# Hydrogenases from Methanogenic Archaea, Nickel, a Novel Cofactor, and $H_2$ Storage

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## Key Words

$H_2$ activation, energy-converting hydrogenase, complex I of the respiratory chain, chemiosmotic coupling, electron bifurcation, reversed electron transfer

## Abstract

Most methanogenic archaea reduce $CO_2$ with $H_2$ to $CH_4$. For the activation of $H_2$, they use different [NiFe]-hydrogenases, namely energy-converting [NiFe]-hydrogenases, heterodisulfide reductase-associated [NiFe]-hydrogenase or methanophenazine-reducing [NiFe]-hydrogenase, and $F_{420}$-reducing [NiFe]-hydrogenase. The energy-converting [NiFe]-hydrogenases are phylogenetically related to complex I of the respiratory chain. Under conditions of nickel limitation, some methanogens synthesize a nickel-independent [Fe]-hydrogenase (instead of $F_{420}$-reducing [NiFe]-hydrogenase) and by that reduce their nickel requirement. The [Fe]-hydrogenase harbors a unique iron-guanylylpyridinol cofactor (FeGP cofactor), in which a low-spin iron is ligated by two CO, one $C(O)CH_2$-, one S-$CH_2$-, and a $sp^2$-hybridized pyridinol nitrogen. Ligation of the iron is thus similar to that of the low-spin iron in the binuclear active-site metal center of [NiFe]- and [FeFe]-hydrogenases. Putative genes for the synthesis of the FeGP cofactor have been identified. The formation of methane from 4 $H_2$ and $CO_2$ catalyzed by methanogenic archaea is being discussed as an efficient means to store $H_2$.

## Contents



## INTRODUCTION

**Black and white smokers:** chimney-like structures formed around hydrothermal vents, where superheated mineral rich water from below Earth's crust comes through the ocean floor

In 1933, Stephenson & Stickland (1) enriched from river sediments methane-forming microorganisms that grow on $H_2$ and $CO_2$ (Reaction 1) and concluded that these methanogens must contain hydrogenases that activate $H_2$ (Reaction 2).

$$4\,H_2 + CO_2 \rightarrow CH_4 + 2\,H_2O \qquad \Delta G^{\circ\prime} = -131\,kJ\,mol^{-1}. \qquad 1.$$

$$H_2 \rightleftharpoons 2\,e^- + 2\,H^+ \qquad E'_o = -414\,mV. \qquad 2.$$

The name hydrogenase was coined in 1931 by Stephenson & Stickland (2, 3) for an activity in anaerobically grown *Escherichia coli* cells mediating the reversible reduction of dyes with $H_2$. Dye reduction was reversibly inhibited by CO, indicating the involvement of a transition metal in $H_2$ activation (4). The transition metal later turned out to be nickel in a binuclear nickel-iron center in the case of [NiFe]-hydrogenases (5–8), iron in a binuclear iron-iron center in the case of [FeFe]-hydrogenases (9–11), and iron in a mononuclear iron center in the case of [Fe]-hydrogenase (12–14), which are the three different types of hydrogenases known to date (**Figure 1**) (15, 16).

From the work of Stephenson, it became evident that methane formation from biomass in river sediments is at least in part the result of the syntrophic interaction of $H_2$-forming bacteria such as *E. coli* and $H_2$-consuming methanogens. And indeed in later studies, it turned out that interspecies hydrogen transfer is a quantitatively important process in the carbon cycle despite the fact that for thermodynamic and kinetic reasons the $H_2$ concentration in anaerobic habitats is generally very low ($pH_2 < 10$ Pa; $E'(H^+/H_2) = -300$ mV) (17, 18). $H_2$ (see the sidebar titled Properties of $H_2$) (19, 20), even at low concentrations, is an ideal electron carrier between organisms because it can freely diffuse through cytoplasmic membranes. Estimates are that approximately 150 million tons of $H_2$ are annually formed by microorganisms and used to fuel methanogens (17). The combustion of 150 million tons $H_2$ yields $18 \times 10^{18}$ J, an energy amount that is 3.75% of the primary energy consumed in 2006 by the world population ($455 \times 10^{18}$ J).

Today on Earth, most of the $H_2$ used by methanogens is of biological origin. Only some of the $H_2$ that sustains the growth of methanogens is geochemically generated, e.g., in black and white smokers. However, in the Archaeozoic (4 to 2.5 billion years back), when the different lineages of microbes on Earth evolved and when the temperatures were much higher than today, geochemically formed $H_2$ probably predominated that of biological origin and

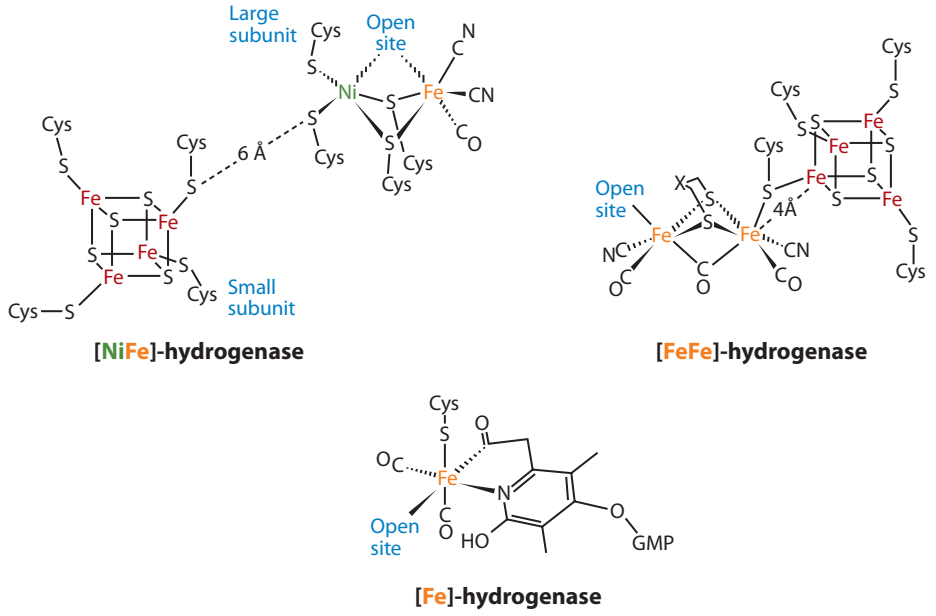


**Figure 1**

The metal sites of the three types of hydrogenases involved in interspecies hydrogen transfer (see **Figure 2**) have unusual structural features in common, such as intrinsic CO ligands. Despite this fact, [NiFe]-hydrogenases (5–8), [FeFe]-hydrogenases (9–11), and [Fe]-hydrogenase (12–14) are not phylogenetically related at the level of their primary structure or at the level of the enzymes involved in their active-site biosynthesis (12). Abbreviation: GMP, guanylyl rest.

fueled the growth of methanogens. Consistently, among recent hydrogenotrophic methanogens, there are many hyperthermophiles, such as *Methanopyrus kandleri* (98°C optimum growth temperature) and *Methanocaldococcus jannaschii* (85°C optimum growth temperature), and these hyperthermophiles branch off the 16S phylogenetic tree relatively early.

This review highlights the properties of the five different hydrogenases found in methanogens within the context of their function in metabolism. Four of the enzymes are [NiFe]-hydrogenases with some properties similar and others dissimilar to those of related [NiFe]-hydrogenases in bacteria. It was in methanogens that nickel was first found to be required for hydrogenase activity (21, 22). The fifth enzyme is a [Fe]-hydrogenase (23) that is unique to methanogens and functional in these only under conditions of nickel limitation. [FeFe]-hydrogenases, which are present in Bacteria and lower Eukarya, have not yet been found in Archaea (15, 16).

## PROPERTIES OF $H_2$

$H_2$ is a colorless gas with a boiling point at 22.28 K (−250.87°C). Its Bunsen coefficient $\alpha$ in water at 20°C is 0.018 (0.8 mM at 1 bar), and its diffusion coefficient $D_w$ in water at 20°C is near $4 \times 10^{-9}$ m$^2$ s$^{-1}$. The homolytic cleavage of $H_2$ in the gas phase is endergonic by +436 kJ mol$^{-1}$, and the heterolytic cleavage in water at 20°C is endergonic by about +200 kJ mol$^{-1}$ ($pK_a$ near 35) (19). The combustion energy of $H_2$ is 120 MJ kg$^{-1}$. The activation of $H_2$ is mechanistically challenging, and the catalytic mechanism is of considerable interest. The $H_2$ generated, e.g., by electrolysis or photolysis of water, is presently discussed as an environmentally clean energy carrier for use in fuel cell-powered electrical cars. Before $H_2$ can be used in fuel cells, cheap catalysts still have to be developed, and it is hoped that the active-site structure of hydrogenases will show how to proceed (20).

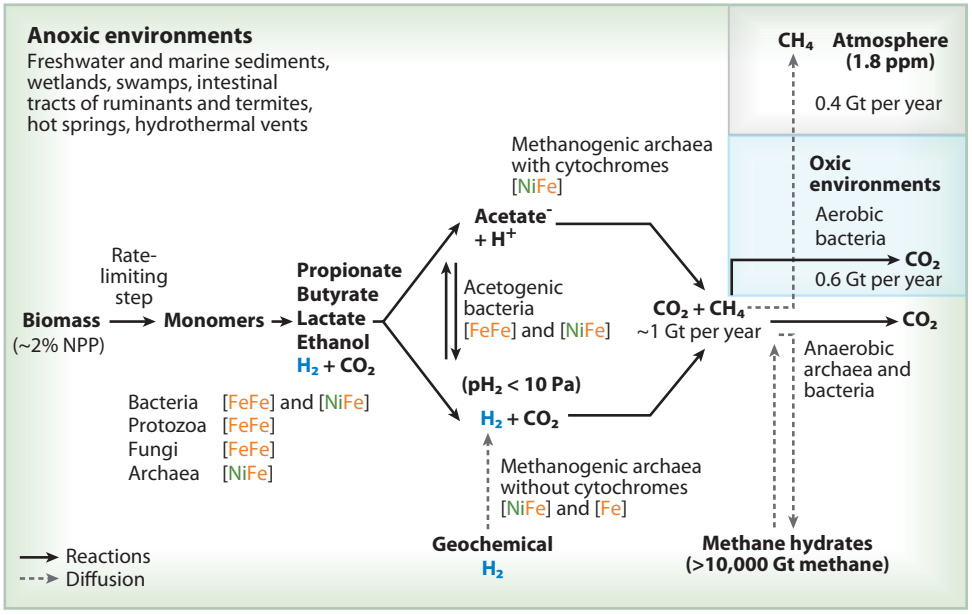


**Figure 2**

Approximately 2% of the net primary production (NPP) of plants, algae, and cyanobacteria are fermented in anoxic environments by a syntrophic association of anaerobic microorganisms with methane, in a process that involves interspecies $H_2$ transfer. The kinetics and thermodynamics of the process are such that the steady-state $H_2$ concentration remains below 0.1 μM (<10 Pa) (17, 18). At these low $H_2$ concentrations, the redox potential of the $H^+/H_2$ couple at pH 7 is near −300 mV. The three types of hydrogenases involved are abbreviated [NiFe], [FeFe], and [Fe], respectively (see **Figure 1**). In the intestinal tract of termites and ruminants, methanogens converting acetate to methane and $CO_2$ are lacking. Therefore, acetate, propionate, butyrate, lactate, and ethanol concentrations build up and can be used by the host as anabolic and catabolic substrates.

