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Review

Redox-driven proton translocation in methanogenic Archaea

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Abstract. Methanogenic archaea of the genus *Methanosarcina* are able to utilize H_2 + CO_2, methylated C_1 compounds or acetate as energy and carbon source, thereby producing methane as the major end product. The methanogenic pathways lead to the formation of a mixed disulfide derived from coenzyme M and coenzyme B. This disulfide is of major importance for methanogens because it is the terminal electron acceptor of a branched respiratory chain. Molecular hydrogen, reduced coenzyme F_{420} or reduced ferredoxin are used as electron donors. Four enzymes are involved in the membrane-bound electron transport system of *Methanosarcina* species, all of which are involved in the generation of an electrochemical proton gradient that is used for ATP synthesis. This review focuses on the membrane-bound electron transport chains of *Methanosarcina* species with respect to the biochemical and genetic characteristics of the unusual energy transducing enzymes. Furthermore, the review addresses questions concerning the relationship between methanogenic proteins and components of respiratory chains found in bacteria and eukarya.

Key words. Methanogens; proton translocation; energy transduction; respiratory chain; NADH dehydrogenase; $F_{420}H_2$ dehydrogenase; hydrogenase; heterodisulfide reductase.

Introduction

Biological methanogenesis is one of the most important processes for the maintenance of the carbon cycle on earth because methanogenic archaea catalyze the terminal step in the mineralization of organic material in many anaerobic environments [1]. Typical habitats of methanogens are freshwater sediments rich in organic matter, swamps and waterlogged soils such as rice paddies, and sewage treatment plants. Methanogenesis is also an important process in fermentations occurring in the intestinal tract of ruminants and termites [1]. The formation of methane from H_2 + CO_2, formate, methylated C_1 compounds (methanol, methylamines, methylthiols) or acetate is the characteristic feature of methanogenic archaea. These substrates are formed in the course of complex degradation processes of organic matter as performed by fermentative and synthrophic bacteria (see [2] for review). The products of methanogenesis, CH_4 and CO_2, are released from anaerobic habitats and can reenter the global carbon cycle. Part of methane is used by aerobic methylotrophic bacteria as a carbon and energy source or is chemically degraded to CO_2 [3]. However, it is worth noting that the CH_4 concentration in the atmosphere has increased more than twofold during the past 150 years [4]. This fact is of great interest for global ecology because methane is one of most the important greenhouse gases and contributes significantly to global warming effects [5]. The process of methanogenesis is not only of ecological importance but has received much attention because the metabolic pathways of methane formation are unique and involve a number of unusual enzymes and coenzymes [6–8]. This review describes the biochemistry of methane formation with special emphasis on developments within the past 3 years concerning membrane-bound enzymes generating primary proton gradients.

Process of methanogenesis

With respect to substrate utilization, two major groups of methanogenic archaea can be distinguished [9]. Obligate hydrogenotrophic methanogens are restricted to $H_2 + CO_2$ and formate. These organisms belong to the orders *Methanobacteriales*, *Methanococcales* and *Methanomicrobiales.* Model organisms of this group are *Methanothermobacter thermoautotrophicus* (formerly *Methanobacterium thermoautotrophicum* strain ΔH) and *Methanococcus jannaschii,* of which the genomes have been sequenced completely. The distantly related methylotrophic methanogens of the order *Methanosarcinales* utilize simple C_1 compounds such as methanol, methylamines and methylthiols. Some of them are also able to grow on $H_2 + CO_2$ and on acetate [9]. Regarding the biochemistry of methylotrophic methanogens, research has concentrated on members of the genus *Methanosarcina*. Recently, the genome of *Methanosarcina mazei* has been sequenced (http://www.g2l.bio.uni-goettingen.de). The organism contains a single circular chromosome consisting of 4.1 mega base pairs. Of a total of 3,371 open reading frames (ORFs) a function could be assigned to 2,450 ORFs. A striking feature of the *Ms. mazei* genome is that 1,043 of the 3,371 ORFs have their highest homologies outside the genus *Methanosarcina* in the bacterial domain [Note added in proof]. Raw genomic sequences are also available for *Methanosarcina barkeri* (http://genome.ornl.gov/microbial/mbar/). In summary, it is evident that genome analysis of methylotrophi methanogens will provide a new research paradigm in the fields of microbiology and molecular genetics.

The metabolic pathways leading to the formation of methane have been elucidated in recent years [10]. Methanogenesis from the above-mentioned substrates proceed according to the following equations [11]:

$CO_2 + 4\ H_2$	→	$CH_4 + 2\ H_2O$	($\Delta G_0' = -130$ kJ/mol CH_4)	(eq. 1)
$4\ HCOOH$	→	$3\ CO_2 + CH_4 + 2\ H_2O$	($\Delta G_0' = -119$ kJ/mol CH_4)	(eq. 2)
$4\ CH_3OH$	→	$3\ CH_4 + 1\ CO_2 + 2\ H_2O$	($\Delta G_0' = -106$ kJ/mol CH_4)	(eq. 3)
$4\ (CH_3)_3\ NH^+ + 6\ H_2O$	→	$9\ CH_4 + 3\ CO_2 + 4\ NH_4^+$	($\Delta G_0' = -76$ kJ/mol CH_4)	(eq. 4)
$2\ (CH_3)_2\ NH_2^+ + 2\ H_2O$	→	$3\ CH_4 + CO_2 + 2\ NH_4^+$	($\Delta G_0' = -75$ kJ/mol CH_4)	(eq. 5)
$4\ CH_3\text{-}NH_3^+ + 2\ H_2O$	→	$3\ CH_4 + CO_2 + 4\ NH_4^+$	($\Delta G_0' = -77$ kJ/mol CH_4)	(eq. 6)
$2\ (CH_3)_2S + 2\ H_2O$	→	$3\ CH_4 + CO_2 + 2\ H_2S$	($\Delta G_0' = -49$ kJ/mol CH_4)	(eq. 7)
$CH_3\text{-}COO^- + H^+$	→	$CH_4 + CO_2$	($\Delta G_0' = -36$ kJ/mol CH_4)	(eq. 8)

Several unusual cofactors are involved in these processes which have been identified and characterized during the last decades (for review see [6, 8, 12]). Coenzyme B (HS-CoB, 7-mercaptoheptanoylthreonine phosphate) and coenzyme F_{420} (a 5-deazaflavin derivative with a midpoint potential of –360 mV) function as electron carriers in the metabolism of methanogens (fig. 1). HS-CoB is the electron donor in the terminal reaction of methanogenesis (see below). F_{420} is the central electron carrier in the cytoplasm of methanogens, which accepts or donates hydride ions [13]. Recently, a unique hydrophobic cofactor was isolated from the cytoplasmic membrane of *Methanosarcina* species and referred to as methanophenazine (fig. 1). The component represents a 2-hydroxy-phenazine derivative that is connected via an ether bridge to a pentaprenyl side chain [14]. It functions as a membrane-integral electron carrier connecting protein complexes of the respiratory chain of *Methanosarcina* strains. Coenzyme M (2-mercaptoethanesulfonate, HS-CoM), methanofuran (MFR), and tetrahydromethanopterin (H_4MPT) are C_1 carriers; the latter component is also found in some methylotrophic bacteria and is chemically related to tetrahydrofuran. MFR is involved in CO_2 fixation and HS-CoM accepts methyl moieties from methyl-H_4MPT and from methylated substrates (methanol, methylamines and methylthiols).

Methyl-S-CoM (2-methylthioethanesulfonate) is the central intermediate in the catabolic metabolism of methanogens (fig. 1). Depending on the substrate, methyl-S-CoM is formed by three different pathways (fig. 2) that are explained below.

CO_2-reducing pathway

In the CO_2-reducing pathway carbon dioxide is bound to MFR and is then reduced to formyl-MFR [15]. The endergonic reaction is catalyzed by a formyl-MFR dehydrogenase and is driven by an electrochemical ion gradient. The direct electron donor of the formyl-MFR dehydrogenase is most probably reduced ferredoxin that is generated by the H_2-oxidizing Ech hydrogenase [16]. In the next reaction, the formyl group is 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 ($\Delta G_0' = -29$ kJ/mol) is coupled to the formation of an electrochemical sodium ion gradient (for review see [17]). In the final reaction methyl-S-CoM reacts with HS-CoB to yield methane and the heterodisulfde (CoM-S-S-

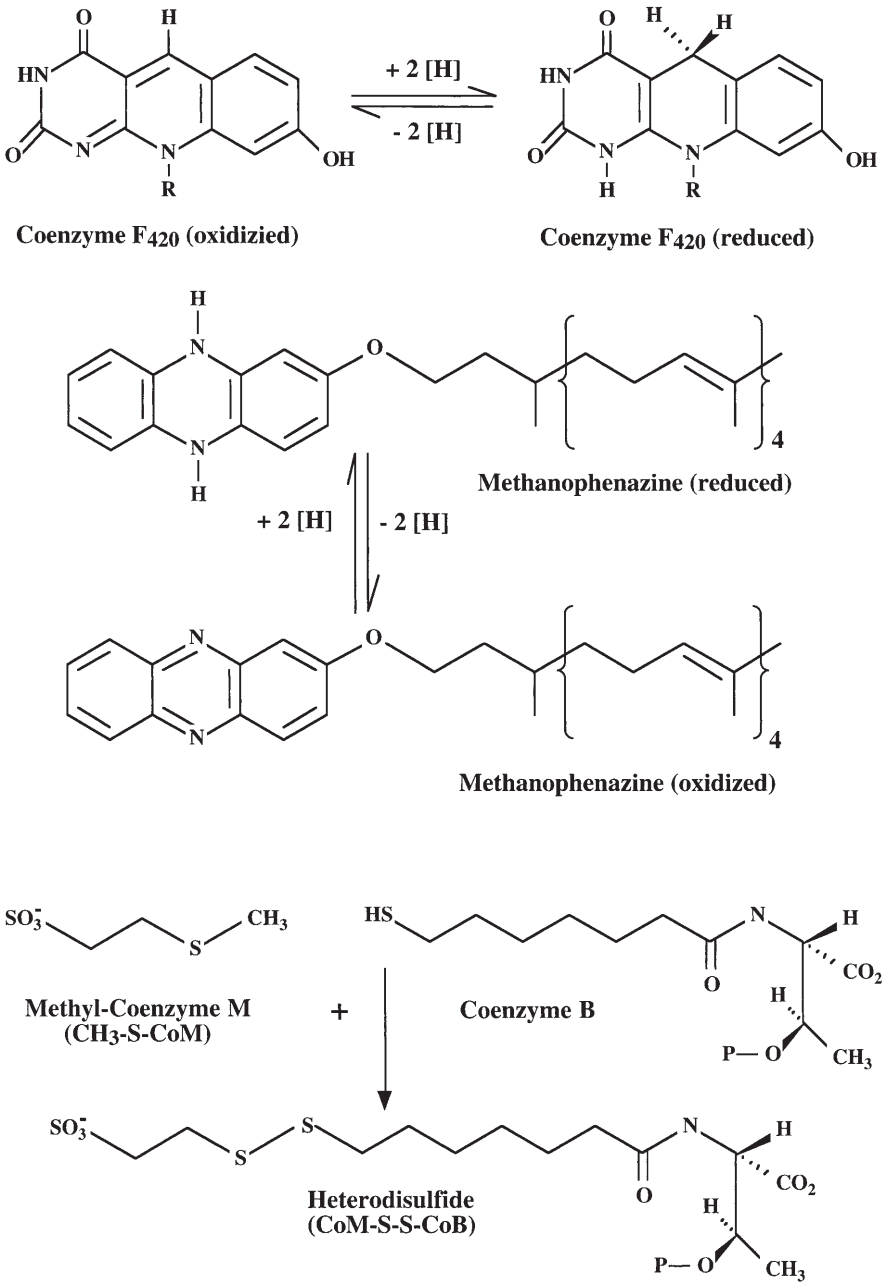

Figure 1. Unusual electron carrier involved in methanogenesis.

