Invited minireview

Novel reactions involved in energy conservation by methanogenic archaea

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Abstract Methanogenic archaea of the order Methanosarcinales which utilize C_1 compounds such as methanol, methylamines or H_2+CO_2, employ two novel membrane-bound electron transport systems generating an electrochemical proton gradient: the H_2:heterodisulfide oxidoreductase and the $F_{420}H_2$:heterodisulfide oxidoreductase. The systems are composed of the heterodisulfide reductase and either a membrane-bound hydrogenase or a $F_{420}H_2$ dehydrogenase which is functionally homologous to the proton-translocating NADH dehydrogenase. Cytochromes and the novel electron carrier methanophenazine are also involved. In addition, the methyl-H_4MPT:HS-CoM methyltransferase is bioenergetically relevant. The enzyme couples methyl group transfer with the translocation of sodium ions and seems to be present in all methanogens. The proton-translocating systems with the participation of cytochromes and methanophenazine have been found so far only in the Methanosarcinales.

Key words: Methanogenesis; Methanophenazine; Proton translocation; Sodium ion translocation; Hydrogenase; $F_{420}H_2$ dehydrogenase

1. Introduction

Methanogenic organisms belong to the kingdom of archaea and are widespread in anoxic environments. They have received much attention due to their ecological and biochemical features. (1) The process of methanogenesis is important for the global carbon cycle since it represents the terminal step in the anaerobic breakdown of organic matter under sulfate-limiting conditions. (2) Large amounts of CH_4 escape into the atmosphere where it acts as a greenhouse gas [1]. (3) The pathway of methanogenesis comprises unusual enzymes and cofactors [2]. (4) The mechanisms of energy conservation involve novel ion-translocating reactions [3].

On the basis of the substrates utilized, methanogens can be divided into two major groups. The first group comprises the organisms of the orders Methanobacteriales, Methanococcales, Methanomicrobiales and Methanopyrales which use only H_2+CO_2 or formate as substrates (hydrogenotrophic methanogens). In contrast, members of the order Methanosarcinales are more versatile and utilize methanol, methylamines, methylthiols or acetate (methylotrophic methanogens); some of them also grow on H_2+CO_2 [4].

2. Methanophenazine – a new cofactor of methanogenesis

Several unusual cofactors are involved in methanogenesis; their structures were elucidated during the last decade [2,5,6]. Methanofuran (MF), tetrahydromethanopterin (H_4MPT) and coenzyme M (HS-CoM, 2-mercaptoethanesulfonate) are C_1 carriers. Coenzyme B (HS-CoB, 7-mercaptoheptanoylthreonine phosphate) functions as electron donor in the terminal reaction of methanogenesis. Coenzyme F_{420} is a 5-deazaflavin derivative ($E_0' = -360$ mV) and is the central electron carrier in the cytoplasm of methanogens. Very recently, a new redox-active component was isolated from the cytoplasmic membrane of *Methanosarcina (Ms.) mazei* Gö1; it is referred to as methanophenazine (Fig. 1). This cofactor represents a 2-hydroxyphenazine derivative which is linked via an ether bridge to a pentaisoprenoid side chain [7]. Methanophenazine is the first example of a phenazine chromophore produced by archaea. Moreover, it is the only phenazine derivative that participates in respiratory chains of living cells (see Sections 4.1 and 4.2).

3. Pathways of methanogenesis

The pathways of methanogenesis from the above-mentioned substrates are summarized in Fig. 2. Methanogenesis from H_2+CO_2 proceeds according to the following equation [2,8]:

$$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O\ (\Delta G_0' = -130\ \text{kJ/mol}) \quad (1)$$

The pathway starts with the H_2- and MF-dependent reduction of CO_2 to formyl-MF. The endergonic reaction is catalyzed by a formyl-MF dehydrogenase and is driven by an electrochemical ion gradient (see Section 4.3). The formyl group is then transferred to H_4MPT and the resulting formyl-H_4MPT is stepwise reduced to methyl-H_4MPT. The electrons are derived from reduced F_{420} ($F_{420}H_2$) which is produced by the F_{420}-reducing hydrogenase. The methyl group of methyl-H_4MPT is then transferred to HS-CoM by the methyl-H_4MPT:HS-CoM methyltransferase. The exergonic reaction ($\Delta G^{0\prime} = -29$ kJ/mol) is coupled to the formation of an electrochemical sodium ion gradient ($\Delta\mu_{Na+}$, see Section 6). The final

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Abbreviations: CoB-SH, 7-mercaptoheptanoylthreonine phosphate; CoM-SH, 2-mercaptoethanesulfonate; F_{420}, (*N*-L-lactyl-γ-L-glutamyl)-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin-5′-phosphate; $F_{420}H_2$, reduced F_{420}; *Mc.*, *Methanococcus*; *Mb.*, *Methanobacterium*; *Ms.*, *Methanosarcina*; MF, methanofuran; H_4MPT, tetrahydromethanopterin; $\Delta\mu_{H+}$, electrochemical proton gradient; $\Delta\mu_{Na+}$, electrochemical sodium ion gradient