## $H_2$ AS AN INTERMEDIATE IN $CH_4$ FORMATION AND THE ORGANISMS INVOLVED

Approximately 2% of the net primary production of plants, algae, and cyanobacteria (70 billion tons C per year) are remineralized via methane in anoxic environments such as freshwater and marine sediments, wetlands, swamps, sewage digesters, landfills, hot springs, and the intestinal tract of ruminants and termites (**Figure 2**). From the biomass, which consists of 60%–70% cellulose, approximately one billion tons of methane are generated per year; 60% is oxidized to $CO_2$ by microorganisms, and 40% escapes into the atmosphere, where its concentration almost doubled within the last hundred years (17). This is of concern since methane is an effective greenhouse gas.

**Geochemically formed $H_2$:** $H_2$ generated abiotically from $H_2S$ or by reaction of $H_2O$ with ultramafic rocks (serpentinization)

In a rate-limiting step, the biomass is degraded by extracellular hydrolytic enzymes excreted by anaerobic bacteria and protozoa to monomers, which after uptake by these microorganisms are primarily fermented to lactic acid, propionic acid, butyric acid, ethanol, and acetic acid with the concomitant formation of $CO_2$, formic acid, and some $H_2$. This process also involves anaerobic fungi in the rumen and anaerobic archaea in hot springs. Of these products, lactic, propionic, and butyric acids and ethanol serve syntrophic bacteria as substrates, which ferment them to acetic acid, $CO_2$, $H_2$, and formic acid.

From acetic acid, $H_2$, $CO_2$, and formic acid, methane is then formed by methanogenic archaea, of which there are two types, those with and those without cytochromes. Acetic

acid is converted to $CO_2$ and methane only by the methanogens with cytochromes, and $H_2$, $CO_2$ and formate are converted to methane mainly by those without cytochromes. None of the methanogens can use lactic, propionic, or butyric acid as energy substrates. But by consuming $H_2$, acetic acid and formic acid, the methanogens keep the $H_2$ partial pressure between 1 Pa and 10 Pa and the acetic and formic acid concentrations well below 0.1 mM, enabling the syntrophic bacteria to convert lactic, propionic, and butyric acid and ethanol to acetic acid, $H_2$, and $CO_2$. Only at low concentrations of $H_2$ and acetic acid are the fermentations of the syntrophs exergonic enough to sustain their growth (18).

In the intestinal tract of ruminants and termites, methanogens with cytochromes are not present, and therefore methanogenesis from acetate does not occur. The reason for this is probably that the growth rate of acetoclastic methanogens is generally lower than the dilution rate in the intestinal tract, and therefore, the acetoclastic methanogens are continuously washed out. Because of the lack of methanogenesis from acetic acid the concentration of acetic acid builds up considerably (>10 mM) with the result that, for the thermodynamic reasons discussed above, lactic, propionic, and butyric acid, therefore also increase in their concentrations. The organic acids are resorbed by the ruminants and insects from their intestinal tracts and used for gluconeogenesis (lactic and propionic acid) and ATP synthesis (acetic acid and butyric acid).

In sediments of hot springs, in which cellulose is completely converted to methane and $CO_2$, surprisingly, at temperatures above 60°C, acetoclastic methanogens are absent for reasons not yet fully understood. Methanogens with cytochromes growing above 60°C have yet to be found. In hot sediments, acetic acid is converted to two $CO_2$ and four $H_2$ by bacteria related to acetogenic bacteria, and the $H_2$ and $CO_2$ thus formed are then converted to methane by methanogens without cytochromes, which have many thermophilic and hypothermophilic species. Thus, in hot springs, the conversion of glucose from cellulose to three $CO_2$ and three $CH_4$ involves 12 $H_2$ as intermediates, which underlines the quantitative importance of $H_2$ as electron carrier between fermenters and methanogens.

All methanogens are known to belong to the domain of Archaea and to the kingdom of Euryarchaeota. From the latter lineage, the Methanopyrales branch off first, followed by the orders Methanococcales and Methanobacteriales, and then by Methanomicrobiales and Methanosarcinales. Only the members of the Methanosarcinales contain cytochromes and can use acetic acid as methanogenic substrate. Methanogenesis from acetate is therefore believed to be a late invention. The ability to use acetate as methanogenic substrate was associated with a change in the mechanism of energy conservation, as electron transport now involves cytochromes. The altered mechanism allowed the methanogens with cytochromes to also use methanol, methylamines, and methylthiols as energy substrates. But it also had a price, namely the loss of the ability to use $H_2$ down to partial pressures below 10 Pa (for an explanation, see below), which is a characteristic of methanogens without cytochromes that are specialized on $H_2$ plus $CO_2$ and/or formate as energy sources. Members of the Methanosarcinales that can grow on $H_2$ and $CO_2$ do this only at significantly higher $H_2$ concentrations than the members of the other orders, which lack cytochromes. This is why the Methanosarcinales do not contribute to methane formation from $H_2$ and $CO_2$ in most anoxic environments (**Figure 2**) (17).

## HYDROGENASES FOUND IN METHANOGENS AND THEIR FUNCTION

The genomes of several members of each of the five known orders of methanogens have been sequenced, and the genes putatively encoding hydrogenases have been identified. Biochemical studies of the hydrogenases have concentrated mainly on a few

**EchA-F, EhaA-T, EhbA-Q, and MbhA-N:** energy-converting [NiFe]-hydrogenases

**MvhADG:** heterodisulfide reductase-associated [NiFe]-hydrogenase

**HdrABC and HdrDE:** heterodisulfide reductases

**VhtACG:** methanophenazine-reducing [NiFe]-hydrogenase

**FrhABG:** an $F_{420}$-reducing [NiFe]-hydrogenase

species, namely *Methanothermobacter thermautotrophicus*, *Methanothermobacter marburgensis*, *Methanococcus maripaludis*, *Methanosarcina barkeri*, *and Methanosarcina mazei*. Genetic analyses have been restricted to *Methanococcus voltae*, *M. maripaludis*, *Methanosarcina acetivorans*, *M. mazei*, and *M. barkeri*. From these studies, a partially coherent picture has emerged.

Four different subtypes of [NiFe]-hydrogenases and one [Fe]-hydrogenase are found in methanogens. The four [NiFe]-subtypes are (*a*) the membrane-associated, energy-converting [NiFe]-hydrogenases (EchA-F, EhaA-T, EhbA-Q, and MbhA-N) for the reduction of ferredoxin with $H_2$; (*b*) the cytoplasmic [NiFe]-hydrogenase (MvhADG) associated with the heterodisulfide reductase (HdrABC) for the coupled reduction of ferredoxin and of the heterodisulfide CoM-S-S-CoB with $H_2$; (*c*) the membrane-associated, methanophenazine-reducing [NiFe]-hydrogenase (VhtACG); and (*d*) the cytoplasmic coenzyme $F_{420}$-reducing [NiFe]-hydrogenases (FrhABG). Not all five hydrogenases are found in all methanogens. Thus, the methanophenazine-reducing [NiFe]-hydrogenase is restricted to methanogens with cytochromes, and the cytoplasmic [Fe]-hydrogenase, which together with $F_{420}$-dependent methylenetetrahydromethanopterin dehydrogenase substitute for the $F_{420}$-reducing [NiFe]-hydrogenase under nickel-limiting growth conditions (see below), is only present in some methanogens without cytochromes. Genes for [Fe]-hydrogenase synthesis are lacking in methanogens with cytochromes and in most members of the Methanomicrobiales (15, 23).

The function of the different [NiFe]-hydrogenases in methanogenesis from $H_2$ and $CO_2$ in methanogens with cytochromes can be deduced from **Figure 3*a*** and in methanogens without cytochromes from **Figure 3*b***. The differences outlined in the two schemes are based, among many other observations, on the finding that the growth yield of cytochrome-containing methanogens on $H_2$ and $CO_2$ (maximally 6.4 g per mole $CH_4$) is more than twice as high as that of methanogens without cytochromes (maximally 3 g per mole $CH_4$), indicating that the ATP gain per mole methane is approximately 0.5 in methanogens with cytochromes and 1 to 1.5 in methanogens without cytochromes (17). The low ATP gain of 0.5 allows the methanogens without cytochromes to grow on $H_2$ and $CO_2$ at $H_2$ partial pressures of 5 Pa at which methanogenesis from $CO_2$ and $H_2$ is exergonic by −25 kJ per mole, which is just sufficient to drive the synthesis of 0.5 mole ATP ($\Delta G' = -50$ kJ per mole ATP). Conversely, an ATP gain of 1 to 1.5 is only thermodynamically possible if the $H_2$ concentration is >100 Pa ($\Delta G < -63$ kJ per mole $CH_4$). And indeed, methanogens with cytochromes are known to have a much higher $H_2$ threshold concentration (>100 Pa) than methanogens without cytochromes <10 Pa) (17). The relatively high threshold concentration for $H_2$ can explain why members of the Methanosarcinales are generally not involved in methanogenesis from $H_2$ and $CO_2$ in most methanogenic habitats (**Figure 2**) and why in some members, e.g., in *M. acetivorans*, transcription of the genes for the hydrogenases are permanently turned off (24).

For an understanding of the function of the different hydrogenases in the proposed two metabolic schemes (**Figure 3*a*,*b***) the energetics of ferredoxin reduction with $H_2$ are of special importance. Under physiological standard conditions ($pH_2 = 10^5$ Pa; pH 7; $Fd_{ox}/Fd_{red} = 1$), the reduction of ferredoxin ($E'_o = -420$ mV) with $H_2$ ($E'_o = -414$ mV) is neither endergonic nor exergonic. However, under in vivo conditions ($pH_2 = 10$ Pa; pH 7; $Fd_{ox}/Fd_{red} < 0.01$), the reduction of ferredoxin with $H_2$ is strongly endergonic with $E'$ of the $H^+/H_2$ couple = −300 mV and that of the $Fd_{ox}/Fd_{red}$ couple = −500 mV. In methanogens, fully reduced ferredoxin is required for the reduction of $CO_2$ to formylmethanofuran (CHO-MFR) ($E'_o = -500$ mV), which is the first step in methanogenesis from $CO_2$ (**Figure 3**), for the reduction of $CO_2$ to CO ($E'_o = -520$ mV), for the reduction of acetyl coenzyme A (acetyl-CoA) and $CO_2$ to pyruvate ($E'_o = -500$ mV), and—in most methanogens—for the reduction

