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Molecular Biology of Microbial Hydrogenases

P.M. Vignais and A. Colbeau

CEA Grenoble, Laboratoire de Biochimie et Biophysique des Systèmes Intégrés, UMR CEA/CNRS/UJF n° 5092, Département de Réponse et Dynamique Cellulaires, 17 rue des Martyrs, 38054 Grenoble Cedex 9, France

Abstract

Hydrogenases (H_2ases) are metalloproteins. The great majority of them contain iron-sulfur clusters and two metal atoms at their active center, either a Ni and an Fe atom, the [NiFe]-H_2ases, or two Fe atoms, the [FeFe]-H_2ases. Enzymes of these two classes catalyze the reversible oxidation of hydrogen gas (H_2 <--> 2 H^+ + 2 e^-) and play a central role in microbial energy metabolism; in addition to their role in fermentation and H_2 respiration, H_2ases may interact with membrane-bound electron transport systems in order to maintain redox poise, particularly in some photosynthetic microorganisms such as cyanobacteria. Recent work has revealed that some H_2ases, by acting as H_2-sensors, participate in the regulation of gene expression and that H_2-evolving H_2ases, thought to be involved in purely fermentative processes, play a role in membrane-linked energy conservation through the generation of a protonmotive force. The Hmd hydrogenases of some methanogenic archaea constitute a third class of H_2ases, characterized by the absence of Fe-S cluster and the presence of an iron-containing cofactor with catalytic properties different from those of [NiFe]- and [FeFe]-H_2ases. In this review, we emphasise recent advances that have greatly increased our knowledge of microbial H_2ases, their diversity, the structure of their active site, how the metallocenters are synthesized and assembled, how they function, how the synthesis of these enzymes is controlled by external signals, and their potential use in biological H_2 production.

Introduction

The decrease in fossil energy resources will lead to the emergence of new energy sources. Sustained energy is the energy that comes from renewable sources, i.e. sunlight, water, biomass. Among the new fuel to come, hydrogen gas is one of the most promising, since its combustion yields water and water can be used to regenerate H_2. Numerous microorganisms can produce H_2 by reactions linked to their energy metabolism. These microorganisms use the protons from H_2O as electron acceptors to dispose of excess reducing power in the cell and to reoxidize their coenzymes in the absence of oxygen. However, most of the organisms able to produce H_2 are also able to consume it, i.e. to oxidize H_2. The key enzyme involved in the metabolism of H_2 is the **hydrogenase** (H_2ase) enzyme.

H_2ases catalyze the simplest chemical reaction:
2 H^+ + 2 e^- <--> H_2.
The reaction is reversible and its direction depends on the redox potential of the components able to interact with the enzyme. In the presence of an electron acceptor, a H_2ase will act as a H_2 uptake enzyme, while in the presence of an electron donor, the enzyme will produce H_2. It is necessary to understand how microorganisms metabolize H_2 in order to induce them to produce H_2 continuously and in large amounts.

The combination of X-ray crystallography, molecular biology and spectroscopic techniques has markedly accelerated the pace of study of the H_2ase enzymes. The prospect of understanding how these enzymes function is the creation of biomimetic and biotechnological devices for energy production. More than one hundred H_2ases have been characterized genetically and/or biochemically. By comparing their amino acid sequences, it has been possible to identify classes and subgroups of enzymes, to compare and correlate genetic, physiological and biochemical information relative to members of the subgroups, independently from their origin and their various roles in energy metabolism. Here we aim to provide a brief overview on H_2ases, their diversity, their biosynthesis, their mechanism of action and provide an exploratory outlook of their biotechnological use for biological H_2 production. Complementary and more detailed information on this topic may be found in the book "Hydrogen as a fuel. Learning from Nature" (Cammack *et al*., 2001), and in the special issue of the International Journal of Hydrogen Energy, "Biohydrogen 2002", (van Niel *et al*., 2002).

Diversity of hydrogenases

Most of the known H_2ases are iron-sulfur proteins with two metal atoms at their active site, either a Ni and an Fe atom (in [NiFe]-H_2ases) (Volbeda *et al*., 1995; Higuchi *et al*., 1997) or two Fe atoms (in [FeFe]-H_2ases) (Peters *et al*., 1998; Nicolet *et al*., 1999). A different type of H_2ases was discovered in some methanogens (Zirngibl *et al*., 1992), which function as H_2-forming methylenetetrahydrometha nopterin dehydrogenases, abbreviated Hmd (Thauer *et al*., 1996). They contain no Fe-S cluster and no Ni and were termed "metal-free" H_2ases (Berkessel and Thauer, 1995). Recently, the group of Thauer (Lyon *et al*., 2004) has demonstrated that Hmd activity depends on an iron-containing cofactor. Thus, this type of H_2ase is more correctly described as an iron-sulfur cluster-free H_2ase. Evidence from sequences and structures indicates that the [NiFe]-, the ("iron-only") [FeFe]- and the "iron-sulfur cluster-free" H_2ases are phylogenetically distinct classes of proteins (Vignais *et al*., 2001).

*For correspondence. Email paulette.vignais@cea.fr.

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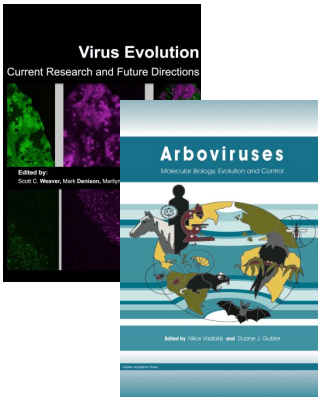

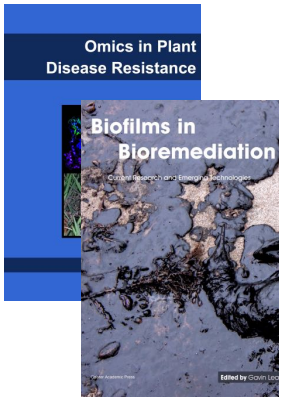

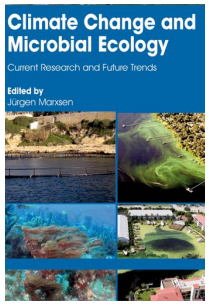

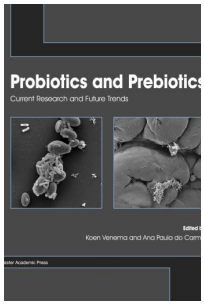

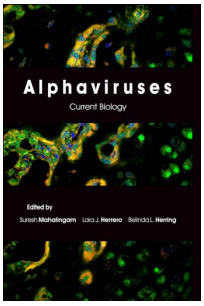

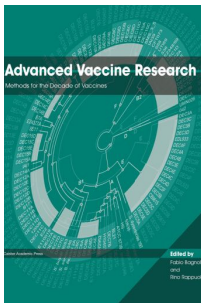

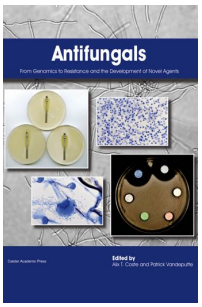

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The [NiFe]-H_2ases

The most numerous and best studied class of H_2ases have been the [NiFe]-H_2ases from the domain of life *Bacteria*. [NiFe]-H_2ases have also been isolated from *Archaea*, archaeal genes have been cloned, and recently several new [NiFe]-H_2ases revealed by archaeal genome sequencing have been identified and isolated (reviewed by Wu and Mandrand, 1993; Vignais *et al*., 2001 and Frey, 2002). The core enzyme consists of an $\alpha\beta$ heterodimer with the large subunit (α-subunit) of ca 60 kDa hosting the bimetallic active site and the small subunit (β-subunit) of ca 30 kDa, the Fe-S clusters. Crystal structures of *Desulfovibrio* H_2ases have revealed the general fold and the nature of the binuclear NiFe active site; they have shown that the two subunits interact extensively through a large contact surface and form a globular heterodimer (Volbeda *et al*., 1995, 1996; Higuchi *et al*., 1997, 1999; Garcin *et al*., 1999; Matias *et al*., 2001). The bimetallic NiFe center is deeply buried in the large subunit; it is coordinated to the protein by four cysteines. Further studies using infrared spectroscopy revealed the presence of three non-protein ligands, 1 CO and 2 CN^-, bound to the Fe atom (Volbeda *et al*., 1996; Happe *et al*., 1997). The small subunit contains up to three Fe-S clusters which conduct electrons between the H_2-activating center and the physiological electron acceptor (or donor) of H_2ase. The [4Fe-4S] cluster that is proximal to the active site (within 14 Å) is "essential" to H_2 activation (Volbeda *et al*., 1995; Fontecilla-Camps *et al*., 1997). Hydrophobic channels linking the active site to the surface of the molecule have been suggested to facilitate gas access to the active site (Fontecilla-Camps *et al*., 1997; Montet *et al*., 1997). Based on the full sequence alignments of the small and large subunits it has been shown that the two subunits of [NiFe]-H_2ases evolved conjointly. This analysis led to a classification of [NiFe]-H_2ases into four groups, which is consistent with the functions of the enzymes (Vignais *et al*., 2001) (Figure 1).

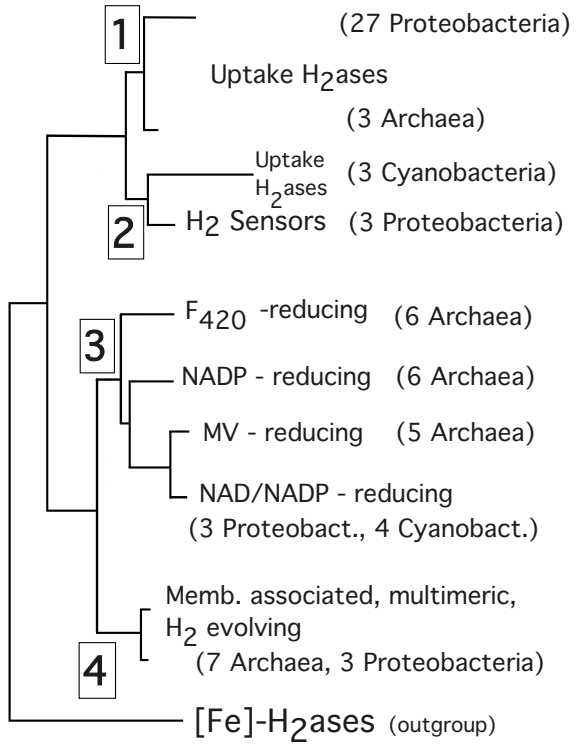

Figure 1. Schematic representation of the phylogenetic tree of [NiFe]-H_2ases based on the complete sequences of the small and the large subunits (the same tree was obtained with each type of subunit) originally established by Vignais *et al*. (2001).