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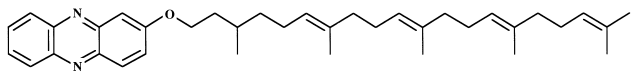

Fig. 1. Structure of methanophenazine.

step in methanogenesis is the reduction of CH_3-S-CoM to CH_4 which can be divided into two partial reactions. First methyl-S-CoM is reductively cleaved by the methyl-S-CoM reductase using HS-CoB as reductant. The reaction results in the formation of methane and a heterodisulfide (CoM-S-S-CoB) from HS-CoM and HS-CoB. In a second reaction CoM-S-S-CoB is reduced by the heterodisulfide reductase. The reducing equivalents are derived from H_2 and channeled to the heterodisulfide reductase by a membrane-bound electron transport system (see Section 4.1).

When cells grow on methanol, methylamines or methylthiols, the substrates undergo disproportionation to CH_4 and CO_2 [3], e.g. methanol is converted according to the following equation:

$$4\ CH_3OH \rightarrow 3\ CH_4 + 1\ CO_2 + 2\ H_2O\ (\Delta G_0' = -106\ kJ/mol) \quad (2)$$

In the oxidative branch of the pathway (Fig. 2) one out of four methyl groups is oxidized to CO_2 by the reversed CO_2 reduction route. The series of reactions starts with the formation of CH_3-S-CoM and methyl group transfer to H_4MPT as catalyzed by the methyl-H_4MPT:HS-CoM-methyltransferase. The endergonic reaction ($\Delta G^{0\prime} = 29$ kJ/mol) is driven by an electrochemical sodium ion gradient (see Section 6). The formation of methyl-H_4MPT is followed by the stepwise oxidation to formyl-H_4MPT. Reducing equivalents derived from these reactions are used for F_{420} reduction. After transfer of the formyl group to MF the formyl-MF dehydrogenase catalyzes the oxidation of CHO-MF to CO_2 and MF (see Section 4.3).

In the reductive branch of the pathway three out of four methyl groups are transferred to HS-CoM by methanol-specific methyltransferases. Again, the HS-CoB-dependent reduction of methyl-S-CoM leads to the formation of CH_4 and CoM-S-S-CoB. The latter component, however, is reduced by an electron transport system which does not occur in hydrogenotrophic methanogens, the membrane-bound $F_{420}H_2$:heterodisulfide oxidoreductase (see Section 4.2). Methylamines and methylthiols are converted in the same manner (Fig. 2) with the exception that methyl transfer to HS-CoM is catalyzed by substrate-specific methyltransferases [8]. Methanogenesis from acetate is not reviewed here. Suffice it to say that the methyl group of acetate enters the methanogenic pathway at the level of methyl-H_4MPT and that reducing equivalents for heterodisulfide reduction are provided by oxidation of CO to CO_2 (for review see [8,9]).

4. Redox-driven ion translocation in methylotrophic methanogens and function of methanophenazine

Transmembrane electrochemical ion gradients used for ATP synthesis in methanogenic archaea are generated by three different electron transport systems which will be described below.

4.1. H_2:heterodisulfide oxidoreductase

When methanogens are grown on H_2+CO_2 molecular hydrogen is used for the reduction of CoM-S-S-CoB (Fig. 2). In *Methanosarcina* strains this reduction is catalyzed by the membrane-bound H_2:heterodisulfide oxidoreductase system [3,9]. The oxidation of H_2 by the F_{420}-non-reducing hydrogenase is the initial reaction (Fig. 3). This enzyme was isolated from *Ms. barkeri* [10] and *Ms. mazei* Gö1 [3]. The purified proteins consist of two different subunits with molecular masses of approximately 60 and 40 kDa and contain a redox-active Ni ion and FeS clusters but no flavins. *Ms. mazei* Gö1 contains two sets of genes (the *vho* and the *vht* operons) which encode F_{420}-non-reducing hydrogenases ([11], see also Section 4.3). The *vhoGAC*-encoded enzyme is believed to be part of the H_2:heterodisulfide oxidoreductase [3]. The deduced amino acid sequences revealed that the small (VhoG) and the large subunit (VhoA) of the protein are homologous to corresponding polypeptides of membrane-bound NiFe hydrogenases from several bacteria [12]. A third gene (*vhoC*) belonging to the hydrogenase operon from strain Gö1 encodes a *b*-type cytochrome (cyt b_1) which probably functions as primary electron acceptor of the core enzyme consisting of VhoG and VhoA [13]. The heterodisulfide reductase is the second component of the H_2:heterodisulfide oxidoreductase and reduces CoB-S-S-CoM. The enzyme from *Methanosarcina*

