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# Biochemistry of Methanogenesis

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**ABSTRACT:** Methane is a product of the energy-yielding pathways of the largest and most phylogenetically diverse group in the *Archaea*. These organisms have evolved three pathways that entail a novel and remarkable biochemistry. All of the pathways have in common a reduction of the methyl group of methyl-coenzyme M ($CH_3$-S-CoM) to $CH_4$. Seminal studies on the $CO_2$-reduction pathway have revealed new cofactors and enzymes that catalyze the reduction of $CO_2$ to the methyl level ($CH_3$-S-CoM) with electrons from $H_2$ or formate. Most of the methane produced in nature originates from the methyl group of acetate. CO dehydrogenase is a key enzyme catalyzing the decarbonylation of acetyl-CoA; the resulting methyl group is transferred to $CH_3$-S-CoM, followed by reduction to methane using electrons derived from oxidation of the carbonyl group to $CO_2$ by the CO dehydrogenase. Some organisms transfer the methyl group of methanol and methylamines to $CH_3$-S-CoM; electrons for reduction of $CH_3$-S-CoM to $CH_4$ are provided by the oxidation of methyl groups to $CO_2$.

**KEY WORDS:** methanogenesis, one-carbon metabolism, metalloproteins, electron transport, *Archaea*.

## I. INTRODUCTION

Biological methanogenesis occurs in a diversity of anaerobic habitats such as the rumen, the lower intestinal tract, sewage digestors, landfills, freshwater sediments of lakes and rivers, rice paddies, hydrothermal vents, and coastal marine sediments. The conversion of complex organic matter to methane requires a microbial food chain (consortium) composed of at least three interacting metabolic groups of anaerobic microorganisms. The fermentative bacteria degrade polymers to $H_2$, $CO_2$, formate, acetate, and higher volatile fatty acids, while the acetogenic bacteria convert the latter to acetate and either $H_2$ or formate. Methanogenic organisms constitute the final group in the consortium. About two thirds of the methane produced in nature derives from reduction of the methyl group of acetate, and about one third from reduction of $CO_2$ with electrons from $H_2$ or formate. Lesser amounts of methane are produced by the oxidative and reductive dismutation of methanol or methylamines. Recently, methanogenic organisms have been described that produce methane from dimethyl sulfide or reduce $CO_2$ with primary, secondary, and cyclic alcohols as electron donors.[1–5] All of the pathways are variations on the theme of methyl group reduction to methane, the major energy-conserving step. Methanogenic microorganisms represent the largest and most diverse group within the *Archaea*.[6,7]

Several excellent reviews have appeared recently on the biochemistry of methanogenesis from specific substrates or specific organisms and the bioenergetics of methanogenesis.[8–11] This review includes advances from the past 5 years on the biochemistry of methanogenesis from each of the major substrates. Other recent reviews have covered the genetics and general aspects of methanogenic organisms.[6,12–14]

## II. REDUCTION OF CARBON DIOXIDE TO METHANE

The reduction of $CO_2$ to $CH_4$ with $H_2$ or

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formate as the electron donor (Reactions 1 and 2) was the first pathway of methanogenesis to be studied. The

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$

$$\Delta G^{\circ\prime} = -130.4 \text{ kJ/mol } CH_4 \quad (1)^{15}$$

$$4HCOO^- + 4H^+ \rightarrow CH_4 + 3CO_2 + 2H_2O$$

$$\Delta G^{\circ\prime} = -119.5 \text{ kJ/mol } CH_4 \quad (2)^{15}$$

$CO_2$-reduction pathway (Figure 1) is derived mostly from studies with *Methanobacterium thermoautotrophicum* strains ΔH and Marburg; although they are classified as strains of the same species, the fact that they are only distantly related may explain some differences reported between them. Studies with these organisms have revealed several novel cofactors (Figure 2) involved in the $CO_2$-reduction pathway and other pathways for methanogenesis; the structure and function of these cofactors is the subject of a recent review.[16]

## A. Reduction of Carbon Dioxide to the Formyl Level

The reduction of $CO_2$ to the formyl level is catalyzed by formyl-methanofuran dehydrogenase. The structure of methanofuran (MF) is shown in Figure 2;[17] variations in structure are dependent on the genus.[16] Formyl-MF is the first stable intermediate in the pathway. The reaction, unlike all other $CO_2$ fixation reactions, involves bonding of $CO_2$ to a primary amine followed by a two-electron reduction (Reaction 3). Enzyme activity in the reverse

$$CO_2 + MF + H_2 \rightarrow \text{formyl-MF} + H_2O$$

$$\Delta G^{\circ\prime} = +16 \text{ kJ/mol} \quad (3)^{18}$$

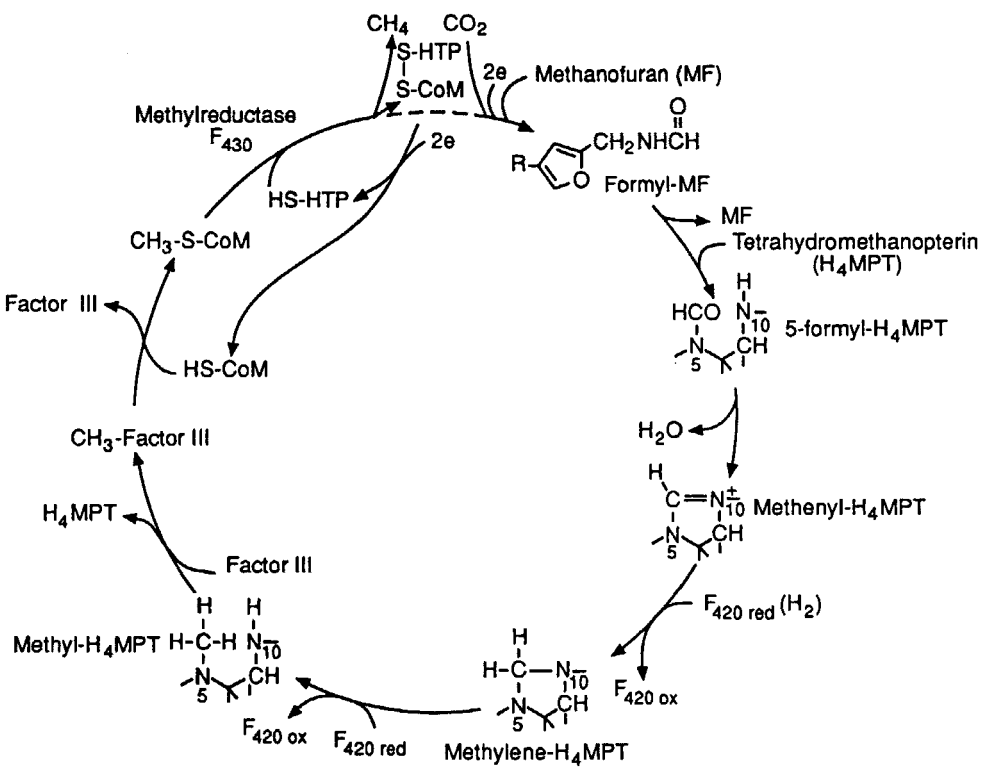


**FIGURE 1.** The pathway of $CO_2$-reduction to $CH_4$. (Modified from DiMarco et al.[16]) (See Figure 2 for the complete structures of cofactors.)

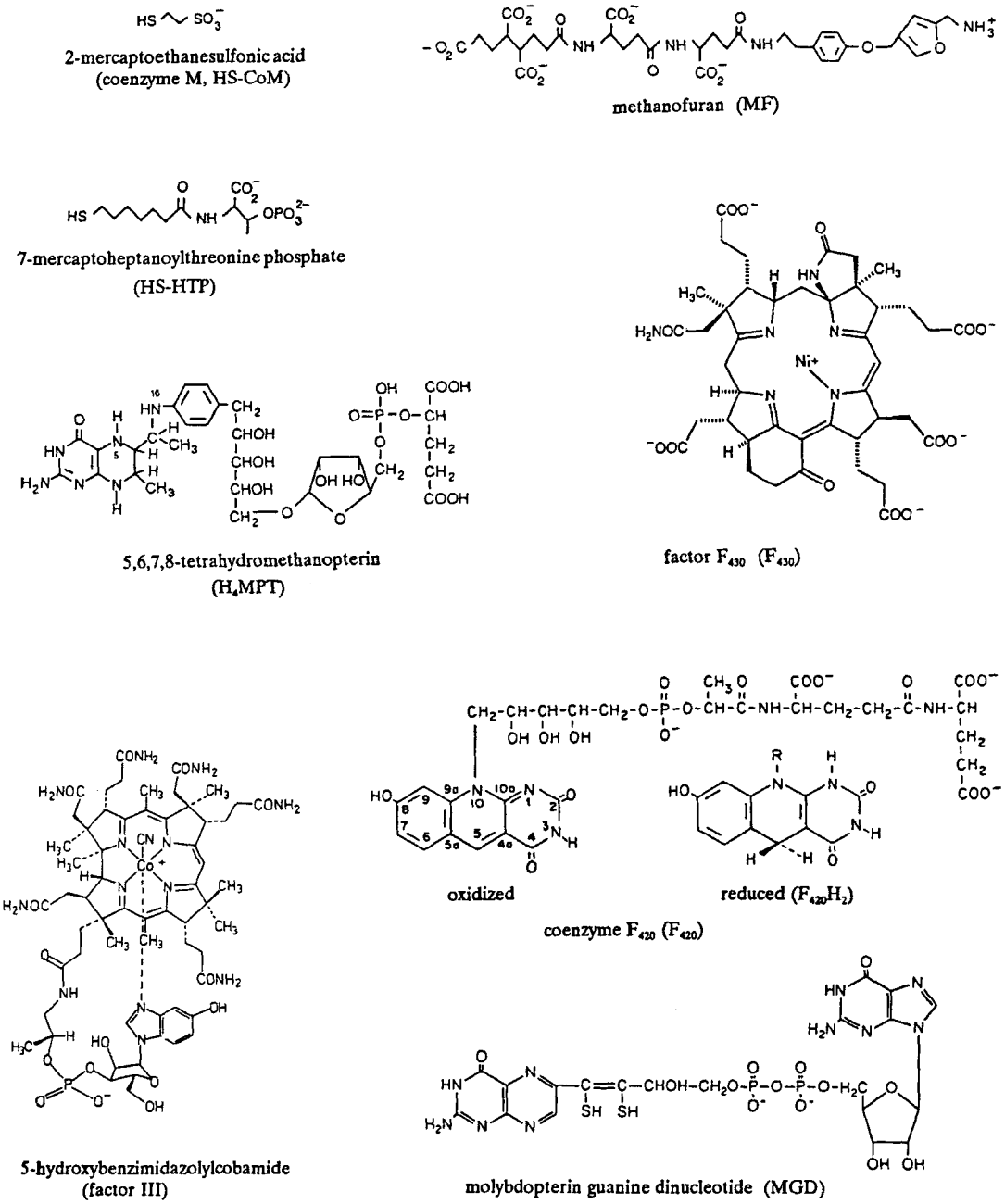


**FIGURE 2.** The structures of one-carbon carriers and cofactors involved in methanogenic pathways.

direction is linked to the reduction of either methylviologen or coenzyme $F_{420}$ in cell extracts of *M. thermoautotrophicum* strain Marburg; the natural electron donor for the physiologically relevant direction is unknown.[19] The minimum structure recognized by the enzyme is the furfurylamine moiety of MF.[20] Enzyme activity is rapidly inhibited by cyanide, an observation that is consistent with the presence of a cyanide-sensitive metal site.[19] The formyl-MF dehydrogenase purified from *M. thermoautotrophicum* strain Marburg is a 110,000-Da iron-sulfur enzyme that contains at least two subunits with apparent molecular masses of 60,000 and 45,000 Da.[21] The enzyme also contains one molecule of either molybdopterin adenine dinucleotide, molybdopterin hypoxanthine dinucleotide, or molybdopterin guanine dinucleotide (Figure 2).[22] More than 60%

of formyl-MF dehydrogenase activity is associated with the pellet obtained after ultracentrifugation of *M. thermoautotrophicum* cell extracts.[19] Further studies are necessary to determine the location of the enzyme; however, its association with the membrane would support the proposal that the endergonic reduction of $CO_2$ to formyl-MF with $H_2$ (Reaction 3) is driven by a sodium gradient as opposed to hydrolysis of ATP.[23–26] In cell extracts of *M. thermoautotrophicum* strain ΔH, the synthesis of formyl-MF from $H_2$ and $CO_2$ requires activation by the heterodisulfide (CoM-S-S-HTP) of the two coenzymes involved in the final step (Reaction 10) of the pathway.[27] Thus, $CO_2$-reduction to $CH_4$ is depicted as a circular pathway (Figure 1) to indicate that the first and last steps are linked through CoM-S-S-HTP. Results suggest that CoM-S-S-HTP activates an unknown electron carrier required for electron flow from $H_2$ for the reduction of $CO_2$ to formyl-MF;[28] ferredoxin is a likely candidate because the unknown carrier can reduce metronidazole.

