outlined in Figure 9.6, may give us some insight into the biosynthesis of the deazaflavin of Factor 420. The biosynthesis of flavins and deazaflavins in eubacteria and fungi has been recently reviewed (Bacher et al., 1987; Bacher, 1991). In the biosynthetic pathway to riboflavin, the pyrimidine ring of the riboflavin originates from the purine ring of GTP. The C_6-aromatic ring and its two attached methyl groups are assembled from two identical C_4 units, which are currently thought to arise from 3,4-dihydroxybutanone-4-phosphate (Volk and Bacher, 1988) (Figure 9.6). The 3,4-dihydroxybutanone-4-phosphate in turn, is derived from the intact carbon chain of a pentose via an intramolecular rearrangement in which the carbon atom 4 of the pentose is removed and the C-3 and C-5 carbons are connected intramolecularly. The mechanism of the formation of 3,4-dihydroxybutanone-4-phosphate from ribulose 5-phosphate, and the subsequent formation of the 6,7-dimethyl-8-ribityl-lumazine were proposed by Volk and Bacher in 1988. The biosynthesis of riboflavin in *Methanobacterium thermoautotrophicum* was recently studied by Eisenreich et al. (1991), and was found to be identical to that in eubacteria and fungi.

Considering that both the deazaflavin of F_{420} and riboflavin contain the same structure for the pyrimidine ring and its attached ribitol side chain, it is possible that these two molecules could be derived from a common intermediate which

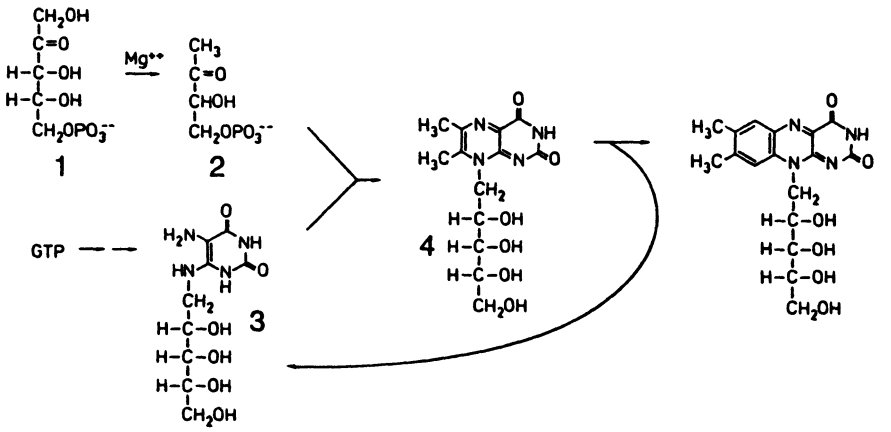

Figure 9.6. Biosynthesis of riboflavin (from Volk and Bacher, 1988).

contains these structural features. The likely choice for this molecule would be 5-amino-6-ribitylamino-2,4(1H,3H)pyrimidinedione (Compound 3 in Figure 9.6) or its 5′-phosphate. In support of this idea, Jaenchan et al. (1984) demonstrated that [2-^{14}C]guanine, but not [8-^{14}C]guanine, was incorporated into F_{420} by *M. thermoautotrophicum*, and that one mole of guanine was incorporated per mole of F_{420}. This was the first experimental proof that the pyrimidine ring of the deazaflavin arose from the pyrimidine of the guanine, as it does during riboflavin biosynthesis (Figure 9.6). This idea was further supported by the observed incorporation of [1-^{14}C]glycine into C_{10a} of deazaflavin (Scherer et al., 1984), and [1-^{13}C]acetate into 4a of deazaflavin (Le Van et al., 1985), respectively, as illustrated in Figure 9.7. In addition, the pattern of labeling of the pyrimidine ring of F_{420} derived from ^{13}C-labeled acetate or pyruvate was identical to that of riboflavin and guanosine present in the *M. thermoautotrophicum*, confirming that they share a common precursor (Eisenreich et al., 1991).

Studies done by Schwarzkopf et al. (1990) showed that the ribityl moiety of F_{420} is derived from the reduction of the ribose side chain of a purine precursor. In fact, studies with [1′-^{14}C]-adenosine showed that the pyrimidine carbon atoms of the purine was transferred together with the attached ribose unit to yield the ribityl side chain of F_{420} and riboflavin. The pattern of incorporation of [1-^{13}C]acetate into this ribityl moiety of F_{420}, riboflavin, and the ribose moiety of nucleosides, was identical and consistent with the carbohydrate metabolism in methanogens (Le Van et al., 1985).

The determination of the origin of the hydroxylated C_6-aromatic ring and C-5 of F_{420} are presently the critical aspects yet to be resolved in establishing the complete pathway for the biosynthesis of the deazaflavin portion of this coenzyme. However, if the complexity of the last steps in the biosynthesis of riboflavin are any guide, these last steps in the biosynthesis of F_{420} could be quite complicated. At present only a limited amount of work has been directed toward determining the nature of these last critical steps. Jaenchan et al. (1984) demonstrated that [U-^{14}C]phenylalanine, [U-^{14}C]tyrosine, and [methyl-^{14}C]methionine failed to be incorporated into F_{420} by *M. thermoautotrophicum*. Since these amino acids were found to be readily incorporated into the cellular phenylalanine, tyrosine, and F_{430} by these cells, this was taken as strong evidence that the aromatic ring and C-5 of F_{420} are not derived from either phenylalanine, tyrosine, or the methyl group of methionine. Studies reported by Le Van et al. (1985), however, showed that the ^{13}C- enrichment pattern of F_{420} from [1-^{13}C]acetate was identical to that of the tyrosine present in the cells. The observed labeling pattern, which is outlined in Figure 9.7, was also consistent with tyrosine being biosynthesized via the shikimate pathway which had been established earlier by other workers (Ekiel et al., 1983). These two results suggest that a metabolite of the shikimate pathway formed prior to the formation of tyrosine, is a likely precursor for both the C_6-aromatic ring, and possibly even the C-5 of the dea-

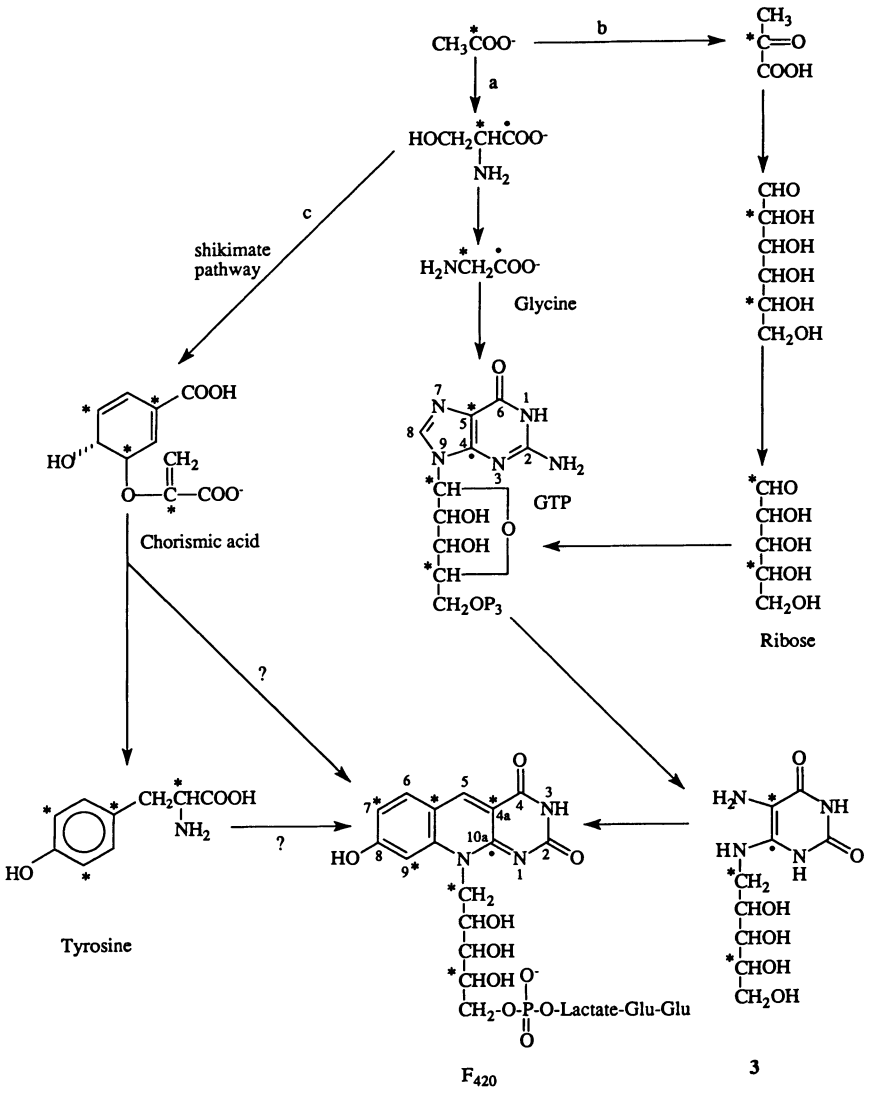

Figure 9.7. Biosynthetic pathway and positions of incorporation of labeled acetates into GTP, tyrosine, and F_{420}.

zaflavin. These authors also indicated that since the labeled F_{420} derived from [1-^{13}C]acetate showed a quasi-symmetrical isotope distribution, the direct precursor must be a symmetrical aromatic molecule, such as 4-hydroxybenzoate. However, labeled F_{420} derived from [2-^{13}C]acetate and [1,2-$^{13}C_2$]acetate indicated that the C-5 of deazaflavin is derived from the C-2 of acetate, whereas the carboxyl group of 4-hydroxybenzoate is derived from the C-1 of pyruvate, not the C-2 of acetate, thus eliminating the intermediacy of 4-hydroxybenzoate (Eisenreich et al., 1991). Prephenate is also a less likely precursor because of its prochiral character.

The above results are to be contrasted with the recent report from Bacher's laboratory (Reuke et al., 1992) which has shown that both tyrosine and its transaminated product, 4-hydroxyphenylpyruvate, are readily incorporated into F_{420} by *M. thermoautotrophicum*, which was stimulated to produce excess deazaflavin by growing the cells in the presence of 5-amino-6-ribitylamino-2,4(1H,3H)pyrimidinedione (Compound 3 in Figure 9.6). This finding is consistent with all the observed labeling experiments and provides an explanation for the origin of all of the carbons of the deazaflavin.

When the biosynthesis of the deazaflavin of F_{420} is established, the route for its incorporation into the complete F_{420} structure must be established to complete the pathway for the biosynthesis of the Factor 420. One possible means for the incorporation of the deazaflavin into the F_{420} is that the deazaflavin, produced from GTP as the triphosphate ester, condenses directly with the hydroxyl of lactate, either free or bound, in the completed side chain as L-lactyl-γ-L-glutamyl-L-glutamic acid, to form the phosphodiester bond of Factor 420. Conceivably, the triphosphate in this deazaflavin product could originate from the original GTP. If the condensation should occur with lactate, then the addition of the γ-L-glutamyl-γ-L-glutamic acid moiety would have to occur as the next step, which would complete the biosynthesis. It is interesting to note that, since γ-L-glutamyl-L-glutamic acid is also a component of the methanofuran, it could be biosynthesized and used as a common precursor in the biosynthesis of each of these coenzymes.

9.5 7-Mercaptoheptanoylthreonine Phosphate

7-Mercaptoheptanoylthreonine phosphate (HS-HTP), originally designated as component B before it was fully characterized, was first identified as a low molecular weight, heat-stable cofactor required for the reductive demethylation of methyl coenzyme M in cell extracts of *M. thermoautotrophicum* (Gunsalus and Wolfe, 1980). The structure of component B was assigned as the mixed

disulfide of 7-mercaptoheptanoylthreonine phosphate and 2-mercaptoethanol by Noll et al. (1986) and has been confirmed by chemical synthesis (Noll et al., 1987). [Recent work has indicated that the true cofactor for the methyl reductase may be a much larger molecule containing a uridine derivative in addition to several other sugars (Keltjens et al., 1989; König et al., 1989; Sauer et al., 1990). Biosynthetic aspects of this molecule have not been reported to date and will not be discussed here.] The 7-mercaptoheptanoyl moiety of HS-HTP has two structural features that make it interesting from the biosynthetic point of view. First the structure contains a polymethylene carbon chain like that found in fatty acids. The presence of such a structure in bacteria that produces fatty acids would not be particularly noteworthy, but its presence in methanogens, which contain mostly isoprenyl lipids, makes one wonder just how the carbon chain of this molecule arises. The second interesting feature of this structure is that a hydrolytic fragment of the HS-HTP, 7-mercaptoheptanoic acid, is the C_7 homolog of one of the precursors of lipoic acid, 7-mercaptooctanoic acid (White, 1980c). Considering the close structural relationship between these two molecules, and that the mechanism used in the biosynthesis of the thiols of lipoic acid are unknown (Parry, 1983), the determination of the biosynthesis of the 7-mercaptoheptanoyl moiety of HS-HTP could be helpful in establishing how lipoic acid is biosynthesized.

As with studies on the biosynthesis of the other methanogenic coenzymes, the biosynthesis of 7-mercaptoheptanoic acid was first explored by measuring the incorporation of stable isotopically labeled precursors into the 7-mercaptoheptanoic acid isolated by acid hydrolysis of HS-HTP (White, 1989a). The derivative of the 7-mercaptoheptanoic acid chosen for this work was the *S*-methyl ether methyl ester. The mass spectrum of this derivative contained an intense molecular ion that allowed for the total label incorporated into the molecule to be measured, as well as intense fragment ions from which the position of incorporation of the label could be measured. Deuterium from [2,2,2-$^2H_3$]acetate was found to be incorporated into four separate positions of the 7-mercaptoheptanoic acid by growing cells of *Methanococcus volta*. One deuterium was equally distributed between the C-2 and C-3, and the remaining three at carbons 4-6. The extent of incorporation at the C-2 and C-3 positions was the same as that observed for the incorporation of [2,2,2-$^2H_3$]-acetate into the α-ketoglutarate produced by the cells. Carbon-13 from [1,2-$^{13}C_2$]acetate was incorporated into four separate sites of the 7-mercaptoheptanoic acid. An intact acetate unit was incorporated at C-2 and C-3, and single carbons of the acetate were incorporated at C-5, C-6, and C-7. The results of these acetate incorporation experiments are outlined in Figure 9.8 and indicate that acetate is incorporated into 7-mercaptoheptanoic acid via at least two separate pathways. One of these pathways supplies the deuterium and ^{13}C incorporated at C-2 and C-3, and the other pathway supplies the carbons incorporated at C-5, C-6, and C-7 and the three single deuteriums incorporated at C-4, C-5, and C-6, respectively. A biochemically plausible explanation for

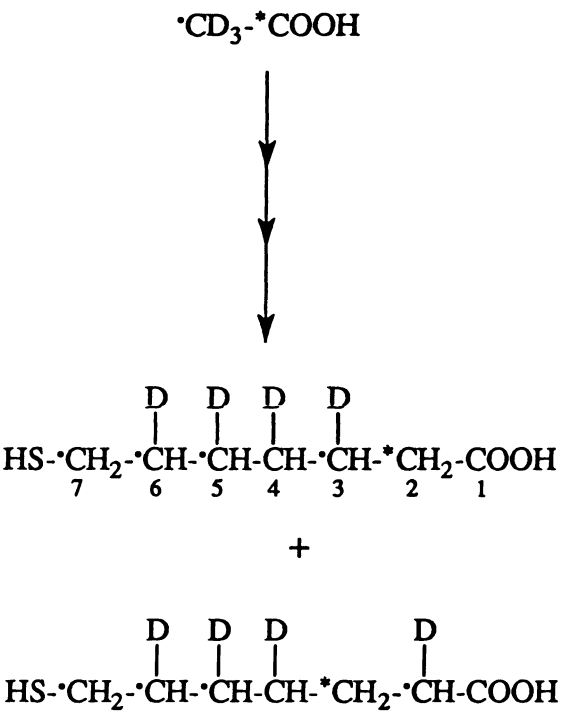

Figure 9.8. Positions of incorporation of ^{2}H and ^{13}C from labeled acetates into 7-mercaptoheptanoic acid.

these observations would be the conversion of α-ketoglutarate, which is derived from acetate via succinate, to α-ketosuberate by repeated α-keto acid chain elongation, as shown in Figure 9.9. The α-ketosuberate would then serve as a precursor for the formation of the 7-mercaptoheptanoic acid by a series of reactions analogous to those found in the biosynthesis of coenzyme M (see below).

The first set of reactions in the α-keto acid chain elongation would produce α-ketoadipic acid from α-ketoglutaric acid via homocitric, homoaconitic, homoisocitric, and oxalglutarate acids, which are all intermediates in the biosynthesis of lysine by the aminoadipic acid pathway (Rodwell, 1969). The second set of reactions would generate α-ketopimelate from α-ketoadipic acid, and a subsequent chain elongation of the α-ketopimelate would yield α-ketosuberate. The known mechanism for this chain elongation process completely explains the observed incorporation of deuterium that is shown in Figure 9.8. The occurrence of these series of reactions in methanogens was confirmed by the GC-MS identification of the expected series of α-keto acids in the three different methanogens examined (White,

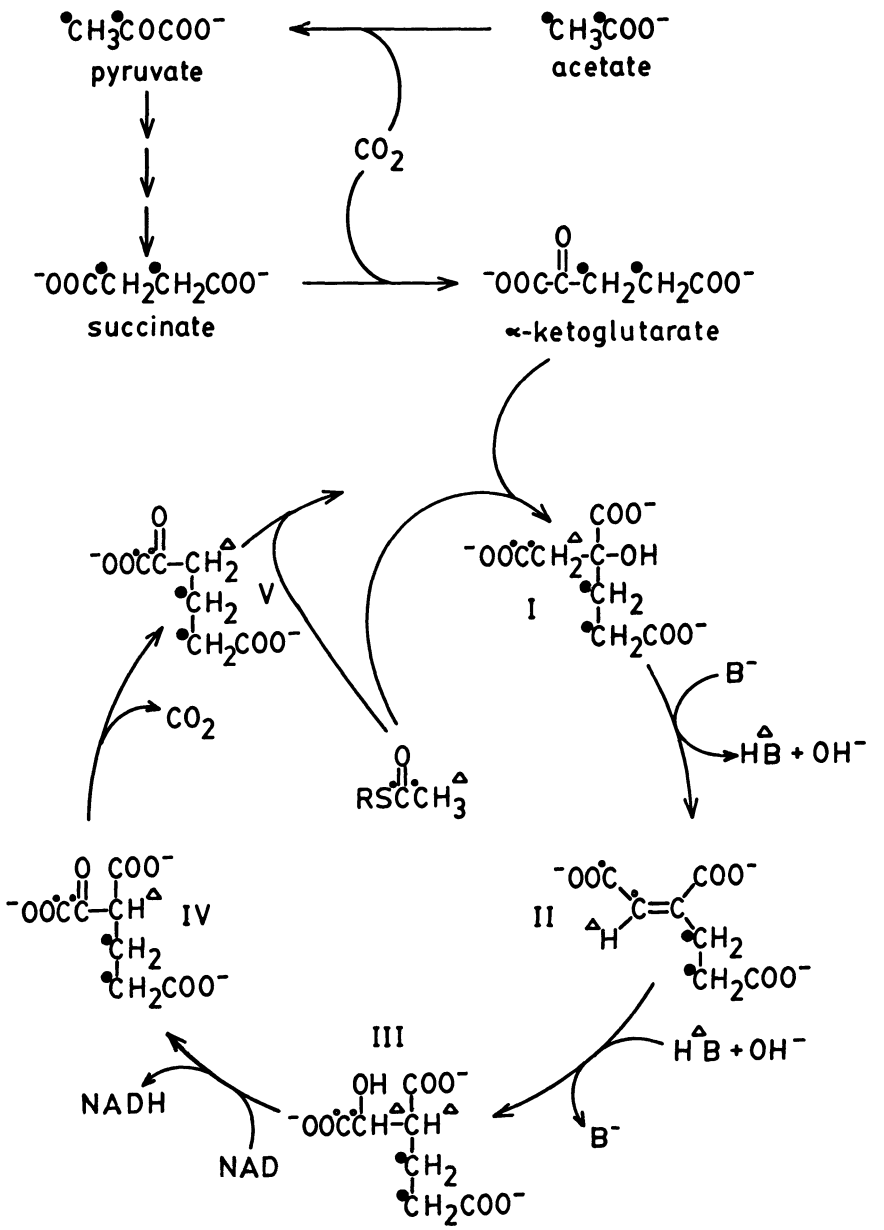

Figure 9.9. Conversion of acetate into α-ketodiacids in methanogenic bacteria. The solid circles represent the intact acetate unit incorporated via α-ketoglutarate and the open circles represent the intact acetate unit which is cleaved during the α-ketodiacid chain elongation steps. (from White, 1989b).

1989b). Labeling experiments with [1,2-$^{13}C_2$]-acetate also confirmed the sequential biosynthetic production of each of the α-keto acids from the preceding acid with the incorporation of only a single labeled carbon (White, 1989b).

The α-keto group of the α-ketosuberate resulting from these series of reactions would then be converted into an alkyl thiol by the same sequence of reactions known to occur in the conversion of sulfopyruvate to coenzyme M (White, 1985c; 1986c; 1988c). These reactions would involve the nonoxidative decarboxylation of α-ketosuberate to 7-oxoheptanoic acid, reaction of the resulting aldehyde with cysteine to form the thiazolidine adduct, and reductive cleavage of the heterocyclic C(2)-N bond in this adduct to form 7-(*S*-cysteinyl)heptanoic acid, which upon elimination of pyruvate and ammonia would form the 7-mercaptoheptanoic acid.

These steps in the conversion of α-ketosuberate to 7-mercaptoheptanoic acid were tested by incubating a series of labeled precursors with cell extracts of either *Methanococcus volta* or rumen isolate 10-16B and measuring their conversion into 7-mercaptoheptanoic acid by GC-MS (White, 1989c). In this series of experiments, [4,4,6,6-$^2H_4$]-2-oxosuberic acid, [7-^{2}H]-7-oxoheptanoic acid, [2-^{2}H]-2(RS)-(5-carboxypentyl)thiazolidine-4(R)-carboxylic acid, and *S*-(6-carboxyhexyl)cysteine were each shown to be converted into the corresponding labeled 7-mercaptoheptanoic acid. These findings were consistent with the biosynthesis of the thiol of 7-mercaptoheptanoic acid originating in the same fashion as does the thiol present in coenzyme M. If this mechanism for the formation of the thiol were indeed operating, then the C-2 and sulfur of the thiazolidine should also be incorporated as an intact unit as was proposed for the biosynthesis of coenzyme M. Therefore, cell extracts were incubated with a mixture of 2(RS)-(5-carboxypentyl)thiazolidine-4(R)-carboxylic acid and [2-^{2}H]-2(RS)-(5-carboxypentyl)-[^{34}S]thiazolidine- 4(R)-carboxylic acid, and the pattern of label incorporated into 7-mercaptoheptanoic acid was measured to determine if the carbon-sulfur bond remained intact during the transformation. The result of this measurement showed that both the ^{34}S and ^{2}H of the thiazolidine were incorporated into 7-mercaptoheptanoic acid, but only after dissociation of the thiazolidine into cysteine and [7-^{2}H]-7-oxoheptanoic acid. Furthermore, analysis of the data established that the sulfur from the cysteine was incorporated into the thiol, only after its elimination from the cysteine and subsequent mixing with an unlabeled sulfur source that had a molecular weight of sufficient size that it was excluded from a Sephadex G-25 size exclusion column. Hydrogen sulfide was indirectly shown to supply the sulfur for the production of the 7-mercaptoheptanoic acid in a reaction that obtained its reducing equivalents from hydrogen via an F_{420}-dependent hydrogenase. Consideration of all of these observations led to the proposal of the pathway for the biosynthesis of HS-HTP shown in Figure 9.10 (White, 1989c).

The remaining work on the biosynthesis of HS-HTP must address the question of the order and mechanism in which threonine, phosphate, and 7-mercaptoheptanoic acid are combined. Considering the close structural similarities between

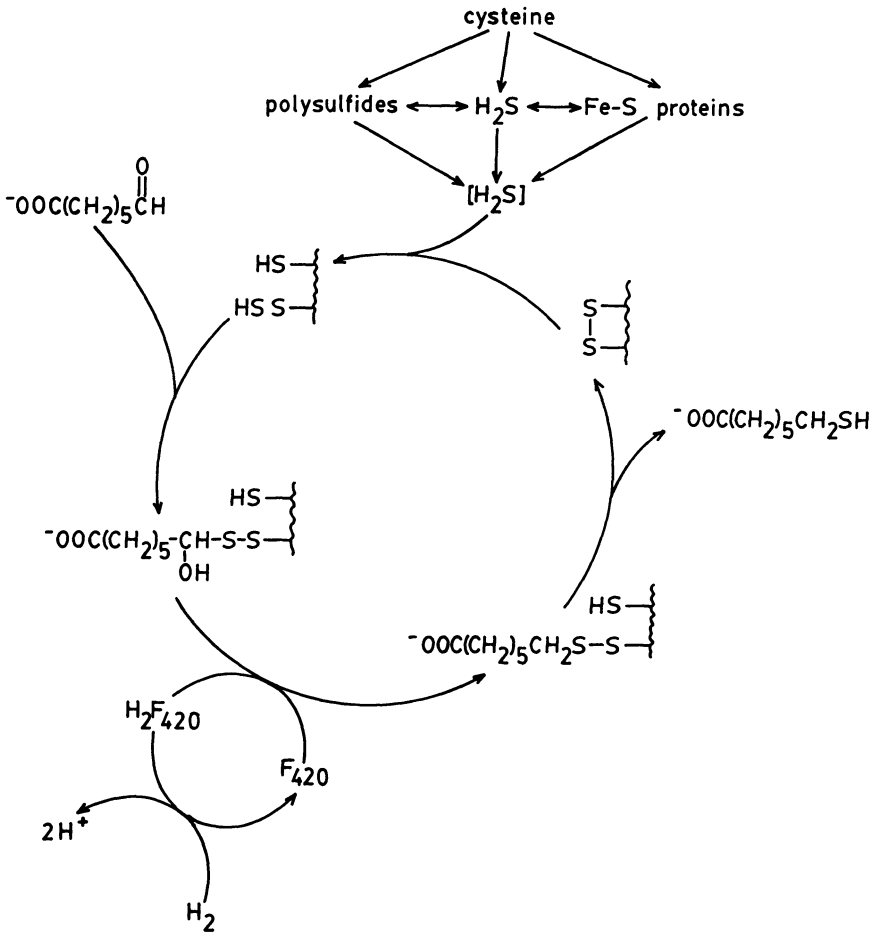

Figure 9.10. Proposed pathway for the biosynthesis of 7-mercaptoheptanoic acid moiety of HS-HTP (from White, 1989b).

biotin, lipoic acid, and 7-mercaptoheptanoic acid, and our present knowledge of the mechanisms used for the coupling of biotin (Kasow and Lane, 1962) or the coupling of lipoic acid (Leach, 1970; Reed et al., 1958) to the amino group of lysine in proteins, a sequence of reactions that could be used for the coupling of 7-mercaptoheptanoic acid to either threonine or threonine-P can be predicted. The first reaction in this two-step sequence would involve the adenylation of 7-mercaptoheptanoic acid by ATP. The resulting 7-mercaptoheptanoyl adenylate could then react either with threonine-P to form HS-HTP directly, or indirectly

by reaction with free threonine to form 7-mercaptoheptanoylthreonine, which would then be easily converted into HS-HTP by a simple kinase enzyme reaction. That 7-mercaptoheptanoic acid can be activated and incorporated into HS-HTP is supported by the observed incorporation of [7,7-$^2H_2$]-7-mercaptoheptanoic acid into HS-HTP by incubation with growing cells of methanogens (White, 1989a).