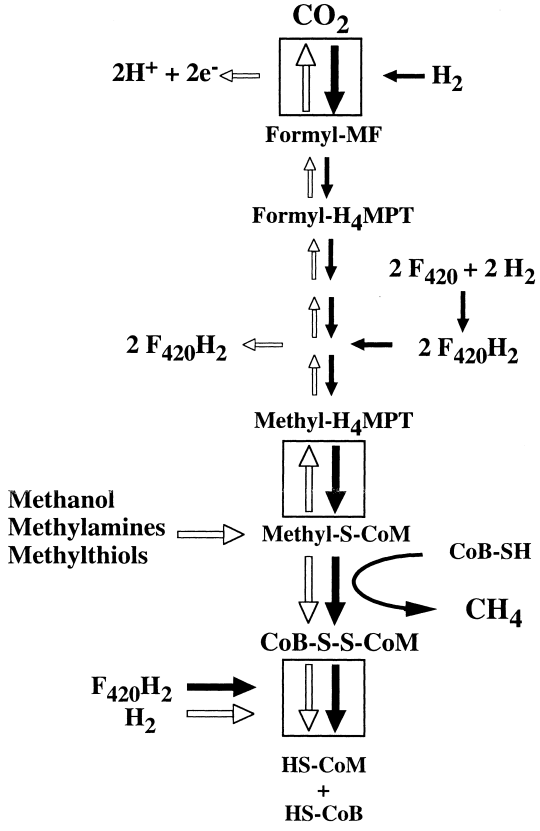

Fig. 2. Pathways of methanogenesis from C_1 compounds. Black arrows, methane formation from H_2+CO_2; open arrows, methanogenesis from methylated substrates. Reactions catalyzed by energy transducing enzymes are boxed. MF, methanofuran; H_4MPT, tetrahydromethanopterin; F_{420}, oxidized form of coenzyme F_{420}; $F_{420}H_2$, reduced F_{420}; HS-CoM, 2-mercaptoethanesulfonate; HS-CoB, 7-mercaptoheptanoylthreonine phosphate; CoB-S-S-CoM, heterodisulfide of HS-CoM and HS-CoB.

species is composed of two subunits (HdrDE) [9,14]. HdrE represents a membrane-integral *b*-type cytochrome (cyt b_2) and contains two distinct heme groups. HdrD is the catalytic subunit of the heterodisulfide reductase and contains two Fe_4S_4 clusters.

After the elucidation of the composition and properties of the key components of the H_2:heterodisulfide oxidoreductase the most interesting question concerned the nature of the electron carrier mediating electron transfer from the F_{420}-non-reducing hydrogenase to the heterodisulfide reductase. This problem was solved with the discovery of methanophenazine (see Section 2). The component is very hydrophobic and cannot be employed for in vitro assays in aqueous buffer systems. Fortunately, the water-soluble 2-OH-phenazine can be used instead. It is reduced by molecular hydrogen as catalyzed by the F_{420}-non-reducing hydrogenase. Furthermore, the membrane-bound heterodisulfide reductase uses dihydro-2-OH-phenazine as electron donor for the reduction of CoB-S-S-CoM [7]. Thus, the process of electron transfer from molecular hydrogen to the heterodisulfide can be divided into two partial reactions:

$$H_2 + \text{2-OH-phenazine} \rightarrow \text{dihydro-2-OH-phenazine} \quad (3)$$

$$\text{dihydro-2-OH-phenazine} + \text{CoM-S-S-CoB} \rightarrow \text{HS-CoM} + \text{HS-CoB} + \text{2-OH-phenazine} \quad (4)$$

Both exergonic reactions ($\Delta G_0'_{\text{eq. 3}} = -31.8$ kJ/mol, $\Delta G_0'_{\text{eq. 4}} = -10.6$ kJ/mol) are coupled by washed inverted vesicles of *Ms. mazei* Gö1 with the transfer of protons across the cytoplasmic membrane [15]. The maximal $H^+/2e^-$ ratio was 0.9 for each reaction. Thus, the H_2:heterodisulfide oxidoreductase system comprises two different proton-translocating segments. The first one involves the 2-OH-phenazine-dependent hydrogenase and the second one the heterodisulfide reductase.

The $H^+/2e^-$ stoichiometries of reactions 3 and 4 add up to 1.8 which corresponds to the efficiency of the overall electron transport from H_2 to CoM-S-S-CoB as determined earlier [3]. However, 3–4 $H^+/2e^-$ were transferred by whole cell preparations of *Ms. barkeri* during methane formation from methanol+H_2 [16]. This discrepancy is explained by the fact that about 50% of the membrane structures present in the vesicle preparations of *Ms. mazei* Gö1 catalyze electron transport not coupled to the generation of a proton gradient [15].

