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# Energetics of Methanogenesis Studied in Vesicular Systems

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Methanogenesis is restricted to a group of prokaryotic microorganisms which thrive in strictly anaerobic habitats where they play an indispensable role in the anaerobic food chain. Methanogenic bacteria possess a number of unique cofactors and coenzymes that play an important role in their specialized metabolism. Methanogenesis from a number of simple substrates such as $H_2 + CO_2$, formate, methanol, methylamines, and acetate is associated with the generation of transmembrane electrochemical gradients of protons and sodium ions which serve as driving force for a number of processes such as the synthesis of ATP via an ATP synthase, reverse electron transfer, and solute uptake. Several unique reactions of the methanogenic pathways have been identified that are involved in energy transduction. Their role and importance for the methanogenic metabolism are described.

**KEY WORDS:** Methanogenesis; $\Delta\tilde{\mu}_{H+}$; $F_{420}H_2$ dehydrogenase; hydrogenase; heterodisulfide reductase; $\Delta\tilde{\mu}_{Na+}$; formyl-methanofuran dehydrogenase; methyl-$H_4$MPT:HS-CoM methyltransferase; ATP synthase.

## 1. INTRODUCTION

Methanogenesis is an important process in nature because it represents the terminal step in the anaerobic breakdown of organic matter under sulfate-limiting conditions. Global biological methane production is thought to exceed the current production of natural gas (Daniels *et al.*, 1984) and must be more than one billion tons per year. The microorganisms responsible for the production of methane became known through the pioneering work of Barker (Barker, 1936) and Hungate (Hungate, 1969). Since the methods became available to isolate and handle these oxygen-sensitive organisms, more than 60 species have been described belonging to the three orders Methanobacteriales, Methanococcales, and Methanomicrobiales (Garcia, 1990).

Methanogenic bacteria constitute the largest branch of one of the two prokaryotic kingdoms, the Archaea, and have received attention due to the peculiarities of their composition and their transcriptional and translational apparatus (Woese *et al.*, 1990). But also their biochemistry is unique. This is true for the enzymes and coenzymes involved in methanogenesis as well as their way of conserving energy.

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## 2. BIOCHEMISTRY OF METHANOGENESIS

Before discussing the bioenergetic aspects of methanogenesis, we will outline the pathways which give rise to the formation of methane from the most prominent methanogenic substrates.

### 2.1. The Substrates of Methanogenic Bacteria

Methanogens utilize only a very limited number of substrates, the most common being $H_2 + CO_2$. Other important substrates are formate, methanol, methylamines (mono-, di-, trimethylamine), and acetate. The utilization of the latter three substrates is limited to members of one family, the Methanosarcinaceae. The conversion of these substrates to methane (and other products) is given in the following



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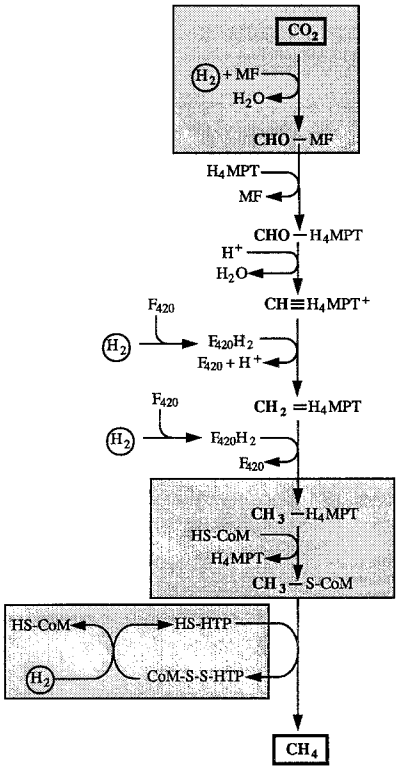



**Fig. 1.** Pathway of methanogenesis from $H_2 + CO_2$. MF, methanofuran; $H_4$MPT, tetrahydromethanopterin; $F_{420}$, oxidized form of coenzyme $F_{420}$; $F_{420}H_2$, reduced form of $F_{420}$; HS-CoM, coenzyme M (2-mercaptoethanesulfonate); HS-HTP, 7-mercaptoheptanoylthreonine phosphate; CoM-S-S-HTP, heterodisulfide of coenzyme M and 7-mercaptoheptanoylthreonine phosphate. Reactions occurring at the membranes are boxed in gray.

equations:

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O,$$
$$\Delta G^{0'} = -130\,\text{kJ/mol } CH_4 \quad (1)$$

$$4HCOOH \rightarrow 3CO_2 + CH_4 + 2H_2O,$$
$$\Delta G^{0'} = -119\,\text{kJ/mol } CH_4 \quad (2)$$

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O,$$
$$\Delta G^{0'} = -106\,\text{kJ/mol } CH_4 \quad (3)$$

$$4(CH_3)_3NH^+ + 6H_2O \rightarrow 9CH_4 + 3CO_2 + 4NH_4^+,$$
$$\Delta G^{0'} = -76\,\text{kJ/mol } CH_4 \quad (4)$$

$$CH_3COOH \rightarrow CH_4 + CO_2,$$
$$\Delta G^{0'} = -32\,\text{kJ/mol } CH_4 \quad (5)$$

## 2.2. The Pathway of $CO_2$ Reduction

The $CO_2$ reduction pathway (Fig. 1) has been extensively reviewed elsewhere (Rouvière and Wolfe, 1988); so we will restrict ourselves to a short summary. The reduction of $CO_2$ to $CH_4$ does not involve the free $C_1$ intermediates formate, formaldehyde, and methanol, but the $C_1$ moieties remain bound to a number of unique cofactors such as methanofuran (MF), tetrahydromethanopterin ($H_4$MPT) and coenzyme M (HS-CoM) (Fig. 2). Whereas $H_4$MPT is a functional analogue of tetrahydrofolate, MF is rather unique from both the structural and functional point of view. The conversion of $CO_2$ to methane starts with the binding of $CO_2$ to methanofuran (MF) with formation of the unstable carboxy-MF intermediate and its subsequent reduction to formyl-methanofuran (HCO-MF) (Fig. 3):

$$\mathbf{CO_2} + H_2 + MF \rightarrow \mathbf{HCO}\text{–}MF + H_2O,$$
$$\Delta G^{0'} = +16\,\text{kJ/mol} \quad (6)$$

The electrons for this reduction stem from $H_2$ which is taken up by hydrogenase, but the physiological electron donor is not yet known. Reaction (6) is catalyzed by formyl-MF dehydrogenase which has been purified from *Methanosarcina barkeri* (Karrasch *et al.*, 1989). The enzyme has a molecular mass of 220 kDa, is composed of six nonidentical subunits, contains nonheme iron and acid-labile sulfur, and catalyzes *in vitro* the following reaction:

$$\mathbf{HCO}\text{–}MF + H_2O + 2\ \text{methyl viologen}_{\text{oxidized}} \rightarrow \mathbf{CO_2} + 2\ \text{methyl viologen}_{\text{reduced}} + MF + 2H^+ \quad (7)$$

The formyl-methanofuran dehydrogenase from *Ms. barkeri* was shown to contain a molybdopterin guanine dinucleotide as a prosthetic group (Karrasch *et al.*, 1990).

Then, the formyl moiety of formyl-MF is transferred to $H_4$MPT giving rise to formyl-$H_4$MPT which subsequently undergoes cyclization to methenyl-$H_4$MPT (Fig. 4):

$$\mathbf{HCO}\text{–}MF + H_4MPT \rightarrow \mathbf{HCO}\text{–}H_4MPT + MF,$$
$$\Delta G^{0'} = -5\,\text{kJ/mol} \quad (8)$$

$$\mathbf{HCO}\text{–}H_4MPT + H^+ \rightarrow \mathbf{CH}\equiv MPT^+ + H_2O,$$
$$\Delta G^{0'} = -2\,\text{kJ/mol} \quad (9)$$

Reaction (8) is catalyzed by formyl-methanofuran: $H_4$MPT formyltransferase which was purified from *Methanobacterium thermoautotrophicum* and *Ms. barkeri* (Breitung and Thauer, 1990; Donnelly and Wolfe, 1986). The enzyme from both organisms is specific for $N^5$-HCO-$H_4$MPT. The gene coding for the enzyme

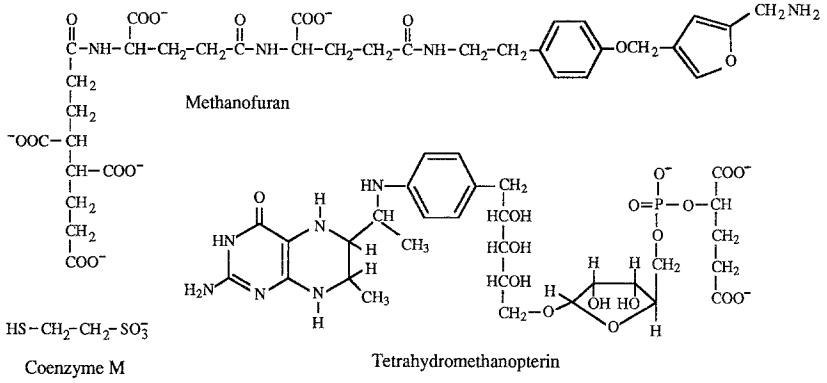



**Fig. 2.** Structures of methanofuran (MF), coenzyme M (HS-CoM), and tetrahydromethanopterin ($H_4$MPT), unique coenzymes involved in methanogenesis.

from *Mb. thermoautotrophicum* was cloned and sequenced (DiMarco *et al.*, 1990); the molecular mass (31 kDa) as deduced from the amino acid sequence is in good agreement with that of the *Ms. barkeri* enzyme (32 kDa) as determined by SDS gel electrophoresis. The native enzyme consists of one polypeptide and is devoid of prosthetic groups (Breitung and Thauer, 1990). Reaction (9) is catalyzed by $N^5$,$N^{10}$-methenyl-$H_4$MPT cyclohydrolase. The enzyme was purified from *Mb. thermoautotrophicum*, and shown to be specific for $N^5$-formyl-$H_4$MPT rather than $N^{10}$-formyl-$H_4$MPT (DiMarco *et al.*, 1986). The 82-kDa protein is composed of two identical subunits of 41 kDa.