9.6 Coenzyme M

The involvement of coenzyme M (2-mercaptoethanesulfonic acid) in the terminal steps of methane biosynthesis was demonstrated in 1971 by McBride and Wolfe and its structure was determined in 1974 by Taylor and Wolfe. Among coenzymes, coenzyme M is unique in that it occurs only in methanogens (Balch and Wolfe, 1979), is the smallest (MW-142), and also contains the highest percentage of sulfur (45%); it is also one of the few sulfonic acids found in nature.

The first study on the biosynthesis of coenzyme M was reported in the thesis of Balch (1979). His experiments were conducted by incubating either growing cells or mid-log-phase grown cells of methanogens, with a series of radiolabeled compounds, and measuring the incorporation of label into coenzyme M and methyl coenzyme M by means of 2-dimensional chromatography thin layer chromatography and thin layer electrophoresis. Using cells of *M. ruminantium*, strains PS and M1, and *M. thermoautotrophicum*, strain ΔH, he was unable to detect the incorporation of the following radiolabeled compounds into coenzyme M: [^{14}C]-amino acid mixture (1 mCi/mg), $Na_2{}^{35}SO_4$ (69 mCi/mmol), [^{14}C]cysteine (25 mCi/mmol), [^{35}S]cysteine (600 Ci/mmol) or [^{14}C]acetate (55 mCi/mmol). In contrast to these findings, $Na_2{}^{35}S$ and $Na_2{}^{35}SO_3$ were found to be readily incorporated into both coenzyme M and methyl coenzyme M when incubated with cells of strains PS and ΔH. Although this work gave some information about the possible sulfur sources used by the cell for the biosynthesis of the coenzyme, it gave no information about the origin of the carbon atoms used in the biosynthesis of the coenzyme.

By means of the metabolic labeling method and the use of the methyl ester derivative of methyl coenzyme M to measure the label incorporated into the coenzyme, it was established that acetate was a precursor to the carbons of coenzyme M (White, 1985c). In the three different strains of methanogens examined, growth in the presence of [2,2,2-$^2H_3$]acetate led to the pro-duction of coenzyme M with from zero to two deuteriums on the C-1 carbon. The distribution of deuterium on this carbon was the same as that calculated for the phosphoenolpyruvate (PEP) present in the cells. In addition, [1,2-$^{13}C_2$]acetate was found to be incorporated into the coenzyme as a unit. The results of these labeling experiments are shown in Figure 9.11.

In addition to acetate, DL-[3,3-$^2H_2$]sulfolactate and $H^{34}SO_3^-$ were also found

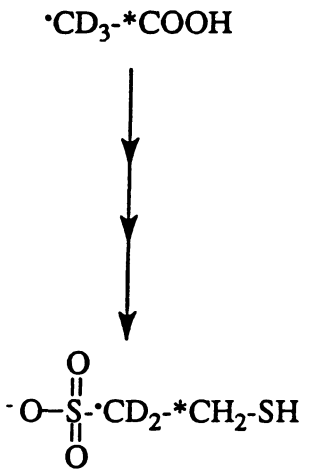

Figure 9.11. Positions of incorporation of ^{2}H and ^{13}C from labeled acetates into coenzyme M.

to be incorporated, whereas [1,1,2,2-$^{2}H_4$]taurine, [2,2-$^{2}H_2$]sulfoacetic acid, L-[3,3-$^{2}H_2$]cysteic acid, DL-[3,3-$^{2}H_2$]cysteine, [1,1,2,2-$^{2}H_4$]isothionate, and $^{34}SO_4^{=}$ were found not to be incorporated. On the basis of these findings, it was concluded that the pathway for the biosynthesis of coenzyme M was as shown in Figure 9.12. In this pathway, the first committed step in the formation of coenzyme M is the reaction of PEP with bisulfite to form sulfolactic acid. Oxidation of the sulfolactic acid to sulfopyruvate, followed by nonoxidative decarboxylation, produces sulfoacetaldehyde. Reaction of the sulfoacetaldehyde with cysteine generates 2-(sulfomethyl)thiazolidine-4-carboxylic acid, which undergoes reductive cleavage of the heterocyclic C(2)-N bond to form *S*-(2-sulfoethyl)cysteine, which, in turn, undergoes β-elimination to produce coenzyme M.

Evidence supporting this pathway consisted of the direct identification of several of the intermediates involved in the pathway (White, 1986c) and the determination, with stable isotope labeled intermediates, of their conversion into labeled coenzyme M in cell-free extracts of *Methanobacterium formicicum* (White, 1988c). Thus, incubation of cell-free extracts of *M. formicicum* with phosphoenolpyruvate, bisulfite, and cysteine were shown to readily catalyze the enzymatic formation of the coenzyme; incubation with pyruvate instead of phosphoenolpyruvate produced no coenzyme M. Analysis of cell extracts of *M. formicicum* incubated with these substrates, for the presence of sulfonic acids by GC-MS, confirmed the presence of sulfolactic acid, sulfopyruvate and sulfoacetaldehyde. Sulfopyruvate was also shown to be readily converted into sulfoacetaldehyde and coenzyme M by these cell-free extracts. Sulfoacetaldehyde, in turn,

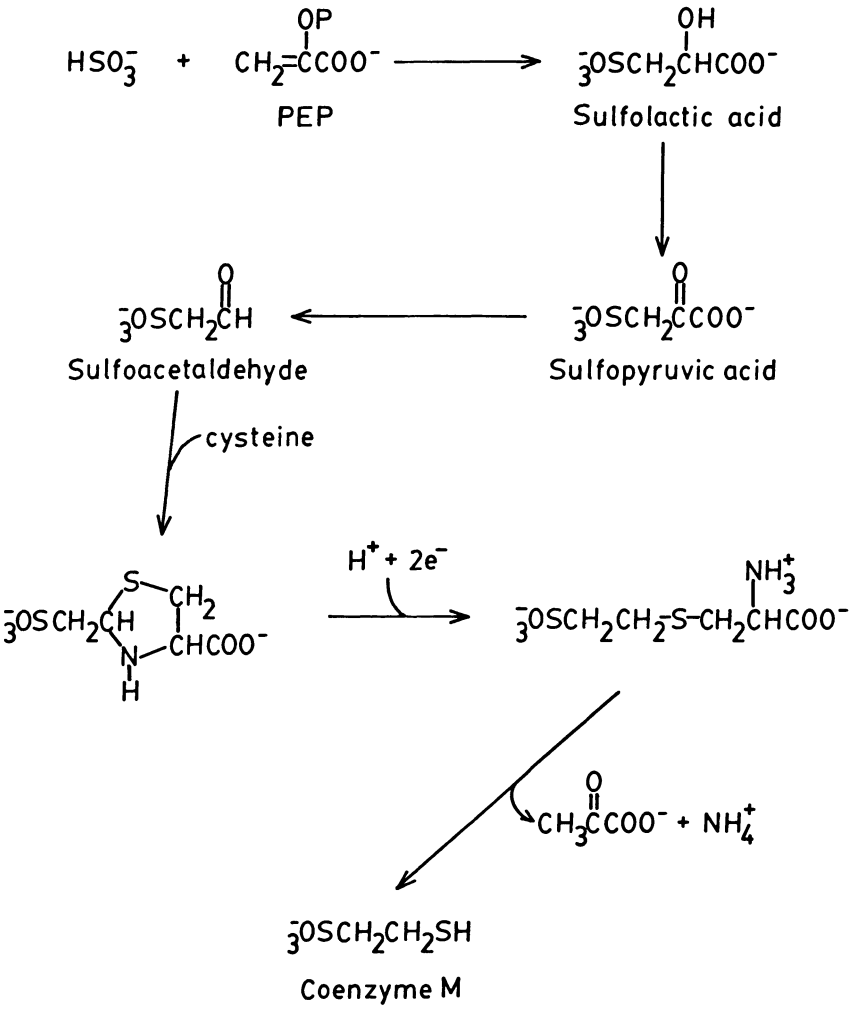

Figure 9.12. Proposed pathway for the biosynthesis of coenzyme M (from White, 1988c).

was shown to be converted enzymatically into coenzyme M by cell extracts of several different methanogens. The conversion of sulfoacetaldehyde into coenzyme M was greatly stimulated by the addition of L-cysteine to the extracts and/or by incubating the extracts under hydrogen; incubation in the presence of sulfide greatly reduced coenzyme M synthesis. Incubation of cell extracts with [2,2-$^2H_2$]sulfoacetaldehyde and L-[^{34}S]cysteine led to the production of dideuterated coenzyme M in which greater that 90% of the thiol sulfur was derived from the cysteine sulfur. Finally, [*ethylene*-$^2H_4$]-*S*-(2-sulfoethyl)cysteine was found to be readily cleaved to $^2H_4$-coenzyme M by cell-free extracts.

All of the enzymatic steps involved in the biosynthesis of coenzyme M, except for the conversion of sulfoacetaldehyde to 2-sulfoethylcysteine, have analogous reactions that occur in other biochemical systems, and do not represent any fundamentally new biochemistry. The reactions converting sulfoacetaldehyde to 2-sulfoethylcysteine and finally to coenzyme M, however, represent a previously undescribed sequence of biochemical reactions for the generation of a thiol. Current evidence also indicates that the biochemical mechanism for generation of the thiol in HS-HTP (discussed previously) is fundamentally different from that used for the production of the thiol found in coenzyme M, and represents a new sequence of biochemical reactions for the generation of a thiol. Thus, the methanogens have evolved at least two new reactions for the biochemical generation of thiols.

9.7 Factor F_{430}

The presence of a nonfluorescent yellow compound, with an absorbance maximum at 430 nm, in extracts of *M. thermoautotropicum,* was first reported in the literature by Gunsalus and Wolfe in 1978. This compound, Factor 430 (F_{430}), was later shown to contain Ni (Whitman and Wolfe, 1980) and to be present only in methanogens (Diekert et al., 1981). Because the Ni was found to be tightly bound in this compound, it was proposed early on that F_{430} may contain a tetrapyrrole ring system (Diekert, Jaenchen, et al., 1980). Since all of the naturally occurring tetrapyrroles examined to date have been found to be biosynthesized from succinate via δ-aminolevinic acid (δ-ALA) and uroporphyrinogen III, the biosynthesis of F_{430} was examined as part of the effort to establish the chemical structure of F_{430} (Diekert, Jaenchen, et al., 1980; Diekert, Gilles, et al., 1980). The results of this biosynthetic work strongly supported the idea that F_{430} was indeed a tetrapyrrole, and was instrumental in establishing the structure as that shown in Figures 9.1 and 9.15. This final structure of F_{430} was established only after extensive analysis of the ^{1}H NMR and ^{13}C NMR spectra of the pentamethyl ester of the F_{430}, which was shown to contain a chromophore of a tetrahydro-derivative of the corphin ring system, considered to be a structural hybrid between porphyrins and corrins.

The initial biosynthetic work by Diekert, Gilles, et al. (1980) showed that [2,3-^{14}C]succinate was incorporated into F_{430}, and that 8 to 9 moles of succinate were incorporated per mole of Ni. Based on the fact that methanogens incorporate succinate only into the α-ketoglutarate-derived amino acids, that is, glutamic acid, arginine and proline, but not into the acetylCoA-derived compounds (i.e., sugars, alanine, etc.), the discovery that 8 to 9 moles of succinate were incorporated into one mole of F_{430} and that the specific activity of the cellular glutamic acid was one-eighths of that of the F_{430}, strongly suggested a tetrapyrrole structure for Factor 430.

The involvement of δ-ALA, an established intermediate in the biosynthesis of tetrapyrroles, in F_{430} biosynthesis, was first demonstrated by the incorporation of [4-^{14}C]-δ-ALA into F_{430} by growing cells of *M. thermoautotrophicum* (Diekert, Jaenchen, et al., 1980). These data also showed that eight moles of δ-ALA were incorporated per mole of Ni, thus again supporting the idea that F_{430} was a tetrapyrrole. This finding was confirmed by the work of Pfaltz et al. (1982), who measured by ^{13}C NMR the incorporation of [2-^{13}C]-, [3-^{13}C]-, [4-^{13}C]-, and [5-^{13}C]-δ-ALA into the pentamethylester of Factor 430. These ^{13}C NMR experiments not only established that F_{430} originated from δ-ALA, as is the case for other tetrapyrroles, but also were vital in establishing the complete structure of Factor 430.

The pathway for the biosynthesis of δ-ALA from succinate in *M. thermoautotrophicum* has also been studied (Gilles et al., 1983). The first experiments to be reported showed that labeled succinate rather than labeled glycine was incorporated into the δ-aminolevinic acid. This was accomplished by measuring the incorporation of label in the δ-ALA excreted into the medium when the labeled compounds were incubated with growing cells of *M. thermoautotrophicum*. This finding showed that δ-ALA is produced from α-ketoglutarate in the methanogens, by the same C-5 pathway (Fig. 9.13) that is operational in higher plants, green algae, cyanobacteria, and some anaerobic eubacteria. In this pathway, δ-ALA synthesis begins from α-ketoglutarate, with either glutamate or 4,5-dioxovaleric acid as the proposed intermediate. This can be compared to the so-called Shemin pathway (Jordan and Shemin, 1972), which is observed in animals, yeast, and many eubacteria, where δ-ALA is formed from succinyl-CoA and glycine, catalyzed by δ-ALA synthase. Additional support for the presence of the C-5 pathway in this methanogen was obtained by the demonstration of L-alanine:4,5-dioxovaleric acid aminotransferase activity and the absence of δ-ALA synthase activity in these cells (Gilles et al., 1983). Thus, from the present available data, the initial steps in the biosynthesis of F_{430} are most likely as outlined in Figure 9.13.

The intermediacy of uroporphyrinogen III in the biosynthesis of F_{430} was established by Gilles and Thauer in 1983. They demonstrated that *M. thermoautotrophicum* cells accumulate ^{14}C-uroporphyrinogen III when growing on Ni-free medium, supplemented with ^{14}C-δ-ALA, and that the accumulated ^{14}C-uroporph-

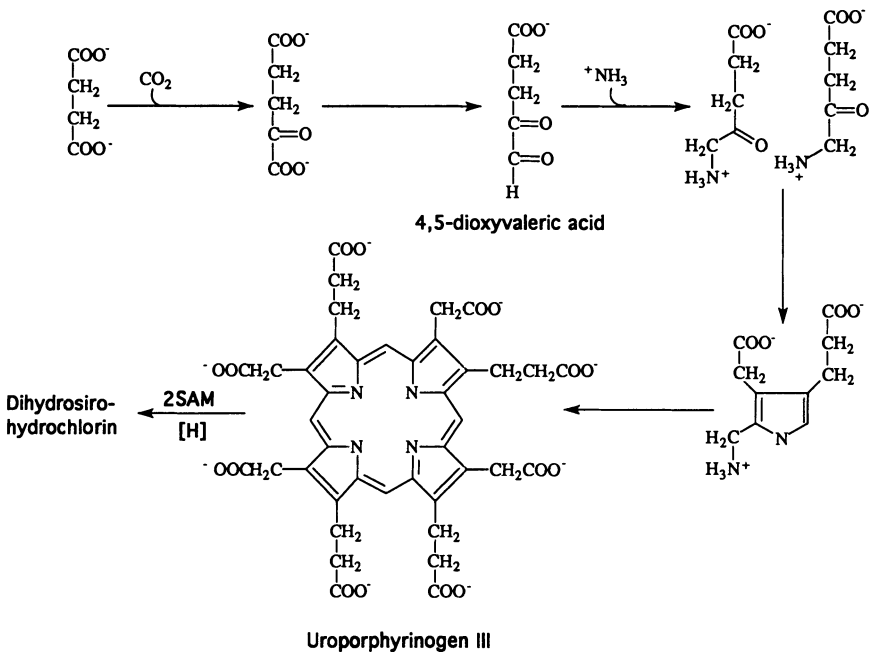

Figure 9.13. The early steps in the biosynthesis of F_{430}.

yrinogen III was quantitatively converted to F_{430} when the cells were incubated in δ-ALA-free medium supplemented with Ni. The newly synthesized F_{430} has the same specific radioactivity as labeled uroporphyrinogen III. Furthermore, enzymes catalyzing the synthesis of uroporphyrinogen III from δ-ALA, δ-ALA dehydratase, hydroxymethylbilane synthase, and uroporphyrinogen III synthase, were found in permeabilized cells of *M. thermoautotrophicum*, and these enzymes were sufficiently active to account for the rate of tetrapyrrole synthesis in the cells growing with a doubling time of 2 hours. The discovery of uroporphyrinogen III as an intermediate in the biosynthesis of F_{430} is consistent with the present dogma that all naturally occurring tetrapyrroles are biosynthesized from uroporphyrinogen III, and that uroporphyrinogen III is the last common intermediate for the biosynthesis of the different tetrapyrrole molecules. Thus, as outlined in Figure 9.14, chlorophylls, hemes, and cytochromes are derived from protoporphyrin IX, which is formed by decarboxylation of uroporphyrinogen III; whereas, siroheme and vitamin B_{12} are derived from sirohydrochlorin, which is formed by the dimethylation of uroporphyrinogen III. Since F_{430} contains two methionine-derived methyl groups (Jaenchen et al., 1981) in the same positions as found in siroheme and vitamin B_{12}, this suggested that the tetrapyrrole of F_{430} is also derived

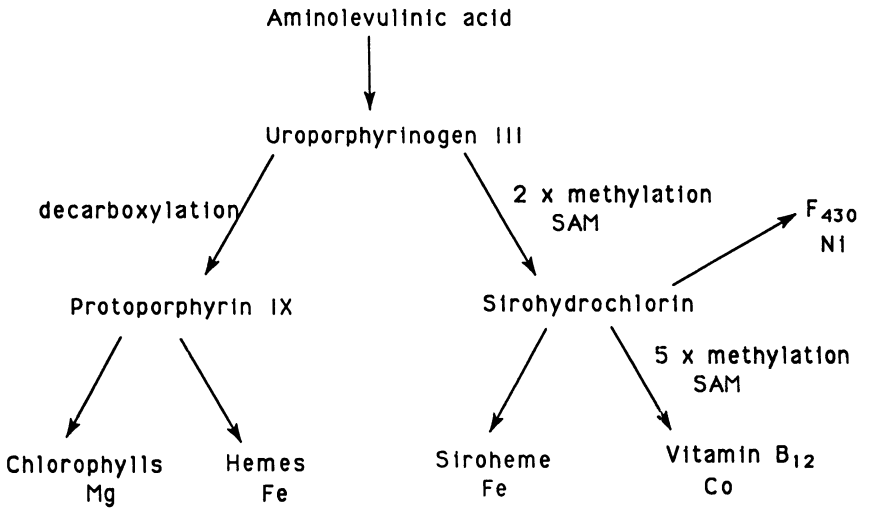

Figure 9.14. Relationship between the biosynthesis of F_{430} and other tetrapyrroles.

from sirohydrochlorin. The involvement of sirohydrochlorin or its reduced form, 15,23-dihydrosirohydrochlorin (Structure 7 in Figure 9.15), in the biosynthesis of F_{430} was established by Mucha et al. (1985), who found that ^{14}C- or ^{3}H-sirohydrochlorin was converted to F_{430} in a cell-free extract of *M. thermoautotrophicum*. This provided strong evidence that sirohydrochlorin or its reduced form is an intermediate in F_{430} biosynthesis. More recently, Pfaltz et al. (1987) identified a Ni-containing compound, 15,17^3-seco-F_{430}-17^3-acid (Structure 3 in Figure 9.15), as an intermediate in F_{430} biosynthesis. This compound was isolated from cells of *M. thermoautotrophicum* grown in a ^{63}Ni-containing medium. The amount of this compound in the cells increased when the cells were grown with increasing amounts of δ-ALA (0–10 mM). Both [^{14}C]-δ-ALA and [*methyl*-$^{3}H_3$]methionine were incorporated into this compound and F_{430}, and the specific radioactivity of the 15,17^3-seco-F_{430}-17^3-acid was identical to that of F_{430}, which was 8-fold that of the incorporated δ-ALA. Furthermore, this compound was demonstrated to be converted to F_{430} in pulse-chase labeling experiments, by growing *M. thermoautotrophicum* with $^{63}NiCl_2$ and chasing with $^{58}NiCl_2$. This work leaves little doubt that 15,17^3-seco-F_{430}-17^3-acid is an intermediate in the biosynthesis of Factor 430. Based on its structure which requires only the formation of the carbocyclic ring for its transformation into F_{430}, this acid must be the last intermediate in the biosynthesis of Factor 430. With this consideration in mind, Pfaltz et al. (1987) proposed the pathway shown in Figure 9.15.

As can be seen in Figure 9.15, many of the individual reactions in this proposed pathway for the biosynthesis of F_{430} are yet to be resolved. These reactions include

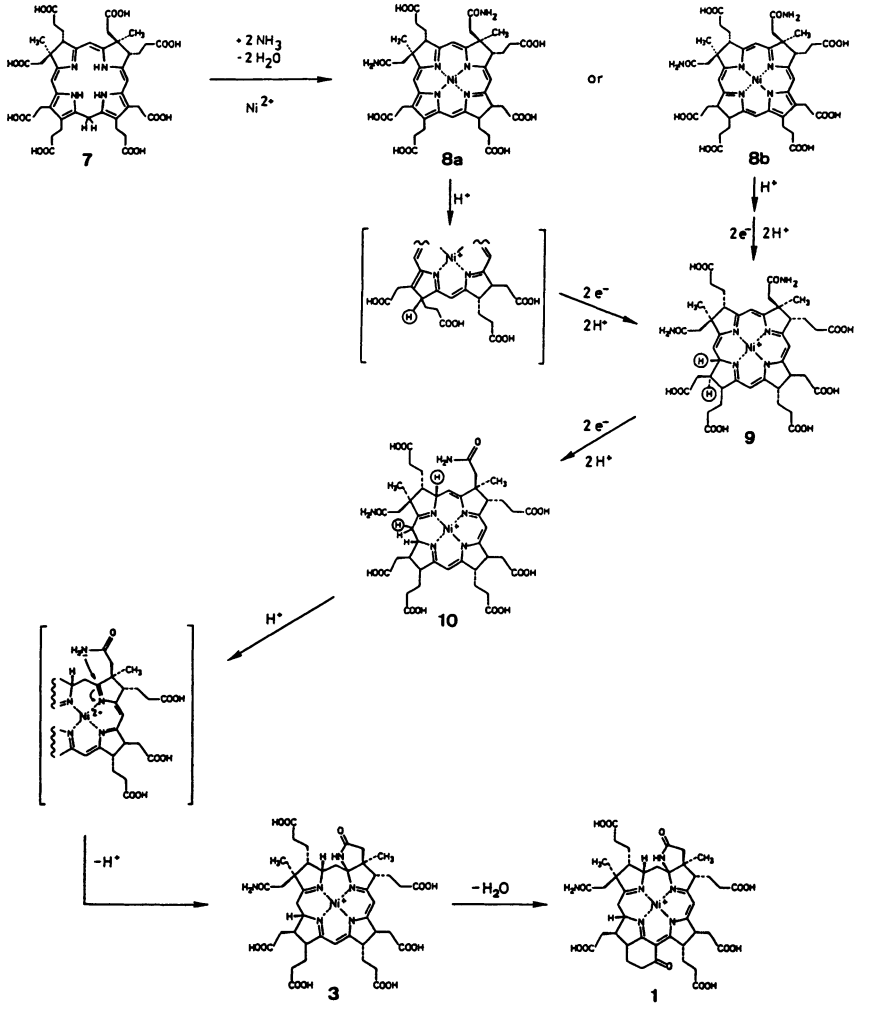

Figure 9.15. Proposed pathway for the biosynthesis of coenzyme F_{430}(**1**) from 15,23-dihydrosirohydrochlorin (**7**) (from Pfaltz et al., 1987).

the positions at which the Ni is inserted into the ring, the positions and order of the introduction of the amide groups, and the positions at which many reduction steps occur. The resolution of the order of these steps will require the isolation and characterization of the intermediates involved. This process may, in fact, require the use of genetically-engineered methanogens, in analogy with the recent work on establishing the latter steps in the biosynthesis of vitamin B_{12} (Weaver et al., 1991).

9.8 Vitamin B_{12}-Factor III

The existence of modified cobamides in methanogenic bacteria was first noted in 1965 by Lezius and Barker. At present two modified cobamides have been characterized, both of which consist of the normal vitamin B_{12} corrinoid ring structure modified by the substitution of an alternate α-ligand. In the case of factor III, this α-ligand is 5-hydroxybenzimidazole, and in the case of pseudo-B_{12}, it is adenine. To date only work on the biosynthesis of factor III has been reported (Scherer et al., 1984). In this work Scherer demonstrated that [2-^{14}C]-5-hydroxybenzimidazole added to growing cultures of *Methanosarcina barkeri* was readily incorporated into factor III. This observation suggested that the free base was likely involved in the biosynthesis of factor III. Interestingly, alternate bases could be incorporated simply by growing cells in their presence with no effect on the cells.

[1-^{13}C]Glycine was found to be incorporated into C-3a of the 5-hydroxybenzimidazole moiety of factor III as measured by ^{13}C NMR spectroscopy (Scherer et al., 1984). This position of incorporation is consistent with the previous observed incorporation of glycine into the 5-methoxybenzimidazole in *Clostridium thermoaceticum* and the formation of 5,6-dimethylbenzimidazole portion of vitamin B_{12}, by anaerobic bacteria (Höllriegl et al., 1982), and would indicate that glycine also supplies N-1 and C-7a of the 5-hydroxybenzimidazole. No information is currently available about the biosynthetic origin of the remaining four carbons present in the phenol ring of the 5-hydroxybenzimidazole. These carbons could presumably arise from a tetrose as has been observed in the biosynthesis of 5,6-dimethylbenzimidazole in the anaerobic bacteria, *Eubacterium limosum* (Vogt et al., 1988).

9.9 Other Coenzymes

Although no work has been reported on the biosynthesis of the more established coenzymes, such as NAD, NADP, and thiamine pyrophosphate, in the methanogens, establishing their pathways in these cells could be most revealing. This

stems from the observation that many of the coenzymes are biosynthesized by different pathways in different organisms, and a knowledge of these different pathways in the methanogens could shed additional light on their evolutionary relationships.