The course of electron transport and the sites of H^+ translocation of the H_2:heterodisulfide oxidoreductase system are summarized in Fig. 3. As already mentioned the F_{420}-non-reducing hydrogenase from *Ms. mazei* Gö1 is highly homologous to corresponding enzymes of several bacteria. Thus, some features of the bacterial proteins might also be important for the hydrogenase from strain Gö1. The mechanism of H^+ release and electron transfer became evident from several biophysical and genetic studies [17] as well as from the analysis of the three-dimensional structure of the periplasmic hydrogenase from *Desulfovibrio gigas* [18]. According to these studies, the active center located in the large subunit contains a bimetallic Ni-Fe cluster which catalyzes the heterolytic cleavage of molecular hydrogen. Three FeS clusters present in the small subunit are distributed along a straight line and provide an electron channel going from the active center to

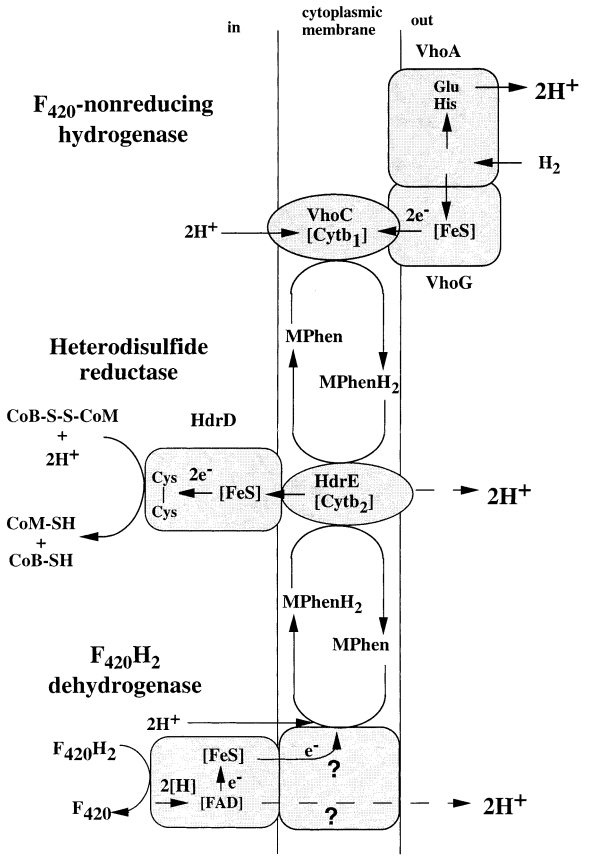

Fig. 3. Tentative scheme of the proton-translocating H_2:heterodisulfide oxidoreductase and the $F_{420}H_2$:heterodisulfide oxidoreductase of *Methanosarcina* strains. MPhen, methanophenazine; $MPhenH_2$, dihydromethanophenazine; VhoG, 40-kDa subunit of the F_{420}-non-reducing hydrogenase; VhoA, 60-kDa subunit of the F_{420}-non-reducing hydrogenase; VhoC, cytochrome b_1 encoded by the third gene (*vhoC*) of the hydrogenase operon; [Fe-Ni], bimetallic Fe-Ni cluster; [FeS], iron-sulfur cluster; HdrDE, subunits of the heterodisulfide. The question marks in the membrane-integral part of the $F_{420}H_2$ dehydrogenase indicate that the mechanisms of electron transfer and H^+ translocation are unknown.

the protein surface where the reduction of the electron acceptor takes place. His and Glu residues which are arranged between the active center and the surface of the protein are involved in the release of protons into the periplasm in the course of the reaction cycle. These amino acids are highly conserved among NiFe hydrogenases and are also present in the F_{420}-non-reducing hydrogenase from *Ms. mazei* indicating that the mechanism of proton release may be identical in this methanogenic organism.

Taking together these findings the mechanism of redox-driven H^+ translocation as catalyzed by the F_{420}-non-reducing hydrogenase from *Ms. mazei* Gö1 can be described as follows (Fig. 3): H_2 is oxidized by VhoA which contains the bimetallic Ni-Fe center. Two protons are transferred via histidine and glutamate residues to the surface of VhoA and are released in the periplasm. The electrons are transferred to subunit VhoG and channeled via FeS clusters to VhoC. From inhibitor studies with diphenyleneiodonium chloride it is known that the heme *b*-containing subunit and methanophenazine interact directly [13]. Thus, it is reasonable to assume that the active center for methanophenazine reduction is lo-

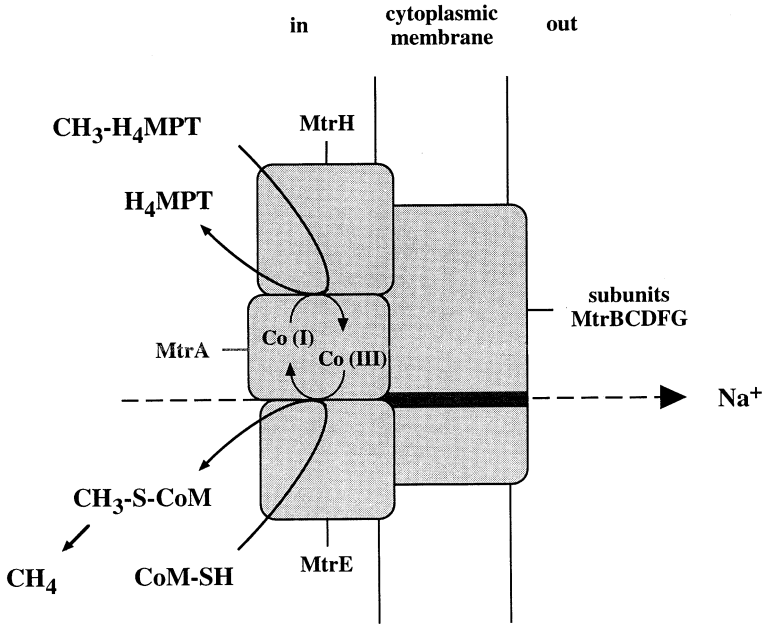

Fig. 4. Hypothetical scheme of the organization of the membrane-bound N^5-methyl-H_4MPT:CoM methyltransferase. Data on the localization of MtrH were taken from [49] and of MtrA from [47]. The localization of all subunits was confirmed by mini cell expression experiments in *E. coli* [45].

cated on VhoC. The protons necessary for the reduction of methanophenazine may be derived from the cytoplasm so that this segment of the electron transport chain results in the generation of two scalar protons.