In two $F_{420}H_2$-dependent reactions, methenyl-$H_4$MPT is subsequently reduced to methylene-$H_4$MPT and further to methyl-$H_4$MPT (Fig. 4):

$$\mathbf{CH}\equiv H_4MPT^+ + F_{420}H_2 \rightarrow \mathbf{CH_2}=H_4MPT + F_{420} + H^+, \quad \Delta G^{0'} = +6.5\,kJ/mol \qquad (10)$$

$$\mathbf{CH_2}=H_4MPT + F_{420}H_2 \rightarrow \mathbf{CH_3}\text{-}H_4MPT + F_{420}, \quad \Delta G^{0'} = -5\,kJ/mol \qquad (11)$$

Coenzyme $F_{420}$ ([*N*-L-lactyl-γ-L-glutamyl]-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin-5′-phosphate) is a deazaflavin which serves as hydrogen carrier in methanogens (Fig. 5). Methylene-$H_4$MPT dehydrogenase catalyzing reaction (10) was purified from *Mb. thermoautotrophicum* and

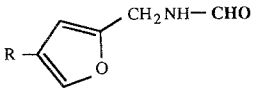


**Fig. 3.** Structure of formyl-methanofuran (CHO-MF). The formyl moiety is indicated in bold letters.

*Ms. barkeri* (Enßle *et al.*, 1991; Hartzell *et al.*, 1985). The native enzyme from both organisms has molecular masses of 240 and 32 kDa, respectively. The *Mb. thermoautotrophicum* protein is composed of eight identical polypeptides of 31 kDa, whereas the *Ms. barkeri* enzyme consists of only one subunit.

In addition to the $F_{420}H_2$-dependent enzyme, *Mb. thermoautotrophicum* contains a $H_2$-linked methylene-$H_4$MPT dehydrogenase (Zirngibl *et al.*, 1990):

$$\mathbf{CH}\equiv H_4MPT^+ + H_2 \rightarrow \mathbf{CH_2}=H_4MPT + H^+, \quad \Delta G^{0'} = -5.5\,kJ/mol \qquad (12)$$

The $H_2$-dependent enzyme is composed of one polypeptide with a molecular mass of 43 kDa. Chromophoric prosthetic groups were not detected in any of the methylene-$H_4$MPT dehydrogenases purified. The

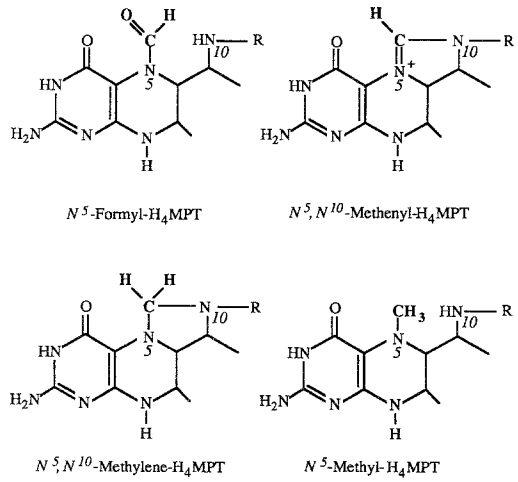



**Fig. 4.** Structures of tetrahydromethanopterin derivatives playing a role in methanogenesis. The respective bound $C_1$ moiety is indicated in bold letters.

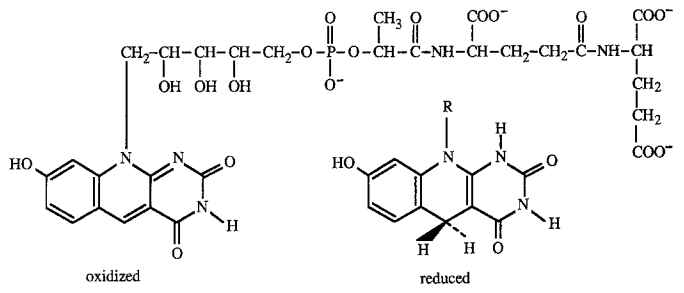



**Fig. 5** Structure of the hydrogen carrier coenzyme $F_{420}$ ([*N*-L-lactyl-γ-L-glutamyl]-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin-5′-phosphate) in the oxidized and reduced form.

physiological significance of the presence of the two enzymes in the same organism is still unknown. However, it appears advantageous for growth on $H_2$ + $CO_2$ that the electrons from $H_2$ may be directly used and not by way of an $F_{420}$-dependent hydrogenase.

Reaction (11) is catalyzed by methylene-$H_4$MPT reductase which has been purified from *Mb. thermoautotrophicum* strains ΔH and Marburg as well as from *Ms. barkeri* (Ma and Thauer, 1990a, b; Te Brömmelstroet *et al.*, 1990). The enzymes from all three organisms are composed of one type of subunit of 36 kDa and seem to be devoid of prosthetic groups.

The $F_{420}H_2$ oxidized by methenyl-$H_4$MPT dehydrogenase and methylene-$H_4$MPT reductase [reactions (10) and (11)] has to be regenerated. This is accomplished by an $F_{420}$-dependent hydrogenase:

$$H_2 + F_{420} \rightarrow F_{420}H_2, \quad \Delta G^{0'} = -13.5\,\text{kJ/mol} \tag{13}$$

$F_{420}$-dependent hydrogenases have been purified from a number of methanogens including *Mb. thermoautotrophicum*, *Methanobacterium formicicum*, *Ms. barkeri*, and *Methanococcus voltae* (Fauque *et al.*, 1984; Fox *et al.*, 1987; Jin *et al.*, 1983; Muth *et al.*, 1987). All of these $F_{420}$-dependent hydrogenases contain flavin and nickel, and their primary structure shows a high degree of similarity with that of other prokaryotic hydrogenases (Reeve and Beckler, 1990). The $F_{420}$-dependent hydrogenases from methanogens are found in the cytoplasm after cell breakage, but a membrane association could be demonstrated in three of these organisms with the immunogold labelling technique (Baron *et al.*, 1989; Lünsdorf *et al.*, 1991; Muth, 1988). An involvement of the $F_{420}$-dependent hydrogenase in energy transduction is still a matter of debate.

The methyl group from methyl-$H_4$MPT is then transferred to another unique cofactor called coenzyme M (HS-CoM), giving rise to methyl-CoM:

$$\mathbf{CH_3}\text{-}H_4\text{MPT} + \text{HS-CoM} \rightarrow \mathbf{CH_3}\text{-S-CoM} + H_4\text{MPT}, \quad \Delta G^{0'} = -29\,\text{kJ/mol} \tag{14}$$

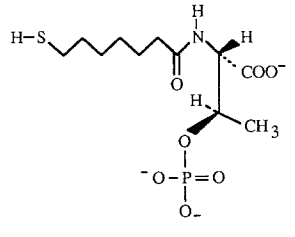
The enzyme catalyzing this reaction was recently purified from *Mb. thermoautotrophicum* (Kengen *et al.*, 1992), and there are indications for a novel type of energy transduction associated with this reaction (see Section 5.1).

The final step of methanogenesis involves two partial reactions:

$$\mathbf{CH_3}\text{-S-CoM} + \text{HS-HTP} \rightarrow \mathbf{CH_4} + \text{CoM-S-S-HTP}, \quad \Delta G^{0'} = -43\,\text{kJ/mol} \tag{15}$$

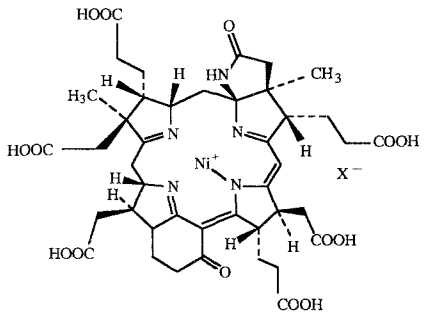
$$\text{CoM-S-S-HTP} + H_2 \rightarrow \text{HS-CoM} + \text{HS-HTP}, \quad \Delta G^{0'} = -42\,\text{kJ/mol} \tag{16}$$

In the first partial reaction (15) methyl-S-CoM is reduced with 7-mercaptoheptanoylthreonine phosphate (HS-HTP), another unique cofactor in methanogens (Fig. 6) giving rise to methane and CoM-S-S-HTP. The reaction is catalyzed by the methyl-CoM



*N*-7-Mercaptoheptanoylthreonine phosphate (HS-HTP)

**Fig. 6.** Structure of 7-mercaptoheptanoylthreonine phosphate (HS-HTP), which serves as electron carrier in methanogenesis.