CoB) of the two methanogenic thiol-containing coenzymes. This reaction is catalyzed by the soluble methyl-CoM reductase [18].

Methylotrophic pathway

When cells grow on methylated C_1 compounds (methylotrophic pathway of methanogenesis), the substrates are converted to CH_4 and CO_2 (fig. 2) [19]. In the oxidative branch 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 membrane-bound methyl-H_4MPT:HS-CoM-methyltransferase. The endergonic reaction ($\Delta G_0' = 29$ kJ/mol) is driven by an electrochemical sodium ion gradient [20]. The formation of methyl-H_4MPT is followed by 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 MFR, the formyl-MFR dehydrogenase catalyzes oxidation to CO_2 and MFR. In the reductive branch of the pathway three out of

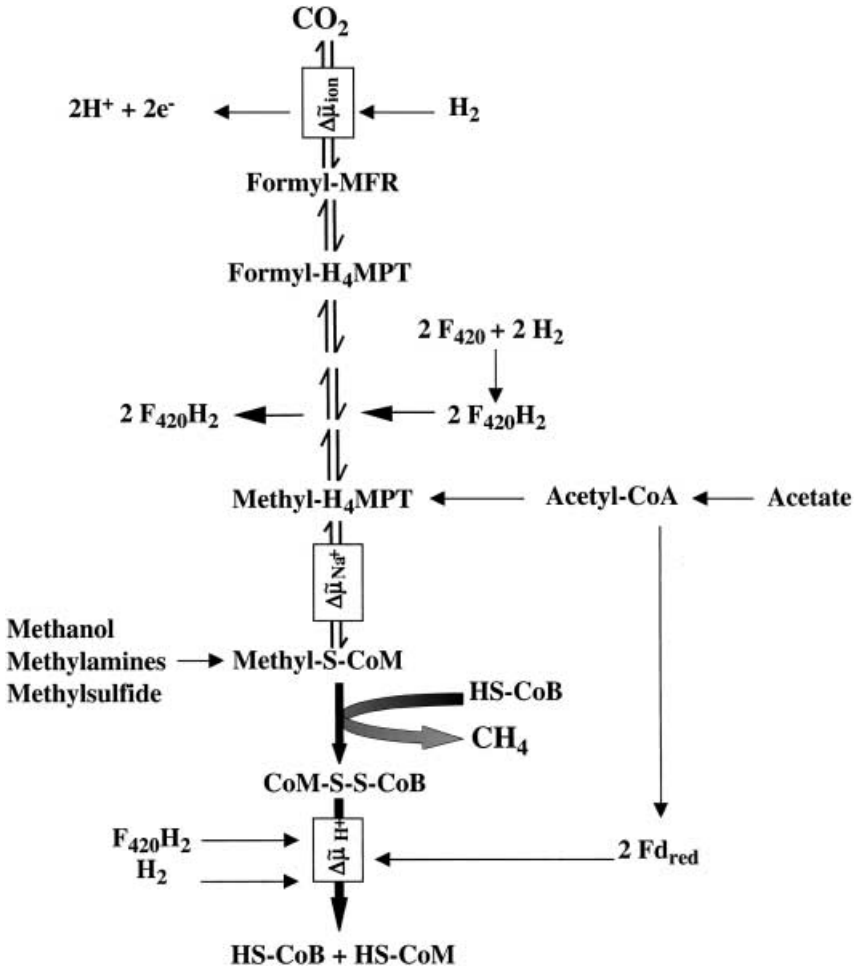

Figure 2. Schematic overview of the methanogenic pathways. $\Delta\tilde{\mu}_{H^+}$, electrochemical proton gradient; $\Delta\tilde{\mu}_{Na^+}$, electrochemical sodium ion gradient; $\Delta\tilde{\mu}_{ion}$, electrochemical ion gradient (either Na^+ or H^+ are used as coupling ions). Reducing equivalents for the reduction of CO_2 are probably derived from the catalytic activity of the Ech hydrogenase that oxidizes molecular hydrogen.

four methyl groups are transferred to HS-CoM by substrate-specific methyltransferases. Again, the HS-CoB-dependent reduction of methyl-S-CoM leads to the formation of CH_4 and CoM-S-S-CoB.

Aceticlastic pathway

The major part of methane production in nature derives from the mineralization of acetate (aceticlastic pathway), which is carried out only by the genera *Methanosarcina* and *Methanosaeta* [21]. After entering the cell, acetate is either activated by an acetate kinase and a phosphotransacetylase (*Methanosarcina* strains) or by an acetyl-CoA synthetase (*Methanosaeta* species). The resulting product acetyl-CoA is cleaved by a CO dehydrogenase/acetyl-CoA synthase complex. In the course of the reaction enzyme-bound CO is oxidized to CO_2, and the electrons are used for ferredoxin reduction. The methyl moiety is transferred to H_4MPT. The resulting methyl-H_4MPT is converted to methane by the catalytic activities of the sodium-translocating methyl-H_4MPT:HS-CoM methyltransferase and methyl-S-CoM reductase as described above.

Reactions coupled to proton translocation in methanogenic Archaea

As evident from the former paragraph, the formation of the heterodisulfide (CoM-S-S-CoB; fig. 1) marks the end of all processes leading to methane formation (fig. 2). As mentioned above, the disulfide-containing compound is of major importance for methanogens because it is the terminal electron acceptor of a branched respiratory chain. CoM-S-S-CoB is reduced by the membrane-bound heterodisulfide reductase that functions as a terminal respiratory reductase. It has been shown for the methylotrophic methanogen *Methanosarcina mazei* that the source of reducing equivalents necessary for the reduc-

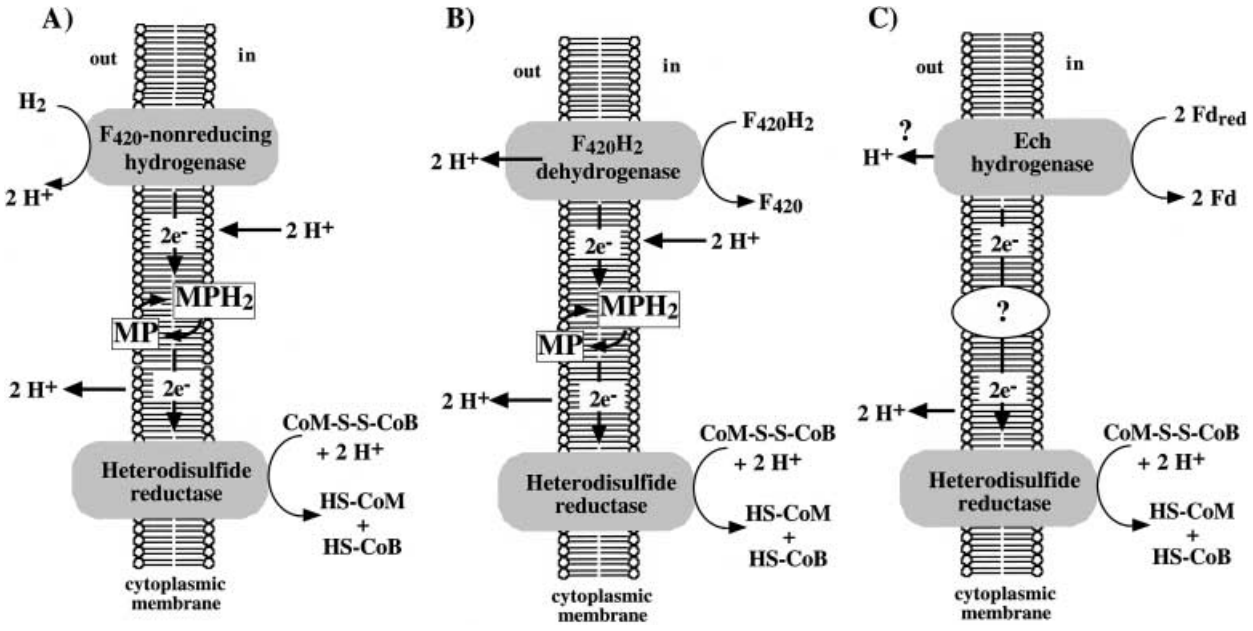

Figure 3. Membrane-bound electron transport in *Methanosarcina* strains. (*A*) H_2: heterodisulfide oxidoreductase system, (*B*) $F_{420}H_2$: heterodisulfide oxidoreductase system, (*C*) reduced ferredoxin:heterodisulfide oxidoreductase system; MP, methanophenazine; MPH_2, reduced methanophenazine; Fd_{red}, reduced ferredoxin.

tion of the heterodisulfide depends on the growth substrate. If molecular hydrogen is present, a membrane-bound hydrogenase channels electrons to the heterodisulfide reductase, which reduces the terminal electron donor [22]. This electron-transport system is referred to as a H_2: heterodisulfide oxidoreductase system (fig. 3a). H_2-dependent heterodisulfide reductases have also been identified in *Ms. barkeri* [23], *Methanothermobacter marburgensis* (formerly *Mb. thermoautotrophicum* strain Marburg [24]), and *Methanococcus voltae* [25]. When *Methanosarcina* strains are grown on methylated C_1 compounds in the absence of molecular hydrogen, some of the methyl groups are oxidized to CO_2 and reducing equivalents are transferred to coenzyme F_{420}. In this case the membrane-bound $F_{420}H_2$: heterodisulfide oxidoreductase system is responsible for the oxidation of $F_{420}H_2$ and reduction of the heterodisulfide (fig. 3b) [26]. $F_{420}H_2$: heterodisulfide oxidoreductase systems have also been identified in members of the genus *Methanolobus* [27]. In the final step of 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 to as a reduced ferredoxin : heterodisulfide oxidoreductase system (fig. 3c) [22, 28].