The reduction of CoB-S-S-CoM is catalyzed by the heterodisulfide reductase (Fig. 3). The reaction resembles the process of sulfur/polysulfide respiration as performed by several bacteria and archaea. It is proposed that the reaction mechanism of the heterodisulfide reductase is similar to that of the ferredoxin-thioredoxin reductase from plants and cyanobacteria [9]. In contrast to many other flavin-containing disulfide reductases, the active centers of the above-mentioned enzymes only possess Fe_4S_4 clusters and a redox-active disulfide. The ferredoxin-thioredoxin reductase catalyzed the reduction of thioredoxin with reduced ferredoxin by two one-electron transfer reactions indicating a one-electron-reduced intermediate in the reaction cycle [19].

As depicted in Fig. 3 it is assumed that dihydromethanophenazine binds to the membrane-integral subunit HdrE of the heterodisulfide reductase where the reduced electron carrier is stepwise oxidized. Electrons are transferred to the heme groups of the polypeptide and two protons are released at the outer aspect of the cytoplasmic membrane. In a second step the electrons are channeled to FeS-clusters of the membrane-associated subunit HdrD and CoM-S-S-CoB is reduced to HS-CoM and HS-CoB. Protons necessary for this reaction are provided by the cytoplasm.

In summary, both the reduction of methanophenazine as catalyzed by the F_{420}-non-reducing hydrogenase and the oxidation of dihydromethanophenazine as catalyzed by the heterodisulfide reductase contribute to the generation of an electrochemical proton gradient ($\Delta\mu_{H^+}$) which can be taken advantage of by the A_1A_0-type ATP synthase [20].

4.2. $F_{420}H_2$:heterodisulfide oxidoreductase

As already mentioned $F_{420}H_2$ and CoM-S-S-CoB are generated in methylotrophic methanogens during methanogenesis from methanol and methylamines (see Section 3). The $F_{420}H_2$:heterodisulfide oxidoreductase, which is composed of a $F_{420}H_2$ dehydrogenase and the heterodisulfide reductase, reoxidizes $F_{420}H_2$ at the expense of CoM-S-S-CoB reduction (Fig. 3). The overall reaction has been shown to be competent in driving proton translocation across the cytoplasmic membrane [3]. Similar to the H_2:heterodisulfide oxidoreductase, electron transport and H^+ translocation are strictly coupled, as indicated by stoichiometries of 4 $H^+/2e^-$. Recent studies revealed that 2-OH-phenazine is reduced by the membrane-bound $F_{420}H_2$ dehydrogenase from *Ms. mazei* Gö1 with $F_{420}H_2$ as electron donor. The resulting dihydro-2-OH-phenazine acts again as substrate for the heterodisulfide reductase (see Section 4.1) indicating that the $F_{420}H_2$-dependent heterodisulfide reduction consists of two partial reactions [7]:

$$F_{420}H_2 + \text{2-OH-phenazine} \rightarrow F_{420} + \text{dihydro-2-OH-phenazine} \quad (5)$$

$$\text{dihydro-2-OH-phenazine} + \text{CoM-S-S-CoB} \rightarrow \text{HS-CoM} + \text{HS-CoB} + \text{2-OH-phenazine} \quad (4)$$

As in the H_2-dependent system both reactions contribute to energy conservation with $H^+/2e^-$ ratios of 1.8 each (unpublished results). The sum of these values is in the same range as the ratio of 4 $H^+/2e^-$ determined earlier for the whole system [3].

All key enzymes of this methanogenic electron transport chain have been isolated and characterized. The $F_{420}H_2$ dehydrogenase from *Ms. mazei* Gö1 with a molecular mass of 115 kDa contains iron-sulfur clusters and FAD [3]. The enzyme is very similar to the corresponding protein from *Methanolobus tindarius* [21]; the solubilized complex is composed of five

different subunits with molecular masses of 40, 37, 22, 20 and 16 kDa. A $F_{420}H_2$ dehydrogenase has also been isolated from the sulfate-reducing archaeon *Archaeoglobus fulgidus* [22]. Recently, the $F_{420}H_2$:heterodisulfide oxidoreductase could be reconstituted by the combination of purified $F_{420}H_2$ dehydrogenase from *Ms. mazei* Gö1, 2-OH-phenazine and purified heterodisulfide reductase from *Ms. thermophila* [23].

