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Energy-Converting [NiFe] Hydrogenases From Archaea and Extremophiles: Ancestors of Complex I

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[NiFe] hydrogenases are well-characterized enzymes that have a key function in the H_2 metabolism of various microorganisms. In the recent years a subfamily of [NiFe] hydrogenases with unique properties has been identified. The members of this family form multisubunit membrane-bound enzyme complexes composed of at least four hydrophilic and two integral membrane proteins. These six conserved subunits, which built the core of these hydrogenases, have closely related counterparts in energy-conserving NADH:quinone oxidoreductases (complex I). However, the reaction catalyzed by these hydrogenases differs significantly from the reaction catalyzed by complex I. For some of these hydrogenases the physiological role is to catalyze the reduction of H^+ with electrons derived from reduced ferredoxins or polyferredoxins. This exergonic reaction is coupled to energy conservation by means of electron-transport phosphorylation. Other members of this hydrogenase family mainly function to provide the cell with reduced ferredoxin with H_2 as electron donor in a reaction driven by reverse electron transport. As complex I these hydrogenases function as ion pumps and have therefore been designated as energy-converting [NiFe] hydrogenases.

KEY WORDS: Hydrogen; [NiFe] hydrogenase; NADH:quinone oxidoreductase; complex I; methanogenic archaea; carbon monoxide dehydrogenase; iron–sulfur proteins.

INTRODUCTION

Hydrogenases are found in a wide variety of microorganisms. They catalyze the simplest chemical reaction in nature: $H_2 \leftrightarrow 2H^+ + 2e^-$. Hydrogenases enable some organisms to use H_2 as a source of reducing equivalents under both aerobic and anaerobic conditions. In other organisms the enzyme is used to reduce protons to H_2, thereby releasing the reducing equivalents obtained from the anaerobic degradation of organic substrates (Schwarz and Friedrich, 2003). On the basis of the transition-metal content, hydrogenases can be divided into two major classes (Vignais *et al.*, 2001): the [Fe]-hydrogenases (Nicolet *et al.*, 2002) and the [NiFe] hydrogenases (Albracht, 1994; Garcin *et al.*, 1998). The basic module conserved in all [NiFe] hydrogenases is formed by two subunits, frequently called "hydrogenase large" and "hydrogenase small" subunit. The large subunit harbors the binuclear [NiFe] active site, which is coordinated by two conserved CxxC motifs, one located in the N-terminal region and the second located in the C-terminal region of the polypeptide (Albracht, 1994). The small subunit of all [NiFe] hydrogenases displays a conserved amino-acid sequence pattern, $CxxCx_nGxCxxxGx_mGCPP$ ($n = 61$–106, $m = 24$–61) (Albracht, 1994), binding one [4Fe–4S] cluster. This cluster is within 14 Å from the active site (Volbeda *et al.*, 1995) and is called the proximal cluster. In most, but not all enzymes, the small subunit contains six to eight additional cysteine residues, which ligate two more clusters, in the *Desulfovibrio gigas* enzyme being a second [4Fe–4S] cluster (distal cluster) and a [3Fe–4S] cluster (medial

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Key to abbreviations: Ech, energy-converting hydrogenase; Coo, components of the CO-oxidizing:H_2-forming enzyme system in *Rhodospirillum rubrum* or *Carboxydothermus hydrogenoformans*; Eha and Ehb, energy-converting [NiFe] hydrogenases A and B from *Methanothermobacter* species; Mbh, membrane-bound [NiFe] hydrogenase from *Pyrococcus furiosus*; complex I, energy-conserving NADH:quinone oxidoreductase.

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cluster). The combination of the [NiFe] active site and the proximal [4Fe–4S] cluster seem to be important for the catalytic action of [NiFe] hydrogenases (Garcin *et al.*, 1998).

In the recent years a group of multisubunit membrane-bound [NiFe] has been identified in several microorganisms. These enzymes form a distinct group within the large family of [NiFe] hydrogenases (Vignais *et al.*, 2001). Members of this hydrogenase family include hydrogenases 3 from *Escherichia coli* (Böhm *et al.*, 1990; Sauter *et al.*, 1992), CO-induced hydrogenase from *Rhodospirillum rubrum* and *Carboxydothermus hydrogenoformans* (Fox *et al.*, 1996b; Soboh *et al.*, 2002), Mbh hydrogenase from *Pyrococcus furiosus* (Sapra *et al.*, 2000; Silva *et al.*, 2000), Eha and Ehb hydrogenases from *Methanothermobacter* species (Tersteegen and Hedderich, 1999), and Ech hydrogenase from *Methanosarcina barkeri* (Künkel *et al.*, 1998; Meuer *et al.*, 1999). The hydrogenases large and small subunit of these enzymes show surprisingly little sequence similarity to other (standard) [NiFe] hydrogenases, except for the conserved residues coordinating the active site and the proximal [Fe–S] cluster. In addition to the hydrogenase large and small subunit these enzymes contain at least four other subunits, two hydrophilic proteins, and two integral membrane proteins. These six subunits form the basic structure of these hydrogenases conserved in all members of this hydrogenase subfamily. These conserved subunits show a striking amino-acid sequence similarity with six subunits of energy conserving NADH:quinone oxidoreductase, also called complex I (Fig. 1) (Albracht and Hedderich, 2000; Friedrich and Scheide, 2000; Friedrich and Weiss, 1997; Yano and Ohnishi, 2001). Complex I is present in the inner mitochondrial membrane and in the cytoplasmic membrane of numerous bacteria. It catalyzes electron transfer from NADH to ubiquinone or menaquinone and couples this reaction to the translocation of protons or sodium ions across a membrane (Brandt *et al.*, 2003). The bacterial enzymes are formed by 13 to 14 subunits also conserved in the mitochondrial enzymes (Yagi *et al.*, 1998). These conserved subunits form the catalytic core of complex I. From sequence comparisons it became evident that the catalytic core of complex I has a highly modular architecture (Friedrich and Weiss, 1997). The electron input domain of the enzyme is formed by three subunits catalyzing the oxidation of NADH. This module contains FMN and most of the iron–sulfur clusters of the enzyme. The subunits of this module are sequence related to other NAD(P)-dependent enzymes. Highest sequence identity has been found to the diaphorase part of NAD^+-reducing [NiFe] hydrogenases (Tran-Betcke *et al.*, 1990) and to $NADP^+$-reducing [Fe] hydrogenases (Malki *et al.*, 1995). This NADH dehydrogenase module mediates the electron transfer to the central part of the enzyme