In recent years, the membrane-bound electron transfer of *Ms. mazei* Gö1 has been analyzed in detail. To elucidate the coupling between electron transport and ion translocation, a subcellular system of this organism was developed, which consisted of washed inverted membrane vesicles free from cytoplasmic components [29, 30]. This preparation has the advantage that enzymes located at the inner site of the membrane are accessible to the highly charged substrates F_{420} and CoM-S-S-CoB because of the inverted orientation of these vesicles. Using these vesicle preparations, it has been shown that electron transport as catalyzed by the $F_{420}H_2$: heterodisulfide oxidoreductase system and the H_2: heterodisulfide oxidoreductase system was accompanied by proton translocation across the cytoplasmic membrane into the lumen of the inverted vesicles. The electrochemical proton gradient thereby generated was used for ATP synthesis from ADP + P_i catalyzed by an A_1A_0-type ATP synthase [22, 31]. Both systems exhibited stoichiometries of about $4H^+/2e^-$ and about $1ATP/2e^-$ [26, 32]. Protonophores dissipated the electrochemical H^+ gradient and inhibited ADP phosphorylation as catalyzed by washed inverted vesicles of *Ms. mazei* Gö1; however, electron transport was stimulated. *N,N′*-dicyclohexylcarbodiimide (DCCD), an ATP synthase inhibitor, prevented ATP synthesis and decreased the electron transport rate. The uncoupler SF 6847 reversed the latter effect. The addition of ADP was stringent for ATP formation and stimulated the reductive cleavage of CoM-S-S-HTP. These effects are comparable to the phenomenon of respiratory control as observed in mitochondria. The experiments clearly demonstrated that ATP is formed by electron transport-driven phosphorylation of ADP and that the proton-translocating oxidoreductases discovered in *Ms. mazei* are of principal importance for all methylotrophic methanarchaea. Hence, already in the

early 1990s the similarities of the methanogenic electron transport with aerobic respiration (using $F_{420}H_2$ instead of $NADH_2$) and with S^O-reducing bacteria (using organic -S-S- bridges instead of S^O) became evident. And until today the membrane-bound electron transport systems in methanogens are of particular interest in the context of the evolution of respiratory chains.

Further investigation of the respiratory chains of *Ms. mazei* revealed that three enzymes are involved in electron transfer [7]. It has been shown that the H_2: heterodisulfide oxidoreductase system is composed of a membrane-bound hydrogenase (referred to below as F_{420}-nonreducing hydrogenase) and the heterodisulfide reductase (fig. 3a). The latter protein is also part of the $F_{420}H_2$: heterodisulfide oxidoreductase system. The second component of the F_{420}-dependent system was named $F_{420}H_2$ dehydrogenase (fig. 3b). Since other components of the system were unknown at that time, only the overall electron transport reaction could be investigated. With the discovery of the novel cofactor methanophenazine and the use of its water soluble analogue 2-hydroxyphenazine (2-OH-phenazine), electron transport in *Ms. mazei* could be analyzed in more detail [33]. 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 electron donor for the reduction of CoM-S-S-CoB [34, 35].

Washed inverted vesicles of this organism were found to couple both processes with the transfer of two protons across the cytoplasmic membrane, indicating that the H_2:heterodisulfide oxidoreductase system comprises two proton-translocating segments. The first one involves the F_{420}-nonreducing hydrogenase and the second one the heterodisulfide reductase [35]. 2-OH-phenazine is also a mediator of electron transfer within the $F_{420}H_2$: heterodisulfide oxidoreductase system [34]. 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. The second reaction of this electron transport system is again catalyzed by the heterodisulfide reductase that uses dihydro-2-OH-phenazine as 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 protons. Thus, the $H^+/2e^-$ stoichiometries of both electron transport chains add up to 4 and support the value of four $H^+/2e^-$ translocated in the overall electron transport from $F_{420}H_2$ and from H_2 to the heterodisulfide, respectively [26, 32]. In agreement with these findings, three to four $H^+/2e^-$ were transferred by whole cell preparations of *Ms. barkeri* when methane formation from methanol + H_2 was analyzed [36].

Using the model compound 2-OH-phenazine and its reduced form, first experiments on the biological function of phenazines in membrane-bound electron transport were performed. As mentioned above, all key enzymes react with the artificial electron carrier [14]. After completion of the total synthesis of methanophenazine, similar tests could also be performed with the natural product [33]. The results clearly indicated that methanophenazine serves as an electron acceptor of both the membrane-bound hydrogenase and the $F_{420}H_2$ dehydrogenase if H_2 and F_{420} are added, respectively (fig. 3a/b). In addition, the heterodisulfide reductase uses the reduced form of methanophenazine as an electron donor for the heterodisulfide reduction. 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. Furthermore, the experiments showed that its role in the energy metabolism of methanogens is similar to that of ubiquinone in mitochondria and bacteria. In summary, the overall electron transport from $F_{420}H_2$ and from H_2 to the heterodisulfide can be described as follows:

$$^{\circ}F_{420}H_2 + MP + 2\,H^+_{in} \xrightarrow{F_{420}H_2\ \text{dehydrogenase}} F_{420} + MPH_2 + 2\,H^+_{out} \quad \text{(eq. 9)}$$

$$^{\circ}H_2 + MP + 2\,H^+_{in} \xrightarrow{F_{420}\text{-nonreducing hydrogenase}} MPH_2 + 2\,H^+_{out} \quad \text{(eq. 10)}$$

$$CoM\text{-}S\text{-}S\text{-}CoB + MPH_2 + 2\,H^+_{in} \xrightarrow{\text{Heterodisulfide reductase}} HS\text{-}CoM + HS\text{-}CoB + MP + 2\,H^+_{out} \quad \text{(eq. 11)}$$

In contrast to H_2-dependent and $F_{420}H_2$-dependent heterodisulfide reduction, information concerning energy transduction in the course of CoM-S-S-CoB reduction by reduced ferredoxin is scarce [37, 38]. Direct measurements of proton translocation activity of the Fd_{red}: heterodisulfide oxidoreductase system have not yet been done. However, indirect evidence suggests that the third electron transport system in *Methanosarcina* species is able to generate electrochemical proton gradients, too (fig. 3c).

Characteristics of proton-translocating enzymes in methanogens

The data described above clearly indicate that the F_{420}-nonreducing hydrogenase, the heterodisulfide reductase and the $F_{420}H_2$-dehydrogenase are redox-driven proton-translocating enzymes showing maximal energetic efficiencies of about two H^+ translocated per $2e^-$ transported. In the aceticlastic pathway of methanogenesis another protein is believed to participate in energy transduction that is referred to as Ech hydrogenase [39]. The current knowledge about these enzymes will be discussed in the following sections.

F_{420}-nonreducing hydrogenase

Hydrogenases play an important role in methanogenic archaea during growth on $H_2 + CO_2$ [40]. They generate reducing equivalents from H_2 for reductive biosynthesis and for electron transport phosphorylation. Hydrogenases are also synthesized by members of the family *Methanosarcinaceae* during growth on methanol or methylamines. Under these conditions, reducing equivalents are derived from the oxidation of methyl groups. A function of the hydrogenases in this metabolic pathway is not immediately obvious. In methylotrophic methanogens, three types of nickel-iron hydrogenases have been described: (i) The so-called F_{420}-dependent hydrogenase reduces the central electron carrier F_{420} as well as artificial dyes such as methylviologen [41]. In *Methanosarcina* species the protein is located in the cytoplasm and is obviously not involved in energy transduction. (ii) The second type of hydrogenase reacts best with methylviologen as electron acceptor and does not reduce F_{420} (F_{420}-nonreducing hydrogenase [42]). (iii) Recently, a third group of NiFe hydrogenases has been discovered in methanogens that are named Ech hydrogenases [39]. Generally, the core of all these enzymes is composed of a small electron transfer subunit and a large catalytic subunit harboring the active nickel-iron center for H_2 cleavage. Besides the core subunits, methanogenic [NiFe] hydrogenases are equipped with several other polypeptides depending on their physiological function.

The membrane-bound F_{420}-nonreducing hydrogenases from *Ms. mazei* and *Ms. barkeri* have been purified, using detergents for solubilization [43, 44]. The purified F_{420}-nonreducing hydrogenase from *Ms. mazei* was composed of two polypeptides with molecular masses of 60 and 40 kDa (fig. 4). It contained about 13 mol S^{2-}, 14 mol Fe and 0.8 mol Ni/mol enzyme. The structural genes of the F_{420}-nonreducing hydrogenases were cloned and sequenced [45, 46]. 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. 5a). Northern blot analysis revealed that the structural genes form an operon,

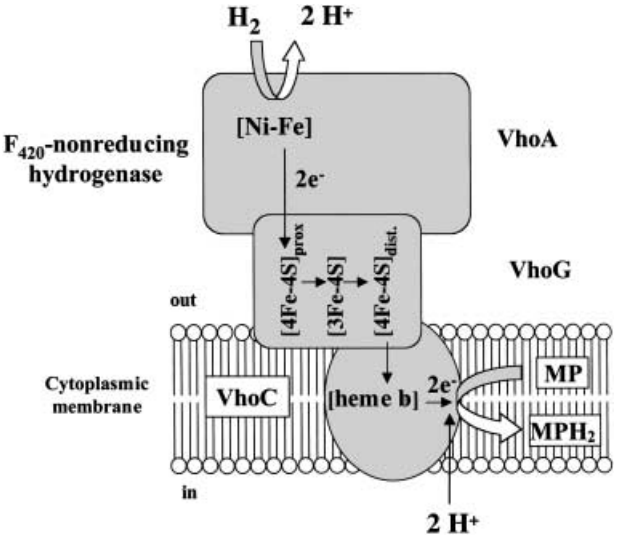

Figure 4. Model of the membrane-bound, F_{420}-nonreducing hydrogenase. MP, methanophenazine; MPH_2, reduced methanophenazine; [FeS], iron-sulfur cluster; [Ni-Fe], bimetallic nickel-iron cluster of the active site; VhoA, large subunit (encoded by *vhoA*); VhoG, small subunit (encoded by *vhoG*); VhoC represents the cytochrome b subunit containing heme b1.

containing one additional open reading frame (*vhoC)* which codes for a membrane-spanning cytochrome b (fig. 5a). Interestingly, highly homologous hydrogenases are found in several bacteria. The crystal structure of the nickel-iron hydrogenase from *Desulfovibrio gigas* revealed that the small subunit contains three iron-sulfur clusters [95]. They are distributed along a straight line with the [3Fe-4S] cluster located halfway between the two [4Fe-4S] clusters that are named proximal and distal based on their distance to the NiFe center. All amino acid residues involved in the ligation of the iron-sulfur clusters are conserved in the small subunit (VhoG) of the F_{420}-nonreducing hydrogenase from *Ms. mazei*, indicating that the [3Fe-4S] cluster and both [4Fe-4S] clusters are present in the methanogenic enzyme. Furthermore, it has been shown for the hydrogenase from *Wolinella succinogens* that the b-type cytochrome acts as the primary electron acceptor of the core enzyme [47]. It is most likely that the heme b-containing subunit of the hydrogenase from *Ms. mazei* and other *Methanosarcina* strains has the same function (fig. 4). Comparison of the sequencing data of *vhoG* from *Ms. mazei* with the experimentally determined N-terminus of the small subunit indicates the presence of a leader peptide in front of the polypeptide [46] that contains a typical twin arginine motif. Hence, it is most likely that the small subunit is translocated across the cytoplasmic membrane by the Sec-independent, twin-arginine pathway [48]. The large subunit lacks a signal sequence, and no evidence for an internal signal has so far been obtained. This polypeptide is most probably cotranslocated together with the small subunit to the periplasmic side of

