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Life Close to the Thermodynamic Limit: How Methanogenic Archaea Conserve Energy

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Abstract Methane-forming archaea are strictly anaerobic, ancient microbes that are widespread in nature. These organisms are commonly found in anaerobic environments such as rumen, anaerobic sediments of rivers and lakes, hyperthermal deep sea vents and even hypersaline environments. From an evolutionary standpoint they are close to the origin of life. Common to all methanogens is the biological production of methane by a unique pathway currently only found in archaea. Methanogens can grow on only a limited number of substrates such as $H_2 + CO_2$, formate, methanol and other methyl group-containing substrates and some on acetate. The free energy change associated with methanogenesis from these compounds allows for the synthesis of 1 (acetate) to a maximum of only 2 mol of ATP under standard conditions while under environmental conditions less than one ATP can be synthesized. Therefore, methanogens live close to the thermodynamic limit. To cope with this problem, they have evolved elaborate mechanisms of energy conservation using both protons and sodium ions as the coupling ion in one pathway. These energy conserving mechanisms are comprised of unique enzymes, cofactors and electron carriers present only in methanogens. This review will summarize the current knowledge of energy conservation of methanogens and focus on recent insights into structure and function of ion translocating enzymes found in these organisms.

1 Introduction

The methanogenic microbes belong to the domain *Archaea* and produce methane as the major end product of their metabolism. Methanogens are widespread in anoxic environments such as fresh water sediments, swamps, tundra areas, rice fields, intestinal tracts of ruminants and termites, and anaerobic digesters of sewage treatment plants (Garcia et al. 2000). Biological methanogenesis is an important process for the maintenance of the carbon cycle on Earth because methanogenic archaea catalyze the terminal step in the breakdown of organic material in many anaerobic environments. The substrates mentioned above are formed in the course of complex degradation processes of organic matter as performed by fermentative and syntrophic

bacteria (Schink 1997). The products of methanogenesis, CH_4 and CO_2 are released from anaerobic habitats and can reenter the global carbon cycle. Hence, large amounts of these greenhouse gases reach the atmosphere and therefore the process of biological methane formation is also of great interest for the global ecology (Khalil and Rasmusen 1994; Reay 2003). Besides the ecological importance, the process of methanogenesis creates a combustible gas that can be used as an energy source for both domestic or industrial applications. Suitable technologies have yet to be developed for a controlled decomposition of renewable biomass to methane. This process is called biomethanation and has great potential as an important nonpolluting energy source in the face of dwindling supplies of fossil fuels (Kashyap et al. 2003). New aspects of methanogenesis concern microbial methane consumption in anoxic sediments which significantly impacts the global environment by reducing the flux of greenhouse gases (Hallam et al. 2004, Meyerdierks et al. 2005). Current models suggest that relatives of methane-producing archaea developed the capacity to reverse methanogenesis and thereby consume methane to produce cellular carbon and energy (Thauer and Shima 2006). Furthermore, it is thought that biological methane production is involved in the formation of methane hydrates (Brooks et al. 2000). These ice-like structures are found in ocean-floor sediments at water depths greater than about 500 meters. Gas hydrates are solids, composed of rigid cages of water molecules that trap CH_4 molecules (Kvenvolden 1999) and represent a potentially enormous natural gas resource (Wood et al. 2002).

Some methanogens grow under conditions that are, from a human perspective, extreme with respect to temperature or salinity. These conditions resemble those of the early days on Earth and therefore, methanogenic archaea are often considered to be very early life forms. The conversion of substrates like $H_2 + CO_2$ or acetate to methane is close to the thermodynamic limit and, as we will see later, methanogens have developed very elaborate mechanisms to conserve energy. Some of these mechanisms are only found in methanogenic archaea. Therefore, ever since methanogenic archaea have been isolated their unique life style and bioenergetics have attracted much attention.

Members of the orders *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales* and *Methanopyrales* only use $H_2 + CO_2$ as substrates. Most of these species can also oxidize formate to form methane (Boone et al. 1993). The *Methanosarcinales* [e.g. *Methansarcina* (*Ms.*) species] are metabolically the most versatile methanogens, capable of growth on $H_2 + CO_2$, acetate, methanol and other methylated C_1 compounds such as methylamines (mono-, di-, or trimethylamine) and methylated thiols (dimethylsulfide, methanethiol or methylmercaptopropionate) (Deppenmeier 2002a). The genome of three species, *Ms. acetivorans*, *Ms. mazei* and *Ms. barkeri* have been published (Galagan et al. 2002; Deppenmeier et al. 2002). They are the largest yet sequenced genomes among species belonging to the domain *Archaea*, reflecting

their wide range of metabolic capabilities which distinguish these methane-producing microbes from all other archaea. The genomic data reveal, for the first time, new insights into the metabolism and the cellular functions of this interesting group of microorganisms. The metabolic pathways of methane formation are unique and involve a number of unusual enzymes and coenzymes (Wolfe 1986; Thauer 1998; Deppenmeier et al. 1999). Here we describe the biochemistry of methane formation with special interest given to unique membrane-bound enzymes generating or using primary ion gradients.

2 The Process of Methanogenesis

Several unusual cofactors are involved in biological methane formation (Deppenmeier 2002a). Coenzyme B (HS-CoB, 7-mercaptoheptanoylthreonine phosphate) and coenzyme F_{420} (a 5-deazaflavin derivative with a mid point potential of – 360 mV) function as electron carriers in the process of methanogenesis (Fig. 1). F_{420} is the central electron carrier in the cytoplasm of methanogens, which replaces nicotinamide adenine dinucleotides in many reactions (Walsh 1986). HS-CoB is the electron donor in the last reaction of methanogenesis. There is a third electron carrier involved in methane formation which has been discovered only recently (Abken et al. 1998,

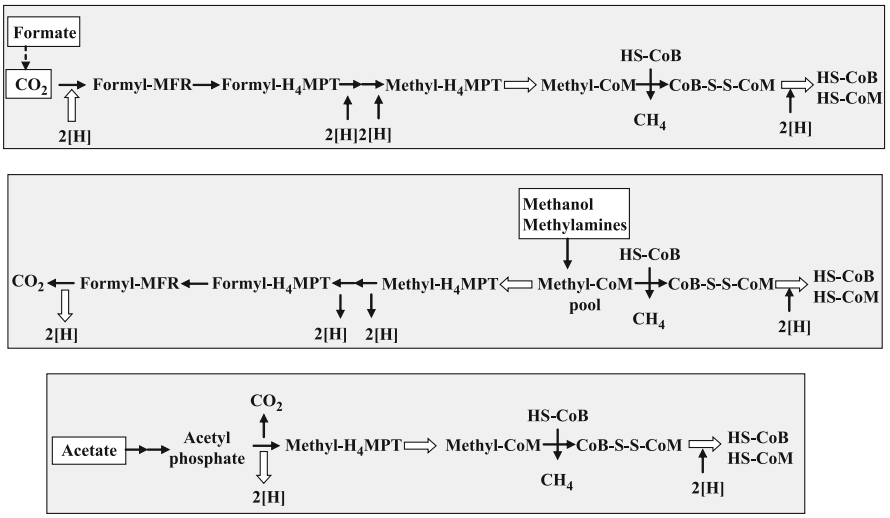

Fig. 1 The process of methanogenesis from formate or H_2 + CO_2 (top), methyl group-containing substrates (*middle*) or acetate (*bottom*). MFR, methanofuran; H_4MPT, tetrahydromethanopterin; CoM, coenzyme M, CoB, coenzyme B. *White arrows* denote reactions catalyzed by membrane-bound, ion-translocating enzymes

Beifuss and Tietze 2005). It is referred to as methanophenazine and represents a 2-hydroxy-phenazine derivative that is connected via an ether bridge to a pentaprenyl side chain (Beifuss et al. 2000). This hydrophobic cofactor has been isolated from the cytoplasmic membrane of *Methanosarcina* species (Deppenmeier 2004) and functions as a membrane integral electron carrier similar to quinones found in bacteria and eukarya. Coenzyme M (2-mercaptoethanesulfonate, HS-CoM), methanofuran (MFR), and tetrahydromethanopterin (H_4MPT) carry C_1-moieties of intermediates in the methanogenic pathways. In *Methanosarcina* species this cofactor is slightly modified and referred to as tetrahydrosarcinapterin (H_4SPT) (van Beelen et al. 1984). MFR is involved in CO_2 reduction and HS-CoM accepts methyl groups from methyl-H_4MPT, methanol and methylamines. The methylated form, methyl-S-CoM (2-methylthioethanesulfonate), is the central intermediate in methane formation (Fig. 1).