## B. Reduction of the Formyl Group to the Formaldehyde Level

Prior to reduction, the formyl group is transferred to 5,6,7,8-tetrahydromethanopterin ($H_4$MPT), as shown in Reaction 4, and then converted to the methenyl derivative by a dehydrating cyclization as shown in Reaction 5.

$$\text{formyl-MF} + \text{H}_4\text{MPT} \rightarrow \text{5-formyl-H}_4\text{MPT} + \text{MF}$$

$$\Delta G^{\circ\prime} = -4.4 \text{ kJ/mol} \qquad (4)^{18}$$

$$\text{5-formyl-H}_4\text{MPT} + \text{H}^+ \rightarrow \text{5,10-methenyl-H}_4\text{MPT}^+ + \text{H}_2\text{O}$$

$$\Delta G^{\circ\prime} = -4.6 \text{ kJ/mol} \qquad (5)^{18}$$

The structure of $H_4$MPT is shown in Figure 2;[29] variations in the structure are found in various genera.[16,30,31] Reaction 4 is catalyzed by formyl-MF:$H_4$MPT formyltransferase (FTR). The ftr gene encoding the oxygen-insensitive enzyme (a tetramer of identical subunits with $M_r$ = 41,000) from *M. thermoautotrophicum* strain ΔH has been sequenced and expressed in *Escherichia coli* in a catalytically active form.[32] Although $H_4$MPT is structurally related to tetrahydrofolates, the amino acid sequence deduced from the ftr gene has no significant identity with other folate-dependent proteins. Conversion of 5-formyl-$H_4$MPT to the 5,10-methenyl derivative (Reaction 5) is catalyzed by cyclohydrolase. The oxygen-stable 5,10-methenyl-$H_4$MPT$^+$ cyclohydrolase purified from *M. thermoautotrophicum* strain ΔH is composed of two identical 41,000-Da subunits;[33] the enzyme has been partially purified from *M. thermoautotrophicum* strain Marburg.[34] The cyclohydrolase purified from the extreme thermophile *Methanopyrus kandlei* is a 42,000-Da monomer that contains no prosthetic groups and requires high concentrations of potassium phosphate for activity.[35]

The reduction of 5,10-methenyl-$H_4$MPT$^+$ to the formaldehyde level with reduced coenzyme $F_{420}$ is shown in Reaction 6a. Coenzyme $F_{420}$

$$\text{5,10-methyl-H}_4\text{MPT}^+ + \text{F}_{420}\text{H}_2 \rightarrow \text{5,10-methylene-H}_4\text{MPT} + \text{F}_{420} + \text{H}^+$$

$$\Delta G^{\circ\prime} = +6.5 \text{ kJ/mol} \qquad (6a)^{36}$$

($F_{420}$) is an obligate two-electron carrier (redox potential near −350 mV) that donates or accepts a hydride ion (structure[37], Figure 2). The 5,10-methylene-$H_4$MPT derivative is also formed nonenzymatically with formaldehyde. The 5,10-methylene-$H_4$MPT dehydrogenase catalyzing Reaction 6a has been purified aerobically from *M. thermoautotrophicum* strain Marburg.[34] When assayed by monitoring the disappearance of 5,10-methylene-$H_4$MPT, activity becomes increasingly dependent on $F_{420}$ as an electron acceptor during the purification procedure or after exposure to air. This behavior is explained by the recent discovery that a genetically distinct dehydrogenase ($H_2$-forming) utilizes protons as an electron acceptor replacing $F_{420}$.[38,39] In addition, $H_2$ serves as an electron donor in the forward direction (Reaction 6b). Thus, the

$$\text{5,10-methenyl-H}_4\text{MPT}^+ + \text{H}_2 \rightarrow \text{5,10-methylene-H}_4\text{MPT} + \text{H}^+$$

$$\Delta G^{\circ\prime} = -5.5 \text{ kJ/mol} \qquad (6b)^{18}$$

$H_2$-forming dehydrogenase from *M. thermoautotrophicum* strain Marburg has reversible hydrogenase activity; however, the enzyme is unable to reduce $F_{420}$ or methylviologen and does not appear to contain metal centers or cofactors, properties that suggest this is a new class of hydrogenase. In contrast to the $F_{420}$-reducing enzyme, which is composed of eight identical subunits with an apparent molecular mass of 32,000 Da, the $H_2$-forming dehydrogenase from strain Marburg is a 43,000-Da monomer unable to reduce $F_{420}$ with 5,10-methylene-$H_4$MPT.[38] The $H_2$-forming dehydrogenase from *M. kandleri* is similar in properties to the $H_2$-forming enzyme from *M. thermoautotrophicum* strain Marburg except it is stable for 60 min at 90°C.[36] Recently, the $F_{420}$-reducing 5,10-methylene-$H_4$MPT dehydrogenase was purified from *M. thermoautotrophicum* strain ΔH with high specific activity.[40] The aerobically purified enzyme is strictly dependent on $F_{420}$ for the reversible oxidation of 5,10-methylene-$H_4$MPT. It is purified as an apparent hexamer of six identical 36,000-Da subunits and contains no cofactors or metal centers. Steady-state kinetic studies indicate that the reaction occurs by a ternary complex mechanism in agreement with a direct hydride transfer to and from $F_{420}$. The 5,10-methylene-$H_4$MPT dehydrogenases from either strain of *M. thermoautotrophicum* do not appear to be integral membrane proteins.

## C. Reduction of the Methylene Group to the Methyl Level

The 5,10-methylene-$H_4$MPT reductase utilizes reduced $F_{420}$ ($F_{420}H_2$) as the physiological electron donor for Reaction 7. This oxygen-stable

$$\text{5,10-methylene-}H_4\text{MPT} + F_{420}H_2 \rightarrow \text{5-methyl-}H_4\text{MPT} + F_{420}$$

$$\Delta G^{\circ\prime} = -5.2 \text{ kJ/mol} \qquad (7)^{18}$$

enzyme, purified from *M. thermoautotrophicum* strain ΔH, is composed of a single subunit of $M_r$ = 35,000.[41] The reaction proceeds in either direction; however, the physiologically relevant methylene reduction is thermodynamically favored. In addition, the velocity of the forward reaction is 26-fold greater than that of the reverse. No flavins or iron-sulfur clusters are present, in contrast to 5,10-methylene-tetrahydrofolate reductases from eukaryotic and eubacterial sources. With $H_2$ as the source of electrons (Reaction 8), the reduction

$$\text{5,10-methylene-}H_4\text{MPT} + H_2 \rightarrow \text{5-methyl-}H_4\text{MPT}$$

$$\Delta G^{\circ\prime} = -14 \text{ kJ/mol} \qquad (8)^{41}$$

is exergonic and therefore could be associated with the generation of a primary electrochemical potential. The reductase from *M. thermoautotrophicum* strain ΔH is present in the soluble fraction, an observation that argues against this proposal; however, it cannot be ruled out that the enzyme is loosely associated with the membrane and is dislodged during cell disruption. The reductase isolated from *M. thermoautotrophicum* strain Marburg has properties similar to those described from strain ΔH except the native enzyme has an apparent molecular mass of 150,000 Da.[42] The reductase from strain Marburg can also be purified in a single step by affinity chromatography with Blue Sepharose CL-6B® and binding is competitive with $F_{420}$.[43] Recently, the reductase has been purified from the extreme thermophile *M. kandleri*;[44] the enzyme is similar to those described for strains of *M. thermoautotrophicum* except it has maximum activity at 90°C and requires high concentrations of sulfate or phosphate for activity.

## D. Conversion of the Methyl Group to Methane

### *1. Transfer of the Methyl Group to Coenzyme M*

Prior to reduction, the methyl group of 5-methyl-$H_4$MPT is transferred to coenzyme M (HS-CoM), as depicted in Reaction 9. The structure of HS-CoM is

$$\text{5-methyl-}H_4\text{MPT} + \text{HS–CoM} \rightarrow \text{CH}_3\text{–S–CoM} + H_4\text{MPT}$$

$$\Delta G^{\circ\prime} = -29.7 \text{ kJ/mol} \qquad (9)^{18}$$

shown in Figure 2.[45] Methanogenic organisms contain high concentrations of two types of corrinoids that are involved in methyltransfer reactions: factor III (Coα-[α-(5-hydroxybenzimidazolyl)]-Coβ-cyanocobamide) and pseudo vitamin $B_{12}$ (Coα-[α-(7-adenyl)]-Coβ-cyanocobamide).[46,47] Factor III (Figure 2) predominates in *Methanobacterium*.[46] The conversion of formaldehyde to $CH_3$-S-CoM in cell extracts of *M. thermoautotrophicum* strain ΔH has been exploited as a means to study methyl transfer;[48] formaldehyde reacts nonenzymatically with $H_4$MPT to form 5,10-methylene-$H_4$MPT. When electrons for the reduction of 5,10-methylene-$H_4$MPT are supplied by $H_2$, the conversion of formaldehyde to $CH_3$-S-CoM requires hydrogenase, 5,10-methylene-$H_4$MPT reductase, and a 5-methyl-$H_4$MPT:HS-CoM transferase system. The intermediary formation of $CH_3$-factor III is detected during $CH_3$-S-CoM formation, a fact consistent with the involvement of a factor III-containing methyltransferase that accepts the methyl group from 5-methyl-$H_4$MPT.[48] Indeed, a methyltransferase was purified recently from *M. thermoautotrophicum* strain ΔH.[49] The enzyme is a high-molecular-weight complex of 100,000 Da units comprised of subunits with apparent molecular masses of 35,000, 33,000, and 31,000 Da. The methyltransferase contained factor III that could be methylated with 5-methyl-$H_4$MPT. The formation of $CH_3$-factor III, coupled with the previously reported methylcobalamin:HS-CoM methyltransferase present in $CO_2$-reducing species, indicates that two enzymes may be involved in transfer of the methyl group from 5-methyl-$H_4$MPT to HS-CoM. The 5-methyl-$H_4$MPT:HS-CoM transferase system is oxygen sensitive and requires reactivation dependent on catalytic amounts of ATP and a $H_2$ atmosphere, both of which can be replaced with the strong reductant titanium(III)citrate.[50,51] These properties are consistent with reduction of the cobalt atom of factor III to Co(I) (a supernucleophile) that is necessary to accept the methyl group of 5-methyl-$H_4$MPT. More recently, it was shown that CoM-S-S-HTP strongly diminishes the requirement for ATP in reductive activation of transferase activity.[52] The mechanism by which ATP and CoM-S-S-HTP assist the reactivation is unknown; however, it is conceivable that a CoM-S-S-HTP-dependent electron carrier is required, as postulated for the activation and reduction of $CO_2$ to formyl-MF.[28] Interestingly, the formaldehyde to $CH_3$-S-CoM activity is soluble; however, it cannot be ruled out that the system is loosely bound to the membrane and becomes soluble only on cell disruption. Methyl transfer to HS-CoM has been investigated in *M. thermoautotrophicum* strain ΔH, utilizing $CH_3$-S-CoM methylreductase as a reagent to produce $CH_4$ that is very sensitive to detection by flame-ionization gas chromatography.[53] The methylreductase complements a membrane-associated enzyme complex, which allows conversion of the methyl group of 5-methyl-$H_4$MPT to $CH_4$. These results indicate that the complex contains a 5-methyl-$H_4$MPT:HS-CoM methyltransferase (MT) system. A new activity, methyl-$B_{12}$:$H_4$MPT methyltransferase ($MT_{1b}$), can be detected that is the reverse of the physiological direction. Additional resolution of the MT system yields a fraction that converts methyl-$B_{12}$ to $CH_3$-S-CoM ($MT_2$ activity). These results provide additional support to the proposal that a two-enzyme system operates in transfer of the methyl group of 5-methyl-$H_4$MPT to HS-CoM with $CH_3$-factor III as an intermediate.[50] However, unlike the formaldehyde to $CH_3$-S-CoM system,[50] the $CH_3$-S-CoM methylreductase-coupled assay localizes the MT system to the membrane.[53] The physiological role for membrane association of the MT system is obscure; however, since the complex also contains 5,10-methylene-$H_4$MPT reductase, it is postulated that the essentially irreversible Reaction 9 may be thermodynamically coupled to Reaction 7 for the purpose of generating an electrochemical potential.[53] In this respect, it is interesting to note that a factor III-containing integral membrane protein has been purified and characterized in *M. thermoautotrophicum* strain Marburg.[54] The 33,000-Da protein is present in a complex with a molecular mass of 500,000 Da that comprises about 8% of the total membrane protein. The 33,000-Da protein contains two Fe atoms for each Co atom. No function can be assigned, but it is interesting that polyclonal antiserum raised against the factor III-containing 33,000-Da protein from strain Marburg cross reacts with two subunits of an enriched fraction from strain Δ H that contains 5-methyl-$H_4$MPT:factor III methyltransferase activity.[55] In addition, it is also hypothesized that the factor III-containing membrane protein could