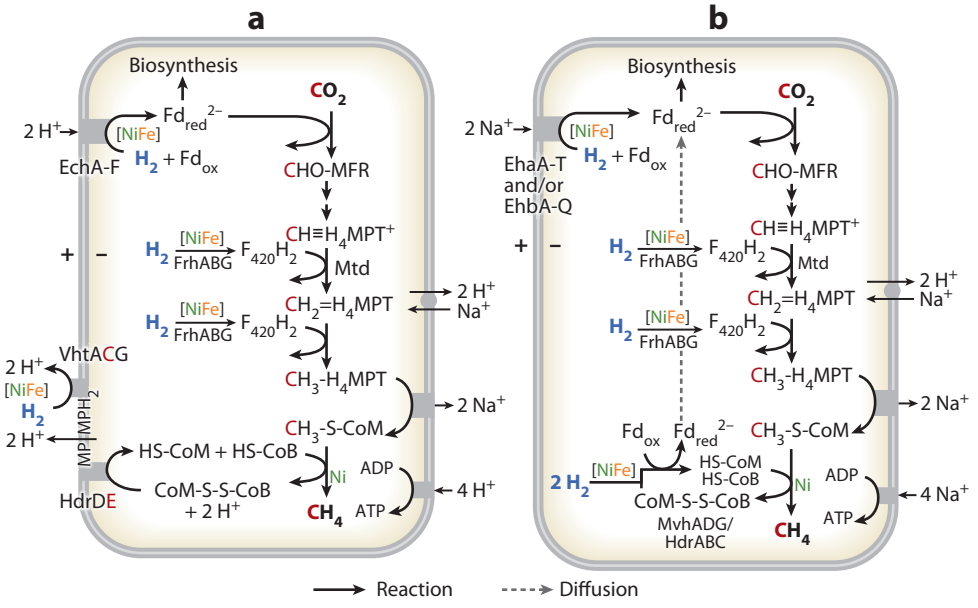


**Figure 3**

The proposed function and localization within the cell of the [NiFe]-hydrogenases involved in methanogenesis from $H_2$ and $CO_2$ are shown for methanogens (*a*) with cytochromes and (*b*) without cytochromes. The cations translocated and the exact stoichiometry of the translocation reactions are still a matter of dispute, and the stoichiometry of electron bifurcation in the MvhADG/HdrABC complex remains to be ascertained (17). In some members of the Methanomicrobiales, genes for MvhA and MvhG are not found, and energy-converting hydrogenases other than Eha or Ehb can be present (25). For interpretations of these novel findings, see the section Heterodisulfide Reductase-Associated [NiFe]-Hydrogenase MvhADG, below. Members of the Methanosarcinales and Methanococcales contain tetrahydrosarcinapterin rather than tetrahydromethanopterin ($H_4$MPT). The two pterins have identical functions in $C_1$-unit transformation. Abbreviations: CHO-MFR, formylmethanofuran; $CH{\equiv}H_4MPT^+$, methenyltetrahydromethanopterin; $CH_2{=}H_4MPT$, methylenetetrahydromethanopterin; $CH_3$-$H_4MPT$, methyltetrahydromethanopterin; CoB-SH, coenzyme B with its thiol group; CoM-SH, coenzyme M with its thiol group; CoM-S-S-CoB, heterodisulfide; EchA-F, EhaA-T, and EhbA-Q, energy-converting [NiFe]-hydrogenases; Fd, ferredoxin with two [4Fe4S]-clusters; FrhABG, $F_{420}$-reducing [NiFe]-hydrogenase; HdrABC and HdrDE, heterodisulfide reductases; MP, methanophenazine; MvhADG, heterodisulfide reductase-associated [NiFe]-hydrogenase; VhtACG, methanophenazine-reducing [NiFe]-hydrogenase; VhtC and HdrE, *b*-type cytochromes.

of succinyl-CoA and $CO_2$ to 2-oxoglutarate ($E'_o = -500$ mV). In methanogens growing on $H_2$ and $CO_2$, the latter three reduction reactions participate in autotrophic $CO_2$ fixation. All of these ferredoxin-dependent oxidoreductase reactions are catalyzed by cytoplasmic enzymes. Therefore, it is the reduction of ferredoxin with $H_2$ that must be energy driven and the site of energy coupling. As outlined below, in energy-converting [NiFe]-hydrogenases, the mechanism of energy coupling is chemiosmotic (**Figure 3*a*,*b***), and in the MvhADG/HdrABC complex, the mechanism of coupling is by electron bifurcation (**Figure 3*b***).

Whereas the reduction of ferredoxin with $H_2$ in methanogens is strongly endergonic, that of methanophenazine ($E'_o = -170$ mV) and of the heterodisulfide CoM-S-S-CoB ($E'_o = -140$ mV) with $H_2$ ($E'_o = -414$ mV) is a strongly exergonic reaction (26). Consistently, methanophenazine and CoM-S-S-CoB reduction with $H_2$ are coupled with energy conservation (**Figure 3*a*,*b***). Of the hydrogenase-catalyzed reactions, only the reduction of

**Electron bifurcation:** the disproportionation of two electrons at the same redox potential to one electron with a higher and one with a lower redox potential

coenzyme $F_{420}$ ($E'_o = -360$ mV) with $H_2$ is not coupled with energy conversion (27). Under in vivo conditions ($pH_2 = 10$ Pa; $F_{420}/F_{420}H_2 < 0.1$), the free energy change associated with the reaction is essentially zero. The energetic differences of the hydrogenase-catalyzed reactions in methanogenesis from $H_2$ and $CO_2$ can therefore explain why there are at least three different hydrogenases in hydrogenotrophic methanogens.

## THE FOUR SUBTYPES OF [NiFe]-HYDROGENASES IN METHANOGENS

The crystal structures of the [NiFe]-hydrogenases found in methanogens have not been determined. Currently, only structures of [NiFe]-hydrogenases from sulfate-reducing bacteria are available (5–8). However, on the basis of sequence comparisons, all [NiFe]-hydrogenases appear to be phylogenetically related, although the sequence similarity is sometimes restricted to the sequences around the N-terminal and C-terminal CxxC motifs, RxCGxCxxxH and DPCxxCxxH/R, respectively, involved in [NiFe]-center coordination. Nevertheless, it is generally assumed that the active-site structures of all [NiFe]-hydrogenases are very similar (**Figure 1*a***); but in one case (soluble [NiFe]-hydrogenase from *Ralstonia eutropha*), there is spectroscopic evidence that the ligand structure could be substantially different (28, 29).

[NiFe]-hydrogenases are minimally composed of two subunits, a large one (40–68 kDa) and a small one (16–30 kDa). The large subunit harbors the [NiFe]-binuclear active-site center. The small subunit generally contains three linearly arranged and evenly spaced iron-sulfur clusters, a proximal and a distal [4Fe4S]-cluster, and one central [3Fe4S]-cluster (8). In energy-converting [NiFe]-hydrogenases, the small subunit contains only the proximal [4Fe4S]-cluster, which appears to be necessary and sufficient for [NiFe]-hydrogenase function. In the heterodimer, the [NiFe]-center is buried and located close to the large interface between the two subunits and close to the proximal [4Fe4S]-cluster of the small subunit (**Figure 1*a***). A gas channel connects the surface with the active site (30).

The large subunit of most [NiFe]-hydrogenases is synthesized as a preprotein. The C-terminal extension after H/R of the DPCxxCxxH/R motif is clipped off proteolytically in the maturation process (31–33). The gene coding the large subunit of some of the energy-converting hydrogenases (34, 35) and some of the $H_2$-sensory [NiFe]-hydrogenases (36) ends with a stop codon directly after the nucleotide sequence for the DPCxxCxxH/R motif. Therefore, synthesis of these [NiFe]-hydrogenases appears to be independent of this proteolytic maturation step.

In the next paragraphs, we summarize what is known about the four different subtypes of [NiFe]-hydrogenases found in methanogens: (*a*) energy-converting [NiFe]-hydrogenases, (*b*) heterodisulfide reductase-associated [NiFe]-hydrogenase, (*c*) methanophenazine-reducing [NiFe]-hydrogenase, and (*d*) $F_{420}$-reducing [NiFe]-hydrogenase.

### Energy-Converting [NiFe]-Hydrogenases

Energy-converting [NiFe]-hydrogenases from methanogens are membrane associated and catalyze the reversible reduction of ferredoxin ($E' \approx -500$ mV) with $H_2$ ($E' = -300$ mV), driven by a proton or sodium ion motive force (Reaction 3) (**Figure 4**) (34, 35).

$$Fd_{ox} + H_2 + \Delta\mu H^+/Na^+ \rightleftharpoons Fd_{red}^{2-} + 2\,H^+. \quad 3.$$

Related enzymes are found in some hydrogenotrophic bacteria and in some $H_2$-forming bacteria and archaea. In the $H_2$-forming microorganisms, the enzyme catalyzes the reverse of Reaction 3. Most convincing is the energy-converting function of the [NiFe]-hydrogenase in the gram-negative *Rhodospirillum rubrum* (37) and *Rubrivivax gelatinosus* (38) as well as in the gram-postive *Carboxidothermus hydrogenoformans* (39). These anaerobic bacteria can grow chemolithoautotrophically on

CO, with $H_2$ and $CO_2$ being the only catabolic end products formed ($CO + H_2O \rightleftharpoons CO_2 + H_2$ $\Delta G^{\circ\prime} = -20$ kJ $mol^{-1}$) (40). The fermentation, which involves only a cytoplasmic carbon monoxide dehydrogenase (CooS), a cytoplasmic polyferredoxin (electron transfer protein) (CooF), and a membrane-associated, energy-converting [NiFe]-hydrogenase (CooHKLMUX), is coupled with chemiosmotic energy conservation as evidenced by growth and uncoupling experiments (37–39).

The energy-converting hydrogenases (EchA-F, EhaA-T, EhbA-Q, and MbhA-N) from methanogens contain six conserved core subunits (**Figure 4**) and up to 14 additional subunits. The six core subunits show sequence similarity to the six subunits of the carbon monoxide dehydrogenase (CooS)-associated [NiFe]-hydrogenase (CooHKLMUX) from bacteria, to the five subunits of the formate dehydrogenase-associated [NiFe]-hydrogenase (HycCDEFG) from *E. coli*, and to six of the core subunits of the NADH:ubiquinone oxidoreductase (NuoA-N) (complex I of the respiratory chain) from *E. coli* (41). Of the conserved subunits, two are integral membrane proteins (the larger one most probably involved in cation translocation), and four are hydrophilic proteins (**Figure 4**). Of the hydrophilic proteins, one is the [NiFe]-hydrogenase large subunit, one the hydrogenase small subunit with only one [4Fe4S]-cluster (the proximal one), one an iron-sulfur protein with two [4Fe4S]-clusters, and one a subunit without a prosthetic group. In complex I, the subunit NuoD homologous to the [NiFe]-hydrogenase large subunit lacks the N- and C-terminal CxxC motifs for [NiFe]-center formation (35).

None of the genes encoding energy-converting [NiFe]-hydrogenases in Bacteria or Archaea show a twin arginine translocation (Tat) motif-encoding sequence. The lack of the Tat motif-encoding sequence (42) indicates that the large subunit and the small subunit of the energy-converting [NiFe]-hydrogenases are not translocated from the cytoplasm to the periplasm and are therefore oriented toward the cytoplasm.

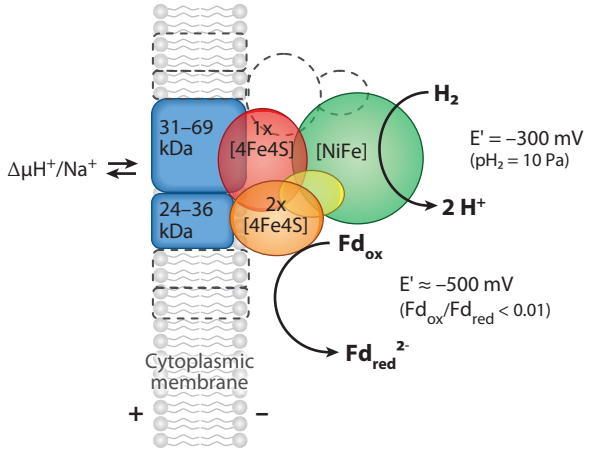


**Figure 4**

Schematic representation of the structure and function of the energy-converting [NiFe]-hydrogenases EchA-F, EhaA-T, EhbA-Q, and MbhA-N found in methanogenic archaea. The energy-converting hydrogenase EchA-F is composed only of the six conserved core subunits, which are highlighted in color. The energy-converting hydrogenases EhaA-T, EhbA-Q, and MbhA-N also contain several hydrophobic and hydrophilic subunits of unknown function. These subunits are symbolized by areas with dashed boundaries. Abbreviation: Fd, ferredoxin with two [4Fe4S]-clusters.