Methanogenesis from $H_2 + CO_2$, formate, methylated C_1-compounds and acetate proceeds by a central, and in most parts reversible, pathway (Deppenmeier 2002a). When cells grow on CO_2 in the presence of molecular hydrogen (Shima et al. 2002), carbon dioxide is bound to MFR and is then reduced to formyl-MFR (Fig. 1). This endergonic reaction is driven by the electrochemical ion gradient across the cytoplasmic membrane, which will be discussed in the following sections. In the next step the formyl group is transferred to H_4MPT and the resulting formyl-H_4MPT is stepwise reduced to methyl-H_4MPT. Reducing equivalents are derived from reduced F_{420} ($F_{420}H_2$), which is produced by the F_{420}-reducing hydrogenase using hydrogen as a reductant. The methyl group of methyl-H_4MPT is then transferred to HS-CoM by a membrane-bound methyl-H_4MPT:HS-CoM-methyltransferase. This exergonic reaction ($\Delta G'_0 = -29$ kJ/mol) is coupled to the formation of an electrochemical sodium ion gradient (see below). Coenzyme B (HS-CoB) functions as the electron donor for the reduction of methyl-S-CoM in the final reaction that is catalyzed by methyl-coenzyme M reductase (Ermler 2005), producing methane and a mixed disulfide of coenzyme M and coenzyme B (heterodisulfide, CoM-S-S-CoB) (Fig. 1). Finally, the heterodisulfide is reduced by the catalytic activity of a membrane-bound electron transfer system that will be discussed in Sect. 3. In total, one mol of carbon dioxide is reduced to methane:

$$CO_2 + 4H_2 = CH_4 + 2H_2O \quad \Delta G'_0 = -131\ \text{kJ/mol}\ CH_4\ . \tag{1}$$

During growth on methylated C_1-compounds in the absence of molecular hydrogen, parts of the methyl-group must be oxidized to gain reducing power for the reduction of CoM-S-S-CoB. Hence, the substrates are degraded to CH_4 and CO_2. With methanol as a substrate one out of four methyl groups is oxidized to CO_2 and three methyl moieties are reduced to methane (Fig. 1):

$$4CH_3OH \rightarrow 3CH_4 + 1CO_2 + 2H_2O \quad \Delta G'_0 = -106\ \text{kJ/mol}\ CH_4\ . \tag{2}$$

Many *Methanosarcina* strains can grow on trimethylamine, dimethylamine and monomethylamine, the substrates being metabolized to CH_4, CO_2 and NH_3:

$$4CH_3 - NH_3^+ + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_4^+ \quad (3)$$
$$\Delta G_0' = -77\ \text{kJ/mol}CH_4\ .$$

The methyl groups are channeled into the central pathway by substrate-specific soluble methyltransferases that catalyze the methyl group transfer to coenzyme M (Paul et al. 2000). The nonhomologous genes encoding the full-length methyltransferases for methylamine utilization each possess an in-frame UAG (amber) codon that does not terminate translation (Krzycki 2004). The amber codon is decoded by a dedicated tRNA, and corresponds to the novel amino acid pyrrolysine, indicating pyrrolysine as the 22nd genetically encoded amino acid (Krzycki 2005). The methyl group transfer to H_4MPT is catalyzed by the membrane-bound methyl-H_4MPT:HS-CoM-methyltransferase (see Sect. 4.5; Gottschalk and Thauer 2001). The next steps involve the stepwise oxidation of methyl-H_4MPT to formyl-H_4MPT. Reducing equivalents derived from the oxidation reactions are used for F_{420} reduction. After transfer of the formyl group to MFR the formyl-MFR dehydrogenase catalyzes the oxidation to CO_2 and MFR (Fig. 1). Hence, the oxidative branch in the methylotrophic pathway of methanogenesis is the reversal of CO_2 reduction to methyl-CoM as found in the CO_2-reducing pathway of methane formation. In the reductive branch of this pathway three out of four methyl groups are transferred to HS-CoM. Again, the HS-CoB-dependent reduction of methyl-S-CoM leads to the formation of CH_4 and CoM-S-S-CoB (Fig. 1).

Most of the methane in anaerobic food chains is derived from the methyl group of acetate. This process is referred to as the aceticlastic pathway of methanogenesis (Fig. 1) and is carried out only by the genera *Methanosarcina* and *Methanosaeta* (Ferry 1997). In *Methanosarcina* species, acetate is activated by conversion to acetyl-coenzyme A (CoA), which is cleaved by the nickel-containing CO dehydrogenase/acetyl-CoA synthase, yielding enzyme-bound methyl and carbonyl groups (Drennan et al. 2004). Following cleavage, the methyl group of acetate is transferred to H_4MPT. In the course of the reaction enzyme-bound CO is oxidized to CO_2 and the electrons are used for ferredoxin reduction. The resulting methyl-H_4MPT is converted to methane by the catalytic activities of the Na^+-translocating methyl-H_4MPT:HS-CoM methyltransferase and methyl-S-CoM reductase as described above (Fig. 1). As in the case of the other methanogenic pathways, an additional product of the reaction catalyzed by methyl-CoM reductase is the heterodisulfide CoM-S-S-CoB, which is reduced by a membrane-bound electron transport system to regenerate the reactive sulfhydryl forms of the coenzymes.

As mentioned above, all methanogens use methanogenesis for energy conservation. Recently however, evidence was presented that *Ms. acetivorans* is

able to employ the acetyl-CoA pathway to conserve energy for growth (Rother and Metcalf 2004). This organism was isolated from a bed of decaying kelp, algae known to accumulate up to 10% CO in their float cells (Sowers et al. 1984; Abbott and Hollenberg 1976). In contrast to many other methanogens, this methanogen is devoid of significant hydrogen metabolism due to the lack of functional hydrogenases. With carbon monoxide the cells grew to high densities with a doubling time of 24 h. Surprisingly, acetate and formate, rather than methane, were the major metabolic end products of metabolism. Moreover, methane production decreased with increasing CO partial pressures, consistent with inhibition of methanogenesis by CO. In spite of these facts, methanogenesis was still required for growth on CO, because the potent methyl-CoM reductase inhibitor BES abolished growth on CO (Rother and Metcalf 2004). Not much is known about the biochemistry of CO as a growth substrate for *Ms. acetivorans*. However, mutations in the operon encoding phosphotransacetylase and acetate kinase failed to use CO as a growth substrate, indicating that these enzymes are required for acetate formation and ATP synthesis via substrate level phosphorylation.