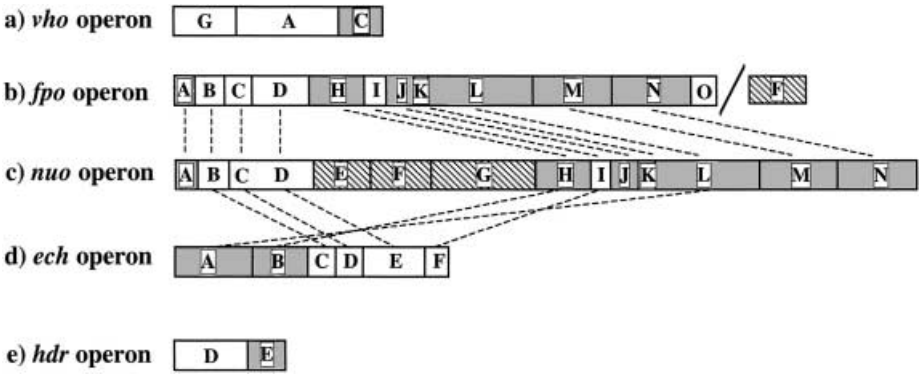

Figure 5. Operon structure of key enzymes of the respiratory chain from *Ms. mazei*. Genes of the *fpo* and the *ech* operon that are homologous to genes encoding complex I (e. g. *nuo* operon from *E. coli)* are linked by broken lines. Genes encoding membrane-integral subunits are shown in grey. Genes encoding subunits of the input module of the $F_{420}H_2$ dehydrogenase and of complex I are hatched.

the cytoplasmic membrane. In accordance, immunogold-labeling experiments showed that the active center of the enzyme from *Ralstonia eutropha* is exposed towards the periplasm [49]. It is believed that the presence of a C-terminal hydrophobic domain in the small subunit of membrane-bound hydrogenases is responsible for membrane localization of these enzymes. Evidence in favor of this assumption is the fact that periplasmic hydrogenases do not contain such a hydrophobic domain. The cytochrome b subunit is also important for localization of membrane-bound hydrogenases because a lesion of the corresponding gene in the hydrogenase from *Ralstonia eutropha* led to the production of a soluble cytoplasmic protein [50]. It is important to note that the cellular location of hydrogenases with respect to the membrane is of great importance for the mechanism of H^+ translocation.

Taking into account all the information, it is possible to construct a tentative model for electron and proton transfer within the F_{420}-nonreducing hydrogenase from *Methanosarcina* strains (fig. 4). In the first step, molecular hydrogen is oxidized by the bimetallic NiFe center of the large subunit. The remaining protons are channeled from the active site to the surface, where they are released into the periplasmic space. The iron-sulfur clusters in the small subunit accept the electrons and transfer them to the heme groups present in the membrane-integral cytochrome b subunit. To complete the reaction, the cytochrome b subunit should accept two protons from the cytoplasm for reduction of methanophenazine. Thus, the overall reaction would lead to the production of two scalar protons. This prediction is in agreement with H^+/e^- values obtained for the H_2-dependent 2-OH-phenazine reduction as catalyzed by inverted vesicles from *Ms. mazei*. Further evidence for the proposed reaction cycle came from inhibitor studies with diphenyleneiodonium chloride, which indicated that phenazine derivatives directly interact with the heme b-containing subunit of F_{420}-nonreducing hydrogenase (fig. 4) [51].

Heterodisulfide reductase

As mentioned above, the heterodisulfide reductase catalyzes the final step in the electron transport chain of methanogens, namely the two-electron reduction of CoM-S-S-CoB to the free thiols HS-CoB and HS-CoM (fig. 6). The enzymes from the methylotrophic methanogens *Ms. barkeri* [52, 53] and *Ms. thermophila* [54] consist of two subunits (HdrD, HdrE). The membrane-integral subunit HdrE represents a b-type cytochrome and contains a low-spin and a high-spin heme. The large subunit HdrD contains two distinct [4Fe-4S] clusters and harbors the active site for disulfide reduction. It is thought that only one [4Fe-4S] cluster and a redox-active disulfide made from two cysteine residues form the active site of the reaction center [55]. Evidence has been presented that the physiological electron donor of the heterodisulfide reductase is the membrane-bound cofactor

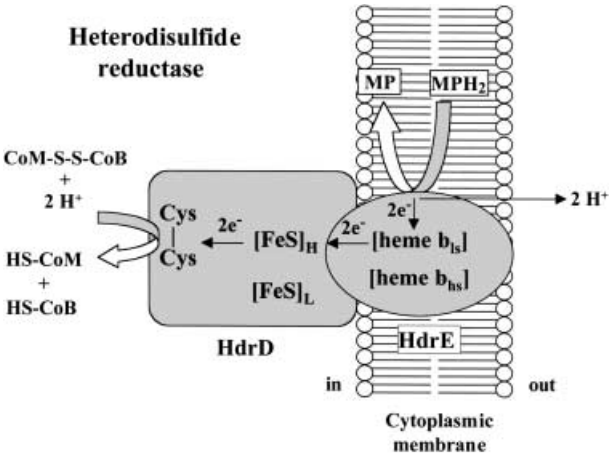

Figure 6. Model of the heterodisulfide reductase. Cys-Cys represents the redox-active disulfide in the active center. HdrE contains the high-spin and low-spin heme b groups (heme b_{hs} and heme b_{ls}). $[FeS]_L$ indicates the low potential iron-sulfur cluster, and $[FeS]_H$ represents the high potential iron sulfur cluster within subunit HdrD.

methanophenazine [33]. The enzyme also reacts with the water-soluble analog 2-OH-phenazine [51]. It is tempting to speculate that the mechanism of energy conservation is based on scalar proton transfer as in case of the F_{420}-nonreducing hydrogenase. According to this hypothesis reducing equivalents from reduced phenazine derivatives are transferred to the one-electron-accepting prosthetic groups of the enzyme (heme b and FeS clusters) and protons are released at the outer phase of the cytoplasmic membrane (fig. 6). Then, the electrons enter the reactive center, where CoM-S-S-CoB is reduced. It is believed that the protons necessary for this reaction are derived from the cytoplasm [35]. Recently, a possible catalytic mechanism for the heterodisulfide reductase was published by R. Hedderich and co-workers [55]. Detailed electron paramagnetic resonance (EPR) studies of the enzymes from *Mb. marburgensis* and *Ms. barkeri* led to the discovery of a paramagnetic species generated by an unusual FeS cluster that is connected to one of the cysteines of the nearby active-site disulfide or by the sulfur of HS-CoM. Also, the pathway of electron transfer from reduced 2-OH-phenazine to the heterodisulfide has been studied by steady-state and transient kinetics using the purified heterodisulfide reductase from *Ms. thermophila* [56]. Stopped-flow experiments showed that the low-potential heme (midpoint potentials = –180 mV) participates in reduction of CoM-S-S-CoB. Furthermore, spectroscopic and kinetic studies with the inhibitor diphenylene iodonium indicated that only the high-potential [4Fe-4S] cluster (midpoint potentials = –100 mV) is involved in electron transfer to CoM-S-S-CoB (fig. 6).

Characteristics of the $F_{420}H_2$ dehydrogenase

The $F_{420}H_2$ dehydrogenase is one of the most fascinating enzymes found in methanogens (fig. 7). As described above, the protein catalyzes the $F_{420}H_2$-dependent reduction of phenazine derivatives, thereby transferring two protons across the cytoplasmic membrane [34]. Thus, the enzyme represents a novel kind of a proton-translocating complex in methanogenic archaea. Because of this interesting feature the protein is of major interest with respect to subunit composition, cofactor content and reaction mechanisms. First attempts to purify the $F_{420}H_2$ dehydrogenase were performed in 1990 using washed cytoplasmic membranes from the methanogenic organism *Methanolobus (Ml.) tindarius*. The results led to the assumption that the protein was composed of only one subunit with a mass of 37 kDa that was able to oxidize reduced F_{420} using viologen dyes as electron acceptors. The subunit contained about 6 mol of non-heme iron and acid-labile sulfur per mole of protein, indicating that at least two FeS clusters were bound to the polypeptide [57]. Further investigations using *Ml. tindarius* and *Ms. mazei* revealed that four additional subunits with molecular masses of 40, 22, 20 and 16 kDa which had been lost in the initial purification procedure were present in the protein [58, 59]. These additional subunits also contained FeS clusters because the values for non-heme iron and acid-labile sulfur increased to 16 mol/mol protein. In contrast to the $F_{420}H_2$ dehydrogenase from *Ml. tindarius*, low amounts of flavin were detected in the *Ms. mazei* enzymes. A similar enzyme was isolated from *Archaeoglobus (A.) fulgidus* [60, 61]. This organism is a sulfate-reducing hyperthermophilic archaeon that uses e.g. lactate plus sulfate as sole carbon and energy sources. *A. fulgidus* seems to be a missing link between methanogens and sulfate reducers [62]. In fact, lactate is converted to acetyl-coenzyme A (CoA) that is cleaved by the CO dehydrogenase. The methyl group is transferred to the methanogenic cofactor H_4MPT, and CO is oxidized to CO_2 in the same manner as described for acetate-growing methanogens (fig. 2). Moreover, the pathway of methyl-group oxidation is very similar to the oxidative branch of methanogenesis from methanol and methylamines as carried out by organisms belonging to the order *Methanosarcinales* (fig. 2). *A. fulgidus* transfers part of the reducing equivalent to coenzyme F_{420}, which is reoxidized by a membrane-bound $F_{420}H_2$: quinone oxidoreductase [39]. Hence, it is not a surprise that *A. fulgidus* possesses a $F_{420}H_2$ dehydrogenase which is highly homologous to the corresponding methanogenic enzyme. The protein has been purified from the cytoplasmic membrane and contains FAD, acid-labile sulfur and non-heme iron [61].