**EchA-F.** Genes for this type of energy-converting [NiFe]-hydrogenase are found in *M. barkeri* and *M. mazei* but not in *M. acetivorans*. They are also present in the genome of all members of the Methanomicrobiales. The enzyme in these organisms is most similar to the energy-converting hydrogenase CooHKLMUX from *R. rubrum* (37) and *C. hydrogenoformans* (39). EchA-F differs, however, in not forming a tight complex with its ferredoxin and functionally associated oxidoreductase. The lack of complex formation is because in methanogens the reduced ferredoxin, generated by the energy-converting hydrogenase, is used in electron transfer to more than one oxidoreductase. A 6 kDa 2[4Fe4S]-ferredoxin from *M. barkeri*, which is most probably the ferredoxin reduced by $H_2$ via the energy-converting hydrogenase EchA-F, has been characterized (43).

The enzyme complex EchA-F has been purified from *M. barkeri* and is composed of six different subunits (**Figure 4**) encoded by the *echA-F* operon (44, 45). EchA (69 kDa) and EchB (32 kDa) are the two integral

**Tat:** twin arginine translocation

membrane proteins. EchA is predicted to have 17 membrane-spanning α-helices and shows 30% sequence identity to a putative $Na^+/H^+$ translocator component in *Bacillus subtilis* (44). EchE is the large subunit, EchC is the small subunit, and these harbor the [NiFe]-center and a [4Fe4S]-cluster, respectively. EchF contains two [4Fe4S]-clusters. EchD is the soluble subunit without a prosthetic group. Chemical analyses of the purified complex have revealed the presence of nickel, nonheme iron, and acid-labile sulfur in a ratio of 1:12.5:12, substantiating the presence of three [4Fe4S]-clusters in addition to the [NiFe]-center. Like CooH, the large subunit EchE is synthesized without a C-terminal extension; the gene ends directly after the DPCxxCxxR motif with a stop codon. The evidence for reversed electron transfer in ferredoxin reduction with $H_2$ comes from biochemical and genetic studies. Cell suspensions of *M. barkeri* catalyze the reduction of $CO_2$ to CO ($E'_o = -520$ mV) with $H_2$ ($E'_o = -414$ mV), involving a cytoplasmic CO dehydrogenase, ferredoxin, and EchA-F. The reaction is driven by a proton motive force (46). The cells also catalyze the reverse reaction, the dehydrogenation of CO to $CO_2$ and $H_2$, which is coupled with the buildup of a proton motive force (47, 48). Δ*ech* mutants did not catalyze the forward or the backward reaction and also did not catalyze one of the other ferredoxin-dependent reductions mentioned above (49, 50). There are conflicting reports with respect to the coupling ion used by Ech in *M. barkeri.* The experiments investigating the reversible conversion of $H_2$ and $CO_2$ to CO and $H_2O$ are more in favor of protons (46–48), whereas those addressing the reduction of $CO_2$ with $H_2$ to formylmethanofuran are more in favor of sodium ions (51, 52).

The iron-sulfur centers in the EchA-F complex have been characterized by electron paramagnetic resonance spectroscopy, revealing that two of the [4Fe4S]-clusters show pH-dependent redox potentials (53) and indicating that these clusters mediate electron and proton transfer. The [4Fe4S]-clusters were assigned to the individual subunits via site-directed mutants (54). Insights into the mechanism of ion translocation come also from inhibition experiments with dicyclohexylcarbodiimide (DCCD), which specifically modifies protonated carboxyl residues located in a hydrophobic environment. Labeling studies of Ech with [$^{14}$C]-DCCD showed that the inhibition of the enzyme was associated with a specific labeling of the two integral membrane subunits EchA and B, particularly of EchA (35). The inhibition of Ech by DCCD indicates that the electron transfer reaction in this enzyme is strictly coupled to cation translocation.

**EhaA-T and EhbA-Q.** The [NiFe]-hydrogenase EhaA-T and EhbA-Q differ from the energy-converting hydrogenase EchA-F by having up to 14 additional subunits; many of these subunits are integral membrane proteins, and some are iron-sulfur proteins (55). A function of the additional subunits is difficult to envisage. Interestingly, the number of subunits of complex I of the respiratory chain also varies significantly without an apparent change in properties; thus, complex I in *E. coli* is composed of 14 subunits and in mitochondria of more than 40 subunits (56, 57).

Genes encoding EhaA-T and/or EhbA-Q are found in *M. kandleri*, in all members of the Methanococcales and Methanobacteriales, and in some members of the Methanomicrobiales (but not in *Methanosphaerula palustris* and *Methanoregula boonei*), and *eha* and/or *ehb* genes are not found in members of the Methanosarcinales. In some methanogens, e.g., in *M. marburgensis*, the genes are clustered and form a transcription unit, and in others, e.g., *M. jannaschii*, some of the genes are in separate loci.

The *eha* operon (12.5 kb) in *M. marburgensis* is composed of 20 open reading frames that form a transcription unit (55). Sequence analysis indicates that four of the genes encode proteins with high sequence similarity to four of the six different subunits characteristic for energy-converting hydrogenases (**Figure 4**): *ehaO* encodes the large [NiFe]-center harboring subunit as a preprotein; *ehaN* encodes the small subunit with only one [4Fe4S]-cluster.

*ehaH* and *ehaJ* encode the two conserved integral membrane proteins (24 kDa and 31 kDa); EhaJ is probably involved in ion translocation. A gene encoding the subunit with two [4Fe4S]-clusters appears to be lacking. Instead, the gene cluster harbors a gene for a 6[4Fe4S]-polyferredoxin (EhaP) and one for a 10[4Fe4S]-polyferredoxin (EhaQ). In addition to these subunits, the *eha* operon encodes four nonconserved hydrophilic subunits and ten nonconserved integral membrane proteins.

The *ehb* operon (9.6 kb) in *M. marburgensis* is composed of 17 open reading frames (55). The gene *ehbN* is predicted to encode the large subunit as a preprotein and *ehbM* the small subunit; *ehbF* and *ehbO* encode the two integral membrane proteins (53 kDa and 36 kDa), with the larger one probably involved in ion translocation; *ehbL* encodes a 2[4Fe4S]-cluster-containing protein (**Figure 4**). The *ehb* operon additionally encodes a 14[4Fe4S]-polyferredoxin (EhbK), two nonconserved hydrophilic subunits, and nine nonconserved integral membrane proteins.

Deletion of the *ehb* genes in *M. maripaludis* revealed a function of Ehb in autotrophic $CO_2$ fixation. The mutant was an acetate auxotroph. Deletion of the *eha* genes was not possible (58).

In Methanomicrobiales, in contrast to Methanobacteriales, the *ehaO* gene encoding the large subunit lacks the 3′ extension; it ends with the sequence motif DPCxxCxxR. The subunit with the [NiFe]-center in Methanomicrobiales is therefore predicted not to be synthesized as a preprotein.

**MbhA-N.** Genes encoding MbhA-N are found in two members of the Methanomicrobiales (*Methanospirillum hungatei* and *Methanocorpusculum labreanum*) but not in members of the four other orders (25). The *mbhA-N* gene cluster found in *M. hungatei* and *M. labreanum* is composed of 14 open reading frames. Six of the deduced proteins correspond to subunits conserved in all energy-converting hydrogenases (**Figure 4**): MbhL (large subunit with [NiFe]-center), MbhJ (small subunit with one [4Fe4S]-cluster), MbhN (subunit with two [4Fe4S]-clusters), MbhK (hydrophilic subunit without a prosthetic group), and MbhM and MbhH (conserved integral membrane proteins of 36 kDa and 56 kDa, respectively). The other eight deduced proteins all appear to be nonconserved integral membrane proteins. The gene *mbhL* for the large subunit encodes a protein with a C-terminal extension.

An enzyme complex with a similar composition was partially purified from *Pyrococcus furiosus*, which ferments glucose at 100°C to two acetic acids, two $CO_2$, and four $H_2$ (59). The enzyme complex was shown to use ferredoxin as electron donor. Addition of reduced ferredoxin to inverted membrane vesicles resulted in the generation of a proton or sodium motive force (inside positive) that could drive ATP synthesis (60). The ATPase involved was shown to translocate sodium ions (61). *P. furiosus* is a member of the order Thermococcales, which—like the five orders of methanogenic archaea—belong to the kingdom of Euryarchaeota. As in the Methanomicrobiales, the large [NiFe]-center harboring subunit (MbhL) in *P. furiosus* is synthesized as a preprotein.

## Heterodisulfide Reductase-Associated [NiFe]-Hydrogenase MvhADG

This cytoplasmic heterodisulfide reductase-associated [NiFe]-hydrogenase (MvhADG) catalyzes the reduction of dyes such as methyl viologen with $H_2$. The physiological electron acceptor is most probably the cytoplasmic heterodisulfide reductase HdrABC (Reaction 4) with which MvhADG forms a tight complex (**Figure 5*a***). In *Methanothermobacter* grown under nickel-limiting growth conditions, almost all of the MvhADG is found in complex with HdrABC (62). When the cells are grown in media with excess nickel, in addition to the MvhADG/HdrABC complex, free MvhADG and HdrABC are also present in varying amounts (62, 63). The MvhADG/HdrABC complex (62, 64) as well as MvhADG (62, 65) and HdrABC (66, 67) have also been purified and characterized.

$$H_2 + \mathrm{HdrABC_{ox}} \rightleftharpoons \mathrm{HdrABC_{red}}^{2-} + 2\,H^+. \quad 4.$$