The uptake [NiFe]-H_2ases (Group 1)

These are membrane-bound respiratory enzymes, which allow the cells to use H_2 as an energy source. They link the oxidation of H_2 to the reduction of anaerobic electron acceptors, such as NO_3^-, SO_4^{2-}, fumarate or CO_2 (anaerobic respiration) or to O_2 (aerobic respiration), with recovery of energy in the form of a protonmotive force. They are connected to the quinone pool of the respiratory chain in the membrane by a third subunit, a di-heme cytochrome *b*, which, together with the hydrophobic C-terminus of the small subunit, anchors the H_2ase dimer to the membrane. Electrons from H_2 are donated to the quinone pool and the energy of H_2 oxidation is recovered by vectorial proton transfer (reviewed by Vignais *et al*., 2004). Being linked to redox components of potentials higher than that of the H_2/H^+ couple, these uptake H_2ases, found in Proteobacteria and recently characterized in *Aquifex aeolicus* (Brugna-Guiral *et al*., 2003), serve to consume H_2. A similar membrane-bound uptake H_2ase has been identified in the methanogenic archaeon *Methanosarcina mazei* Gö1 (Ide *et al*., 1999). It uses the methanogenic methanophenazine, which fulfills the same function as quinones in bacteria. Its third subunit (also a cytochrome *b*), donates the electrons from H_2 to the formation of methane. This electron transfer system is coupled to vectorial proton transfer, leading to the creation of a protonmotive force.

Another type of uptake H_2ase is represented by the periplasmic H_2ase of *Desulfovibrio* species. It is able to interact with low-potential *c*-type cytochromes and a transmembrane redox protein complex encoded by the *hmc* operon (Rossi *et al*., 1993) and to participate in the creation of a proton gradient across the membrane for energy conservation (reviewed by Fauque *et al*., 1988 and Vignais *et al*., 2001). However, Hmc does not appear to be the only complex capable of coupling electron transport to proton pumping (Dolla *et al*., 2000) and some additional redox partners, not yet identified, seem to be required for the establishment of proton gradient across the membrane for energy conservation during H_2 oxidation; a second Hmc-related complex, as identified in *Desulfovibrio vulgaris* (Valente *et al*., 2001), might participate in the creation of a protonmotive force in the membrane. Other uptake H_2ases, e.g. H_2ase-2 of *E. coli* encoded by the *hybOABCDEFG* operon (Dubini *et al*., 2002), and H_2ase-1 of *Thiocapsa roseopersicina* encoded by the *hydSisp1isp2hydL* operon (Rákhely *et al*., 1998), have subunits that share significant degrees of identity with two subunits of the Hmc complex.

The uptake H_2ases are characterized by the presence of a long signal peptide (30-50 amino acid residues) at the N-terminus of their small subunit. The signal peptide contains a conserved (S/T)RRxFxK motif recognized by a specific protein translocation pathway known as the membrane targeting and translocation (Mtt) (Weiner *et*

al., 1998) or twin-arginine translocation (Tat) (Sargent *et al*., 1998) pathway, and serves as signal recognition to target the fully folded heterodimer to the membrane and the periplasm (Wu *et al*., 2000a,b; Berks *et al*., 2000; Vordouw 2000; Vignais *et al*., 2001; Sargent *et al*., 2002). The Tat pathway is structurally and mechanistically similar to the ΔpH-dependent pathway used to import chloroplast proteins into the thylakoid (cf Bogsch *et al*., 1998; Wu *et al*., 2000a; Berks *et al*., 2003). Homologues of Tat proteins are found in many archaea, bacteria, chloroplasts and mitochondria (Yen *et al*., 2002). Several H_2ases of group 1, *E. coli* H_2ase-1 and H_2ase-2 (Sargent *et al*., 1998; Bogsch *et al*., 1998; Chanal *et al*., 1998; Rodrigue *et al*., 1999; Dubini *et al*., 2002), the membrane-bound H_2ase of *Wolinella succinogenes* (Gross *et al*., 1999) and of *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) (Bernhard *et al*., 2000) have been shown to be exported by this so-called hitchhiker mechanism of cotranslocation of the two subunits.

The cytoplasmic H_2 sensors and the cyanobacterial uptake [NiFe]-hydrogenases (Group 2)

The small subunit of the enzymes of group 2 does not contain a signal peptide at its N-terminus; these H_2ases are not exported but remain in the cytoplasm. Two representatives of group 2, the *Rhodobacter capsulatus* HupUV H_2ase (Vignais *et al*., 2000) and the *R. eutropha* HoxBC H_2ase (Kleihues *et al*., 2000) are indeed soluble, cytoplasmic proteins. They are not directly involved in energy transducing reactions. Their role is to detect the presence of H_2 in the environment and to trigger a cascade of cellular reactions controlling the synthesis of respiratory [NiFe]-H_2ases (see below).

Group 2 also includes the so-called uptake H_2ases (HupSL) of the cyanobacteria *Nostoc* (Oxelfelt *et al*., 1998) and *Anabaena variabilis* (Happe T. *et al*., 2000). These enzymes, which are induced under N_2 fixing conditions, are localised on the cytoplasmic side of either the cytoplasmic or thylakoid membrane (Appel and Schulz, 1998; Tamagnini *et al*., 2002). The third H_2ase of *A. aeolicus*, a soluble enzyme which belongs to group 2 has been proposed to provide reductant to the reductive TCA cycle for CO_2 fixation (Brugna-Guiral *et al*., 2003).

The bidirectional heteromultimeric cytoplasmic [NiFe]-hydrogenases (Group 3)

In group 3, the dimeric H_2ase module is associated with other subunits that are able to bind soluble cofactors, such as cofactor 420 (F_{420}, 8-hydroxy-5-deazaflavin), NAD or NADP (Figure 1). They are termed bidirectional because, physiologically, they function reversibly and can thus reoxidize the cofactors under anaerobic conditions by using the protons of water as electron acceptors. Many members of this group are found in *Archaea*. They include the trimeric F_{420}-reducing H_2ases, the tetrameric bifunctional H_2ases of hyperthermophiles, able to reduce S° to H_2S *in vitro* and to use NADPH as electron donor (Ma *et al*., 1994) and the F_{420}-non-reducing H_2ases. It has recently been shown that the physiological role of the F_{420}-non-reducing H_2ase (Mvh) from *Methanothermobacter marburgensis* is to provide reducing equivalents for heterodisulfide reductase (Stojanowic *et al*., 2003). In *Methanosarcina mazei*, the energy-conserving electron transfer from H_2 involves a [NiFe]-H_2ase, a *b*-type cytochrome and $F_{420}H_2$ dehydrogenase. The $F_{420}H_2$ dehydrogenase, encoded by the *fpo* genes, is a redox-driven proton pump sharing similarities with the proton-translocating NADH:quinone oxidoreductase of respiratory chains (Bäumer *et al*., 2000; reviewed by Deppenmeier *et al*., 1999).

Bidirectional NAD(P)-linked H_2ases are also found in bacteria and cyanobacteria. The first NAD-dependent, tetrameric [NiFe]-H_2ase was isolated from *R. eutropha* (*A. eutrophus*) (Schneider and Schlegel, 1976; Tran-Betcke *et al*., 1990); homologous enzymes were later discovered in cyanobacteria (Schmitz *et al*., 1995, 2002; Appel and Schulz, 1996) and recently in the photosynthetic bacterium *T. roseopersicina* (Rákhely *et al*., 2004). These bidirectional H_2ases are composed of two moieties: the heterodimer [NiFe]-H_2ase moiety encoded by the *hoxY* and *hoxH* genes, and the diaphorase moiety, encoded by the *hoxU, hoxF* (and also *hoxE* in cyanobacteria and in *T. roseopersicina*) genes, which is homologous to subunits of complex I of the mitochondrial and bacterial respiratory chains and contains NAD(P), FMN and Fe-S binding sites (Figure 2) (reviewed by Friedrich and Schwartz, 1993; Appel and Schulz, 1998; Vignais *et al*., 2001; 2004; Tamagnini *et al*., 2002). The bidirectional H_2ase may function in cyanobacteria as an electron valve for disposal of low-potential electrons generated at the onset of illumination (Appel *et al*., 2000).

The H_2-evolving, energy-conserving, membrane-associated hydrogenases (Group 4)

These multimeric (six subunits or more) enzymes reduce protons from water in order to dispose of excess reducing equivalents produced by the anaerobic oxidation of C_1 organic compounds of low potential, such as carbon monoxide or formate. *E. coli* H_2ase-3, the prototype of this group, encoded by the *hyc* operon, is part of the formate hydrogen lyase complex (FLH-1) (encoded by *hycBCDEFGHI)* (Böhm *et al*., 1990; Sauter *et al*., 1992), which metabolizes formate to H_2 and CO_2 (Böck and Sawers, 1996; Sawers, 1994). At another locus, the *hyf* operon of *E. coli* encodes a putative 10-subunit hydrogenase complex (H_2ase-4). Seven genes of the *hyf* operon (*hyfABCGHIJ)* encode homologues of seven Hyc subunits of H_2ase-3. Three additional genes (*hyfD, hyfE* and *hyfF*) that have no counterpart in the Hyc complex are capable of encoding integral membrane proteins, two of them sharing similarities with subunits which play a crucial role in proton translocation and energy coupling in the NADH:quinone oxidoreductase (complex I) (see below). Originally, Andrews *et al*. (1997) proposed that the *hyf* operon encodes a novel H_2ase complex (H_2ase-4) which combines with formate dehydrogenase H (Fdh-H) to form a second formate hydrogenlyase (FHL-2) and that, unlike FHL-1, FHL-2 is an energy-conserving proton-translocating system. However, up to now, no Hyf-derived H_2ase or formate dehydrogenase activity could be detected and no Ni-containing protein corresponding to HyfG, the large subunit of H_2ase-4, was observed (Skibinski *et al*., 2002). In addition, *E. coli* H_2ase-3 (Hyc) closely resembles the CO-induced H_2ase (Coo) from *Rhodospirillum rubrum*, which is energy conserving.