To provide a basis for future investigations on structure/function relationships of the above-mentioned protein, it was necessary to identify the genes encoding the $F_{420}H_2$ dehydrogenase. Based on the N-terminal amino acid sequences of the subunits, Westenberg and co-workers [63] were able to clone and sequence the gene encoding the 40-kDa polypeptide of the $F_{420}H_2$ dehydrogenase from *Ml. tindarius*. However, open reading frames encoding the other subunits were not identified. A detailed insight into the organization of the genes encoding the $F_{420}H_2$ dehydrogenase had to await the genome sequencing project of *Ms. mazei*. Taking advantage of the N-terminal amino acid sequences of the subunits, one DNA fragment comprising 12 genes was identified that encoded the 40-, 22-, 20- and 16-kDa subunits of the purified $F_{420}H_2$ dehydrogenase from *Ms. mazei* [34]. The gene locus was named *fpo* for $\underline{F}_{420}H_2$: phenazine oxidoreductase and the genes, referred to as *fpo A, B, C, D, H, I, J, K, L, M, N and O*, were shown to be organized in one operon (fig. 5b). The deduced N-terminal amino acid sequences of *fpo B, C, D and I* were identical to the N-termini of four subunits of the purified $F_{420}H_2$ dehydrogenase. The fifth subunit is encoded by the *fpoF* gene that will be described below. Hydropathy plots revealed that the deduced subunits from *fpo A, H, J, K, L, M* and *N* are membrane-integral components comprising more than 50 transmembrane he-

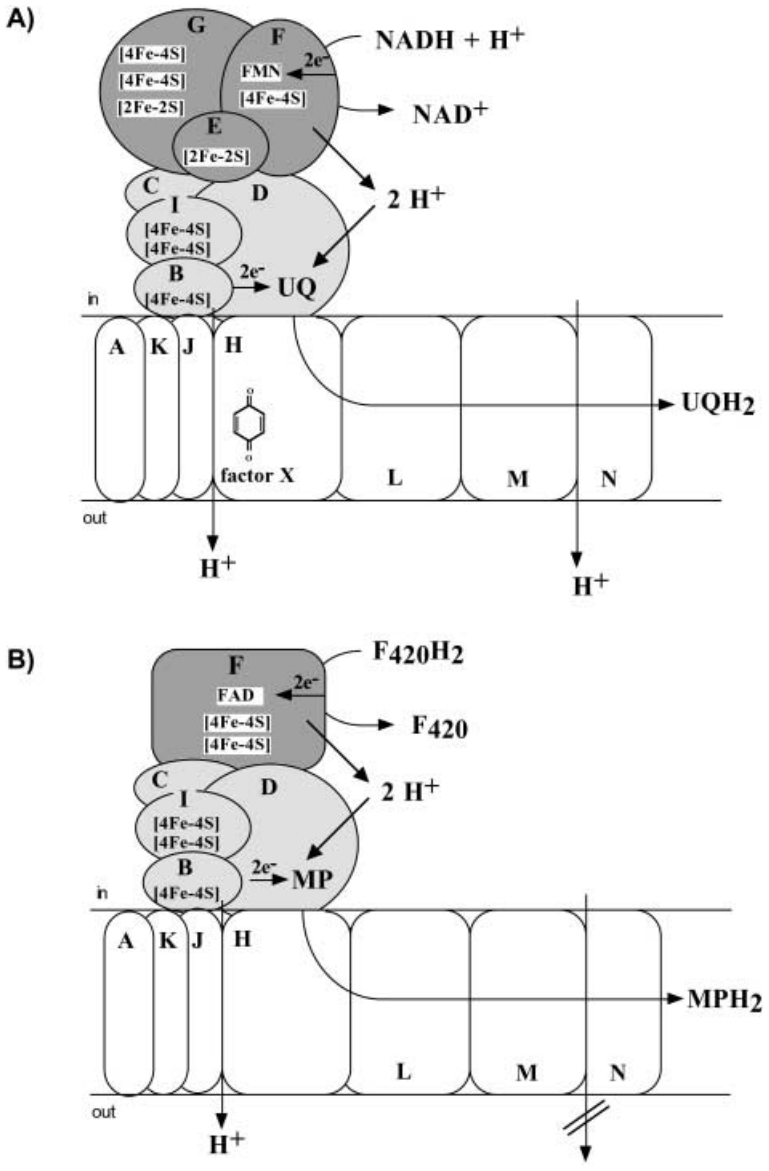

Figure 7. Tentative scheme of complex I from bacteria and methanogenic $F_{420}H_2$ dehydrogenase. (*A*) The NADH dehydrogenase module (NuoEGF) oxidizes NADH and transfers electrons to the connecting module (light gray) via FMN and FeS clusters (N1a, N1b, N1c, N3, N4 and N5). The electrons are accepted by FeS cluster N6a/b located on NuoI. Further electron transport to cluster N2 and finally to ubiquinone is coupled to redox-driven H^+ translocation. The overall electron transport might cause conformational changes within the membrane-integral module, leading to additional H^+ transfer (conformational driven coupling site [103]). The position of the quinoid factor X is arbitrary because it is not known which subunit harbors this prosthetic group. (*B*) The $F_{420}H_2$ dehydrogenase subunit FpoF oxidizes $F_{420}H_2$ via FAD and two [4Fe-4S] clusters. As in case of complex I, electrons are transferred to clusters N6a/b located on FpoI and to cluster N2 probably located on subunit FpoB. FpoO might be involved in electron transfer to methanophenazine that is bound at the interface of FpoD/B, the hydrophobic part being fixed within FpoH.

lices. The subcomplex purified thus far has a molecular mass of 115 kDa and is composed of only five hydrophilic subunits (FpoBCDFI [59]). In this context it is worth noting that the stability of multisubunit complexes in solution is critically dependent on the detergent used for solubilization. The procedure leading to the purification of the 115-kDa subcomplex of the $F_{420}H_2$ dehydrogenase was performed using the detergent CHAPS, which is known to cause fragmentation of proteins. Thus, it is most likely that the membrane-integral polypeptides were lost in the course of purification. In agreement with this assumption is the finding that a $F_{420}H_2$ dehydrogenase complex with a size of 400 kDa could be partially purified when the membrane was solubilized with the mild detergent octylglycoside instead of CHAPS. In contrast to the 115-kDa enzyme, the 400-kDa complex is able to catalyze a $F_{420}H_2$-dependent methanophenazine reduction, indicating the structural integrity of the $F_{420}H_2$ dehydro-

genase in the octylglycoside preparations [unpublished results].

DNA sequence analysis and amino acid sequence alignments have revealed that all subunits of the $F_{420}H_2$ dehydrogenase (with the exception of FpoO) show high similarities to distinct subunits of proton-translocating NADH: quinone oxidoreductases from prokaryotes and eukaryotes (from here on referred to as complex I). In this context it is important to note that the $F_{420}H_2$ dehydrogenase and complex I have some interesting features in common [34, 64]:

1) The flavin and iron-sulfur-containing enzymes reveal a complex subunit composition and are the initial enzymes of membrane-bound electron transport systems.
2) The electron donors $F_{420}H_2$ and NADH are similar because both cofactors are reversible hydride donors with comparable midpoint potentials.
3) The enzymes use small hydrophobic nonproteinous electron acceptors, namely quinones in the case of NADH dehydrogenases and methanophenazine in the case of $F_{420}H_2$ dehydrogenases.
4) It was shown that mitochondrial and bacterial NADH: ubiquinone oxidoreductases as well as $F_{420}H_2$ dehydrogenase were inhibited by diphenyleniodonium chloride.
5) Both enzymes show redox-driven proton-translocating activity.

In order to speculate about reaction mechanisms of the methanogenic protein and about structure/function relationships of its subunits, it is first necessary to describe the composition of complex I (see table 1 for nomenclature). This multisubunit enzyme catalyzes the electron transfer from NADH to quinones present in the inner membrane of mitochondria and in the cytoplasmic membrane of many bacteria (fig. 7a). The redox reaction is linked to proton translocation across the membrane and can be summarized as follows:

$$NADH + H^+ + Q + 4\ H^+_{in} \rightarrow NAD^+ + QH_2 + 4\ H^+_{out}$$

Whereas the mitochondrial complex I is composed of up to 43 different polypeptides, the bacterial protein comprises only 13–14 subunits [65]. The proton-translocating efficiency of the bacterial enzyme is similar to that of the mitochondrial complex I. Therefore, the bacterial complex I is considered to be a minimal structural form of a multisubunit NADH: ubiquinone oxidoreductase and is an ideal model to study the characteristics of this proton-translocating enzyme (fig. 7a). In *E. coli* the complex I genes are organized in the so-called *nuo* operon (fig. 5c) [66]. It comprises 13 genes that are referred to as *nuoA, nuoB, nuoCD* (a fusion of gene C and D), *nuoF, nuoG, nuoH, nuoI, nuoJ, nuoK, nuoL, nuoM* and *nuoN* (see table 1 for homologous genes in eukaryotes, bacteria and archaea).

The corresponding subunits of the *E. coli* enzyme are organized in three different modules [72]:

1) NuoEFG catalyze the oxidation of NADH. This input module of complex I is also called the NADH dehydrogenase fragment. It contains the FMN and NADH binding sites and several EPR-detectable FeS clusters.
2) The subunits NuoBCDI form the amphipathic fragment (membrane-associated module) and connect the input module to the membrane fragment. Two different FeS clusters have been identified in this module, indicating its function as a mediator of electron transfer from module 1 to 3.
3) NuoAHJKLMN form the membrane-integral module and are involved in quinone reduction and H^+ transfer. Very recently a quinoide prosthetic group was detected

Table 1. Nomenclature of subunits from complex I-like proteins.

Bos taurus[a]		*E. coli*[b] *R. capsulatus*[d]	*T. thermophilus*[c] *P. denitrificans*[f]	*Ms. mazei*[e]	*A. fulgidus*[g]
Mito[h]	Nucleo[i]				
nd1		NuoH	Nqo8	FpoH	FqoH
nd2		NuoN	Nqo14	FpoN	FqoN
nd3		NuoA	Nqo7	FpoA	FqoA
nd4		NuoM	Nqo13	FpoM	FqoM
nd4L		NuoK	Nqo11	FpoK	FqoK
nd5		NuoL	Nqo12	FpoL	FqoL
nd6		NuoJ	Nqo10	FpoJ	FqoJ
	49 kD	NuoD	Nqo4	FpoD	FqoD
	TYKY	NuoI	Nqo9	FpoI	FqoI
	30 kD	NuoC	Nqo5	FpoC	FqoC
	PSST	NuoB	Nqo6	FpoB	FqoB
	24 kD	NuoE	Nqo2	–	–
	51 kD	NuoF	Nqo1	–	–
	75 kD	NuoG	Nqo3	–	–

[a][71]; [b][66]; [c][69]; [d][67]; [e][34]; [f][70]; [g][68]; [h]Proteins encoded by mitochondrial genes; [i]Proteins encoded by the nucleus.

in this subcomplex that is most probably involved in electron transfer to ubiquinone or menaquinone [84].

Because of the complexity of the NADH dehydrogenase each module will be described separately in the next sections and will be compared to its respective counterpart in the $F_{420}H_2$ dehydrogenase.

The input module

The water-soluble NADH dehydrogenase fragment of complex I from bacteria is composed of subunits NuoE, F and G (fig. 7a). It is capable of transferring electrons from NADH to ferricyanide, and therefore it represents the electron-input part of complex I [73]. The fragment is evolutionarily related to the diaphorase part of bacterial NAD-reducing hydrogenase [74]. The enzyme of *Ralstonia eutropha* is made up of four subunits (HoxFUYH) and comprises two subcomplexes [75, 76]. Subunits HoxH and HoxY form the hydrogenase part and the dimer from subunit HoxF and HoxU functions as diaphorase. The relationship to complex I is evident from the finding that the N-terminus of HoxF is homologous to the 24-kDa subunit of complex I from bovine (equivalent of NuoE), its C-terminus to the 51-kDa subunit of bovine complex I (NuoF; see table 1) [77]. Subunit HoxU is homologous to the N-terminal part of the 75-kDa subunit (NuoG), [78].