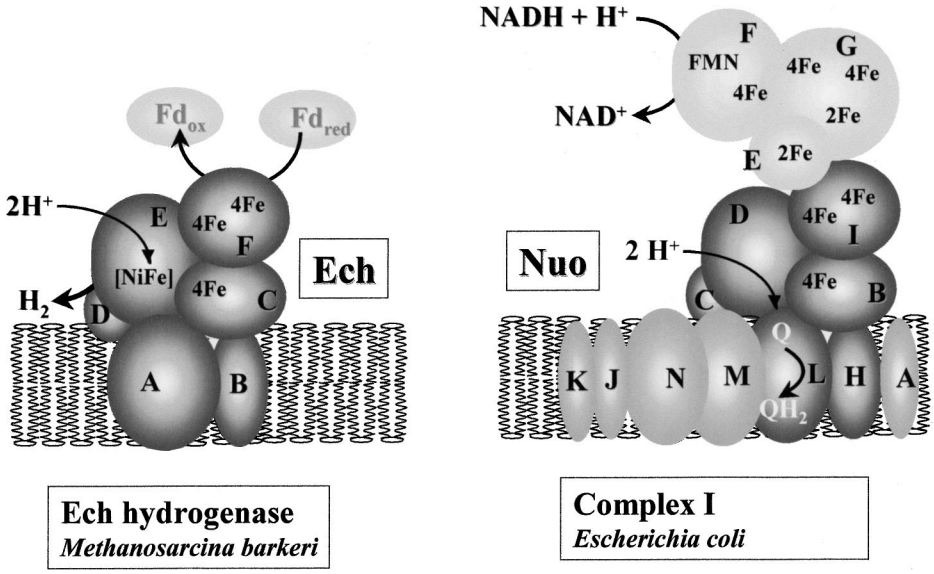

Fig. 1. Schematic representation of the common protein modules in energy-converting [NiFe] hydrogenases and complex I. Ech from *M. barkeri* and complex I from *E. coli* (Friedrich and Scheide, 2000) are shown as examples. Subunits conserved between both enzymes are shown in the same gray color. Capital letters indicate subunits of the enzymes. Abbreviations: [NiFe]: binuclear NiFe-active site of hydrogenase; 4Fe: [4Fe–4S] cluster; 2Fe: [2Fe–2S] cluster; FMN: flavin mononucleotide; Q: ubiquinone or menaquinone.

formed by four hydrophilic subunits. These subunits form the contact site to the membrane part of the enzyme. There is experimental evidence that these subunits participate in the reduction of the quinone (Brandt *et al.*, 2003). The four hydrophilic subunits in this central part of complex I are highly homologous to the four hydrophilic subunits of membrane-bound [NiFe] hydrogenases. Furthermore, the two membrane-bound subunits present in these hydrogenases are closely related to subunits present in the membrane part of complex I (Fig. 1). The evolutionary relationship between complex I and the membrane-bound [NiFe] hydrogenases has been addressed in recent reviews (Albracht and Hedderich, 2000; Friedrich and Scheide, 2000; Yano and Ohnishi, 2001). From growth experiments with *R. rubrum* and *C. hydrogenoformans* (Kerby *et al.*, 1995; Svetlichny *et al.*, 1991), from cell-suspension experiments and genetic studies with *M. barkeri* (Bott and Thauer, 1989; Meuer *et al.*, 2002), and from experiments with inverted vesicles of *P. furiosus* (Sapra *et al.*, 2003) it can be inferred that the [NiFe] hydrogenases in these organisms probably pump protons or sodium-ions as well. They have therefore been designated energy-converting [NiFe] hydrogenases (Vignais *et al.*, 2001). The purpose of this review is to summarize our current knowledge about these membrane-bound [NiFe] hydrogenases with a special focus on the architecture of these enzymes, their physiological function in the different organisms, and their relationship to complex I. Most of the enzymes identified thus far are from archaea or from organisms, which exhibit an extremophilic life style.

Ech HYDROGENASE FROM *Methanosarcina barkeri*

Biochemical Properties

From a biochemical perspective, the most thoroughly studied member of the family of energy-converting [NiFe] hydrogenase is Ech hydrogenase found in the methanogenic archaeon *M. barkeri*. This enzyme will therefore be described in more detail.