The CO-induced H_2ase of *R. rubrum*, another member of this group, is a component of the CO-oxidizing system that allows *R. rubrum* to grow in the dark with CO as sole energy source. CO-dehydrogenase and the H_2ase (CooLH) encoded by the *coo* operon oxidize CO to CO_2 with concomitant production of H_2. It has been proposed that the H_2ase component of the oxidizing system constitutes the energy coupling site, since the CO dehydrogenase is a peripheral membrane protein (Fox *et al*., 1996a,b). *E. coli* H_2ase-3 and *R. rubrum* CooLH H_2ase are labile enzymes; the exact number of their subunits is still unknown. On the other hand, a homologous enzyme complex was isolated from the thermophilic Gram-positive bacterium *Carboxydothermus hydrogenoformans* (Soboh *et al*., 2002; reviewed by Hedderich, 2004). It comprises a Ni-containing CO-dehydrogenase (CooS), an electron transfer protein containing four [4Fe-4S] clusters (CooF) and a membrane-bound [NiFe]-H_2ase composed of four hydrophilic subunits and two membrane integral subunits (CooL,X,U,H and CooM,K), which couple the conversion of CO to CO_2 and H_2 to energy conservation.

The majority of H_2ases assigned to group 4 have been found in *Archaea* (Figure 1), including *Methanosarcina barkeri* (Künkel *et al*., 1998), *Methanobacterium thermoautotrophicum* strain Marburg (now called *Methanothermobacter marburgensis)* (Tersteegen and Hedderich, 1999) and *Pyrococcus furiosus* (Sapra *et al*., 2000; Silva *et al*., 2000). The membrane-bound [NiFe]-H_2ase found in the acetate-grown methanogenic archaeon *M. barkeri*, catalyzes H_2 formation from reduced ferredoxin, generated by the oxidation of the carbonyl group of acetate to CO_2, by an energy-conserving mechanism (Künkel *et al*., 1998; Meuer *et al*., 1999, 2002). The enzyme, purified from acetate-grown cells, consists of six subunits encoded by the *echABCDEF* operon. The EchA and EchB subunits are predicted to be integral membrane-spanning proteins while the other four are expected to extrude into the cytoplasm. Electron paramagnetic resonance (EPR) studies of the Ech enzyme have assigned two [4Fe-4S] clusters to the EchF subunit and one [4Fe-4S] cluster to the EchC subunit (Figure 2). Redox titrations at different pH values demonstrated that the proximal cluster (in the EchC subunit) and one of the clusters in the EchF subunit have a pH-dependent mid-point redox potential (Kurkin *et al*., 2002), a result which supports the hypothesis that the Fe-S clusters are involved in an electron-transfer driven proton-pumping unit as was already suggested by Albracht and Hedderich (2000). The use of a *M. barkeri* mutant lacking Ech H_2ase (Δ*ech*) revealed that this enzyme is absolutely required for the reduction of CO_2 to formylmethanofuran by H_2. Ech catalyzes the reduction of a low-potential ferredoxin by H_2 and the reduced ferredoxin serves as electron donor for the synthesis of formylmethanofuran (Meuer *et al*., 2002). The authors suggested that the thermodynamically unfavorable reduction of ferredoxin by H_2 is coupled to the consumption of a membrane ion gradient, the Ech H_2ase functioning as an ion pump. It is to note that a membrane-bound Ech [NiFe]-H_2ase has recently been identified in *Desulfovibrio gigas* (Rodrigues *et al*., 2003). The methanogenic archaeon *M. thermoautotrophicum,* which is able to grow on CO_2/H_2 as carbon and energy source contains, in addition to F_{420}-reducing and F_{420}-non-reducing H_2ases, two gene groups designated "energy converting H_2ase A" (*eha*) and "energy converting H_2ase B" (*ehb*), which encode putative, multisubunit, membrane-bound H_2ases homologous to *E. coli* H_2ase-3 and *R. rubrum* CooHL H_2ase (Tersteegen and Hedderich, 1999). The hyperthermophilic archaeon *P. furiosus* contains two cytoplasmic H_2-evolving H_2ases (I and II) (Pedroni *et al*., 1995; Ma *et al*., 2000), members of group 3, and a membrane-bound H_2ase (MBH), member of group 4, encoded by a 14-gene operon (Schut *et al*., 2001) termed *mbh* (either *mbh1-14* (Sapra *et al*., 2000) or *mbhA-N* (Silva *et al*., 2000)). Four gene products of this operon share similarities with subunits of complex I. These multimeric membrane-bound H_2ase complexes comprise transmembrane subunits homologous to complex I subunits involved in proton pumping and energy coupling and appear to be able to couple the oxidation of a carbonyl group (originating from formate, acetate or carbon monoxide) with the reduction of protons to H_2 (reviewed by Hedderich, 2004). Indeed, MBH from *P. furiosus* was shown recently to couple electron transfer from reduced ferredoxin to both proton reduction and proton translocation, i.e. to couple the production of H_2 to ATP synthesis (Sapra *et al*., 2003).

The [FeFe]-hydrogenases

Unlike [NiFe]-H_2ases, composed of at least two subunits, many [FeFe]-H_2ases are monomeric and consist of the catalytic subunit only (although dimeric, trimeric and tetrameric enzymes are also known (Vignais *et al*., 2001)). The smallest [FeFe]-H_2ases (ca 45-48 kDa) have been found in green algae (Happe and Naber, 1993; Happe *et al*, 1994). The catalytic subunit of [FeFe]-H_2ases, in contrast to those of Ni-containing enzymes, vary considerably in size. Besides the conserved domains of ca 350 residues containing the active site (H-cluster, Adams, 1990), they often comprise additional domains, which accommodate Fe-S clusters. The H-cluster consists of a binuclear [FeFe] center bound to a [4Fe-4S] cluster by a bridging cysteine and attached to the protein by four cysteine ligands. Non-protein ligands, CN^- and CO, are attached to both iron atoms (Peters *et al.*, 1998; Peters, 1999; Nicolet *et al.*, 1999). Similarly to [NiFe]-H_2ases (Montet *et al.*, 1997; Frey *et al*., 2001), hydrophobic H_2 channels and H^+ channels lined with hydrophilic side chains, connecting the buried active site to the surface of the protein, have been identified in [FeFe]-H_2ases (Peters *et al.*, 1998; Nicolet *et al.*, 1999; 2000).

This type of enzyme is found in anaerobic prokaryotes, such as clostridia and sulfate reducers (reviewed by Adams, 1990; Atta and Meyer, 2000 and Vignais *et al*., 2001) and in eukaryotes (Akhmanova *et al*., 1998; Horner *et al*., 2000, 2002; Florin *et al*., 2001, Wünschiers *et al*., 2001b, Happe and Kaminsky, 2002; Happe *et al*., 2002; Voncken *et al*., 2002; Forestier *et al*., 2003). [FeFe]-H_2ases are the only type of H_2ase to have been found in eukaryotes, and they are located exclusively in membrane-limited organelles, i.e. in chloroplasts or in hydrogenosomes. While [NiFe]-H_2ases tend to be involved in H_2 consumption, [FeFe]-H_2ases are usually involved in H_2 production. However, the periplasmic [FeFe]-H_2ase of *D. vulgaris* has been demonstrated to function as an uptake H_2ase (Pohorelic *et al*., 2002). Recently, components of an [FeFe]-H_2ase

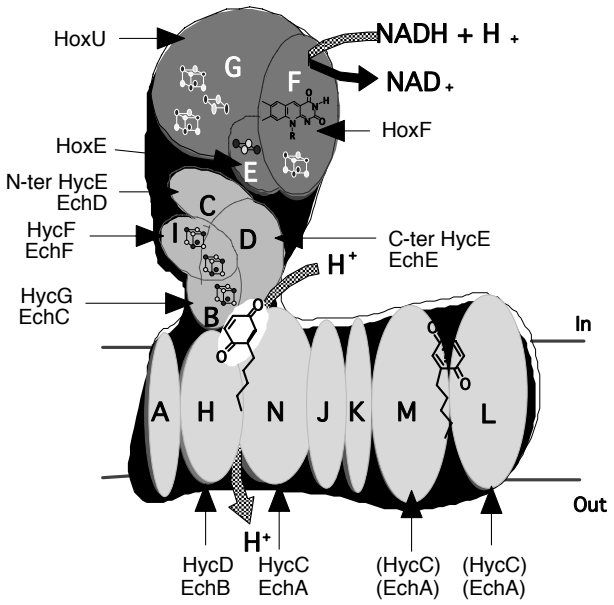

Figure 2. Relationship of *R. capsulatus* complex I subunits (NuoA-N) to [NiFe]-H_2ases subunits from S*ynechocystis* PCC 6803 (HoxEFUYH) (Appel and Schulz, 1996; Schmitz *et al.* 2002), *E. coli* H_2ase-3 (HycCDEFG) (Sauter *et al*., 1992) and *Methanosarcina barkeri* (EchABCDEF) (Künkel *et al*., 1998) (Adapted from Dupuis *et al*., 2001; Holt *et al*., 2003; Hedderich 2004). N-ter: N-terminus; C-ter: C-terminus. The [4Fe-4S] or [2Fe-2S] clusters are shown in the appropriate subunits.

have been found associated with formate dehydrogenases from *Eubacterium acidaminophilum* (Graentzdoerffer *et al*., 2003). In green algae, the [FeFe]-H_2ase is an electron "valve" that enables the algae to survive under anaerobic conditions (Happe *et al*., 2002).