potentially be involved in electron transport.[54] The inability of reduced vitamin $B_{12}$ to convert, 5,10-methylene-$H_4$MPT to 5-methyl-$H_4$MPT suggests that this reduction is not mediated by corrinoids.[53] Interestingly, corrinoids are implicated in the reductive dechlorination of one-carbon compounds and CO is a product of the reaction.[56,57]

### 2. Reductive Demethylation of $CH_3$-S-CoM to Methane

The $CH_3$-S-CoM methylreductase catalyzes Reaction 10. In the final reductive step of the pathway, CoM-S-S-HTP is reduced to the respective sulfhydryl cofactors (Reaction 11).[58,59]

$$CH_3\text{–}S\text{–}CoM + HS\text{-}HTP \rightarrow CH_4 + CoM\text{–}S\text{–}S\text{–}HTP$$

$$\Delta G^{\circ\prime} = -45 \text{ kJ/mol} \qquad (10)^{60}$$

$$CoM\text{–}S\text{–}S\text{–}HTP + H_2 \rightarrow HS\text{–}CoM + HS\text{–}HTP$$

$$\Delta G^{\circ\prime} = -40 \text{ kJ/mol} \qquad (11)^{18}$$

Methylreductases have been studied extensively from both the ΔH and Marburg strains of *M. thermoautotrophicum*. The enzyme purified from both strains is 300,000 Da and is composed of three different subunits with molecular masses of 65,000 Da (α), 46,000 Da (β), and 35,000 Da (γ) in an $\alpha_2\beta_2\gamma_2$ configuration. Electron microscopy of the enzyme from strain ΔH indicates that the subunits are arranged as an eclipsed pair of open trimers with a central stain-penetrating region.[61] The 300,000-Da enzyme from *M. thermoautotrophicum* strain Marburg migrates with an apparent $M_r$ = 150,000 on polyacrylamide gels under nondenaturing conditions; however, the cofactor content and activity of the $M_r$ = 150,000 species was not reported.[59] The native (300,000-Da) enzyme contains two molecules of coenzyme $F_{430}$ ($F_{430}$), which are tightly, but not covalently, bound. Methylreductase purified from the extreme thermophile *M. kandleri* has properties similar to the enzyme from the moderately thermophilic *M. thermoautotrophicum*.[62] The electron donor for the methylreductase isolated from *M. thermoautotrophicum* strains ΔH and Marburg is 7-mercaptoheptanoylthreonine-phosphate (HS-HTP);[59,63,64] the structure is shown in Figure 2.[65] The enantiomer containing the D-form of threonine is inactive.[66] Analog pairs of HS-CoM and HS-HTP in which the number of methylene or side carbons were changed (but not the total number of carbons in the heterodisulfide) did not substitute for the native cofactors, a result that indicates that the overall length of each cofactor is important for catalytic competence.[67] Recently, MRF (methyl reducing factor) was described from *M. thermoautotrophicum*, which contains HS-HTP bound to a UDP-disaccharide through a carboxylic-phosphoric anhydride linkage;[68] the structure is uridine 5′-[*N*-(7-mercaptoheptanoyl)-*O*-3-phosphothreonine-*P*-yl(2-acetamido-2-deoxy-β-mannopyranuronosyl) (acid anhydride)]-(1 → 4)-*O*-2-acetamido-2-deoxy-α-glucopyranosyl diphosphate. It is postulated that hydrolysis of the unstable anhydride linkage could result in the release of HS-HTP during purification.[68] MRF has a sixfold lower $K_m$ than HS-HTP and a 50% greater $V_{max}$, suggesting that the UDP-disaccharide moiety may be important in binding MRF to $CH_3$-S-CoM methylreductase. Inhibition of methylreductase activity with an oxidized uridine-5′-diphospho-*N*-acetylglucosamine derivative supports involvement of the UDP-disaccharide in cofactor binding.[69] However, an improved method for purification of methylreductase from *M. thermoautotrophicum* strain Marburg yields an enzyme with HS-HTP-dependent activity equal to that in whole cells, suggesting derivatives of HS-HTP are not required for maximum activity.[70]

$F_{430}$ is a yellow, nickel-containing porphinoid (Figure 2).[71] The cofactor is present in cells in both the free and methylreductase-bound form but only the latter is active. $F_{430}$ is slowly oxidized in air, yielding the blue-colored 12,13-didehydro form ($F_{560}$); apparently, the oxidation occurs *in vivo* since methanogenic organisms contain an enzyme(s) that reduces $F_{560}$ to $F_{430}$.[72] The mechanism involving $F_{430}$ in the reductive demethylation of $CH_3$-S-CoM is unknown. Recent spectroscopic investigations have addressed the stereochemistry and conformation of $F_{430}$ as well as the mode of coordination it assumes in the free and enzyme-bound form.[73–81] The $F_{430}$

skeleton has considerable flexibility that is needed to accommodate the structural changes that accompany reduction to Ni(I). Electron paramagnetic resonance (EPR) spectroscopy of cell extracts and whole cells of *M. thermoautotrophicum* strain Marburg reveals six different signals attributable to Ni in $F_{430}$.[82] Two of these signals appear in whole cells under $H_2$, indicating that Ni is redox active. The Ni(I) state exists in two forms, one of which is postulated to result from the addition of HS-HTP (see below) to form an axial ligand to Ni. It is thought that this Ni-S-HTP form reacts with $CH_3$-S-CoM, yielding CoM-S-S-HTP plus $CH_4$ and a return to the nonliganded Ni(I) state. Recently, a mechanism was proposed that focuses on the formation of CoM-S-S-HTP.[83] In the mechanism, it is assumed that reduction to Ni(I) is thermodynamically possible, based on model studies. It is proposed that Ni(II) is reduced to Ni(I) with electrons from $^-$S-HTP yielding the thiyl radical ($^\cdot$S-HTP) that then reacts with $CH_3$-S-CoM to form the sulfuranyl radical (CoM-S-($CH_3$)-S-HTP). Demethylation of the sulfuranyl radical by Ni(I) results in the Ni(II)-$CH_3$ species and CoM-S-S-HTP. This step is consistent with the reductive demethylation of sulfonium salts by $F_{430}$ in the Ni(I) state and the likely formation of a Ni(II)-$CH_3$ species.[84,85] In the final step of the proposed mechanism,[83] protonation of Ni(II)-$CH_3$ yields $CH_4$ and Ni(II). The mechanism is consistent with an overall reductive displacement of the sulfur of $CH_3$-S-CoM by hydrogen that proceeds with net inversion of configuration.[86] Interestingly, $F_{430}$ is also a catalyst for the reductive dechlorination of one-carbon compounds.[87]

Progress has been inhibited because studies on the mechanism of methyltransferase have utilized enzyme preparations that contained less that 5% of the *in vivo* activity; however, a purification procedure was reported recently for *M. thermoautotrophicum* strain marburg that yielded enzyme with a high specific activity that should facilitate future studies.[70] The methylreductase, as purified from *M. thermoautotrophicum* strain ΔH, requires additional proteins and ATP for activity.[88] With $H_2$ as the electron donor, protein fractions A1, A2, $A3_a$, $A3_b$, and catalytic amounts of ATP and FAD are required in addition to HS-HTP.[89,90] A1 is a crude protein fraction that is proposed to contain $F_{420}$-reducing hydrogenase and a CoM-S-S-HTP heterodisulfide reductase to regenerate HS-HTP. It is likely that FAD is required for either of the flavin-containing enzymes $F_{420}$-reducing hydrogenase or heterodisulfide reductase or both.[91,92] Protein A2 and fraction $A3_a$ are thought to reactivate methylreductase by reduction of Ni(II) to Ni(I) in $F_{430}$ by an unknown mechanism that requires ATP. It is proposed that ATP may bind to $A3_a$ or methylreductase to induce a conformational change and modify its redox potential.[90,93] Protein A2 is a colorless, air stable monomer ($M_r$ = 59,000).[94] Fraction $A3_b$ contains a MV-hydrogenase (see Section II.E) distinct from the $F_{420}$-reducing hydrogenase in component A1. Fraction $A3_b$ is thought to supply electrons for the ATP-dependent reductive activation of methylreductase by A2 and $A3_a$. When titanium(III)citrate is the electron donor, fractions A1 and $A3_b$ are no longer required. Recently, the methylreductase from *M. thermoautotrophicum* strain ΔH was reactivated with light above 400 nm, a procedure that bypasses the requirements for A2, $A3_a$, $A3_b$, and ATP and greatly simplifies the system for future studies.[95] Light activation requires HS-HTP, $CH_3$-S-CoM, and titanium(III)citrate; $CH_4$ and CoM-S-S-HTP are products, suggesting the activation is linked to enzyme turnover. However, since titanium(III)citrate and methylreductase both absorb light above 400 nm, the site of reactivation is unknown. A compound similar in structure to the UDP-disaccharide component of MRF, as well as other UDP-sugars, stimulates the $H_2$-dependent reduction of $CH_3$-S-CoM in cell extracts of *M. thermoautotrophicum* strain ΔH;[96] presumably, the UDP-sugars replace the requirement for ATP. Unlike *M. thermoautotrophicum* strain ΔH, the homogeneous methylreductase from strain Marburg does not require reactivation and catalyzes the reduction of $CH_3$-S-CoM to $CH_4$ and CoM-S-S-HTP, with HS-HTP as the only requirement.[59,97] The reaction is stimulated by the presence of dithiothreitol and vitamin $B_{12}$ or titanium(III)citrate that nonenzymatically reduces the heterodisulfide and regenerates HS-CoM.