Most methanogenic archaea contain a set of two distinct standard [NiFe] hydrogenases, called F_{420}-reducing and F_{420}-nonreducing hydrogenase. These enzymes have been purified and characterized from several organisms (Deppenmeier *et al.*, 1996; Thauer, 1998) (see also review by Uwe Deppenmeier in this issue). Some methanogens in addition contain a so-called metal-free hydrogenase, not containing a redox-active metal center (Thauer *et al.*, 1996). Studies with the membrane fraction of *M. barkeri* in the recent years led to the discovery of third type of [NiFe] hydrogenase in this organism, designated as Ech hydrogenase (Künkel *et al.*, 1998; Meuer *et al.*, 1999). The enzyme is an integral membrane protein, which, when purified, is composed of six subunits, corresponding to the products of the *echABCDEF* operon. Ech hydrogenase is only distantly related to the two standard [NiFe] hydrogenases of *M. barkeri*. The subunits of this enzyme are more closely related to members of a small group of membrane-bound [NiFe] hydrogenases, such as hydrogenase 3 from *E. coli* and the CO-induced hydrogenase from *R. rubrum*. The EchA and EchB subunits of the enzyme are predicted to be integral, membrane-spanning proteins, while the other four subunits are expected to extrude into the cytoplasm (Fig. 1). Amino-acid sequence analysis of the cytoplasmic subunits points to the presence of two classical [4Fe–4S] clusters in EchF and one [4Fe–4S] cluster in EchC. The EchC subunit belongs to the family of the small subunits in [NiFe] hydrogenases. However, EchC and its homologous in other energy-converting [NiFe] hydrogenases is significantly smaller than the corresponding subunit of standard [NiFe] hydrogenases and only contains the cysteine ligands for the proximal [4Fe–4S] cluster. The EchE subunit shows the characteristic binding-motif for the [Ni-Fe] site found in the large subunits of all [NiFe] hydrogenases. Chemical analysis revealed the presence of Ni, non-heme Fe and acid-labile S in a ratio of 1:12.5:12 (Meuer *et al.*, 1999), corroborating the presence of three [Fe–S clusters]. This was also confirmed by EPR spectroscopic studies of purified Ech (see below).

A low-potential, soluble two [4Fe–4S] ferredoxin ($E^{0'} = -420$ mV) isolated from *M. barkeri* was identified as electron donor/acceptor of Ech. The enzyme catalyzed the formation of hydrogen with reduced ferredoxin as electron donor. The catalytic efficiency coefficient was calculated to be $3.7 \times 10^7\ M^{-1}\ s^{-1}$. To determine the rate of ferredoxin reduction by H_2 the metronidazole assay was used. In this assay reduced ferredoxin is oxidized in a fast chemical reaction by metronidazole. Ech hydrogenase catalyzed the reduction of metronidazole by H_2 only in the presence of the ferredoxin, which indicates that the ferredoxin is a direct electron acceptor of the enzyme. A catalytic efficiency coefficient (k_{cat}/K_m) of $1.5 \times 10^8 M^{-1}\ s^{-1}$ was calculated for this reaction. The apparent K_m for H_2 was 5 μM. These kinetic data strongly indicate that the ferredoxin is the physiological electron donor or acceptor of Ech hydrogenase (Meuer *et al.*, 1999).

The metal centers of Ech were spectroscopically characterized. A primary structure analysis of Ech hydrogenase and related enzymes reveals the presence of three binding motifs for [4Fe–4S] clusters in addition to the binding motif for the [NiFe] center. This

agrees well with the determined Ni, Fe, and acid-labile sulfur content of purified Ech. The data are also consistent with the spectroscopic characterization of the enzyme.

A characterization of the [NiFe] center of the enzyme by EPR and FTIR spectroscopies revealed the characteristic features obtained with standard [NiFe] hydrogenases indicating an active-site architecture of Ech similar to that of standard [NiFe] hydrogenases (Kurkin, S., Hedderich, R., and Albracht, S. P. J., unpublished results).

The iron–sulfur centers of Ech hydrogenase have been characterized in detail by EPR spectroscopy (Kurkin *et al.*, 2002). From the EPR line shape and the temperature dependence of spectra from H_2-reduced Ech, it was concluded that signals due to three different $S = 1/2$ species from reduced [4Fe–4S] clusters are present. These were labeled as the $g = 1.92$ signal, the $g = 1.89$ signal, and the $g = 1.96$ signal. Redox titrations indicated that the $g = 1.96$ signal has the lowest redox potential (well below -420 mV at pH 7); therefore this cluster could only partly be reduced. This is in line with the maximal amount of spins detected in the spectra of the reduced [Fe–S] clusters (about 2–2.6 spins per enzyme molecule at pH 8). Magnetic interaction of the 1.89 signal with an unpaired electron localized on the [Ni–Fe] site indicated that this is the proximal cluster as found in all [NiFe] hydrogenases. Hence, this cluster was tentatively assigned to be located in the EchC subunit. The other two clusters could therefore be assigned to be bound to the EchF subunit. Redox titrations at different pH values demonstrated that there is a pH dependence of the midpoint potentials of [Fe–S] clusters responsible for the $g = 1.92$ and $g = 1.89$ signals. For the $g = 1.92$ signal the $E^{\circ'}$ value decreased by 53 mV per pH unit, for the $g = 1.89$ signal it decreased by 62 mV per pH unit. The obtained values for both signals were reasonably close to the theoretical value of -59 mV per pH unit for a redox titration involving a stoichiometric amount of electrons and protons. $E^{\circ'}$ values with such a large pH dependence are rare in the in the field of [Fe–S] clusters. The pH dependence for the proximal cluster ($g = 1.89$ signal) is in agreement with the pH dependence of the $E^{\circ'}$ value of the proximal cluster in standard [NiFe] hydrogenases. The g values ($g_z = 2.05$ and $g_{xy} = 1.92$) and pH dependence (-53 mV per pH unit) of the 1.92 signal, ascribed to one of the [4Fe–4S] clusters of EchF, is reminiscent to the g values ($g_z = 2.054$ and $g_{xy} = 1.922$) and the pH dependence (-60 mV per pH unit) of the signal ascribed to the cluster(s) N-2 of bovine complex I (Ingledew and Ohnishi, 1980). Because of its unique redox properties cluster N2 is considered to be an important component of the ion pump (Albracht and Hedderich, 2000).