The $F_{420}H_2$ dehydrogenase is functionally homologous to the NADH:ubiquinone oxidoreductase. Both enzymes reveal a complex subunit composition and contain FeS clusters and flavins [24]. $F_{420}H_2$ and NADH are reversible hydride donors with comparable mid-point potentials. Electrons derived from the oxidation process are transferred to quinones and methanophenazine, respectively, which are similar in possessing isoprenoid side chains that enable them to move in the hydrocarbon phase of the cytoplasmic membrane. It has been shown that mitochondrial and bacterial NADH:ubiquinone oxidoreductases as well as the $F_{420}H_2$ dehydrogenase are inhibited by diphenyleneiodonium chloride [13,25]. The sequencing of the entire genome of *Ms. mazei* Gö1 is currently in progress (Genomics Laboratory Göttingen). First results indeed unravel a close relationship of NADH dehydrogenase and $F_{420}H_2$ dehydrogenase. The study of the enzymes is especially interesting in the view of their proton-translocating activity.

From the results obtained so far, we suggest that the $F_{420}H_2$ dehydrogenase transfers reducing equivalents from $F_{420}H_2$ to protein-bound FAD (Fig. 3). The cofactor mediated a $2e^-/1e^-$ switch and channels electrons to FeS clusters. The remaining protons are released to the periplasm by an unknown mechanism. Reduced FeS clusters are reoxidized and the electrons are transferred to the site of methanophenazine reduction which is envisaged to be located in the lipophilic environment of the cytoplasmic membrane. The localization of the active center would require the existence of a proton conducting pathway within the protein for H^+ movement from the cytoplasm to the site of methanophenazine reduction. The proposed mechanism would lead to the generation of a $\Delta\mu_{H^+}$ and would explain the stoichiometry of about $2H^+/2e^-$. Further electron transport within the $F_{420}H_2$:heterodisulfide oxidoreductase is performed by the proton-translocating heterodisulfide reductase as described above.

4.3. Formyl-methanofuran dehydrogenase system

The endergonic formyl-MF formation from CO_2 and H_2 ($\Delta G_0' = +16$ kJ/mol) involves a membrane-bound hydrogenase, the formyl-MF dehydrogenase, and probably several electron carriers. The driving force of the reaction is the transmembrane electrochemical ion potential. It is not clear yet whether H^+ or Na^+ function as coupling ions [3].

The MF dehydrogenase from *Ms. barkeri* is composed of five distinct subunits and contains molybdopterin guanine dinucleotide and several FeS clusters [2]. The genes encoding the subunits are organized in one operon (*fmdEFACDB*). Subunit FmdB harbors the molybdenum-containing active site and probably one Fe_4S_4 cluster. FmdF is predicted to be a polyferredoxin containing eight Fe_4S_4 clusters and is tightly bound to the enzyme complex, indicating the important function of this polypeptide in the reaction cycle [26]. As described in Section 4.1, *Ms. mazei* Gö1 contains two operons encoding F_{420}-non-reducing hydrogenases which share high homology. It has been proposed that the *vht*-encoded hydrogenase from *Ms. mazei* Gö1 is connected to the formyl-MF dehydrogenase [3]. According to these findings, the formation of formyl-MF could proceed as follows: (1) H_2 is oxidized by the *vht*-encoded hydrogenase. The electrons are transferred to the heme *b*-containing subunit VhtC. (2) The polyferredoxin FmdF could function as mediator of electron transfer from VhtC to the formyl-MF dehydrogenase. The latter protein then reduces enzyme-bound carboxy-MF to formyl-MF. The redox potential difference between cytochrome *b* and FmdF may be overcome by an unknown mode of reversed electron transport driven by $\Delta\mu_{H^+}$ or $\Delta\mu_{Na^+}$. The formyl-MF dehydrogenase system also catalyzes the reverse reaction during methanogenesis from methanol or methylamines, where it is involved in the dehydrogenation of formyl-MF to CO_2 and MF (Fig. 2). In this pathway the enzyme functions as a primary ion pump, thereby energizing the cytoplasmic membrane of the organisms. It is still unknown how electrons derived from this reaction are channeled to the heterodisulfide reductase.

5. Redox-driven energy conservation in hydrogenotrophic methanogens

In contrast to methylotrophic methanogens, information on the mechanism of energy conservation in obligate hydrogenotrophic methanogens is scarce. Dybas and Konisky [27] showed that methanogenesis from methyl-S-CoM and H_2 is coupled to the translocation of sodium ions across the cytoplasmic membrane of *Methanococcus (Mc.) voltae*, indicating that ATP synthesis is mediated by a chemiosmotic mechanism. The authors proposed that a membrane-associated H_2-dependent heterodisulfide reductase functions as a primary sodium ion pump. Such an H_2:CoB-S-S-CoM oxidoreductase complex has been isolated from *Methanobacterium (Mb.) thermoautotrophicum* which contains heterodisulfide reductase, F_{420}-non-reducing hydrogenase and several other proteins with unknown functions [28]. However, evidence for an ion-translocating activity has not been obtained yet. It also was shown that washed membranes of H_2+CO_2- or formate-grown *Mc. voltae* cells exhibit $F_{420}H_2$-dependent heterodisulfide reductase activity [29]. The F_{420}-reducing hydrogenase and the heterodisulfide reductase participate in this electron transport system. Whether the electron transfer from $F_{420}H_2$ to the heterodisulfide is coupled to energy conservation has not been studied yet.