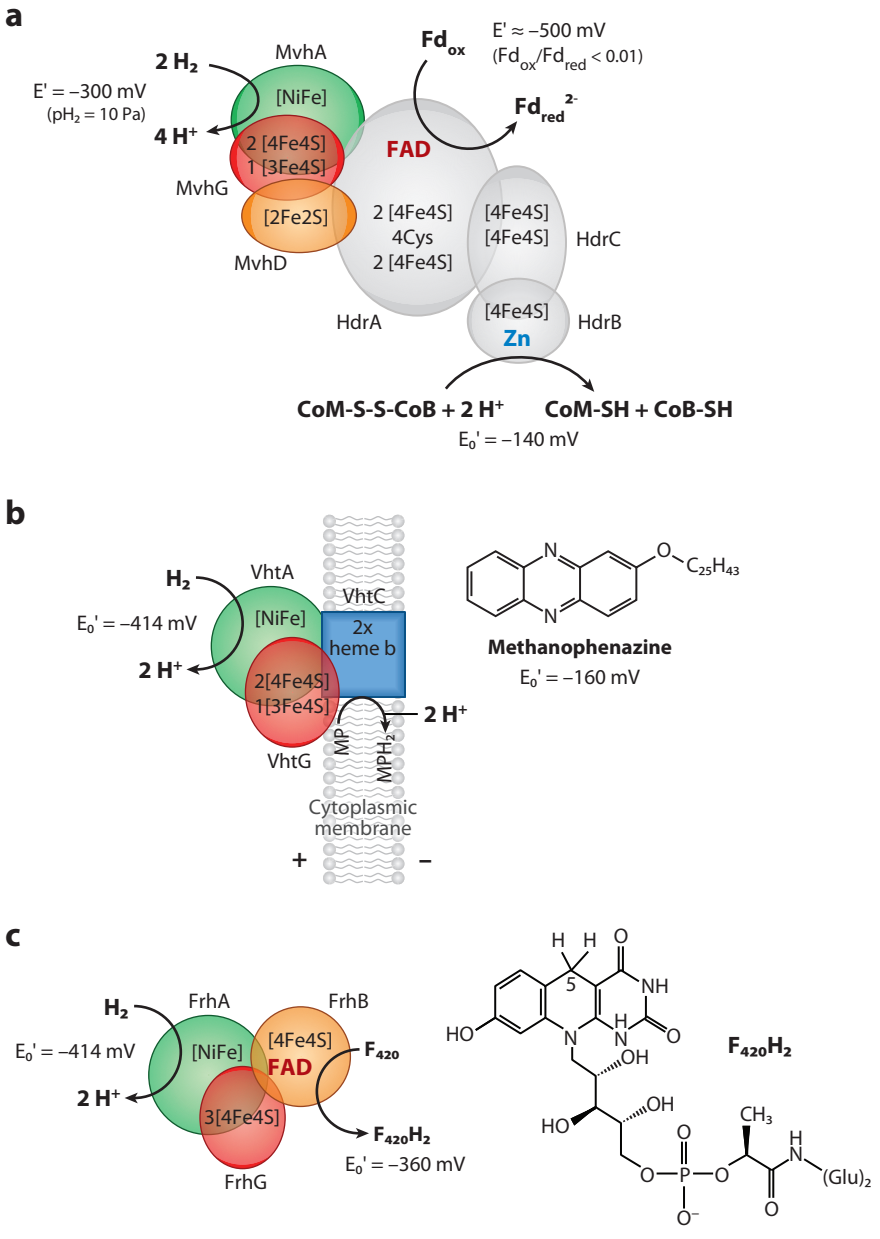


**Figure 5**

The structures and functions of (*a*) the MvhADG/HdrABC complex, (*b*) the VhtACG complex, and (*c*) the FrhABG complex involved in $H_2$ uptake in methanogenic archaea are schematically shown. The Mvh/Hdr complex is found mainly in methanogens without cytochromes, and the Vht complex is found only in methanogens with cytochromes. The stoichiometry of the MvhADG/HdrABC-catalyzed reaction has not yet been ascertained. Abbreviations: CoM-SH, coenzyme M with its thiol group; CoB-SH, coenzyme B with its thiol group; $F_{420}$, coenzyme $F_{420}$; Fd, ferredoxin with two [4Fe4S]-clusters; FrhA, FrhB, and FrhG, $F_{420}$-reducing [NiFe]-hydrogenase subunits; HdrA, HdrB, and HdrC, heterodisulfide reductase subunits; MP, methanophenazine; MvhA, MvhD, and MvhG, heterodisulfide reductase-associated [NiFe]-hydrogenase subunits; VhtA, VhtC, and VhtG, methanophenazine-reducing [NiFe]-hydrogenase subunits.

MvhA is the large subunit with the [NiFe]-center. It is synthesized as a preprotein. MvhG is the small subunit with one [3Fe4S]-cluster and two [4Fe4S]-clusters. MvhD is a subunit with one [2Fe2S]-cluster that mediates electron transfer from MvhG to HdrABC (**Figure 5*a***). None of the other [NiFe]-hydrogenases from methanogens contain a subunit with a [2Fe2S]-cluster. The presence of [2Fe2S]-clusters in iron-sulfur proteins of Archaea is the exception.

HdrB harbors the active site for CoM-S-S-CoB reduction. It contains two cysteine-rich sequence motifs $Cx_{31-39}CCx_{35-36}CxxC$ designated as CCG domains. The C-terminal CCG domain is involved in the binding of an unusual [4Fe4S]-cluster, and the N-terminal one is involved in zinc binding (68). HdrC harbors two [4Fe4S]-clusters, and HdrA contains four [4Fe4S]-clusters and a FAD that is only loosely bound but essential for activity. In addition, a conserved sequence motif with four cysteines is found. In HdrA from *Methanococcus* species, one of the four cysteines is a selenocysteine (**Figure 5*a***).

The purified MvhADG/HdrABC complex catalyzes the reduction of the heterodisulfide CoM-S-S-CoB with $H_2$ at only low specific activity (62, 64). The complex also catalyzes a CoM-S-S-CoB-dependent reduction of clostridial ferredoxin with $H_2$ at high specific activity. In the presence of ferredoxin, the specific rate of CoM-S-S-CoB reduction is increased (A.-K. Kaster, unpublished results). The complex thus appears to couple the endergonic reduction of ferredoxin ($E' \approx -500$ mV) with $H_2$ ($E'_o = -414$ mV) to the exergonic reduction of CoM-S-S-CoB ($E'_o = -140$ mV) (26) with $H_2$. The coupling probably involves the FAD in HdrA as the center of electron bifurcation (17, 69, 70). The exact stoichiometry of the reaction has not yet been ascertained but is, analogous to a ferredoxin-dependent crotonyl-CoA reduction with NADH (70), presently assumed to be two $H_2$ that reduce one clostridial ferredoxin (with two one-electron-accepting [4Fe4S]-clusters) and one CoM-S-S-CoB (**Figure 5*a***).

In *Methanothermobacter*, the ferredoxin reduced by the MvhADG/HdrABC complex is most probably the 12[4Fe4S] polyferredoxin encoded by the *mvhB* gene of the *mvhDGAB* operon (71). The polyferredoxin partially copurifies with the MvhADG/HdrABC complex (72, 73), which is why the MvhADG/HdrABC preparations always contain polyferredoxin—albeit in substoichiometric amounts (64, 65).

Genes encoding the MvhADG/HdrABC complex are also found in some cytochrome-containing methanogens, e.g., *M. barkeri*, *M. mazei*, and in Rice cluster I methanogens, and in some nonmethanogenic archaea, e.g., *Archaeoglobus fulgidus* (17). In *M. barkeri* and *A. fulgidus*, the homolog of *mvhD* is fused to the 3′ end of an *hdrA* homolog (62). In the Methanobacteriales, Methanopyrales, and Methanococcales, the genes are generally organized in three transcription units, *mvhDGAB*, *hdrBC*, and *hdrA*, which are not located adjacent to one another (67). In the Methanomicrobiales, the three *hdr* genes are juxtapositioned.

In the *Methanococcus* species and *M. kandleri*, there are two versions of MvhADG, one designated VhuADG in which the large [NiFe]-center harboring subunit A shows a C-terminal DPUxxCxxH motif (U for selenocysteine) and one abbreviated VhcADG in which the subunit A shows a C-terminal DPCxxCxxH motif (74, 75). When sufficient selenium is in the medium, only the [NiFeSe]-hydrogenase VhuADG is formed (76). In *Methanocaldococcus* species, there is only the selenoprotein version. The three other orders of methanogens do not contain selenoproteins (77).

Interestingly, in *Methanococcus* species, the gene for the large subunit of the [NiFeSe]-hydrogenase is split, and therefore the large subunit consists of two polypeptides, each contributing two ligands to the [NiFeSe]-center. A fusion of the two proteins was shown to be without effect on the kinetic and spectroscopic properties of the [NiFeSe]-hydrogenase VhuADG (78).

The genomes of most members of the cytochrome-less Methanomicrobiales—an

exception being *Methanoculleus marisnigri*—lack the genes for MvhA and MvhG but contain the genes for MvhD and HdrABC, which are juxtapositioned. It has therefore been proposed that in these methanogens a MvhD/HdrABC complex is associated with one of the energy-converting hydrogenases EchA-F, EhaA-T, or MbhA-N (25). As a consequence, in most Methanomicrobiales, heterodisulfide reduction with $H_2$ would be energy consuming. This is the consequence of the finding that the different energy-converting hydrogenases all have the same topology and should therefore have the same function, namely to catalyze the oxidation of $H_2$ in a reaction requiring, rather than generating, energy. Consistent with this interpretation is that *M. hungatei* (one of the Methanomicrobiales without *mvhA* and *mvhG* genes) is known to grow on $H_2$ and $CO_2$ at very low $H_2$ partial pressures (79, 80), indicating a very low ATP gain (17).

An alternative hypothesis is that, in cytochrome-less methanogens lacking the genes for MvhA and MvhG, these two subunits are substituted by FrhA (large [NiFe]-hydrogenase subunit) and FrhG (small [NiFe]-hydrogenase subunit) of the $F_{420}$-reducing hydrogenase (FrhABG) (see below). All methanogens without cytochromes contain genes for this enzyme. FrhA and G would thus be present both in a putative FrhAG/MvhD/HdrABC complex and in the FrhABG complex. There are precedents for such subunit sharing. Thus, the molybdenum-containing formylmethanofuran dehydrogenase FwdA/FmdBC and the tungsten-containing formylmethanofuran dehydrogenase FwdABC from *M. marburgensis* share the subunit FwdA and the pyruvate dehydrogenase complex, and the 2-oxoglutarate dehydrogenase complex from *E. coli* shares the lipoamide dehydrogenase subunit (81). Interesting in this respect is that in the genome of *M. boonei* putative genes for a large subunit (NCBI Mboo_2023) and one for a small subunit (NCBI Mboo_1398) of the $F_{420}$-reducing [NiFe]-hydrogenase are found in addition to the *frhADGB* transcription unit.

## Methanophenazine-Reducing [NiFe]-Hydrogenase VhtACG

This membrane-associated, cytochrome *b*-containing [NiFe]-hydrogenase catalyzes the reduction of methanophenazine with $H_2$ (Reaction 5) and couples this reaction with the buildup of an electrochemical proton potential (**Figure 5*b***). Methanophenazine is a 2-hydroxyphenazine derivative that is connected via an ether bridge to a pentaprenyl side chain (**Figure 5*b***) (82, 83). Like ubiquinone ($E'_o$ = +110 mV) and menaquinone ($E'_o$ = −80 mV), methanophenazine ($E'_o$ = −170 mV) (26) is a lipid-soluble electron and proton carrier; the difference is that methanophenazine's redox potential is much lower. Methanophenazine (shown as MP in Reaction 5) is only found in the Methanosarcinales, i.e., in methanogens that contain cytochromes (84, 85).

$$H_2 + MP \rightarrow MPH_2 \quad \Delta G^{\circ\prime} = -50\,kJ\,mol^{-1}. \qquad 5.$$

The methanophenazine-reducing hydrogenase VhtACG from *M. barkeri* has been characterized (**Figure 5*b***) (86). VhtA is the [NiFe]-center harboring large subunit, which is synthesized as a preprotein. VhtG is the [4Fe4S]/[3Fe4S]/[4Fe4S]-cluster harboring small subunit. VhtC is a cytochrome *b* that is integrated into the membrane. The gene *vhtG* contains at its 5′ end a sequence encoding a Tat signal (DRRTFM/I). Genetic and biochemical studies indicate that in such cases the large subunit is cotranslocated with the small subunit across the cytoplasmic membrane (87, 88). The subunits with the [NiFe] active site thus face the periplasm. As a consequence, the protons generated upon $H_2$ oxidation are released outside. VhtACG thus has a topology similar to that described for [NiFe]-hydrogenase-1 and [NiFe]-hydrogenase-2 in *E. coli* and for the membrane-associated [NiFe]-hydrogenase in *Ralstonia* (32).

In some *Methanosarcina* species, e.g., *M. mazei*, the genome harbors two sets of genes, *vhtGACD* and *vhoGAC* (the latter encodes a Vht isoenzyme); each set is a transcription unit. In the *vho* operon, a *vhtD*-like

gene is not present. *vhtD* is homologous to *hoxM* from *R. eutropha* and to *hyaD* from *E. coli*, which encode specific maturation endopeptidases. In *M. mazei*, the *vho* operon is transcribed constitutively, whereas the *vht* operon is transcribed only during growth on methanol and $H_2/CO_2$ rather than on acetate (89, 90).