3
Reactions and Compounds of the Methanogenic Electron Transport Chains

The synthesis of CH_4 and the formation of the heterodisulfide (CoM-S-S-CoB; Fig. 1) mark the end of all pathways leading to methane formation (Deppenmeier 2002b). In *Methanosarcina* species CoM-S-S-CoB is the electron acceptor of a branched respiratory chain and is reduced to the thiol-containing cofactors HS-CoM and HS-CoB by an enzyme referred to as heterodisulfide reductase (Fig. 2). The protein is membrane-bound and functions as a terminal respiratory reductase (Hedderich et al. 2005). The source of reducing equivalents necessary for the reduction of the heterodisulfide depends on the growth substrate. In the presence of hydrogen a membrane-bound hydrogenase (F_{420}-nonreducing hydrogenase) channels electrons to the heterodisulfide reductase. This electron transport system is referred to as H_2:heterodisulfide oxidoreductase system (Fig. 2a) (Ide et al. 1999). When *Methanosarcina* strains grow on methylated C_1 compounds part of the methyl groups are oxidized to CO_2 and electrons are transferred to coenzyme F_{420}. Under this growth condition the membrane-bound $F_{420}H_2$:heterodisulfide oxidoreductase system catalyzes the oxidation of $F_{420}H_2$ and the reduction of the heterodisulfide (Fig. 2b) (Deppenmeier et al. 1991). The key enzyme of this electron transport chain is the $F_{420}H_2$ dehydrogenase which is responsible for the oxidation of the reduced cofactor (Bäumer et al. 2000). In the aceticlastic pathway the heterodisulfide reductase reduces CoM-S-S-CoB which derives electrons from reduced ferredoxin by a third membrane-bound electron transport chain referred

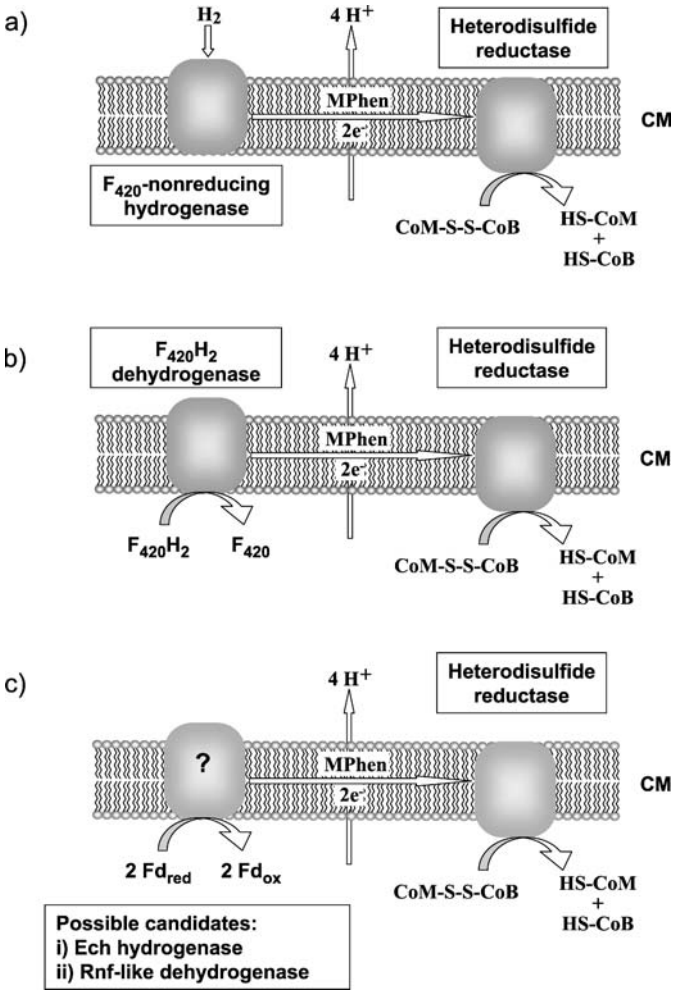

Fig. 2 Membrane-bound electron transport systems operating during methanogenesis from H_2 + CO_2 (**a**) methyl groups (**b**) or acetate (**c**). Mphen, methanophenazine; Fd, ferredoxin. For further explanations, see text

to as reduced ferredoxin:heterodisulfide oxidoreductase system (Fig. 2c) (Deppenmeier 2002a).

Using inverted vesicle preparations from *Ms. mazei*, it has been shown that electron transport is catalyzed by the H_2:heterodisulfide oxidoreductase system and the $F_{420}H_2$:heterodisulfide oxidoreductase system is coupled to proton translocation across the cytoplasmic membrane (Deppenmeier 2004). An A_1A_O ATP synthase (Sect. 5) catalyzes ATP synthesis from ADP + P_i thereby taking advantage of the electrochemical proton gradient (Müller et al. 1999). Additional experiments using uncouplers and ATP synthase inhibitors

clearly demonstrated that ATP is formed by electron transport-driven phosphorylation of ADP (Deppenmeier et al. 1990, 1991).

After the elucidation of the enzymes involved in energy conservation the question arose how electrons are channeled from one protein complex to the other. In the cytoplasmic membrane of methanogens only tocopherolquinones in very low concentration were identified (Hughes and Tove 1982), which had obviously no function in the electron transport chain. A comprehensive search for other redox-active, lipid-soluble components indicated the presence of one major factor in the membranes of *Ms. mazei*. Detailed NMR analysis revealed the aromatic structure as a phenazine derivative connected at C-2 to an unsaturated side chain via an ether bridge (Abken et al. 1998; Beifuss et al. 2000). The lipophilic side chain is responsible for the anchorage in the membrane and consists of five isoprene units linked to each other in a head-to-tail manner. The redox active product was referred to as methanophenazine and was the first phenazine isolated from archaea. After completion of the total synthesis of methanophenazine (Beifuss et al. 2000), electron transport in *Ms. mazei* could be analyzed in more detail. It was shown that methanophenazine is reduced by molecular hydrogen or $F_{420}H_2$, catalyzed by the F_{420}-nonreducing hydrogenase and the $F_{420}H_2$ dehydrogenase, respectively (Fig. 2a,b). Furthermore, the membrane-bound heterodisulfide reductase was able to use dihydro-methanophenazine as an electron donor for the reduction of CoM-S-S-CoB (Bäumer et al. 2000; Ide et al. 1999). Therefore, the cofactor is able to mediate the electron transport between the membrane-bound enzymes. Hence, methanophenazine was characterized as the first phenazine derivative involved in the electron transport of biological systems. More features of the electron transport chains could be analyzed using the water soluble analogue 2-hydroxyphenazine (2-OH-phenazine). Washed inverted vesicles of this organism were found to couple electron transfer processes with the transfer of four protons across the cytoplasmic membrane. It was shown that 2-OH-phenazine is reduced by molecular hydrogen as catalyzed by the F_{420}-nonreducing hydrogenase. Furthermore, the membrane-bound heterodisulfide reductase was able to use dihydro-2-OH-phenazine as the electron donor for the reduction of CoM-S-S-CoB (Bäumer et al. 2000; Ide et al. 1999) according to:

$$H_2 + \text{2-OH-phenazine} + 2H^+_{in} \rightarrow \text{dihydro-2-OH-phenazine} + 2H^+_{out} \quad (4)$$

$$\text{dihydro-2-OH-phenazine} + \text{CoM-S-S-CoB} + 2H^+_{in} \rightarrow \text{2-OH-phenazine} + \text{HS-CoM} + \text{HSCoB} + 2H^+_{out}\,. \quad (5)$$

There are two proton-translocating segments. The first one involves the F_{420}-nonreducing hydrogenase (Eq. 4) and the second one the heterodisulfide reductase (Eq. 5). Thus, the $H^+/2e^-$ stoichiometry of the electron transport chain adds up to four and supports the value of four $H^+/2e^-$ translocated in

the overall electron transport from H_2 to the heterodisulfide (Deppenmeier et al. 1991). 2-OH-phenazine was also a mediator of electron transfer within the $F_{420}H_2$:heterodisulfide oxidoreductase system (Bäumer et al. 1998). It has been shown that reducing equivalents are transferred from $F_{420}H_2$ to 2-OH-phenazine by the membrane-bound $F_{420}H_2$ dehydrogenase. Also, this process is coupled to proton translocation across the cytoplasmic membrane exhibiting a stoichiometry of about two protons translocated per two electrons transferred (Bäumer et al. 2000). The second reaction of this electron transport system is again catalyzed by the heterodisulfide reductase that uses dihydro-2-OH-phenazine as the electron donor for CoM-S-S-CoB reduction. Just as in the H_2-dependent system, both partial reactions of the $F_{420}H_2$:heterodisulfide oxidoreductase system are coupled to the translocation of two protons (Deppenmeier 2004):

$$F_{420}H_2 + \text{2-OH-phenazine} + 2H^+_{in} \rightarrow \text{dihydro-2-OH-phenazine} + F_{420} + 2H^+_{out} \quad (6)$$

$$\text{dihydro-2-OH-phenazine} + \text{CoM-S-S-CoB} + 2H^+_{in} \rightarrow \text{2-OH-phenazine} + \text{HS-CoM} + \text{HSCoB} + 2H^+_{out}\,. \quad (7)$$