Factor $F_{430}$

**Fig. 7.** Structure of coenzyme $F_{430}$, the prosthetic group of the methyl-coenzyme M reductase.

reductase which contains a nickel tetrapyrrole called coenzyme $F_{430}$ (Fig. 7) (Pfaltz *et al.*, 1984). In the active enzyme the nickel is most likely in the monovalent state (Cheesman *et al.*, 1989). The enzyme from *Mb. thermoautotrophicum* has a molecular mass of 300 kDa, binds 2 mol of $F_{430}$/mol enzyme, and is composed of three different subunits with molecular masses of 65, 46, and 35 kDa in an $\alpha_2\beta_2\gamma_2$ arrangement (Ellefson and Wolfe, 1981; Ellermann *et al.*, 1988). Up to 10% of the cellular protein consists of this enzyme, indicating that the methyl-CoM reductase represents the rate-limiting step in methanogenesis (Rouvière and Wolfe, 1988). The genes coding for the three polypeptides of the enzyme have been cloned from a number of methanogenic bacteria and shown to have a high degree of similarity (Allmansberger *et al.*, 1986; Weil *et al.*, 1988). Interestingly, *Mb. thermoautotrophicum* contains a second methylreductase called MCRII which is genetically distinct from the enzyme described above (MCRI) (Rospert *et al.*, 1990). MCRII is also composed of three subunits, but differs with respect to the N-termini of the polypeptides and the size of the $\gamma$-subunit. MCRI is mainly produced when $H_2 + CO_2$ is limiting.

In the second partial reaction (16), CoM–S–S–HTP which was formed in the methyl-CoM reductase reaction is reduced in order to regenerate HS–CoM and HS–HTP. Evidence has been presented that this reaction involves electron transport along membrane-bound electron carriers coupled with proton translocation across the cytoplasmic membrane (see further below). Depending on the growth substrate, either $H_2$ or $F_{420}H_2$ serves as the reductant in this reaction (Deppenmeier *et al.*, 1991). An enzyme catalyzing the reduction of CoM–S–S–HTP with reduced viologen dyes and the oxidation of HS–CoM and HS–HTP with methylene blue was purified from the soluble fraction of *Mb. thermoautotrophicum* after cell breakage (Hedderich *et al.*, 1990). This enzyme named heterodisulfide reductase has an apparent molecular mass of 550 kDa, is composed of three different subunits (80, 36, and 21 kDa), and contains 4 mol FAD and 72 mol FeS per mole of native enzyme. In *Methanosarcina* strains, the heterodisulfide reductase is membrane-bound and has to be solubilized with detergents (U. Deppenmeier, unpublished results).

### 2.3. Methanogenesis from Substrates other than $H_2 + CO_2$

With a few exceptions, the conversion of methanogenic substrates other than $H_2 + CO_2$, involves the same reactions discussed above for the $CO_2$ reduction pathway. Formate utilization starts with its oxidation by formate dehydrogenase:

$$HCOOH \rightarrow CO_2 + 2e^- + 2H^+ \qquad (17)$$

The $CO_2$ thereby produced enters the $CO_2$ reduction pathway outlined above (Fig. 1) with the exception that the reducing equivalents are derived from formate and not from $H_2$. Although produced in small quantities, $H_2$ itself does not appear to be an obligate intermediate in methanogenesis from formate (Schauer and Ferry, 1980). The formate dehydrogenase purified from *Mb. formicicum* contains nontheme iron, molybdopterin guanine dinucleotide as a cofactor, and requires FAD for activity (Johnson *et al.*, 1991; Schauer and Ferry, 1986). The $F_{420}$-linked enzyme has a molecular mass of 177 kDa and is composed of two subunits of 85 and 53 kDa. The corresponding genes were cloned, sequenced, and expressed in *Escherichia coli* (Shuber *et al.*, 1986). *Methanococcus vannielii* was reported to contain two $F_{420}$-reactive formate dehydrogenases, a selenium-dependent and a selenium-independent one (Jones and Stadtman, 1981). The $F_{420}H_2$ produced in the formate dehydrogenase is reoxidized in the $F_{420}$-linked reactions of the $CO_2$ reduction pathway [reactions (10) and (11)], but it is not yet clear whether $F_{420}H_2$ also serves as electron carrier in the first $CO_2$ reduction step (formyl-MF dehydrogenase) and in the heterodisulfide reduction during growth on formate. If this turns out to be true, formate-utilizing methanogens would also be expected to use the $F_{420}H_2$-dependent heterodisulfide reductase system (Section 4.2).

The conversion of **methanol** or **methylamines** differs from the $CO_2$ reduction pathway in that the

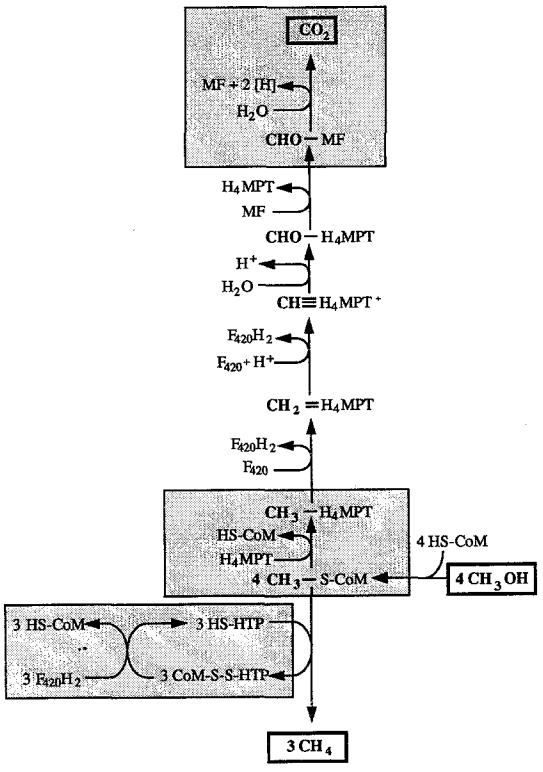



**Fig. 8.** Pathway of methanogenesis from methanol. MF, methanofuran; $H_4$MPT, tetrahydromethanopterin; $F_{420}$, oxidized form of coenzyme $F_{420}$; $F_{420}H_2$, reduced form of $F_{420}$; HS-CoM, coenzyme M (2-mercaptoethanesulfonate); HS-HTP, 7-mercaptoheptanoylthreonine phosphate; CoM-S-S-HTP, heterodisulfide of coenzyme M and 7-mercaptoheptanoylthreonine phosphate. Reactions occurring at the membranes are boxed in gray.

methyl groups from these substrates are disproportionated, i.e., their conversion consists of an oxidative and a reductive branch (Fig. 8):

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

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

The oxidation of methanol involves the transfer of the methyl group to $H_4$MPT followed by the stepwise oxidation of the resulting methyl-$H_4$MPT to $CO_2$. It is not yet clear whether methyl-CoM is an obligate intermediate or whether the methyl group from the respective substrate is directly transferred to $H_4$MPT (see also Section 5.1.2). The oxidation of methyl-$H_4$MPT to $CO_2$ follows the same route outlined above for the $CO_2$ reduction pathway, but in the reverse direction [reactions (6) and (8)–(11)]. The electrons thereby generated are used to reduce methanol to methane (Fig. 8). Methanol enters the reductive branch by transfer of the methyl group to coenzyme M as catalyzed by two methyl transferases, one of them being a corrinoid enzyme (Van der Meijden *et al.*, 1983). During growth on trimethylamine, a specific methyltransferase is expressed (Naumann *et al.*, 1984). Methyl-CoM reduction occurs via methyl-CoM reductase [reaction (15)] and heterodisulfide reductase [reaction (16)], but in contrast to growth on $H_2$ + $CO_2$, the electrons for this reduction are not derived from $H_2$ but from $F_{420}H_2$ which is generated during the methyl group oxidation (in the methylene-$H_4$MPT reductase and the methylene-$H_4$MPT dehydrogenase reactions). The $F_{420}H_2$-dependent heterodisulfide reductase will be discussed in Section 4.2. It is not yet clear how the electrons formed during the oxidation of formyl-$H_4$MPT are channelled to the heterodisulfide reductase, since the physiological electron acceptor in this reaction is not yet known.

Methanogenesis from **acetate** starts with its activation to acetyl-CoA which is achieved by the combined action of acetate kinase and phosphotransacetylase (in *Methanosarcina* species) or by acetate thiokinase (in *Methanothrix* species). The exact mechanism of the acetate cleavage is still unclear, but there is general agreement that the carbon monoxide dehydrogenase complex plays a central role in this process. Acetyl-CoA cleavage can be considered as a decarbonylation giving rise to enzyme-bound CO and a methyl moiety which is transferred to $H_4$MPT and subsequently to HS–CoM (Laufer *et al.*, 1987). The enzyme-bound CO is oxidized and the electrons thereby generated are used to reduce methyl-CoM to methane. Again, this involves the reduction by HS–HTP and the formation of CoM–S–S–HTP which is then reduced by the electrons derived from CO oxidation. Recent investigations indicate the involvement of ferredoxin in this electron transfer (Fischer and Thauer, 1990). The conversion of CO to $H_2$ and $CO_2$ was shown in *Ms. barkeri* to be coupled to the generation of $\Delta\tilde{\mu}_{H^+}$ and ATP synthesis (Bott *et al.*, 1986).

## 3. THERMODYNAMIC CONSIDERATIONS OF METHANOGENESIS

### 3.1. The Reactions from $CO_2$ to Methyl-CoM

Under standard conditions, corresponding to a hydrogen partial pressure ($p_{H2}$) of 1 atm, the free energy change ($\Delta G^{0'}$) of the first step in methanogenesis from $H_2$ + $CO_2$ is endergonic (+ 16 kJ/mol). Since the

$p_{H_2}$ is natural habitats of methanogens is much lower than that, the actual $\Delta G^{0'}$ of the formyl-MF formation is $> +16$ kJ/mol. Consequently, in order to make the reaction proceed in the direction of the synthesis of formyl-MF, it can be assumed that this reaction is "pulled" by the exergonic reactions that follow or that a certain form of energy is invested. We will discuss this topic in more detail in Section 5.2.