The major difference between methanogens of the order Methanosarcinales and members of the orders Methanobacteriales, Methanococcales and Methanomicrobiales is that the latter organisms do not contain cytochromes [30]. It is, therefore, evident that redox-driven energy conservation has to be different. The key enzymes of the electron transport systems described in Sections 4.1 and 4.2 are also present in obligate hydrogenotrophic methanogens. However, all of them differ in structure and composition from their counterparts found in methylotrophic methanogens.

F_{420}-non-reducing hydrogenases from obligate hydrogenotrophic methanogens are very similar with respect to protein composition and genetic organization (for review see [12]). The enzyme from *Mb. thermoautotrophicum* has been analyzed in detail and can serve as a model [31]. The protein is encoded by the *mvhDGAB* operon. The small subunit (MvhG) and the large subunit (MvhA) of the core enzyme are homologous to the corresponding polypeptides in methylotrophic

methanogens (see Section 4.1). However, a signal sequence found in the gene encoding the small subunit of the F_{420}-non-reducing hydrogenase from *Ms. mazei* Gö1 is not present in *mvhG*. The function of MvhD, which co-purifies with the F_{420}-non-reducing hydrogenase, is unknown. A polyferredoxin containing eight Fe_4S_4 clusters is encoded by *mvhB*. The function of this protein is still a matter of debate. It has been suggested that the protein acts as electron acceptor of the hydrogenase, a hypothesis which indicates that it may replace the *b*-type cytochromes found in the corresponding enzymes of the Methanosarcinales. However, there is still no direct evidence that the polyferredoxin functions as an electron carrier in the H_2:CoB-S-S-CoM oxidoreductase system [9].

The heterodisulfide reductase from *Mb. thermoautotrophicum* is an iron-sulfur protein composed of the subunits HdrA (80 kDa), HdrB (36 kDa) and HdrC (21 kDa) [9]. In contrast to the enzyme from *Methanosarcina* strains, the protein of the hydrogenotrophic organism contains FAD which is bound to HdrA. Binding motifs for FeS clusters were found in HdrC and HdrA. Parts of HdrB and HdrC are homologous to HdrD from *Ms. barkeri* and probably form the active site of the enzyme from *Mb. thermoautotrophicum* [9]. It was proposed that the mechanism of S-S-bond cleavage of CoM-S-S-CoB is similar in the heterodisulfide reductases from hydrogenotrophic methanogens and from methylotrophic methanogens which was described in Section 4.1. In contrast, the pathway of electron transfer to the active site has to be different.

A distinct $F_{420}H_2$ dehydrogenase as found in *Methanosarcina* strains was not detectable in *Mc. voltae* [29]. Furthermore, there is no indication for such an enzyme from the analysis of the genomes from *Mc. jannaschii* and *Mb. thermoautotrophicum* [32,33]. Other enzymes are probably involved in channeling reducing equivalents from $F_{420}H_2$ to membrane-integral electron carriers in hydrogenotrophic methanogens. Suitable candidates for this function are F_{420}-reducing hydrogenases. In *Mc. voltae* two operons were identified which encode F_{420}-reducing hydrogenases of the NiFe type (*frcADGB*) and the NiFeSe type (*fruADGB*), respectively [34]. The purified Fru isoenzyme is composed of three subunits (43, 37 and 27 kDa) and contains FAD and FeS clusters. Immunogold labeling revealed that the enzyme is predominantly located in the cytoplasmic membrane [35]. Recently, this enzyme has been isolated from the membrane fraction of *Mc. voltae* and it was shown that the protein is involved in the $F_{420}H_2$-dependent heterodisulfide reduction in this organism [29].

Despite the fact that the major pathways of metabolism in methylotrophic methanogens and hydrogenotrophic methanogens are very similar, the phylogenetic differences between these groups are reflected in the composition of their electron transport systems.

6. The methyl-H_4MPT:HS-CoM methyltransferase – generation of an electrochemical sodium gradient

First interest in sodium ions participating in the pathway of methanogenesis arose when it was discovered that growth of all methanogenic archaea tested so far and formation of methane are dependent on the presence of sodium ions [36,37]. Early investigations using various combinations of substrates narrowed the sodium dependence to the reactions between methylene-H_4MPT and methyl-S-CoM [37]. Later experiments identified the methyl-H_4MPT:HS-CoM methyltransferase as the sodium-translocating, membrane-bound, corrinoid-containing protein (Fig. 4) that catalyzes the methyl group transfer from N^5-H_4MPT to HS-CoM [38,39]. Further investigations focused on the methyl-H_4MPT:HS-CoM methyltransferase of two organisms: *Mb. thermoautotrophicum* strain Marburg, a hydrogenotrophic methanogen, and the methylotrophic *Ms. mazei* Gö1 (sequence data are also available from *Mc. jannaschii* [32] and *Methanopyrus kandleri* [40]).