The iron-sulfur cluster free-hydrogenases, Hmd

The H_2ases found in some methanogenic archaea, called Hmd, do not contain Fe-S clusters or nickel (Zirngibl *et al.,* 1992). This type of H_2ase is induced in cells grown under nickel limitation (Afting *et al*., 1998; 2000). The enzyme consists of two identical subunits of ca 40 kDa; it catalyzes the reversible reduction of N^5,N^{10}-methenyltetrahydrome thanopterin (methenyl-H_4MPT$^+$) with H_2 to methylenetetr ahydromethanopterin (methylene-H_4MPT) and a proton. It is active only in the presence of its second substrate, methenyl-H_4MPT$^+$. It does not mediate the reduction of dyes such as methylviologen or methylene blue nor the exchange of protons with hydrogen isotopes (unless the substrate methenyl-H_4MPT$^+$ is present) (Schwörer *et al.,* 1993). It was shown recently to bind a cofactor, which can reconstitute active Hmd from the apoenzyme (Buurman *et al*., 2000). The enzyme and the cofactor are sensitive to UV-A (320-400 nm) or blue (400-500 nm) light. The Hmd purified in the dark contains one Fe per monomer, which is released from the enzyme and from the isolated cofactor upon light inactivation. High CO concentrations inhibit Hmd and protect it against light inactivation. For all these reasons, it was concluded by the group of Thauer (Lyon *et al*., 2004) that the iron has a functional role in the enzyme, which can no longer be called a "metal-free" H_2ase. Upon light inactivation or heat inactivation a stable product with a molecular mass of 542 Da is formed from the active cofactor. The proposed structure for the inactivated cofactor is 6-carboxymethyl-3,5-dimethyl 4-guanylyl-2-pyridone (Shima *et al*., 2004).

In short, the Hmd enzymes differ from the [NiFe]- and [FeFe]-H_2ases not only by the primary and tertiary structures but also by the fact that the iron, required for enzyme activity is not redox active. They are associated with a specific cofactor, and have catalytic properties different from those described for [NiFe]- and [FeFe]-H_2ases, in particular they do not catalyze the reversible reaction: 2 H^+ + 2 e^- <--> H_2.

Hydrogenases and complex I

Sequence similarities between H_2ases and complex I (NADH dehydrogenase) were first reported by Böhm *et al.* (1990) and by Pilkington *et al*. (1991) and have been emphasized in several subsequent reports (see, e.g. Albracht and de Jong, 1997; Friedrich and Weiss, 1997; Friedrich and Scheide, 2000; Albracht and Hedderich, 2000; Dupuis *et al*., 2001; Friedrich, 2001; Yano and Ohnishi, 2001; Vignais *et al*., 2001, 2004; Hedderich, 2004). To compare the subunits of H_2ases to those of complex I, we have adopted the nomenclature used for *E. coli* and *R. capsulatus* enzymes (Friedrich, 1998; Dupuis *et al*., 1998) (Figure 2). Subunits NuoE, NuoF, NuoI and the N-terminal Fe-S binding domain (ca 220 residues) of NuoG have homologous counterparts in accessory subunits and domains of soluble [NiFe]-H_2ases (see above) and [FeFe]-H_2ases (Malki *et al*., 1995; Verhagen *et al*, 1999). In addition, three subunits located within the connecting module of complex I share similarities with subunits of [NiFe]-H_2ases, the NuoB subunit with the small H_2ase subunit, the NuoC and NuoD subunits (fused as a single NuoCD protein in *E. coli*) with the large H_2ase subunit. Furthermore, hydrophobic subunits of multimeric, membrane-bound [NiFe]-H_2ases belonging to group 4 (reviewed by Hedderich, 2004), namely *E. coli* Hyc, *R. rubrum* Coo, *M. barkeri* Ech, *M. marburgensis* Eha and Ehb and *P. furiosus* Mbh, are also homologous to transmembrane subunits of complex I (NuoH, NuoL, NuoM, NuoN). It should be noted that these H_2ases of group 4 are also ion (H^+ or Na^+) pumps. The nature of the coupling ion used by these enzymes is still elusive. Thus, the presumed evolutionary links between H_2ases and complex I concern not only the electron transferring subunits but also the ion pumping units, i.e. the coupling between electron transport and energy recovery by a chemiosmotic mechanism.

On the basis of the similarities between [NiFe]-H_2ases and the NuoB-NuoD dimer of the connecting module, Dupuis *et al*. (2001) have proposed that the [NiFe] active site of H_2ases was reorganized into a quinone-reduction site, carried by the NuoB-NuoD dimer and a hydrophobic subunit such as NuoH (Figure 2), and that NuoD might provide both the quinone gate and a potential proton channel entry for a minimal "H^+ (or Na^+) pumping" module, composed of subunits NuoB, NuoD, NuoI, NuoH and NuoL (Friedrich and Scheide, 2000; Dupuis *et al*., 2001; see also Kashani-Poor *et al*., 2001). Subunit NuoL, (or NuoM, or NuoN, which apparently evolved by triplication of an ancestral gene related to bacterial H^+/K^+ antiporters (Fearnley and Walker, 1992; Friedrich and Weiss, 1997)),

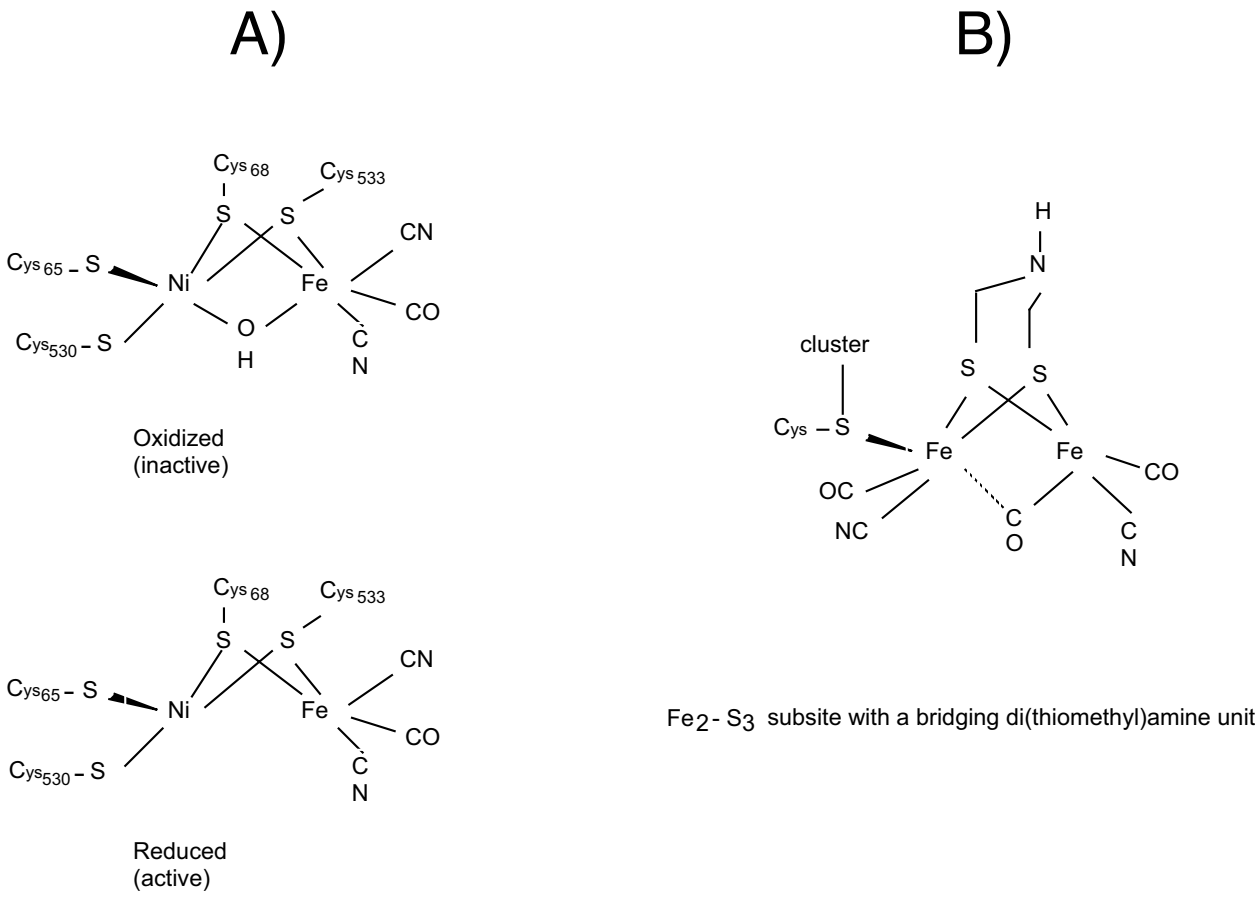

Figure 3. Schematic structures of the active site of [NiFe]- and [FeFe]-H_2ases. A) [Ni-Fe]-Hases: the oxidized inactive form corresponds to the Ni-A state and the reduced active form to the Ni-S state (see Figure 4). B) [FeFe]-H_2ases: the Fe_2-S_3 sub-site of the H-cluster. The diatomic ligand, CO, is seen in a bridging position in *C. pasteurianum* H_2ase (CpI) (Peters *et al*., 1998) while in the *D. desulfuricans* H_2ase (DdH) it appears rather asymetrically bound to Fe2 (distal to the [4Fe-4S] cluster) (Nicolet *et al*., 1999, 2000, 2001).

would have provided the transmembrane channel required to complete the proton (or Na^+) pump (Dupuis *et al*., 2001; Mathiesen and Hagerhall, 2002; Hedderich, 2004).

Active sites and model compounds

Active sites

The X-ray crystallographic studies of *[NiFe]-H_2ases* isolated from *Desulfovibrio gigas* (Volbeda *et al*., 1995, 1996), *Desulfovibrio vulgaris* Miyazaki (Higuchi *et al*., 1997, 1999), *Desulfovibrio fructosovorans* (Volbeda *et al*., 2002), *Desulfomicrobium norvegium* (formerly *Desulfomicrobium baculatum*) (Garcin *et al*., 1999) and *Desulfovibrio desulfuricans* ATCC 27774 (Matias *et al*., 2001) have revealed the unique dinuclear [NiFe] active site of these enzymes (Figure 3). The nickel atom is coordinated by four cysteinate-sulfur atoms, two of which bridge to the iron atom (in the *D. norvegium* enzyme, a selenocysteine instead of a cysteine coordinates the Ni). In the aerobically isolated inactive form of the enzyme, there is an additional μ-oxo or hydroxo (Garcin *et al*., 1999; Volbeda *et al*., 2002) or sulfido group (Higuchi *et al*., 1997; Matias *et al*., 2001) bridging the Ni and Fe atoms. In addition, as demonstrated by crystallography and spectroscopy, the iron atom is bound to non-protein diatomic ligands, normally poisonous molecules, two CN^- and one CO (Volbeda *et al*., 1996; Happe *et al*., 1997; Pierik *et al*., 1999), or SO, CO and CN^- in *D. vulgaris* (Higuchi *et al*., 1997, 2000). The FTIR and EPR properties of the metallocenter of the cytoplasmic NAD-reducing H_2ase of *R. eutropha* (SH) suggested the presence of two additional CN^- ligands, so that SH may have a $Ni(CN)Fe(CN)_3(CO)$ at its active site (Happe R.P. *et al*., 2000).