The conversion of formyl-MF to methyl-$H_4$MPT [reactions (8)–(11)] under standard conditions is slightly exergonic, but in principle reversible. All of the enzymes catalyzing this transformation are located in the cytoplasm and there are no indications for a coupling with ion translocations across the cytoplasmic membrane. In contrast, the methyl group transfer from methyl-$H_4$MPT to HS–CoM is highly exergonic under standard conditions. As we shall see in Section 5.1, experimental evidence has been presented that this reaction occurs at the cytoplasmic membrane and is coupled with the extrusion of sodium ions.

### 3.2. The Central Role of the Methyl-CoM Reduction in Methanogenesis from All Substrates

The free energy available in methyl-CoM reduction depends on the electron donor utilized. The reductive demethylation of $CH_3$–S–CoM with $H_2$ as electron donor is the energetically most favorable reaction in the $CO_2$ reduction pathway ($\Delta G^{0'} = -85$ kJ/mol). Using $CH_3OH + H_2$ as a substrate, it was indirectly demonstrated that resting cells of *Ms. barkeri* couple the $H_2$-dependent methyl-CoM reduction with the generation of a transmembrane electrochemical gradient of protons ($\Delta\tilde{\mu}^{H^+}$) which is taken advantage of for ATP synthesis (Blaut and Gottschalk, 1984). The results from this study were further supported by experiments with inverted crude vesicles of *Methanosarcina* strain Göl which clearly demonstrated ATP synthesis from ADP and $P_i$ in response to either artificial pH gradients or the addition of $H_2$ and methyl-CoM; both processes were susceptible to uncouplers (Peinemann *et al.*, 1989).

When it became evident that the methyl-CoM reduction consists of two partial reactions, the question arose which of the two was associated with the generation of a $\Delta\tilde{\mu}_{H^+}$. Both partial steps are associated with a standard free energy change of more than $-40$ kJ/mol and could therefore be theoretically coupled with energy transduction. However, only the reduction of the heterodisulfide could be demonstrated in *Methanosarcina* strain Göl to occur at the membranes and to be coupled with ATP synthesis, whereas the methyl-CoM reductase does not appear to be directly involved in energy transduction (Peinemann *et al.*, 1990). The standard redox potential of the HS–CoM, HS–HTP/CoM–S–S–HTP couple is on the order of $E_{m,7} = -200$ mV (Hauska, 1988), so that electron transport-driven phosphorylation with $H_2$ as electron donor ($E_{m,7} = -420$ mV) is possible. However, it seems very likely that the actual redox potential ($E_h$) is more positive under cellular steady-state conditions. This would be the case if the [CoM–S–S–HTP]/[HS–CoM] [HS–HTP] ratio exceeds 1. At ratios of 10 and 100 the actual redox potentials would be $E_h = -140$ mV, $-80$ mV, respectively. This would enlarge the redox span ($\Delta E$) between $H_2$ and the heterodisulfide considerably. Consequently, $\Delta G'$ for the heterodisulfide reduction would become $-54$ kJ/mol and $-65$ kJ/mol, respectively. The [CoM–S–S–HTP]/[HS–CoM] [HS–HTP] ratio is highly influenced by reaction (15), both thermodynamically and kinetically. Since the formation of CoM–S–S–HTP and $CH_4$ from $CH_3$–S–CoM and HS–HTP is at least thermodynamically highly favored ($K_{eq} = 34.5 \times 10^6$), a $\Delta G'$ considerably more negative than $\Delta G^{0'}$ can be envisaged for the $H_2$-dependent heterodisulfide reduction.

## 4. CELL-FREE ENERGY-TRANSDUCING ELECTRON TRANSPORT SYSTEMS OF METHANOGENIC BACTERIA

The conversion of each methanogenic substrate involves the reductive demethylation of methyl-CoM to methane with the concomitant formation of the heterodisulfide and its subsequent reduction. The latter reaction takes place at the cytoplasmic membrane and is directly associated with energy transduction. Which reductant is used in this reaction depends on the substrate utilized. So far, two heterodisulfide reductase systems were described: a $H_2$-dependent and an $F_{420}H_2$-dependent one. The former was suggested to be operative during growth on $H_2 + CO_2$, and the latter during growth on methanol (Deppenmeier *et al.*, 1991). The electron donor in the heterodisulfide reductase reaction during growth on acetate has not been identified; however, recent experiments suggest the involvement of ferredoxin (Fischer and Thauer, 1990).

### 4.1. Methane Formation from Methyl-CoM and $H_2$ Coupled with ATP Synthesis in a Cell-Free System

The first cell-free system catalyzing ATP synthesis in response to artificially imposed pH gradients or to methane formation from methyl-CoM and $H_2$ consisted of inverted vesicles of *Methanosarcina* strain Göl still containing cytoplasmic components (Peinemann *et al.*, 1989). ATP synthesis but not methanogenesis was inhibited by protonophores. However, the coupling of this system was very poor: 1 mol of ATP synthesized per 100 mol of $CH_4$ produced.

### 4.2. The $F_{420}H_2$-Dependent Heterodisulfide Reductase System

After it became evident that there is no functional necessity for a membrane association of the methyl-reductase, attempts were made to establish a vesicle system free of cytoplasmic proteins which was capable of energy transduction. Such a system became available with the discovery that washed inverted vesicles of *Methanosarcina* strain Göl synthesize ATP in response to $F_{420}H_2$-dependent heterodisulfide reduction (Deppenmeier *et al.*, 1990). The electron transport from $F_{420}H_2$ to CoM-S-S-HTP was shown to be coupled with the translocation of protons across the membrane into the lumen of the vesicles, resulting in the generation of a $\Delta\tilde{\mu}_{H^+}$. Protonophores prevent the establishment of $\Delta\tilde{\mu}_{H^+}$ and the synthesis of ATP from ADP and $P_i$, but stimulate the electron transport. The decrease of the electron transport rate upon addition of *N,N'*-dicyclohexylcarbodiimide (DCCD), an inhibitor of the ATP synthase, and the reversal of this inhibition by protonophores, as well as the stimulation of electron transport by ADP, indicated a stringent coupling between electron transport and ATP synthesis. These results are analogous to the effects of ADP, dinitrophenol, and oligomycin on mitochondrial electron transport and indicate that the electron transport in these vesicles is under respiratory control. The electron transport system exhibits a stoichiometry of $2H^+$ translocated or 0.4 ATP synthesized per $F_{420}H_2$ oxidized.

What is now the role of the $F_{420}H_2$-dependent heterodisulfide reductase system? It has been proposed that the heterodisulfide reductase plays an indispensable role in methanogens that grow on methanol or methylamines (Deppenmeier *et al.*, 1990). During growth on these substrates, $F_{420}H_2$ is formed in the process of methyl group oxidation to $CO_2$ [reactions (10) and (11) in the reverse direction]. This situation is depicted in Fig. 9. The $F_{420}H_2$ generated has to be oxidized and the reducing equivalents have to be channelled to the heterodisulfide. This is accomplished by the $F_{420}H_2$-dependent heterodisulfide reductase. It will be of interest to find out whether the $F_{420}H_2$-dependent heterodisulfide reductase is found in all methanogens or whether it is restricted to the methylotrophic methanogens.

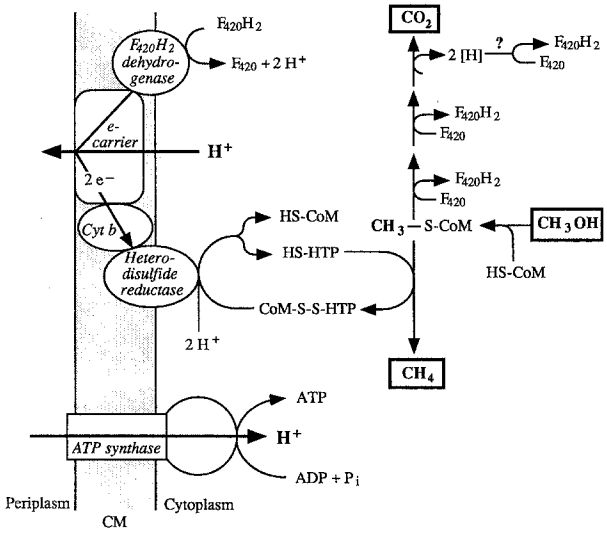



**Fig. 9.** Scheme of electron transfer from $F_{420}H_2$ to the heterodisulfide of coenzyme M and 7-mercaptoheptanoylthreonine phosphate (CoM-S-S-HTP) coupled with proton translocation and ATP synthesis as catalyzed by *Methanosarcina* strain Göl. CM, cytoplasmic membrane.

#### 4.2.1. The $F_{420}H_2$ Dehydrogenase

The $F_{420}H_2$-dependent heterodisulfide reductase can be divided into two partial reactions: in the first one, $F_{420}H_2$ is oxidized by $F_{420}H_2$ dehydrogenase and the electrons are then transferred to an unknown electron carrier. The activity of this reaction can be assayed *in vitro* by using an artificial electron acceptor system such as metronidazole in the presence of methyl viologen as mediator:

$$F_{420}H_2 + \text{metronidazole}_{oxidized} \rightarrow F_{420} + \text{metronidazole}_{reduced} \quad (20)$$

The enzyme catalyzing this reaction was purified from *Methanolobus tindarius*, an obligate methylotrophic methanogen, after solubilization from the membrane with the detergent CHAPS (Haase *et al.*, 1992). The apparent molecular mass of the native

enzyme is 120 kDa and it consists of five different subunits of 45, 40, 22, 18, and 17 kDa. The purified enzyme contains 16 mol Fe and 16 mol S per mole of enzyme, but flavin was not detectable. The absence of flavin is interesting with respect to the question how the enzyme manages the 2-electron/1-electron transition from the hydrogen carrier $F_{420}H_2$ to the one-electron carrier methylviologen which serves as mediator in this system.