Thauer and coworkers detected eight different subunits in the purified methyl-H_4MPT:HS-CoM methyltransferase of *Mb. thermoautotrophicum*; their apparent molecular masses are 34 (MtrH), 28 (MtrE), 24 (MtrC), 23 (MtrA), 21 (MtrD), 13 (MtrG), 12.5 (MtrB), and 12 kDa (MtrF) [39,41]. The *mtr* genes are organized in a 4.9-kbp operon in the order of *mtrEDCBAFGH*. The native enzyme forms a tetrameric complex of 670 kDa containing 7.6 mol 5-hydroxybenzimidazolyl cobamide, 37 mol non-heme iron, and 34 mol acid-labile sulfur [39]. The corrinoid prosthetic group is bound to the 23-kDa subunit [39]. The dependence of enzyme activity on sodium ions was demonstrated [42], but the energy-conserving nature of the protein could not be studied because a method to generate vesicular proteasomes from *Mb. thermoautotrophicum* cells is not yet available. For the purified methyl-H_4MPT:HS-CoM methyltransferase from *Ms. mazei* Gö1 we reported that the enzyme is composed of at least six different polypeptides [43] containing also a cobamide and a $[4Fe\text{-}4S]^{2+/1+}$ iron-sulfur cluster with an E_0' value of –426 mV [44]. The purified active enzyme was reconstituted in ether lipid liposomes of *Ms. mazei* Gö1 and shown to translocate sodium ions across the membrane coupled to the methyl transfer reaction. Stoichiometry studies have shown that approximately 2 Na^+ are translocated per methyl group transferred [43]. Genetic analysis of the methyl-H_4MPT:HS-CoM methyltransferase from *Ms. mazei* Gö1 revealed likewise eight coding genes organized in the same order as the operon of *Mb. thermoautotrophicum* [45].

Inhibition of the methyl transfer reaction and the concomitant vectorial Na^+ translocation by propylation of the central cobalt atom of the cobamide [38] gave first evidence for the importance of the corrinoid prosthetic group in the mechanism of methyl transfer. Since further experiments demonstrated that the methyl-H_4MPT:HS-CoM methyltransferase is only active when the corrinoid exists in the cob(I) form, it is assumed that analogous to the methionine synthase of *Escherichia coli* [46] the methyl transfer proceeds in a two-step reaction:

(a) $CH_3\text{-}H_4MPT + E : Cob(I)amide \rightarrow$

$E : CH_3\text{-}Cob(III)amide + H_4MPT$

(b) $HS\text{-}CoM + E : CH_3\text{-}Cob(III)amide \rightarrow$

$CH_3\text{-}S\text{-}CoM + E : Cob(I)amide$

In the first step an enzyme-bound cob(I)amide serves as an attacking nucleophile that displaces the methyl group from CH_3-H_4MPT, resulting in a methylated enzyme and H_4MPT. The second step corresponds to the demethylation of the enzyme-bound corrinoid and the methylation of HS-CoM.

Although both reaction steps are accompanied by a free energy change of −15 kJ/mol only the second step is apparently sodium ion-dependent [42]. Sodium translocation experiments with the methyl-H_4MPT:HS-CoM methyltransferase confirmed that only in the presence of HS-CoM a translocation of sodium ions across the membrane occurs [43]. Various investigations demonstrated that the coordination of the corrinoid base to the central cobalt atom affects the reactivity of the cobamide. For the methyl-H_4MPT:HS-CoM methyltransferase of *Mb. thermoautotrophicum* it was demonstrated that the corrinoid is bound in the base-off form combined with a histidine ligation to the protein [47]. Recent results revealed that histidine in position 84 is the active-site histidine in the MtrA subunit of the *Mb. thermoautotrophicum* protein [48]. EPR spectra of the *Ms. mazei* Gö1 methyltransferase yielded signal characteristics for a base-on cobamide [44]. However, it was stressed that the lower ligand could be the benzimidazole base or a protein-derived ligand such as a histidine. In light of sequence homology it can be concluded that cobamide binding in MtrA of *Ms. mazei* Gö1 also proceeds 'base off/his on'.

Recent studies on the methyl-H_4MPT:HS-CoM methyltransferase are concerned with the function of single subunits of the enzyme (Fig. 4). As reported above the 23-kDa subunit, MtrA, harbors the essential corrinoid prosthetic group which is methylated and demethylated in the catalytic cycle. The 34-kDa subunit MtrH was recently demonstrated to catalyze the transfer of the methyl group from CH_3-H_4MPT to the corrinoid of MtrA [49]. Further on, sequence comparisons of the integral membrane subunit MtrD (21 kDa) indicate the presence of conserved structural elements suggesting a participation in sodium ion translocation [45].

Taking into account the sequence data of four methanogenic species and the protein data of two species it can be concluded that the components involved in sodium ion translocation have been highly conserved among the methanogenic archaea. This is in contrast to the components involved in proton translocation.

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