Physiological Function of Ech Hydrogenase

Methanosarcina species are able to utilize a broader range of energy substrates in comparison to other groups of methanogens (Deppenmeier *et al.*, 1996). They can reduce CO_2 to CH_4 with H_2 as electron donor, can reduce methanol or methylamines to CH_4 using H_2 as electron donor, are able to convert methanol or methylamines to CO_2 and CH_4 and can convert acetate to CO_2 and CH_4. RNA hybridization and immunoblotting experiments indicate that the *ech* operon is expressed at similar levels during growth on these substrates (Künkel *et al.*, 1998; Meuer *et al.*, 1999).

To elucidate the physiological function of Ech hydrogenase in these metabolic pathways, a mutant lacking this enzyme was constructed and characterized (Meuer *et al.*, 2002). This Δ*ech* mutant was unable to grow on methanol/H_2/CO_2, H_2/CO_2, or acetate as carbon and energy sources, but showed wild-type growth rates with methanol as sole substrate. Addition of pyruvate to the growth medium restored growth on methanol/H_2/CO_2, but not on H_2/CO_2 or acetate. Results obtained from the physiological characterization of the Δ*ech* mutant allowed the following conclusions to be made.

(1) One essential role for Ech is to provide reducing equivalents for the first step of methanogenesis from H_2/CO_2, the reduction of CO_2 to formylmethanofuran (CHO-MFR). Because of the low midpoint potential of the CO_2 + methanofuran/formylmethanofuran couple ($E^{\circ'} = \sim -500$ mV) this reaction becomes endergonic with H_2 as electron donor ($E^{\circ'} = -414$ mV) (Bertram and Thauer, 1994). This is even more pronounced at the low hydrogen partial pressures prevailing in the natural habitats of methanogens. In freshwater sediments, for example, the H_2 partial pressure is in the order of 5 Pa corresponding to an E' for the $2H^+/H_2$-couple of -286 mV. Therefore, this unfavorable reaction requires an additional input of energy to proceed. Cell suspension experiments with wild-type *M. barkeri* and *M. mazei* had provided evidence that reduction of CO_2 to CHO-MFR by H_2 is driven by an electrochemical proton or sodium ion gradient (Kaesler and Schönheit, 1989a,b; Winner and Gottschalk, 1989). The coupling of thermodynamically unfavorable redox reactions to the consumption of a membrane ion gradient, often referred to as reverse electron transport, is an important process in many anaerobic microorganisms but poorly understood on a mechanistic level. It requires

that at least one of the enzymes involved is an integral membrane protein. From theoretical perspectives it has been assumed that the reduction of CO_2 + methanofuran to formylmethanofuran by H_2 involves a hydrogenase, electron transfer components and a specific oxidoreductase. The latter enzyme, called formylmethanofuran dehydrogenase, has been biochemically characterized. It is a soluble enzyme belonging to the family of molybdopterin-containing dehydrogenases (Vorholt *et al.*, 1996). The hydrogenase and the electron transfer protein involved were until recently unknown.

The characterization of the *M. barkeri* Δ*ech* mutant revealed that Ech hydrogenase is absolutely required for the reduction of CO_2 to formylmethanofuran by H_2. The data obtained strongly indicate that Ech hydrogenase catalyzes reduction of ferredoxin by H_2 and that the reduced ferredoxin thus generated functions as low potential electron donor for the synthesis of formylmethanofuran. It is assumed that reverse electron transport drives the energetically unfavorable reduction of the ferredoxin by H_2 and that Ech hydrogenase functions as the ion pump (Meuer *et al.*, 2002).

(2) Ech hydrogenase in addition provides the cell with reduced ferredoxin required as electron donor of oxidoreductases in biosynthetic pathways. One example is the biosynthesis of pyruvate from acetyl-CoA and CO_2 catalyzed by pyruvate:ferredoxin oxidoreductase, a central reaction in the anabolic CO_2 fixation pathway of methanogens (Bock *et al.*, 1996). This explains why the Δ*ech* mutant is not able to grow with methanol/H_2 as energy substrates, unless the medium is supplemented with pyruvate (Meuer *et al.*, 2002).

(3) In acetoclastic methanogenesis Ech is proposed to catalyze the reverse reaction, the production of H_2 with reduced ferredoxin as electron donor. In this pathway acetate is first activated to acetyl-CoA and subsequently split into methyltetrahydrosarcinapterin (CH_3-H_4SPT) and enzyme-bound CO via the acetyl-CoA synthase/CO dehydrogenase complex. Enzyme-bound CO is oxidized to CO_2 via this complex with concomitant production of reduced ferredoxin, providing reducing equivalents for the reduction of the methyl group to CH_4 (Ferry, 1997). The electrons from reduced ferredoxin may flow through H_2 via the action of a hydrogenase. This has been concluded from experiments with intact cells. Cell suspensions of wild-type *M. barkeri* convert CO quantitatively to CO_2 and H_2 (Bott *et al.*, 1986). Cell suspensions of Δ*ech* catalyzed the oxidative half of the acetoclastic pathway (conversion of CO to CO_2 and H_2) at a significantly lower rate than the wild type indicating that Ech is the hydrogenase involved in this reaction (Meuer *et al.*, 2002). Importantly, the conversion of CO to CO_2 and H_2 was found to be coupled to the generation of a proton motive force (Bott and Thauer, 1989). This is consistent with the putative ion-translocating activity of Ech. Also *C. hydrogenoformans* couples the conversion of CO to CO_2 and H_2 to energy conservation (see below).