Genes encoding the subunits NuoE, F and G are not present in the *fpo* gene cluster or anywhere else on the *Ms. mazei* chromosome. This is not surprising, because the input module of the methanogenic enzyme should be adapted to the oxidation of $F_{420}H_2$. In fact, strong evidence has been presented that the 37-kDa subunit FpoF functions as an electron-feeding device for the $F_{420}H_2$ dehydrogenase (fig. 7b) [60]. The polypeptide is homologous to the β subunit of F_{420}-reducing hydrogenases and to subunits of the $F_{420}H_2$ dehydrogenase from *Ml. tindarius* (FfdB [63]) and *A. fulgidus* (FqoF [60]). In the latter organism the *fpoF*-homolog gene *fqoF* is part of the operon encoding the $F_{420}H_2$ dehydrogenase [68]. All FpoF-like polypeptides contain motifs for the binding of two [4Fe-4S] clusters (in FpoF from *Ms. mazei*: $C^{33}xxC^{36}xxC^{39}xxxC^{43}P$ and $C^{64}xxC^{67}xxC^{70}xxxC^{74}P$). Recently, FqoF from *A. fulgidus* was overexpressed in *E. coli*, and the corresponding polypeptide was purified to homogeneity [60]. The subunit contained non-heme iron, acid-labile sulfur and FAD and was able to oxidize $F_{420}H_2$ when the artificial electron acceptor methylviologen was added. Since FpoF from *Ms. mazei* and FfdB from *Ml. tindarius* are structurally equivalent to FqoF, it is most likely that the subunits of the methanogenic $F_{420}H_2$ dehydrogenases also function as an electron-input module. Keeping in mind these findings, one observation described above can now be explained. In the course of the first efforts to purify the $F_{420}H_2$ dehydrogenase from *Ml. tindarius*, a single subunit with a size of 37 kDa was isolated [57]. This polypeptide most probably represented subunit FfdB, which is able to oxidized reduced F_{420} using viologen dyes as electron acceptors. All other subunits were obviously separated and got lost during the purification procedure.

As indicated above, the input modules from $F_{420}H_2$ dehydrogenases and from NADH dehydrogenases are not homologous. However, both modules catalyze the oxidation of obligate hydride donors ($F_{420}H_2$ in case of the $F_{420}H_2$ dehydrogenase and NADH in case of complex I) and contain flavins and FeS clusters. The electron transfer from the two-electron donors $F_{420}H_2$ and NADH to iron-sulfur clusters requires a $2e^-/1e^-$ switch, which is managed by flavins present in the input devices of both enzymes [79].

The membrane-associated module

The second module is constituted of four proteins, namely NuoB, C, D and I in *E. coli* [66] and FpoB, C, D and I in *Ms. mazei* [34] (equivalent subunits in other organisms are indicated in table 1). Structurally, the membrane-associated subunits connect the NADH-oxidizing unit to the membrane-integral subcomplex. Comprehensive EPR analysis of the connecting module from *E. coli* indicated the presence of three distinct FeS clusters (fig. 7a). The first one, named cluster N2, is characterized by a high and pH-dependent E_0' value and its sensitivity to the electron chemical proton gradient [80, 81]. Furthermore, it shows mutual magnetic interacts with semiquinone species [82], indicating its direct role in quinone reduction and proton translocation (see below). The location of this redox active compound is still a matter of debate. However, several lines of experimental data and homology analysis favor the NuoB subunit bearing FeS cluster N2 ([83] see below). Very recently, it was shown by means of combined ultraviolet-visible (UV/vis) and EPR spectroscopy that the connecting module contains two additional tetranuclear FeS clusters (N6a and N6b) located on NuoI [84]. Electrochemical titration revealed a pH-independent midpoint potential of –270 mV. UV/Vis reduced-minus-oxidized difference spectra indicate a close relationship to FeS clusters found in 8Fe-ferredoxins. In agreement, sequence comparison indicated that two repeats of typical binding motifs for [4Fe-4S] clusters ($CxxCxxCxxxCP(x)_{27-28}CxxCxxCxxx$ CP) are present in all NuoI-like proteins.

Besides the electron transfer function, the membrane-associated module might also be involved in quinone reduction. Mutational analysis, inhibitor studies and sequence comparison indicated that the very C-terminal strand of the NuoD subunit might be the binding site of the polar head of quinones, whereas the hydrophobic tail of the electron acceptor is bound to the membrane-inte-

gral subunit NuoH [85]. In fact, by comparison of amino acid sequences a 'consensus quinone-binding motif' of the form aliphatic-$(X)_3$-H-$(X)_{2,3}$-(L/T/S) has been detected in almost all ubiquinone-dependent enzymes of respiratory and photosynthetic systems [86]. A closer look to complex I from bacteria and mitochondria indicate that this signature is found in many NuoD-like proteins (fig. 8, sequence 1) [87]. However, the last position of this consensus motif is not conserved in the N-terminus of NuoD from menaquinone-reducing complex I as found in *E. coli* and *Thermus thermophilus* (fig. 8) [66, 68]. The same observation was made for complex I-like enzymes of choroplasts, which probably react with plastoquinone [88]. Similarly, the conserved amino acid triade was present in the ubiquinone-dependent succinate dehydrogenase of *E. coli*, but not conserved in the closely related fumarate dehydrogenase (a menaquinol-oxidizing enzyme) [89]. In the case of FpoD as part of the $F_{420}H_2$ dehydrogenase from *Ms. mazei,* only one amino acid of the 'Rich'-motif is conserved (fig. 8, sequence 1). This finding is not a surprise, because the electron acceptor of the enzyme, methanophenazine, is structurally different from quinones. In summary, the consenus sequence aliphatic-$(X)_3$-H-$(X)_{2,3}$-(L/T/S) allows a structural classification of complex I-like enzymes. It is found in ubiquinone-dependent enzymes but is modified in proteins using alternative electron acceptors such as menaquinone or methanophenazine. However, the sequence is an important factor to localize the quinone reactive site in complex I.

Further evidence that NuoD encompasses at least part of the active center for quinone reduction came from the identification of *Rhodobacter capsulatus* mutants that conferred resistance of complex I activity to piericidin and rotenone [90]. Surprisingly, a single point mutation was detected in these mutants, altering a valine residue (V407) in the very C-terminal strand of the NuoD subunit (fig. 8, sequence 3). Further studies indicated that other changes of amino acid in the C-terminal end of NuoD gave rise to mutants resistent to certain inhibitors. In addition, mutational analysis of the 49-kDa subunit of complex I from *Yarrowia lipolytica* revealed a decreased sensitivity towards ubiquinone analogous inhibitors [91]. It is known that many inhibitors targeting complex I such as rotenone, piericidin and pyridaben bind at, or close to, the quinone binding site(s) [92]. Since quinones and complex I inhibitors share a hydrophobic nature, the binding site for these species has long been considered to be buried in the NuoH subunit as part of the membrane domain. However, the simplest way to reconcile these data is to postulate that the inhibitor/quinone binding site is located at the interface between the connecting module and membrane-integral module of complex I and involves both the

Organism	Subunit	Enzyme	Seq. 1	Seq. 2	Seq. 3
Sinorhizobium melioliti	NuoD	NADH:UQ-OR	^{51}PH**I**GYL**H**RG**T**EK	^{77}DRLDYLCPP	^{387}DMIAVLGSLDPVMAEVDK
Rickettsia prowazekii	NuoD	NADH:UQ-OR	^{37}PH**I**GLL**H**RG**T**EK	^{63}DRLDYVSPM	^{372}DVITIIATLDIVFGEIDR
Rhodobacter capsulatus	Nqo4	NADH:UQ-OR	^{58}PH**I**GLL**H**RG**T**EK	^{84}DRLDYVAPM	^{396}DVSAIIGTMDV**V**F**G**EI**D**R
Paracoccus denitrificans	Nqo4	NADH:UQ-OR	^{58}PH**I**GLL**H**RG**T**EK	^{84}DRLDYVAPM	^{395}DVPAIIATLDFVMSDVDR
Bos taurus (mito)	49k	NADH:UQ-OR	^{78}PH**I**GLL**H**RG**T**EK	^{104}DRLDYVSMM	^{413}DVVAIIGTQDIVFGEVDR
Neurospora crassa (mito)	49k	NADH:UQ-OR	^{126}PH**V**GLL**H**RG**T**EK	^{152}DRLDYVSMM	^{461}DAVAVIGTMDLVFGEVDR
Arabidopsis thaliana (mito)	49k	NADH:UQ-OR	^{42}PH**I**GSL**H**RG**T**EK	^{68}DRSDYVSMM	^{377}DVVTIIGTQDIVFGEVDR
Yarrowia lipolytica (mito)	49k	NADH:UQ-OR	^{114}PH**V**GLL**H**RG**T**EK	^{140}DRL**D**YVSMM	^{449}DAVAIIGTM**D**L**V**FG**E**VDR
Oryza sativa (chl)	NhdH	NADH:PQ-OR ?	^{42}PI**L**GYL**H**RGMEK	^{68}TRWDYLATM	^{376}DIMTILGSIDIIMGEVDR
Arabidopsis thaliana (chl)	NdhH	NADH:PQ-OR ?	^{42}PI**L**GYL**H**RGMEK	^{68}TRWDYLATM	^{376}DIMTILGSIDIVFGEVDR
Synechocystis PCC 6803	NdhH	?	^{42}PV**I**GYL**H**RGMEK	^{68}SRWDYAAGM	^{377}DIMAILGSIDIIMGSVDR
E. coli	NuoD	NADH:Q-OR	^{54}PD**I**GYH**H**RGAEK	^{80}DRIEYLGGC	^{390}DLIVYLGSIDFVMSDVDR
Thermus thermophilus	Nqo4	NADH:Q-OR	^{57}PH**I**GYL**H**TGFEK	^{83}PRMDYLHSF	^{392}DMVAIIASLDPVMAEVDK
Methanosarcina mazei	FpoD	$F_{420}H_2$:Mphen-OR	^{41}VEMGYI**H**KGIEK	^{67}DRI$\underline{C}$YLVAL	^{357}DMVSISGSMDG$\underline{C}$TSEVDR
Rhodospirillum rubrum	CooH	H2ase	^{35}ITAGHVHRGIEY	^{67}ERV$\underline{C}$SL$\underline{C}$SN	^{344}DIPLIVNSIDP$\underline{C}$IS$\underline{C}$TER
Methanosarcina barkeri	EchE	H2ase	^{34}PSLGYVHRGLET	^{60}ERI$\underline{C}$GI$\underline{C}$SA	^{341}DVPIVVLTIDP$\underline{C}$VS$\underline{C}$TER
E. coli	HyfG	H2ase	^{213}YRLFYVHRGMEK	^{240}DRV$\underline{C}$GI$\underline{C}$GF	^{522}DAPLIIGSLDP$\underline{C}$YS$\underline{C}$TDR
E. coli	HycE	H2ase	^{217}YRLFYVHRGMEK	^{238}DRV$\underline{C}$GI$\underline{C}$GF	^{520}DAPLIIGSLDP$\underline{C}$YS$\underline{C}$TDR

Figure 8. Partial alignment of sequences of NuoD-like proteins of complex I-homologs and of the [NiFe] containing subunit of hydrogenases. Seq. 1: Residues forming the quinone binding triade are shown in bold. Seq. 2 and 3: Critical cysteine residues responsible for the coordination of the NiFe-cluster of hydrogenases are underlined. Mutated amino acids leading to inhibitor resistance are marked in bold.