As mentioned above, the oxidation of H_2 and $F_{420}H_2$ as well as the reduction of CoM-S-S-CoB are catalyzed by membrane-bound electron transport chains that couple the redox reaction with the translocation of protons. The mid-point potentials of the electron carriers were determined to – 420 mV for $H_2/2H^+$, – 360 mV for $F_{420}H_2/F_{420}$ (Walsh 1984), – 165 mV for methanophenazine/dihydromethanophenazine and – 143 mV for CoM-S-S-CoB/HS-CoM + HS-CoB (Tietze et al. 2003). Thus, the change of free energy ($\Delta G^{o\prime}$) coupled to the H_2- and $F_{420}H_2$-dependent methanophenazine reduction (Eqs. 4 + 6) is – 49.2 kJ/mol and – 42.1 kJ/mol, respectively. Taking into account that the membrane potential Δp is about – 180 mV (Peinemann 1989), the translocation of two protons per reaction cycle is feasible. In contrast, the change of free energy for the dihydromethanophenazine-dependent reduction of the heterodisulfide (Eq. 7) under standard conditions is only – 4.2 kJ/mol. However, the reductive demethylation of methyl-CoM as catalyzed by the methyl-CoM reductase, is highly exergonic (– 32 kJ/mol). Hence, it is tempting to speculate that the formation of CH_4 is the driving force for proton translocation at the second coupling site of the membrane-bound electron transport systems.

There is another enzyme system that has to be discussed in respect to methanogenesis from $H_2 + CO_2$ or methanol. A novel hydrogenase (Ech) was discovered in acetate-grown cells of *Ms. barkeri* (Meuer et al. 1999) that is homologous to hydrogenase 3 and 4 from *E. coli* and to the CO-induced hydrogenase from *Rhodospirillum rubrum* (Sauter et al. 1992; Fox et al. 1996). The enzyme catalyzed the H_2-dependent reduction of a two [4Fe – 4S] ferredoxin and is also able to perform the reverse reaction, namely hydrogen formation

from reduced ferredoxin (Fig. 2c). The Ech hydrogenase could be involved in the formation and degradation of formyl-MFR (Stojanowic and Hedderich 2004). In the CO_2-reducing pathway H_2 is oxidized by the membrane-bound Ech hydrogenase and electrons are transferred to a ferredoxin, which in turn is used by the formyl-MFR dehydrogenase to catalyze the reduction of CO_2. Taking into account the low hydrogen pressures found in the natural environments of methanogens, the H_2-dependent reduction of the ferredoxin is an endergonic process (Hedderich and Forzi 2005). It has been proposed that the electrochemical proton gradient is the energy source for the Ech hydrogenase to drive this reduction (Hedderich and Forzi 2005; Hedderich 2004). Thus, H_2-dependent ferredoxin reduction is enabled by the influx of protons or sodium ions through the enzyme. During growth on methanol the formyl-MFR dehydrogenase and the Ech hydrogenase may function in the reverse direction. In this case, the oxidation of formyl-MFR is coupled to the formation of reduced ferredoxin. The Ech hydrogenase could then oxidize reduced ferredoxin and release H_2. The overall process might be coupled to the translocation of protons or sodium ions and could contribute to the formation of the electrochemical ion gradient (Meuer et al. 2002). Molecular hydrogen is not an end product but is used as an electron donor for the H_2:heterodisulfide oxidoreductase system (Deppenmeier 2004).

The smallest change of free energy $\Delta G'_0 = -36\,\mathrm{kJ/molCH_4}$ of all substrates is coupled to methane formation from acetate. Therefore, the organisms must possess efficient energy-conserving systems to cope with this thermodynamic limitation. In *Methanosarcina* strains one ATP is used in acetate activation to form acetyl-CoA. As mentioned above the reactions of the aceticlastic pathway of methanogenesis results in the formation of the intermediates methyl-H_4MPT and reduced ferredoxin (Fd_{red}). The methyl moiety is transferred to H_4MPT and then to CoM by the Na^+-motive methyltetrahydromethanopterin:coenzyme M methyltransferase (Sect. 4.5). Reduced ferredoxin is used as the electron donor for the reduction of CoM-S-S-CoB catalyzed by the Fd_{red}:heterodisulfide oxidoreductase (Fig. 2c). It is very possible that this membrane-bound electron transport system is also able to generate an electrochemical proton gradient (Deppenmeier 2004):

$$2Fd_{red} + \text{CoM-S-S-CoB} \rightarrow 2Fd_{ox} + \text{HS-CoM} + \text{HS-CoB} \quad (\Delta G'_0 \sim -50\,\mathrm{kJ/mol})\,. \tag{8}$$

The composition of this third electron transport system is still a matter of debate and two scenarios are discussed:

a) It has been proposed that the Ech hydrogenase and the heterodisulfide oxidoreductase are involved in this process (Hedderich et al. 1999). Molecular hydrogen would be an intrinsic intermediate formed by the Ech hydrogenase at the expense of reduced ferredoxin. H_2 would be reoxi-

dized by the F_{420}-nonreducing hydrogenase and the electrons channeled via methanophenazine to the heterodisulfide reductase. Studies using *Ms. barkeri* mutants lacking the Ech hydrogenase confirmed these results (Meuer et al. 2002; Stojanowic and Hedderich 2004).

b) The genome of *Ms. acetivorans* does not contain genes encoding a functional Ech hydrogenase (Galagan et al. 2002), suggesting alternative electron transport components involved in the transfer of electrons to CoM-S-S-CoB. Compared to methanol-grown cells, acetate-grown *Ms. acetivorans* synthesized greater amounts of subunits of the potential ion-translocating Rnf electron transport complex previously characterized from bacteria (Li et al. 2006). Combined with sequence and physiological analyses, these results suggest that *Ms. acetivorans* replaces the H_2-evolving Ech hydrogenase complex with the Rnf complex. The subunits of the Rnf complex from *R. capsulatus* (Rc-Rnf) have been previously characterized (Jouanneau et al. 1998; Kumagai et al. 1997). In *Ms. acetivorans* the Rnf complex is presumably composed of a membrane-bound subcomplex containing a cytochrome *c* subunit that functions as an ion channel for the translocation of either protons or sodium and catalyzes the electron transfer to methanophenazine (Li et al. 2006). A membrane associated subcomplex containing FeS clusters is thought to interact with reduced ferredoxin and an electron-transfer module probably mediates electron transfer between the subcomplexes mentioned before. Subunits of the Rnf complex are similar to subunits of the Na^+-translocating NADH-quinone-reductase (Nqr) from *Vibrio* species (Steuber et al. 2002) indicating that Rnf-type enzymes catalyze ion transport coupled to electron transport. However, it is important to note that ion transport by Rnf-type enzymes has not yet been demonstrated. It is also interesting to note that the genomes of the freshwater organisms *Ms. mazei* and *Ms. barkeri* do not contain genes homologous to the Rnf complex. This may indicate that there may be differences in the pathway for acetate conversion to methane especially in the ferredoxin-dependent electron-transport chains in *Methanosarcina* species.

4 Structure and Function of Ion-Translocating Enzymes

Comprehensive reviews have been published recently that describe the features of the proton translocating proteins in great detail ($F_{420}H_2$ dehydrogenase, Deppenmeier 2004; NiFe hydrogenases, Vignais and Colbeau 2004; Ech hydrogenase, Hedderich and Forzi 2006; heterodisulfide reductase, Hedderich et al. 2005). Therefore, we discuss these enzymes only briefly and will focus on the methyltetrahydromethanopterin:coenzyme M methyltransferase and the A_1A_O ATP synthase from methanogens.