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Note added in proof: After this chapter was prepared a paper appeared (Eisnreich, W., and Bacher, A. 1992. Biosynthesis of methanofuran in *Methanobacterium thermoautophicum*. *J. Biol. Chem.* **267**: 17574–17580) showing an incorporation of ^{13}C-labeled acetate into the TCA moiety of methanofuran by *Methanobacterium thermoautophicum* which differed from that discussed in this chapter. From the reported data these authors concluded that malonate *was not* a precursor to TCA in this methanogen.

10

Anabolic Pathways in Methanogens

Peter G. Simpson and William B. Whitman

10.1 Introduction

Even though the methanogens are morphologically and nutritionally diverse, every major phylogenetic group includes some autotrophic species. Even among methanogens that require an organic carbon source, acetate is usually the major carbon source, and acetyl-CoA is also the major product of autotrophic CO_2 fixation. These observations are consistent with the hypothesis that all methanogens are either autotrophs or recently evolved from autotrophic ancestors. Because their biosynthetic pathways originate from simple carbon compounds, the role of the central metabolic pathways is the elaboration of precursors for macromolecular biosynthesis, and precursor biosynthesis is a major component of the cellular energy budget. The bioenergetic importance of precursor biosynthesis is further compounded by the low energy yields of methanogenesis and the low partial pressures of H_2 that are typical of most methanogenic habitats. This situation is fundamentally different from that of many heterotrophs, where precursors are readily obtained from catabolic intermediates, and it is likely to have profound consequences. Even though these consequences are poorly understood, our examination of the anabolic pathways in methanogens will utilize this perspective to obtain insights into why and how methanogens use the pathways they do.

10.2 Central Anabolic Pathways

10.2.1 Autotrophic Acetyl-CoA Biosynthesis

Autotrophic methanogens assimilate carbon dioxide by a unique variation of the Ljungdahl-Wood acetogenic pathway that was first discovered in the

homoacetogenic clostridia (Figure 10.1). In the clostridial pathway, acetyl-CoA is the first product of CO_2 fixation, and the two carbons of the acetyl group are biosynthesized by different routes (Ljungdahl, 1986). The methyl carbon is derived from CO_2 via the reductions in the tetrahydrofolate pathway. The carbonyl of acetyl-CoA is obtained by the reduction of CO_2 catalyzed by carbon monoxide dehydrogenase. This enzyme also catalyzes the oxidation of CO to CO_2 in the presence of methyl viologen and other artificial electron acceptors. Because it also catalyzes the condensation of the methyl group, CO, and HS-CoA to form acetyl-CoA, it is also called acetyl-CoA synthase. In methanogens, the methyl group is obtained by the reduction of CO_2 via the tetrahydromethanopterin path-

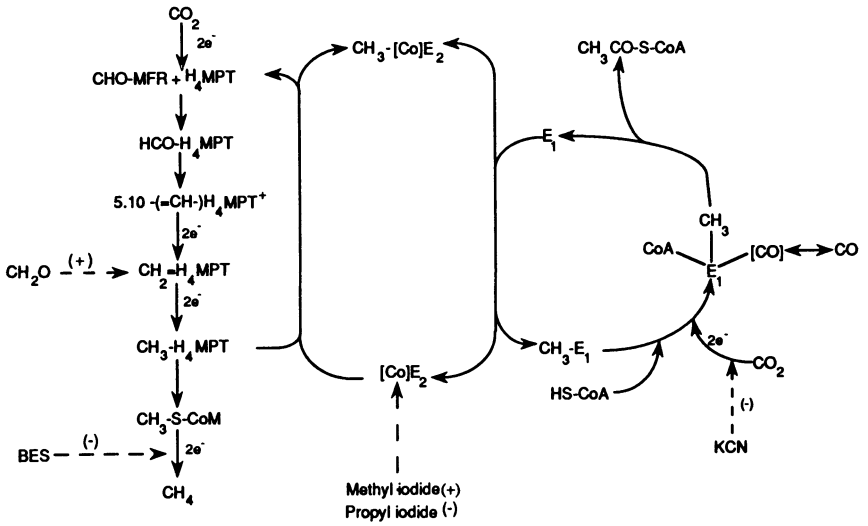

Figure 10.1. The modified Ljungdahl-Wood pathway of autotrophic methanogens. The tetrahydromethanopterin (H_4MPT) pathway of CO_2 reaction to CH_4 is the precursor of the methyl group of acetyl-CoA. Formaldehyde reacts spontaneously with H_4MPT to form methylenetetrahydromethanopterin (CH_2=H_4MPT), an intermediate of the pathway. Methyltetrahydromethanopterin (CH_3—H_4MPT) probably donates the methyl group to a corrinoid Fe/S protein, [Co]E_2. [Co]E_2 is inactivated by propyl iodide, and low concentrations of methyl iodide are methyl donors in vitro. Therefore, the cobamide, [Co], of E_2 may be the methyl carrier. The methyl group is then probably transferred to E_1, the acetyl-CoA synthase. The reversible reduction of CO_2 to the oxidation state of CO is also catalyzed by this enzyme, which is also called carbon monoxide dehydrogenase. The carbon monoxide dehydrogenase activity is inhibited by cyanide, but cyanide has no effect on its ability to bind free carbon monoxide. Other abbreviations: CHO-MFR, formylmethanofuran; HCO-H_4MPT, formyl tetrahydromethanopterin; 5,10-(=CH—)H_4MPT$^+$, methenyltetrahydromethanopterin; CH_3-S-CoM, methyl coenzyme M; BES, bromoethanesulfonate.

way, which is the central pathway of methane biosynthesis (Figure 10.1). The carbonyl group appears to be formed in a manner analogous to the clostridial pathway. Evidence for the pathway in methanogens is three-fold. One, other autotrophic pathways common in bacteria are absent. Two, isotopic labeling of whole cells indicate that acetate or acetyl-CoA is the first product of CO_2 fixation. Three, the enzymatic activities associated with the Ljungdahl-Wood pathway are present in cell extracts. This evidence is reviewed below.

Historically, the two well established pathways of autotrophy in bacteria are the Calvin cycle (reductive pentose phosphate pathway) and the reverse tricarboxylic acid cycle (Evans et al., 1966; Buchanan et al., 1972; McFadden, 1973). Other, less well known routes of carbon dioxide or C-1 fixation include the serine pathway and ribulose monophosphate cycle for formaldehyde assimilation in methylotrophs, the acetogenic pathway present in homoacetogenic clostridia, and the hydroxypropionate pathway in *Chloroflexus* (Lange et al., 1961; Johnson and Quayle, 1965; Ljungdahl, 1986; Holo and Sirevag, 1986; Holo, 1989; Quayle, 1972). With the exception of the last pathway, there is strong evidence that none of the above CO_2 fixation pathways are operative in methanogens. 3-Phosphoglycerate, the predominant short term fixation product of the Calvin cycle, is not an early product of CO_2 assimilation in whole cells of *Methanobacterium thermoautotrophicum, Methanobrevibacter ruminantium*, and *Methanosarcina barkeri*. In addition, the key enzyme of this pathway, ribulose bisphosphate carboxylase, is not detectable in cell extracts of *M. thermoautotrophicum, M. barkeri*, or *Methanococcus maripaludis* (Daniels and Zeikus, 1978; Weimer and Zeikus, 1978; Yu and Whitman, unpublished data). Therefore, the Calvin cycle is almost certainly absent from these microorganisms. Similarly, all of the enzymes required for a reductive tricarboxylic acid cycle have been demonstrated in vitro for *M. thermoautotrophicum* with the important exceptions of isocitrate dehydrogenase and citrate lyase (Zeikus et al., 1977). Similarly, isocitrate dehydrogenase is also absent from extracts of *M. maripaludis* (Shieh and Whitman, 1987). Thus, it is unlikely that a complete reductive TCA cycle is present in these methanogens. Because extracts of *M. thermoautotrophicum* and *M. barkeri* lack activities for hexulose phosphate synthase and hydroxypyruvate reductase, the major pathways of formaldehyde assimilation, the ribulose monophosphate cycle and the serine pathway, are also absent (Taylor et al., 1976; Weimer and Zeikus, 1978). Activities of enzymes associated with the clostridial acetogenic pathway, formyl tetrahydrofolate synthetase and methylene tetrahydrofolate dehydrogenase, are also too low in extracts of a number of methanogens to be involved in a major CO_2 fixation pathway (Ferry et al., 1977). Lastly, while the possibility of the hydroxypropionate pathway has not been examined in methanogens, little evidence has accumulated to contradict the hypothesis that a modified Ljungdahl-Wood is the major pathway. Therefore, it is unlikely that this pathway is significant in the methanogens that have been investigated.

Whole cells of *M. thermoautotrophicum* incorporate label from ^{14}C-acetate into cellular components derived from both acetate and pyruvate, indicating that acetate or acetyl-CoA play a central role in the carbon metabolism of this organism (Fuchs and Stupperich, 1980; Rhulemann et al., 1985). Importantly, radiolabeled pyruvate is not incorporated into compounds derived exclusively from acetyl-CoA, like the N-acetyl moieties of pseudomurein. Therefore, pyruvate is not a precursor of acetyl-CoA biosynthesis, and the reductive TCA cycle cannot be a major pathway of autotrophic CO_2 fixation.

The major early products of $^{14}CO_2$ incorporation by whole cells of *M. thermoautotrophicum* are alanine, aspartate, glutamate, and intermediates of methanogenesis (Daniels and Zeikus, 1978). After 2 s of radiolabeling, the distribution of radiolabel between the C-1, C-2, and C-3 carbon atoms of alanine are 61%, 23%, and 16%, respectively (Stupperich and Fuchs, 1981). This distribution is consistent with a CO_2 assimilation pathway that involves the formation of acetyl-CoA from two molecules of CO_2 via condensation of one carbon intermediates. Reductive carboxylation of acetyl-CoA by pyruvate oxidoreductase is responsible for the high specific activity of the C-1 carbon (Zeikus et al., 1977; Fuchs et al., 1978). Acetyl-CoA itself is not easily detected because its concentration in cells is very low; after 1.5 and 25 seconds of radiolabeling, acetyl-CoA accounts for about 0.6% and 0.1% of the radiolabel incorporated, respectively (Rhulemann et al., 1985). Importantly, the acetyl-CoA pool is fully radiolabeled within 5–10 s, whereas the alanine pool is not fully radiolabeled within 25 seconds. The rapid decline in the percentage of total radiolabel in acetyl-CoA and the rapid saturation of the acetyl-CoA pool with radiolabel from $^{14}CO_2$ are consistent with it being an early product. Importantly, while the carbonyl and methyl carbons of acetyl-CoA are equally radiolabeled after 10 seconds, the carbonyl carbon is radiolabeled preferentially at earlier time points (Rhulemann et al., 1985). Therefore, the two carbons of acetyl-CoA are derived from different pathways.

The sources for each of the carbons of acetyl-CoA was further elucidated by a series of experiments with whole cells and cell extracts (Figure 10.1). At the time that these experiments were initiated, it was known that *M. thermoautotrophicum* grew slowly on carbon monoxide and contained carbon monoxide dehydrogenase (Daniels et al., 1977). In addition, it was known that carbon monoxide dehydrogenase is associated with acetate biosynthesis by whole cells and in highly purified enzyme fractions of the homoacetogen *Clostridium thermoaceticum* (Diekert and Thauer, 1978; Drake et al., 1981). The role of carbon monoxide dehydrogenase in *M. thermoautotrophicum* was established by the following observations. During autotrophic growth on H_2 and CO_2, small amounts of CO are produced (Conrad and Thauer, 1983). CO production is inhibited by low concentrations of cyanide, which are also inhibitory to carbon monoxide dehydrogenase (Eikmanns et al., 1985). In growing cells, propyl iodide stimulates CO

production 10-fold. Alkyl halides inhibit acetyl-CoA biosynthesis, probably because they inactivate the corrinoid Fe-S protein; but they have little effect on the CO dehydrogenase activity (Lu et al., 1990). Therefore, the increase in CO production may be attributed to inhibition of a major sink for CO (Eikmanns et al., 1985). Importantly, CO production is also sensitive to uncouplers and requires a membrane potential (Eikmanns et al., 1985). During growth, radiolabeled CO is also incorporated into cell material, and the C-2 of alanine is preferentially radiolabeled (Stupperich et al., 1983). In cell extracts, acetyl-CoA biosynthesis from CO_2 is also inhibited by cyanide, and activity is restored by CO (Stupperich and Fuchs, 1983). Under this condition, the carbonyl of acetyl-CoA is not radiolabeled by $^{14}CO_2$, suggesting that it is derived directly from CO (Stupperich and Fuchs, 1984b). This conclusion was confirmed by demonstrating directly that ^{14}CO is incorporated into the carbonyl of acetyl-CoA by cell extracts (Lange and Fuchs, 1987).

Evidence for the source of the methyl carbon of acetyl-CoA comes largely from in vitro experiments. In cell extracts of *M. thermoautotrophicum*, methane formation from carbon dioxide and H_2 is stimulated by the addition of methyl coenzyme M, a phenomenon known as the RPG effect (Gunsalus and Wolfe, 1980). Methane production is also strongly inhibited by the coenzyme M analogue bromoethanesulfonate, halogenated alkanes like carbon tetrachloride, and N_2O. Significantly, methyl coenzyme M also stimulates acetyl-CoA biosynthesis in cell extracts of *M. thermoautotrophicum*, suggesting that the two processes are either coupled or possess one or more common intermediates (Stupperich and Fuchs, 1983, 1984a ; Holder et al., 1985; Lange and Fuchs, 1987). Likewise, bromoethanesulfonate, carbon tetrachloride, and N_2O inhibit both methanogenesis and acetyl-CoA biosynthesis equally (Stupperich and Fuchs, 1984a,b). In these cell extracts, carbon from methanogenic substrates such as CO_2, formaldehyde, and the C-3 of serine are incorporated into both methane and the methyl carbon of acetyl-CoA (Holder et al., 1985). Carbon monoxide stimulated the incorporation of radiolabel from [3-^{14}C]serine into acetate. Thus, there is convincing evidence to suggest that these two processes share common intermediates in the carbon dioxide reduction pathway. To identify the branch point between the two pathways, the incorporation of radiolabel from intermediates in the pathway of CO_2 reduction was examined. [^{14}C-Methenyl]tetrahydromethanopterin labels both methane and the methyl of acetyl-CoA, whereas [^{14}C-methyl]coenzyme M radiolabels only methane (Stupperich and Fuchs, 1984b; Lange and Fuchs, 1985). In addition, [^{14}C-methyl]tetrahydromethanopterin also radiolabels acetyl-CoA (Lange and Fuchs, 1987). Although these results suggest that methyltetrahydromethanopterin is the branch point between methanogenesis and acetyl-CoA biosynthesis, these experiments were performed in extracts so it is still possible that a more oxidized C-1 intermediate is the actual proximal donor. However, this

possibility seems unlikely by analogy to the clostridial system, where methyltetrahydrofolate is the methyl donor in a system composed of homogeneous enzymes.

Similar evidence has demonstrated a modified Ljungdahl-Wood pathway in *Methanococcus*. The carbon monoxide dehydrogenase, purified to homogeneity from autotrophically grown *Methanococcus vannielii*, is similar in quaternary structure and metal content to the enzyme from the aceticlastic methanogen *M. barkeri* (DeMoll et al., 1987). Because it lacks the acetyl-CoA biosynthetic activity associated with the purified enzymes from *C. thermoaceticum* and *Methanosarcina thermophila*, it is probably only one component of a larger enzyme complex. However, acetyl-CoA biosynthetic activity has been demonstrated in cell extracts of the facultative autotroph *M. maripaludis* (Shieh and Whitman, 1988). Autotrophic $^{14}CO_2$ assimilation is detected by trapping [^{14}C]pyruvate as [^{14}C]lactate. Following degradation, the percentages of radiolabel in the C-1, C-2 and C-3 positions of lactate are 73%, 33%, and 11%, respectively. This distribution is expected if the C-1 of lactate (pyruvate) is derived from CO_2 by the reductive carboxylation of acetyl-CoA, and the C-2 and C-3 of lactate are derived from the carbonyl and methyl groups of acetyl-CoA, respectively. As expected, ^{14}CO is preferentially incorporated into the C-2 of lactate; and [^{14}C]formaldehyde, which reacts spontaneously with tetrahydromethanopterin in cell extracts to form [^{14}C-methylene]tetrahydromethanopterin, preferentially radiolabels the C-3 of lactate. Further support for these conclusions is obtained by examination of acetate auxotrophs of *M. maripaludis* (Ladapo and Whitman, 1990). These mutants simultaneously lose: (1) the ability to grow autotrophically, (2) in vitro methyl viologen-linked carbon monoxide dehydrogenase activity, and (3) in vitro acetyl-CoA biosynthetic activity as demonstrated by the lactate trap. In spontaneous revertants, all three activities are restored. Therefore, carbon monoxide dehydrogenase is essential for autotrophic growth.

There is also significant evidence for a modified Ljungdahl-Wood pathway in other methanogens. During methylotrophic growth, *M. barkeri* preferentially incorporates [^{14}C]methanol into the C-3 of alanine, which is derived from the C-2 of acetyl-CoA (Kenealy and Zeikus, 1982). In extracts of H_2:CO_2 grown cells, ^{14}CO is also incorporated into acetate. This activity is dependent on methylcobalamin, which can also serve as a methyl donor to the clostridial enzyme (Kenealy and Zeikus, 1982). Low concentrations of cyanide also inhibit autotrophic growth of *M. barkeri* on CO_2 or methanol (Smith et al., 1985). Likewise, carbon monoxide dehydrogenase is abundant in extracts of a wide variety of methanogens that grow autotrophically, but it is absent from many methanogens that require acetate as a carbon source (Bott et al., 1985).

While the evidence for a modified Ljungdahl-Wood pathway of autotrophic CO_2 assimilation in methanogens is compelling, most of the enzyme components have not been purified from an autotroph. For this reason, interpretations of the

physiological experiments in whole cells and cell extracts rely heavily on the detailed biochemical studies of enzymes from the homoacetogenic clostridia or aceticlastic methanogens. Thus, the evidence for a corrinoid Fe/S protein is based largely on sensitivity to inhibitors like iodopropane and the ability of iodomethane to serve as a methyl donor (Holder et al., 1985; Shieh and Whitman, 1988). While details on the active site of carbon monoxide dehydrogenase from autotrophs are unknown, the sensitivity to cyanide and ability of CO to serve as a carbonyl donor suggest strong similarities to the catabolic enzymes.

The last point for discussion is why? Clearly, there are bioenergetic advantages of the modified Ljungdahl-Wood pathway over some of the other autotrophic pathways (Fuchs, 1986). In methanogens, there is no ATP requirement for acetyl-CoA biosynthesis. However, the initial reduction of CO_2 in the tetrahydromethanopterin pathway and the reduction catalyzed by carbon monoxide dehydrogenase are probably coupled to a proton or sodium motive force (Thauer, 1990). Therefore, acetyl-CoA biosynthesis requires some energy input, and, although the magnitude is not known with certainty, it is likely to be less than or equal to two "ATP equivalents," or one for each coupling site. For comparison, acetate biosynthesis from H_2 + CO_2 is the primary means of energy generation for the autotrophic homoacetogenic bacteria. However, autotrophic growth of the homoacetogens is only thermodynamically favorable at partial pressures of H_2 higher than those usually found in methanogenic habitats. Moreover, if acetyl-CoA is the product instead of acetate, there is an additional cost of the one ATP normally obtained via the phosphotransferase and acetate kinase reactions. Therefore, it is unlikely that autotrophic acetyl-CoA biosynthesis is actually accomplished without cost in methanogens, but the cost is probably low. In contrast, the Calvin cycle requires 3 ATP for every CO_2 fixed. Similarly, the reductive TCA cycle and the serine pathway require at least 2 ATP per acetyl-CoA formed. The reductive TCA cycle further requires two unfavorable reductions for pyruvate and -ketoglutarate biosynthesis. However, the ribulose monophosphate pathway of formaldehyde assimilation produces pyruvate without a requirement for ATP and contains only two enzymes not previously identified in methanogens, the hexulose phosphate synthase and hexulose phosphate isomerase. If free formaldehyde could be obtained from an intermediate in methanogenesis, like methylenetetrahydromethanopterin, this pathway could potentially be very efficient and would require only the initial reduction of CO_2 in the tetrahydromethanopterin pathway. For this reason, the modified Ljungdahl-Wood pathway in methanogens cannot be rationalized solely on bioenergetic grounds.

10.2.2 Pyruvate Biosynthesis

Acetyl-CoA enters the major biosynthetic pathways of methanogens via a reductive carboxylation catalyzed by pyruvate oxidoreductase (Fuchs and Stup-

perich, 1986). The biosynthetic activity of this enzyme, the carboxylation of acetyl-CoA, is present in cell-free extracts of *M. maripaludis* and *M. voltae*, which represent autotrophic and heterotrophic methanococci, respectively (Shieh and Whitman, 1987). While only low biosynthetic activities were obtained, this result was expected due to the extreme oxygen sensitivity and general lability of this enzyme in extracts. In addition to these two methanococci, high activities of the catabolic reaction associated with pyruvate oxidoreductase, the oxidation of pyruvate to acetyl-CoA and CO_2 coupled with the reduction of methyl viologen, have been demonstrated in cell extracts of several other methanogens including *M. thermoautotrophicum* and *M. barkeri* (Zeikus et al., 1977; Weimer and Zeikus, 1979; Jansen et al., 1982). Unlike the ferredoxin- dependent eubacterial pyruvate oxidoreductases, the enzymes from methanogens appear to be linked to coenzyme F_{420} in cell extracts (Zeikus et al., 1977). However, whether or not this result is an artifact of the in vitro activity is not known. Cell extracts of *M. thermoautotrophicum* also catalyze an exchange reaction between CO_2 and pyruvate, an activity commonly associated with bacterial pyruvate oxidoreductases (Zeikus et al., 1977).

Enrichment and labeling patterns of cellular components extracted from *Methanospirillum hungatei*, *Methanothrix concilli*, *M. thermoautotrophicum*, and *M. voltae* incubated with stable or radioactive isotopes have also given results that are consistent with the presence of an anabolic pyruvate oxidoreductase in these bacteria (Fuchs et al., 1978; Ekiel et al., 1983; Ekiel, Jarrell, et al., 1985; Ekiel, Sprott, et al., 1985; Evans, Tolman, et al., 1986; Jansen et al., 1982). Therefore, the pathway of pyruvate formation from acetyl-CoA and CO_2 appears to be common to several distantly related methanogens and is probably widely distributed throughout the methanogens.

Although the pyruvate oxidoreductase activity is fully reversible in vitro, the catabolic activity is inhibited in whole cells during growth on $H_2 + CO_2$ (Yang and Whitman, 1992). Thus, acetate auxotrophs of *M. maripaludis* are unable to grow with pyruvate in place of acetate. However, resting cells in the absence of H_2 utilize pyruvate as an electron donor for methanogenesis and excrete acetate. Presumably, the catabolic activity of pyruvate oxidoreductase is inhibited to prevent a futile cycle of pyruvate synthesis and oxidation. However, the mechanism of this regulation is not known.

10.2.3 Incomplete Tricarboxylic Acid Cycles

Attempts to demonstrate a complete reductive or oxidative tricarboxylic acid (TCA) cycle in methanogenic bacteria have been unsuccessful. Nevertheless, both oxidative and reductive incomplete cycles are present, and they are responsible for

supplying cells with key biosynthetic intermediates (Figure 10.2). Physiologically, the distribution of these pathways can be rationalized on the basis of the bioenergetics of α-ketoglutarate biosynthesis. Both pathways require the biosynthesis of one molecule of oxaloacetate and are equivalent up to that point. The oxidative incomplete TCA cycle requires a second acetyl-CoA and would be inefficient in an organism where there is a high premium for autotrophically derived acetyl-CoA. In fact, the oxidative pathway appears to be restricted to acetotrophic methanogens where acetate is supplied exogenously. In contrast, the reductive incomplete TCA cycle requires two additional reductions and a reductive carboxylation to form α-ketoglutarate from oxaloacetate. Because it is the only pathway found in hydrogenotrophic methanogens, this pathway may be favored when electron donors, like H_2, are abundant. While these rationales are logical, they cannot be the entire story. For instance, hydrogenotrophic methanogens grown with exogenous acetate still use the reductive pathway. Because the oxidative incomplete TCA cycle has only be found in the *Methanosarcinales*, the strategy of α-ketoglutarate biosynthesis may also be conserved on phylogenetic lines.

Two lines of investigation have established the incomplete reductive TCA cycle in *M. thermoautotrophicum*. First, enzymes necessary for a reductive pathway, phosphoenolpyruvate carboxylase for oxaloacetate synthesis, malate dehydrogenase, fumarase, fumarate reductase, succinyl CoA synthetase, and α-ketoglutarate oxidoreductase, have high activities in cell extracts (Zeikus et al., 1977; Fuchs and Stupperich, 1986). Likewise, the labeling patterns of aspartate and glutamate and, therefore, their keto acid precursors from cultures grown with ^{14}C-acetate are consistent with oxaloacetate formation from phosphoenolpyruvate and CO_2 and α-ketoglutarate synthesis from succinyl-CoA and CO_2. Thus, these results confirm the in vitro activities of phosphoenolpyruvate carboxylase and α-ketoglutarate oxidoreductase (Fuchs et al., 1978). In the complete reductive cycle, succinate contributes to the carbon skeleton of both pyruvate and oxaloacetate, and the formation of these products relies upon the presence of isocitrate dehydrogenase. Thus, it is significant that exogenous ^{14}C-succinate is incorporated into α-ketoglutarate but not pyruvate and oxaloacetate (Fuchs and Stupperich, 1978). In addition, isocitrate dehydrogenase and citrate lyase activities appear to be absent in cell extracts of *M. thermoautotrophicum* (Zeikus et al., 1977).