NuoH and NuoD subunits [90, 93, 94]. In fact, the C-terminal part of nuoD-like subunit is highly conserved in complex I from bacteria and mitochondria, indicated by the consensus sequence D-xxx-I/V-I/L/-A/G-S/T-x-D-x-V-M/F-A/G-E/D-I/V-D-R (fig. 8, sequence 3). NADH dehydrogenases believed to interact with menaquinone are slightly different (e.g. NuoD from *E. coli* and Nqo4 from *T. thermophilus*). It is interesting to note that the quinone binding motif is not conserved in subunit FpoD that is the equivalent of NuoD in the $F_{420}H_2$ dehydrogenase. This observation further strengthens the idea that the polar head pocket of the quinone binding site addressed by rotenone and piericidin is borne by the NuoD subunit. In the case of $F_{420}H_2$ dehydrogenase, the domain in FpoD is obviously modified and adapted for the reduction of methanophenazine (fig. 7b).

As pointed out above, strong evidence has been presented that the binding site of the polar head of the electron-accepting quinone is located in NuoD close to subunit NuoB [80]. Inhibitor studies using different mutants indicated that the C-terminal domain of NuoD might be essential for the binding process [90]. In contrast to ubiquinone, the polar head of methanophenazine is composed of three aromatic rings (fig. 1), indicating that the binding cavity has to be larger compared with NuoD. Thus, it not a surprise that the corresponding amino acid sequence of the C-terminus of FpoD is different from NuoD. Notably, Gly367 (fig. 8, sequence 3) might be of special importance because this small amino acid might provide the space necessary for the binding of methanophenazine. In NuoD-like subunits large aliphatic amino acids (Val, Ile or Phe) or Pro are found in this position. The different structural composition of the methanophenazine binding cavity, which is probably located in FpoD, might be the reason why all complex I inhibitors tested so far have no effect on the activity of the $F_{420}H_2$ dehydrogenase [unpublished results].

It is important to note that part of the membrane associated module obviously evolved from ancient hydrogenases [95, 96]. The NuoB/FpoB subunits display striking similarities to the small subunit of [NiFe] hydrogenases. Furthermore, the large subunits of these hydrogenases share several sequence motifs with NuoD/FpoD. The large catalytic subunit harbors a NiFe-center that is ligated to the protein by one motif in the amino-terminal portion of the polypeptide (RxCxxCxxxH; fig. 8, sequence 2) and a second one at the carboxyl-terminal region (DPCxx CxxH/R; fig. 8, sequence 3). Homologous sequences are found in the N-terminus and the C-terminus of NuoD. However, the four cysteine residues of the motifs that coordinate the nickel atom are not found in the NuoD protein family (fig. 8, sequence 2/3). Interestingly, two of the four cysteines are conserved in the FpoD subunit of the $F_{420}H_2$ dehydrogenase from *Ms. mazei*. Thus, the corresponding regions are clearly reminiscent of the active site of hydrogenases. However, the enzyme does not possess any measurable hydrogenase activity, indicating that two conserved cysteine residues are not able to form a NiFe cluster. In summary, the proton reduction site of the [NiFe] large subunit might have evolved to a quinone-reduction site (or methanophenazine-reduction site), using the preexisting protons and electrons pathways of ancient hydrogenases.

As depicted in X-ray structures of the periplasmic hydrogenase from *Desulfovibrio gigas* the NiFe site is located at the interface between the large and small subunit adjacent to the proximal FeS cluster of the small subunit [97]. Regions containing ligand cysteine residues of a [4Fe-4S] proximal cluster are highly conserved among the [NiFe] hydrogenases ($CxxC(x)_{32-33}GxCxxxG(x)_{23-24}PGC$). Three of the cysteines coordinating the proximal FeS cluster are conserved in NuoB/FpoB and are expected to ligate cluster N2 [91, 93]. It remains an unsolved question which residue may serve as the fourth ligand of this FeS cluster. Based on structural homologies to hydrogenases one is tempted to speculate that the fourth ligand of N2 actually resides on subunit NuoD (or FpoD in case of $F_{420}H_2$ dehydrogenase) [80, 98]. The identity of this ligand residue remains to be determined.

The membrane-integral module

The membrane-integral part of the $F_{420}H_2$ dehydrogenase complex (FpoA, FpoH and FpoJ to FpoN) shows high similarities to the corresponding module of bacterial NDH-1 with respect to composition and homology of the amino acid sequences [34]. In the bacterial *nuo/nqo* operons known so far the genes encoding hydrophobic subunits are clustered at the 3′ end of the operon (nuoH, nuoJ-N in case of *E. coli* [66] and nqo8, nqo10–14 in case of *Paracoccus denitrificans* [70]). The only exception is *nuoA/nqo7,* which is located at the very beginning of the operon (fig. 5c). The same organization is given in the *fpo* gene cluster from *Ms. mazei* Gö1 (fig. 5b). It is important to note that the hydrophobic subunits of bacterial NDH-1 and of the $F_{420}H_2$ dehydrogenase have their counterparts in mitochondrially encoded complex I subunits from Eukarya (nd1-6 and nd4L) [71].

It is widely accepted that the membranous part of complex I is involved in quinone binding and proton translocation. As mentioned above, NuoH is probably responsible for binding the ‘isoprenyl tail’ of ubiquinone [90, 93, 94]. The overall organization of the quinone binding site can be proposed to be constituted of a hydrophobic pocket borne by the NuoH subunit and a polar head borne by the NuoD subunit. Because of the homologies of the subunits FpoD and FpoH of the $F_{420}H_2$ dehydrogenase, these subunits might have the same function. Thus, they might be responsible for binding the cofactor methanophenazine (fig. 7b).

The mechanism of electron and proton transfer within the membrane-integral module of complex I is still unclear. Moreover, even the number of binding sites for quinones is an unsolved question subject to intense controversy [93, 99]. Based on experiments with labeled quinones and inhibitors, up to three sites have been proposed. In addition, EPR studies have revealed that three ubisemiquinone species with distinct spectroscopic and thermodynamic properties are detectable in complex I (called SQNf, SQNs and SQx) that should function as electron/proton translocators [82, 99]. Among these, SQNf is postulated to be bound at close distance from center N2. Very recently, evidence has been presented for the existence of a novel redox group located in the membrane arm of the complex (called factor X) [84]. Electrochemical titration in combination with UV/Vis redox difference spectroscopy revealed a midpoint potential of –80 mV. It has been proposed that this group may have a quinoid structure and may represent a posttranslational modified amino acid. It might be involved in electron transfer from the FeS cluster N2 to ubiquinone.

Keeping in mind these findings, same important conclusion can be drawn when thinking about the relationship of complex I and $F_{420}H_2$ dehydrogenases: On one hand, the subunit composition of the membranous part of the $F_{420}H_2$ dehydrogenase and complex I is identical, and the corresponding subunits are highly homologous; on the other hand, it is worth mentioning that typical quinones such as ubiquinone and menaquinone are not found in methanogenic archaea [100]. Thus, quinone binding sites and semiquinone radicals cannot exist in the methanogenic enzyme. Furthermore, the midpoint potential of quinoid-factor X (–80 mV), as found in the *E. coli* enzyme, is not consistent with the redox demands of methanophenazine with a midpoint potential of about –250 mV [101]. Thus, it is very questionable whether such a factor is involved in the catalytic mechanism of the $F_{420}H_2$ dehydrogenase. In summary, the electron transport pathway through the membrane-integral module of the $F_{420}H_2$ dehydrogenase must be different compared with complex I and must involve the electron carrier methanophenazine rather than quinones.

Proposed reaction mechanism of the $F_{420}H_2$ dehydrogenase

The overall similarity of $F_{420}H_2$ dehydrogenase and complex I invites speculation about the reaction mechanism of the archaeal enzyme. It is evident that reduced coenzyme F_{420} is oxidized by FpoF that functions as the electron input module of the protein complex (fig. 7b). From the $F_{420}H_2$-oxidizing device the electrons are then channeled to the amphipathic connecting fragment composed of FpoBCDI that is highly homologous to the corresponding module of complex I. Since all FeS signatures are conserved in FpoB/FpoD and FpoI, it is reasonable to assume that FeS clusters comparable to N2 and N6 are present in these subunits. As outlined above, the hydrophilic head of the electron acceptor methanophenazine might be bound to a cavity within FpoD (fig. 7b). In contrast to the binding site for quinones in NuoD, the cavity of FpoD must be enlarged to allow the entrance of a phenazine derivative that is composed of three aromatic rings. In accordance to this hypothesis, several complex I inhibitors, known to interact with quinone binding sites, have no effect on the catalytic activity of the $F_{420}H_2$ dehydrogenase [unpublished results].

In contrast to all the subunits mentioned above, the deduced polypeptide of the last gene in the *fpo* operon (FpoO) has no counterpart in complex I. It is a hydrophilic subunit and contains a motif for the binding of one [2Fe-2S] cluster, indicating that it might be involved in electron transfer within the $F_{420}H_2$ dehydrogenase. Because FpoO is not found in complex I, it is tempting to speculate that the polypeptide participates in the reduction of methanophenazine. Electrons derived from cluster N2 could be transferred to the FeS cluster of subunit FpoO, which in turn could reduce the reactive head of methanophenazine that is bound at FpoD. If this hypothesis is correct, FpoO would represent a special device needed for the reduction of the alternative cofactor. In summary, the proposed electron transport pathway within the methanogenic enzyme is:

$$F_{420}H_2 \rightarrow FAD_{FpoF} \rightarrow 2[4Fe\text{-}4S]_{FpoF} \rightarrow N6_{FpoI} \rightarrow N2_{FpoB/FpoD} \rightarrow Nx_{FpoO}(?) \rightarrow MP_{FpoD/FpoH}$$

There are still two questions to discuss that concern the function of the membrane-integral module and the mechanism of proton translocation. Complex I contains both hydrogen carriers (flavin and ubiquinone) and electron carriers ([Fe-S] clusters). According to a classical loop mechanism, the association of these two types of cofactors allows a net transport of two protons across the membrane per molecule of NADH oxidized. However, it has been shown that complex I transfers four or even more protons across the membrane per reaction cycle [102]. Thus, complex I must function according to a more sophisticated mechanism.

Several models concerning the mechanism of H^+ translocation have been published [103–105]. According to Friedrich [106], complex I contains two coupling sites and is a chimera of a redox-driven and a conformation-driven proton pump. The redox-driven part would be composed of the membrane-associated module (NuoBCDI) and part of the membrane-integral module (NuoHL) with the redox reaction of cluster N2 as the molecular switch for proton translocation. The conformation-driven part would be formed by NuoA, J, K, M and N as the second coupling site within the complex.