The *[FeFe]-H_2ases* from *Clostridium pasteurianum* (Peters *et al*., 1998) and *D. desulfuricans* (Nicolet *et al*., 1999) contain at their active site an unprecedented metal cluster called the H-cluster. The latter consists of a binuclear iron sub-site ($[Fe_2S_3]$) bound to a conventional [4Fe-4S] cluster by a bridging cysteinyl sulfur. To each Fe atom a terminal carbon monoxide, a bridging carbon monoxide and a cyanide ligand are bound. The Fe atoms also share two bridging sulfur ligands of a 1,3-propanedithiolate (or rather the related di(thiomethyl)amine molecule, $HN(CH_2\text{-}S^-)_2$, (Nicolet *et al*. 2002)). The Fe atom distal to the [4Fe-4S] cluster (Fe2) has a vacant coordination site and is occupied by carbon monoxide, a competitive inhibitor, in the CO-inhibited form of the enzyme (Lemon and Peters, 1999; Bennet *et al*., 2000; Chen *et al*., 2002); it is therefore thought to be the position where hydride/dihydrogen is bound during enzyme turnover.

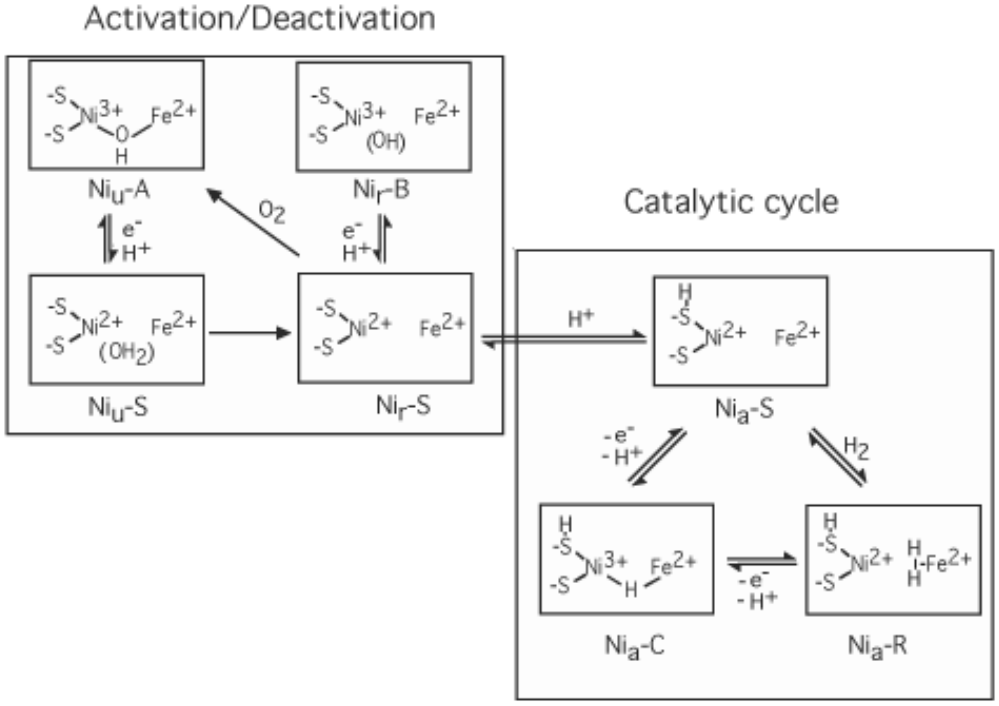

Figure 4. Proposed enzymatic mechanism of [NiFe]-H_2ases. The scheme illustrates the spectroscopically characterized states and coordination of the NiFe active site. The bridging sulfur ligands and the diatomic ligands to Fe are omitted, only the thiol groups of terminal cysteines liganding Ni are shown. The scheme combines current views of H_2ase activation and catalysis deduced from potentiometric titrations (Cammack *et al*., 1987; Roberts and Lindahl, 1994; Volbeda *et al*., 1996; De Lacey *et al*., 1997), kinetics measurements (Happe *et al*, 1999; De Lacey *et al*., 2000a) and from the crystal structures of oxidized (Volbeda *et al*., 1995, 1996, 2002) and reduced (Garcin *et al*., 1999; Higuchi *et al*., 1999) forms of [NiFe]-H_2ases. The oxidized inactive form of the enzyme yields the Ni_u-A state, unready to catalyze H_2 activation (i.e. the splitting of the H-H bond). In absence of O_2, the oxidized enzyme in the Ni_r-B state is ready and able to enter quickly in a catalytic cycle. Upon reduction, Ni-B yields the EPR-silent intermediate Ni-S. Ni-S occurs in two states in equilibrium, the Ni_r-S (ready) state and the Ni_a-S (active) state, supposed to be the protonated form of Ni_r-S (De Lacey *et al*., 1997). Each of the active states, Ni_a-S, Ni_a-C and Ni_a-R have been proposed to be involved in the catalytic cycle (Cammack, 2001; Roberts and Lindahl, 1995; De Lacey *et al*., 2000a). Only the Ni-C/Ni-R couple is in thermodynamic equilibrium with H_2. The competitive inhibitor CO was proposed to block electron and proton transfer at the active site by binding to the Ni atom and thereby displacing H_2 and stabilizing a Ni-S(CO) reduced state (Happe *et al*., 1999; De Lacey *et al*., 2002) (Scheme adapted from De Lacey *et al*., 2000a; Bleijlevens *et al*., 2001 and Jones *et al*., 2003). The subscript "u" stands for unready, "r" for ready and "a" for active. The Ni-A, Ni-B and Ni-C states produce EPR signals, the Ni-S and Ni-R (fully reduced) states are EPR silent. Ni-C is represented with a bridging hydride ligand coordinated between the Ni and Fe atoms (Foerster *et al*., 2003).

Although the [NiFe]- and the [FeFe]-H_2ases have completely different structures and are evolutionary unrelated, they share a common feature, namely the presence of endogenous CO and CN^- ligands bound to a Fe center in the active site. The presence of these ligands stabilizes iron in a low oxidation and spin state and makes it resemble transition metals (Ru, Pd or Pt) known to be good catalysts for H_2 splitting (Adams and Stiefel, 2000). Another common feature is the presence of an Fe-S cluster proximal to the dinuclear metallocenter, which is then wired to the surface for electron exchange with its partner redox proteins by a conduit of iron-sulfur clusters. Finally, both types of enzymes contain hydrophobic gas channels. In the *D. desulfuricans* ATCC 7757 [FeFe]-H_2ase, there is a single channel that runs from the molecular surface to the buried active site and points at the vacant coordination site of one of the two Fe atoms of the sub-site (Nicolet *et al*., 1999). This channel is also present in iron-only hydrogenase I from *C. pasteurianum* (Nicolet *et al*., 2002). These structural informations are very useful to identify amino acid residues to be modified to render the enzyme more efficient and chiefly less O_2 sensitive.

Model compounds

The discovery of the enzyme active site structure and the knowledge of its mechanism of action have guided the design of organometallic compounds able to activate or to produce H_2. Synthetic chemical compounds capable of mimicking the [NiFe] active site (e.g. Alvarez *et al*., 2001; Sellman *et al*., 2002) and the di-iron subsite of the H-cluster of [FeFe]-H_2ases (Zhao *et al*., 2001, 2002; George *et al*., 2002; Gloaguen *et al*., 2001, 2002; Darensbourg *et al*., 2003; Kayal and Rauchfuss, 2003; Ott *et al*., 2003; Nehring and Heinekey, 2003; Salyi *et al*., 2003) have been assembled. The chemistry of these synthetic [NiFe] and [FeFe] systems and their pertinence as new electrocatalytic systems for hydrogen production or uptake is described and discussed in the review by Evans and Pickett (2003). These synthetic chemical systems (and theoretical investigations of them (see e.g. Bruschi *et al*., 2002) may contribute to the identification of factors indispensable for efficient intramolecular electron transfer, stability and high turnover of the metallocenter in its supramolecular protein framework.

The enzymatic mechanism for [NiFe]-hydrogenases

Numerous studies using various biophysical approaches (e.g. Mössbauer, electron paramagnetic resonance (EPR) spectroscopy and Fourier transform infrared (FTIR) spectroscopy coupled to potentiometric measurements) have been used to identify the redox intermediates generated in the H_2ase enzyme under hydrogen (cf reviews by Albracht, 1994, 2001; Frey *et al*., 2001; Cammack, 2001). In the oxidized aerobic state, most [NiFe]-H_2ases are inactive due to the presence of a bridging ligand between the Ni and Fe atoms. Upon reductive activation, the ligand, depicted as a hydroxo species in Figure 4, is removed via reduction to water and the Ni ion is reduced from Ni^{3+} to Ni^{2+}. The enzyme in the active state (Ni_a-S) can bind H_2 to produce Ni-R (EPR silent) where dihydrogen is suggested to be bound side-on to the iron. The catalytic cycle then progresses with the heterolytic cleavage of molecular hydrogen at the metal center. Ni-R oxidizes by one electron forming the EPR-active Ni-C state in which a hydride is bound to the NiFe center. The question of whether the hydride is bound either to the nickel, or to the iron, or forms a bridge between the two metals, is not solved. The amino acid residue which is protonated during activation or turnover has also not been clearly identified (see commentaries by Maroney and Bryngelson, 2001; Siegbahn *et al*., 2001; Fan and Hall, 2001). A further one-electron oxidation of the NiFe center in the Ni-C state releases this hydride as a proton and regenerates Ni-S (Ni^{2+}), completing the cycle (Figure 4). The proximal [4Fe-4S] cluster receives the electrons from the NiFe center and transfers them one at a time to the distal [4Fe-4S] cluster, which is close to the surface of the molecule and is able to transfer the electrons one at a time to an external acceptor or donor. In the absence of a redox partner, the enzyme still activates molecular hydrogen, as can be seen by the use of hydrogen isotopes. In the presence of D_2 gas, the enzyme splits the D_2 molecule into a deuteron (D^+) and a deuteride (D^-), which by exchange with the protons of the solvent (H_2O) leads to the formation of HD and H_2. The same pathway is used for H_2 evolution in the H^+/D_2 exchange reaction and in the reduction of protons by an exogenous electron donor (McTavish *et al*., 1996; Bertrand *et al*., 2000; Vignais *et al*., 2002). Catalysis of H^+/D_2 exchange is a requirement for functional models of H_2ase active site (Georgakaki *et al*., 2003). Fast passage to the active site of hydrons (H^+/D^+) from the exterior of the H_2ase was revealed by measurement of H/D exchangeable ENDOR signals, but it was not clear whether this phenomenon occurs during enzyme turnover or activation (Müller *et al*., 2002)