Oxaloacetate synthesis in *M. thermoautotrophicum* is dependent upon the carboxylation of phosphoenolpyruvate. Consequently, to support anabolism by an incomplete reductive TCA cycle, substantial amounts of phosphoenolpyruvate must be produced. In methanogens that are assimilating carbon primarily as CO_2 or acetate, glycolysis is not a potential source, and another route of phosphoenolpyruvate production must exist. The partial purification and characterization of phosphoenolpyruvate synthetase from *M. thermoautotrophicum* indicates that the phosphoenolpyruvate demand is met by its unidirectional synthesis from pyruvate

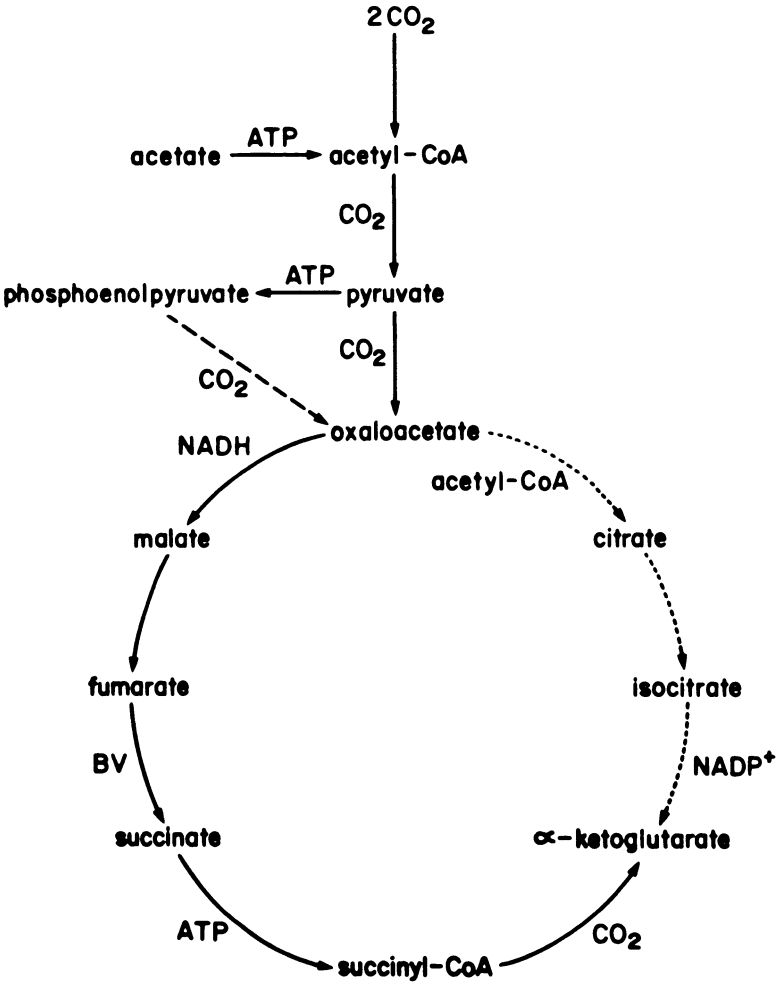

Figure 10.2. Reductive and oxidative incomplete tricarboxylic acid cycles in methanogenic bacteria. Enzymes demonstrated in *Methanococcus maripaludis* are shown by the solid lines. *Methanobacterium thermoautotrophicum* contains these enzymes, except that phosphoenolpyruvate carboxylase substitutes for pyruvate carboxylase. *Methanosarcina barkeri* contains the oxidative branch of the TCA cycle shown by the broken lines. Used with permission from Shieh and Whitman (1987).

(Eyzaguirre et al., 1982). This enzyme, which has sufficiently high in vitro activity to account for its in vivo function, has a low affinity for phosphoenolpyruvate and is inhibited by AMP, ADP, and α-ketoglutarate. Therefore, it may also represent a regulatory step in the incomplete reductive TCA cycle of this methanogen (Eyzaquire et al., 1982).

The enzymes of the reductive branch of the TCA cycle have also been demonstrated in cell extracts of *M. maripaludis* and *M. voltae* (Shieh and Whitman, 1987). The pathway is similar to that reported for *M. thermoautotrophicum* except for an alternate pathway of oxaloacetate formation (Figure 10.2). In the two methanococci, phosphoenolpyruvate carboxylase is absent, and high activities are found for pyruvate carboxylase instead (Shieh and Whitman, 1987). In addition, enrichment patterns of amino acids from cultures of *M. voltae* grown on [2-^{13}C]acetate are consistent with the operation of an incomplete reductive TCA cycle for α-ketoglutarate biosynthesis (Ekiel and Jarrell et al., 1985). Stable isotope labeling experiments have provided evidence that *Methanospirillum hungatei* also uses the incomplete reductive TCA cycle for the production of biosynthetic intermediates (Ekiel et al., 1983). In addition the malate dehydrogenase has been extensively characterized from *M. hungatei* (Sprott et al., 1979; Storer et al., 1981). Because of its low affinity of NAD^+, the enzyme is nearly unidirectional for oxaloacetate reduction. Similar characteristics have also been described for the malate dehydrogenase from *Methanothermus fervidus* (Honka et al., 1990).

Cell extracts of *Methanosarcina barkeri* lack α-ketoglutarate oxidoreductase. Therefore, this methanogen is unable to synthesize α-ketoglutarate by the reductive pathway. However, the presence of citrate synthase, aconitase, and isocitrate dehydrogenase in cell extracts suggests that the demand for α-ketoglutarate is met by an incomplete oxidative TCA cycle (Weimer and Zeikus, 1979). In support of this hypothesis, ^{14}C tracer studies indicate that acetate is incorporated into glutamate as predicted by the incomplete oxidative TCA cycle (Weimer and Zeikus 1979). In addition, ^{13}C NMR spectroscopy of amino acids following growth with [^{13}C]acetate has confirmed that the incomplete oxidative TCA cycle is active in the phylogenetically related acetotroph *Methanosaeta* (*Methanothrix*) *concilii* (Ekiel, Sprott, et al., 1985).

10.3 Precursor Biosynthesis

10.3.1 Amino Acid Biosynthesis

Several lines of evidence indicate that the amino acid biosynthetic pathways in methanogenic bacteria closely resemble their eubacterial and eukaryotic counterparts. Early ^{14}C-labeling studies of whole cells demonstrated that alanine,

aspartate, and glutamate are produced from pyruvate, oxaloacetate, and α-ketoglutarate in *Methanobacterium* spp. and *M. barkeri* (Fuchs et al., 1978; Kenealy et al., 1982). A series of very thorough ^{13}C-labeling studies on the amino acids biosynthesized by whole cells of *M. voltae*, *M. hungatei*, and *M. concilii* demonstrated similar mechanisms in these methanogens (Ekiel et al., 1983; Ekiel, Jarrell, et al., 1985; Ekiel, Sprott, et al., 1985). These studies also examined the labeling of the remaining amino acids.

The ^{13}C-labeling of serine from acetate and CO_2 is consistent with its synthesis from pyruvate, presumably via 3-phosphoglycerate, and the labeling of glycine is consistent with its synthesis from serine (Ekiel et al., 1983). Likewise, in *M. thermoautotrophicum*, serine and glycine are radiolabeled to a high specific activity by exogenous [2-^{14}C]pyruvate, and only serine is radiolabeled by [3-^{14}C]pyruvate (Holder et al., 1985).

A serine transhydroxylmethylase from *M. thermoautotrophicum* has been isolated and characterized (Hoyt et al., 1986). The enzyme, responsible for biosynthesis of glycine from serine, requires pyridoxal phosphate and magnesium ions for activity and is similar in this respect to eucaryotic and eubacterial transhydroxylmethylases. Significantly, the methanogen enzyme is dependent upon tetrahydromethanopterin as the acceptor of C-1 units from serine. Tetrahydrofolate does not substitute, which may reflect the unique coenzyme content of methanogenic bacteria (Hoyt et al., 1986). Since tetrahydromethanopterin is a coenzyme directly associated with methane evolution, it is possible that serine may be a methanogenic substrate. Indeed, cell extracts of *M. thermoautotrophicum* convert serine to methane and glycine (Holder et al., 1985). However, there is no evidence that this activity is significant in vivo. *M. thermoautotrophicum* also incorporates exogenous glycine into serine, presumably by the reverse of this reaction (Taylor et al., 1976).

The branched-chain amino acids (BCAA), leucine, valine, and isoleucine, are synthesized by the acetohydroxy acid pathway (Ekiel et al., 1983). Of particular note is the biosynthetic route leading to the production of α-ketobutyrate, a precursor of isoleucine. Although some methanogens can also produce α-ketobutyrate from propionate and CO_2, ^{14}C and ^{13}C isotopic studies reveal that in many methanogens, the α-ketobutyrate used for biosynthesis of isoleucine is formed from pyruvate and acetyl-CoA via the citramalate pathway and not from the typical route through threonine (Figure 10.3; Eikmanns, Jaenchen, et al., 1983; Eikmanns, Linder, et al., 1983; Ekiel et al., 1984). Isoleucine is then produced from α-ketobutyrate by the addition of an active aldehyde in a manner common to the acetohydroxy acid pathway (Ekiel et al., 1983).

The enzymes of the BCCA biosynthetic pathway have been partially purified from the methanococci and characterized (Xing and Whitman, 1987, 1991, 1992). These studies not only confirmed the route of BCAA biosynthesis but also provided valuable information about the relationship between archaebacterial,

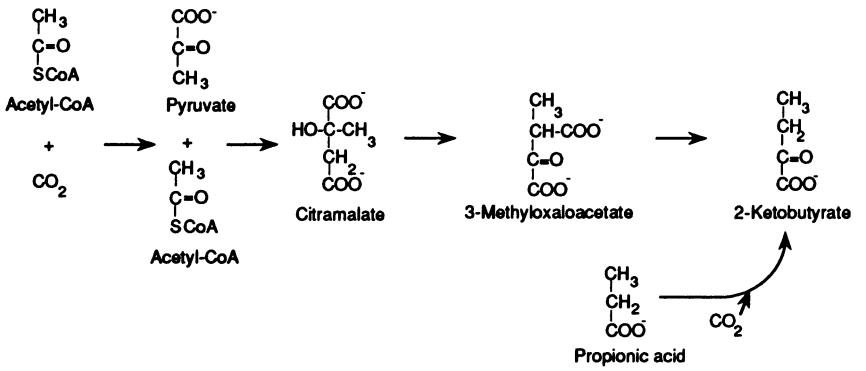

Figure 10.3. Pathway of α-ketobutyrate biosynthesis in methanogens. α-Ketobutyrate, a precursor of isoleucine, is biosynthesized from acetyl-CoA and CO_2 via the citramalate pathway. When propionate is present, it may also be formed by reductive carboxylation.

eubacterial, and eucaryotic amino acid biosynthesis. The four enzymes of the pathway, acetohydroxy acid synthase, acetohydroxy acid isomeroreductase, dihydroxy acid dehydratase, and branched-chain aminotransferase are present in cells at levels necessary to support growth. In addition, their kinetic properties are similar to their eubacterial counterparts (Xing and Whitman, 1991, 1992). The archaebacterial acetohydroxyacid synthase is also susceptible to herbicides to a similar degree as the eubacterial enzyme (Xing and Whitman, 1987). Interestingly, the methanococcal acetohydroxyacid synthase and dihydroxyacid dehydratase are oxygen labile. While the eubacterial enzymes are also sensitive to oxygen, the methanococcal enzymes are inactivated by lower partial pressures. Thus, the methanococcal enzymes probably lack the oxygen protection mechanisms of the facultative aerobic eubacteria that have been examined. This hypothesis is in agreement with the evolution of an oxygen atmosphere late in the earth's history so that ancient anaerobic bacteria may not have evolved a complete oxygen protection system for these enzymes (Xing and Whitman, 1991).

In *M. hungatei*, stable isotopic labeling suggests that lysine is biosynthesized by the diaminopimelic acid pathway (Ekiel et al., 1983). Cell extracts of *M. thermoautotrophicum* also contain activities of two enzymes of this pathway, dihydropicolinate synthase and diaminopimelate decarboxylase (Bakhiet et al., 1984). The alternate route of lysine biosynthesis, the aminoadipate pathway, is absent in *M. thermoautotrophicum* because cell extracts lack saccharopine dehydrogenase.

In *M. hungatei*, the labeling of arginine by stable isotopes closely resembles that of glutamate (Ekiel et al., 1983). The enzymes ornithine acetyltransferase, *N*-acetylglutamate 5-phosphotransferase, and ornithine carbamoyltransferase are

also present in extracts of *Methanobrevibacter arborphilus*, *Methanococcus vannielii*, and *M. thermoautotrophicum*. Therefore, methanogens utilize the ornithine acetyltransferase pathway for arginine biosynthesis instead of the route involving *N*-acetylglutamate synthase (Meile and Leisinger, 1984). The latter pathway is more energy consuming because acetyl groups are not recycled between ornithine and glutamate. Because of the low energy yield associated with methanogenesis, it is not surprising that the more economical route of arginine biosynthesis is utilized. It has also been suggested that the ornithine transacetylase route is the more ancient of the two arginine biosynthetic pathways (Meile and Leisinger, 1984). In addition, genes for two enzymes of the pathway have been cloned and sequenced (Morris and Reeve, 1988). The genes for the argininosuccinate synthetase from *M. barkeri* and *M. vannielii* share about 38% sequence similarity to the human gene on the amino acid level. The gene for carbamyl phosphate synthetase from *M. barkeri* has 45 and 31% sequence similarity on the amino acid level to the genes from *E. coli* and *Saccharomyces cerevisiae* (Morris and Reeve, 1988).

The incorporation of stable isotopes into histidine suggests that it is synthesized by the normal pathway via phosphoribosylpyrophosphate and ATP (Ekiel et al., 1983). This hypothesis is confirmed in principle by the demonstration of high sequence similarity between the eubacterial and methanococcal genes for two enzymes in the pathway, phosphoribosylformimino-5-amino-1-phosphoribosyl-4-imidazolecarboxamide isomerase and phosphoribosyl-AMP cyclohydrolase (Beckler and Reeve, 1986; Cue et al., 1985).

The incorporation of stable isotopes suggests that the aromatic amino acids, phenylalanine and tyrosine, are produced via the shikimate and chorismate pathway (Ekiel et al., 1983). Likewise, the entire tryptophan biosynthetic gene cluster of *M. thermoautotrophicum* has been cloned and sequenced, and a portion of the operon has been sequenced from *M. voltae* (Meile et al., 1991; Sibold and Henriquet, 1988). On the amino acid level, these genes generally have 30–50 % sequence similarity to the eubacterial and eucaryotic enzymes.

Lastly, the labeling patterns of stable isotopes into threonine and methionine are consistent with their biosynthesis from aspartate (Ekiel et al., 1983; Holder et al., 1985). Likewise, glutamate is a precursor of proline (Ekiel et al., 1983). These labeling patterns are expected from the common biosynthetic pathways.

10.3.2 Carbohydrate Biosynthesis

Carbohydrate biosynthesis in methanogens proceeds from pyruvate via reactions commonly associated with gluconeogenesis (Figure 10.4). In addition to the requirement for pentose biosynthesis, carbohydrates are components of pseudomurein in the *Methanobacteriales*, glycoproteins in the envelopes of numerous

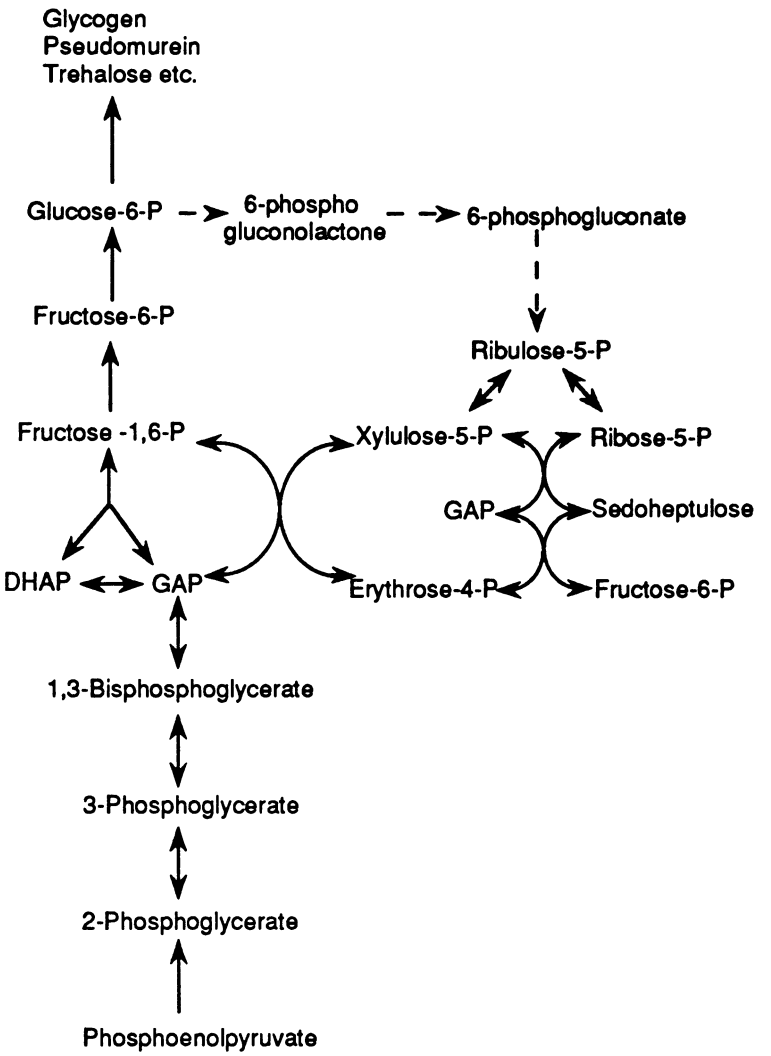

Figure 10.4. Pathway of hexose and pentose biosynthesis. In all methanogens examined, hexoses are probably formed by gluconeogenesis from phosphoenolpyruvate. In *Methanococcus* and *Methanobacterium*, pentoses are formed by transketolase and transaldolase (solid lines). In *Methanospirillum*, pentoses are formed by oxidation of glucose-6-phosphate (broken lines).

methanogens, the heteropolysaccharide in *Methanosarcina*, and the reserve polysaccharides glycogen and trehalose.

Equal amounts of radioactivity from 2-^{14}C pyruvate are incorporated into the C-2 of alanine and into the C-5 and C-2 of hexosamine by whole cells *M. thermoautotrophicum* and *Methanobrevibacter smithii*. This labeling pattern predicts a glycogenic pathway involving an aldolase mediated head to head condensation between two triose phosphates (Jansen et al., 1982). Short term $^{14}CO_2$ labelling studies in the same bacteria have supported this conclusion since radiolabel appears in triose phosphates before it is detected in hexoses (Jansen et al., 1982). In addition, the enzymes required for the pathway, phosphoenolpyruvate synthetase, enolase, phosphoglycerate mutase, phosphoglycerate kinase, glyceraldehyde 3-phosphate dehydrogenase, aldolase, and fructose-1,6-bisphosphate phosphatase have all been demonstrated in *M. thermoautotrophicum* (Jansen et al., 1982; Fuchs et al., 1983). However, the aldolase activity was only detected in the anabolic direction towards the synthesis of fructose-1,6-bisphosphate, suggesting that carbohydrate metabolism is unidirectional and that carbohydrate fermentation probably does not occur in this methanogen. Moreover, organisms which simultaneously perform glycolysis and gluconeogenesis typically regulate fructose-1,6-bisphosphate phosphatase both allosterically with AMP and by substrate inhibition with fructose-1,6-bisphosphate. Such regulation is not observed for the *Methanobacterium* enzyme. Likewise, the regulation of the phosphoenolpyruvate synthetase is consistent with the unidirectional biosynthesis of phosphoenolpyruvate from pyruvate (Eyzaguirre et al.,1982). In contrast to these results, incorporation of ^{13}C-glucose into triose phosphates by this bacterium suggests that these reactions are at least in isotopic equilibrium (Evans, Raleigh, et al., 1986).

The gluconeogenic enzymes have also been demonstrated in *Methanosaeta* (*Methanothrix*) and *M. maripaludis* (Pellerin et al., 1987; Shieh and Whitman, 1987; Yu and Whitman, unpublished data). For these methanogens, the pathway is very similar to that found for *Methanobacterium*. In addition, the genes for glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase have been cloned and sequenced from *Methanobacterium* spp. and *Methanothermus* (Fabry et al., 1989, 1990). Stable isotope incorporation into glucose in *M. hungatei* is also consistent with this pathway (Ekiel et al., 1983). However, in *M. maripaludis* both the catabolic activity of aldolase in the direction of triose formation as well as the anabolic activity are present (Yu and Whitman, unpublished data). In addition, low levels of pyruvate kinase and 6-phosphofructose kinase are detectible. Thus, the pathway is also glycolytic. This result is somewhat surprising because nonmethanogenic archaebacteria utilize variations of the Entner-Doudoroff pathway for glucose oxidation (Jones et al., 1987).

(c) Pentose Biosynthesis

In *M. hungatei*, the labeling of pentoses by stable isotopes is consistent with their formation by an oxidative decarboxylation of hexoses, presumably via the

oxidative pentose phosphate pathway (Figure 10.4; Ekiel et al., 1983). However, the enzymes of this pathway are not detectible in *M. maripaludis*, and pentoses appear to be formed from fructose bisphosphate and glyceraldehyde-3-phosphate by the transketolase and transaldolase reactions (Yu and Whitman, unpublished data). In *M. thermoautotrophicum*, the radiolabeling of pentoses with acetate is also most consistent with their biosynthesis via the transketolase and transaldolase reactions (Fuchs and Stupperich, 1980). Thus, two pathways of pentose biosynthesis have been identified in methanogens.

In *M. thermoautotrophicum*, the formation of deoxynucleotides by ribonucleotide reductase has also been examined. Like other bacteria, the nucleotide diphosphate is the substrate for the reduction (Sprengel and Follman, 1981). Nucleotide triphosphates are positive allosteric effectors (Hogenkamp et al., 1987). However, many properties of the methanogen enzyme are unique and distinguish it from the iron- and manganese-containing as well as the cobalamin-dependent ribonucleotide reductases characterized from eubacteria (Sprengel and Follman, 1981; Sze et al., 1992).

Cyclic 2,3-diphosphoglycerate (cDPG) is an abundant small molecule in the *Methanobacteriales* which is correlated with thermoadaptation (Hensel and König, 1988). The role of this compound is reviewed in Part II Chapter 11. In *M. fervidus*, the two enzymes required for cDPG biosynthesis have been purified and characterized (Lehmacher et al., 1990). The first, 2-phosphoglycerate kinase utilizes ATP to form 2,3-diphosphoglycerate. The second enzyme, cDPG synthetase, requires hydrolysis of one additional ATP for the cyclization of 2,3-diphosphoglycerate. In *M. thermoautotrophicum*, a third enzyme has been identified that hydrolyses cDPG to 2,3-diphosphoglycerate (Sastry et al., 1992). In extracts of *M. thermoautotrophicum*, 2,3-diphosphoglycerate is metabolized to phosphoenolpyruvate via 3-phosphoglycerate and 2-phosphoglycerate by the combined action of a phosphatase and mutase (van Alebeek et al., 1991).

10.3.3 Purine and Pyrimidine Biosynthesis

In *M. hungatei*, the pathways of purine and pyrimidine biosynthesis have been examined by ^{13}C-labeling studies (Ekiel et al., 1983). The labeling of the pyrimidines is consistent with their biosynthesis from aspartate by the usual pathway. Similar results have been obtained in radiotracer studies with *M. thermoautotrophicum* (Holder et al., 1985). For purines, glycine is a precursor of C-5 and C-6 in *M. hungatei*, as expected for the usual pathway (Ekiel et al., 1983). However C-2 and C-8 are obtained from the C-2 of acetate. These carbons are obtained from the tetrahydrofolate pathway in eubacteria. In *M. hungatei*, the tetrahydromethanopterin pathway could be labeled by the C-2 of acetate via serine and serine transhydroxymethylase. However, this label should be rapidly diluted during CO_2 reduction to CH_4. Because it was not, more than one pool of C-1 intermediates may exist. Similar results have been obtained in *M. thermoautotro-*

phicum where [^{14}C]formate is incorporated specifically into purines and not into methane (Taylor et al., 1976). The 5′-phosphoribosyl-5-aminoimidazole carboxylase gene of the purine biosynthetic pathway has also been cloned from *M. thermoautotrophicum* and *Methanobrevibacter smithii* (Hamilton and Reeve, 1985a,b). This result also confirms the presence of the usual pathway in methanogens.

The salvage pathway and purine metabolism is described elsewhere in this volume and is not discussed here (Part III, Chapter 11).

10.3.4 Lipid Biosynthesis

The membrane polar lipids of methanogenic bacteria consist of a diverse collection of diphytanyl glycerol diethers and dibiphytanyl diglycerol tetraethers. While this high degree of diversity is beyond the bounds of this discussion, (for reviews see Langworthy, 1985; De Rosa et al., 1986), the presence of isoprenoid hydrocarbons as a major component of this lipid suggests that a common biosynthetic pathway exists for this constituent (De Rosa et al., 1986; Jones et al., 1987). Moreover, the ubiquity of isoprenoid hydrocarbons throughout the three domains of life indicates that one universal biosynthetic pathway exists for all organisms (Jones et al., 1987).

In eucaryotes and eubacteria isoprenoid biosynthesis typically proceeds through the mevalonate pathway, and studies upon nonmethanogenic archaebacteria clearly show the same route of biosynthesis. It is, therefore, not unreasonable to expect this path to be present in the methanogenic branch of the archaeobacteria (Kate et al., 1968; De Rosa et al., 1977; Langworthy et al., 1985). The enrichment patterns of diphytanyl glycerol diethers and bidiphytanyl diglycerol tetraethers purified from *M. hungatei* and *Methanosaeta concilli* following growth on ^{13}C-enriched acetate are in agreement with this expectation (Ekiel et al., 1983; Ekiel and Sprott, 1985). The labeling is fully consistent with the condensation of three acetate molecules to form one mevalonate molecule as an intermediate of isoprenoid synthesis, and an identical labeling pattern was seen with the thermoacidophilic archaebacterium *Calderiella* (De Rosa et al., 1986).

The C_5 isopentenyl skeleton is the product of the mevalonate pathway, and higher isoprenoids, such as C_{10} (geranyl), C_{15} (farnesyl), and C_{20}(geranyl-geranyl), are the products of condensations between isopentenyl skeletons. Even more complex isoprenoids are synthesized through reactions unique to the archaebacteria and may be described as head to head, tail to tail, and head to tail condensations. Examples of these syntheses include the tail to tail condensation of farnesyl units to produce squalene (C_{30}); C_{25}, abundant in methanogens, is produced by head to tail condensations of C_{20} and C_5 moieties or via tail to tail condensations of C_{15} and C_{10} moieties (Langworthy, 1985).

10.4 Polymer Biosynthesis

10.4.1 Glycogen Biosynthesis and Catabolism

Glycogen is a widespread reserve polysaccharide in methanogens and has been reported in *Methanolobus*, *Methanococcus*, *Methanosarcina*, and *Methanosaeta* (*Methanothrix*) (König et al., 1985; Murray and Zinder, 1984; Pellerin et al., 1987; Ratner et al., 1989). Glycogen is distributed within the cytoplasm as densely packed particles and, in the case of *Methanosaeta* and *Methanosarcina thermophila*, can represent up to 2% of the total dry weight of the cell (Murray and Zinder, 1984; Pellerin et al., 1987). In *Methanosaeta*, UDP-glucose is the preferred substrate for the glycosyl transferase activity associated with glycogen biosynthesis in cell extracts (Pellerin et al., 1987). In this respect, the activity resembles the eubacterial and not the *Sulfolobus* enzyme. In addition, the same cell extracts contain branching enzyme activity, which may also play a role in glycogen biosynthesis (Pellerin et al., 1987).