The cytoplasmic membrane of all methanogens with cytochromes contains an associated methanophenazine-dependent heterodisulfide reductase, HdrDE (**Figure 3*a***). The subunit HdrE is a cytochrome *b* that is integrated into the membrane, and HdrD is the peripheral subunit that catalyzes CoM-S-S-CoB reduction. HdrD combines the sequences of HdrB and HdrC of the cytoplasmic heterodisulfide reductase HdrABC. The gene encoding HdrD lacks a Tat sequence, indicating that the HdrD subunit faces the cytoplasm, which is consistent with its function as a catalyst of the reduction of CoM-S-S-CoB, generated by methyl-coenzyme M reduction with coenzyme B in the cytoplasm (91, 92).

Thus, whereas the active-site-harboring subunit of the methanophenazine-reducing hydrogenase is located on the periplasmic side of the membrane, that of the methanophenazine-oxidizing heterodisulfide reductase is located on the cytoplasmic side (**Figure 3*a***). Both complexes are electrically connected via the lipid-soluble methanophenazine, which is reduced by the cytochrome *b* of the hydrogenase and is reoxidized by the cytochrome *b* of the heterodisulfide reductase. Experimental evidence has been provided that per heterodisulfide reduced with $H_2$ in this system four electrogenic protons are generated, which can be used to drive the synthesis of one ATP via a proton-translocating $A_1A_0$-ATPase (84, 85, 93, 94).

In Rice cluster I, which belongs to the Methanosarcinales, the gene for HdrE (cytochrome *b*) is lacking, indicating that in this methanogen the methanophenazine reduced by VhtACG cannot be reoxidized. In agreement with this prediction is the finding that in Rice cluster I the genes for a functional MvhADG/HdrABC complex are present (17).

## Coenzyme $F_{420}$-Reducing [NiFe]-Hydrogenases FrhABG

This cytoplasmic [NiFe]-hydrogenase catalyzes the reversible reduction of coenzyme $F_{420}$ with $H_2$ (Reaction 6). Coenzyme $F_{420}$ is a 5-deazaflavin (**Figure 5*c***) found in high concentrations in methanogenic archaea and in *A. fulgidus* and in low concentrations also in other archaea and in some bacteria. Although structurally resembling a flavin, $F_{420}$ is functionally more like the pyridine nucleotide NAD(P) in transferring two electrons plus a proton (a hydride) rather than single electrons. The functional difference from NAD(P) is, however, that the redox potential of the $F_{420}/F_{420}H_2$ couple is −360 mV and thus 40 mV more negative than that of the NAD(P)/NAD(P)$H_2$ couple (27).

$$H_2 + F_{420} \rightleftharpoons F_{420}H_2 \quad \Delta G^{\circ\prime} = -11\,\mathrm{kJ\,mol^{-1}}. \qquad 6.$$

In methanogenic archaea, $F_{420}H_2$ is involved in two reduction steps of methanogenesis from $CO_2$ (**Figure 3**) and also in several anabolic reduction reactions, e.g., in the $F_{420}H_2$:NADP oxidoreductase reaction, the $F_{420}$-dependent glutamate synthase reaction, the $F_{420}$-dependent sulfite reductase reaction, and the $F_{420}H_2$:$O_2$ oxidoreductase reaction. Under conditions of $H_2$ limitation, transcription of the genes for the $F_{420}$-reducing hydrogenase are upregulated (95, 96) and under conditions of nickel limitation downregulated (97).

During growth of methanogenic archaea on formate, $F_{420}$ reduction is catalyzed by a cytoplasmic $F_{420}$-dependent formate dehydrogenase FdhABC. Under these conditions, the $F_{420}$-reducing hydrogenase catalyzed the formation of $H_2$ (Reaction 6) with the $H_2$ used via intraspecies hydrogen transfer as electron donor for the coupled reduction of ferredoxin and heterodisulfide catalyzed by the cytoplasmic MvhAGD/HdrABC complex (**Figure 5*a***) (98, 99).

The FrhABG complex has been purified, and the encoding genes have been determined (100). The genes are organized in a transcription unit *frhADGB*, where *frhA* encodes

the large subunit with the [NiFe]-center, *frhG* encodes the small subunit with three [4Fe4S]-clusters, and *frhB* encodes an iron-sulfur flavoprotein with one [4Fe4S]-cluster and one FAD, which functions as a one electron/two electron switch in $F_{420}$ reduction (**Figure 5*c***). *frhD* encodes an endopeptidase (homologous to HycI from *E. coli*), which is required to clip off the C-terminal extension in the FrhA preprotein.

In the genome of *M. barkeri*, a *frhADGB* operon and a *freAEGB* operon are found, the latter encoding a Frh isoenzyme. The *freAEGB* operon lacks a gene homolog of *frhD* for the endopeptidase (101). Genetic evidence has recently been found that *freAEGB* is expressed functionally only if the *frhADGB* operon is simultaneously expressed, indicating that FrhD is also involved in FreA maturation (102). The function of the *freE* gene (123 bp) is not known.

The small subunit of most [NiFe]-hydrogenases harbors two [4Fe4S]-clusters and one central [3Fe4S]-cluster. However, in the small subunit of the $F_{420}$-reducing hydrogenase, the central cluster is always a [4Fe4S]-cluster. Mutational studies, in which the middle cluster was converted to a [3Fe4S]-cluster, revealed significant changes in electron transport rates (103).

In the cytoplasm of methanogenic archaea, FrhABG is aggregated to a complex with a molecular mass of >900 kDa (63, 104). Upon ultracentrifugation of cell extracts, the $F_{420}$-reducing hydrogenase is recovered in the membrane fraction, which is why it was long believed that this enzyme is membrane associated.

In most *Methanococcus* species and *M. kandleri*, there are two versions of $F_{420}$-reducing hydrogenases, FrcABG and FruABG. The large subunit FrcA has a C-terminal DPCxxCxxH motif, and the large subunit FruA has a C-terminal DPUxxCxxH motif (U for selenocysteine) (74, 75). When selenium is in the medium, only the [NiFeSe]-hydrogenase (FruABG) is formed (76). In *Methanococcus aeolicus* and in *Methanocaldococcus* species, there is only the selenoprotein version.

## Genes Involved in [NiFe]-Hydrogenase Maturation

In *E. coli*, for the synthesis of the [NiFe]-center in the large subunit of hydrogenase-3 at least six proteins are required: HypA and HypB for nickel insertion, HypE and HypF for the synthesis of the cyanide ligand from carbamoyl phosphate, and HypC and HypD for the transfer of the cyanide to the active site (31, 32, 105–107). The *hyp* genes are also found in all methanogenic archaea, although not clustered as in *E. coli*, e.g., in *M. marburgensis* only the *hypAB* genes form a transcription unit. Despite this fact, it is very likely that in methanogens the synthesis of the [NiFe]-center proceeds in principle as has been described for hydrogenase-3 from *E. coli*.

It is not yet known how in *E. coli* the CO ligand of iron in the [NiFe]-hydrogenases is generated. Carbamoyl phosphate was excluded as a precursor, and free CO was shown to be incorporated (108, 109). Labeling experiments with acetate indicate that in *Allochromatium vinosum* the CO in the [NiFe]-center is derived from the carboxyl group of acetate (110). A hypothesis is that the iron, which at the end carries two cyanide ligands and one CO ligand (**Figure 1*a***), reacts with acetyl-CoA, yielding a acetyl-iron complex ($CH_3CO$-Fe), which after methyl group migration affords the CO iron complex and methanol. An acyl iron complex (-$CH_2CO$-Fe) is found in [Fe]-hydrogenase (**Figure 1*c***).

Some methanogens, examples include *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*, growing on $H_2$ and $CO_2$ as energy sources require acetate as a carbon source. These methanogens lack genes for carbon monoxide dehydrogenase and acetyl-CoA synthase/decarbonylase but contain active [NiFe]-hydrogenases, indicating that the two nickel enzymes are not involved in the synthesis of CO for the [NiFe]-center.

The large subunits of most of the [NiFe]-hydrogenases in methanogens are synthesized as preproteins from which a C-terminal extension has to be clipped off after completion of

[NiFe]-center synthesis. The endopeptidase gene *vhtD*, required for the maturation of methanophenazine-reducing hydrogenase, was found in the *vhtGACD* operon, and the gene *frhD* for the maturation of the $F_{420}$-reducing hydrogenase was found in the *frhADGB* operon. Whereas VhtD shows the best hits to the endopeptidase HyaD from *E. coli* (involved in hydrogenase-1 maturation) and HoxM from *Ralstonia* (involved in membrane-bound hydrogenase maturation), FrhD is more similar to the endopeptidase HycI from *E. coli* (involved in hydrogenase-3 maturation) (111, 112). The endopeptidase genes for the other [NiFe]-hydrogenases in methanogens have not yet been found. The *mvhDGAB* operon lacks a gene for an endopeptidase, and none of the genes in the *eha* or *ehb* operons show homology to genes for known endopeptidases or proteases.

In the genomes of some methanogens, aside from the gene clusters for the various hydrogenases, an open reading frame predicted to encode for an endopeptidase is found. The putative endopeptidase has sequence similarity to HycI involved in hydrogenase-3 maturation in *E. coli*. This gene is not associated with any other gene cluster from which a function could be deduced. Whether the *hycI* homolog outside the hydrogenase gene clusters has a function in [NiFe]-hydrogenase maturation remains to be shown.

## Nickel Regulation

Nickel is a relatively abundant metal, although its concentration in freshwater and marine environments can be very low (<10 nM). Because the requirement of microorganisms for nickel is generally also low and because most microorganisms including methanogens have active, high-affinity nickel-uptake transporters (113), it was long overlooked that nickel is an essential trace element for most prokaryotes. It was the finding in 1979 that growth of methanogens is dependent on nickel that changed the picture (114). In addition to the [NiFe]-hydrogenases, methanogens contain three other nickel enzymes for methanogenesis and autotrophic $CO_2$ fixation, namely methyl-coenzyme M reductase (**Figure 3**), carbon monoxide dehydrogenase, and acetyl-CoA synthase/decarbonylase. The nickel enzymes are required in such high concentrations that nickel has to be added to growth media in over 1 μM concentrations in order for nickel not to become growth limiting. Therefore, in their natural habitats, methanogens have to continuously cope with the problem of nickel famine, and they probably have had to do so for the past 2.4 billion years, since the time of the so-called Great Oxidation Event. Recent evidence indicates that this was when the concentration of nickel in the oceans dropped from 400 nM to below 200 nM within 100 million years and subsequently to the modern day value of 9 nM by 550 Mya (115). It is argued that, as the rate of methanogenesis became nickel limited, the high concentrations of methane in the Precambrian atmosphere decreased, allowing the atmospheric $O_2$ concentration to build up. Methane reacts in the atmosphere with $O_2$ in a photochemical reaction cycle to become $CO_2$ and $H_2O$.

**Great Oxidation Event:** Earth's atmospheric oxygen rose from $<10^{-5}$ PAL (present atmospheric level) to between 0.1 and 0.2 PAL

Methanogenic archaea respond to changing nickel concentrations in the growth medium. Under conditions of nickel limitation, for example, the transcription of the genes for [Fe]-hydrogenase and $F_{420}$-dependent methylene-tetrahydromethanopterin dehydrogenase are upregulated, and those for $F_{420}$-reducing hydrogenase (FrhABG) are downregulated. This has been shown for *M. marburgensis* (97, 116) as well as for *M. maripaludis* and *M. jannaschii* (A.-K. Kaster, unpublished results).