RELATED HYDROGENASES IN OTHER METHANOGENS

Energy-converting [NiFe] hydrogenases are also found in the other phylogenetic groups of methanogens. *Methanothermobacter thermoautotrophicus* and *Methanothermobacter marburgensis* (formerly *Methanobacterium thermoautotrophicum* strain ΔH and strain Marburg) each encode two multisubunit membrane-bound [NiFe] hydrogenase, designated Eha and Ehb (Tersteegen and Hedderich, 1999). The length of the transcription units was determined using reverse transcription polymerase chain reactions (RT-PCR). The *eha* operon (12.5 kb) and the *ehb* operon (9.6 kb) were found to be composed of 20 and 17 open reading frames, respectively. Sequence analysis of the deduced proteins indicated that the *eha* and *ehb* operons each encode a [NiFe] hydrogenase large subunit, a [NiFe] hydrogenase small subunit, and two conserved integral membrane proteins. These proteins show high sequence similarity to subunits of Ech hydrogenase from *M. barkeri*. In addition to these four subunits, the *eha* operon encodes a 6[4Fe–4S] polyferredoxin, a 10[4Fe–4S] polyferredoxin, four nonconserved hydrophilic subunits, and ten nonconserved integral membrane proteins; the *ehb* operon encodes a 2[4Fe–4S] ferredoxin, a 14[4Fe–4S] polyferredoxin, two nonconserved hydrophilic subunits, and nine nonconserved integral membrane proteins. *Methanothermobacter* species can only utilize H_2/CO_2 as energy substrates. Since *Methanothermobacter* species only grow with H_2/CO_2 as energy substrates it has been proposed that these membrane-bound [NiFe] hydrogenases catalyze the reduction of a low-potential ferredoxin or polyferredoxins by H_2 in a reaction driven by reverse electron transport, in analogy to the function of Ech

hydrogenase in *M. barkeri* when the organism is cultivated on H_2/CO_2. A purification of these enzymes has not been achieved thus far (Stojanowic, A., and Hedderich, R., unpublished data).

Also *Methanococcus jannaschii* and *Methanopyrus kandleri* contain a predicted operon closely resembling the *eha* operon of *Methanothermobacter* species as deduced from the completely sequenced genomes of these organisms. The genome of *M. jannaschii* also has homologues of the *ehb* genes, these are however not organized in one operon. In contrast, the genome of *M. kandleri* does not encode a second membrane-bound hydrogenase (Bult *et al.*, 1996; Slesarev *et al.*, 2002).

FORMATE HYDROGENLYASE REACTION IN *Escherichia coli*

Hydrogenase 3 from *E. coli* was the first enzyme of the family of energy-converting [NiFe] hydrogenases that had been discovered. This hydrogenase was found to be essential for the formation of H_2 in fermenting *E. coli*. Together with a formate dehydrogenase (FDH_H) this hydrogenase catalyzes the conversion of formate to CO_2 and H_2, in the so-called formate hydrogenlyase reaction (Böhm *et al.*, 1990; Sauter *et al.*, 1992). The subunits of the hydrogenase are encoded by the *hyc* operon. The *hyc* operon encodes homologous of the six subunits present in purified Ech hydrogenase, however in the *E. coli hyc* operon the homologous of *echD* and *echE* are fused. The *hyc* operon in addition encodes an electron transfer protein (HycB) with four predicted [4Fe–4S] clusters. HycB is related to the CooF protein described in the next chapter. It is assumed to function as electron carrier between formate dehydrogenase and hydrogenase. Unfortunately, the hydrogenase 3 is unstable and could not be purified as intact enzyme. But much of which is known today about the synthesis of the [NiFe] center in hydrogenases was deduced from experiments with this hydrogenase (Blokesch *et al.*, 2002).

The physiological function of the formate hydrogenlyase reaction is to prevent an acidification of the cytoplasm by converting formic acid to CO_2 and H_2. Under standard substrate concentrations the formate hydrogenlyase reaction is not an exergonic process. At low H_2 partial pressures and low pH, the in vivo conditions, the reaction becomes exergonic (~ -20 kJ/mol). Thus, from a thermodynamic point of view the reaction could be coupled to energy conservation.

In addition to the *hyc* operon, which encodes hydrogenase 3, the *E. coli* genome encodes a second multisubunit membrane-bound [NiFe] hydrogenase. The so-called *hyf* operon encodes homologous of all the Hyc proteins but in addition encodes three membrane proteins that have no direct counterparts in the *hyc* operon (Andrews *et al.*, 1997). The *hyf* operon was found to resemble the *hyc* operon in being induced under anaerobic conditions by formate at low pH. No mutant phenotype could be assigned to Δ*hyf* mutants and no *hyf* operon-encoded proteins could be detected so far. Hence, the physiological role of the Hyf system remains unknown (Skibinski *et al.*, 2002).

THE CO-OXIDIZING:H_2-FORMING SYSTEM IN *Rhodospirillum rubrum* AND *Carboxydothermus hydrogenoformans*

Only a few microorganisms are known that can grow anaerobically with CO under chemolithoautotrophic conditions. These organisms couple the oxidation of CO to CO_2 with the reduction of protons to H_2.

$$CO + H_2O \rightarrow CO_2 + H_2, \quad \Delta G^{\circ\prime} = -20 \text{ kJ mol}^{-1}$$

Organisms known to grow at the expense of this reaction are the gram-negative bacteria *R. rubrum* and *Rubrivax gelatinosus* (Kerby *et al.*, 1995; Uffen, 1976) and the gram-positive bacterium *C. hydrogenoformans* (Svetlichny *et al.*, 1991).