The genes encoding the three subunits of methylreductase have been sequenced from five different species representing four genera.[98–103] Comparisons reveal a high degree of similarity

in the deduced amino acid sequences. No conclusions can be drawn that relate structure to function; however, comparisons show regions of high identity within subunits that may define functional domains required for cofactor and substrate binding, subunit interaction, or protein folding. Two genes (*mcrC* and *mcrD*) are apparently cotranscribed with the three genes (*mcrA*, *mcrB*, and *mcrG*) encoding the α, β, and γ subunits of the methylreductase. Antibodies raised against the products of *mcrC-lacZ* and *mcrD-lacZ* fusions expressed in *E. coli* can be used to show that *mcrC* and *mcrD* are expressed in *M. thermoautotrophicum* strain Marburg.[104] The *mcrC* and *mcrD* gene products do not copurify with the active methylreductase, and addition of the *mcrC-lacZ* and *mcrD-lacZ* gene products does not stimulate activity. *mcrD* is also expressed in *Methanococcus vannielii*.[105] All three subunits of the *M. vannielii* methylreductase coprecipitate with *gpmcrD-lacZ*, indicating an *in vitro* association; however, the association appears to be weak because the methylreductase and *gpmcrD-lacZ* separate during nondenaturing gel electrophoresis. Thus, the function of the *mcrC* and *mcrD* gene products is unknown.

Although mechanical disruption of cells yields methylreductase in the soluble fraction, immunocytochemical methods indicate attachment to the cytoplasmic membrane of *M. thermoautotrophicum* strain Marburg and *Methanococcus voltae*.[106,107] The methylreductase of *M. thermoautotrophicum* strain ΔH is often randomly distributed in the cell;[107] however, this distribution may result from an overproduction of the enzyme when cells are grown in a medium with excess nickel.[106] The *M. voltae* enzyme is arranged in a high-molecular-weight complex similar to the membrane-associated "methanoreductosome" described for the methanol-utilizing organism strain Gö1.[108] The methylreductase is not involved in electron transport; thus, a function for membrane attachment is not immediately obvious.

A second methylreductase has been detected in both strains of *M. thermoautotrophicum* that elutes from anion exchange columns in a lower salt concentration than the previously characterized enzyme and differs in the N-terminal amino acid sequence of all three subunits as well as containing a smaller α subunit.[60] The physiological significance of these isofunctional enzymes is unknown, but relative amounts of the two methylreductases vary with the growth conditions.

## E. Electron Transport and Bioenergetics

An excellent review of the energetics of methanogenesis has appeared recently.[11] The existing evidence points to an electrochemical mechanism for the generation of ATP during the reduction of $CO_2$ to $CH_4$, but the identity of the coupling ion is not clear. Interestingly, a novel vanadate-sensitive and DCCD-insensitive ATPase has been described from *M. voltae*;[109] however, the amino acid sequence deduced from the cloned gene has no significant identity with known ion-transporting ATPases.[110] Only Reactions 8 and 11 (the reduction of 5,10-methylene-$H_4$MPT and CoM-S-S-HTP with $H_2$) are sufficiently exergonic for a coupled electron transport phosphorylation. Little is understood regarding electron transport in $CO_2$-reducing species, and much is inferred from studies on $H_2$-oxidizing, methanol-reducing organisms (see Section IV.C).

### 1. Electron Carriers

It is clear that $F_{420}$ is an electron donor in the reactions (6a and 7) catalyzed by 5,10-methenyl-$H_4$MPT$^+$ dehydrogenase and 5,10-methylene-$H_4$MPT reductase. On exposure to oxygen, $F_{420}$ is converted to the 8-OH-AMP and 8-OH-GMP esters termed $F_{390}$-A and $F_{390}$-G owing to a shift in absorbance to shorter wavelengths. Reestablishment of anaerobiosis in *M. thermoautotrophicum* strain Marburg returns the nucleotide derivatives to $F_{420}$, a finding that supports the hypothesis that conversion to $F_{390}$ may function to restrict the deleterious interaction of reduced electron carriers with oxygen.[111] Cell extracts of *M. thermoautotrophicum* strain ΔH catalyze the $H_2$-dependent reduction of $F_{390}$ (midpoint reduction potential = −325 mV),[112] but at a rate approximately 40- fold lower than with $F_{420}$, a result that further supports the hypothesis.[113,114] In addition, extracts catalyze the hydrolysis of $F_{390}$ to AMP and $F_{420}$ under opposite conditions for maximal $F_{390}$ synthesis.[114] However, the reversible conversion does not seem to be universal among

$H_2$-utilizing species.[115] The levels of $F_{420}$ and three other $F_{420}$ analogs (containing three, four, or five glutamic acid residues in the side chain) change in relative proportions during batch growth of *M. barkeri*; however, the significance of the interconversion of these analogs during growth is unknown.[116]

Carriers other than $F_{420}$ that participate in electron transfer from $H_2$ or formate have not been identified. Oxidation of the $H_2$-reduced particulate fraction of *M. thermoautotrophicum* strain ΔH, by the addition of $F_{420}$ or $CH_3$-S-CoM, yields EPR signals attributable to the iron-sulfur clusters of hydrogenase and unknown iron-sulfur proteins.[117] The electron carrier proposed to be involved in formyl-MF synthesis is able to reduce metronidazole, a result that is consistent with a ferredoxin-like protein.[118] A thermostable 2 × [4Fe-4S] ferredoxin from the $CO_2$-reducing *Methanococcus thermolithotrophicus* has been described, but it is thought to be involved in reductive biosynthesis.[119,120] Although CoM-S-S-HTP reductase from *M. thermoautotrophicum* strain Marburg has been characterized, the physiological electron donor is unknown.[92] The enzyme has an apparent molecular mass of 550,000 Da and contains three different subunits with apparent molecular masses of 80,000 Da and contains three different subunits with apparent molecular masses of 80,000, 36,000, and 21,000 Da. The native reductase contains 4 FAD and 72 Fe-S. The electron donor used to assay activity is reduced methylviologen; in addition, the enzyme catalyzes the reduction of methylene blue with the substrates HS-HTP plus HS-CoM and generates CoM-S-S-HTP as the product. The CoM-S-S-CoM reductase that has been purified from *M. thermoautotrophicum* strain ΔH utilizes NADPH and NADH as electron donors.[121] The monomeric enzyme ($M_r$ = 64,000) contains one flavin and has low CoM-S-S-HTP reducing activity. It is postulated that the enzyme may function to regenerate HS-CoM trapped in CoM-S-S-CoM, an unusable form of the cofactor.

## 2. Hydrogenase

The $F_{420}$-reducing hydrogenase is one of the best-studied $F_{420}$-dependent enzymes. The enzyme from *M. thermoautotrophicum* strain ΔH ($M_r$ = 115,000) contains three subunits $\alpha_1\beta_1\gamma_1$ with $M_r$s of 47,000, 31,000, and 26,000, and exists primarily in a $M_r$ = 800,000 aggregate.[122] Electron micrographs indicate that the aggregate is assembled as two stacked rings, each containing four $\alpha_1\beta_1\gamma_1$ trimers.[61] The hydrogenase is purified aerobically, but requires an anaerobic reductive activation with $H_2$. The $M_r$ = 115,000 species contains 0.6–0.7 nickel atoms, 0.8–0.9 FAD, and 13–14 iron atoms that are present in 4Fe-4S clusters. Studies with $D_2$ and $D_2O$ reveal that no steps involving D transfer are substantially rate determining;[123] further, there is a complete exchange of H from $H_2$ with solvent before final transfer of a hydride ion to $F_{420}$. The *frhA*, *frhB*, and *frhG* genes encoding the α, β, and γ subunits of the $F_{420}$-dependent hydrogenase from strain ΔH have been cloned and sequenced.[124] The deduced sequence of the *frhG* gene encoding the γ subunit of the $F_{420}$-reducing hydrogenase from *M. thermoautotrophicum* contains eight cysteines arranged in a bacterial ferredoxin-like sequence suggestive of 2 × [4Fe-4S] clusters.[124] The genes are tightly linked to a fourth gene (*frhD*) and arranged in an apparent transcriptional unit of *frhADGB*. All four polypeptides are expressed in *E. coli*; however, the *frhD* gene product is not detected in the active α,β,γ, enzyme purified from *M. thermoautotrophicum*. It is of interest that the nickel-containing $F_{420}$-reducing hydrogenase purified from *M. voltae* is composed of four subunits with molecular masses of 55,000, 45,000, 37,000, and 27,000 Da.[125]

The $F_{420}$-reducing hydrogenase from *Methanobacterium formicicum* is similar in composition and properties to that of the *M. thermoautotrophicum* enzyme.[91] The FAD dissociates during reductive activation of the *M. formicicum* hydrogenase resulting in a deflavoenzyme able to reduce methylviologen with $H_2$ but incompetent in the reduction of $F_{420}$; reconstitution of the deflavoenzyme with FAD restores the ability to reduce $F_{420}$. The results support the proposal that flavin shuttles electrons between one-electron iron-sulfur clusters and the obligate two-electron acceptor $F_{420}$. The *M. formicicum* enzyme is bidirectional, evolving $H_2$ from $F_{420}H_2$ at approximately one third the rate of $H_2$ uptake with $F_{420}$ as the electron acceptor. The $H_2$ evolving activity

of the hydrogenase is demonstrated by reconstitution of a formate hydrogenlyase system (oxidation of formate and evolution of $H_2$ plus $CO_2$) with the hydrogenase, $F_{420}$, and a $F_{420}$-reducing formate dehydrogenase purified from *M. formicicum*.[126] The $F_{420}$-reducing hydrogenase from *M. formicicum* is loosely associated with the cytoplasmic side of the membrane, apparently through hydrophobic interactions.[127,128] Thus, it is unlikely that protons generated by this hydrogenase are translocated across the cytoplasmic membrane to generate a proton gradient unless assisted by another protein. The $F_{420}$-reducing hydrogenase from *M. voltae* is also loosely associated with the membrane and is similar to the nickel-containing iron-sulfur enzymes isolated from *methanobacterium*, except it contains four subunits and selenium.[125,129] Acetylene is a poor inhibitor of the *M. voltae* hydrogenase, consistent with the observation that Ni-Fe hydrogenases are inhibited 10- to 50-fold more than are the Ni-Fe-Se hydrogenases.[130] The nickel-containing $F_{420}$-reducing hydrogenase from *Methanospirillum hungatii* is also loosely associated with the cytoplasmic membrane.[131] Although electron micrographs of the *M. hungatii* enzyme are similar to those of the *M. thermoautotrophicum* $F_{420}$-reducing hydrogenase, the subunit composition ($\alpha_1\beta_3$, $\alpha$ = 50,700 Da and $\beta$ = 30,700 Da) is very different. Apparently, FAD is not present in the *M. hungatii* enzyme, in contrast to other $F_{420}$-reducing hydrogenases.