#### *4.2.2. The Heterodisulfide Reductase*

The second element in the $F_{420}H_2$-dependent electron transport system is the heterodisulfide reductase. As already mentioned above, there are differences in the location of this protein depending on the organism. The heterodisulfide reductase from *Mb. thermoautotrophicum* is found in the cytoplasmic fraction after cell breakage (Hedderich *et al.*, 1990); its characteristics were discussed in Section 2.2. In contrast, in *Methanosarcina* and *Methanolobus* species the heterodisulfide reductase is found in the membrane fraction, so that detergents have to be employed to detach the enzyme from the membranes. Unfortunately, the purification of this enzyme from any of these organisms has not yet been accomplished.

#### *4.2.3. Physiological Electron Carriers in the $F_{420}H_2$-Dependent Heterodisulfide Reductase Reaction*

An important question aims at 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). Little is known about how tight 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 *Mb. thermoautotrophicum* in the soluble fraction contradict an intrinsic membrane location. As a consequence, the presence of a membrane-intrinsic electron carrier which is engaged in electron transport and proton translocation has to be postulated (Deppenmeier *et al.*, 1990). 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 Göl contain two *b*- type and two *c*-type cytochromes with midpoint potentials ($E_{m,7}$) 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 Methanosarcinaceae (Kühn *et al.*, 1983), the same group of organisms that is assumed to have an $F_{420}H_2$-dependent heterodisulfide reductase.

#### *4.2.4. Possible Mechanisms of $H^+$ Translocation*

Taking into consideration what is known about the $F_{420}H_2$-dependent electron transport system, it is very difficult to formulate a mechanistic concept of how proton translocation is accomplished. A Q-cycle type of mechanism appears unlikely because both the electron donor and the electron acceptor system are located on the cytoplasmic side of the membrane and no hydrogen carrier such as a quinone appears to be involved. Scalar proton production at the periplasmic side, which would be theoretically possible if a periplasmic hydrogenase was involved, cannot occur either. A participation of cytochromes in proton translocation is conceivable. From the present point of view it appears most likely that proton translocation is brought about by a redox-driven proton pump. However, a final answer will only be available after the identification and functional reconstitution of all components involved.

### 4.3. The $H_2$-Dependent Heterodisulfide Reductase System

Besides the $F_{420}H_2$-dependent electron transport system a second energy-conserving system was described in *Methanosarcina* strain Göl (Deppenmeier *et al.*, 1991): 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 vesicle lumen. The $\Delta\mu_{H^+}$ thereby generated drives ATP synthesis from ADP and $P_i$. The system exhibits maximal stoichiometries of 1 $H^+$ translocated/$e^-$ and 1 ATP synthesized/4 $e^-$. Protonophores stimulate

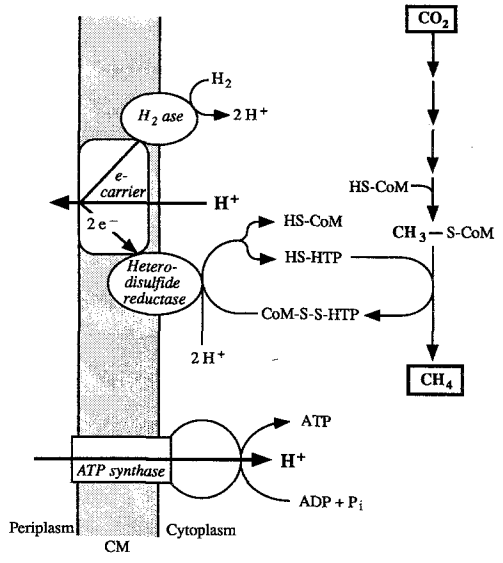



**Fig. 10.** Scheme of electron transfer from $H_2$ to the heterodisulfide of coenzyme M and 7-mercaptoheptanoylthreonine phosphate (CoM-S-S-HTP) coupled with proton translocation and ATP synthesis as catalyzed by *Methanosarcina* strain Göl. CM, cytoplasmic membrane; $H_2$ase, hydrogenase.

electron transport, but dissipate $\Delta\mu_{H^+}$, and inhibit the synthesis of ATP. Electron transport and ATP synthesis are stringently coupled as described above for the $F_{420}H_2$-dependent system (Section 4.2). Figure 10 depicts the role of the $H_2$-dependent heterodisulfide reductase system in energy transduction during growth on $H_2 + CO_2$.

### *4.3.1. Physiological Role of the $H_2$dependent Heterodisulfide Reductase*

The $H_2$-dependent heterodisulfide reductase system was shown to be independent of $F_{420}H_2$. Analysis of the distribution of the $F_{420}H_2$- and the $H_2$-dependent system in two additional methanogens revealed that membranes of the obligate methylotrophic *Ml. tindarius* are devoid of the $H_2$-dependent heterodisulfide reductase, but contain the $F_{420}H_2$-dependent system. The situation encountered in the obligate hydrogenotrophic *Methanococcus thermolithotrophicus* is ambiguous: membranes from this organism are essentially devoid of benzylviologen-dependent heterodisulfide reductase. This activity is almost completely recovered from the cytoplasmic fraction as reported for *Mb. thermoautotrophicum* (Hedderich and Thauer, 1988). Since the membranes from *Mc. thermolithotrophicus* contain in contrast to those from *Methanosarcina* strain Göl and *Ml. tindarius* relatively high $F_{420}$-dependent hydrogenase activity, it is uncertain whether the observed metronidazole/methylviologen-dependent $F_{420}H_2$ oxidation is due to $F_{420}H_2$ dehydrogenase or hydrogenase activity. Based on these results, it was speculated that methanogenic bacteria growing exclusively at the expense of $H_2 + CO_2$ rely on the $H_2$-dependent system, whereas those growing only on methanol and methylamines use the $F_{420}H_2$-dependent system; organisms such as *Methanosarcina* strain Göl which are capable of utilizing methanol or $H_2 + CO_2$ contain both systems (Deppenmeier *et al.*, 1991).

### *4.3.2. The $F_{420}$-Nonreactive Hydrogenase*

We already discussed the role of the $F_{420}$-dependent hydrogenase in the $CO_2$ reduction pathway; it is predominantly involved in the generation of reduced $F_{420}H_2$ which serves as electron donor in reactions (10) and (11). The low activity of this enzyme in washed membranes of *Methanosarcina* strain Göl argues against a role of this hydrogenase in the $H_2$-dependent heterodisulfide reduction (Deppenmeier *et al.*, 1992). In contrast, an $F_{420}$-nonreactive hydrogenase, often referred to as methylviologen-reducing hydrogenase, remains bound to the membranes. The enzyme reduces viologen dyes, but the physiological electron donor is not known. The enzyme is thought to be part of the $H_2$-dependent heterodisulfide reductase system. Very recently, the $F_{420}$-nonreactive hydrogenase was purified from membranes of *Methanosarcina* strain Göl after solubilization with the detergent CHAPS (Haase *et al.*, 1992). SDS gel electrophoresis revealed two polypeptides with molecular masses of 40 and 60 kDa. The enzyme contains 13 mol acid-labile S, 15 mol Fe, and 1 mol Ni/mol enzyme, but no flavin. Interestingly, the *N*-termini of the polypeptides show a higher degree of similarity with the $H_2$-uptake hydrogenase of some eubacteria than to the $F_{420}$-dependent enzyme from *Mb. thermoautotrophicum* and *Mb. formicicum*. The enzyme from *Methanosarcina* strain Göl shares a number of features with several eubacterial periplasmic or membrane-bound hydrogenases such as the number of subunits, the presence of nickel, and the noncytoplasmic location (Deppenmeier *et al.*, 1992). The $F_{420}$-nonreactive hydrogenases isolated from other methanogens such as *Mb. formicicum* and *Mb. thermoautotrophicum* are as well composed of two subunits and contain Ni, but there are no indications for a periplasmic or membrane location (Jin *et al.*, 1983; Kojima *et al.*, 1983). The genes encoding the

large and small subunit of the $F_{420}$-nonreactive hydrogenase from *Mb. 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 can be speculated that the products of these genes may act as membrane anchors for the two catalytic subunits and participate in electron transport.

#### *4.3.4. Electron Carriers Involved in $H_2$Dependent Electron Transport*

Since cytochromes were only detected in the Methanosarcinaceae the majority of the methanogenic species must rely on other electron carriers catalyzing the electron transport-driven $H^+$ translocation. Moreover, even in the methylotrophic species which harbor cytochromes, there are indications that the $H_2$-induced cytochrome reduction as observed in *Ms. barkeri* is unphysiological (Kamlage and Blaut, 1992; Kemner *et al.*, 1987). It is conceivable that the polyferredoxin and the aforementioned protein of unknown function (Section 4.3.3) play a role in this process.

#### *4.3.5. Mechanism of $H^+$ Translocation in the Process of $H_2$-Dependent Heterodisulfide Reduction*

In contrast to the $F_{420}H_2$-dependent heterodisulfide reductase system (Section 4.2.3), a theoretical mechanism of $\Delta\tilde{\mu}_{H^+}$ formation can be formulated if it is assumed that the hydrogenase has a periplasmic location. Under such conditions, uptake of $H_2$ would result in the liberation of scalar protons on the periplasmic side of the 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 of 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. (2) Isotope fractionation studies performed with *Mb. 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 *Ms. barkeri* for proton translocation in response to methanogenesis from methanol $+H_2$ cannot be accounted for by this mechanism (Blaut *et al.*, 1988). Presently it does not appear to be useful to suggest a mechanistic scheme of $H^+$ translocation as long as the identity and the location of the components participating in this electron transport are unknown.