There has been some speculation on the physiological purpose of the reserve polysaccharide. In *Methanolobus* and *Methanococcus*, the glycogen content of cells increases under nitrogen-limiting conditions and, after transfer to a medium devoid of any carbon and energy source, the intracellular glycogen levels decrease (König et al., 1985). Methanogenesis is concomitant with the decrease in glycogen, suggesting that this polymer may be a substrate for methanogens during starvation (König et al., 1985). Similarly, *M. thermophila* degrades glycogen when shifted to different methanogenic substrates (Murray and Zinder, 1987). This methanogen makes the transition from acetate to methanol utilization more readily when intracellular glycogen granules are present. When glycogen is present, the intracellular level of ATP is elevated at the onset of methanol utilization compared to cells devoid of glycogen. Thus, by mobilizing its reserve of glycogen, the energy needed for the biosynthesis of proteins required for methanol utilization can be produced (Murray and Zinder, 1987).

10.4.2 Biosynthesis of Cellular Wall Components

Methanogens display considerable diversity in the constituents of their cell envelopes, which can be grouped into three categories; pseudomurein, glycoprotein or protein layers, and heteropolysaccharides (Jones et al., 1987).

Pseudomurein is common only among the *Methanobacteriales* and is analogous in structure and function to eubacterial murein (Kandler and König, 1985). However, close inspection of pseudomurein reveals significant differences between it and eubacterial peptidoglycan. In pseudomurein, L-talosaminouronic acid replaces muramic acid found in murein, the peptide bridges between the glycan strands have a different amino acid sequence, and the peptide bridges do

not contain D-amino acids (König and Kandler, 1979). Moreover, the secondary structure of pseudomurein and eubacterial peptidoglycan is very different (Leps et al., 1984).

Even though the structure of pseudomurein is significantly different from murein, it is not possible to decide whether or not pseudomurein is homologous to the eubacterial peptidoglycans based upon this information alone. Because eubacterial peptidoglycans are also diverse, pseudomurein could be an extreme 'variation on a theme' so often encountered in microbial physiology. Alternatively, the similarities of the methanogen and eubacterial peptidoglycans could be an example of convergent evolution.

Because the biosynthetic pathway for pseudomurein is significantly different from eubacterial peptidoglycan, it is unlikely that these polymers have a common origin (Hartmann and König, 1990b). In eubacterial peptidoglycan biosynthesis, *N*-acetylmuramic acid is biosynthesized from UDP-*N*-acetylglucosamine early in the pathway, and the pentapeptidyl side chain is biosynthesized by the sequential addition of amino acids to UDP-*N*-acetylmuramic acid. The UDP-*N*-acetylmuramic acid-pentapeptide is then transferred to the isoprenoid carrier lipid undecaprenol pyrophosphate. The completed monomer composed of the disaccharide and the pentapeptide is formed by the addition of *N*-acetylglucosamine from UDP-*N*-acetylglucosamine to the lipid precursor. In *Methanobacterium thermoautotrophicum*, the UDP-disaccharide of *N*-acetylglucosamine and *N*-acetyltalosaminuronic acid is readily detected (König et al., 1989). Because UDP-*N*-acetyltalosaminuronic acid is not detected and UDP-*N*-acetylgalactosamine is abundant, *N*-acetyltalosaminuronic acid is probably formed from the UDP-disaccharide of *N*-acetylglucosamine and *N*-acetylgalactosamine. Therefore, the distinctive sugar derivative of pseudomurein is formed at a late stage of biosynthesis, and disaccharide formation occurs prior to addition of the peptide side chain and transfer to the lipid carrier. Because UDP-glutamate and UDP-pentapeptide are also detected, the biosynthesis of the pentapeptide side chain probably occurs on UDP prior to transfer to the UDP-disaccharide (Hartman et al., 1990). Other intermediates detected include the UDP-disaccharide-pentapeptide and its derivatives of the lipid carrier undecaprenol monophosphate (Hartmann et al., 1989; Hartmann and König, 1990a). Finally, because alanine specifically accumulates in the medium during growth, a transpeptidation reaction between pentapeptidyl side chains that would release the C-terminal alanine may be involved in crosslinking of the glycan strands (Schonheit and Thauer, 1980).

M. barkeri produces an extracellular wall component that is structurally similar to chondroitin sulfate. Named methanochondroitin, it is a polymer composed of a trisaccharide repeating unit, [→4)-β-D-GlcA-(1→3)-β-D-GalNAc-(1→3 or 4)-β-D-GalNAc-(1→]$_n$ (Kreisl and Kandler, 1986). After extraction of whole cells, five potential intermediates were identified (Hartmann and König, 1991). These included a UDP-disaccharide, UDP-trisaccharide, and a undecaprenyl pyrophos-

phate-trisaccharide. Thus, methanochondroitin biosynthesis appears to require formation of the UDP-activated oligosaccharide prior to transfer to the lipid carrier undecapronyl pyrophosphate (Hartmann and König, 1991). This pathway resembles pseudomurein biosynthesis and is quite different from the pathways of chondroitin sulfate or eubacterial extracellular polymer biosynthesis.

A complex pathway of glycoprotein biosynthesis has also been partly elucidated in *Methanothermus fervidus* (Hartmann and König, 1989). Like pseudomurein and methanochondroitin, UDP-activated oligosaccharides are intermediates. After the completion of oligosaccharide biosynthesis on UDP, it is transferred to the lipid carrier, which is dolichyl-phosphate. Dolichol is also the lipid carrier in eucaryotic glycoprotein biosynthesis. However, in eucaryotes the oligosaccharide is biosynthesized at the lipid carrier stage. Interestingly, *M. fervidus* also contains undecaprenol, which probably functions in pseudomurein biosynthesis.

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11

Nitrogen and Phosphorus Metabolism of Methanogens

Edward DeMoll

11.1 Nitrogen Metabolism

11.1.1 Acquisition of NH_4^+ from Sources Other Than N_2

Methanogens as a group are able to use a variety of compounds as sources of nitrogen, however individual species are relatively restricted in their choices. All methanogens may use NH_4^+, many will fix nitrogen if deprived of NH_4^+, some can deaminate amino acids, some hydrolyze urea, others metabolize methylamines, and some degrade purines or pyrimidines. However, to date no single methanogen has been shown to possess all of these capabilities. Figure 11.1 is a summary of nitrogen metabolism in methanogens based on both unpublished and published experiments.

Methanogens use nitrogen in the biosynthesis of amino acids, purines, pyrimidines, and other basic biochemicals by employing most of the same reactions that are used by eubacteria. These topics are discussed in Part III, Chapters 9 and 10. Methanogens also use nitrogen in the synthesis of polyamines. The distribution of polyamines is variable and appears to be quite specific for each family (Scherer and Kneifel, 1983). The *Methanosarcinaceae* contain putrescine and symhomospermidine, the *Methanomicrobiaceae* have putrescine, spermidine, and symhomospermidine, the *Methanococcaceae* have putrescine and spermidine. The *Methanobacteriaceae* were found to have very low levels of polyamines (Scherer and Kneifel, 1983).

Methanogens also synthesize several nitrogen containing osmolytes which are used to support growth in environments with high salinity. The type synthesized is dependent on the salinity of the medium (Robertson and Roberts, 1991).

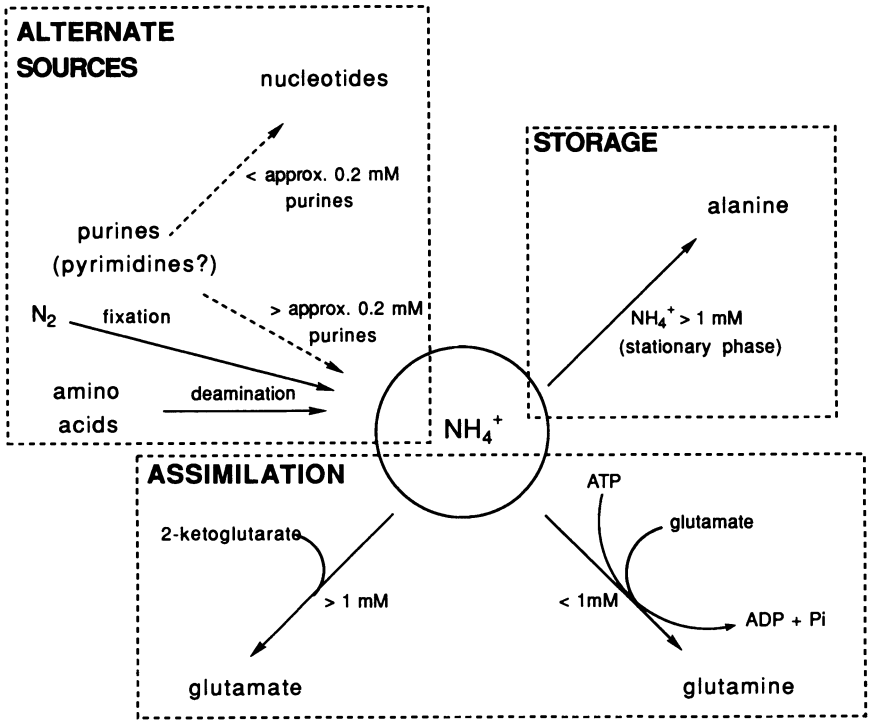

Figure 11.1. Nitrogen metabolism in methanogens. > 1 mM and < 1 mM refer to concentrations of NH_4^+.

Osmolytes which have been isolated include betaine, α- and β-glutamate, N^{ε}-acetyl-β-lysine, and β-glutamine (Robertson and Roberts, 1991). Although betaine was isolated from several families, in the case of *Methanogenium cariaci*, betaine does not appear to be synthesized. The organism instead accumulates betaine through uptake from the medium (Robertson et al., 1990). Some extreme halophiles, which grow in media where the sodium chloride concentration is above approximately 1.7 M, synthesize betaine (M.-C. Lai and R. P. Gunsalus, unpublished results). Apparently, all other osmolytes are synthesized by methanogens. N^{ε}-acetyl-β-lysine appears to be synthesized when the external medium reaches high (greater than 6%) NaCl concentrations (Robertson and Roberts, 1991). The routes of biosynthesis for these compounds are not known at the present time.

Ammonia assimilation by methanogens is similar to that seen in eubacteria. At levels of NH_4^+ above 1–2 mM the main mechanism of assimilation is the glutamate dehydrogenase-catalyzed synthesis of glutamate from NH_4^+ and 2-

ketoglutarate. Below 1–2 mM the active pathway uses glutamine synthetase to catalyze the ATP-dependent synthesis of glutamine from NH_4^+ and glutamate. This was first demonstrated in *Methanobacterium thermoautotrophicum* (Kenealy et al., 1982) by assay of cell extracts, and has been confirmed by isotopic labeling studies (Choi et al., 1986). Glutamine synthetase from *Methanobacterium ivanovi* has been purified and characterized (Bhatnagar et al., 1986). The enzyme is a dodecamer of subunits of approximately 50 kDa, as are most eubacterial enzymes. However, unlike some eubacterial glutamine synthetases, the enzyme is not regulated by adenylylation (Bhatnagar et al., 1986). This property is one shared by gram positive bacteria (Tronick et al., 1973) and *Bacteroides fragilis* (Hill et al., 1989). The glutamine synthetase gene from *Methanococcus voltae* has been cloned and sequenced (Possot et al., 1989). This enzyme has a predicted subunit size of 50 kDa. When the nucleotide sequence of the *M. voltae glnA* gene is compared to other known bacterial *glnA* genes, the highest similarity is to that of the gram positive organisms, *Bacillus subtilis* and *Clostridium acetobutylicum*. From the predicted amino acid sequence it may be seen that glutamine synthetase from *M. voltae* does not have the tyrosine residue that is adenylated in some eubacteria. These results are consistent with those mentioned previously with glutamine synthetase from *M. ivanovi* (Bhatnagar et al., 1986), and indicate that the methanogen glutamine synthetase is more similar in sequence and function to the gram positive eubacterial enzyme than it is to the *Escherichia coli* enzyme. At present, one can only speculate as to whether this close similarity is due to some past gene transfer between anaerobic methanogens (or some progenitor) and Gram positive anaerobes that shared the same environmental niche, to anaerobic environmental pressures, or to the divergence of the Gram negative glutamine synthetase gene.

The methanogen glutamine synthetase is repressed under conditions where there is sufficient NH_4^+ (Bhatnagar et al., 1986; Possot et al., 1989), thus saving the one **ATP** that is spent in the reaction catalyzed. Under conditions of NH_4^+ sufficency, and when cells are near stationary phase, NH_4^+ is directed into alanine dehydrogenase-catalyzed alanine synthesis, and alanine begins to accumulate (Choi, et al., 1986; Kenealy, et al., 1982; Roberts, et al., 1990). Arginine catabolism also becomes active in near stationary phase cells of *M. thermoautotrophicum*. The catabolism of arginine begins with the deamination of arginine, thus producing 2-ketoarginine (Choi et al., 1986). These results are consistent with results we have obtained with *Methanococcus vannielii* where we have shown that alanine and arginine may both provide nitrogen for growth (Auffenberg and DeMoll, unpublished results).

To date *M. vannielii* is the only methanogen reported to be able to utilize purines or pyrimidines as its sole nitrogen source (DeMoll and Tsai, 1986a). However, there is at least one report of purine degradation by *M. voltae* (Bowen and Whitman, 1987). A 6-mercaptopurine resistant mutant of *M. voltae* was

found to be able to degrade this hypoxanthine analog. This ability was detected by the disappearance of the insoluble 6-mercaptopurine around colonies of 6-mercaptopurine resistant mutants. This observation implies that *M. voltae* contains xanthine dehydrogenase, and xanthine amidohydrolase, at least if an analogy to the *M. vannielii* or the anaerobic eubacterial systems is valid. Xanthine dehydrogenase would catalyze the oxidation of 6-mercaptopurine to 6-mercaptoxanthine, and xanthine amidohydrolase would catalyze the hydrolysis of 6-mercaptoxanthine by the introduction of a water molecule between the 1-nitrogen and 6-carbon, thus producing the comparatively water soluble 4-ureido-5-imidazolecarboxylic acid or its sulfur containing analog. If however, *M. voltae* also has the next enzyme in the purine degrading pathway, 4-ureidoimidazole-5-carboxylic acid amidohydrolase, then the organism might be able to use purines as a source of nitrogen, since that enzyme catalyzes the release of one mole of NH_4^+. However, additional experiments indicate that *M. voltae* does not degrade purines to a large extent (see below). In the previously mentioned study of purine utilization by *M. voltae* (Bowen and Whitman, 1987), the organism was grown in the presence of either 0.1 mM adenine, guanine, or hypoxanthine. When *M. vannielii* was grown in the presence of 0.2 mM guanine and 19 mM NH_4^+, guanine degradation was not conspicuous either. Most of the exogenous guanine was incorporated directly into GTP (Auffenberg and DeMoll, unpublished results), as was reported for *M. voltae* (Bowen and Whitman, 1987) and *M. thermoautotrophicum* (Jaenchen et al., 1984; Worrell and Nagle, 1990) grown under similar conditions. This level of purine is approximately enough to just satisfy the cellular purine requirement, so it would be very inefficient for the methanogen to degrade, for example, guanine, only to have to resynthesize it. This is especially true when the medium contains substantial NH_4^+, as it did in those studies. Purine degradation in *M. vannielii* was demonstrated by assaying for the enzymes of degradation and by showing that purines could serve as sole nitrogen source when supplied at 4–5 mM concentrations (DeMoll and Tsai, 1986a).

The fact that a mutant of *M. voltae* degrades 6-mercaptopurine quite well hints of the possibility that the organism does indeed degrade purines. However, when grown in the presence of purines under conditions reported for purine degradation in *M. vannielii*, *M. voltae* apparently did not degrade purines to a large enough extent so that they could serve as sole nitrogen source for growth (W. B. Whitman, personal communication).

In studies of the biosynthesis of coenzyme F_{420} in *M. thermoautotrophicum*, guanine with various isotopic labels was added to cultures (Jaenchen et al., 1984). In all cases, a significant amount of nonspecific labeling was detected. Although it was not considered in the report, this might be accounted for by degradation of the added guanine.

M. vannielii apparently only degrades purines when they are present in amounts

greater than necessary to accomodate the cellular purine requirement (DeMoll and Tsai, 1986a). At low (approximately 200 μM or lower) purine concentrations *M. vannielii* simply incorporates the purines into guanine and adenine nucleotides. Under these conditions there also is almost no interconversion of adenine and guanine nucleotides (Auffenberg and DeMoll, unpublished results). However, when the purine or pyrimidine levels are high enough, in the low millimolar range, this methanogen may use the purines guanine, xanthine, hypoxanthine, or uric acid, but not adenine, and the pyrimidines, uracil or thymine, but not cytosine, as sole nitrogen source (DeMoll and Tsai, 1986a). Xanthine is the purine which is degraded. All of the other purines which are eventually degraded are first converted to xanthine (DeMoll and Tsai, 1986b). Although the pyrimidine degrading system has not been fully characterized, unpublished experiments indicate that it is at least similar to the anaerobic pathway of pyrimidine degradation characterized in other organisms (Vogels and van der Drift, 1976). Evidently *M. vannielii* cannot deaminate either adenine or cytosine, and therefore these compounds cannot be metabolized to xanthine in the first case, or to uracil in the latter. The same enzyme might be catalyzing the degradation of uracil, thymine, and xanthine, since the architecture of the molecules at the site of ring cleavage and the mechanisms of ring cleavage are the same with both purines and pyrimidines. This is also consistent with the observation that adenine and cytosine are not degraded, since those compounds have identical structures around the would-be site of ring cleavage.

Before purines may be degraded, they must be converted to xanthine. The reversible reduction of uric acid to xanthine and then to hypoxanthine is catalyzed by xanthine dehydrogenase. The enzyme from *M. vannielii* has a higher specific activity in cells grown with uric acid as sole nitrogen source than in cells grown with NH_4^+. This implies that uric acid, which is excreted by birds and bony fish, is a likely source of nitrogen for *M. vannielii* in the environment. The conversion of guanine to xanthine and NH_4^+ is catalyzed by guanine deaminase.

Except for xanthine dehydrogenase, all of the enzymes involved in purine degradation by *M. vannielii* were found in cells grown with NH_4^+ as sole nitrogen source. Either they are constitutively expressed, or they are induced by low levels of purines released as a result of cell lysis during growth.

M. thermoautotrophicum may use urea as its sole nitrogen source (Bhatnagar, et al., 1984; Choi and Roberts, 1987; Kenealy, et al., 1982). The enzyme that catalyzes the decomposition of urea to NH_4^+ and CO_2 has not been purified. Ordinarily it would be assumed that this would be accomplished by urease, however if *M. thermoautotrophicum* has purine degrading enzymes, this reaction might be catalyzed by 4-ureido-5-imidazolecarboxylic acid amidohydrolase, which catalyzes an analogous reaction. Although this enzyme has not been well characterized, it is known that the enzymes from *Clostridium cylindrosporum* (Rabinowitz and Pricer, 1956) and from *M. vannielii* (Auffenberg and DeMoll,

unpublished data) both require either Mn^{2+} or Fe^{2+} for activity. Urease contains Ni^{2+}, so it is evident that hydrolysis of the ureido moiety in either urease or in the purine dedegradation product, 4-ureido-5-imidazolecarboxylic acid, requires a divalent cation. Whether or not urea is a substrate for the methanogen purine degrading enzyme has not been tested.

Certain amino acids may also serve as sole nitrogen sources for growth of methanogens. Alanine has been reported to be used by *M. voltae* (Whitman et al., 1987). *M. vannielii* can also use alanine to supply its nitrogen requirement. Several other amino acids substantially spare NH_4^+ for growth of *M. vannielii*. However, when supplied as the sole nitrogen source without added NH_4^+, individual amino acids allow only slow or poor growth. Of those other amino acids, arginine is the best nitrogen source. The highest levels of growth obtained in the absence of NH_4^+ are obtained when a mixture of 16 amino acids is included in the medium. (Auffenberg and DeMoll, unpublished results). This decrease in the growth rate, seen when single amino acids are the sole nitrogen source, is not matched by a comparable decrease in methanogenesis (Auffenberg and DeMoll, unpublished results). The transaminases responsible for generating NH_4^+ from free amino acids in *M. vannielii* have not been studied. However, four amino transferases from *M. aeolicus* have been identified (Xing and Whitman, 1992).

11.1.2 Nitrogen Fixation

Nitrogen fixation in methanogens has recently been extensively reviewed by Lobo and Zinder (Lobo and Zinder, 1992), to which the reader is referred for a more detailed discussion of the topic.

The first report of nitrogen fixation by a methanogen was by Pine and Barker (1954) for *Methanobacterium omelianskii*. However, since this "organism" was later shown to be a mixture of two different species of bacteria, the report of nitrogen fixation was discounted. Ironically, Belay et al. (1988) have since shown that the methanogenic component of *M. omelianskii*, *Methanobacterium bryantii* strain M.o.H., does indeed carry out nitrogen fixation.

Thirty years after the report of nitrogen fixation by *M. omelianskii*, the phenomenon of nitrogen fixation in methanogens was independently discovered by several different groups. The ability of *Methanosarcina barkeri* strain 227 to use N_2 as a nitrogen source was suspected by Murray and Zinder (1984) when they noted that the expected growth limitation did not occur in cultures containing low levels of NH_4^+. They demonstrated that *M. barkeri* 227 does indeed fix nitrogen. This was accomplished by following the incorporation of $^{15}N_2$ into total cellular nitrogen. They found that $^{15}N_2$ assimilation could be effectively eliminated by addition of 20 mM NH_4^+. In a similar instance Belay, Sparling, and Daniels (1984) noticed that control cultures of *Methanococcus thermolithotrophicus* grew in the absence of NH_4^+. They provided evidence that this growth was due to

nitrogen fixation by showing that growth of the organism could take place in the absence of NH_4^+, but with N_2 supplied to the cultures. However, if N_2 was replaced with Ar, no growth was seen. Further experiments showed that cultures of *M. thermolithotrophicus* could reduce acetylene to ethylene at a level of acetylene (0.023 μmol per ml gas phase) which does not appreciably inhibit methanogenesis.

Bomar, Knoll, and Widdel (1985) discovered that a culture they were enriching for nitrogen fixing organisms contained *M. barkeri* strain Fusaro as its principal component. They then demonstrated that in a pure culture this strain of *M. barkeri* can incorporate $^{15}N_2$ into cellular nitrogen, that the organism can grow with N_2 as its sole nitrogen source, and that cultures are capable of reducing acetylene to ethylene, thus showing that *M. barkeri* Fusaro does indeed fix nitrogen.

The *nifHDK* genes encode the three polypeptides of nitrogenase. Sibold et al. (1985) demonstrated with Southern blotting experiments that DNA from these genes, isolated from both *Klebsiella pneumoniae* and *Anabaena* strain 7120, hybridizes to DNA of *M. ivanovi*, *M. thermoautotrophicum*, *M. voltae*, and *M. barkeri*. The extent of hybridization is greatest for *nifH* with DNA from all of the methanogens tested. Hybridizations to *nifD* or *nifK* were detectable only under conditions of low stringency (10% formamide) and only with *M. voltae* and *M. ivanovi* DNA.

Since these initial reports, nitrogen fixation has also been reported to be carried out by *Methanosarcina acetivorans* (Lobo and Zinder, 1992), *M. thermoautotrophicum* strain Marburg (Fardeau et al., 1987), *M. ivanovi* (Magot et al., 1986), *Methanolobus tindarius* (König et al., 1985) and *Methanobacterium formicicum* (Magingo and Stumm, 1991). It should be noted that in the instance cited above, *M. thermoautotrophicum* was grown in a chemostat. There is at least one report that this methanogen does not grow with N_2 as sole nitrogen source in batch cultures (Lobo and Zinder, 1992).

(c) Genetics and Regulation of Nitrogen Fixation

There are two regions of *nifH* sequence similarity found in each of the *M. ivanovi*, *M. thermolithotrophicus* and *M. barkeri* genomes. However, at least in *M. thermolithotrophicus*, only one of the genes, designated *nifH1*, is expressed under conditions which induce nitrogen fixation (Sibold et al., 1985). The other, *nifH2*, is not. Also found in the region which contains the *nifH1* gene of *M. thermolithotrophicus*, are *nifD* and *nifK* plus two genes which encode two different, but related polypeptides, each with approximately 50% deduced amino acid sequence identity or similarity to the *glnB* gene product, P_{II}, of some eubacteria. These two *glnB*-like genes are are also found in *M. ivanovi* and near both *nifH* loci in *M. barkeri* 227 (Sibold et al., 1991). The *nifD* gene was also found on the genomes of these organisms. To date not enough of the nitrogen fixing locus

of *M. ivanovi*, nor enough of that of *M. barkeri* 227 has been sequenced to be able to determine whether both have *nifK*, although it is assumed they must. For the same reason, it is not known whether both or just one *nif* loci of *M. barkeri* 227 has *nifD*. The nitrogen fixing genes in *M. thermolithotrophicus* are transcribed in the order *nifH1*, *glnBa*, *glnBb*, *nifD* and *nifK*. The two genes which encode the P_{II}-like proteins are designated *glnB*a and *glnB*b. The termination codon of *nifD* ends eight nucleotides into the start of the coding sequence for *nifK*. From what is known about *M. barkeri* 227 and *M. ivanovi*, it is likely that they will have a similar genetic arrangement for the *nif* genes. There is a typical archaebacterial promoter upstream of *nifH1*.

As mentioned previously, methanogens do not appear to regulate glutamine synthetase by covalent modification catalyzed by P_{II}, as do most eubacteria. However, based upon the findings of Sibold and his colleagues, one may easily imagine that methanogens do use a P_{II}-like protein to regulate a different aspect of nitrogen metabolism, nitrogen fixation. Since P_{II} is tetrameric, Sibold et al. (1991) speculated that the two different P_{II}-like polypeptides of methanogens possibly arose through a gene duplication, and that they might form an $\alpha_2\beta_2$ tetramer, similar to the eubacterial homotetramer. Furthermore, this protein would be expected to have activity toward nitrogenase in much the same way the eubacterial P_{II} has for glutamine synthetase.

It is clear that nitrogen fixation in methanogens is tightly regulated. This is not surprising in light of the high energy cost required to reduce N_2 (Lobo and Zinder, 1988). Those investigators also showed that 5 mM NH_4^+ halts the ability of cell suspensions of *M. barkeri* 227 to carry out acetylene reduction. Later they demonstrated that even 10 μM NH_4^+ causes transient loss of this acetylene reducing ability. This loss of nitrogenase activity is not due to loss of the protein, since antiserum to component 2 still detects the protein three hours after addition of 5 mM NH_4^+ to growing cultures. The stained band is seen at approximately the same intensity as before addition of NH_4^+.

It remains to be proven how this regulation is affected. Do the *glnB*-like genes give rise to a P_{II}-like protein, which in turn ADP-ribosylates one of the components of nitrogenase? If so, how is the activity or expression of this protein regulated?