*M. thermoautotrophicum* strain ΔH contains a second distinct hydrogenase unable to reduce $F_{420}$, and for this reason it is named MV-hydrogenase for the nonphysiological electron acceptor methylviologen. The MV-hydrogenase appears to function in the reductive reactivation of the $CH_3$-S-CoM methylreductase (see Section II.D), but the physiological electron acceptor for this hydrogenase is unknown. The genes *mvhA*, *mvhD*, and *mvhG* encoding the $\alpha$, $\delta$, and $\gamma$ subunits of the enzyme have been cloned and sequenced.[132] The products of *mvhA*, *mvhD*, and *mvhG* have calculated molecular masses of 53,000, 15,800, and 33,000 Da. The genes are tightly linked to a fourth gene (*mvhB*) in the order *mvhDGAB* and appear to form an operon. The *mvhB* gene product is predicted to encode a 44,000-Da protein with six tandemly repeated bacterial ferredoxin-like domains and, therefore, is named polyferredoxin. A polyferredoxin-encoding gene is also linked to the gene encoding the large subunit of the MV-hydrogenase in the hyperthermophile *Methanothermus fervidus*.[133] Recently, the putative polyferredoxin has been purified from *M. thermoautotrophicum*; however, the function is unknown.[134] The *mvhA* gene products encoding the largest ($\alpha$) subunits of the MV-hydrogenases from *M. thermoautotrophicum* and *M. fervidus* have a striking identity with the *frhA*-encoded large ($\alpha$) subunit of the $F_{420}$-reducing hydrogenase from *M. thermoautotrophicum* and the largest subunits of eubacterial nickel-containing hydrogenases. The fact that the regions of conservation are very defined suggests that they are the minimum structures needed for hydrogenase function. Particularly interesting are two pairs of cysteines located near the amino and carboxy termini that are potential thiol ligands to nickel.[135] The redox behavior of nickel in the MV-hydrogenase from *M. thermoautotrophicum* strain Marburg has been studied by EPR spectroscopy.[136] It is proposed that the aerobically prepared enzyme contains Ni(III) and Ni(II) in forms unable to promote activation by $H_2$ (the "unready" state of the enzyme). On reductive reactivation at low redox potentials, Ni(III) and Ni(II) are converted to different forms that allow activation by $H_2$ (the "ready" state of the enzyme). The active enzyme contains the Ni(I) responsible for $H_2$ binding. The distinct EPR characteristics of Ni(III) in the "unready" and "ready" forms of the enzyme indicate that the coordination of nickel changes. The requirement for a low redox potential to convert the enzyme from the "unready" to the "ready" form suggests the possible redox transition of a ligand to nickel.

### 3. Formate Dehydrogenase

Most $CO_2$-reducing species also utilize formate as an electron donor. The molybdenum-containing $F_{420}$-reducing formate dehydrogenases from *Methanococcus vannielii* and *M. formicicum* are characterized extensively.[137] The *M. formicicum* enzyme also contains a dissociable FAD that is required for $F_{420}$ reduction, and molyb-

denum is present in a molybdopterin guanine dinucleotide cofactor (Figure 2).[138] Recent EXAFS (extended X-ray analysis of fine structure) studies indicate O (or N) and S ligands to molybdenum;[139] the Mo-S bond length suggests a typical interaction between the molybdopterin side chain and Mo. Synthesis of formate dehydrogenase protein in *M. formicicum* is directly dependent on the level of molybdenum in the growth medium, a fact that suggests a transcriptional or posttranslational regulation by molybdenum.[140] Addition of tungstate to the medium results in the synthesis of an inactive enzyme containing the metal-free molybdopterin cofactor. The *fdhAB* genes encoding the two subunits ($\alpha_1\beta_1$) of the *M. formicicum* enzyme have been cloned and sequenced.[141] The deduced amino acid sequence of *fdhC* upstream of *fdhA*, and cotranscribed with *fdhAB*, contains seven hydrophobic membrane-spanning regions characteristic of channel proteins and has high identity with the predicted *nirC* gene product required for nitrite reduction in *E. coli*.[142] The amino acid sequence deduced from the *fdhB* gene, encoding the smaller (β) subunit, contains two CXXCXXCXXXCP sequences capable of participating in the formation of two 4Fe-4S centers previously characterized by EPR spectroscopy. Interestingly, the deduced sequence for the β subunit shows high identity with the sequence for the β subunit of the $F_{420}$-reducing hydrogenase of *M. thermoautotrophicum* strain ΔH. Since both the formate dehydrogenase and hydrogenase reduce $F_{420}$ mediated by $FADH_2$, it is postulated that this ability is reflected in the conserved amino acids of the respective subunits.[124] The amino acid sequence deduced from the *fdhA* gene encoding the larger (α) subunit of the formate dehydrogenase from *M. formicicum* shows regions of high identity to the largest subunits of five eubacterial enzymes that bind molybdopterin cofactors.[143]

The predicted *fdhA* gene product has high sequence identity with that of the *fdhF* gene from *E. coli* in the region flanking the selenocysteine residue. The *M. formicicum* enzyme does not contain selenium, but the deduced sequence contains a cysteine residue (Cys-132) in the position corresponding to selenocysteine in the *E. coli* enzyme; however, the function of the cysteine residue in the *M. formicicum* enzyme is unknown. The incorporation of selenocysteine into formate dehydrogenase from *E. coli* is directed by an opal UGA codon and requires a stem-loop structure in the m-RNA flanking UGA on the 3′ side. Introduction of an UGA for the UGC cysteine codon in the *fdhA* gene, and stable stem-loop structure, is not sufficient for decoding for selenocysteine; instead, incorporation of selenocysteine requires replacement of the immediately adjacent portion of the stem-loop with a sequence identical to that present in the *E. coli* *fdhF* mRNA structure.[145]

The $F_{420}$-reducing formate dehydrogenase from *M. formicicum* is loosely associated with the inner aspect of the cytoplasmic membrane and analysis of the deduced amino acid sequences reveal no hydrophobic membrane spanning domains;[128] thus, similar to the $F_{420}$-reducing hydrogenase from this organism, it is unlikely that the formate dehydrogenase alone is involved in the generation of an electrochemical potential. In addition to supplying electrons for $CO_2$ reduction to $CH_4$, the enzyme is a component of the formate hydrogenlyase system that also requires $F_{420}$ and the $F_{420}$-reducing hydrogenase from *M. formicicum*.[126]

### 4. Alcohol Dehydrogenase

Several methane-producing organisms oxidize primary, secondary, and cyclic alcohols that provide electrons for the reduction of $CO_2$ to $CH_4$. A $F_{420}$-dependent secondary alcohol dehydrogenase isolated from *Methanogenium thermophilum* oxidizes 2-propanol to acetone.[144] The native enzyme ($M_r$ = 65,000) is a homodimer with a subunit $M_r$ of 39,000. The $F_{420}$-dependent alcohol dehydrogenase from *Methanogenium liminatans*,[146] an enzyme that catalyzes the oxidation of various secondary and cyclic alcohols to the corresponding ketones, has a native molecular mass of 150,000 Da and is composed of four identical 39,000-Da subunits. A $NADP^+$-dependent alcohol dehydrogenase from *Methanobacterium palustre* has been described that also catalyzes the oxidation of various secondary and cyclic alcohols.[146] The $NADP^+$-dependent enzyme (175,000 Da) is a tetramer similar to the $F_{420}$-dependent enzyme from *M. liminatans* ex-

cept the native *M. palustre* enzyme contains 4 to 8 zinc. There is no significant identity between the N-terminal amino acid sequences of the $NADP^+$- and $F_{420}$-dependent enzymes.

## III. CONVERSION OF ACETATE TO CARBON DIOXIDE AND METHANE

The conversion (Reaction 12) is restricted to *Methanosarcina* and *Methanothrix*. In both genera, acetate is activated to acetyl-CoA followed

$$CH_3COO^+ + H^+ \rightarrow CH_4 + CO_2$$

$$\Delta G^{\circ\prime} = -36 \text{ kJ/mol} \quad (12)^{15}$$

by decarbonylation and methyl transfer to HS-CoM. The reductive demethylation of $CH_2$-S-CoM to $CH_4$ is similar to that described for $CO_2$-reducing species except that electrons for reduction of CoM-S-S-HTP derive from oxidation of the carbonyl group of acetate to $CO_2$. The current understanding of the pathway in *Methanosarcina thermophila* is shown in Figure 3.

### A. Activation of Acetate to Acetyl-CoA

Several lines of evidence indicate that acetate is first activated to acetyl-CoA prior to cleavage of the C-C bond. Cell extracts of acetate-grown *Methanosarcina* contain high acetate kinase (Reaction 13) and phosphotransacetylase (Reaction 14) activities.[147,148] These activities are not present in acetate-grown *Methanothrix soehngenii*, but this organism contains high levels of acetyl-CoA synthetase (Reaction 15).[149]

$$CH_3COO^- + ATP \rightarrow CH_3CO_2PO_3^{2-} + ADP \quad (13)$$

$$CH_3CO_2PO_3^{2-} + CoA \rightarrow CH_3COSCoA + Pi \quad (14)$$

$$CH_3COO^- + CoA + ATP \rightarrow CH_3COSCoA + AMP + PPi \quad (15)$$

Coenzyme A stimulates $CH_3$-S-CoM synthesis, and acetyl phosphate is converted to methane, in extracts of *M. barkeri*.[147,150,151] Western blot analysis and two-dimensional electrophoresis show that the synthesis of acetate kinase and phosphotransacetylase in *M. thermophila* increases severalfold when the growth substrate is switched from methanol to acetate.[152–154] The acetate kinase purified from *M. thermophila* is an $\alpha_2$ homodimer with a subunit $M_r$ of 53,000 and is present in the soluble fraction after cell lysis.[152] Activity with TTP, ITP, UTP, and GTP is greater than 80% of the activity with ATP. The $K_m$ for acetate is 22 m*M*, consistent with the relatively high $K_s$ for acetate uptake by this organism. The monomeric ($M_r$ = 42,000) phosphotransacetylase purified from *M. thermophila* is also present in the soluble fraction.[153] Potassium or ammonium ions are required for maximum activity, while phosphate, arsenate, and sulfate are inhibitory. The acetyl-coenzyme A synthetase purified from *M. soehngenii* is an $\alpha_2$ homodimer with a subunit molecular mass of 73,000 Da.[149] The $K_m$ for acetate is 0.86 m*M*, reflecting the high affinity of this organism for acetate. The amino acid sequence, which was deduced from the gene encoding the synthetase, shows significant identity with consensus ATP binding sites.[155]

### B. Decarbonylation of Acetyl-CoA

All acetate-utilizing species contain high levels of CO dehydrogenase that is postulated to catalyze breakage of the C-C and C-S bonds of acetyl-CoA in reverse analogy to the well-characterized CO dehydrogenase (acetyl-CoA synthase) of *Clostridium thermoaceticum* that synthesizes acetyl-CoA from CoA, CO, and a methylated corrinoid/Fe-S protein.[156,157] The ability of the CO dehydrogenase complex from *M. thermophila* to catalyze the synthesis of acetyl-CoA from CoA, CO, and $CH_3I$ strongly supports the proposed function.[158] CO can be replaced with $CO_2$ and the reductant titanium(III)citrate as a consequence of the CO dehydrogenase activity of the complex. Recently,[159,160] the CO dehydrogenases from *M. thermophila* and *M. soehngenii* were shown to catalyze an exchange of CO with the carbonyl