The biochemical process underlying the conversion of CO to CO_2 and H_2 has been intensively studied in *R. rubrum*. The carbon monoxide oxidation system (Coo) is encoded by the *coo* regulon, which consists of two gene clusters regulated by the *cooA* gene (Shelver, *et al.*, 1997). One gene cluster encodes the catalytic subunit (CooS) of the CO dehydrogenase, an electron transfer protein (CooF), and proteins required for the insertion of Ni into the enzyme (CooC, T, and J) (Kerby *et al.*, 1992, 1997). The CO dehydrogenase is a nickel iron–sulfur protein that, when purified, is only composed of the CooS protein which forms a homodimer (Bonam and Ludden, 1987). The crystal structure of this enzyme has been determined (Drennan *et al.*, 2001). Under certain purification conditions, CooS copurifies with the iron–sulfur protein CooF, which mediates the electron transfer from CooS to a membrane-bound hydrogenase (Ensign and Ludden, 1991). The second gene cluster *cooMKLXUH* encodes a hydrogenase (Fox *et al.*, 1996a,b), with high sequence similarity to Ech hydrogenase from *M. barkeri* and to hydrogenase 3 from *E. coli*. However, this hydrogenase turned out to be extremely labile preventing its purification and biochemical characterization.

The thermophilic gram-positive bacterium *C. hydrogenoformans* is also able to utilize CO as sole energy

source. Recently the purification and characterization of a membrane-bound enzyme complex catalyzing the conversion of CO to CO_2 and H_2 at high rates [450 U (mg protein)$^{-1}$]has been reported (Soboh *et al.*, 2002). This enzyme complex, which was designated as CO-oxidizing:H_2-evolving enzyme complex, was found to be composed of eight polypeptides, the encoding genes were identified in the preliminary genome sequence of *C. hydrogenoformans* (see http://www.tigr.org.). From the sequence analysis it was deduced that the enzyme complex is composed of a Ni-containing carbon monoxide dehydrogenase (CooS), an electron transfer iron–sulfur protein (CooF) and a membrane-bound [NiFe] hydrogenase. The latter enzyme is composed of four hydrophilic subunits and two membrane integral subunits (CooM, K, L, X, U, and H). All subunits of the complex show high sequence identity to protein sequences deduced from the corresponding *R. rubrum* genes. The subunits forming the hydrogenase part of the enzyme complex also show high sequence identity to Ech hydrogenase and other members of this hydrogenase family. The CooS protein, which is the catalytic subunit of CO dehydrogenase has also been purified from the soluble fraction of *C. hydrogenoformans* as a homodimer (Svetlitchnyi *et al.*, 2001). Not all the CooS protein present in the cell is tightly associated with the hydrogenase. A possible explanation could be that the synthesis of CO dehydrogenase and hydrogenase are not completely coregulated resulting in an excess of CooS in the cell. From *C. hydrogenoformans* the CooS dimer of a second CO dehydrogenase (CO dehydrogenase II) has been purified (Svetlitchnyi *et al.*, 2001). The crystal structure of CO dehydrogenase II has been solved (Dobbek *et al.*, 2001).

On the basis of the data described above the architecture of the CO-oxidizing:H_2-forming enzyme complex shown in Fig. 2 is suggested. Each monomer of the CO dehydrogenase dimer contains one [Ni–4Fe–5S] active-site that is electrically connected to the [NiFe] center of the hydrogenase via an "iron wire" formed by several [4Fe–4S] clusters. According to this scheme the electron transfer reaction only involves the hydrophilic, peripheral subunits of the enzyme complex. This process somehow has to be coupled to energy conservation since *C. hydrogenoformans* grows with CO as sole energy source. Since the free energy associated with this reaction is only −20 kJ/mol direct synthesis of ATP via substrate-level phosphorylation can be excluded.

ENERGY-CONVERTING HYDROGENASES IN FERMENTING ORGANISMS

Members of the family of energy-converting hydrogenases have recently also been identified in organisms thought to have a purely fermentative metabolism. This includes the hyperthermophilic archaeon *P. furiosus* (Sapra *et al.*, 2000; Silva *et al.*, 2000) and the thermophilic gram-positive bacterium *Thermoanaerobacter tengcongensis* (Bao *et al.*, 2002). The genome of *P. furiosus* contains the putative *mbhA-N* operon encoding for a 14-subunit membrane-bound [NiFe] hydrogenase complex. Six of the deduced proteins correspond to subunits conserved in Ech hydrogenase, CO-induced hydrogenase, and *E. coli* hydrogenase 3. The *mbh* operon in addition encodes eight small proteins each predicted to form membrane-spanning helices. This is reminiscent of the *eha* and *ehb*

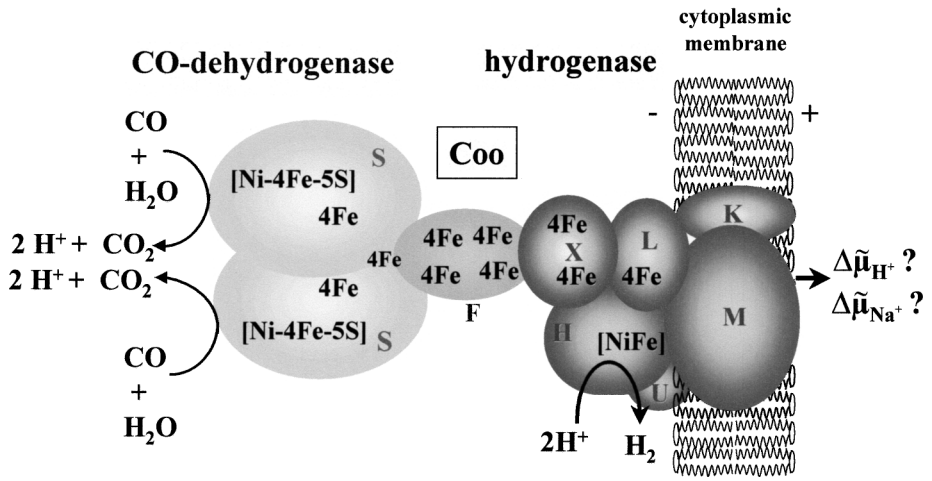

Fig. 2. Proposed CO-oxidizing: H_2-forming enzyme complex from *Carboxydothermus hydrogenoformans*. Abbreviations: [NiFe]: binuclear NiFe-active site of hydrogenase; 4Fe: [4Fe–4S] cluster; [Ni-4Fe–5S]: active-site of CO dehydrogenase. Capital letters indicate the subunits of the enzyme complex.