In *E. coli*, there are two nickel-responsive transcriptional regulators, NikR, which suppresses transcription only in the presence of nickel (117), and RcnR, which only allows transcription in the presence of nickel (118). NikR and RcnR bind nickel reversibly with high affinity. Genes for only one of the two transcriptional regulators, namely NikR, are found in the genomes of methanogens. In many methanogens, several copies for NikR

*hmd*: gene encoding [Fe]-hydrogenase

are present. The hypothesis therefore is that NikR is involved in the transcriptional regulation of the synthesis of [Fe]-hydrogenase and of $F_{420}$-dependent methylenetetrahydromethanopterin dehydrogenase, which are upregulated under conditions of nickel limitation.

The nucleotide sequence in the promoter region, to which NikR binds, has been mapped in *E. coli*. In this γ-proteobacteria, the NikR box is a 28-bp palindromic operator sequence (GTATGA-$N_{16}$-TCATAC) (119). In other taxonomic groups, the palindrome sequences differ somewhat, the palindrome can be less complete, and the space between the dyad-symmetric consensus sequences can be 12 bp to 16 bp (120). In some cases, the genes regulated by NikR do not even contain identifiable symmetric recognition sites (121). With this caveat, a putative NikR box was identified in the promoter region of the *hmd* gene in *M. marburgensis*, *M. maripaludis* and *M. jannaschii* (A.-K. Kaster, unpublished results).

How the synthesis of the $F_{420}$-reducing hydrogenase in methanogens is downregulated under nickel-limiting growth conditions is not known to date. In the nickel-limited growth of *M. marburgensis*, neither the enzyme nor the transcript of the *frhADGB* operon was found (97).

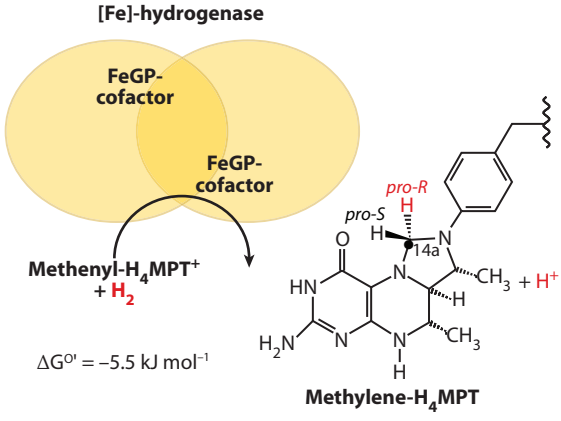


**Figure 6**

The structure and function of the homodimeric [Fe]-hydrogenase are schematically shown with its two active sites. For the structure of the iron-guanylylpyridinol cofactor (FeGP cofactor) see **Figure 1*c***. [Fe]-hydrogenase catalyzes the reversible transfer of a hydride from $H_2$ into the *pro-R* side of methenyl-tetrahydromethanopterin (methenyl-$H_4MPT^+$) yielding methylene-$H_4MPT$ (122).

## [Fe]-HYDROGENASE IN METHANOGENS WITHOUT CYTOCHROMES

When methanogens without cytochromes grow under conditions of nickel limitation, some of them synthesize the nickel-free [Fe]-hydrogenase instead of the $F_{420}$-reducing [NiFe]-hydrogenase (97, 116). [Fe]-hydrogenase catalyzes the reversible transfer of a hydride from $H_2$ to methenyltetrahydromethanopterin (methenyl-$H_4MPT^+$), which is reduced to methylene-$H_4MPT$ (Reaction 7) (**Figure 6**) (122, 123).

$$H_2 + \text{methenyl-}H_4MPT^+ \rightleftharpoons \text{methylene-}H_4MPT + H^+ \quad \Delta G^{\circ\prime} = -5.5\ \text{kJ mol}^{-1} \qquad 7.$$

Together with the $F_{420}$-dependent methylenetetrahydromethanopterin dehydrogenase (Reaction 8), [Fe]-hydrogenase catalyzes the reduction of $F_{420}$ with $H_2$ (Reaction 6) (116). Consistent with this function are the findings that the synthesis of both [Fe]-hydrogenase and $F_{420}$-dependent methylenetetrahydromethanopterin dehydrogenase are upregulated under nickel-limiting growth conditions (116) and that in *M. maripaludis* it has been possible to knock out the genes for $F_{420}$-reducing hydrogenase or the gene for [Fe]-hydrogenase or that for $F_{420}$-dependent methylenetetrahydromethanopterin dehydrogenase with only minor effects on growth on $H_2$ and $CO_2$, but it has not been possible to knock out two of these genes (99, 124).

$$\text{Methylene-}H_4MPT + F_{420} + H^+ \rightleftharpoons \text{methenyl-}H_4MPT^+ + F_{420}H_2 \quad \Delta G^{\circ\prime} = -5.5\ \text{kJ mol}^{-1}. \qquad 8.$$

[Fe]-hydrogenase has a more than 20-fold higher $K_m$ for $H_2$ (0.2 mM) than the $F_{420}$-reducing [NiFe]-hydrogenase (0.01 mM). As a compensation, cells grown with limited

nickel have more than 40 times the specific [Fe]-hydrogenase activity (65 μmol $min^{-1}$ mg $protein^{-1}$) than nickel-sufficient cells have $F_{420}$-reducing hydrogenase activity (1.6 μmol $min^{-1}$ mg $protein^{-1}$) (values for *M. marburgensis*) (116). Thus the catalytic efficiency of $H_2$ uptake is maintained more or less constant (15).

## Structural Properties

When discovered in 1990, [Fe]-hydrogenase was found to contain two moles iron per mole homodimer of 76 kDa but not to contain iron-sulfur clusters (23) and was therefore named iron-sulfur-cluster-free hydrogenase (125). The single iron per subunit is low spin and not redox active. A catalytic mechanism was proposed that did not require a redox active iron (126). Therefore, the iron was initially thought not to have a catalytic function, which is why the enzyme was dubbed "metal-free hydrogenase" (123, 126). However, it had been overlooked that the enzyme is inhibited by CO, albeit only at relatively high concentrations ($K_i$ >0.5 mM), indicating an involvement of the iron in $H_2$ activation (127).

It is now known that [Fe]-hydrogenase harbors a novel iron-guanylylpyridinol (FeGP) cofactor covalently bound to the [Fe]-hydrogenase only via the thiol/thiolate group of a cysteine residue (**Figure 1*c***). In the cofactor, a low-spin iron (II) is ligated by two CO, one $C(O)CH_2$-, one S-$CH_2$-, and a $sp^2$-hybridized nitrogen of the pyridinol ring (12–14, 127–131). After protein unfolding, the cofactor can be released from the protein in the presence of thiol reagents under mild alkaline conditions or in the presence of acids in the absence of thiols (S. Shima, unpublished). When the FeGP cofactor is added to apoenzyme heterologously produced in *E. coli*, an active holoenzyme is formed (132), which has allowed the investigation of [Fe]-hydrogenase from methanogens that are difficult to grow and the performance of genetic analysis of the active-site amino acids involved in catalysis (13, 131). In [FeFe]-hydrogenases, the [FeFe]-center is also covalently attached to the protein only via a single cysteine residue (**Figure 1*b***). However, until now, it has not been possible to reversibly detach the center from this enzyme.

## Catalytic Properties

As mentioned above, [Fe]-hydrogenase is reversibly inhibited by CO as are most [NiFe]- and [FeFe]-hydrogenases. [Fe]-hydrogenase is also reversibly inhibited by cyanide ($K_i$ = 0.1 mM) and by isocyanides. With cyclohexylisocyanide, a specific, highly effective inhibitor with a $K_i$ of <0.1 μM was recently found (S. Shima, unpublished result). Cyanide and isocyanides do not appear to inhibit [NiFe]-hydrogenases or [FeFe]-hydrogenases.

All three types of hydrogenases are rapidly inactivated by copper ions and by the superoxide anion radical $O_2^{\cdot-}$ ($E'_o$ of the $O_2^{\cdot-}/H_2O_2$ couple = +890 mV). Whereas, in the presence of $H_2$, [FeFe]- and most [NiFe]-hydrogenases are rapidly inactivated by $O_2$, most probably in part owing to the reduction of $O_2$ with $H_2$ to $O_2^{\cdot-}$ ($E'_o$ of the $O_2/O_2^{\cdot-}$ couple = −330 mV), purified [Fe]-hydrogenase remains active in the presence of $O_2$ both in the presence and absence of $H_2$ (15, 127).

[Fe]-hydrogenase shows a ternary complex catalytic mechanism (127). It does not catalyze the reduction of dyes with $H_2$, the exchange of protons of water into $H_2$, or the conversion of *para*-$H_2$ to *ortho*-$H_2$ (spin isotopomers of $H_2$), three reactions characteristically catalyzed by [FeFe]- and [NiFe]-hydrogenases. However, in the presence of its substrate methenyl-$H_4MPT^+$, [Fe]-hydrogenase catalyzes the exchange of $H^+$ from water into $H_2$ and the conversion of *para*-$H_2$ to *ortho*-$H_2$, with kinetics almost indistinguishable from those of the two other types of hydrogenases (133). The enzyme also catalyzes a stereospecific exchange of the *pro-R* hydrogen of methylene-$H_4MPT$ (see **Figure 6**) with protons of water (134). A catalytic mechanism consistent with these results was recently deduced from the crystal structure of the [Fe]-hydrogenase-methylene-$H_4MPT$ complex (14).

*hcg*: *hmd* co-occurring genes

**SAM:** *S*-adenosylmethionine

The *hmd* gene for [Fe]-hydrogenase is present in the genomes of *M. kandleri*, all members of the Methanococcales, most members of the Methanobacteriales, and only one member of the Methanomicrobiales (*M. labreanum*). The *hmd* gene has not yet been found in the genome of one of the members of the Methanosarcinales.

## Genes Involved in FeGP Cofactor Biosynthesis

The genes involved in the biosynthesis of the FeGP cofactor (**Figure 1*c***) have not yet been determined experimentally. However, an *in silico* analysis indicates that there are seven genes present in all methanogens with a *hmd* gene, with one exception (see below). These genes are tentatively designated *hcg* genes (*hmd* co-occurring genes). In many of the methanogens, the seven *hcg* genes neighbor the *hmd* gene and are clustered (**Figure 7**). Despite being juxtapositioned to *hcgABCDEF* in *M. marburgensis*, the *hmd* gene is transcribed monocistronically (97). The exception is *M. hungatei*. This methanogen lacks a *hmd* gene but harbors a *hcgCDEFG* gene cluster without, however, having the genes *hcgA* and *hcgB*.

The gene *hcgA* is predicted to encode a protein with a sequence similar to the radical-SAM (*S*-adenosylmethionine) iron-sulfur protein BioB, which is involved in sulfur insertion in biotin biosynthesis. However, HcgA lacks the N-terminal signature $CX_3CX_2C$ motif or $CX_4CX_2C$ motif that is characteristic for the radical-SAM protein superfamily and that coordinates a [4Fe4S]-cluster essential for radical formation (135, 136). Instead, HcgA universally harbors a unique $CX_5CX_2C$ motif (137). Some radical-SAM enzymes have a function in methylation reactions (138). One of the two methyl groups attached to the pyridinol ring in the FeGP cofactor is derived from the methyl group of methionine (see below). It is therefore likely that HcgA is involved in this methylation reaction. Interestingly, a BioB homolog is also involved in [FeFe]-hydrogenase maturation (139–141).