### 4.4. ATP Synthase

$\Delta\tilde{\mu}_{H^+}$-driven ATP synthesis from ADP and $P_i$ in inverted vesicles of *Methanosarcina* strain Göl necessitates the presence of an ATP synthase. Inatomi (1986) isolated the catalytic part of an ATPase from membranes of *Ms. barkeri* and sequenced the corresponding genes. The catalytic part consists of two subunits of 62 kDa ($\alpha$) and 49 kDa ($\beta$) associated in a 1 : 1 ratio. The deduced amino acid sequence from the cloned genes revealed more than 50% identity with the corresponding subunits of the vacuolar ATPase from *Neurospora crassa*, the ATPase from the archaebacterium *Sulfolobus acidocaldarius*, and 20% identity with the corresponding subunits of the $F_1F_0$-type ATPase from *Escherichia coli* (Inatomi *et al.*, 1989a). In accordance with these results, sequence comparisons revealed that the vacuolar ATPase (so-called V-type ATPase) is closely related to the $F_1F_0$-type ATPase (Zimniak *et al.*, 1988). This suggests that all three types of proteins (the archaebacterial, the V-type, and the $F_1F_0$-type ATPase) were derived from a common ancestral protein (Inatomi *et al.*, 1989a). The physiological role of the vacuolar ATPases is ATP-driven $H^+$ translocation, whereas $F_1F_0$-type ATPases serve in most bacteria as ATP synthases. The expected role as an ATP synthase in *Ms. barkeri* and *Ml. tindarius* is supported by the finding that the complete ATPase complex contains in addition to the membrane-peripheral $\alpha$ and $\beta$ subunits four additional membrane-intrinsic polypeptides of 40, 27, 23, and 6 kDa, the latter of which binds DCCD (Inatomi *et al.*, 1989b). Whereas $F_1F_0$-ATPases are known to contain a DCCD-binding protein of about 7 kDa, corresponding polypeptides in vacuolar ATPases are typically 17–19 kDa. The presence of a DCCD-binding proteolipid of 5.5 kDa in membranes from *Ml. tindarius* and the purification of a membrane ATPase from this organism suggest that this type of ATPase is typical of methylotrophic methanogenic bacteria (Scheel and Schäfer, 1990). The available data suggest that the archaebacterial ATPases exhibit at least from the functional point of view a higher similarity with the $F_1F_0$-type ATPases than with the V-type ATPases although the catalytic subunits are more closely related to the latter class of enzymes.

It is still an open question whether hydrogenotrophic organisms also contain the same type of enzyme. Recent sequence comparisons of an amplified DNA fragment from the hydrogenotrophic organism *Mc. thermolithotrophicus* with the amino acid sequence of the ATPase genes from *Sulfolobus acidocaldarius* and from *Neurospora crassa* revealed a high degree of similarity (Gogarten *et al.*, 1989). This indicates that the type of ATPase found in *Ms. barkeri* and *Ml. tindarius* is also present in hydrogenotrophic methanogens and, moreover, in other archaebacteria. A completely different type of ATPase was found in *Mc. voltae* (Dharmavaram and Konisky, 1987). The possible role of this enzyme will be discussed in Section 6.

## 5. GENERATION OF PRIMARY ELECTROCHEMICAL SODIUM ION GRADIENTS IN METHANOGENIC BACTERIA

Growth of all methanogenic bacteria tested so far is strictly dependent on $Na^+$in the medium (Perski *et al.*, 1982). Since this is also true for methane formation as carried out by resting cells, a participation of this ion in the pathway of methanogenesis and the mechanism of energy transduction was envisaged. Recently, the role of $Na^+$ was clarified using whole cells of *Ms. barkeri* and *Mb. thermoautotrophicum* as well as vesicles of *Methanosarcina* strain Göl. These studies led to the description of a new mechanism for the generation of a primary $\Delta\tilde{\mu}_{Na^+}$.

### 5.1. The Methyl-$H_4$MPT : HS–CoM Methyltransferase Is a Primary Sodium Pump

A first clue to the role of $Na^+$ came from a study of the bioenergetics of methane and carbon dioxide formation from methanol. Using different substrates and substrate combinations, it was demonstrated that methane formation from methanol + $H_2$ as well as the simultaneous increase in the intracellular ATP content is independent of the $Na^+$ concentration of the buffer system (Blaut *et al.*, 1985). This finding argues against an involvement of $Na^+$ in the mechanism of ATP synthesis coupled to the heterodisulfide reductase reaction. However, the observed sodium dependence was shown to be due to the conversion of methanol to the formal redox level of formaldehyde (i.e., methylene-$H_4$MPT); this reaction is endergonic under standard conditions and dependent on $Na^+$ as well as on an energized membrane (Blaut *et al.*, 1985).

This dependence is the result of a $\Delta\tilde{\mu}_{Na^+}$-driven formation of methylene-$H_4$MPT from methanol (Müller *et al.*, 1988a). The sodium gradient is established via a $Na^+/H^+$ antiporter with the $\Delta\tilde{\mu}_{H^+}$ produced in the $F_{420}H_2$-heterodisulfide reductase reaction (Müller *et al.*, 1987). Although the actual sodium-driven reaction could not be identified using this approach, an electrogenic sodium influx coupled to methanol oxidation was unequivocally demonstrated.

The sodium-translocating system was analyzed in more detail in the reverse direction, the reduction of formaldehyde to the level of methanol (Müller *et al.*, 1988b). Methanogenesis from $H_2$ + HCHO but not from $H_2$ + $CH_3OH$ is strictly dependent on $Na^+$, indicating that not only the oxidative reaction sequence, but also the reductive conversion of methylene-$H_4$MPT to the formal redox level of methanol, is sodium-dependent. Studies using $^{22}Na^+$ revealed that methanogenesis from $H_2$ + HCHO is accompanied by the generation of a primary, electrogenic sodium ion gradient.

The conversion of formaldehyde, i.e., methylene-$H_4$MPT, to the formal redox level of methanol is mediated by the two enzymes, methylene-$H_4$MPT reductase and methyl-$H_4$MPT : HS–CoM methyltransferase, which cannot be distinguished from each other using the approach mentioned above. This problem was solved by analyzing the individual reactions using inverted vesicles of *Methanosarcina* strain Göl (Becher *et al.*, 1992a). Washed membranes convert methylene-$H_4$MPT to methyl-CoM in the presence of $H_2$ (as electron donor), $F_{420}$ (as electron carrier between $H_2$ and the methylene-$H_4$MPT reductase), and HS–CoM (as a methyl group acceptor) with a rate of 86 nmol $min^{-1}$ mg $protein^{-1}$. The overall reaction is accompanied by a primary, electrogenic $Na^+$ translocation into the lumen of the vesicles. Of the individual reactions tested, 83% of the methylene-$H_4$MPT reductase is found in the cytoplasmic and 17% in the membrane fraction (Becher *et al.*, 1992b); however, the membrane-bound activity is not coupled to $^{22}Na^+$ transport. On the other hand, 88% of the methyl-$H_4$MPT : HS–CoM methyltransferase is localized in the membrane fraction. This activity is strictly dependent on $Na^+$ in the buffer system and accompanied by the generation of a primary $\Delta\tilde{\mu}_{Na^+}$. Interestingly, the enzyme can also use methyl-tetrahydrofolate as a methyl group donor for HS–CoM reduction, which is of importance for practical reasons. This was the first description of the actual sodium translocating system in methanogens and is, to the best of our knowledge,

the first example on an ion-translocating methyltransferase.

In this context it is interesting to note that the $\Delta\tilde{\mu}_{Na^+}$-generating methyl-$H_4$MPT : HS–CoM methyltransferase represents a second site of energy transduction not only during methane formation from $H_2 + CO_2$ but also during methanogenesis from all other substrates which are converted via methyl-$H_4$MPT. This includes the substrates formate and acetate. For the latter it is now obvious because recent investigations demonstrated that the methyl group of acetate is transferred from the enzyme CO dehydrogenase via soluble corrinoid proteins to $H_4$MPT (Cao and Krzycki, 1991; Fischer and Thauer, 1989; Grahame, 1991). This finding implies that the sodium-motive enzyme is also involved in the pathway of methane formation from acetate and readily explains the early observation that protonophores are not able to completely dissipate the $\Delta\Psi$ produced during this fermentation. This is only observed upon the combined action of protonophores with sodium ionophores (Peinemann *et al.*, 1988), which is in accordance with the presence of two primary ion gradients, i.e., a $\Delta\tilde{\mu}_{Na^+}$ and a $\Delta\mu_{H^+}$.

### *5.1.1. Biochemistry of the Methy-$H_4$MPT : HS-CoM Methyltransferase*

Methanogenic bacteria contain high amounts of membrane-bound corrinoids (Dangel *et al.*, 1987; Schulz and Fuchs, 1986) and the characteristic corrinoid found is 5-hydroxybenzimidazolyl cobamide ($B_{12}$HBI, Factor III) (Pol *et al.*, 1982). A membrane-bound corrinoid protein, which had to be solubilized by 10 mg Triton X-100/mg protein, was purified from *Mb. thermoautotrophicum* strain Marburg after labeling cells with $^5$Co (Schulz *et al.*, 1988). It has an apparent molecular mass of 500 kDa and consists of subunits of 33, 28, 26, and 23 kDa with the 33-kDa subunit carrying the corrinoid (since a physiological test was not available, it is not certain whether all of these proteins belong to a functional unit). The corrinoid content is $\geqslant 8$ mol $B_{12}$HBI per mole of the 500-kDa protein. Although a function was not ascribed to this protein, recent experiments demonstrate that at least the 33-kDa subunit may be part of the membrane-bound, sodium-motive methyltransferase. This is based on several lines of evidence. The methyl-$H_4$MPT : HS-CoM methyltransferase of *Mb. thermoautotrophicum* as measured with an indirect approach was shown to contain a corrinoid as a cofactor (Poirot *et al.*, 1987); in addition, the methyltransferase in *Methanosarcina* strain Gö1 is a membrane-bound, corrinoid-containing enzyme (Becher *et al.*, 1992a,b). Furthermore, there is immunological evidence for a strong homology (or identity?) of the membrane-bound corrinoid as isolated from *Mb. thermoautotrophicum* with a methyl-$H_4$MPT : HS-CoM methyltransferase as purified from the cytoplasmic fraction of *Mb. thermoautotrophicum* strain ΔH (Stupperich *et al.*, 1990).