4.1
$F_{420}H_2$ Dehydrogenase

Genes encoding the $F_{420}H_2$ dehydrogenases are found in all genomes of methylotrophic methanogens sequenced so far, and the protein was purified from several archaea (Abken and Deppenmeier 1997; Haase et al. 1992; Kunow et al. 1994). In methanogens the enzyme catalyzes the $F_{420}H_2$-dependent reduction of phenazine derivatives, thereby transferring two protons across the cytoplasmic membrane (Fig. 3a) (Bäumer et al. 2000). Thus, the enzyme represents a novel kind of a proton-translocating complex in methanogenic archaea (Deppenmeier et al. 2002). The protein from *Ms. mazei* is encoded by the *fpo* cluster that comprises 12 genes which were designated *fpo* A, B, C, D, H, I, J, K, L, M, N, O (Bäumer et al. 2000). The gene *fpoF* is not part of the *fpo* operon and is located elsewhere on the chromosome. The enzyme subunits are highly homologous to proton translocating NADH dehydrogenases of respiratory chains from bacteria (NDH-1) and eukarya (complex I). In this context it is important to note that the $F_{420}H_2$ dehydrogenase and bacterial NADH dehydrogenases have some in-

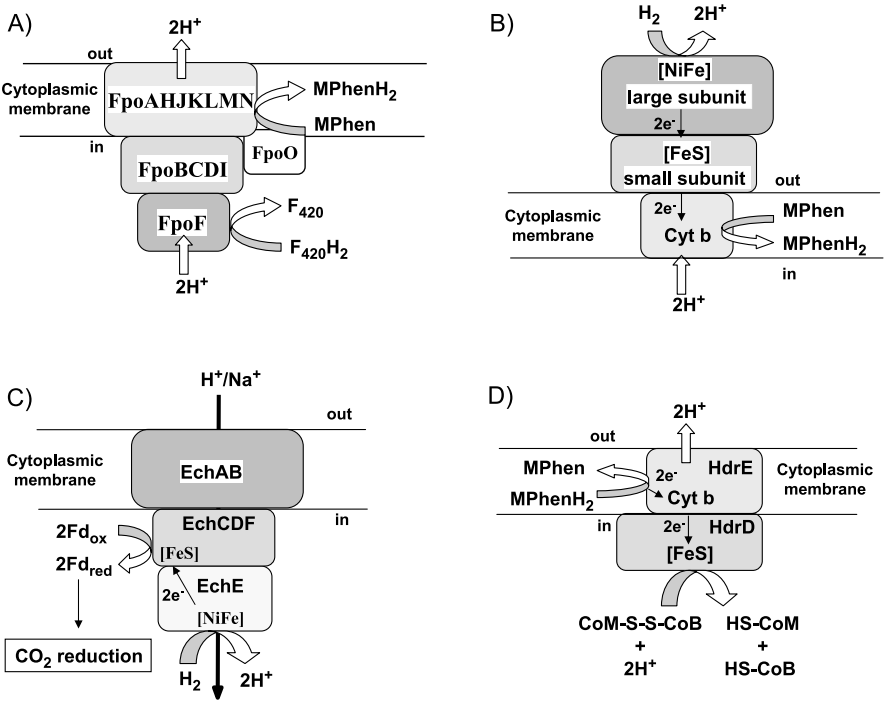

Fig. 3 Ion-translocating redox enzymes in methanogens. **A** F_{420} dehydrogenase. **B** F_{420} non-reducing hydrogenase. **C** Ech hydrogenase. **D** Heterodisulfide reductase

teresting features in common (Friedrich and Scheide 2000). Both enzymes have a complex subunit composition and contain flavin and iron-sulfur centers. They are the initial enzymes of membrane-bound electron transport systems. The electron donors $F_{420}H_2$ and NADH are similar in that both cofactors are reversible hydride donors with comparable mid-point potentials. The enzymes use small hydrophobic electron acceptors, namely quinones in the case of NADH dehydrogenases, and methanophenazine in the case of $F_{420}H_2$ dehydrogenases. Moreover, both enzymes show redox-driven proton-translocating activity (Friedrich et al. 2005). The overall similarity of the $F_{420}H_2$ dehydrogenase to complex I allows for speculation on the reaction mechanism of the archaeal enzyme. The proteins are essentially composed of three subcomplexes (Fig. 3a). The electron donors NADH and $F_{420}H_2$ are oxidized by the input module, which is formed by NuoEFG (complex I from *E. coli*) and FpoF ($F_{420}H_2$ dehydrogenase from *Ms. mazei*), respectively. Electrons are channeled to the membrane integral subcomplex (NuoAHIJKLMN, *E. coli*; FpoAHJKLMN, *Ms. mazei*) that is involved in the reduction of the species-specific electron acceptor (ubiquinone, *E. coli*; methanophenazine, *Ms. mazei*). The membrane-associated module (NuoBCDI, *E. coli*; FpoBCDI, *Ms. mazei*) connects the above-mentioned subunits and catalyzes electron transfer between the modules.

4.2 F_{420}-nonreducing Hydrogenase

The membrane-bound F_{420}-nonreducing hydrogenases from *Ms. mazei* and *Ms. barkeri* were purified and found to be composed of a small and a large subunit (Deppenmeier et al. 1992; Kemner and Zeikus 1994). The genes, arranged in the order *vhoG* and *vhoA* were identified as those encoding the small and the large subunit of the NiFe hydrogenases (Fig. 3b). The third gene in the *vho* operon (*vhoC*) encodes a membrane-spanning cytochrome *b* (Deppenmeier et al. 1995). The crystal structure of the highly homologous nickel-iron hydrogenase from *Desulfovibrio gigas* revealed that the large subunit harbors the binuclear [NiFe]-active site, which is coordinated by two conserved CxxC motifs (Volbeda et al. 1995). This polypeptide is likely co-translocated with the small subunit to the periplasmic side of the cytoplasmic membrane. The small subunit contains three iron-sulfur clusters, forming an electron transfer line from the [NiFe]-center of the large subunit to the prosthetic heme groups of the *b*-type cytochrome. Hence, the third subunit acts as the primary electron acceptor of the core enzyme (Bernhard et al. 1997).

A proposed model for electron and proton transfer within the F_{420}-nonreducing hydrogenase comprises hydrogen oxidation by the bimetallic Ni/Fe center of the large subunit, thereby separating electrons and protons (Fig. 3b) (Deppenmeier 2004). Protons are released on the outside of the cytoplasmic membrane, and the iron-sulfur clusters in the small subunit accept

the electrons. In the next step, electrons are transferred to the heme groups of the cytochrome *b* subunit, which then completes the reaction by accepting two protons from the cytoplasm for the reduction of methanophenazine. Thus, the overall reaction would lead to the production of two scalar protons.

4.3 Ech Hydrogenase

A membrane-bound [NiFe]-hydrogenase was isolated from *Ms. barkeri* (Meuer et al. 1999) that belongs to the class of energy-converting [NiFe] hydrogenases (Fig. 3c). These enzymes are found in several anaerobic or facultatively anaerobic microorganisms (Hedderich and Forzi 2006). From growth characteristics of *Rhodospirillum rubrum* and from cell-suspension experiments with *Ms. barkeri*, it can be concluded that the [NiFe]-hydrogenases in these organisms probably act as a proton or sodium ion pump (Hedderich and Forzi 2006). The purified Ech hydrogenase consists of six subunits encoded by genes organized in the *echABCDEF* operon (Kuenkel et al. 1998; Meuer et al. 1999). The six subunits show a striking amino acid sequence similarity to the catalytic core of complex I (Nuo BCDIHL) and the $F_{420}H_2$ dehydrogenase (FpoBCDIHL) (Hedderich 2004). The evolution of complex I and energy-converting hydrogenases has been discussed in recent reviews (Friedrich and Scheide 2000; Brandt et al. 2003; Hedderich 2004; Vignais and Colbeau 2004).