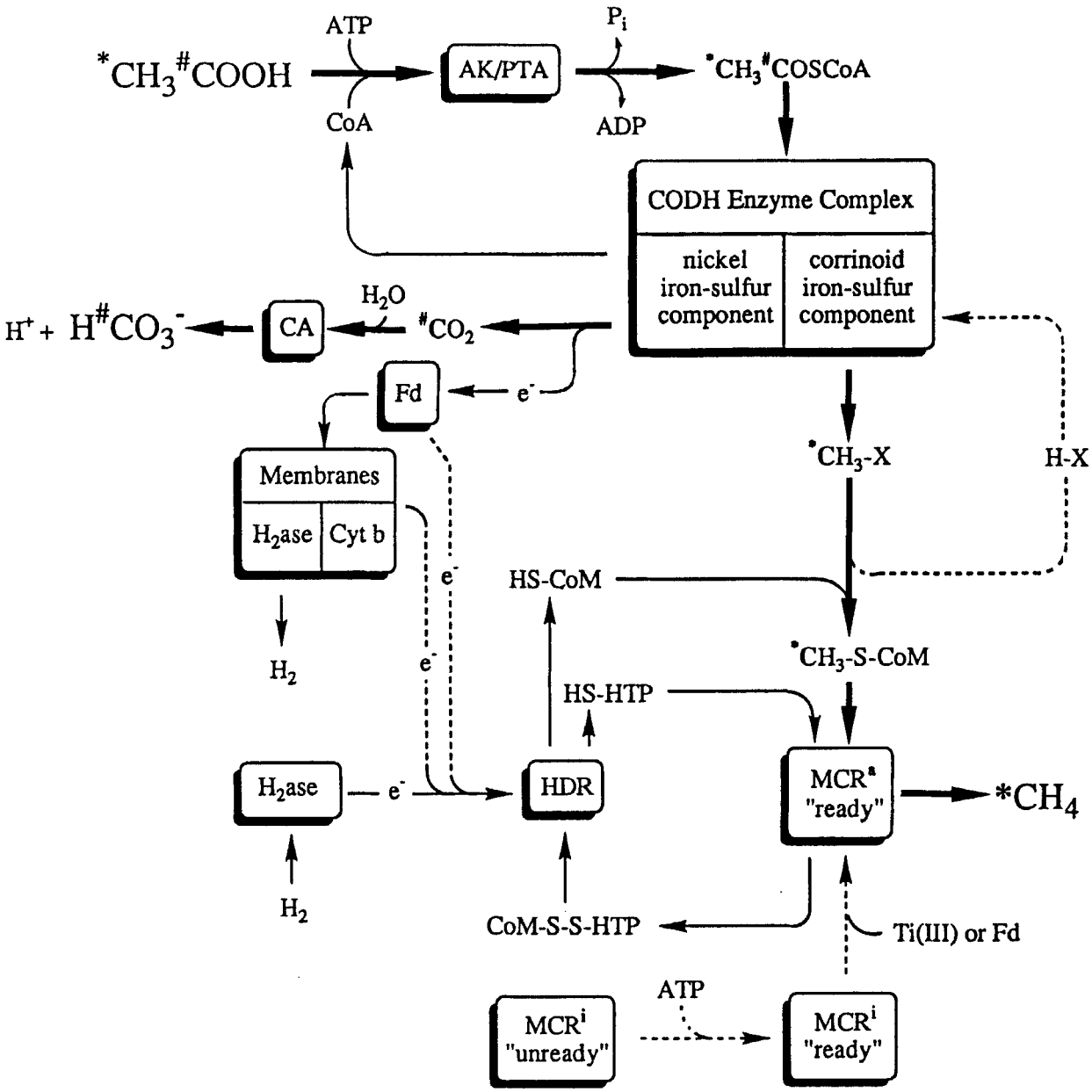


**FIGURE 3.** Proposed pathway for the conversion of acetate to $CO_2$ and $CH_4$ in *Methanosarcina thermophila*. (Modified from Jablonski and Ferry.[179]) AK, acetate kinase; PTA, phosphotransacetylase; HX, proposed methyl carrier; CA, carbonic anhydrase; $MCR^i$, inactive methylreductase; $MCR^a$, active methylreductase; HDF, heterodisulfide (CoM-S-S-HTP) reductase; Fd, ferredoxin; CODH, carbon monoxide dehydrogenase enzyme complex; cyt b, cytochrome b; $H_2$ase, hydrogenase. Dashed lines represent gaps in understanding of the pathway. Evidence suggests that the methyl carrier "X" in *M. barkeri* is $H_4$MPT (see text).

group of acetyl-CoA, a finding that demonstrates the C-C and C-S bond cleavage activity and implicates involvement of this enzyme in the $CO_2$/$CH_3COOH$ exchange activity of *M. barkeri* cell extracts.[162] In addition, the *M. thermophila* enzyme complex catalyzes an exchange of CoA with acetyl-CoA at rates fivefold greater than the *C. thermoaceticum* acetyl-CoA synthase.[159] The difference may reflect the function of the *M. thermophila* enzyme in degrading acetyl-CoA, whereas the *C. thermoaceticum* enzyme functions exclusively for biosynthesis of acetyl-CoA.

The properties of the CO dehydrogenase complex from *M. thermophila* strongly support an acetyl-CoA cleavage mechanism that is analogous to a reversal of the mechanism proposed for synthesis catalyzed by the well-characterized *C. thermoaceticum* acetyl-CoA synthase.[156,157] The *M. thermophila* complex contains two components: a Ni/Fe-S protein (approximately 200,000 Da) containing 89,000- and 19,000-Da subunits and a Co/Fe-S protein (approximately 100,000 Da) containing 60,000- and 58,000-Da subunits.[163] The CO-reduced Ni/Fe-S component contains a Ni-Fe-C center with an EPR spectrum indistinguishable from the spin-coupled Ni-Fe-C center of the acetyl-CoA synthase from *C. thermoaceticum*.[148] The Ni-Fe-C center is the proposed site for synthesis of the acetyl group of acetyl-CoA.[157] In addition, the Ni-Fe-C EPR signal from both enzymes is perturbed on incubation with acetyl-CoA.[148] Both the Ni/Fe-S component and the acetyl-CoA synthase have CO dehydrogenase activity, reduce ferredoxin, and contain Fe-S centers. The acetyl-Co-A synthase from *C. thermoaceticum* associates with a two-subunit corrinoid/Fe-S protein that donates a methyl group to the synthase;[156] thus, it is proposed that methyl transfer occurs between the Co/Fe-S and Ni/Fe-S components of the *M. Thermophila* complex, in analogy to the clostridial system.[163] The Co/Fe-S component of *M. thermophila* 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;[163] in this redox state, factor III is methylated with $CH_3I$ supporting the proposed function. Indeed, whole-cell studies with acetate-grown *M. barkeri* indicate that a corrinoid protein is required for synthesis of acetyl-CoA from CoA, CO, and $CH_3I$.[164] In addition, inhibitor studies with *M. barkeri* cell extracts suggest that a corrinoid accepts the methyl group after cleavage of the C-C bond in acetyl-CoA.[162] It also has been shown that a corrinoid protein is methylated after cleavage of acetyl-CoA in extracts of *M. barkeri*.[151] Thus, it is likely that during methanogenesis from acetate the Ni/Fe-S component of the *M. thermophila* complex cleaves acetyl-CoA followed by oxidation of the carbonyl group to $CO_2$ and transfer of the methyl group to the Co/Fe-S component. The carbonyl group does not exchange with CO during methanogenesis, indicating that free CO is not an intermediate.[165] The component proteins from the *M. thermophila* complex have been characterized in more detail using EPR spectroscopy in combination with electrochemistry.[166] Three species of Fe-S clusters are detected in the CO-oxidizing Ni/Fe-S component. The dominant species ($E_m$ = −444 mV) has apparent g values at 2.05, 1.94, and 1.89 ($g_{av}$ = 1.94) attributable to a 4Fe-4S cluster. A $g_{av}$ = 1.97 species appears at very low redox potentials (−540 mV) with g values at 2.05, 1.97 and 1.90. A third, fast relaxing species ($g_{av}$ = 1.85) with g values of 2.02, 1.82, and 1.71 has a higher redox potential (−150 mV). The results are similar to those reported for the Fe-S centers in the *C. thermoaceticum* acetyl-CoA synthase.[167,168] EPR spectroscopy of the as-isolated Co/Fe-S component indicates a low spin $Co^{2+}$; there is no superhyperfine splitting from the nitrogen nucleus (I = $^1/_2$) of the 5-hydroxybenzimadazole base in factor III, indicating a base-off configuration that is thought to stabilize the $Co^{1+}$ redox state required to accept a methyl group.[163,169] Redox titration of the $Co^{2+/1+}$ couple shows an $E_m$ of −515 mV similar to that reported for the corrinoid/Fe-S protein from *C. thermoaceticum*.[169] The Co/Fe-S component of *M. thermophila* contains a 4Fe-4S center with g values of 2.06, 1.94, and 1.84 ($g_{av}$ = 1.94) and an $E_m$ of −502 mV.[166] Although the functions of the Fe-S centers are yet to be determined, the striking resemblance between the enzyme components of the *M. thermophila* and *C. thermoaceticum* systems further support common catalytic mechanisms.

The CO dehydrogenase from *M. barkeri* is purified as an $\alpha_2\beta_2$ structure composed of subunits with $M_r$s of approximately 90,000 and 19,000 similar to the CO-oxidizing Ni/Fe-S component of the *M. thermophila* complex;[170,171] however, a recent report indicates that the *M. barkeri* $\alpha_2\beta_2$ CO dehydrogenase is also associated with a corrinoid protein in an enzyme complex.[172] Although the *M. barkeri* CO dehydrogenase contains Ni and Fe, no EPR signal attributable to a Ni center can be detected. Core extrusion experiments indicate 6 × [4Fe-4S] clusters per tetramer.[171] At least one of the clusters in the dithionite-reduced enzyme has g values of 2.05, 1.94, and 1.90 and an $E_m$ of −390 mV

similar to the *M. thermophila* Ni/Fe-S component. A second EPR signal is obtained from the reduced enzyme with apparent g values of 2.005, 1.91, and 1.76 and an $E_m$ of −35 mV; the g = 1.76 feature shifts to 1.73 on incubation with CO. This signal, and the shift to g = 1.73, is seen in whole cells of *M. barkeri* during methanogenesis, indicating that the cleavage of acetate yields a moiety that CO dehydrogenase recognizes as CO.[173] In addition, transient electron flow through Fe-S clusters can be correlated with methanogenesis.

The CO dehydrogenase purified from *M. soehngenii* has properties nearly identical to the enzyme from *M. barkeri*.[174] The amino acid sequence deduced from the DNA sequence of the largest (α) subunit shows homology with acyl-CoA oxidases; however, no specific residues involved in acetyl-CoA or CoA binding can be assigned.[175] The amino acid sequence of the largest subunit also contains eight cysteine residues with spacings that could accommodate 2 × [4Fe-4S] centers. The anaerobically purified CO dehydrogenase shows two low-temperature $S = {}^1/_2$ EPR signals.[160,161] One, with g values of 2.05, 1.93, and 1.865, has an $E_m$ of −410 mV attributable to a magnetically isolated 4Fe-4S center. The other has g values of 2.005, 1.894, and 1.733 with an $E_m$ of −230 mV; this signal partially disappears when the enzyme is incubated with CO. In addition, EPR signals are present in the oxidized enzyme with g values at g = 14.5 and 5.5 ascribed to a $S = {}^9/_2$ system and g values at g = 9.6, 4.6, 4.2, and 3.8 ascribed to a $S = {}^5/_2$ system.[161] Both sets of signals disappear on reduction with an $E_m$ = −280 mV. Thus, the CO dehydrogenases from *M. soehngenii* and *M. barkeri* vary from the Ni/Fe-S component of *M. thermophila* and the acetyl-CoA synthase of *C. thermoaceticum* in two ways: (1) a Ni EPR signal and a very low potential Fe-S signal have yet to be detected for either the *M. soehngenii* or *M. barkeri* CO dehydrogenases, and (2) a novel high-spin system is reported for the *M. soehngenii* enzyme. These differences could be the result of different methods used for purification of the enzymes.