(b) Enzymology of Nitrogenase

Nitrogen fixation in methanogens is catalyzed by a nitrogenase that appears similar in activity and structure to those seen in eubacteria. The eubacterial nitrogenase component 1 is a tetramer of two subunits each of 55 and 60 kDa, while component 2 exists in dimeric form and is componsed of a single polypeptide of 30–35 kDa (Postgate, 1982). Magot et al. (1986) found on SDS-polyacrylamide gels proteins that were only present in N_2 grown cells. The proteins from *M. thermolithotrophicus* are of 29 and 55 kDa while those from *M. ivanovi* are

of 38, 54, and 56 kDa. Immunological studies showed that the 29 and 38 kDa and an additional 32 kDa protein from *M. ivanovi* cross react with antiserum to component 2 of *Azotobacter vinelandii*.

Nitrogenase has been partially purified from *M. barkeri* 227 (Lobo and Zinder, 1990). The protein analogous to eubacterial component 1 has two subunits of 55 and 62 kDa which exist in the active form in an $\alpha_2\beta_2$ conformation. The component 2 homolog, which does react with antiserum to component 2 of *A. vinelandii*, apparently exists as a tetramer, rather than a dimer, of a single polypeptide of 31 kDa (Lobo and Zinder, 1990).

The *nifH1* of *M. thermolithotrophicus* has a significant level of sequence similarity to the *nif* genes of eubacteria. However, it is also more closely related to the eubacterial *nifH* genes than it is to *nifH* from *M. voltae* or *M. ivanovi*. Moreover, it is even more dissimilar to *nifH2* from its own genome (Lobo and Zinder, 1992). Also, the *nifH1* gene of *M. thermolithotrophicus* appears more similar genetically to the gene which encodes the second alternative, iron-containing, eubacterial nitrogenases than to the molybdenum-containing eubacterial nitrogenase (S. H. Zinder, personal communication based on the data from Sibold et al., 1991). Lobo and Zinder (1990) have found biochemical similarities such as poor stability, low specific activity, and preference for N_2 over acetylene. However, the protein from *M. barkeri* 227 apparently requires Mo for activity, since addition of 100 μM WO_4^{2-}, an analog of MoO_4^{2-} inhibits growth of N_2-grown cells, while it has no visible effect on ammonia-grown cells (Lobo and Zinder, 1988). Eubacterial nitrogenase catalyzes the ATP-dependent production of hydrogen. The enzyme from *M. barkeri* 227 has been tested for this ability but the results were inconclusive (S. H. Zinder, personal communication).

The ability to convert N_2 to NH_4^+ is widespread and possibly ancient among bacteria. That the ability is widespread is not in dispute. Proof of an ancient origin for nitrogen fixation is often based on DNA sequence data, but while these comparisons are consistent with an ancient origin, they cannot take into account lateral transfer of genetic material (Lobo and Zinder, 1992).

11.2 Phosphorus Metabolism

Methanogens obtain their phosphorus, as do most living organisms, in the form of inorganic phosphate. Most of the details of phosphate metabolism, such as in nucleotide synthesis, are also identical to those seen in other organisms. There are, however, several aspects of phosphate metabolism in methanogens, which are either unique or that bear emphasis.

The most abundant form of phosphate in *M. thermoautotrophicum* is cyclic 2,3-diphosphoglycerate (cDPG). This compound was first reported by Seely and Fahrney (1983) and Kanodia and Roberts (1983). When grown in batch cultures

under normal conditions, cDPG is found intracellularly at levels as high as 100–200 mM (M. F. Roberts, personal communication).

It was once thought that this compound is found in pseudomurein-containing organisms of the genera *Methanobacterium*, *Methanothermus* and *Methanobrevibacter* but not *Methanococcus*, *Methanospirillum*, or *Methanosarcina* (Tolman, et al., 1986). Recently, however, cDPG has been found in *Methanosarcina frisia* (Rudnick, et al., 1990). *Methanosphaera stadtmanae* also contains cDPG, but it is found in comparatively small amounts (Sastry et al., 1992).

Since its discovery, a definitive role for cDPG in the cell has been the subject of much speculation. It has been proposed that this compound is involved in energy metabolism (Kanodia and Roberts, 1983), thermostabilization (Hensel and König, 1988), in lipid synthesis (Seely and Fahrney, 1984), in phosphate storage (Krueger et al., 1986; Seely and Fahrney, 1984), or as a counterion to high intracellular cation concentrations; however, it seems most likely that the primary role is in carbohydrate metabolism (Evans et al., 1985; Lehmacher et al., 1990; Sastry et al., 1992; Tolman et al., 1986), probably as a storage compound. This hypothesis is based on several observations. First, polyphosphates, which are known to be phosphorus storage compounds, are found together with cDPG in *M. frisia* (Rudnick et al., 1990). Also, *M. thermoautotrophicum* still makes cDPG under phosphate limiting conditions (Krueger et al., 1986), so it is unlikely that the compound is involved in phosphate storage. However, one could make the suggestion that by sequestering inorganic phosphate into cDPG, the chemical gradient of phosphate inside the cell versus phosphate outside the cell is more favorable toward accumulation under conditions of low external phosphate. In unpublished experiments (M. F. Roberts, personal communication) it has been demonstrated that *M. thermoautotrophicum* increases cDPG only from 0.12 to 0.2 M over a ten-fold increase in external sodium chloride from 0.0.04 to 0.4 M. This, coupled with the rapid turnover of labelled cDPG pools (Evans et al., 1985; Rabinowitz and Pricer, 1956; Rühlemann et al., 1985), makes it seem unlikely that cDPG is acting solely as an osmolyte.

Arguments in favor of cDPG being primarily a carbohydrate storage compound begin with an examination of work done with *M. thermoautotrophicum* and *Methanothermus fervidus*, which shows that there is a well regulated system for control of synthesis and breakdown of this compound (Lehmacher et al., 1990; Sastry et al., 1992; M. F. Roberts, unpublished results). Also, 2-phosphoglycerate (2PG), the compound from which the pathway to cDPG branches off, occurs relatively early in the pathway of carbon assimilation in *M. thermoautotrophicum* (Rühlemann et al., 1985), so carbon flow may be easily directed from cDPG into most precursor molecules should the need arise. Finally, as one would expect of a carbohydrate storage compound, the compound is somewhat metabolically sequestered from the mainstream of carbon flow.

It is also perhaps relevant that none of the cells which have been shown to

accumulate glycogen (König et al. 1985), another carbohydrate storage compound, have been demonstrated to contain cDPG.

Figure 11.2 summarizes what is known about methanogen metabolism related to cDPG synthesis and breakdown. This scheme is based on work with *M. fervidus* and *M. thermoautotrophicum*. Lehmacher et al. (1990) have purified two enzymes from *M. fervidus* which are required for the biosynthesis of cDPG. The first, 2-phosphoglycerate kinase (2PGK), catalyzes the formation of 2,3-diphosphoglycerate (2,3-DPG) from 2PG at the expense of one mole of ATP. The second, cyclic 2,3-diphosphoglycerate synthetase, then catalyzes the synthesis of cDPG from 2,3-DPG also with the hydrolysis of one mole of ATP to ADP and P_i. A third enzyme of approximately 33 kDa, which specifically catalyzes the degradation of cDPG to 3-phosphoglycerate (3PG), has been purified from *M. thermoautotrophicum* (Sastry, et al., 1992) and partially characterized (Sastry et al., 1992, M. F. Roberts, unpublished results). The hydrolysis of cDPG, catalyzed by this enzyme, is inhibited by the product of the reaction, 2,3-DPG. Also, it has recently been reported that this enzyme is regulated by phosphorylation (M. F. Roberts, unpublished results). This was discovered because treatment of crude extract with alkaline phosphatase greatly increased the specific activity. Later experiments showed that the covalent attachment of $^{32}PO_4^{2-}$ to this enzyme was catalyzed by cell free extracts. This enzyme was affinity purified from crude extracts by elution with cDPG from blue dextran agarose. Phosphorylation of the enzyme inhibited the elution from this column. If, instead, 2,3-DPG was used to free protein from the column, an otherwise uncharacterized protein of approximately 40 kDa (determined by SDS-polyacrylamide gels) eluted along with lesser amounts of the cDPG hydrolyzing enzyme. Interestingly, in the final step in the purification of cDPG synthetase, a 145 kDa protein with subunits of approximately 38 kDa was eluted from 2,3-DPG agarose (Lehmacher et al., 1990). The question that arises is whether the 40 kDa peptide from *M. thermoautotrophicum* and the 38 kDa protein from *M. fervidus* are analogous.

Several methanogens have been reported to accumulate phosphate in the form of polyphosphates (König et al., 1985; Rudnick et al., 1990). One may speculate whether this ability reflects some ancient form of high energy phosphate bond storage, or whether it serves simply as a phosphate reservoir.

Methyl-coenzyme M dependent pyrophosphate (PP_i) synthesis by cell-free extracts of *M. thermoautotrophicum* (Keltjens et al., 1988) to levels as high as 40 mM has been described. This high level of PP_i was reported even in the presence of a pyrophosphatase, which is inhibited by levels of PP_i greater than 1 mM. This level of PP_i should be obvious in ^{31}P-NMR experiments, however it has not been detected in *M. thermoautotrophicum* strain Marburg or strain ΔH (M. F. Roberts, personal communication).

Recently a pyrophosphatase from *Methanothrix soehngenii* was isolated and characterized (Jetten et al., 1992). This enzyme, too, is inhibited, although

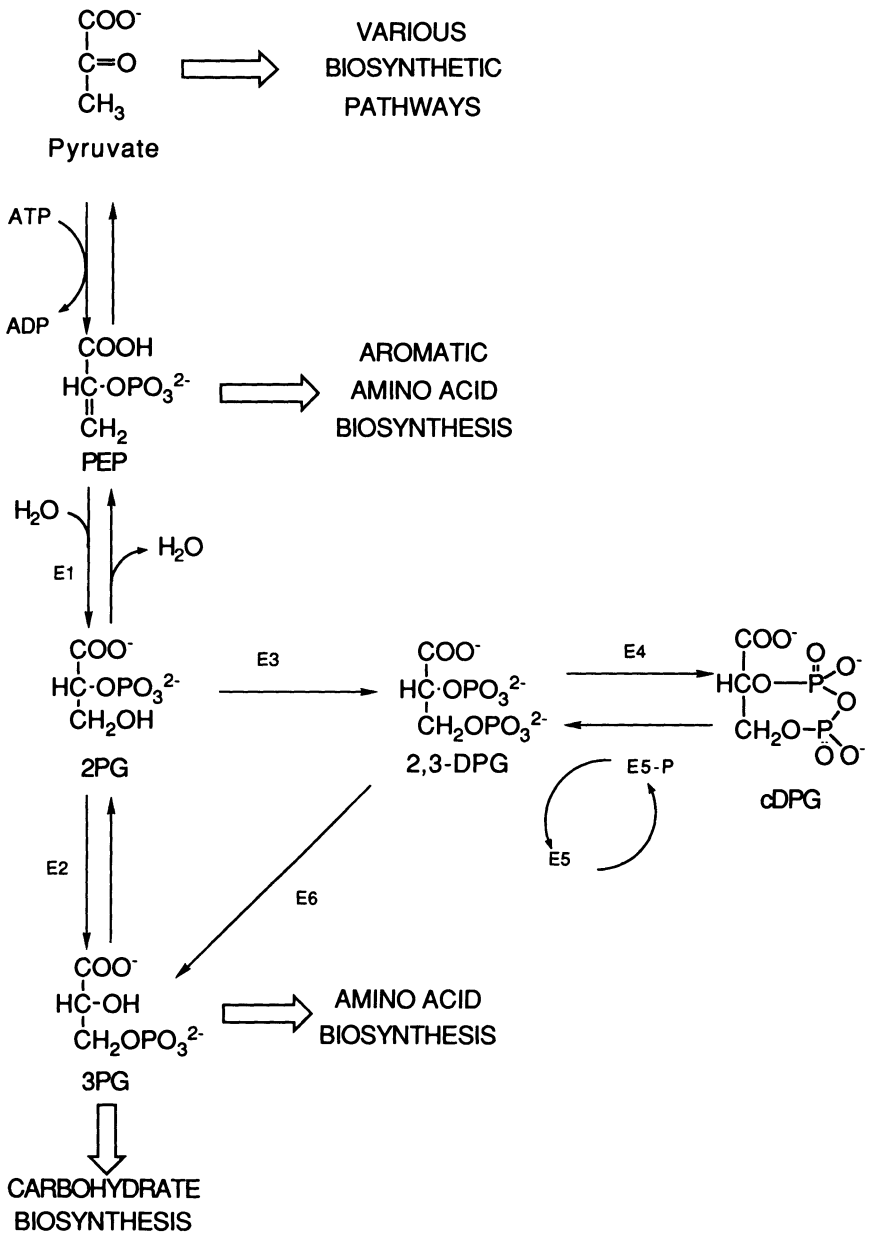

Figure 11.2. Metabolism of cDPG. E1, enolase; E2, phosphoglycerate mutase; E3, 2-phosphoglycerate kinase; E4, cyclic 2,3-diphosphoglycerate synthetase; E5, cyclic 2,3-diphosphoglycerate hydrolase; E6, 2,3-diphosphoglycerate phosphatase. E5 is interconverted between its active and inactive forms by phosphorylation and dephosphorylation.

apparently to not as great an extent as the enzyme from *M. thermoautotrohpicum* (Keltjens, et al., 1988), by PP_i. Since hydrolysis of one mole of PP_i can potentially represent the loss of the equivalent of one mole of ATP, it is thought that there might be some mechanism through which the hydrolysis is coupled to energy production (Jetten et al., 1992; Keltjens et al., 1988), however to date such a coupling has not been demonstrated.

11.3 Growth Effects of Nitrogen and Phosphorus Availability

When nitrogen and phosphorus sources are limiting, methanogens slow their growth, but they do not slow methanogenesis by a comparable amount. Instead, the ratio of methanogenesis to growth increases. This was reported for phosphorus limitation in the growth of *M. barkeri* and *M. thermoautotrophicum* (Archer, 1985; Seely and Fahrney, 1984). Similarly, growth, but not methanogenesis, of *M. thermoautotrophicum* Marburg is inhibited by certain purine analogs (Worrell and Nagle, 1990). Additionally, when given only a single amino acid from which to derive all of its nitrogen by deamination of that amino acid, *M. vannielii* slows its growth, but does not alter methanogenesis to nearly as great as an extent (Auffenberg and DeMoll, unpublished data). In studies of nitrogen fixation, cells switched to N_2 as sole nitrogen source exhibit responses comparable to those seen in cases of nitrogen or phosphate limitation. Growth slows, but the rate of methanogenesis is not reduced by a comparable amount (Belay et al., 1988; Fardeau et al., 1987; König et al., 1985). This has been attributed to an increased requirement for ATP in N_2 reduction to NH_4^+ (2). However, since König et al. (1985) have shown that methanogens grown under nitrogen limiting conditions synthesize glycogen, and that this polymer serves as an energy reservoir, it would be difficult to assign all of the increase in the ratio of methanogenesis to growth to the energy requirements of nitrogen fixation. Some of the energy may be converted to an energy rich polymer. This possibility should also be considered in light of the observations that growth and methanogenesis conditions, similar to those seen when methanogens fix nitrogen, may be generated by limiting either nitrogen or phosphorus. Perhaps at least some of the increase in the ratio of methanogenesis to growth seen under conditions which allow nitrogen fixation may be attributed to the acquisition of nitrogen being the growth limiting step; NH_4^+ is not generated as fast from N_2 as it is demanded by cellular metabolism.

In summary, when there is more carbon than is necessary to meet the decreased growth needs, methanogens do not wastefully slow down carbon acquisition or methanogenesis. Instead, they direct carbon into at least one storage compound, glycogen, which may serve as a reservoir of both energy and carbon. Whether or not cDPG may act in a comparable role is unknown.

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IV

GENETICS

12

Structure and Organization of Genes

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12.1 Introduction

Recognition of the *Archaea* as a separate and coherent group of prokaryotes (Woese et al. 1978, 1990), distinct from the *Bacteria*, has led to searches for differences and novelties in their molecular biology. Very little regard has been given to the fact that the *Archaea*, which includes all methanogens (Balch, et al., 1979) are, nevertheless, prokaryotes. They *are* single-celled microorganisms with a nucleus (genome) that is a single circular DNA molecule not separated from the cytoplasm by a nuclear membrane. DNA replication, transcription and translation all occur in the same cellular compartment allowing coupling of transcription and translation and immediate access of regulatory molecules to regulatory sites on the genome. It is therefore perhaps not surprising that, as reviewed in this chapter, the organization, structure and mechanisms of expression of genes in methanogens appear superficially very similar to their bacterial counterparts. Cloning and sequencing has, however, revealed that many methanogen genes are more closely related, in evolutionary terms, to eukaryal than bacterial genes (Brown et al., 1989; Reeve, 1992). These evolutionary relationships, and current knowledge of the genes unique to methanogens, i.e., genes involved in methanogenesis, are also discussed in this Chapter.

12.2 Genome Structure

12.2.1 DNA Replicons

Based on their denaturation and renaturation kinetics methanogen genomic DNAs were predicted to be molecules with prokaryotic complexities, comprised

predominantly of unique sequences and somewhat smaller than the genome of *Escherichia coli* (Mitchell et al., 1979; Klein and Schnorr, 1984). The accuracy of these predictions has now been confirmed for the *Methanococcus voltae* genome with the publication of its physical map (Sitzmann and Klein, 1991). This genome is a single, circular, double-stranded DNA molecule, approximately 1.9 Mbp in length or ~45% the size of the *E. coli* genome. Southern hybridization experiments have located genes all around the *M. voltae* genome. As in *Bacteria*, some genes with related functions are clustered whereas others involved in the same biochemical pathway, are not physically linked. In contrast to the situation in some halophilic *Archaea*, which contain large numbers of mobile insertion sequences (Sapienza et al., 1982), construction of the *M. voltae* map gave no evidence for frequently occurring, repetitive sequences. Only one mobile repetitive element has, in fact, so far been identified and characterized from a methanogen. This element, designated ISM1, was cloned fortuitously adjacent to a *proC* gene from *Methanobrevibacter smithii* (Hamilton and Reeve, 1985a). It appears to be a typical, prokaryotic insertion sequence. ISM1 is 1183 bp in length, has 29 bp terminal inverted repeat sequences, is present in ~10 copies per *M. smithii* genome and duplicates 8 bp at the sites of its insertion. One long open reading frame (ORF) occupies ~87% of the length of ISM1 and seems likely to encode its transposase. In addition to genomic DNA, some methanogens also contain plasmid DNAs but, so far, phenotypes associated with the presence of these plasmids have not been identified. The complete 4439 bp DNA sequence of the plasmid pME2001, isolated from *Methanobacterium thermoautotrophicum* strain Marburg, has been obtained (Bokranz et al., 1990) revealing several ORFs and a sequence that is highly transcribed *in vivo* (Meile et al., 1988). Nevertheless, *M. thermoautotrophicum* strain Marburg cells lacking pME2001 are fully viable.

12.2.2 Chromatin

All cells face the problem of how to compact their genomic DNA within the limited nuclear space available. In *Eukarya*, chromosomal DNA is compacted by histones into well-defined nucleosomes, which then further assemble to form chromatin. Abundant and conserved DNA-binding proteins have also been identified in *Bacteria* but there is no convincing evidence for a universally conserved complex, within bacterial cells, analogous to the eukaryal nucleosome. This appears to be a major difference between the *Eukarya* and the *Bacteria*, and determining how archaeal genomic DNA is packaged *in vivo* is therefore now important. A related issue has arisen from the discovery of hyperthermophilic archaeal species, some of which are methanogens (Stetter et al., 1981; Huber et al., 1989). These organisms live at temperatures at which their genomic DNAs, *in vitro*, would be single stranded. These genomic DNAs must therefore not only be compacted *in vivo* to accommodate space limitations, but must also be pro-

tected from heat denaturation. The DNA binding proteins, designated HMf and HMt, that have been isolated and characterized from *Methanothermus fervidus* (Krzycki et al., 1990) and *Methanobacterium thermoautotrophicum* strain ΔH (Musgrave et al., 1992), respectively, could play roles in both these activities. These proteins contain two very similar, small (~7 Kd) subunit polypeptides (HMf1+HMf2 and HMt1+HMt2) with amino acid sequences that are closely related to the sequences of eukaryal histones (Sandman et al., 1990). HMf and HMt binding to DNA molecules *in vitro* results in the formation of nucleosome-like structures (NLS) (Sandman et al., 1990) which are calculated to contain ~150 bp of DNA (Musgrave et al., 1991), consistent with experiments that demonstrate that only DNA molecules longer than ~120 bp can form electrophoretically stable complexes with HMf (Figure 12.1). The DNA molecule in these archaeal NLS is wrapped in a positive toroidal supercoil (Figure 12.2) in contrast to the negatively supercoiled DNA molecule in an eukaryal nucleosome (Musgrave et al., 1991,1992). Formation of the NLS does increase the resistance of the bound DNA molecule to heat denaturation *in vitro* but the importance of this *in vivo* is uncertain. The cytoplasm of *M. fervidus*, which grows optimally at 83°, contains ~1 M potassium 2′3′(cyclic)-diphosphoglycerate (K_3cDPG)(Hensel and König, 1988). This unusual salt, at this concentration, increases the functional half-lives of *M. fervidus* enzymes and should also protect its genomic DNA from heat denaturation. Separating the DNA strands of the *M. fervidus* genome at such a high internal salt concentration for replication and transcription could, in fact, pose a problem. Having a substantial portion of the *M. fervidus* genome constrained by HMf in positive toroidal supercoils *in vivo*, should increase the negative plectonemic superhelicity of the remainder of the genome and could therefore facilitate strand separation. As there is sufficient HMf *in vivo* to wrap ~25% of the genome in positive supercoils and HMf does bind to DNA at temperatures >80° in the presence of the *in vivo* concentration of K_3cDPG, this may be an important function of HMf (Stroup and Reeve, 1992). *M. fervidus*, together with other hyperthermophiles, also contains reverse gyrase, (Bouthier de la Tour et al., 1990). This enzyme introduces positive plectonemic supercoils into circular DNA molecules *in vitro* and has also been suggested as likely to play a role in providing heat resistance to DNA molecules. In *M. fervidus*, reverse gyrase activity might balance the effects of HMf binding. The negative plectonemic superhelicity introduced into HMf-free regions of the genome, as a consequence of HMf binding, could be reduced by reverse gyrase activity. This cannot, however, be essential as *M. thermoautotrophicum* cells (growth temperature ~65°C) contain HMt, which forms NLS *in vitro* that are virtually identical to those formed by HMf, with DNA molecules constrained in positive toroidal supercoils, but *M. thermoautotrophicum* cells do not contain reverse gyrase (Musgrave et al., 1992).

Several related members of an entirely different family of small (~10 Kd),

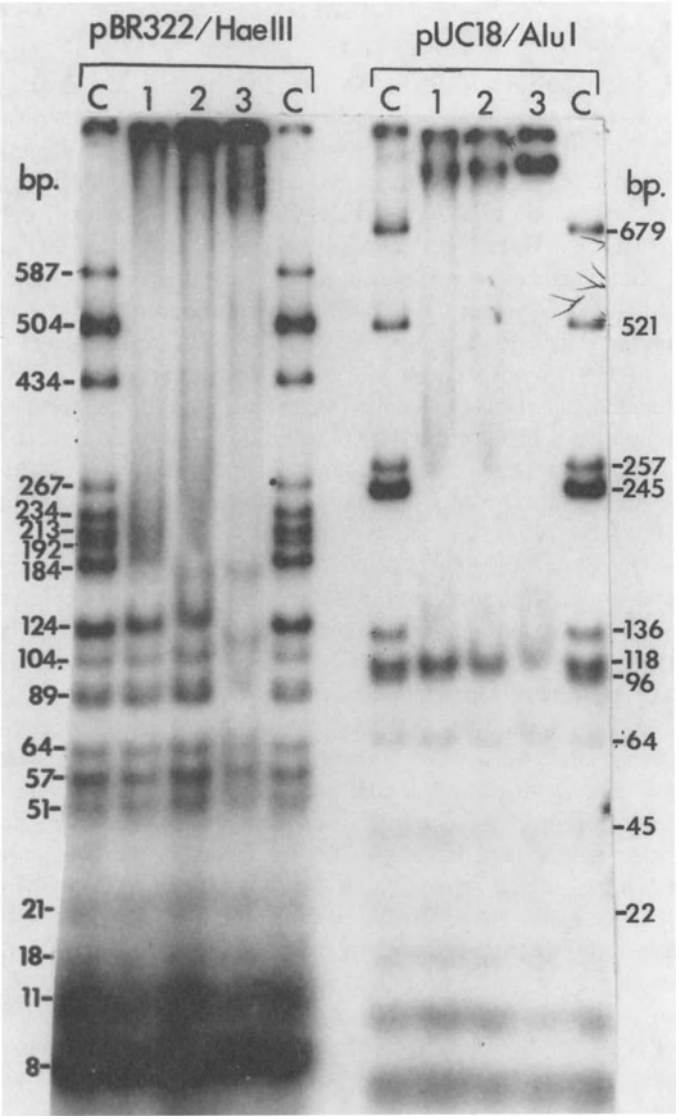

Figure 12.1. HMf binding to restriction fragments. Restriction fragments generated by *Hae*III digestion of pBR322 or *Alu*I digestion of pUC18 were ^{32}P-end-labeled, separated by electrophoresis through a 5.5% w/v polyacrylamide gel and visualized by autoradiography (Control tracks labeled C). The DNA fragments in the tracks labeled 1,2 and 3 were allowed to bind 32, 64 and 128 ng of HMf (Krzycki *et al.*, 1990) before the electrophoresis. The electrophoretic mobilities of restriction fragments shorter than ~120bp were not effected by HMf. This appears therefore to be the minimal length of DNA that can form an electrophoretically stable complex with HMf. The experimental results shown were provided by Dr. J.A. Krzycki (Krzycki *et al.*, 1990).