The obligate hydrogenotrophic methanogens *Methanococcus (Mc.) maripaludis* and *Methanothermobacter* (*Mt.*) *marburgensis* contain genes for two separate multisubunit energy-conserving hydrogenases, Eha and Ehb, which are composed of 20 and 17 subunits, respectively (Tersteegen and Hedderich 1999; Hendrickson et al. 2004). Some of the subunits are also homologous to the NADH-ubiquinone oxidoreductase. Like the Ech hydrogenase from *Methanosarcina* species, these enzymes may be necessary to reduce low-potential ferredoxins for the reduction of CO_2 as catalyzed by the formyl-MFR reductase. A deletion in the *ehb* operon of *Mc. maripaludis* showed the mutant strain had severely impaired growth in minimal medium. Both acetate and yeast extract were necessary to restore growth to nearly wild-type levels, suggesting that Ehb is involved in anabolic CO_2 assimilation (Porat et al. 2006).

4.4 Heterodisulfide Reductase

As mentioned above the heterodisulfide reductase has a key function in the energy metabolism of methanogens (Fig. 3d). The enzyme catalyzes the reduction of CoM-S-S-CoB generated in the final step of methanogenesis. Two types of heterodisulfide reductases were characterized from distantly related

methanogens (Hedderich et al. 1990; Heiden et al. 1994; Simianu et al. 1998). In *Mt. marburgensis*, and other obligate hydrogenotrophic methanogens the enzyme is a soluble iron–sulfur flavoprotein composed of three subunits HdrA, HdrB and HdrC (Hedderich et al. 1994). HdrA contains a FAD-binding motif and four binding motifs for [4Fe – 4S] clusters. HdrC contains two binding motifs for [4Fe – 4S] clusters. Evidence has been presented that the catalytic center is formed by subunits HdrC and HdrB. The second type of heterodisulfide reductase is found in *Methanosarcina* species (Fig. 3d) (Heiden et al. 1994; Simianu et al. 1998). It is composed of two subunits, a membrane-anchoring *b*-type cytochrome (HdrE) and a hydrophilic iron–sulfur protein (HdrD). The latter subunit is regarded as a hypothetical fusion protein of subunits HdrC and HdrB of the *Mt. marburgensis* enzyme (Kuenkel et al. 1997) and comprises the catalytic center in *Methanosarcina* species. The active site harbors a [4Fe-4S] cluster, which is directly involved in the disulfide cleavage reaction (Hedderich et al. 2005). The two types of heterodisulfide reductases use different physiological electron donors. HdrDE receives reducing equivalents from the reduced methanophenazine pool via its *b*-type cytochrome subunit whereas HdrABC forms a complex with the F_{420}-nonreducing hydrogenase (Mvh) which is located in the cytoplasm after cell lysis (Setzke et al. 1994). The mechanism of energy conservation in obligate hydrogenotrophic methanogens is still unknown.

4.5
Structure and Function of the Methyltetrahydromethanopterin: Coenzyme M Methyltransferase, a Primary Sodium Ion Pump

Growth as well as methanogenesis is strictly sodium ion dependent (Perski et al. 1981, 1982) and the methyltetrahydromethanopterin:coenzyme M methyltransferase was identified to be responsible for the Na^+ dependence of the pathway (Müller et al. 1988b; Gärtner et al. 1993; Weiss et al. 1994; Lienard et al. 1996). The enzyme couples the methyltransfer from methyltetrahydromethanopterin to CoM with vectorial transport of Na^+ across the cytoplasmic membrane, and is the first example of a methyltransferase catalyzing ion transport across a membrane. Because the central pathway is reversible, this enzyme functions as a generator of a sodium ion potential during methanogenesis from CO_2 or acetate (Fig. 1), but the reaction is endergonic and driven by the sodium ion potential in the course of methyl-group oxidation (Müller et al. 1988a). Unlike the cytochromes and the resulting differences in the electron-transport chains, the methyltransferase is found in every methanogen and there is no reason to assume different reaction mechanisms.

The energetics of the methyltransferase was first investigated using cell suspensions of *Ms. barkeri* and the substrate combination H_2 + HCHO. Upon addition of the substrate, sodium ions were actively extruded from the cy-

toplasm, resulting in the generation of a transmembrane Na^+ gradient of – 60 mV (Müller et al. 1988b). A Na^+/formaldehyde stoichiometry of 3 to 4 was determined using cell suspensions (Kaesler and Schönheit 1989), but proteoliposomes containing the purified methyltransferase from *Ms. mazei* catalyzed an electrogenic Na^+ transport with a stoichiometry of 1.7 mol Na^+ per mol methyl-H_4MPT demethylated (Lienard et al. 1996).

The methyltransferase was purified from *Mt. thermoautotrophicus* and *Ms. mazei* Gö1 (Gärtner et al. 1993; Lienard et al. 1996). Six subunits were found in the *Ms. mazei* enzyme, with apparent molecular masses of 34, 28, 20, 13, 12 and 9 kDa. It contains a [4Fe – 4S] cluster with a E_0' of – 215 mV and a "base on" cobamide with a standard reduction potential of – 426 mV for the $Co^{2+}/^{1+}$ couple (Lu et al. 1995; Lienard et al. 1996). In *Mt. thermoautotrophicus*, eight subunits were found, with apparent molecular masses of 34 (MtrH), 28 (MtrE), 24 (MtrC), 23 (MtrA), 21 (MtrD), 13 (MtrG), 12.5 (MtrB), and 12 kDa (MtrF) (Fig. 4). The purified enzyme contains two mol corrinoid, eight mol nonheme iron, and eight mol acid-labile sulfur (Gärtner et al. 1993; Harms et al. 1995). The encoding genes have been sequenced from a number of methanogens, and they are organized in an operon in the order *mtrEDCBAFGH*. Hydrophobicity plots indicate that all of the subunits except MtrA and MtrH are hydrophobic and potentially membrane-bound. The membrane localization of MtrD was confirmed experimentally for *Ms. mazei* Gö1, *Mt. thermoautotrophicus* and *Methanocaldococcus (Mc.) jannaschii* (Ruppert et al. 2001). This subunit may be directly involved in Na^+ transport (Lienard and Gottschalk 1998).

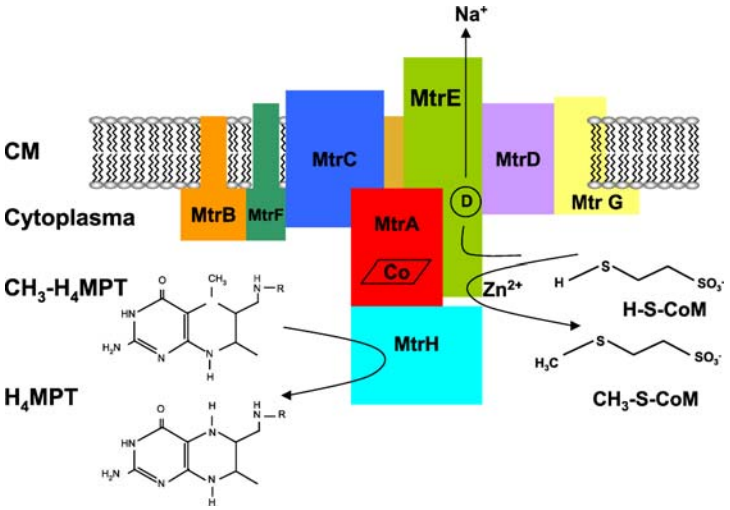

Fig. 4 The Na^+-motive methyltetrahydromethanopterin:coenzyme M methyltransferase of methanogenic archaea. For explanations see text. Adapted from Gottschalk and Thauer 2001

The corrinoid that catalyzes the methyltransfer is Coα-[α-(5-hydroxybenzimidazolyl)]-cobamide (factor III) (Poirot et al. 1987; Gärtner et al. 1993, 1994). The cofactor in its super-reduced Co(I) form accepts the methyl group from methyltetrahydromethanopterin, giving rise to a methyl-Co(III) intermediate. In the second partial reaction, this methyl-Co(III) is subjected to a nucleophilic attack, probably by the thiolate anion of CoM, to give rise to methyl-CoM and regenerated Co(I) (Fischer et al. 1992; Gärtner et al. 1994).

$$CH_3 - H_4MPT + E : Co(I) \rightarrow H_4MPT + E : CH_3 - Co(III) \quad (9)$$

$$E : CH_3 - Co(III) + HS - CoM \rightarrow CH_3\text{-S-CoM} + E : Co(I)\,. \quad (10)$$

Reaction 9 has a free-energy change of – 15 kJ/mol and is not stimulated by sodium ions. On the other hand, demethylation of the enzyme-bound corrinoid (Eq. 10) is also accompanied by a free-energy change of – 15 kJ/mol, and this reaction was sodium-ion-dependent, with half-maximal activity obtained at approximately 50 μM Na^+. This finding indicates that the demethylation of the enzyme-bound corrinoid is coupled to sodium-ion translocation (Weiss et al. 1994) (Fig. 4).