The crystal structure of HcgB from *M. thermautotrophicus* has been determined within a structural genomics project (142). The homodimeric protein contains three bound

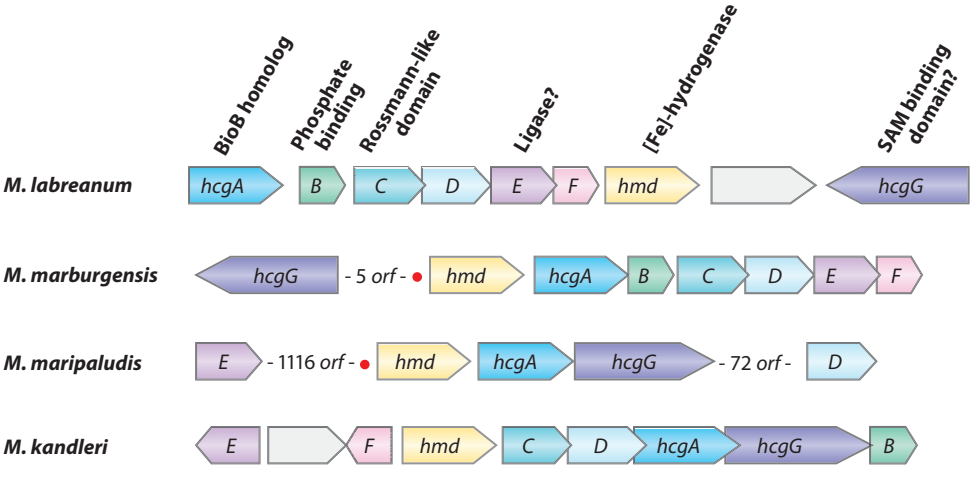


**Figure 7**

Genes neighboring the *hmd* gene for [Fe]-hydrogenase in *Methanocorpusculum labreanum*, *Methanothermobacter marburgensis*, *Methanococcus maripaludis*, and *Methanopyrus kandleri* are shown. The red dot indicates the presence of a putative NikR box to which the transcriptional regulator NikR of methanogens could bind and stop transcription when nickel is present. *hcg* is the abbreviation for *hmd* co-occurring genes.

phosphates and shares structural similarities with pyrophosphatases. The gene *hcgC* encodes a hypothetical protein with a putative NAD(P)-binding Rossmann-like domain. The presumed protein HcgD has a sequence similar to a protein that in yeast interacts with the transcriptional activator NGG1p. HcgE shows sequence similarity to proteins that catalyze ubiquitin activation with ATP. The gene *hcgF* is without a recognizable function. HcgG is annotated as a fibrillarin-like protein with a C-terminal domain that could bind SAM.

All the *hcg* genes and the *hmd* gene in *M. labreanum* (the only member of the Methanomicrobiales with these genes) show higher sequence similarity at the protein level to the respective genes in *M. marburgensis* than to the respective genes in *M. kandleri*, in the Methanococcales, and in *M. smithii*. These findings are interpreted to indicate that the *hcg* gene cluster in *M. labreanum* (**Figure 7**) has been acquired by this methanogen from a *Methanothermobacter* species via lateral gene transfer (M. Schick, unpublished results). On the same basis, this is also likely for the gene cluster *hcgCDEFG* in *M. hungatei*.

Labeling studies with [1-$^{13}$C]-acetate, [2-$^{13}$C]-acetate, [1-$^{13}$C]-pyruvate and L-[methyl-$D_3$]-methionine, performed mainly with the acetate auxotroph *M. smithii*, have revealed via mass spectrometry that of the nine carbons in the pyridinol moiety of the FeGP cofactor (**Figure 1*c***) three are derived from C1 of acetate, two from C2 of acetate, one from the methyl group of methionine, two from the carboxyl group of pyruvate, and one from $CO_2$ (M. Schick, unpublished results).

In the genomes of some methanogens that contain an *hmd* gene, one or two genes homologous to *hmd* are found (97). The two encoded proteins, designated HmdII and HmdIII, show only low sequence identity (<20%) to [Fe]-hydrogenase but share high sequence identity (80%) with each other. The homologs are not found in methanogens without an *hmd* gene. Structure predictions indicate that HmdII and HmdIII have an intact site for FeGP cofactor binding (14, 15). Consistently, HmdII was found to bind the FeGP cofactor. However, neither HmdII nor HmdIII catalyzed the reduction of methenyl-$H_4$MPT$^+$ with $H_2$. These results were interpreted to indicate that HmdII and HmdIII could be scaffold proteins involved in FeGP cofactor biosynthesis (15). In [NiFe]-hydrogenase maturation, there is a precedent for this. The synthesis of the [NiFe]-center of the membrane-associated hydrogenase from *R. eutropha* involves HoxV, which is a HoxA homolog (large subunit carrying the [NiFe]-center), as the scaffold (143).

*M. labreanum* and *M. smithii*, which both can synthesize active [Fe]-hydrogenase (M. Schick, unpublished data), do not contain *hmdII* or *hmdIII* genes, which does not support the scaffold hypothesis. However, in *Ralstronia*, the scaffold (HoxV) is required only for the maturation of the membrane-bound hydrogenase and not for the soluble one, and in *E. coli*, a scaffold protein homologous to the large subunit is not involved in the synthesis of any of the three [NiFe]-hydrogenases. It could therefore be that in some methanogens [Fe]-hydrogenase synthesis is independent of Hmd homologous putative scaffold proteins.

## $H_2$ STORAGE VIA $CH_4$ FORMATION

The formation of methane from 4 $H_2$ and $CO_2$ (Reaction 1) catalyzed by methanogenic archaea is being discussed as an efficient means to store $H_2$ (144). The combustion of 4 $H_2$ with 2 $O_2$ to 4 $H_2O$ yields 949 kJ mol$^{-1}$ and that of $CH_4$ with 2 $O_2$ to $CO_2$ and 2 $H_2O$ yields 818 kJ mol$^{-1}$ free energy. Thus, most of the combustion energy of $H_2$ is conserved in methane. Compared to $H_2$, methane is relatively easy to store and to transport. From methane, $H_2$ can be regenerated in a reforming process ($CH_4 + H_2O \rightarrow 3\ H_2 + CO$), followed by the shift reaction ($CO + H_2O \rightarrow CO_2 + H_2$), which is standard technology.

The chemical reduction of $CO_2$ with $H_2$ to methane requires very high temperatures and pressures. By contrast, methanogenic archaea catalyze the process at room temperature and

at $H_2$ pressures way below 1 bar. The rate of biological $CO_2$ reduction to methane in cell suspensions, e.g., of *M. marburgensis*, can be as high as 3 μmol per minute and milligram of cells (dry mass) (145). Thus, with 100 g cells of this methanogen per day, approximately 7 kg methane can be formed from 4 $H_2$ and $CO_2$; this is equivalent to 350 MJ energy, which is only somewhat lower than the amount of primary energy consumed by an average person each day in Germany (474 MJ per person per day) (144).

The idea is to use $CO_2$ from coal power plants and $H_2$ generated either via reforming of biomass or via photolysis or electrolysis of water (the electricity required for $H_2O$ electrolysis could be provided by solar or wind energy). After storage, when the methane is burned, no more $CO_2$ is released into the atmosphere than was used in the formation of methane from $H_2$ and $CO_2$.

In contrast to the [Fe]-hydrogenase, all the [NiFe]-hydrogenases present in methanogens are rapidly inactivated by $O_2$ in the presence of $H_2$ and even the [Fe]-hydrogenase is inactivated by the $O_2$-reduction product $O_2^{\cdot-}$. Therefore, there is no potential to employ these enzymes in vitro in large-scale technical processes. However, within the cells, the hydrogenases are much more robust because methanogens contain enzymes that reduce $O_2$ and $O_2^{\cdot-}$ to $H_2O$ (96, 146–148). There are even some methanogens that can thrive on $H_2$ and $CO_2$ in the presence of $O_2$ (146, 148). The use of methanogenic archaea in converting energy from $H_2$ to methane is thus not an illusion. The economic feasibility, however, remains to be shown.

## SUMMARY POINTS

1. There are three types of enzymes that activate $H_2$, namely [NiFe]-hydrogenases, [FeFe]-hydrogenases, and [Fe]-hydrogenase, which have emerged by convergent evolution. Of the three types, only [NiFe]- and [Fe]-hydrogenases are found in methanogenic archaea.
2. In methanogenic archaea, there are four different [NiFe]-hydrogenases, of which the $F_{420}$-reducing hydrogenase and the heterodisulfide reductase-associated hydrogenase are cytoplasmic, and the energy-converting hydrogenases and methanophenazine-reducing hydrogenase are membrane-associated proteins. The [NiFe]-center harboring subunit of the energy-converting hydrogenases faces the cytoplasm, and the active-site harboring subunit of the methanophenazine-reducing hydrogenase is oriented toward the periplasm.
3. The energy-converting [NiFe]-hydrogenases are proton or sodium ion pumps from which complex I of the respiratory chain has evolved. The reduction of ferredoxin with $H_2$, catalyzed by the energy-converting hydrogenases, is energy consuming.
4. The $F_{420}$-reducing hydrogenase in methanogens is unique in that its small subunit contains three [4Fe4S]-clusters, the energy-converting hydrogenase is unique in that its small subunit contains only one [4Fe4S]-cluster, and the heterodisulfide reductase-associated hydrogenase is unique in that it contains a subunit, MvhD, which harbors a [2Fe2S]-cluster seldomly found in archaeal proteins.
5. The gene clusters encoding $F_{420}$-reducing hydrogenase and methanophenazine-reducing hydrogenase each harbor a gene for an endopeptidase involved in [NiFe]-hydrogenase maturation. The gene clusters encoding the energy-converting hydrogenases and the heterodisulfide reductase-associated hydrogenase lack such a gene. Maturation of some of the energy-converting [NiFe]-hydrogenases appears not to require a protease step.

6. In all methanogens investigated, homologs of the genes *hypA-F* are found, which in *E. coli* encode proteins necessary, and apparently sufficient, for [NiFe]-center biosynthesis.
7. The [Fe]-hydrogenase is of functional importance in methanogens under nickel-limiting growth conditions, which appear to prevail in many natural habitats. The enzyme harbors a novel iron-guanylylpyridinol cofactor covalently attached to the protein only via one cysteine sulfur ligand to iron. In the presence of thiol reagents or acids, the cofactor can be reversibly detached. Genes putatively involved in cofactor biosynthesis have been identified.
8. Methanogenic archaea can catalyze the formation of methane from $CO_2$ and $H_2$ at specific activities high enough to be considered as catalysts in industrial energy transformation.



## FUTURE ISSUES

1. The crystal structure of the energy-converting hydrogenase of the EchA-F type from methanogens would be of great interest because EchA-F is phylogenetically related to complex I of the respiratory chain whose crystal structure for the complete enzyme is still unknown (57).
2. The crystal structure of the MvhADG/HdrABC complex is required to help understand how the electrons from $H_2$ are bifurcated such that both ferredoxin and heterodisulfide are reduced by $H_2$ in an energy-coupled reaction. The stoichiometry of ferredoxin and heterodisulfide reduction with $H_2$ remains to be ascertained.
3. A crystal structure of [Fe]-hydrogenase in complex with its substrate methylenetetrahydromethanopterin is required in a closed form. The recently published structure is in the open form, in which the bound methylenetetrahydromethanopterin does not interact with the active-site iron.
4. There is a need to isolate the hydrogenase/heterodisulfide reductase complex from *M. hungatei* and from other members of the Methanomicrobiales that lack the genes for MvhA and MvhG. Is there an FrhAG/MvhD/HdrABC complex as speculated in this review?
5. Seven genes co-occurring with the gene *hmd* encoding [Fe]-hydrogenase have been proposed to be involved in the FeGP cofactor biosynthesis. Gene knockout experiments in *M. maripaludis*, for which a genetic system has been developed, could help clarify this point. In parallel, attempts are needed to heterologously express the seven putative cofactor synthesis genes together with the *hmd* gene in *E. coli*.

## DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Annual Review of Biochemistry

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# Contents

**Indexes**



**Errata**

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