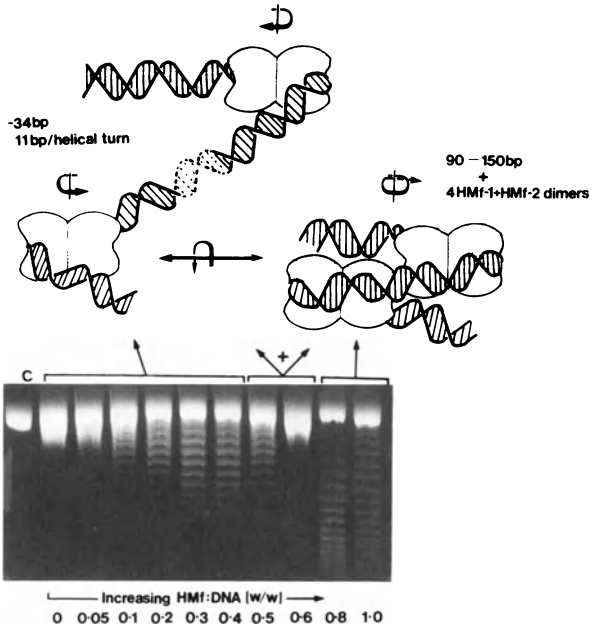

Figure 12.2. Model for HMf-DNA complex formation. Each HMf molecule contains a clear and stippled region representing its two polypeptide subunits (HMf1 and HMf2). At low protein to DNA mass ratios, HMf binding causes kinks (Sandman *et al.*, 1990) which are shown as being formed by an association of two HMf molecules (four polypeptide subunits). Topoisomer analyses indicate that only one intermediate (causing net negative superhelicity) forms *en route* to the complex which contains four HMf molecules (eight subunits) and 90 to 150 bp of positively wrapped DNA (Musgrave et al., 1991). A topoisomer analysis experiment is shown to illustrate how the results of such experiments are reflected in the model. The increasing amounts of HMf indicated were bound to form II DNA of plasmid pBSKS (a pUC18 based recombinant plasmid containing a total of 6888 bp) and the topoisomers remaining after exposure to topoisomerase I and deproteinization were separated by agarose gel electrophoresis. The increasing number of negative topoisomers detected following HMf binding, at protein to DNA ratios up to 0.3 to 1, are explained as a result of the DNA molecules kinked around HMf being underwound (~11bp/helical turn). As there are equal numbers of left-handed and right-handed kinks, kinking *per se* has no net effect on superhelicity. At HMf to DNA ratios >0.3 to 1 HMf molecules already bound to DNA interact and direct the formation of the complexes that contain positive toroidal supercoils. The net effect, as the number of positive toroidal supercoils increases, is first a decrease in the number of assayable negative topoisomers, then an overall balance in positive and negative superhelicity resulting in virtually no assayable topoisomers, followed by a large increase in assayable positive topoisomers as all the DNA-bound HMf molecules enter complexes. At HMf to DNA mass ratios >1 to 1, associations of complexes must occur in which the DNA molecules become increasingly more inaccessible to topoisomerase 1. The net effect is then a decrease in the number of assayable positive topoisomers.

and abundant DNA binding proteins have been isolated and characterized from the *Methanosarcinaceae* (Chartier et al. 1985, 1989a,b; Imbert et al., 1990). These proteins, designated MC1 (methanogen chromosomal protein 1), also appear to play a role in genome compaction and can provide DNA molecules with resistance to heat denaturation *in vitro*. They do not, however, show any evolutionary relationship to eukaryal histones nor to any other group of prokaryotic DNA-binding proteins. Binding of MC1 to dsDNA molecules does not result in the formation of globular NLS but does cause DNA bending and introduces negative superhelicity (Laine et al., 1991). Cross-linking experiments have identified a tryptophan containing region of the MC1 protein from *Methanosarcina* sp. CHTI55 that interacts directly with DNA (Katouzian-Safadi et al., 1991). A second, somewhat larger (~17 Kd) DNA binding protein, designated MC2, has also been identified in *Methanosarcinaceae* (Chartier et al., 1988).

12.2.3 DNA Repair, Replication, and Metabolism

Exposure of methanogens to chemical mutagens or irradiation results in substantial cell death and mutagenesis of the survivors. Error-prone DNA repair systems are therefore likely to be present in methanogens and a photoreactivation system has been identified in *M. thermoautotrophicum* (Kiener et al., 1985) although molecular studies of DNA repair mechanisms have not been reported. A *Methanosarcina mazei* gene, homologous to the *E. coli dna*K heat-shock responsive gene, has been cloned and sequenced (Macario et al., 1991). Despite being obligate anaerobes and having evolved in an anoxic world, methanogens do contain superoxide dismutase (SOD) (Takao et al., 1991). The superoxide radical must therefore also pose a toxicity problem for methanogens and a SOD-encoding gene has been cloned and sequenced from *M. thermoautotrophicum* (Takao et al., 1990). Based on its primary sequence, this would be predicted to be a Mn-SOD but atomic adsorption spectroscopy has demonstrated that this methanogen enzyme is, in fact, a Fe-SOD (Takao et al., 1991).

Several restriction enzymes have been isolated from methanogens and some are now available as commercial products (Schmidt et al., 1984; Thomm, Frey, et al., 1988; Lunnen et al., 1989). The genes encoding the *Mwo*I restriction and modification system, cloned from *Methanobacterium wolfei*, have been expressed at high levels in *E. coli* to facilitate the commercial production of this enzyme (Lunnen et al., 1989). DNA-dependent DNA-polymerases have also been isolated from *M. thermoautotrophicum* (Klimczak et al., 1986) and *Methanococcus vannielii* (Zabel et al., 1985). These enzymes, based on their sensitivities to inhibitors, appear to be more closely related to eukaryal DNA-polymerases than to bacterial DNA polymerases.

12.3 Gene Structure

12.3.1 RNA Polymerases and Promoter Structure

Methanogens, like *Bacteria*, appear to contain only one DNA-dependent RNA-polymerase (RNAP) core enzyme but these archaeal RNAPs contain more polypeptide subunits than do bacterial RNAPs and have no resemblance to the standard bacterial $\alpha_2\beta\beta'\sigma$ subunit configuration (Thomm et al., 1986; Zillig et al., 1988). Based initially on immunological cross-reactivities (Schnabel et al., 1983), and more recently on gene cloning and sequencing, methanogen RNAPs have been shown to be more similar in structure to eukaryal RNAPs than to bacterial RNAPs (Berghöfer et al., 1988; Schallenberg et al., 1988; Zillig et al., 1988; Auer et al., 1989; Klenk et al., 1992). This is also reflected in the sequences that they employ as promoters. The major element determining transcription initiation in methanogens is an eukaryal-like TATA-box, with the consensus sequence 5′ TTTA(T/A)ATA. This was first identified, as a conserved sequence, located upstream of many cloned genes and was designated boxA. A second conserved sequence, designated boxB, with the consensus 5′ ATGC was also recognized ~25 bp downstream from boxA (Wich et al., 1986a,b; Thomm and Wich, 1988; Zillig et al., 1988; Brown et al., 1989; Thomm et al., 1989). It is now known that methanogen RNAPs bind to the TATA-box (boxA) region (Thomm and Wich 1988; Thomm et al., 1988b; Brown and Reeve 1989) and initiate transcription at the boxB sequence, usually at a purine-pyrimidine dinucleotide (Thomm and Wich, 1988; Hausner et al., 1991; Thomm et al., 1992). The development of cell-free systems that initiate transcription accurately *in vitro*, at the same sites as *in vivo*, has facilitated the functional dissection of methanogen promoters (Frey et al., 1990; Knaub and Klein, 1990; Thomm et al., 1990) and an exhaustive study of the $tRNA^{val}$ gene promoter from *Methanococcus vannielii* has been reported (Hausner et al., 1991). The conservation of the TATA box as a functionally important element directing transcription of both stable RNA and protein encoding genes, in two phylogenetically very different methanogens, has been demonstrated directly. RNAP isolated from *Methanococcus thermolithotrophicus* accurately transcribed the *hmfB* and 7S RNA encoding genes cloned from *Methanothermus fervidus* (Thomm et al., 1992; Koller et al., 1992). Methanogen RNAPs do resemble bacterial RNAPs in binding directly to promoter sequences which has allowed footprinting studies to be undertaken (Thomm et al. 1988; Brown and Reeve, 1989) but, as in the *Eukarya*, transcription factors also play a role in transcription initiation in methanogens (Frey et al., 1990). Transcription of *nif* genes *in vivo* occurs only in methanogens grown in the absence of ammonium ions (Souillard and Sibold, 1989) but this regulation could not be duplicated *in vitro*. Transcription of the *nifH1* gene from *M. thermolithotrophicus* was initiated at the correct site *in vitro*, but equally well with RNAP isolated from

M. thermolithotrophicus cells grown diazotrophically or grown in a medium containing ammonium ions (Gohl et al., 1992).

Nucleosome localization can regulate gene expression in *Eukarya* (Simpson, 1990) and preliminary studies indicate that DNA-binding, by proteins such a HMf and MC1, (see above, Section 12.2.2) could regulate gene expression in methanogens. At relatively high protein to DNA mass ratios both of these proteins inhibit transcription *in vitro*. This inhibition is, however, reversed by adding HMf-free competitor DNA to an HMf inhibited systems (Thomm et al., 1992) and, at low protein to DNA ratios, MC1 binding actually stimulates transcription *in vitro* (Chartier et al., 1989b).

12.3.2 Transcription Terminators and mRNA Structure

Transcription terminators downstream of stable RNA and protein encoding genes in methanogens, appear to conform to one of two motifs (Zillig et al., 1988, Brown et al., 1989). In some, which seem to be similar to rho-independent bacterial terminators, transcription terminates following an inverted repeat sequence that presumably forms a stem-loop structure in the transcript to direct termination (Müller et al., 1985; Weil et al., 1989). The second motif appears to be an oligo-T sequence, and several tandemly arranged oligo-T sequences, interspersed with C-residues, have been located downstream of some coding sequences. Only the oligo-T type of transcription terminators have so far been found in the hyperthermophile *M. fervidus*, suggesting that the spontaneous formation of dsRNA structures may not be a very effective regulatory mechanism at high growth temperatures (Weil et al., 1988; Haas et al., 1989, 1990; Sandman et al., 1990; Bröckl et al., 1991).

The synthesis of polycistronic mRNAs in methanogens has usually been inferred by localizing transcription initiation and termination sites, although some polycistronic mRNAs have been demonstrated directly by Northern blotting (Bokranz et al., 1988; Patel and Ferry, 1988). As in *Bacteria*, methanogen mRNAs appear to be relatively short-lived. The average half life was shown to be ~12 min for the population of mRNAs in *Methanococcus vannielii* cells growing with a generation time of ~5 h at 37°C (Brown and Reeve, 1985). Recent studies indicate however, that under these conditions, individual mRNAs in *M. vannielii* cells have substantially different half lives, ranging from 6 min for the *secY* transcript, to 15 min for transcripts of the *mcr* operon, to 30 min for transcripts of the *mvaL1* operon (A. N. Hennigan and J.N. Reeve, unpublished results). Eukaryal mRNAs, which must traverse the nuclear membrane before being translated, usually have a 5′ cap-structure, long 3′ poly-A^+ tails and introns. There is, however, no evidence for 5′ cap structures or introns in methanogen RNAs and although some *M. vannielii* mRNAs do have poly-A^+ tails (Brown

and Reeve, 1985), these are on average only 12 bases in length, typical of bacterial poly-A^+ sequences.

12.3.3 Ribosome Binding Sites and Codon Usage

Immediately preceding virtually every methanogen ORF, including each ORF within a polycistronic transcript, is a sequence which when transcribed would be complementary to the 3′ terminal sequence of the methanogen's 16S rRNA (Brown et al., 1989). These sequences are presumed to be ribosome binding sites (RBS) and, as in *Bacteria*, highly expressed genes have strong RBS, with more bases complementary to the 16S rRNA sequence, than do the RBS of housekeeping genes. The 3′ terminal sequences of the 16S rRNAs of *Bacteria* and methanogens are very similar and it is therefore not surprising that methanogen RBS appear to function correctly when expressed in *E. coli* or in *B. subtilis*. Methanogens employ the "standard" genetic code and codon usage patterns reflect both the overall base composition of the genomic DNA, which ranges in methanogens from 26 to 68% mol G+C (Balch et al., 1979), and the level of gene expression. Highly expressed genes, even in methanogens with high genomic A+T contents, tend to contain codons with a C in the wobble position, when a choice between the synonymous NNC/U pairs of codons is available (Cram et al., 1987; Weil et al., 1989). Although ATG is the most frequently used translation initiation codon, methanogen genes initiated with a GTG or TTG codon have been identified (Bokranz et al., 1987; Souillard et al. 1988; Fabry et al., 1990; Takao et al., 1990; Bröckl et al. 1991; Eggen et al. 1991; Klenk et al., 1992).

12.3.4 Leader (Signal) Sequences

Several methanogen genes have been sequenced that encode proteins which appear to be synthesized initially as precursors, with a N-terminal amino-acid sequence that is not present in the mature protein (Bröckl et al., 1991; Dharmavaran et al., 1991; Kalmokoff and Jarrell, 1991). These leader (signal) peptides are presumably removed during the translocation of the protein to the cell surface or its maturation into an active enzyme. The presence of a system analogous, possibly homologous, to the bacterial signal peptidase-secretion system is suggested by the presence of a *secY* gene in *Methanococcus vannielii* (Auer et al., 1989a; Lechner et al., 1989) which, when expressed in *E. coli*, complements the defective phenotype of an *E. coli secY* mutant (Auer et al., 1991). Although the structure and cleavage sites predicted for the signal peptides of the surface layer (S-layer) proteins of *Methanothermus* species (Bröckl et al., 1991) do conform closely to the consensus for such structures in *Bacteria*, this is not always the case. The amino-terminal peptides removed during the maturation of flagellins

(Kalmodoff and Jarrell, 1991) and an ATPase from *Methanococcus voltae* (Dharmavaram et al., 1991) show no resemblance to bacterial signal sequences.

12.4 Cloned Methanogen Genes

12.4.1 Amino Acid and Purine Biosynthetic Pathways

The discovery that methanogen genes could complement auxotrophic mutations in *E. coli* and *Bacillus subtilis* (Reeve et al, 1992; Wood et al., 1983; Morris and Reeve, 1984) has facilitated the isolation and sequencing of several genes which encode enzymes that participate in amino acid or purine biosynthetic pathways. Complementation of the same *E. coli* mutation, by DNA fragments cloned from different methanogens, has allowed genes with the same function to be sequenced and compared from different methanogens. Methanogen genes, cloned by this procedure, have been given the same designation as that of the complemented *E. coli* gene, namely *hisA, hisI, argG, proC, trpBA* and *purE*. Sequencing the flanking genomic DNAs has, in some cases, identified additional genes with related functions or with functions that have been identified tentatively by the presence of sequences conserved in previously identified genes.

(a) Histidine

HisA genes have been cloned and sequenced from *Methanococcus vannielii*, *Methanococcus voltae* and *Methanococcus thermolithotrophicus* (Cue et al., 1985; Weil et al., 1987). These three *hisA* genes are closely related, coding for proteins with amino acid sequences that are ~70% identical, however the genomic regions flanking these genes have undergone different rearrangements (Weil et al., 1987). The ORFs adjacent to the *hisA* genes do not appear to encode histidine biosynthetic enzymes. ORF547, immediately upstream of the *M. vannielii hisA* gene, encodes 547 amino-acid residues which seem likely to form a protein with both glycoprotease (Abdullah et al., 1991) and kinase (Hanks et al., 1988) or phosphotransferase (Brenner, 1987) activities (Figure 12.3; B. Baum, personal communication). This protein could be a member of the serine-tyrosine family of protein kinases that regulate cellular functions in the *Eukarya* (Hanks et al., 1988).

The promoter sequences that direct transcription of the *M. vannielii hisA* gene *in vivo* in the methanogen and, following cloning, in *E. coli* have been identified (Brown et al., 1988). Transcription in *M. vannielii* is initiated, as expected, at an initiator boxB sequence located at an appropriate distance downstream from a TATA box but transcription of the *hisA* gene is initiated in *E. coli* at an entirely different site. Intergenic regions in methanogens are very A/T rich (Brown et al,

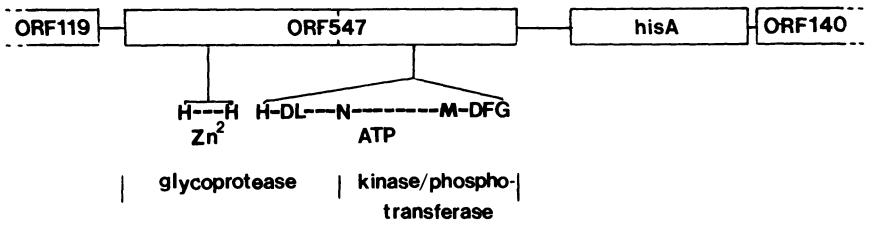

Figure 12.3. The ORF547-*his*A region of the *Methanococcus vannielii* genome. The *hisA* gene was cloned by complementation of an *hisA* auxotroph of *E. coli* (Cue et al., 1987). Sequencing the flanking regions revealed the additional open-reading frames (ORFs) (G.S. Beckler Ph.D. Thesis 1987, The Ohio State University; Weil et al., 1987). Based on comparisons with the sequences of known genes, ORF547 (547 codons) appears to encode a protein with glycoprotease (Abdullah et al., 1991) and kinase (Hanks et al., 1988) or phosphotransferase (Brenner 1987) activities. The first 300 codons appear to encode a polypeptide that is related to bacterial glycoproteases that includes a conserved histidine-x-x-x-histidine zinc ligand (Abdullah et al., 1991). The remainder of ORF547 encodes polypeptide sequences that are conserved in eukaryal protein kinases (Hanks et al., 1988) and bacterial phosphotransferases (Brenner 1987). The conserved domain, implicated in ATP binding, is illustrated. These sequence conservations were recognized and provided by B. Baum.

1989), especially in methanogens such as *M. vannielii*, which have genomic DNA that are overall ~70%mol A+T. These intergenic regions frequently contain sequences that conform to the consensus sequence for *E. coli* promoters and one such sequence appears to direct the transcription of the *M. vannielii hisA* gene in *E. coli* (Brown et al., 1988). Transcription of the *hisA* gene in *M. voltae* is stimulated by the presence of aminotriazole, an inhibitor of histidine biosynthesis in many biological systems (Sment and Konisky, 1986). A *hisI* gene has also been cloned and sequenced from *M. vannielii*. This gene does not appear to be closely linked to the *M. vannielii hisA* gene (Beckler and Reeve, 1986) and unlike *hisI* sequences of *E. coli* and in fungi, this methanogen *hisI* gene is not fused to DNA sequences that encode additional catalytic activities involved in histidine biosynthesis.

(b) Arginine

argG genes have been cloned and sequenced from *M. vannielii* and *Methanosarcina barkeri* (Morris and Reeve, 1984;1988). The encoded argininosuccinate synthetases have amino acid sequences that are 50% identical to each other and 38% identical to the sequence of the human argininosuccinate synthetase. The human argininosuccinate synthetase encoding gene, however, contains at least

nine introns, none of which are retained in the methanogen *argG* genes. Upstream of the *M. barkeri argG* gene is an ORF that appears to encode carbamyl phosphate synthetase, an enzyme which also participates in arginine biosynthesis. This gene has therefore been designated *carB*. The complete sequence of the *M. barkeri carB* gene has been determined (P. Schofield, personal communication) and shown to contain the same large tandem duplication found in all other *carB* genes sequenced to date. An ancient gene duplication event apparently generated a sequence from which all extant *carB* genes have evolved. The intergenic region, 389 bp in length, that separates the *M. barkeri carB* and *argG* genes appears to be a region of complex regulation. It contains six tandemly repeated copies of a 14 bp sequence, three tandemly repeated copies of a 29 bp sequence and a 9 bp inverted repeat. The *M. barkeri argG* gene also complements *argA* mutations in *B. subtilis* (Morris and Reeve, 1984) and an *argG* gene has been cloned, but not yet sequenced from *Methanococcus voltae* (Wood et al., 1983).

(c) Tryptophan

Tryptophan biosynthetic genes have been cloned and sequenced from *M. voltae* (Sibold and Henriquet, 1988) and *M. thermoautotrophicum* strain Marburg (Meile et al., 1991) by *trpBA* complementation. As in *Bacteria*, these *trp* genes are clustered forming polycistronic transcriptional units arranged *trpDFBA* in *M. voltae* and *trpEGCFBAD* in *M. thermoautotrophicum*. Only the order *trpBA*, the genes that encode the α and β subunits of tryptophan synthetase, has been maintained in the all *trp* gene clusters so far investigated in *Bacteria* and *Archaea*. In *M. thermoautotrophicum*, the promoter proximal *trpEG* genes overlap by 2 bp and are separated from the promoter by a DNA sequence with dyad symmetry reminiscent of the *trp* operator region in *E. coli*. In some *Bacteria* tryptophan binding to a conserved amino acid sequence in the *trpE* gene product, the α-subunit of anthranilate synthetase, directs feed-back inhibition. This amino-acid sequence is conserved at approximately the same location within the *trpE* encoded polypeptide of *M. thermoautotrophicum*.

(d) Adenine

Genes cloned from *M. thermoautotrophicum* strain ΔH, and *Methanobrevibacter smithii*, by complementation of mutations in the *purE* locus of *E. coli* (Hamilton and Reeve, 1985a,b) complement mutations in both the *purE* and *purK* genes of *E. coli* (Tiedeman et al., 1989). Sequencing has, however, demonstrated that these methanogen *purE* genes are single ORFs. They encode polypeptides with sequences that are 45% identical to each other, and 38% identical to the *E. coli purE* gene product, that have both 5′-phosphoribosyl-5-aminoimidazole carboxylase activity (*E. coli purE* function) and CO_2 binding ability (*E. coli*

purK function). The *purK* complementing activity has been located, by Tn5 mutagenesis, within the carboxyl region of the *M. smithii* protein (Hamilton and Reeve, 1985a; Tiedermann et al., 1989). Both methanogen *purE* genes are flanked by unidentified ORFs and appear to be within polycistronic transcriptional units.

A truncated ORF, located immediately 3′ to the isoleucyl-tRNA synthetase encoding *ile*S gene in *M. thermoautotrophicum* strain Marburg, has been designated *purL* because its sequence predicts that it encodes formylglycineaminidine ribonucleotide synthetase, the *purL* gene product of *B. subtilis* (Jenal et al., 1991)

(e) Proline

A *proC* complementing gene has been cloned and sequenced from *Methanobrevibacter smithii* which presumably, therefore, encodes 1-pyrroline-5-carboxylate reductase (Hamilton and Reeve, 1985a).

(f) Glutamine

A glutamine synthetase (GS) encoding *glnA* gene has been cloned from *Methanococcus voltae* by hybridization to a bacterial *glnA* sequence (Possot et al., 1989). This archaeal GS appears to be more closely related to bacterial than eukaryal GSs, being ~51% identical to *B. subtilis* GS and 33% identical to *Anabaena* GS but <12% identical to plant and animal GSs. The TATA-box and transcription initiator boxB element, upstream of the *M. voltae glnA* gene, are separated by a 22bp palindromic sequence which is similar to a palindrome located in the promoter region of the *nifH1* gene of *Methanococcus thermolithotrophicus* (Souillard and Sibold, 1989). Adenylation of a conserved tyrosine residue regulates GS activity in enterobacteria however this tyrosine is replaced by a phenylalanine in the *M. voltae* GS indicating that this regulation is not conserved. Several ORFs, related to bacterial *glnB* genes, have been cloned and sequenced from methanogens which also appear to encode proteins involved in the regulation of nitrogen fixation (see Section 12.4.2 below).

12.4.2 Nitrogen Fixation

The ability to fix dinitrogen is widely distributed in methanogens (see Biosynthesis, Part III) and the presence of sequences related to bacterial *nifD, nifH* and *nifK* genes has been demonstrated in many in methanogen genomic DNAs by Southern hybridizations (Sibold et al., 1985; Possot et al., 1986). Two related, but substantially different *nifH* genes, *nifH1* and *nifH2*, have been cloned and sequenced from *Methanobacterium ivanovii*, *Methanosarcina barkeri* and *Methanococcus thermolithotrophicus* (Souillard et al., 1988; Souillard and Sibold, 1989; Sibold et al., 1991) and a single *nifH* gene has been cloned from *M. voltae*

(Souillard and Sibold, 1986) despite the inability of this methanogen to grow diazotrophically. All these methanogen *nifH* genes appear to encode the Fe-protein components of MoFe-nitrogenases. Their amino-acid sequences are however only 47 to 55% conserved, in contrast to the *nifH* gene products of *Bacteria* which have 67% to 97% identical amino-acid sequences. Downstream of the methanogen *nifH* genes are related ORFs which encode amino acid sequences that are ~50% identical to those of the *glnB* encoded P_{II}-proteins that regulate nitrogenase activity in *Bacteria* (Sibold et al., 1991). Transcription of the *nifH1*-ORF105-ORF128-*nifD*-*nifK* cluster of genes in *M. thermolithotrophicus* occurs only in the absence of fixed nitrogen and generates two transcripts, *nifH1*-ORF105-ORF128 and *nifD*-*nifK*. The *nifD* and *nifK* genes overlap by 8bp and encode the α and β subunits of nitrogenase. Accurate transcription of the *nifH1* gene has been demonstrated, *in vitro*, using a cell-free system, although the regulation of transcription by fixed nitrogen could not be reproduced (Gohl et al., 1992). Transcription of the *M. thermolithotrophicus nifH2* gene, was not detected *in vivo* under any growth conditions (Souillard et al., 1988).

12.4.3 Intermediary Metabolism

(a) Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), L-Malate Dehydrogenase (MDH) and 3-Phosphoglycerate Kinase (PGK)

The genes, *gap*, *mdh* and *pgk*, which encode GAPDH, MDH and PGK in the hyperthermophile *Methanothermus fervidus* have been cloned, sequenced and compared with the sequences of related genes from the mesophilic methanogens *Methanobrevibacter bryantii* and *Methanobacterium formicicum* (Fabry and Hensel 1988; Fabry et al., 1989, 1990; Honka et al., 1990) and from *Bacteria* and *Eucarya*. The *M. fervidus gap* gene codes for a protein which is only ~70% identical in sequence to the GAPDHs from the two mesophilic methanogens whereas these enzymes are 90% identical to each other. The *M. fervidus* and *M. bryantii pgk* encoded enzymes similarly have only 61% identical amino-acid residues. Comparisons with related bacterial and eukaryal enzymes indicate that extensive sequence divergence has occurred during the evolution of these intermediary metabolism enzymes (Hensel et al., 1989). Bacterial and eukaryal MDHs have amino acid sequences that are more similar to the sequences of their L-lactate dehydrogenases (LDH) than to the sequences of the methanogen MDHs. Bacterial and eukaryal MDH and LDH encoding genes appear therefore to have diverged from each other after the methanogen MDH genes separated from the common ancestral sequence. Comparisons of the sequences of *M. fervidus* enzymes have also been made with their mesophilic counterparts to identify amino acid substitutions that might confer heat resistance on the *M. fervidus* enzymes. There are discernible trends; however, a definitive correlation of a

particular amino-acid substitution with thermophily has not been possible. Hybrid *gap* genes, constructed by joining together regions of the *M. fervidus* and *M. bryantii gap* genes, have been expressed in *E. coli* (Biro, et al., 1990). The encoded enzymes, synthesized in *E. coli*, retain the inherent heat resistance of *M. fervidus* GAPDH if a short amino-acid sequence, derived from the carboxyl region of the *M. fervidus* enzyme, is present.