MtrA was overexpressed, purified from *E. coli* and successfully reconstituted with cobalamin. EPR spectroscopic studies indicate that the cobalamin is in the "base off" form and that the axial ligand is a histidine residue of MtrA (Harms and Thauer 1996). From this observation, a hypothetical mechanism was formulated for coupling the methyltransfer reaction to ion transport via a long-range conformational change in the protein (Harms and Thauer 1996). It is known that cob(II)alamin and cob(III)alamin, but not cob(I)alamin, carry an axial ligand. Methylation of cob(I)alamin gives rise to a methylcob(III)alamin, which is then able to ligate the histidine residue; demethylation leads to a reversal of this reaction. It is easily conceivable that binding and dissociation of the histidine residue with the corrinoid lead to a conformational change in the hydrophilic part of the enzyme. This change is then transmitted to the membrane-bound subunits, giving rise to Na^+ transport.

5
ATP Synthesis in Methanogens

Methanogens are the only microorganisms known to produce two primary ion gradients, $\Delta\mu_{Na^+}$ and $\Delta\mu_{H^+}$, at the same time (Schäfer et al. 1999; Müller et al. 2005a). They are, therefore, confronted with the problem of coupling both ion gradients to the synthesis of ATP. How this is achieved is still a matter for debate. Inhibitor studies using whole cells suggested the presence of two distinct ATP synthases in *Mt. thermoautotrophicus* and *Ms. mazei*. It was suggested that they have a F_1F_O ATP synthase that couples with Na^+

and a A_1A_O ATP synthase that couples with H^+ (Becher and Müller 1994; Smigan et al. 1994, 1995). However, later it turned out that the genomes of these two species only encode a A_1A_O ATP synthase. The same is true for all other methanogens sequenced so far, except two that contain potential F_1F_O ATP synthase genes in addition to the A_1A_O ATPase. *Ms. barkeri* strain MS contains a gene cluster that potentially encodes proteins with similarity to subunits of a F_1F_O ATP synthase; however, the order of the genes is different from any other F_1F_O ATP synthase gene cluster, the deduced γ subunit is very unusual and presumably nonfunctional, and no gene encoding subunit δ was found. Since a *m*RNA transcript could not be detected in cells grown on methanol it is doubtful that the F_1F_O-like genes are expressed in *Ms. barkeri* (Lemker and Müller, unpublished). A gene cluster potentially encoding proteins with similarity to subunits of a F_1F_O ATP synthase is also present in *Ms. acetivorans* but whether it indeed encodes an active ATPase has not been addressed. Most likely, the F_1F_O ATPase genes present in these two methanogens have arisen from horizontal gene transfer.

The presence of only the A_1A_O ATP synthase poses the question whether the enzyme translocates Na^+, H^+ or both. A definite answer can not be given at the moment due to the lack of a functional proteoliposome system. Although the first entire A_1A_O ATPase has been recently purified (Lingl et al. 2003), so far it could not be reconstituted in a coupled state into liposomes. Also, Na^+ dependence of ATP hydrolysis could not be addressed due to the lack of a suitable buffer system. However, in every methanogen sequenced so far, the membrane embedded rotor subunit, subunit *c*, that catalyzes the ion transport has a motif similar to the Na^+ binding motif identified experimentally in F_1F_O ATP synthase and V_1V_O ATPases (Müller 2004; Meier et al. 2005; Murata et al. 2005). This makes it highly likely that the A_1A_O ATP synthase can use both, Na^+ and H^+. If not, the electrochemical sodium gradient across the cytoplasmic membrane of methanogens such as *Ms. mazei* or *Ms. barkeri* could be converted to a proton potential by action of a Na^+/H^+ antiporter. Interestingly, the genome of *Ms. mazei* encodes three such secondary transporters but their function in cellular bioenergetics has not been studied.

5.1 Structure and Function of the A_1A_0 ATP Synthase from Methanogens

The ATP synthases/ATPases arose from a common ancestor and, therefore, A_1A_O ATP synthases share properties with both the eukaryal V_1V_O ATPase and the F_1F_O ATP synthase as present in bacteria, chloroplasts and mitochondria. The overall subunit composition and the primary sequence of the major subunits A and B is more closely related to V_1V_O ATPases than to F_1F_O ATP synthases but their function clearly is to synthesize ATP. Therefore, archaeal ATP synthases are very unique, ancient energy converters and phylogenetic

analyses clearly revealed that they form a separate class of ATPases, the A_1A_O ATP synthases/ATPases (Müller et al. 1999).

The A_1A_O ATP synthases from methanogens are hitherto the best investigated specimen of A_1A_O ATP synthases. The A_1A_O ATP synthase has at least nine subunits (A_3:B_3:C:D:E:F:H:*a*:c_x) (Müller and Grüber 2003; Müller et al. 2005a,b). The A_1 complex of archaeal ATP synthases has a pseudo-hexagonal arrangement of six peripheral globular masses, reflecting the major subunits A and B, as proposed from two dimensional images of the thermoacidophilic archaea *Sulfolobus acidocaldarius* and *Ms. mazei* Gö1 (Lübben et al. 1988; Wilms et al. 1996). Despite several attempts over the years, so far only one A_1A_O ATP synthase, from *Mc. jannaschii*, could be purified without loss of subunits (Lingl et al. 2003). *Mc. jannaschii* is a hyperthermophile that grows optimally at 85 °C. The ATPase was solubilized by Triton-X-100 and purified by gel filtration and ion exchange chromatography to apparent homogeneity. The first projected structure of an intact A_1A_O ATP synthase was determined by electron microscopy of single particles at a resolution of 1.8 nm (Coskun et al. 2004a). The enzyme has an overall length of 25.9 nm and is organized in an A_1 headpiece (9.4 × 11.5 nm), and a membrane domain A_O (6.4 × 10.6 nm), that are linked by a central stalk about 8 nm in length (Fig. 5). A part of the central stalk is surrounded by a col-

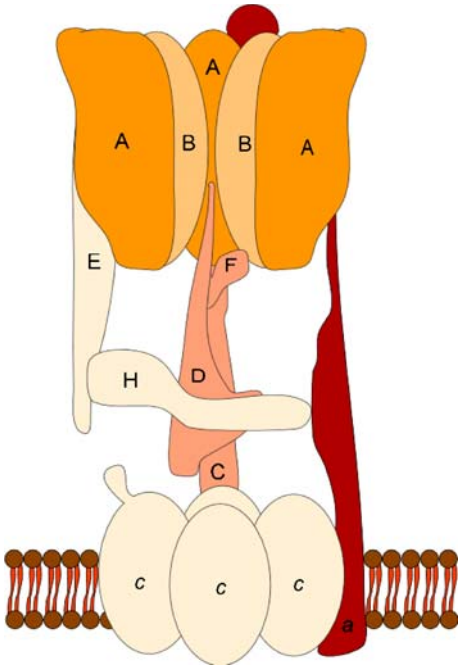

Fig. 5 Structure and subunit topology of A_1A_O ATP synthases. The cartoon shows the enzyme from *Mc. jannaschii* with only five monomers of the rotor subunit

lar. The collar is connected to the top of the A_1 portion via a peripheral stalk, and in addition, there is a second peripheral stalk that connects the A_O with the A_1 domain.