The dynamic changes within the active site during the catalytic cycle are still mostly unknown. Current studies address the question of the nature of the oxygen (sulfur) species bridging the Ni and Fe atoms in the oxidized redox states (Ni-A and Ni-B states), the localization of the substrate hydrogen (or D_2) in the active state of the enzyme and of the hydride (deuteride) after heterolytic cleavage of the molecule. To dissect the mechanism of H_2 activation and identify proton pathways, H_2ase mutants have been prepared by site-directed mutagenesis of amino acid residues proximal to the active site, and the mutants analyzed by FTIR and characterized kinetically (De Lacey *et al*., 2000b). The Glu25 residue of *D. fructosovorans* [NiFe]-H_2ase, which establishes a hydrogen bond with one terminal cysteine ligand of the Ni atom, has been proposed to stabilize the water molecule that is ejected from the active site during activation of the enzyme (Stadler *et al*., 2002). Its replacement by a glutamine suppresses the fast proton transfer from the active site but does not impede the heterolytic cleavage of molecular hydrogen (De Lacey *et al*., 2003). For the soluble, O_2-tolerant, NAD^+-reducing hydrogenase (SH) from *R. eutropha*, a subclass of mutants was identified, which showed O_2-sensitive growth on H_2, due to an O_2-sensitive but catalytically active SH protein (Burgdorf *et al*., 2002). The mutant proteins have been purified and characterized by XAS and FTIR spectroscopy to investigate which features of the [NiFe] site are essential for oxygen tolerance. In the H_2-sensing HoxBC H_2ase, the replacement of a glutamine residue, close to two metal coordinating cysteines, by a glutamate, led to a completely unstable protein, indicating that this glutamine is critical for the stability of the protein (Buhrke *et al*., 2002).

The proton transfer pathway proposed by Peters *et al*. (1998) for the [FeFe]-H_2ase I of *C. pasteurianum*, which involves amino acid residues conserved in all [FeFe]-H_2ases known to date (Nicolet *et al*., 2002), has not yet been tested by site-directed mutagenesis.

Biosynthesis of [NiFe]-hydrogenases

Clustering of hydrogenase genes in Proteobacteria

In Proteobacteria, the genes that encode H_2-uptake H_2ases are clustered. These clusters comprise the structural genes (generally labeled L for large subunit and S for small subunit), accessory genes for maturation and the insertion of Ni, Fe, CO and CN^- at the active site of the heterodimer. In some organisms, the H_2ase gene cluster also comprises regulatory genes that control expression of the structural genes. Not included in these H_2ase gene clusters are the genes encoding nickel transport - for nickel uptake is required for the biosynthesis of [NiFe]-H_2ases in the cytoplasm (Navarro *et al*., 1993; de Pina *et al*., 1999; Olson and Maier, 2000; reviewed by Eitinger and Mandrand-Berthelot, 2000) -, and the genes whose products can translocate folded, mature, proteins across the bacterial cytoplasmic membrane by the specific Tat (twin arginine translocation) protein translocation pathway (Wu *et al*., 2000a,b; Voordouw *et al*., 2000; Berks *et al*., 2000, 2003; Ize *et al*., 2002). The Tat signal-peptide-bearing precursors of *E. coli* H_2ase-1 and -2 have been shown, by a bacterial two-hybrid assay, to interact with the accessory (chaperone) proteins HyaE and HybE, respectively. It was proposed that the latter proteins act at a "proofreading" stage in H_2ase assembly and police the protein transport pathway preventing premature targeting of Tat-dependent H_2ases (Dubini and Sargent, 2003).

Since H_2ase gene clusters encode homologous proteins, it is inferred that analogous biosynthetic mechanisms operate in the various organisms containing those clusters. The correspondence of these genes, designated differently in different organisms, can be found in the reviews of Casalot and Rousset (2001) and Vignais *et al*. (2001). It should be noted that even though H_2ase operons are well conserved and exhibit a high

degree of similarity, each *cis*-acting maturation system is specific to the corresponding structural gene products. This specificity may explain why H_2ases cannot be matured when produced in heterologous hosts.

The biosynthesis of *E. coli* H_2ase-3 has been extensively studied by the group of A. Böck in Munich, Germany. It is described hereafter as a prototype (Figure 5). It begins by the synthesis of the large subunit (HycE) as a precursor protein (pre-HycE) with an extension at the carboxyl-terminus. After insertion of the metallocenter, an endopeptidase removes the C-terminal extension from the precursor of the large subunit. After proteolysis, the large subunit is then capable of binding to the small subunit (Figure 5).

Metallocenter assembly

The maturation of the H_2ase follows a complex pathway, which involves at least seven auxiliary proteins, the products of the so-called *hyp* genes, namely HypA, HypB, HypC, HypD, HypE and HypF, and an endopeptidase. This set of proteins, which directs the synthesis and incorporation into proteins of the metal center, controls the fidelity of insertion of the correct metal into the cognate target protein, maintains a folding state of the protein competent for metal addition and allows protein conformational changes for internalization of the assembled metal center (see reviews by Casalot and Rousset, 2001; Vignais *et al*., 2001; Mulrooney and Hausinger, 2003).

The insertion of Fe and Ni into the metal center is a sequential process in which Fe incorporation precedes that of Ni. The Fe donor is not identified, Fe appears to be incorporated into a complex formed by HypC and HypD (Blokesch and Böck, 2002). Synthesis of the CN^- (and probably also CO) ligands is accomplished by the HypF and HypE proteins (Reismann *et al*., 2003). HypF contains sequence motifs characteristic of *o*-carbamoyl transferases (Paschos *et al*., 2001) and acyl-phosphatases (the acyl-phosphatase domain has been crystallized and structurally characterized (Rosano *et al*., 2002)). HypF accepts carbamoyl phosphate (CP) as a substrate, catalyzes a CP-dependent hydrolysis of ATP into AMP and inorganic pyrophosphate (PPi) and forms an adenylated CP derivative (Paschos *et al*., 2002). CP is necessary for the synthesis of the metal center since mutants lacking CP synthetase activity are unable to mature [NiFe]-H_2ases (Pachos *et al*., 2001). In the presence of HypE and HypF (and CP + ATP), the carbamoyl group of CP is transferred to HypE, to the cysteine residue at the C-terminus of HypE. HypE carbamoylation is followed by the ATP-dependent dehydration of the carbamoyl adduct resulting in cyanated HypE, then the cyano group is transferred to iron. The CO ligand may be derived from hydrolysis of a CN ligand and transfer of the carbamoyl group to iron, followed by deamination (Reissmann *et al*., 2003). The protein-bound ligands are then transferred to Fe, carried by a HypD-HypC complex (HypD contains an Fe-S cluster (Blokesch *et al*., 2002)). The chaperone HypC forms a complex with pre-HycE and is thought to deliver the liganded Fe to pre-HycE. The pre-HycE-HypC complex is then ready to incorporate Ni (Magalon and Böck, 2000a, b). (Either HypC or HybG, the chaperone specific for H_2ase-2, can act in the maturation of *E. coli* H_2ase-1 (Blokesch *et al*., 2001)).

Mutational analysis has shown that the activity of two gene products, HypA and HypB, are required *in vivo* (Jacobi *et al*., 1992; Olson *et al*., 2001). Whereas HypA functions in the maturation of H_2ase-3 of *E. coli*, a homologue encoded by *hybF* in the *hyb* operon operates in H_2ase-1 and -2 maturation (Hube *et al*., 2002). All HypB proteins have a conserved GTP-binding motif at the carboxyl terminus, and GTPase activity has been shown to be essential for Ni insertion (Maier *et al*., 1995; Olson *et al*., 1997; Olson and Maier, 2000). In some organisms, such as *Rhizobium leguminosarum, R. capsulatus, Azotobacter vinelandii* or *Bradyrhizobium japonicum*, a histidine-rich domain

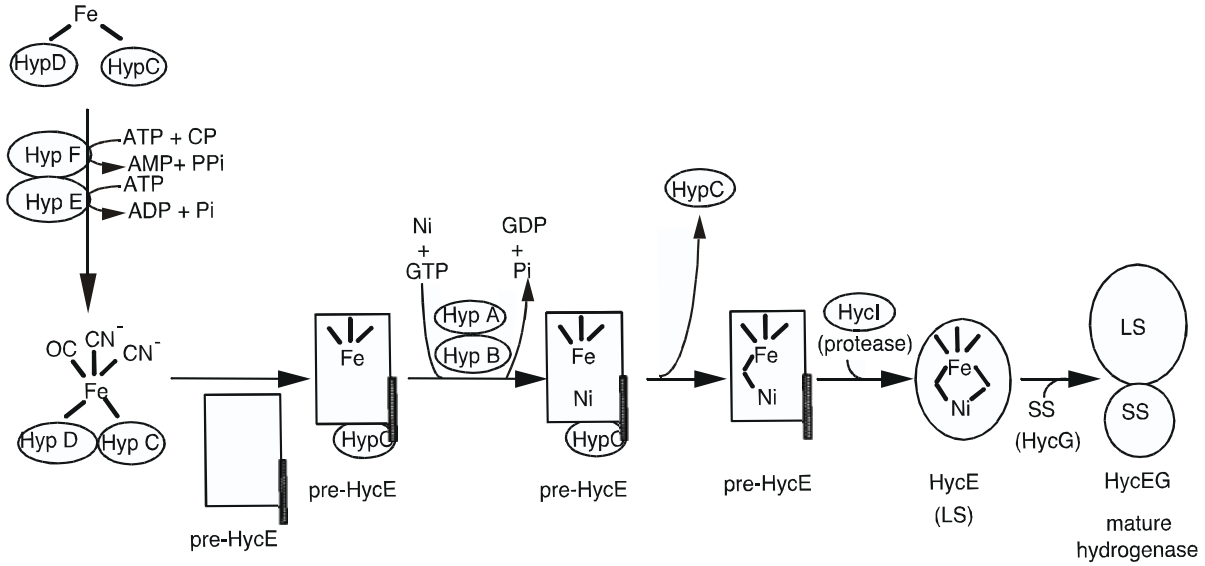

Figure 5. Postulated path of the maturation of *E. coli* H_2ase-3 (from Blokesch et al., 2002; Böck, 2003). CP: carbamoyl phosphate.