### C. Methyl Transfer to HS-CoM

Methane formation from acetate in extracts of *M. barkeri* is dependent on the presence of $H_4$MPT, and methyl-$H_4$MPT accumulates when the methylation of HS-CoM is blocked, suggesting that methyl-$H_4$MPT is an intermediate in the pathway.[176] Indeed, the CO dehydrogenase-corrinoid enzyme complex from *M. barkeri* catalyzes methylation of tetrahydrosarcinapterin with acetyl-CoA.[172] Thus, it is proposed that the methyl group of the methylated corrinoid/Fe-S protein is transferred to $H_4$MPT by a methyltransferase.[176] Transfer of the methyl group from methyl-$H_4$MPT to HS-CoM is likely to involve a second corrinoid-containing methyltransferase in analogy to the $CO_2$-reducing pathway. A methylcobalamin:HS-CoM methyltransferase ($M_r$ = 34,000) from acetate-grown *M. barkeri* has been described;[177] however, it is unknown if methyl-$H_4$MPT can replace methylcobalamin. The acetate-dependent methylation of two corrinoid proteins (480,000 and 29,000 Da) in cell extracts of *M. barkeri* was reported recently.[178] The 480,000-Da protein is methylated at the onset of methanogenesis and demethylated when methanogenesis stops, indicating an involvement in methyltransfer. The 29,000-Da protein is methylated only when reductive demethylation of $CH_3$-S-CoM is inhibited. The partially purified 480,000-Da protein contains subunits with molecular masses of 40,000 and 30,000 Da, similar to the methylcobalamin:HS-CoM methyltransferase described from *M. barkeri*,[177] but this enzyme activity was not assayed.[178]

### D. Reductive Demethylation of $CH_3$-S-CoM to Methane

Resting and methane-producing whole cells of acetate-grown *M. barkeri* display different EPR signals attributable to two forms of Ni(I) in $F_{430}$.[173] The two forms are proposed to arise from an axial ligation of HS-HTP to Ni that is absent during production of methane and CoM-S-S-HTP.[82] The EPR signal of the ligated form of Ni(I) $F_{430}$ is seen in resting cells of *M. barkeri*; how-

ever, when cells are actively converting acetate to methane, the EPR signal of the unligated form predominates, consistent with the proposed mechanism for the *M. thermoautotrophicum* methylreductase.[82]

Unlike most other methylreductases described, the enzyme purified from acetate-grown *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.[179] The native enzyme ($M_r$ = 141,000) contains one mol of $F_{430}$ and utilizes HS-HTP as the electron donor. The as-isolated enzyme is inactive but can be reductively reactivated *in vitro* with ferredoxin purified from *M. thermophila*.[180] The ferredoxin is reduced by the Ni/Fe-S component of the CO dehydrogenase complex;[163] thus, the enzyme system involved in reductive reactivation of the *M. thermophila* methylreductase appears less complex than that for *M. thermoautotrophicum* strain ΔH. ATP is not required but stimulates reactivation of the *M. thermophila* enzyme. 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 reductively reactivated unless converted to the "ready" form by an unknown mechanism that requires ATP (Figure 3).[179] The inactive methylreductase purified from *M. mazei* contains $F_{430}$ and has a native molecular mass of 283,400 Da with subunits of 68,000, 43,215, and 30,500 Da in a $\alpha_2\beta_2\gamma_2$ configuration.[181] The $CH_3$-S-CoM methylreductase from acetate-grown *M. soehngenii* has the same subunit composition as the enzyme from $CO_2$-reducing organisms and utilizes HS-HTP as the electron donor.[182] Similar to the methylreductase from $CO_2$-reducing organisms, the activity of the enzyme purified from *M. soehngenii* is only 7% of the activity in cell extracts. 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 cell interior;[181] apparently, the cells were grown with abundant nickel in the growth medium, growth conditions that may have influenced the amount of cytoplasmic methylreductase relative to membrane-associated enzyme.[106]

The ability of HS-HTP to serve as reductant for the methyltransferases of acetotrophic organisms implies that CoM-S-S-HTP is a product of the reaction that must be reduced to the corresponding sulfhydryl derivatives with electrons derived from the carbonyl group of acetyl-CoA. Indeed, cell extracts of acetate-grown *M. barkeri* contain high levels of heterodisulfide reductase activities comparable with those of $CO_2$-reducing organisms.[183,184]

## E. Electron Transport and Bioenergetics

Whole cells of *M. barkeri* converting acetate to methane generate a proton-motive-force of −120 mV, consistent with a chemiosmotic mechanism for ATP synthesis in this organism.[185] The membranes of *M. thermophila* and *M. barkeri* contain hydrogenase, cytochrome b, multiple Fe-S centers, and possibly rubredoxin;[186,187] thus, it is possible 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. CO-dependent methylreductase activity is stimulated by the addition of membranes to the soluble fraction, a result that supports the involvement of a membrane-bound electron transport chain.[186] Ferredoxin is a direct electron acceptor for the Ni/Fe-S component of *M. thermophila*,[163] and is required for methanogenesis from acetate in extracts of *M. barkeri*,[188] results that strongly implicate an involvement of ferredoxin in electron transport. However, the carriers mediating electron flow from ferredoxin to the heterodisulfide reductase are unknown.

*M. thermophila* contains a CO-oxidizing:$H_2$-evolving system (Reaction 16)

$$CO + H_2O \rightarrow CO_2 + H_2$$

$$\Delta G^{\circ\prime} = -20 \text{ kJ/mol} \quad (16)^{189}$$

in which the CO dehydrogenase complex reduces ferredoxin that in turn transfers electrons to the membrane where $H_2$ is evolved.[186] The evolution of $CO_2$ and $H_2$ from acetyl-CoA in extracts of *M. barkeri* is also dependent on ferredoxin.[188]

The membranes of *M. thermophila* contain a b-type cytochrome reducible with a membrane-bound hydrogenase,[186] as do membranes of *M. barkeri*;[187] thus, oxidation of the carbonyl group of acetate could be coupled to a membrane-bound electron transport chain (Figure 3), an implication of the potential for generation of a proton-motive-force. Indeed, proton translocation is coupled to the oxidation of CO to $CO_2$ and $H_2$ in cell suspensions of acetate-grown *M. barkeri*.[189] In addition, over 50% of the heterodisulfide reductase activity in *M. barkeri* is associated with the membrane fraction.[184] Cell extracts of *M. thermophila* catalyze the reduction of CoM-S-S-HTP with $H_2$;[179] however, it is unknown whether this reaction is required for the conversion of acetate to methane. Although $F_{420}$ is an important electron carrier in the $CO_2$-reducing pathway, it is only present in low levels in acetate-grown cells of *M. barkeri* and is not required for conversion of acetyl-CoA to methane in cell extracts of this organism.[176] However, $F_{420}$ may be involved in oxidation of the methyl group of acetate to $CO_2$ to provide electrons for reductive biosynthesis (see Section III.F). Cell extracts of acetate-grown *M. barkeri* convert $F_{420}$ to factor 390 by an ATP-dependent reaction.[190]

Methane formation from acetate is dependent on sodium and is accompanied by the generation of a secondary sodium ion gradient;[185] however, the sodium requirement is not understood. Two reactions that may be driven by a sodium ion potential are the uptake of acetate and the potentially endergonic cleavage of acetyl-CoA (Reaction 17).

$$CH_3CO\text{–}S\text{–}CoA + HS\text{–}CoM \rightarrow CO + CH_3\text{–}S\text{–}CoM + CoA$$

$$\Delta G^{\circ\prime} = +40.3 \text{ kJ/mol} \quad (17)^{191}$$

### F. Other Enzyme Activities

Acetate-grown cells of *M. barkeri* contain low levels of formyl-MF dehydrogenase and 5,10-methylene-$H_4$MPT dehydrogenase.[184] In addition, acetate-grown *M. thermophila* contains low activities of formyl-MF:$H_4$MPT formyltransferase, 5,10-methenyl-$H_4$MPT$^+$ cyclohydrolase, and $F_{420}$-dependent 5,10-methylene-$H_4$MPT dehydrogenase.[154] These results suggest that enzymes of the $CO_2$-reduction pathway are not directly involved in the conversion of acetate to methane; however, these enzymes may be involved in oxidation of the methyl group of acetate to $CO_2$ to provide electrons for reductive biosynthesis.

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 unknown.[154,192] It is proposed that the formation of carbonic acid may be required in an exchange mechanism for acetate transport.[192]

## IV. DISPROPORTIONATION OF METHANOL OR METHYLAMINES TO METHANE AND CARBON DIOXIDE

The conversion of methanol to $CH_4$ is a disproportionation event in which three methanol molecules are reduced to methane and a fourth molecule of methanol is oxidized to $CO_2$ (Reactions 18 to 20). Methylamines are also disproportionated to $CH_4$ and $CO_2$; however, much more is known concerning the biochemistry of methanol conversion (Figure 4). The methyl group of methanol is transferred to HS-CoM to yield $CH_3$-S-CoM, an intermediate common to all known pathways of methanogenesis. Electrons for reductive demethylation of $CH_3$-S-CoM derive from oxidation of methanol to $CO_2$ by utilizing enzymes of the $CO_2$-reduction pathway.

$$CH_3OH + H_2O \rightarrow CO_2 + 6e^- + 6H^+ \quad (18)$$

$$3CH_3OH + 6e^- + 6H^+ \rightarrow 3CH_4 + 3H_2O \quad (19)$$

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$$

$$\Delta G^{\circ\prime} = -103 \text{ kJ/CH}_4 \quad (20)^{15}$$

### A. Methyl Transfer Reactions Leading to Methane

Methyl transfer from methanol to HS-CoM, as studied in *M. barkeri*, involves two steps cat-

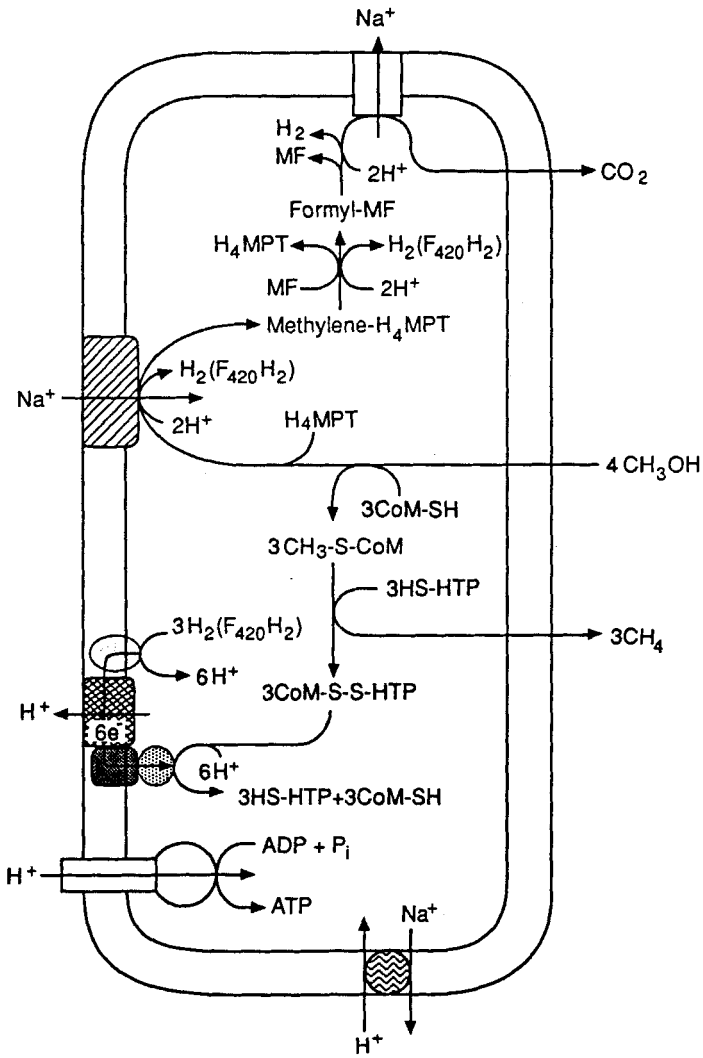


**FIGURE 4.** Proposed reactions in the pathways of carbon and electron flow for the disproportionation of methanol to $CO_2$ and $CH_4$. (Modified from Blaut et al.[11])

alyzed by enzymes $MT_1$ and $MT_2$.[193,194] Methanol:factor III methyl transferase ($MT_1$) transfers the methyl group of methanol to the cobalt atom of enzyme-bound factor III (Reaction 21). The enzyme has a native

$$CH_3OH + H^+ + [Co(I)\text{factor III}]MT_1 \rightarrow [CH_3\text{–}Co(III)\text{factor III}]MT_1 + H_2O \quad (21)$$

molecular weight of 122,000 and contains two different subunits with molecular weights of 34,000 and 53,000. Activation of $MT_1$ requires the presence of ATP, $H_2$, hydrogenase, and ferredoxin and leads to the formation of Co(I), a methyl-accepting supernucleophile. Methyl-factor III:HS-CoM methyl transferase ($MT_2$) transfers the methyl group of methyl-$MT_1$ to HS-CoM (Reaction 22). It is an air-stable, monomeric ($M_r$ = 43,000) enzyme that does not contain

$$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$$
$$\Delta G^{\circ\prime} = -36 \text{ kJ/mol} \quad (22)^{15}$$

a corrinoid cofactor.[193] The enzyme also accepts a methyl group from free methylcobalamin and is alternatively referred to as methylcoba-

lamin:HS-CoM methyl transferase. The relative concentrations of two isozymes of methylcobalamin:HS-CoM transferase change in methanol- vs. acetate-grown cells of *M. barkeri*, suggesting specific transferases for each pathway.[177] The synthesis of chiral methanol has been used to show that transformation of the methyl group into $CH_3$-S-CoM proceeds with net retention of the methyl group configuration,[195] a result consistent with a two-reaction mechanism involving $MT_1$ and $MT_2$.