gene clusters, which in addition to subunits conserved in all energy converting hydrogenases, encode 10 or 9 non-conserved small integral membrane proteins, respectively. Thus far, a purification of the intact Mbh complex has not been achieved. However, washed membranes of *P. furiosus* contained a significant amount of hydrogenase activity. A partial purification of the hydrogenase present in the membrane fraction resulted in a preparation that allowed the determination of the aminoterminal sequences for two of the polypeptides present (Sapra *et al.*, 2000; Silva *et al.*, 2000). A determination of the catalytic properties using the partially purified enzyme revealed that the ratio of H_2 evolution activity to H_2 uptake activity was approximately 250:1. Most other [NiFe] hydrogenases favor hydrogen uptake over evolution. Membranes and partially purified hydrogenase preparations of *P. furiosus* not only catalyzed H_2 production with reduced methylviologen as electron donor but could also use reduced *P. furiosus* ferredoxin as electron donor. The kinetic data obtained indicate that this ferredoxin is the physiological substrate of Mbh (Silva *et al.*, 2000). With inverted membrane vesicles of *P. furiosus* it was recently shown that addition of reduced ferredoxin to the vesicles resulted in the generation of both a ΔpH and a $\Delta\Psi$ which could be coupled to ATP synthesis (Sapra *et al.*, 2003). The experiments performed do not allow distinguishing between a primary H^+ or a primary Na^+ pump mechanism. Hence, the H^+ translocation observed could be due to the conversion of a Na^+ gradient into a H^+ gradient by a Na^+/H^+ antiporter.

In conclusion, *P. furiosus* in addition to substrate-level phosphorylation can gain energy via oxidative phosphorylation. The Mbh hydrogenase present in these membrane vesicles can be assumed to function as a redox-driven ion pump coupling the reduction of protons with electrons derived from the oxidation of a low-potential ferredoxin to the generation of a H^+ (or Na^+) motive force. This also explains why *P. furiosus* has an unusual glycolytic pathway that uses ferredoxin in place of the expected NAD^+ as electron acceptor for glyceraldehyde 3-phosphate oxidation (Sapra *et al.*, 2003).

Thermoanaerobacter tengcongensis also has a fermentative life style. The organism ferments both monosaccharides and polysaccharides yielding H_2, CO_2, ethanol, and acetate as major metabolic end products (Xue *et al.*, 2001). On the basis of the analysis of the genome sequence the organism is proposed to convert glucose to pyruvate via a classical Emden–Meyerhof pathway generating NADH as a source of reducing equivalents. Part of the pyruvate is cleaved via pyruvate:ferredoxin oxidoreductase (POR) to acetyl-CoA and CO_2. Acetyl-CoA is converted to acetate via acetyl-phosphate. Reducing equivalents generated in the POR reaction are transferred to a ferredoxin. In addition to an NADH-oxidizing Fe-only hydrogenase the organism was found to contain a membrane-bound [NiFe] hydrogenase, which was purified and characterized (Soboh, B., and Hedderich, R., unpublished results). The enzyme is composed of six subunits. The encoding genes were identified in the completely sequenced genome of *T. tengcongensis*. They form a putative transcription unit that is organized similarly to the *ech* operon of *M. barkeri* and also the deduced proteins show high sequence identity to the subunits of Ech hydrogenase from *M. barkeri*. The enzyme catalyzes the production of H_2 with a *T. tengcongensis*-ferredoxin as electron donor. The catalytic efficiency coefficient (k_{cat}/K_m) was calculated to be 7.3×10^7 M^{-1} s^{-1} strongly suggesting that this is the physiological reaction catalyzed by this hydrogenase.

In conclusion, *T. tengcongensis* forms two different hydrogenases. One hydrogenase is NAD^+ active and in vivo is proposed to oxidize NADH regenerating NAD^+, the second enzyme oxidizes reduced ferredoxin and thus regenerates the ferredoxin as electron acceptor. As in *P. furiosus* the latter reaction is proposed to be coupled to energy conservation.

A POSSIBLE ENERGY COUPLING MECHANISM

For the discussion of how the exergonic electron transfer reaction in energy-converting hydrogenases is coupled to the generation of a proton or sodium-ion motive force it is essential to understand the electron transfer pathway in these enzymes. From the comparison with known structures of standard [NiFe] hydrogenases it can be inferred that the proximal cluster in the hydrogenase small subunit interacts with the [NiFe] center. Hence, the two [4Fe–4S] clusters located on subunit EchF can be predicted to transfer electrons from the ferredoxin substrate to the proximal cluster. This proposed electron transfer route only involves prosthetic groups in the hydrophilic part of the enzyme (Fig. 1). Unlike complex I quinones or compounds functionally equivalent to quinones, such as the methanogenic methanophenazine, seem not to be involved in the electron transfer reaction of the hydrogenases. This is based on several observations: (1) Quinones or the methanogenic methanophenazine are lacking from some of the organisms with energy-converting hydrogenases, for example *Methanothermobacter marburgensis* (Uwe Deppenmeier, personal communication) or *P. furiosus*. (2) The redox potential of known quinones or methanophenazine does not match the redox potential of the components of an electron transport chain, which operates in the redox range between -450 and -300 mV. (3) Recent studies with complex I strongly indicate that

subunit NuoD (49-kDa subunit) of complex I, which is highly homologous to the [NiFe] center carrying hydrogenase large subunit, forms part of a quinone-binding site (Brandt *et al.*, 2003; Yano and Ohnishi, 2001). This is based on mutant studies, which showed that amino acid residues in the carboxy-terminal part of this subunit affect the binding of ubiquinone antagonists such as piericidin or rotenone, which are strong inhibitors of complex I. The sequence region identified corresponds to the carboxy-terminal region of the hydrogenase large subunit that carries two of the four [NiFe] center-ligating cysteine residues. It has therefore been concluded that a significant part of the quinone-binding pocket of complex I is located within the NuoD (49-kDa subunit) and that the quinone-binding site in complex I has evolved from the [NiFe] site of hydrogenases (Darrouzet *et al.*, 1998; Kashani-Poor *et al.*, 2001).