(b) ATPases

Related genes, *atpA* and *atpB*, have been cloned and sequenced from *Methanosarcina barkeri* that encode the α and β subunits, respectively, of a membrane bound ATPase (Inatomi et al., 1989). These genes, which are separated by only 2bp, are contranscribed in the direction *atpAB* and encode polypeptides that have ~25% identical amino acid sequences. They appear to have evolved from a common ancestor, following a gene duplication event, that also apparently gave rise to several eukaryal vacuolar H^+-ATPases, bacterial F_1 H^+-ATPases and an ATPase in the non-methanogenic archaeon, *Sulfolobus acidocaldarius*. A gene that encodes a completely unrelated vanadate-sensitive ATPase in *Methanococcus voltae* has also been cloned and sequenced (Dharmavaram and Konisky 1989; Dharmavaram et al., 1991). The sequence of this gene predicts that this ATPase is synthesized as a precursor protein, with a N-terminal leader sequence formed by 12 amino-acid residues, that is removed during the maturation of the enzyme.

(c) *S*-Adenosyl-L-Methionine Uroporphyrinogen-III Methyl-Transferase (SUMT)

A SUMT encoding gene (*cofA*) has been cloned and sequenced from *Methanobacterium ivanovii* (Blanche et al., 1991). It contains sequences that are conserved in several SUMT-encoding genes in *Bacteria*. The *cofA* encoded enzyme is presumed to catalyze a step in the biosynthesis of precorrin II, a precursor of the methyl coenzyme M reductase cofactor F_{430}.

12.4.4 Genes Involved in Methanogenesis

Many of the enzymes that participate directly in methanogenesis have been purified (see Part II, Biochemistry) and genes which encode some of the enzymes have now also been isolated and sequenced. Recombinant gene libraries, constructed in *E. coli*, have been screened using either antibodies raised against the purified methanogen enzyme or oligonucleotides synthesized with sequences based on N-terminal peptide sequences. Methane genes cloned from one methanogen have then been used, in some cases, as probes to clone related genes from other methanogens.

(a) Formylmethanofuran:tetrahydromethanopterin Formyl Transferase

Methanogenesis from CO_2 and H_2 proceeds through a series of reductive steps (see Part II, Chapter 4) and C1-moiety transfer reactions. A formyl group is transferred at one stage from the methanogen cofactor methanofuran to a second cofactor, tetrahydromethanopterin, and the gene (*ftr*) which encodes the formyl transferase that catalyzes this reaction has been cloned and sequenced from *M. thermoautotrophicum* strain ΔH (DiMarco et al., 1990). The *ftr* gene product is a 31Kd polypeptide that retains its catalytic activity even when synthesized at 37°C in aerobically grown *E. coli* cells. The *ftr* gene appears to be part of a multigene transcriptional unit, although the adjacent genes have not been identified.

(b) Methyl Coenzyme M Reductase

The final reaction in methanogenesis, from all substrates, reduces the methyl group of CH_3-S-CoM to CH_4. This reaction is catalyzed by methyl coenzyme M reductase (MR), an enzyme that appears to be conserved and abundant in all methanogens. The presence and activity of MR essentially defines a cell as a methanogen and the genes, *mcrA*, *mcrB* and *mcrG* that encode the α, β and γ subunits of this enzyme have been cloned and sequenced from five different methanogens (Cram et al., 1987; Bokranz et al., 1987;1988; Klein et al., 1988; Weil et al., 1988). In every case they are arranged *mcrBDCGA* forming a single transcriptional unit, conventionally designated as the *mcr* operon. The two additional genes, *mcrD* and *mcrC*, that are always present do direct the synthesis of polypeptides in methanogens but their function(s) remain unknown. The *mcrD* gene product is physically associated with MR in *M. vannielii* (Sherf and Reeve, 1990) and antibodies raised against this polypeptide do reduce methanogenesis *in vitro* (D. Stroup and J.N. Reeve, unpublished results). An *mcr* operon has also been cloned from *Methanopyrus kandleri* (Huber et al., 1990) and sequencing has confirmed the presence of the *mcrBDCGA* arrangment in this unusual hyperthermophile (J.R. Palmer, V.J. Steigerwald, C.J. Daniels, J.N. Reeve, unpublished results).

As all the sequenced *mcr* operons appear to have evolved from a common ancestor and encode polypeptides that have retained common functions, comparisons of their sequences should provide valid estimates of evolutionary divergence. The *mcrBCGA* genes appear to have been conserved to approximately the same extent whereas the *mcrD* genes are less conserved (Klein et al., 1988; Weil et al., 1988, 1989). The phylogenetic relationships predicted for methanogens by *mcr* comparisons are consistent with the methanogen relationships established by 16S rRNA sequencing (Balch et al., 1979; Woese 1987). As amino acid sequences are available for MRs from three mesophiles, one thermophile and two hyperthermophiles, their comparisons may also eventually be useful in identifying ther-

mophily related features. Conserved DNA sequences in the *mcr* genes can be used as hybridization probes to demonstrate and quantitate methanogens in environmental samples without their cultivation (Weil et al., 1988).

Members of the *Methanobacteriales* contain two similar but distinct MR isoenzymes, MRI and MRII (Rospert et al., 1990) and two *mcr* operons have been demonstrated by Southern hybridizations in the genomes of *M. thermoautotrophicum* strains Marburg and ΔH, *M. wolfei* and *Methanothermus fervidus* (Hennigan et al., 1991). Rapidly growing cells contain predominantly MRII whereas stationary phase cells contain MRI (Respert et al., 1990). The *mcr* operons, initially cloned and sequenced from *M. thermoautotrophicum* (Bokranz et al., 1988) and *M. fervidus* (Weil et al., 1988) encode the MRI enzymes. The genes encoding the β-subunits of the MRII enzymes have now also been cloned and sequenced, from these two methanogens, and these sequences indicate that the two MRI and two MRII enzymes are more similar to each other than are the MRI and MRII isoenzymes in the same methanogen (Steigerwald et al., 1992; V.J. Steigerwald and J.N. Reeve, unpublished results).

Transcription of the *mcr* operon cloned from *Methanococcus vannielii* (Cram et al., 1987) has been found to be maximal during exponential growth of *M. vannielii* cells, resulting in a steady-state level of ~350 intact *mcr* transcripts per cell (A. Hennigan and J.N. Reeve, unpublished results). These transcripts have a half-life *in vivo* of ~15 min at 37°C and do not appear to be cleaved into smaller mRNAs that could be translated with different efficiencies or have different functional stabilities. The very high levels of synthesis, in *M. vannielii* cells, of the α, β and γ subunits of MR when compared to the amounts of the *mcrC* and *mcrD* gene products must therefore result from differences in RBS strength, and possibly also differences in codon usage, but not from mRNA processing (Cram et al., 1987).

(c) Hydrogenases and Polyferredoxins

Most methanogens can obtain reductant from molecular hydrogen and two nickel and iron containing hydrogenases (NiFe-hydrogenases) have been isolated and characterized from several different methanogens (Reeve and Beckler, 1990; see also Part II, Chapter 7). These enzymes are defined functionally as the factor F_{420}-reducing hydrogenases (FRH) and the methyl viologen-reducing hydrogenase (MVH) even though FRH can also reduce methyl viologen. The genes encoding the polypeptide subunits of these two enzymes have been cloned from *M. thermoautotrophicum* strain ΔH (Reeve et al., 1989; Alex et al., 1990). They form two separate transcriptional units, *frhADGB* and *mvhDGAB*. The α, β and γ subunits of FRH are encoded by *frhA*, *frhB* and *frhG*, respectively, and the α, γ and δ subunits of MVH are encoded by *mvhA*, *mvhG* and *mvhD*, respectively. The *frhA* and *mvhA* sequences and the *frhG* and *mvhG* sequences have apparently evolved

from the same ancestral sequences as the genes that now encode the large and small subunits of NiFe hydrogenases in a wide range of *Bacteria* (Reeve et al., 1989; Reeve and Beckler, 1990). The FRH and MVH operons both contain one ORF, *frhD* and *mvhB* respectively, which encodes a polypeptide that does not co-purify with the hydrogenase activities. The *frhD* gene has sequences in common with the *hydD* gene located in a hydrogenase encoding cluster of genes in *E. coli* (Alex et al., 1990) but the functions of the *frhD* and *hydD* gene products remain unknown. The precise function of the *mvhB* gene product is also unknown however as this protein appears to be a polyferredoxin, a single protein containing six tandemly arranged, contiguous ferredoxin-like domains (Reeve et al., 1989), a function in electron transport seems most likely. Polyferredoxin molecules have been isolated from *M. thermoautotrophicum* strains ΔH (Steigerwald et al., 1992) and Marburg (Hedderich et al., 1992) and shown, as expected, to have very high Fe contents. The polyferredoxin is synthesized in exponentially growing cells of *M. thermoautotrophicum* but is present in only minimal amounts in stationary phase cells (Steigerwald et al., 1992). This pattern of synthesis in growing cells and disappearance on entry into stationary phase is the same as that reported for MRII, and it does appear likely that the syntheses of MVH and MRII are coordinated (Steigerwald et al., 1992). Genes encoding polyferredoxins that are predicted to contain six ferredoxin-like domains have also been cloned and sequenced, from *Methanothermus fervidus* (Steigerwald et al., 1990) and *Methanococcus voltae* (Halboth and Klein, 1992). In all three cases, domain 1 is unusually small and domain 5 contains a conserved, additional sequence (Figure 12.4) however the primary sequences of these polyferredoxins are not, overall, very similar. *M. fervidus* and *M. thermoautotrophicum*, both members of the *Methanobacteriales*, have *mvhB* encoded polyferredoxins that are only 64% identical to each other and <45% identical to the *vhuB* encoded polyferredoxin in *M. voltae*. A di-ferredoxin encoding gene, designated *vhcB*, has also been cloned and sequenced from *M. voltae*, which contains two domains that are most closely related to domains 5 and 6 of the *vhuB* encoded polyferredoxin. The additional amino acids, located in domain 5 of the polyferredoxins, are also retained in the di-ferredoxin. Both the *vhuB* and *vhcB* genes are linked to MVH encoding genes in *M. voltae* (Halboth and Klein, 1992).

The sequences of the intergenic regions upstream of the *mvh* operon in several different *M. thermoautotrophicum* strains have been determined (Reeve and Beckler, 1990). A TATA-box containing sequence, >100 bp in length, is conserved in every case that may contain the regulatory elements that coordinate the syntheses of MVH and MRII (Steigerwald et al., 1992).

(d) Formate Dehydrogenase

The *fdhA* and *fdhB* genes, which encode the α and β subunits of formate dehydrogenase (FDH) in *Methanobacterium formicicum*, have been cloned and

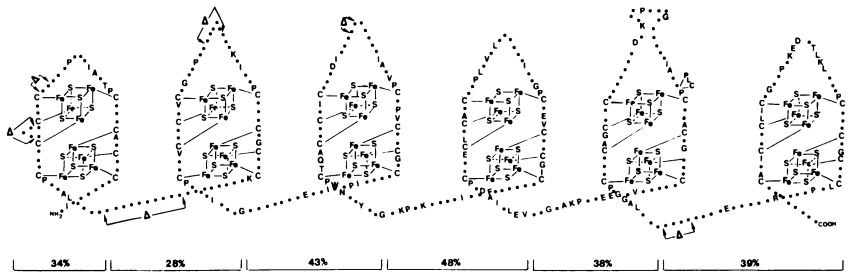

Figure 12.4. Comparison of the polyferredoxins. The amino acid sequences of *mvhB* encoded polyferredoxins from *M. thermoautotrophicum* (Reeve *et al.*, 1989) and *M. fervidus* (Steigerwald *et al.*, 1990) and the *vhuB* encoded polyferredoxin from *M. voltae* (Halboth and Klein, 1992) were aligned and amino acids conserved in all three polyferredoxins are indicated, at their locations, by the appropriate single-letter code. Dots indicate positions occupied by different amino acid residues in the different polyferredoxins. The *M. voltae* sequence does not contain the regions indicated by Δ. The percentage of conserved amino acid residues, in each domain, is listed above the bracketed regions which were used to define the limits of each domain. The two domains of the "diferredoxin" encoded by the *vhcB* in *M. voltae* are 59% and 56% identical respectively, to domains 5 and 6 of the *vhuB* encoded polyferredoxin. The region of additional amino acids shown at the top of domain 5, including the conserved lysine (K), proline (P) and glycine (G) residues, is retained in the *vhcB* gene product (Halboth and Klein, 1992).

sequenced (Shuber et al., 1986). They are arranged *fdhAB*, overlapping by 1 bp, and are cotranscribed with the *fdhC* gene which is upstream of *fdhA*. Transcription is stimulated by molybdenum (White and Ferry, 1992). FDH contains a molybdopterin cofactor so that although the mechanism of Mo regulation of *fdhCAB* transcription is unknown, a biochemical connection between Mo availability and FDH is established. Replacement of Mo with tungsten results in the synthesis of an inactive FDH in *M. formicicum* (May et al., 1988) whereas W is required for optimum growth on formate by *M. vannielii* (Jones and Stadtman, 1977). Several sequences in the regulatory region upstream of the *fdh* operon in *E. coli* are also present further upstream of the *fdhCAB* operon in *M. formicicum*, suggesting a conserved regulatory system (Patel and Ferry, 1988). In methanogens both FDH and FRH reduce the cofactor F_{420} and an amino acid sequence, conserved in the β-subunits of both these enzymes, appears likely to be the site of this common activity (Alex et al., 1990).

(e) Acetyl Coenzyme A Synthetase and Carbon Monoxide Dehydrogenase

Acetate must first be activated to acetyl-CoA before CH_4 can be generated from the CH_3-moiety of acetate (see Part II, Chapter 6). In *Methanothrix soehnge-*

nii, acetyl coenzyme A synthetase (ACS) catalyzes this reaction and an ACS-encoding gene (*acs*) has been cloned and sequenced from *M. soehngenii* and expressed in *E. coli* resulting in the synthesis of an active enzyme (Eggen et al., 1991a). Immediately downstream from this *acs* gene are two ORFs that resulted either from a sequencing error or a frame-shift mutation as combining their sequences generates a second ACS encoding gene (B. Baum, personal communication). The genome of *M. soehngenii* apparently therefore contains, or once contained, two tandemly arranged *acs* genes that could encode similar but not identical ACS enzymes that are separated by a 386bp intergenic region.

Carbon monoxide dehydrogenase (CODH) cleaves the C-C bond of the acetyl moiety of acetyl-CoA to release methane (see Part II, Chapter 6). The genes *cdhA* and *cdhB*, that encode the large and small subunits of CODH, have also been cloned and sequenced from *M. soehngenii* (Eggen et al., 1991b). They are separated by 19 bp and form a single transcriptional unit, *cdhAB*, that is not closely linked to the *acs* gene(s) in the *M. soehngenii* genome. Expression of the *cdhAB* gene cluster in *E. coli* results in the synthesis of the 89 Kd and 21 Kd encoded polypeptides but they lack CODH catalytic activity.

Methanosarcina species can also dissimilate acetate to methane but, unlike *M. soehngenii* which uses only acetate as a carbon and energy source, the *Methanosarcinae* can also catabolize methanol or methylamines to methane (see Part II, Chapter 5). In the presence of these more energetically favorable substrates *Methanosarcina* species do not synthesize CODH (Terlesky et al., 1986; Bhatnagar et al., 1987) and this substrate-regulated synthesis of CODH has been shown to occur at the level of transcription initiation in *Methanosarcina thermophila*. The gene (*cdhA*) that encodes the large subunit of CODH in *M. thermophila* has been cloned and sequenced and its transcription *in vivo* shown to be dependent on the presence of acetate and absence of methanol (K.R. Sowers, T.T. Thai and R.P. Gunsalus, personal communication). Different methyl transferases catabolize methanol, mono-, di- and tri-methylamines in *Methanosarcina barkeri* and these enzymes are also synthesized in response to the availabilty of the different substrates. When cloned, the genes encoding these substate-induced methyltransferases should be very useful as model systems to study gene regulation in *M. barkeri*.

12.5 Genes for Components of the Transcription and Translation Machineries

12.5.1 Elongation Factors, Ribosomal Proteins and RNA Polymerases

Methanogen ribosomes are intermediate in size between bacterial and eukaryal ribosomes. They contain bacterial-sized 5S, 16S and 23S rRNAs but additional

ribosomal proteins (r-proteins), some of which are related to r-proteins found previously only in eukaryal ribosomes (Auer et al., 1989a). Methanogen ribosomes also exhibit a mixed pattern of sensitivities *in vitro* to antibiotics which inhibit translation by modifying or binding to ribosomal components generally thought of as either specifically bacterial or eukaryal. The inhibition of a *M. vannielii* derived *in vitro* translation system by diphtheria toxin (Lechner et al., 1988) predicted the presence of elongation factors (EF) related to eukaryal EFs. This was confirmed when EF2 and EF1α encoding genes were cloned and sequenced from *M. vannielii*. They are located within a cluster r-protein genes, arranged ORF1-ORF2-S12-S7-EF2-EF1α-S10 (Lechner and Böck, 1987; Auer et al., 1989a; Iwake et al. 1989; Lechner et al., 1989) which is similar to that of the *E. coli* streptomycin operon however the *M. vannielii* ORF1 encodes a protein that is not found in *E. coli* but is 37% identical to rat r-protein L-30. Three other r-protein encoding clusters of genes have also been cloned and sequenced from *M. vannielii* that appear to be the counterparts of the *E. coli* spectinomycin, S10 and L1 operons (Auer et al., 1989a,b; Baier et al., 1990). The *M. vannielii* spectinomycin operon contains genes arranged ORFa-ORFb-S17-L14-L24-ORFc-L5-S14-S8-L6-ORFd-ORFe-L18-S5-L30-L15 (Auer et al., 1989b). This is the same order of r-protein genes as in *E. coli* but with the addition of the S17 gene and ORFs, c,d, and e, which encode proteins most closely related to mouse L32, rat L19 and yeast S6. Located immediately upstream of the *M. vannielii* spectinomycin operon is the *M. vannielii* equivalent of the *E. coli* S-10 operon. In the methanogen, this operon actually lacks the S10 encoding gene but, as in *E. coli*, does contain the L22-S3-L29 cluster of r-genes.

In *E. coli*, the L1 gene is cotranscribed with the L11 gene in the sequence L11-L1 (Post et al., 1979) and binding of the L1 r-protein to this transcript prevents its translation. In *M. vannielii*, the transcriptional unit appears to be L1-L10-L12 (Baier et al., 1990) but, as the primary and secondary structures surrounding the L1 binding site are conserved, a L1-based autoregulatory system of translation control of r-protein synthesis may also be present in the methanogen. The L10 and L12 genes are also located directly downstream from the L1 gene in *E. coli* but in a separate transcriptional unit that contains the genes encoding the β and β' subunits of the *E. coli* DNA-dependent RNA-polymerase (RNAP) (Post et al., 1979). In *M. vannielii*, the genes encoding the B″ and H subunits of RNAP are not adjacent to the L10-L12 genes. They are, however, close to r-protein genes, located within a cluster of genes immediately upstream of its spectinomycin operon (Stobel et al., 1988; Auer et al., 1989 a,b; Klenk et al., 1992). Genes encoding the A+C, B″ and B′ subunits of RNAP in *Methanobacterium thermoautotrophicum* have also been cloned and sequenced (Berghöffer et al., 1988; Schallenberg et al., 1988). The primary sequences obtained for the *M. thermoautotrophicum* and the *M. vannielii* RNAP encoding genes confirm that these archaeal RNAPs are more closely related to eukaryal than to bacterial RNAPs

(Berghöffer et al., 1988; Schallenberg, et al., 1988; Zillig et al., 1988; Klenk et al., 1992).

12.5.2 tRNA, rRNA and 7S RNA Genes

Clusters of related tRNA encoding genes have been cloned and sequenced from *M. vannielii*, *M. voltae* and *M. fervidus* (Wich et al., 1984, 1986a,b, 1987; Haas et al., 1989, 1990). The secondary structures predicted for these tRNAs indicate that tRNAs in the hyperthermophile *M. fervidus* have increased numbers of base-pairs and increased numbers of G-C base pairs relative to A-U base-pairs (Haas et al., 1989). Four tRNA genes have also been cloned and sequenced from the unrelated hyperthermophile *Methanopyrus kandleri* (Huber et al., 1990) and these tRNAs are predicted to have secondary structures that are almost exclusively formed from G-C base pairs (J.R. Palmer, C.J. Daniels and J.N. Reeve, unpublished results). The *M. kandleri* tRNA genes also appear to encode the 3′ terminal CCA sequence of the mature tRNAs, a feature not found in many other methanogen or eukaryal tRNA genes but commonly found in bacterial tRNA encoding genes. As in *Bacteria*, there are also tRNA genes associated with methanogen rRNA operons. There is one 16S-23S-5S rRNA operon in the genome of *M. voltae* (Wich et al., 1987), two in *M. thermoautotrophicum* (Willekens et al., 1986; Østergaard et al., 1987), *M. formicicum* (Lechner et al., 1985), *M. fervidus* (Haas et al., 1990) and *M. soehngenii* (Eggen et al., 1989, 1990) and four in *M. vannielii* (Jarsch et al., 1983; Jarsch and Böck 1983, 1985a,b). In most cases, there is a $tRNA^{Ala}$ located between the 16S and 23S rRNA encoding sequences and immediately upstream of one of the two rRNA operons, in *M. thermoautotrophicum* and *M. fervidus*, is a $tRNA^{Ser}$ gene (Østergaard et al., 1987; Haas et al., 1990). Additional 5S rRNA genes have also been located and sequenced within clusters of tRNA genes in *M. vannielii* and *M. voltae* (Wich et al., 1984, 1987). *M. kandleri* contains only one 16S and one 23S rRNA gene and these do not appear to be closely linked, indicating that this methanogen does not contain a 16S-23S-5S rRNA operon (J.R. Palmer, C.J. Daniels and J.N. Reeve, unpublished results).

All *Archaea* so far investigated contain large amounts of a transcript, approximately 300 nucleotide in length, which has been designated the 7S RNA (Luehrsen et al., 1985). In *Halobacterium halobium* the 7S RNA is physically associated with ribosomes synthesizing the membrane protein bacterioopsin (Gropp et al., 1992) and archaeal 7S RNAs have secondary structures predicted to be similar to that of the 7S RNA found in eukaryal signal recognition particles (Haas et al., 1990; Kaine 1990). Both these features adumbrate a role for the archaeal 7S RNA in the synthesis and/or localization of membrane or secreted proteins. The discovery that the 7S RNA encoding gene is located directly upstream of the $tRNA^{ser}$-rRNA operon in *M. fervidus* and *M. thermoautotrophicum* (Haas et al,

1990), and may be cotranscribed with these translation-related stable RNAs (Koller et al., 1992), also hints at a role for the 7S RNA in translation. Genes encoding the 7S RNA have also been cloned and sequenced from *Methanococcus voltae* and *Methanosarcina acetivorans* but their linkage to $tRNA^{Ser}$ or rRNA operons was not reported (Kaine and Merkel, 1989; Kaine, 1990).

Introns have been found in tRNA genes and rRNA genes in non-methanogenic *Archaea* (Daniels et al., 1985, Kjems and Garrett, 1985; Kaine 1987) but there is no evidence, to date, for introns in stable RNA-encoding genes in methanogens.

12.5.3 Isoleucyl-tRNA Synthetase

The wild-type and a mutated version of the *ile*S gene, which encodes isoleucyl-tRNA synthetase in *M. thermoautotrophicum* strain Marburg, have been cloned and sequenced (Jenal et al., 1991). The mutation substitutes an aspartic acid residue for a glycine residue at position 590 of the 1045 amino acid sequence, resulting in an enzyme with a much decreased affinity for pseudomonic acid. This mutation confers pseudomonic acid resistance on *M. thermoautotrophicum* but expression of the mutated *ile*S gene in *E. coli* does not increase the resistance of *E. coli* to this isoleucine analogue. Bacterial and eukaryal isoleucyl-tRNA synthetases form an evolutionarily homologous group (Burbaum and Schimmel, 1991) and the sequence of the *M. thermoautotrophicum ileS* gene demonstrates that this archaeal enzyme is also a member of the class I group of isoleucyl-tRNA synthetases.

12.6 Macromolecular Surface Structures

12.6.1 Flagella

Archaeal flagella are thinner than bacterial flagella and may contain several different flagellin polypeptides (Kalmokoff et al., 1990; Southam et al., 1990). Two flagellin encoding transcriptional units, *flaA* and *flaB1-flaB2-flaB3*, that are physically adjacent have been cloned and sequenced from the *M. voltae* genome (Kalmokoff and Jarrell, 1991). The encoded flagellins have similar but not identical amino acid sequences and appear to be synthesized as precursors, with short N-terminal leader peptides, that are not present in the mature proteins. A similar precursor structure has been documented for the flagellin of non-methanogenic, halophilic *Archaea* (Kalmokoff et al., 1990). The *M. voltae fla* gene sequences indicate that the encoded flagellins have several sites that could be glycosylated although glycosylation *per se* has not been demonstrated.

12.6.2 Surface-Layer Proteins

The surface layers (S-layer) of the closely related, hyperthermophilic methanogens, *Methanothermus fervidus* and *Methanothermus sociabilis* are formed by glycoprotein arrays. The genes (*slgA*) that encode the protein components of these S-layer glycoproteins have been cloned and sequenced (Bröckl et al., 1991). Both *slgA* genes encode 593 amino-acid residues which differ at only three locations. The encoded proteins are predicted to be synthesized as precursors with amino-terminal leader peptides that conform closely to the consensus structure established for bacterial signal sequences. These leader peptides, 22 amino acid residues in length, are cleaved from the mature protein at sites very similar to the consensus cleavage site employed by bacterial signal peptidases. Maturation of these S-layer proteins also requires the addition of mannose, 3-*O*-methyl glucose, galactose, *N*-acetyl-glucosamine and *N*-acetyl-galactosamine moieties which together constitute ~17% of the final mass of the glycoproteins (Hartmann and König, 1989). The intergenic regions upstream of the two *slgA* genes are conserved and contain readily identifiable TATA boxes which direct transcription initiation 33 bp upstream of GTG translation initiation codons. Downstream of the *M. fervidus slgA* gene is an oligo-T sequence, typical of *M. fervidus* transcription terminators, but this sequence is not present downstream of the *M. sociabilis slgA* gene (Bröckl, et al., 1991).

12.7 Gene Regulation

As described in this Chapter, many methanogen genes have now been cloned and sequenced. With improvements in handling procedures and the development of genetic systems, the regulation of expression of these genes in methanogens can now be studied. Determining how growth regulates the expression of the MRI, MRII and MVH operons (Steigerwald, et al., 1992) and how substrate availability controls CODH synthesis (Terlesky et al., 1986; Bhatnagar et al., 1987) are important current topics in methanogenesis that should now be experimentally tractable. Results, perhaps of wider interest, should also soon be forthcoming from studies of *nif* gene regulation (Gohl et al., 1992) and the heat-shock response in methanogens (Herbert et al., 1991, Macario et al., 1991). Analogies and homologies with bacterial and eukaryal paradigms of gene regulation will, undoubtedly, continue to be sought—and should continue to be informative.

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