present at the N terminus of HypB enables the protein to store nickel (Rey *et al*., 1994; Fu *et al*., 1995; Olson *et al*., 1997, Olson and Maier, 2000). In other organisms, such as *E. coli or R. eutropha*, the absence of this nickel-storage function has been assumed to be compensated by a highly efficient nickel transport system (Eitinger and Mandrand-Berthelot, 2000). Evidence has been reported for cooperation between HypA and HypB during insertion of Ni into hydrogenase (Hube *et al*., 2002) and a heterodimer of the two *Helicobacter pylori* proteins has been detected by chemical crosslinking (Mehta *et al*., 2003). In *H. pylori*, these proteins are required for full activity of both hydrogenase and urease (Olson *et al*., 2001; reviewed by Mulrooney and Hausinger, 2003). The result of HypA/HypB-dependent Ni insertion into pre-HycE is a complex of pre-HycE containing Ni and $Fe(CO)(CN^-)_2$ centers with the chaperone HypC (Figure 5).

After dissociation of the chaperone, hydrogenase biosynthesis is completed by proteolytic cleavage of the carboxyl-terminus of pre-HycE and formation of the heterodimer by association of the mature large and small subunits. For C-terminal processing, HypC has to dissociate from the complex, since only a HypC-free, Ni-containing form of pre-HycE is a substrate for the maturation endopeptidase (Magalon and Böck, 2000a,b). (In *T. roseopersicina*, two HypC proteins, the products of $hypC_1$ and $hypC_2$ genes, have been proposed to be involved in the HypC cycle (Maróti *et al*., 2003)). The endopeptidase HycI, responsible for proteolytic maturation of pre-HycE, removes a 32 amino acid fragment from the C-terminus of pre-HycE but only when Ni has been inserted. Nickel serves as a substrate recognition and binding motif for the endopeptidase (Theodoratou *et al*., 2000b). The endopeptidase HybD, the structure of which has been established (Fritsche *et al*., 1999), is specific for the maturation of the precursor of *E. coli* H_2ase-2. The specificity of C-terminal endopeptidases has also been observed in *Anabaena* PCC 7120 (Wünschiers *et al*., 2003). The endopeptidases homologous to HycI/HybD cleave after the conserved His (or Arg) in the CX_2CX_2H/R motif at the carboxyl terminus. The proteolysis is highly specific for Ni, since Zn prevents proteolysis and hinders formation of a protease:precursor complex (Magalon et al. 2001). Furthermore, substitutions of amino acids located close to the cleavage site leads to maturation abortion (Massanz *et al*., 1997; Theodoratou *et al*., 2000a). This cleavage triggers a conformational change, resulting in the closure of the bridge between the two metals by the most C-terminally located cysteine residue, and the formation of the complete hetero-binuclear center (Figure 5) (Fritsche *et al*., 1999; Magalon and Böck, 2000b). The mature large subunit can then be assembled with the mature small subunit to form the functional heterodimer.

Maturation of the H_2 sensor proteins (called HupUV or HoxBC) also involves the participation of *hyp* gene products. The synthesis of *R. capsulatus* HupUV requires at least HypF (Colbeau *et al*., 1998) and HypD (Vignais *et al.,* 2000), and that of *R. eutropha* HoxBC is highly dependent on HypDEF (Buhrke *et al*., 2001). These proteins (and also Ech from *M. barkeri*, Coo from *R. rubrum* and Coo from *C. hydrogenoformans*) lack the carboxyl-terminal extension cleaved in the precursor form of [NiFe]-H_2ase large subunits. Thus no protease is needed for maturation and the mechanism of interaction with a H_2ase-specific chaperone needs to be assessed.

Biosynthesis of Fe-S clusters.

Pioneering studies of the biosynthesis of nitrogenase, which is encoded by the *nif* genes, led to the identification of proteins involved in [Fe-S] cluster assembly (reviewed by Frazzon *et al*., 2002; Frazzon and Dean, 2003). The NifS and NifU proteins of *A. vinelandii* were originally found to be necessary for the synthesis of both components of the nitrogenase enzyme, each of which contains [Fe-S] clusters. It was later shown that NifS is a homodimeric pyridoxal-phosphate dependent enzyme that uses L-cysteine as a substrate to form an enzyme-bound cysteine-persulfide, the proposed activated form of sulfur that is ultimately used for [Fe-S] cluster assembly (Zheng *et al*., 1993; 1994). NifS belongs to a class of proteins (IscS, CsdB, CSD) having cysteine desulfurase activity (Mihara *et al*., 1999; Kurihara *et al*., 2003). Several of them have been analyzed by crystallography (Kaiser *et al*., 2000; 2003; Fujii *et al*., 2000; Lima, 2002). NifU is a modular, homodimeric protein that provides a molecular scaffold for the NifS-directed formation of [Fe-S] clusters. NifU contains one redox-active [2Fe-2S] cluster per subunit, which is stable and is designated the "permanent" cluster. A second type of [2Fe-2S] cluster, highly labile, is assembled on NifU when it is co-incubated with NifS, Fe^{2+} and cysteine. This second, labile cluster type, designated "transient" cluster, is ultimately destined for nitrogenase [Fe-S] cluster formation (Yuvaniyama *et al*., 2000).

Because the inactivation of either *nifS* or *nifU* only decreased, but did not eliminate, nitrogenase activity, non-*nif* genes that encode proteins similar in structure and function to NifS and NifU were searched for. The *iscS* and *iscU* genes ("*isc*" for iron-sulfur-cluster formation) were found in the *iscRSUA-hscBA-fdx* gene cluster within the *A. vinelandii* genome (Zheng *et al*., 1998). The *isc* region is widely conserved among most bacteria. Homologues of proteins encoded by the *isc* gene cluster are also present in eukaryotic organisms (Lill and Kispal, 2000; Mühlenhoff and Lill, 2000; Tong and Rouault, 2000; Tong *et al*., 2003). All the products of the *isc* operon are involved in [Fe-S] cluster biogenesis. In *E. coli*, IscS activity is necessary for the mobilization of S for the maturation of various cofactors and proteins (Takahashi and Nakamura, 1999; Schwartz *et al*., 2000; Tokumoto and Takahashi, 2001; Lauhon and Kampampti, 2000). The crystal structure of IscS has been determined (Cupp-Vickery *et al*., 2003). IscU is a truncated version of NifU, containing the N-terminal domain of NifU (Yuvaniyama *et al*., 2000). IscU provides molecular scaffolds for the IscS-mediated assembly of [Fe-S] clusters (Agar *et al*, 2000a; Wu *et al*., 2002). The mechanism of [Fe-S] cluster assembly involves the formation of a IscS-IscU complex (Urbina *et al*., 2001; Smith *et al*., 2001) in which a covalent disulfide bond is formed between a conserved cysteine residue (Cys_{328}) of IscS and Cys_{63} of IscU (Kato *et al*;, 2002; Kurihara *et al*., 2003; Cupp-Vickery *et al*., 2003*)*. The transient [Fe-S] clusters in IscU are subsequently transferred to target proteins (Nishio and Nakai, 2000; Wu *et al*., 2002). IscA might function as an alternative scaffold for [Fe-S] cluster assembly, as IscA, like IscU,