Comprehensive analysis of the proton-translocating activity of the $F_{420}H_2$ dehydrogenase revealed that the enzyme is only able to translocate two protons in the course of the reaction cycle [34]. Thus, the coupling efficiency is only half of the one of complex I (fig. 7). These findings are also reflected by thermodynamic considerations. The midpoint potential of methanophenazine is in the range of –250 mV [101]. Thus, the change of free energy ($\Delta G_0'$) coupled with the $F_{420}H_2$-dependent methanophenazine reduction is only –20.2 kJ/mol compared with a $\Delta G_0'$ of –80.9 kJ/mol for the NADH-dependent reduction of ubiquinone. It is evident that the Gibbs free energy available from the electron transfer reactions between $F_{420}H_2$ and methanophenazine is just sufficient to drive the transfer of about two protons/2e$^-$. Thus, it seems reasonable to assume that only one of the aforementioned coupling sites is functional in the $F_{420}H_2$ dehydrogenase. Since all components necessary for the formation of the redox-driven H^+ pump are highly conserved in the $F_{420}H_2$ dehydrogenase, it is tempting to speculate that this energy-transducing segment [106] is actually working in the methanogenic enzyme. In contrast, the conformational change mode of proton translocation is probably coupled to the redox activity of semiquinone radicals in the membranous part of complex I. Because quinone radicals are obviously not present in the $F_{420}H_2$ dehydrogenase, this mode of proton translocation might not be functional. Hence, subunits Fpo A, K, J, L, M and N could be arrested in one of the conformational states that allows protonation of methanophenazine from the cytoplasm but no vectorial H^+ transport across the membrane. On the other hand, we cannot exclude that methanophenazine functions as a quinone, thereby forming methanophenazine radicals in the course of the reaction cycle. In this case, a conformational mode of proton translocation might be possible.

Features of the Ech hydrogenase

Recently, a novel multisubunit membrane-bound [NiFe] hydrogenase (Ech) was isolated from acetate-grown cells of *Ms. barkeri* [37, 39] that is most likely part of the reduced ferredoxin:heterodisulfide oxidoreductase system. The purified enzyme is composed of six different subunits (EchABCDEF) and catalyzes the reversible H_2-dependent reduction of a 2[4Fe-4S] ferredoxin from *Ms. barkeri* (fig. 9). Furthermore, it has been shown that reduced ferredoxin (Fd_{red}) produced in the course of acetyl-CoA degradation is reoxidized by the Ech hydrogenase (fig. 3c) [37]. However, the electron transfer pathway from the hydrogenase to CoM-S-S-CoB is still unclear. There are at least two possibilities:

1) The electrons derived from the oxidation of reduced ferredoxin are used for proton reduction. As a result, molecular hydrogen would be produced that could be reoxidized by the F_{420}-nonreducing hydrogenase and channeled via methanophenazine to the heterodisulfide reductase (fig. 3a).
2) The electrons could be transferred from the Ech hydrogenase directly to methanophenazine, which would then be reoxidized by the heterodisulfide reductase. In any case, there is no doubt that the Ech hydrogenase is an intrinsic part of the Fd_{red}: heterodisulfide oxidoreductase system (fig. 3c).

The overall reaction as catalyzed by the Ech hydrogenase is probably coupled to the formation of electrochemical proton gradient (fig. 9) [107]. Evidence in favor of this assumption came from the analysis of the deduced amino acid sequences of the subunits of the Ech hydrogenase (see below). The enzyme shares the highest sequence similarity with the CO-induced hydrogenase from *Rhodospirillum (R.) rubrum* [108]. The organism can grow in the dark on CO, thereby forming H_2 and CO_2. This reaction is coupled to the translocation of protons across the cytoplasmic membrane. Since the CO dehydrogenase in *R. rubrum* is a soluble enzyme, the membrane-bound CO-induced hydrogenase is most likely the site of energy conservation [109].

Recently, it became evident that the Ech hydrogenase may have a second function and could be involved in the formation and degradation of formyl-MFR [R. Hedderich, personal communication]. In the CO_2-reducing pathway (fig. 2) 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 [110]. Taking

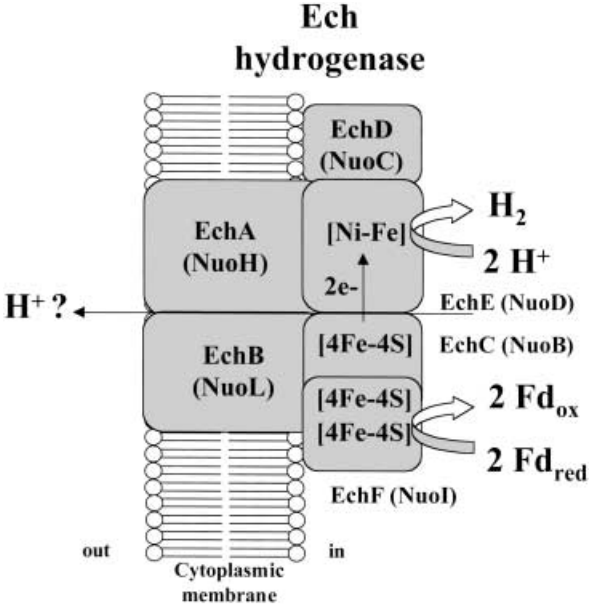

Figure 9. Scheme of the Ech hydrogenase from *Methanosarcina* strains. Homologous subunits found in complex I are shown in brackets.

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. It has been proposed that the electrochemical proton gradient is the energy source for the Ech hydrogenase to drive this reduction. Thus, H_2-dependent ferredoxin reduction is enabled by the influx of protons through the enzyme.

The genes encoding the subunits of the Ech hydrogenase are organized in the *echABCDEF* operon (fig 8d). The deduced amino acid sequences of the genes revealed that the protein belongs to a small group of multisubunit membrane-bound [NiFe] hydrogenases [39]. The list of enzymes includes hydrogenase 3 and 4 from *E. coli* [111, 112], and the above-mentioned carbon monoxide-induced hydrogenase from *R. rubrum* [108, 109]. Two integral membrane proteins and three hydrophilic polypeptides of these multisubunit enzymes are highly conserved. Subunits EchE and EchF represent the homologs of the large subunit and the small subunit of the classical [NiFe] hydrogenases, respectively [37]. Hydropathy plots indicate that EchA and EchB are membrane-integral subunits, whereas EchC and EchD reveal a hydrophilic nature and are probably membrane associated. Most interestingly, these subunits are more closely related to subunits of complex I and of $F_{420}H_2$ dehydrogenases than to subunits of other [NiFe] hydrogenases [37]. The homologies concern the membrane-associated module (fig. 9) and the subunits forming the membrane-integral module. Sequence similarities are found between EchE and NuoD-like proteins (49-kDa subunit of the mitochondrial protein, NuoD/Nqo4/FpoD of the prokaryotic complexes) as well as between EchC- and NuoB-like subunits (PSST and NuoB/Nqo6/FpoB; table 1). The same is true for EchF and NuoI-like proteins (TYKY and NuoI/Nqo9/FpoI). Subunits EchA and EchB are membrane-integral proteins that show similarity to subunits NuoL (nd5/nqo12) and NuoH (nd1/nqo8) of complex I and to FpoL and FpoH of $F_{420}H_2$ dehydrogenases, respectively [34, 37]. The overall composition and the homologies of the subunits of the Ech hydrogenase to complex I led to the hypothesis that multisubunit membrane-bound hydrogenases might be progenitors of complex I and $F_{420}H_2$ dehydrogenases [96]. This ancient progenitor was obviously not equipped with the input modules (NuoEFG, FpoF) found in today's NADH and $F_{420}H_2$ dehydrogenases and did not possess the sophisticated membrane-integral module composed of seven subunits (NuoAHJKLMN and FpoAHJKLMN). However, it contained a hydrogen-oxidizing/electron transfer unit (the equivalent of EchCDEF) and a simple membrane-integral unit (the equivalent of EchAB). Thus, the enzyme may have worked already as a proton-translocating hydrogen:ferredoxin oxidoreductase. It is tempting to speculate that today's Ech hydrogenases retained that function (fig. 9). Thus, ferredoxin itself is the electron-input device of Ech hydrogenase that transfers electrons most probably via the two [4Fe-4S] clusters of subunit EchF (the homolog of NuoI/Nqo9, TYKY/FpoI) and via the FeS cluster of subunit EchC (the homolog of NuoB/Nqo6/PSST/FpoB) to the [NiFe] active site present on subunit EchE (the homolog of NuoD/Nqo4/ 49kD/FpoD), where two protons are reduced to H_2 (fig. 9). The membrane-integral subunits (EchA and EchB) might be involved in proton transfer across the cytoplasmic membrane. The enzyme can also operate in the reverse direction and can reduce the ferredoxin with H_2 as electron donor.

Mechanisms of redox-driven proton translocation in *Methanosarcina* strains: A summary

At least three different modes of redox-driven H^+ transfer across biological membranes are known that are named (i) redox loop, (ii) Q cycle and (iii) proton pump mechanism. From the data presented, the F_{420}-nonreducing hydrogenase and the heterodisulfide reductase could function according to the redox loop mechanism. In this case, the membrane-bound electron transfer system must consist of alternating carriers for hydrogen and for electrons, the topology must be such that catalytic sites are accessible alternately from the cytoplasm and from the periplasm, and the stoichiometry cannot exceed two protons per electron pair passing over the loop. In the course of the reaction, a hydrogen carrier undergoes a protolytic attack (H_2 in case of the hydrogenase and MPH_2 in case of the heterodisulfide reductase). Two protons are discharged at the outside of the membrane, while two electrons are conducted back across the membrane by an electron-carrying wire (FeS cluster and heme group found in the above-mentioned enzymes). The electrons and protons from the cytoplasm are used for the reduction of a second hydrogen carrier (MP in case of the hydrogenase and CoM-S-S-CoB in case of the heterodisulfide reductase). In contrast, the donor ($F_{420}H_2$) and the acceptor (MP) of the $F_{420}H_2$ dehydrogenase are most likely bound to subunits that are oriented towards the cytoplasm (see above). Therefore, the release and the uptake of protons should take place at the cytoplasmic surface of the membrane, respectively. The same might be true for the Ech hydrogenase (formation of protons by ferredoxin-dependent CO oxidation and production of H_2 by the reduction of protons in the cytoplasm of the cell). Thus, the outward transport of protons by the $F_{420}H_2$ dehydrogenase and the Ech hydrogenase cannot be attributed to a redox loop. Instead, the enzymes must be seen as proton pumps. That means the cycle of reduction and oxidation is linked to concurrent changes in protein conformation that in turn control the orientation and affinity of proton binding sites. In contrast to a redox loop where protons and electrons are directly bound to the redox center, the proton

pump mechanism implicates that protons are bound to some other sites whose properties are closely coupled to the redox reaction. Thus, the enzymes require a proton-conducting channel across the membrane and a mechanism coupling electron transfer to the vectorial movement of protons through this.

Note added in proof: During reviewing of this article the complete genome sequence of *Methanosarcina mazei* has been published (Deppenmeier U., Johann A., Hartsch T., Gottschalk G. et al. (2002) The genome of *Methanosarcina mazei:* Evidence for lateral gene transfer between Bacteria and Archaea. J. Mol. Microbiol. Biotechnol. **4:** 453–461). The genome sequence has been deposited in Genbank (http.//www.ncbi.nlm.nih.gov, Acc. No. AE003854) and teh Göttingen Genomics Laboratory database (http.//www.g21.bio.uni-goettingen.de)

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