The location of $CH_3$-S-CoM methylreductase has been studied in methanol-grown strain Göl that has physiological properties similar to the *Methanosarcina* but contains a proteinaceous cell wall and forms protoplasts well suited for membrane studies. Immunoelectron microscopy indicates that the methylreductase is located in a large membrane-associated structure called a "methanoreductosome".[196] The structure contains a sphere-like hollow head piece that contains several copies of the methylreductase that can be reassociated from isolated methylreductase molecules;[108] the molecular mass of the isolated enzyme from Göl is 182,000 Da with an $\alpha_1\beta_1\gamma_1$ configuration. It is proposed that the activity of solubilized methylreductase is dependent on ATP for reassociation of the enzyme molecules into a complex similar to the "methanoreductosome".[197]

## B. Oxidation of the Methyl Group to Carbon Dioxide

Oxidation of the methyl group of methanol supplies electrons for reduction of the CoM-S-S-HTP generated in the reduction of $CH_3$-S-CoM to $CH_4$ when HS-HTP is the electron donor. Cofactor-free extract of *M. barkeri* is unable to oxidize formaldehyde to $CO_2$ unless methanofuran-b and $H_4$MPT-b are added, indicating that the $CO_2$-reducing pathway operates in reverse to oxidize methanol.[198] Methanofuran-b and $H_4$MPT-b are close structural analogs of methanofuran and $H_4$MPT present in *Methanobacterium*.[199,200] In addition, methanol-grown cells of *M. thermophila* and *M. barkeri* contain significant activities of MF- and $H_4$MPT-dependent enzymes in the pathway of $CO_2$ reduction to $CH_3$-$H_4$MPT.[154,184] Recently, the $F_{420}$-dependent 5,10-methylene-$H_4$MPT dehydrogenase and 5,10-methylene-$H_4$MPT reductase have been purified from the soluble fraction of methanol-grown *M. barkeri*.[201] The dehydrogenase is a hexamer of a single 35,000-Da subunit and the reductase is composed of four identical 38,000-Da subunits. Very low levels of MF- and $H_4$MPT-dependent enzymes are present in *Methanosphaera stadtmanae* grown on $H_2$ plus methanol, consistent with the inability of this organism to reduce $CO_2$ to $CH_4$ or convert methanol to $CH_4$ unless $H_2$ is also supplied as a reductant.[184,202] Although it is clear that a reversal of the $CO_2$-reduction pathway operates in methanol oxidation, the point of entry for methyl group oxidation is not understood.

The 5,10-methylene-$H_4$MPT reductase and the 5,10-methylene-$H_4$MPT dehydrogenase, purified from methanol-grown *M. barkeri*, utilize $F_{420}$ as the electron acceptor and have properties nearly identical to the enzyme from *M. thermoautotrophicum*.[203,204] The 5,10-methenyl-$H_4$MPT$^+$ cyclohydrolase purified from *M. barkeri* strain MS appears identical to the *M. thermoautotrophicum* enzyme.[41] Extracts of *M. barkeri* strain Fusaro are unable to convert 10-formyl-$H_4$MPT to 5,10-methenyl-$H_4$MPT$^+$, a result that indicates that 5-formyl-$H_4$MPT, rather than 10-formyl-$H_4$MPT, is an intermediate in methanol oxidation to carbon dioxide in *M. barkeri* strain Fusaro.[205] The formyl-MF dehydrogenase from methanol-grown *M. barkeri* is a molybdo-iron-sulfur enzyme with a native molecular mass of 220,000 Da and contains six subunits with apparent molecular masses of 65,000, 50,000, 37,000, 34,000, 29,000, and 17,000 Da.[206] The native enzyme contains approximately 1 mole of molybdopterin guanine dinucleotide.[207] Activity is assayed with the artificial electron acceptor methylviologen and does not reduce $F_{420}$; thus, the physiological electron acceptor is unknown.[206]

## C. Electron Transport and Bioenergetics

### 1. Methanol Oxidation to $CO_2$

Methanol-grown *M. barkeri* contains multiple forms of hydrogenase,[208-211] and the $F_{420}$-re-

ducing hydrogenase purified from strain Fusaro constitutes nearly 2% of the total cell protein,[210] suggesting a potential role for hydrogenases in methanol conversion to methane. Furthermore, $H_2$ is a product of methanol oxidation when the methylreductase of whole cells is inhibited,[208,212] and whole cells catalyze a $H_2$-dependent reduction of methanol to methane coupled to proton translocation and ATP synthesis.[213] Thus, it is proposed that $H_2$ may be an intermediate during methanogenesis from methanol,[208] but direct evidence is lacking. The $F_{420}$-reducing hydrogenase from methanol-grown *M. barkeri* is located at the periphery of the cytoplasmic membrane and could potentially initiate an electron transport chain with CoM-S-S-HTP as the final electron acceptor.[214]

Sodium is required for the growth of all methanogenic organisms on $H_2/CO_2$, acetate, or methanol. In the case of methanol dismutation to $CO_2$ and $CH_4$, a sodium gradient (high outside) drives the thermodynamically unfavorable oxidation of methanol to the redox level of formaldehyde (ca. 5,10-methylene-$H_4$MPT) in *M. barkeri*;[215,216] thus, $H_2$ and $CO_2$ formation from methanol is strictly dependent on sodium ions.[212] A secondary $Na^+/H^+$ antiport system is responsible for the $Na^+$ extrusion. Methane formation from methanol plus $H_2$ is independent of sodium and evidence for a $Na^+$-translocating ATPase cannot be obtained; thus, involvement of a primary $Na^+$ pump in *M. barkeri* is unlikely.[216] The requirement of a $Na^+$ gradient for methanol oxidation led to the discovery of an electron-transport-driven sodium extrusion coupled with reduction of formaldehyde (ca. 5,10-methylene-$H_4$MPT) to methane.[23] The extrusion is independent of the $Na^+/H^+$ antiporter and a proton gradient, indicative of a primary sodium pump. Another primary sodium pump in *M. barkeri* is coupled to the oxidation of formaldehyde to $H_2$ and $CO_2$; it is proposed that the exergonic oxidation of formyl-MF (Reaction 3) to $CO_2$ and $H_2$ may be the coupling reaction.[25] The oxidation of formaldehyde to $H_2$ and $CO_2$ is also coupled to the phosphorylation of ADP by a proton-motive-force generated with the $Na^+/H^+$ antiporter;[25] thus, 5,10-methylene-$H_4$MPT oxidation during methanol dismutation may conserve energy.

### 2. Methanol Reduction to $CH_4$

Several lines of evidence support a proton-motive-force-driven synthesis of ATP during reduction of methanol to $CH_4$ in *M. barkeri* and *Methanolobus tindarius*.[213,217–219] Sodium ions are not required for ATP synthesis coupled to the reduction of methanol with $H_2$, an argument against the involvement of a $Na^+$ gradient. In addition, the genes encoding the α and β subunits of a DCCD-sensitive ATPase from methanol-grown *M. barkeri* reveal a deduced amino acid sequence with high identity to the vacuolar $H^+$-ATPases of eukaryotes and to a lower extent with the $F_1F_0$; ATPase from *Escherichia coli*.[220] ATP synthesis, and the generation of a transmembrane proton gradient, is coupled to $CH_4$ formation from $CH_3$-S-CoM and $H_2$ catalyzed by vesicles of strain Gö1; these results suggest a requirement for membrane components in electron transport.[217] Furthermore, the addition of membranes to the soluble fraction of methanol-grown strain Gö1 greatly stimulates $CH_4$ formation from $H_2$ and $CH_3$-S-CoM.[221] A role for iron-sulfur centers, and possibly cytochromes, is postulated for electron transport during the $H_2$-dependent reduction of methanol to methane.[221,222] ATP synthesis by vesicles of Gö1 was studied further by examining the reduction of CoM-S-S-HTP with $H_2$;[223] as expected, a transmembrane proton gradient couples ATP synthesis with the transfer of electrons from $H_2$ to the heterodisulfide. The results also show that the heterodisulfide reductase can be localized to the membranes of strain Gö1 when prepared by the gentle lysis of protoplasts. Recently, a $F_{420}$-nonreactive and membrane-bound hydrogenase was purified from methanol-grown *Methanosarcina* strain Gö1.[224] The 79,000-Da enzyme is composed of two subunits with molecular masses of 60,000 and 40,000 Da. The hydrogenase contained nickel, iron, and sulfide, but no flavins were detected. In addition to $H_2$, reduced $F_{420}$ ($F_{420}H_2$) serves as an electron donor to vesicles of strain Gö1 that catalyze the reduction of CoM-S-S-HTP.[225] The $F_{420}H_2$-dependent reduction of the heterodisulfide is coupled to the transfer of protons across the everted vesicles into the lumen, thereby generating an electrochemical potential that drives the phosphorylation of

ADP.[226] The ability of 5,10-methylene-$H_4$MPT dehydrogenase and 5,10-methylene-$H_4$MPT reductase from methanol-grown *M. barkeri* to reduce $F_{420}$ is consistent with these results.[201] Thus, it is proposed that an $F_{420}H_2$-dehydrogenase transfers electrons from $F_{420}H_2$ to the heterodisulfide reductase through membrane-bound electron carriers.[225,226] The presence of a vesicle-associated $F_{420}H_2$-oxidizing hydrogenase that first converts $F_{420}H_2$ to $H_2$ has been ruled out;[225] however, methanol-grown *M. barkeri* contains a membrane-associated $F_{420}$-dependent hydrogenase.[214] Recently, a $F_{420}H_2$-dehydrogenase has been purified from methanol-grown *M. tindarius*.[227] The apparent molecular mass of the native enzyme is 120,000 Da and is composed of five different subunits with apparent molecular masses of 45,000, 40,000, 22,000, 18,000, and 17,000 Da. The dehydrogenase contains iron and acid-labile sulfur but no flavin. Vesicles of Gö1 also catalyze an $F_{420}$-independent $H_2$; CoM-S-S-HTP oxidoreductase activity coupled to proton translocation, thereby driving the phosphorylation of ADP;[222] apparently, an $F_{420}$-independent membrane-bound hydrogenase is involved.[224] The question then arises as to the purpose of two different proton-translocating systems with CoM-S-S-HTP as the electron acceptor.

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This review is dedicated to Professor Ralph S. Wolfe on the occasion of his seventieth birthday. I am grateful to Andrew P. Clements and Peter E. Jablonsky for their helpful comments.

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