Very recently, a methyl-$H_4$MPT : HS-CoM methyltransferase was purified from membranes of *Mb. thermoautotrophicum* strain ΔH (Kengen *et al.*, 1992). As in *Methanosarcina* strain Göl, more than 80% of the enzyme is found in the membrane fraction. It consists of three subunits of 35, 33 and 31 kDa in an $\alpha_1\beta_1\gamma_1$ configuration; the corrinoid is bound to the 33-kDa subunit. The enzyme-bound $B_{12}$HBI is methylated by methyl-$H_4$MPT and, interestingly, the methyltransferase can use free $B_{12}$ instead of HS-CoM as a methyl group acceptor. This is also seen with vesicles of strain Göl where the methyltransferase as well as the coupled $Na^+$ transport is observed with free $B_{12}$ instead of HS-CoM as a methyl group acceptor (Becher *et al.*, 1992b).

### *5.1.2. How Can the Methyl-$H_4$MPT : HS-CoM Methyltransferase Reaction be Coupled to $Na^+$ Transport?*

Based on an analogous reaction which is catalyzed by methionine synthase and where the methyl group is transferred from methyl-tetrahydrofolate to homocysteine (Banerjee and Matthews, 1990), the following working model for the reaction mechanism of the methyl-$H_4$MPR : HS-CoM methyltransferase is developed (Fig. 11): the first subunit of the methyl-$H_4$MPT : HS-CoM methyltransferase binds the substrate methyl-$H_4$MPT which then undergoes a nucleophilic displacement by a nucleophilic attack of a Co(I) species, giving rise to methyl-Co(III) and $H_4$MPT. The corrinoid is bound to the second subunit which spans the membrane; the immunological evidence suggest that the 33-kDa subunit may play this role. As a prerequisite, the corrinoid has to face the cytoplasmic side so that it comes into contact with the methyl-$H_4$MPT bound to the first subunit. The enzyme-bound methyl-Co(III) intermediate is the

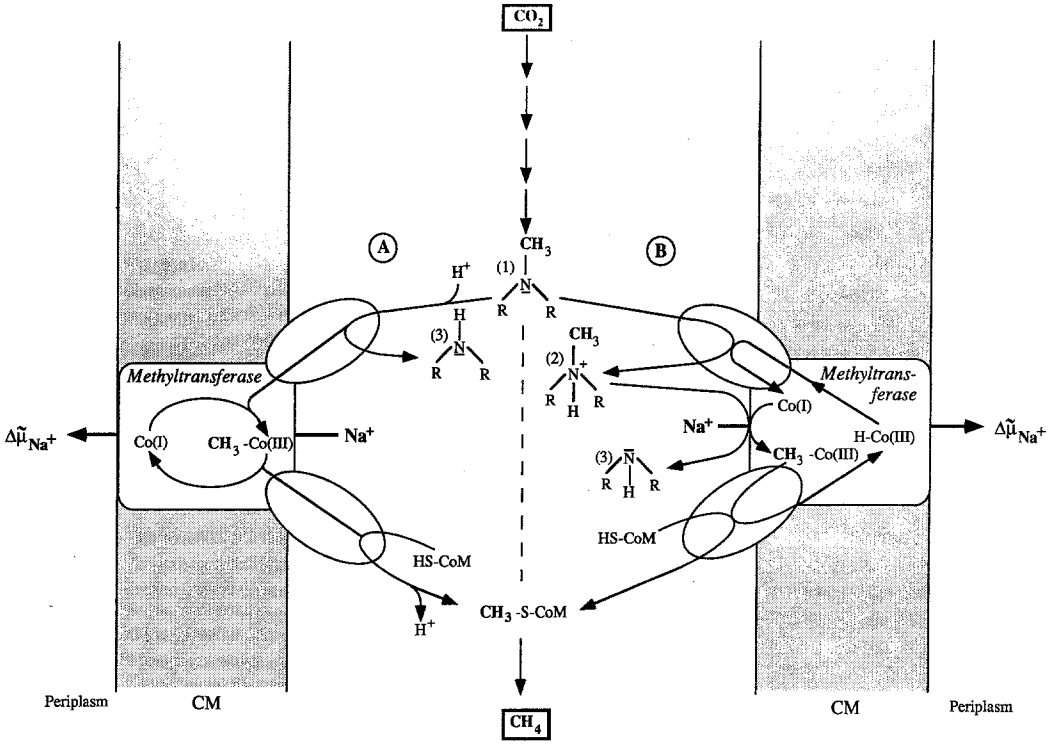



**Fig. 11.** Scheme of the sodium-motive methyl-tetrahydromethanopterin : coenzyme M methyltransferase reaction. A and B represent two different possibilities for the reaction cycle. In A, a general scheme is presented. In B, the involvement of a H-Co(III) species and the activation of the methyl-$H_4$MPT prior to the nucleophilic displacement reaction are indicated. The corrinoid is bound to the membrane-bound subunit of the enzyme. CM, cytoplasmic membrane; HS-CoM, coenzyme M (2-mercaptoethanesulfonate); $CH_3$-S-CoM, methyl-coenzyme M; $H_4$MPT, tetrahydromethanopterin; $\Delta\tilde{\mu}_{Na+}$, electrochemical sodium ion gradient; (1) methyl-$H_4$MPT; (2) activated, quaternized $N^5$ of methyl-$H_4$MPT; (3) $H_4$MPT. In the structural formulas only the methyl group binding $N^5$ of $H_4$MPT is indicated, for the rest (−R) of the molecule, see Fig. 4.

target for a nucleophilic attack by a thiolate anion of HS-CoM. This reaction is catalyzed by the third subunit and gives rise to methyl-CoM and it regenerates Co(I) (Fig. 11A). However, this model does not explain how methyl-$H_4$MPT is activated before the nucleophilic displacement can occur.

A more specified scheme which also takes into account the activation of methyl-$H_4$MPT is presented in Fig. 11B. This model is based on a scheme which was developed recently by Stupperich and co-workers for a soluble, corrinoid-containing methyltransferase involved in methanol conversion in *Sporomusa ovata* (Aulkemeyer and Stupperich, 1992). In this model, the involvement of a hydrido-Co(III)-$B_{12}$HBI in methyl group transfer has been suggested. If we transfer this model to the methyl-$H_4$MPT : HS-CoM methyltransferase, we may speculate that a hydrido-Co(III)-$B_{12}$HBI species transfers a proton to methyl-$H_4$MPT giving rise to Co(I)-$B_{12}$HBI and a quaternized $N^5$ in methyl-$H_4$MPT. The latter facilitates the abstraction of a $CH_3^+$ ion by a nucleophilic attack of the Co(I)-$B_{12}$HBI. The subsequent reaction cycle is analogous to the one described above whereby the carbonium ion is transferred from methyl-Co(III) to the acceptor HS-CoM, giving rise to methyl-CoM and a hydrido-Co(III) cobalamin.

Since the methyl-$B_{12}$HBI : HS-CoM methyltransferase was found to be only 2% in the washed vesicles, an involvement of such an enzyme in the methyl-$H_4$MPT : HS-CoM methyltransferase is highly unlikely; this is in contrast to the soluble methyltransferase involved in methanol conversion. As observed for the methionine synthase, methylation of HS-CoM with methyl-$H_4$MPT as a substrate is directly catalyzed by the methyl-$H_4$MPT : HS-CoM methyltransferase.

The sodium ion might interact with the membrane-spanning subunit as observed for sodium-translocating ATP synthase of *Propionigenium modestum* (Laubinger *et al.*, 1990). Based on the observed $Na^+$ transport in the presence of free $B_{12}$ instead of HS-CoM we can speculate

that free $B_{12}$ can be used as a methyl group acceptor. Although it is not clear whether this is achieved by nonspecificity of the enzyme or by a methyl group exchange reaction, this result argues for the methyl group transfer from methyl-$H_4$MPT to enzyme-bound Co(I) as the sodium-translocating step. As stated above, the sodium motive reaction is reversible and was actually discovered as a $\Delta\tilde{\mu}_{Na+}$-driven reaction during methanol oxidation. Taking this and the other lines of indication, we have to propose that methyl-CoM is an intermediate in $CO_2$ formation from methanol; the conversion of methyl-CoM to methyl-$H_4$MPT as catalyzed by the methyl-$H_4$MPT:HS-CoM methyltransferase is driven by $\Delta\tilde{\mu}_{Na+}$. It is conveivable that the methyl group transfer from enzyme-bound methyl-Co(III) to $H_4$MPT has to be driven by energy input.

However, the question how the $Na^+$ transport is mechanistically coupled to any of the partial reactions remains open and leaves an interesting field for further research.