An electron transfer route only involving the hydrophilic peripheral arm of the enzyme has important consequences for the discussion of how this hydrogenase uses redox-energy to transport charges across the cytoplasmic membrane. A conformational energy transfer mechanism would be most consistent with an electron transfer process not involving membrane-bound electron carriers. With our current knowledge it is purely speculative which step in the electron transfer pathway could induce such a conformational change. One of the iron–sulfur clusters could play a key role. A possible candidate is the iron–sulfur cluster with properties similar to that of cluster N-2 in complex I. In the catalytic mechanism suggested in Fig. 3 this cluster is accessible by two different proton channels. In its oxidized state the cluster is only accessible by a proton channel connecting the cluster with the cytoplasmic space. Reduction of the cluster results in the protonation of an acidic residue in the proximity of this cluster with a proton derived from the cytoplasm. Reduction and/or protonation induces a conformational change of the enzyme making the iron–sulfur cluster now accessible to a second proton channel which is largely formed by acidic residues of the two integral membrane subunits. Oxidation of the cluster results in the translocation of the proton via this transmembrane proton channel. The back-flow of the proton to the cytoplasmic side is prevented. The mechanism depicted in Fig. 3 basically would also allow the translocation of Na^+ instead of H^+.

Two integral membrane subunits are conserved within the family of energy-converting hydrogenases corresponding to EchA and EchB in the *M. barkeri* enzyme. EchB is a homologue of the complex I NuoH (ND1) protein. EchA is a homologue of the complex I NuoL, NuoM, or NuoN (ND2, ND4, or ND5) subunits, which most likely share a common ancestor and arose by gene triplication. These proteins are also related to a novel type of bacterial K^+ or Na^+/H^+ antiporter found in *Sinorhizobium*

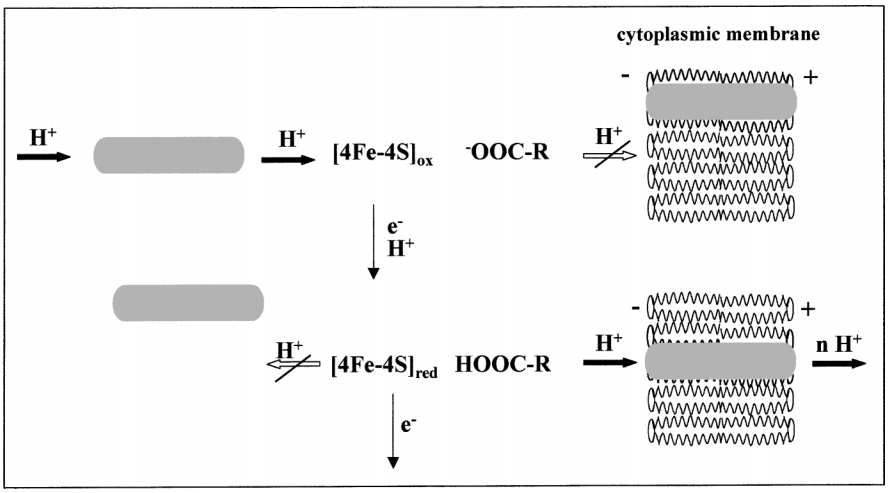

Fig. 3. Proposed conformational change mechanism for coupling of electron transfer to the translocation of H^+ (or Na^+) in energy-converting [NiFe] hydrogenases. In the oxidized enzymeone of the iron–sulfur clusters is accessible by a $H^+(Na^+)$ channel deriving from the cytoplasmic side of the enzyme. After reduction of the cluster a conformational change is induced which blocks the accessibility of the cluster by the cytoplasmic channel but makes the cluster accessible to a second, transmembrane H^+ (Na^+) channel. Upon reoxidation of the cluster the H^+ (Na^+) is translocated across the cytoplasmic membrane via the transmembrane channel. The backflow to the cytoplasmic side is blocked. The proposed ion channels are shown as gray bars.

meliloti and *Bacillus* sp. C-125 (Hamamoto *et al.*, 1994; Putnoky *et al.*, 1998). Both EchA and EchB share highly conserved acidic residues, predicted to be located in transmembrane helices, with the corresponding subunits of complex I. Also the Na^+/H^+ antiporters share the conserved acidic residues present in EchA and its complex I counterparts.

FINAL REMARK

Data obtained in the recent years strongly indicate that complex I and energy-converting [NiFe] hydrogenases share a conserved module that couples an exergonic redox reaction with the electrogenic translocation of a cation across a biological membrane. For the hydrogenases the coupling ion (H^+ or Na^+) and also the $H^+(Na^+)/e^-$ stoichiometry remains to be determined, but for thermodynamic reasons cannot exceed $2H^+(Na^+)/1e^-$. Since energy-converting hydrogenases are found in organisms exhibiting a quite ancient metabolism, such as growth on CO under anaerobic conditions, it is tempting to speculate that these ion pumps first developed in these organisms. Complex I may have evolved from these ion-pumping hydrogenases by the addition of alternative electron-input domains, replacement of the [NiFe] center by a quinone-binding site and the addition of further membrane subunits.

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