The overall structure of an A_1 subcomplex was obtained by small angle X-ray scattering of an ABCDF subcomplex heterologously produced in *E. coli*. It is asymmetric, with a headpiece that is approximately 94 Å long and 92 Å wide and a stalk with a length of approximately 84 Å and 60 Å in diameter (Grüber et al. 2001; Lemker et al. 2001, 2002) (Fig. 5). Global structural alterations occur in the A_1 ATPase due to nucleotide binding. Subunits C and F are exposed stalk subunits, whereas subunit D is the functional homolog of the γ subunit of F_1F_O ATPases (Grüber et al. 2001; Coskun et al. 2002).

Recently, high-resolution structures of the noncatalytic A and the catalytic B subunits of A_1A_O ATP synthases were obtained. The structure of subunit A was determined at a resolution of 2.55 Å and shown to contain four domains (Maegawa et al. 2006). One represents an insertion of about 90 amino acids that is absent in the homologous β subunit of F_1F_O ATP synthases and corresponds to the "knob-like structure" seen in electron micrographs suggested to be involved in connecting the peripheral stalk to the AB-assembly (Coskun et al. 2004a,b). The noncatalytic subunit B binds ADP and ATP, but with a weaker affinity than subunit A. The overall structure, as determined at 1.5 Å resolution, is similar to that of the related α subunit of F_1F_O ATP synthases; however, like in the V_1V_O ATPases, the P-loop is missing in subunit B of the methanoarchaeal ATP synthase (Schäfer et al. 2006b). The first low-resolution shape of subunit F of the A_1A_O ATP synthase from the archaeon *Ms. mazei* Gö1 in solution was determined by small angle X-ray scattering (Schäfer et al. 2006a). The protein is monomeric and has an elongated shape, divided in a main globular part with a length of about 4.5 nm, and a hook-like domain of about 3.0 nm in length. Subunit D can be cross-linked to the catalytic A subunit depending on nucleotide binding. This interaction between A and D involves the N- and C-termini of subunit D (Coskun et al. 2002), whose secondary structures are predicted to be α helical (Wilms et al. 1996), as described for both termini of subunit γ of F_1. Cross-linking studies provide evidence that subunit B and F interact with each other and the contact surface of B–F could be mapped in the high-resolution structure of subunit B of the A1AO ATP synthase. Furthermore, D–E, A–H, and A–B–D crosslinks were obtained in the intact A_1A_O ATP synthase. Taken together, these data suggest the topology of subunits depicted in Fig. 5.

5.2
The Unique Membrane-Embedded Rotor of A_1A_0 ATP Synthases

The A_O domain contains only two membrane-intrinsic subunits, *a* and *c* (cf. Fig. 5). Subunit *a* is the stator and subunit *c* builds the rotor of this membrane-embedded motor. Rotor subunits have been purified and char-

acterized from some archaea and in almost every case they were shown to be of $M_r \approx 8000$ with two transmembrane helices (Müller 2004). This size corresponds to the size of the *c* subunit from F_1F_O ATP synthases and was until now assumed to be the reason for the F_1F_O-like properties of the A_1A_O ATP synthases, i.e. their function as ATP synthases. However, *Mt. thermautotrophicus* has a duplicated and *Mc. maripaludis* and *M. jannaschii* have triplicated *c* subunits. Apparently, these *c* subunits arose by gene duplication and triplication, respectively, with subsequent fusion of the genes (Ruppert et al. 1999, 2001; Müller et al. 2005b, Lewalter and Müller 2006). In the case of *Mt. thermautotrophicus*, the ion binding site is conserved in helix two and four, but in *Mc. jannaschii* and *Mc. maripaludis* it is only conserved in helix four and six, in helix two it is substituted by a glutamine residue. The genome sequence of *Methanopyrus kandleri* revealed another extraordinary feature: the A_1A_O ATP synthase genes are located in one cluster, and the gene encoding the *c* subunit is 13-times the size of the gene encoding an 8-kDa *c* subunit. The sequence predicts a *c* subunit of 97.5 kDa comprising 13 covalently linked hairpin domains (Slesarev et al. 2002)! These domains have a highly conserved sequence (55.9 to 86.3%), and the ion-binding site is conserved in helix two of every hairpin domain. However, post-transcriptional and post-translational modifications can not be excluded and, therefore, the extraordinary size of the *c* subunit has to be verified by other means.

It should be mentioned that the extraordinary variation in *c* subunits in archaea is not restricted to methanogens. The pyrococci *Pyrococcus furiosus*, *Pyrococcus horikoshii* and *Pyrococcus abyssi* are anaerobic archaea that have a fermentative metabolism. Interestingly, their *c* subunit genes arose by duplication and subsequent fusion of a precursor gene coding for one hairpin (Müller 2004). The duplicated *c* subunit with two covalently linked hairpins has an ion binding site in hairpin two but not in one. Therefore, the *c* subunit of the A_1A_O ATP synthases from pyrococci is identical to the 16-kDa *c* subunit of eukaryal V_1V_O ATPases. This finding gives further evidence that 16-kDa *c* subunit with only one ion binding site in two hair pins are not an exclusive feature of eukarya.

The rotor stoichiometry has not been solved for any A_1A_O ATP synthase but two important conclusions regarding the structure of the rotor and the function of the enzyme can be drawn. For structural considerations, it is assumed for the sake of simplicity that the rotor contains 12 hairpins. This would accommodate 12 copies of the 8-kDa *c* subunits from most archaea, six of the one from *Mt. thermautotrophicus* and pyrococci, and four of *M. jannaschii*. These rotors are multimeric, but the number of subunits decreases in this order. A comparison to the optimal and maximal growth temperatures reveals a striking correlation of the number of rotor subunits to the optimal and maximal growth temperatures. The higher the growth temperature of the organisms the fewer the number of subunits per rotor. The extreme is en-

countered in the presumably monomeric rotor of *M. kandleri* that thrives at 110 °C. It should be kept in mind that the rotor subunits are embedded into the membrane and are shielded from heat protective mechanisms present in the cytoplasm. Therefore, they are directly exposed to the heat and it is easily conceivable that the increase of covalently-linked rotor subunits increases the stability and supports the function of the rotor in the cytoplasmic membrane at high temperatures.

For the function of the enzymes, the number of ion-translocating residues per rotor unit is important. The capability to synthesize ATP is directly dependent on the number of ions translocated per ATP synthesized. According to $\Delta G_P = - n \cdot F \cdot \Delta p$, a phosphorylation potential (ΔG_P) of $\sim$ 50 to 70 kJ/mol is sustained by the use of n = 3–4 ions/ATP at a physiological electrochemical ion potential of – 180 mV (Δp). Assuming a rotor with 12 ion-translocating groups and a catalytic domain with three $\alpha\beta$/AB pairs and thus three ATP binding sites, this gives exactly the number of four ions required thermodynamically for ATP synthesis. This is apparently realized in most archaeal ATPases found to date. However, a special case is the rotor of *Mc. jannaschii* and *Mc. maripaludis* that have only eight ion-binding sites (assuming 12 hairpin domains per rotor). Apparently, 2.6 carboxyl groups per catalytic center are already sufficient for ATP synthesis.

6 Concluding Remarks

Archaea are truly fascinating microbes that live under conditions that are, from a human perspective, extreme. Some of them are "ancient" and developed in the early history of life. During evolution, they kept their ecological niches and their physiological properties. Therefore, we have a window through which we can glance at very early life processes, including mechanisms of energy conservation. Methanogens are hitherto the best investigated archaea with respect to energy metabolism, they have very unique enzymes involved in energy conservation only found in methanogens, they employ Na^+- and H^+-based energetics and their ATP synthases have an outstanding variety of rotor subunits. Unfortunately, there is no high-resolution structure of any of the energy conserving enzymes available, this is still a challenging task for future studies. The structure-function analyses are still in their infancy, but genetic techniques have improved and heterologous expression systems may lead to the quantities required for structural analyses. The emerging pictures of the $F_{420}H_2$ dehydrogenase and the ATP synthase shows that the structure determination of energy-conserving enzymes in methanogens is well on its way and promises interesting new structures in the future.

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