### 5.2. The Formyl-MF Dehydrogenase as a Possible Primary Sodium Pump

As outlined above, the generation of formyl-MF from $H_2 + CO_2$ and MF is an energy-dependent reaction. The simplest idea would be a coupling of the formation of formyl-MF with the hydrolysis of ATP in analogy to the formyl-tetrahydrofolate synthetase reaction in acetogens. However, there is no indication for a direct involvement of ATP in the formation of formyl-MF. The second more plausible possibility is that a reverse electron transfer driven by a transmembrane electrochemical gradient of ions (either $H^+$ or $Na^+$) takes place. This is also substantiated by the recent findings that 60% of the enzyme is found in the membrane fraction of *Mb. thermoautotrophicum* (Börner *et al.*, 1989). Reduction of an electron carrier with a midpoint potential more negative than the $H^+/H_2$ couple ($E_{m,7} = -420$ mV) would then shift the thermodynamic equilibrium toward the synthesis of formyl-MF ($E_{m,7} = -497$ mV). After the discovery of a reversible, $\Delta\tilde{\mu}_{Na+}$-utilizing system connected to the oxidative conversion of methanol, the idea arose that the formyl-MF dehydrogenase is also sodium-motive (Müller *et al.*, 1988a).

Strong indications in favor of such a mechamism were obtained with resting cells of *Ms. barkeri.* Methane formation from $H_2 + CO_2$ is only insensitive to protonphores when the $Na^+/H^+$ antiporter is inhibited; under those conditions the $\Delta\tilde{\mu}_{Na+}$ as apparently generated by the methyl-$H_4$MPT:HS-CoM methyltransferase amounts to $-120$ mV, whereas the $\Delta\tilde{\mu}_{H+}$ is zero, excluding $\Delta\tilde{\mu}_{H+}$ as driving force for this reaction (Kaesler and Schönheit, 1989b). Furthermore, methane formation from $H_2 + CO_2$ but not from $H_2$ + HCHO is inhibited by sodium ionophores, and from the $Na^+/CH_4$ ratios determined during methane formation from different substrates it is concluded that 2–3 mol of $Na^+$ are consumed during the formation of 1 mol of formyl-MF.

As seen before with the methanol/formaldehyde system, the $\Delta\tilde{\mu}_{Na+}$-motive formaldehyde conversion is apparently reversible. The oxidation of HCHO to $CO_2$ and 2 $H_2$ is dependent on $Na^+$ and accompanied by a primary electrogenic $Na^+$ translocation with a stoichiometry of 2–3 mol $Na^+$ per mol HCHO consumed (Kaesler and Schönheit, 1989a). $Na^+$ extrusion is not inhibited by protonphores and inhibitors of the $Na^+/H^+$ antiporter and results in the generation of a protonphore-resistant $\Delta\Psi$.

However, it should be noted that experiments with *Ms. barkeri* and *Methanosarcina* strain Göl performed in our laboratory do not support the idea of a sodium-motive formyl-MF dehydrogenase and favor $\Delta\tilde{\mu}_{H+}$ as driving force. For example, a sodium dependence was not observed for formaldehyde oxidation to $CO_2$ and $H_2$ (Winner and Gottschalk, 1989) and studies using the inhibitors also used by Kaesler and Schönheit led to contradictory results. Furthermore, the physiological activities during the transport experiments performed by Kaesler and Schönheit were very low and one has to keep in mind that these experiments were done with resting cells in the presence of bromoethanesulfonate; this compound inhibits the demethylation of methyl-CoM but does not completely rule out the possibility that the observed sodium transport is due to the methyl-$H_4$MPT:HS-CoM methyltransferase reaction. Therefore, a final conclusion has to await experiments with defined systems where a $Na^+$ transport can be directly coupled to the formyl-MF dehydrogenase reaction.

## 6. CAN $\Delta\tilde{\mu}_{Na+}$ SERVE AS DRIVING FORCE FOR ATP SYNTHESIS?

The observed sodium dependence of methanogenesis led very early to the idea that sodium ions are involved in the mechanism of ATP synthesis, but there are up to now contradictory results published in the literature. After the discovery of the simplest type of methane formation, methanogenesis from $H_2$ +

$CH_3OH$, which is coupled to ATP synthesis by electron transport phosphorylation, we could address this question. However, there was no clue that ATP synthesis as coupled to the heterodisulfide reduction involves $Na^+$ (Blaut *et al.*, 1985), and later on it was shown directly that protons are the coupling ions (Blaut *et al.*, 1987; Deppenmeier *et al.*, 1990). In addition, also using *Ms. barkeri*, Kaesler and Schönheit demonstrated that ATP synthesis is not driven directly by $\Delta\tilde{\mu}_{Na^+}$ but by a secondary $\Delta\tilde{\mu}_{H^+}$ produced via the $Na^+/H^+$ antiporter (Kaesler and Schönheit, 1989a).

On the other hand, there are some reports about sodium-stimulated ATPases in methanogenic bacteria. This issue causes some confusion and has to be discussed with special attention paid to two topics. First, one has to clearly differentiate between marine (such as *Methanococcus voltae*) and nonmarine organisms (such as *Ms. barkeri, Methanosarcina* strain Göl and *Mb. thermoautotrophicum*). In the marine and halotolerant organism *Mc. voltae*, a sodium-stimulated ATPase was demonstated (Carper and Lancaster, 1986; Crider *et al.*, 1985). However, this enzyme was supposed to be involved in $Na^+$ export ($Na^+$ homeostasis) coupled to ATP hydrolysis and not ATP synthesis. Subsequent studies by Konisky and co-workers then led to the description of a vanadate-sensitive $E_1E_2$-type ATPase. The enzyme was purified; it consists of one subunit of 74 kDa which forms an acyl phosphate intermediate during catalysis. The enzyme can be removed from the membranes by salt extraction, a property not observed for integral membrane proteins; in addition, the hydropathy profile of the amino acid sequence, which was deduced from the cloned and sequenced gene, does not indicate membrane-spanning segments. Furthermore, the deduced amino acid sequence does not show a significant similarity to other ATPases (Dharmavaram *et al.*, 1991; Dharamavaram and Konisky, 1987, 1989). Again, this enzyme is not supposed to be involved in ATP synthesis and might be involved in $Na^+$ extrusion in marine methanogenic bacteria.

Is there a possibility to reconcile the contradictory reports on $Na^+$-stimulated ATPases in methanogens? After the discovery by Dimroth and coworkers (Laubinger and Dimroth, 1989) of an $F_1F_0$-ATP synthase in *Propionigenium modestrum* that uses $Na^+$ or $H^+$ as a coupling ion one has to consider this possibility also for other organisms. Interestingly, in all cases where ΔpNa-driven ATP synthesis in methanogenic bacteria is found, also a ΔpH-driven ATP synthase activity is demonstrated and, therefore, we might expect also in methanogens the presence of an ATPase translocating $Na^+$ and $H^+$ alternatively or two enzymes with distinct but different ion specificities. If this is indeed true for methanogenic bacteria, why did we not observe a ΔpNa-driven ATP synthase in *Ms. barkeri* and *Methanosarcina* strain Göl? A simple explanation is that the experiments were done with $H_2 + CH_3OH$ as a substrate and since the heterodisulfide reductase is proton motive and couples to the $H^+$-ATP synthase activity, it is not surprising that an effect of $Na^+$ on ATP synthesis is not observed in this system. However, this does not rule out that an ATP synthase activity is present which uses $\Delta\tilde{\mu}_{Na^+}$ produced in the course of the methyl-$H_4$MPT : HS-CoM methytransferase. Although indications for the presence of a $Na^+$-translocating ATP synthase were not oberved by applying ΔpNa pulses to resting cells (Müller *et al.*, 1988b) this does not completely exclude the possible existence of such an enzyme activity. Furthermore, the presence of a $Na^+$ and $H^+$ translocating $F_1F_0$-like ATP synthase in methanogens would also explain the observed stimulation by $Na^+$ of ATP synthesis and ATPase activity in *Mb. thermoautotrophicum* (Al-Mahrouq *et al.*, 1986; Smigan *et al.*, 1988) as well as in marine methanogens. Such an ATP synthase would enable hydrogentorophic methanogens to conserve the energy liberated in the methyl-$H_4$MPT : HS-CoM methyltransferase reaction directly via a $\Delta\tilde{\mu}_{Na^+}$ in the form of ATP.

This leaves us with the question why such an activity has not been observed before with the purified enzyme. From the published data it is clear that such a behavior would not have been observed because the experiments with the purified enzyme from *Ms. barkeri* were done with the $F_1$-analogous part; at least for *P. modestum*, $Na^+$ was shown to interact with $F_0$ (Laubinger *et al.*, 1990). For a final conclusion of this topic, first a more defined system than whole cells has to be used and, second, the enzyme has to be reconstituted into liposomes and ATP-dependent $^{22}Na^+$ transport has to be measured.

## 7. CONCLUDING REMARKS

It is remarkable that protons as well as sodium ions are employed by methanogenic bacteria as coupling ions in energy conservation. The nature of the primary pumps is quite established now although many details still have to be worked out, especially

regarding the redox carriers involved in electron transfer from $H_2$ or $F_{420}H_2$ to the heterodisulfide and the reaction mechanism of the methyl-$H_4$MPT : HS-CoM methyltransferase. Progress has been made in our understanding of how $H^+$ and $Na^+$ circuits are closed. The presence of a $H^+$-translocating ATP synthase in certain methanogens is established, and it is not excluded that this ATP synthase might also use $Na^+$ as coupling ion. In addition, the reaction leading to formyl-MF is a candidate for taking advantage of the electrochemical $Na^+$ gradient. The question remains why different coupling ions are employed in methanogenesis. This might have mechanistic reasons because one has to take into account that $H^+$-coupled systems have to operate at $10^{-7}$ M $H^+$, whereas $Na^+$ is available at concentrations on the order of $10^{-3}$ M.

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