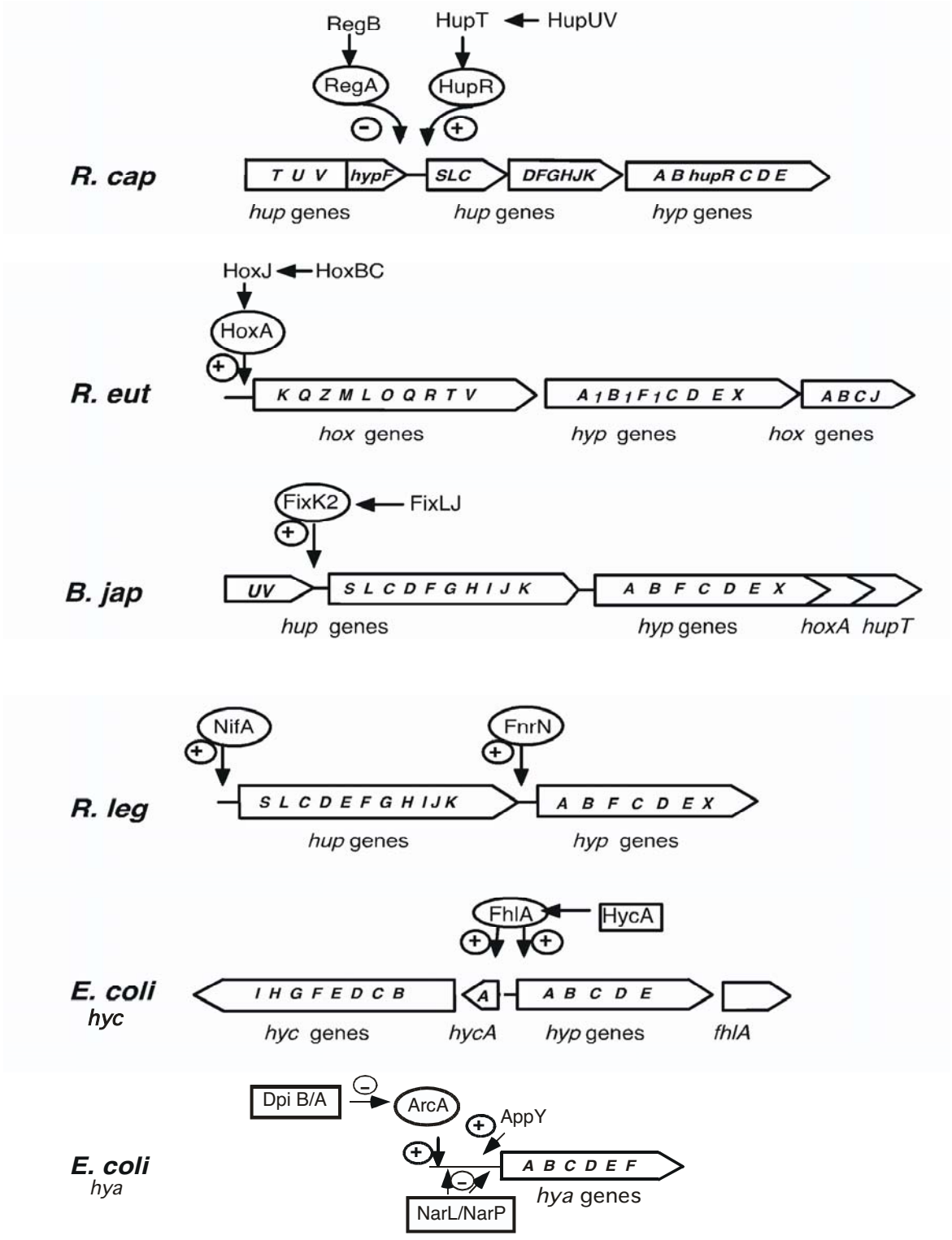

Figure 6. [NiFe]-H_2ase gene clusters and transcriptional factors regulating gene expression in some Proteobacteria. The operons have been termed *hup* (for hydrogen uptake) or *hox* (for hydrogen oxidation) and *hyp* ("p" for pleiotropic). The *hyp* gene products are necessary for the insertion of the Ni and the Fe atom with its diatomic CO and CN^- ligands, at the active site. In each structural operon of uptake H_2ase, the gene encoding the small subunit ("S" or "K") precedes that of the large subunit ("L" or "G"), the third gene, named *hupC* or *hoxZ* encodes a cytochrome *b*. In *E. coli* the *hyc* genes encode H_2ase-3 that is part of formate hydrogen lyase complex and evolves H_2; the *hya* operon encodes H_2ase-1. (See relevant references concerning the function of various accessory H_2ase genes in the reviews of Friedrich and Schwartz 1993; Vignais *et al*., 2001; Casalot and Rousset, 2001). *R. cap (R. capsulatus*), *R. eut* (*R. eutrophus*), *B. jap.* (*B. japonicum*), *R. leg* (*R. leguminosarum*). The direction of transcription is shown by the arrowed bars.

can host a transient [2Fe-2S] cluster (Krebs *et al*., 2001; Ollagnier-de Choudens *et al*., 2001; Wu and Cowan, 2003; Wollenberg *et al*., 2003). However, the crystal structure of IscA (Bilder *et al*., 2004) revealed the presence of a well-orderted fold in contrast to the highly mobile secondary structural elements within IscU (Bertini *et al*., 2003; Mansy *et al*., 2003), suggesting that the two proteins may not have equivalent function. In *Synechocystis*, it is cystine rather than cysteine which is the source of activated S (Leibrecht and Kessler, 1997; Clausen *et al*., 2000; Jaschkowitz and Seidler, 2000; Kaiser *et al*., 2003) and the activated species is free cysteine-persulfide rather than a cysteine persulfide residue bound to an active-site enzyme.

While it is clearly established that sulfur in [Fe-S] clusters is provided by cysteine desulfurases (NifS, IscS, CsdB, SufS, or yeast Nfs1p) via desulfurization of L-cysteine, the iron donor is essentially unknown. It has been reported that human frataxin, present in the mitochondrial matrix, may act as the iron donor for [Fe-S] assembly in ISU, a human IscU homologue (Yoon and Cowan, 2003). Human apofrataxin can bind up to six or seven iron atoms. Holofrataxin then mediates the transfer of iron to the nucleation sites for [2Fe-2S] cluster formation on ISU. Similarly, the yeast frataxin homologue Yfh1 has been shown to physically interact with the core [Fe-S] cluster assembly complex, composed of the scaffold protein Isu1 and the cysteine desulfurase Nfs1, and to be involved in the *de novo* [Fe-S] cluster synthesis on Isu1 (Gerber *et al*., 2003). This suggests that frataxin might play a role in iron loading of Isu1. Although IscU was reported to bind mononuclear iron (Agar *et al*., 2000a,b; Yuvaniyama *et al*., 2000; Nuth *et al*., 2002), association of an [Fe-S] cluster with the homologous yeast protein Isu1p, rather than mononuclear iron, was deduced by Mülenhoff *et al*. (2003). IscA has been proposed recently to act as an iron donor for [Fe-S] clusters in *E. coli*. IscA was shown to be an iron binding protein with an apparent iron association constant of $3.0 \times 10^{19}\ M^{-1}$; the iron-loaded IscA could provide iron for the assembly of transient [Fe-S] clusters in IscU in the presence of IscS and L-cysteine (Ding and Clark, 2004). The authors postulated that the primary function of IscA is to recruit intracellular iron and deliver iron for the assembly of [Fe-S] clusters in proteins. Studies of the interactions of IscA with IscS and IscU will help to elucidate whether delivery of iron **(**Nuth *et al*., 2002) or of sulfur (Urbina *et al*., 2001; Smith *et al*, 2001) is the first step in [Fe-S] cluster assembly on IscU. The *hscA* and *hscB* gene products share a high degree of sequence similarity to the molecular chaperones DnaK and DnaJ, respectively. Interaction of IscU with HscBA greatly stimulates the intrinsic ATPase activity of the HscBA complex, which appears to be intimately involved in [Fe-S] cluster assembly on the IscU scaffold (Hoff *et al*., 2000; 2002; Silberg *et al*., 2001). The study of the yeast chaperone homologues, Ssq1p and Jac1p, substantiated that the two chaperones form a functional unit in [Fe-S] protein biogenesis but, instead of being involved in *de novo* [Fe-S] cluster assembly on Isu1p, the chaperone system would be more likely required for the dislocation of a preassembled [Fe-S] cluster from Isu1 (Mülhenhoff *et al*., 2003). The *iscR* gene encodes a [2Fe-2S]-containing transcription factor, a negative regulator of the expression of all genes contained within the *isc* region (Schwartz *et al*., 2001).

Other genes playing a role in [Fe-S] cluster formation have been identified in *E. coli*, namely the *sufABCSDE* operon (*suf* for mobilization of sulfur) (Patzer and Hantke, 1999). SufS, like IscS, exhibits cysteine desulfurase activity, while SufA shares sequence similarity with IscA, including the three conserved cysteines involved in [Fe-S] cluster assembly. However, there is no homologue of IscU or HscBA in the *suf* operon. The Suf proteins, all located in the cytosol, form a third bacterial system for the assembly of [Fe-S] clusters (Takahasi and Tokumoto, 2002). The Suf machinery can be subdivised into three functional sub-complexes: 1) SufB, SufC and SufD form a complex endowed with ATPase activity, 2) SufS and SufE form a new type of two-component complex exhibiting both cysteine desulfurase and selenocysteine lyase activities, 3) SufA is a scaffold protein on which Fe-S clusters are transiently assembled before being inserted into the target apoprotein (Loiseau *et al*., 2003; Nachin *et al*., 2003; Ollagnier-de Choudens *et al*., 2003). While sulfur is provided by the activity of the SufES complex, the source of iron remains unknown. Physiological and genetic studies show that the Suf system functions under conditions of iron limitation.

Regulation of hydrogenase gene expression: signaling and transcription control

[NiFe]-H_2ases are found in organisms endowed of physiological attributes allowing their growth under very diverse environmental conditions: autotrophic or heterotrophic growth, in the light or in darkness, aerobically or anaerobically. The control of H_2ase synthesis represents a means to quickly and efficiently respond to changes in the environment and in particular to new energy demands. It is exerted at the transcription level; this was demonstrated by the use of transcriptional reporter systems measuring levels of promoter activity of structural operons (see e.g. Colbeau and Vignais, 1992) (Figure 6) (reviewed by Friedrich *et al*., 2001). Transcriptional control involves usually one or several two-component regulatory systems, which may act either positively or negatively. In response to a specific signal, the first component, a sensor histidine kinase, autophosphorylates at a conserved histidine residue and then transphosphorylates the cognate response regulator at a conserved aspartate residue (Hoch and Silhavy, 1995).

H_2ase synthesis responds to several type of signals: *a) molecular hydrogen*, which is also the substrate, activates H_2ase expression in aerobic bacteria (e.g. *R. eutropha)*, in photosynthetic bacteria (e.g. *R. capsulatus*) or in free-living *Rhizobia* (e.g. *B. japonicum).* In all these bacteria, the regulatory cascade sensing H_2 uses the same elements; *b) molecular oxygen*, an inhibitory signal for most of the hydrogenases. In symbiotic *Rhizobia*, the regulation of hydrogenase expression is linked to the expression of nitrogenase; *c) nickel ions,* necessary for the enzyme function; *d) metabolites* functioning as electron donors or acceptors, such as formate, carbon monoxide, nitrate, or sulfur.

1) Response to H_2

An *H_2-specific regulatory cascade* controls the transcription of uptake H_2ase genes (*hupSLC* in *R. capsulatus* and *B.*

Table 1. Components of the H_2-specific regulatory cascade.

Elements	Name of the protein or gene	Strain	References
H_2-sensing hydrogenase	HupUV	*R. capsulatus*	Vignais *et al*., 1997, 2000, 2002; Colbeau *et al*., 1998; Elsen *et al*., 2003.
	HoxBC	*R. eutropha*	Lenz and Friedrich, 1998; Kleihues *et al*., 2000; Bernhard *et al*., 2001; Buhrke *et al*., 2001, 2002; Lenz *et al*., 2002;.
	hupUV	*R. capsulatus*	Elsen *et al*., 1996.
	hupUV	*B. japonicum*	Black *et al*., 1994.
	hoxBC	*R. eutropha*	Lenz *et al*., 1997.
Histidine kinase	HupT	*R. capsulatus*	Elsen *et al*., 1993, 1997, 2003; Dischert *et al*. 1999.
	HupT	*B. japonicum*	Van Soom *et al*., 1999.
	HoxJ	*R. eutropha*	Lenz *et al*., 1997; Lenz and Friedrich, 1998.
Response regulator	HupR	*R. capsulatus*	Richaud *et al*., 1991; Toussaint *et al*., 1997; Dischert *et al*., 1999.
	HoxA	*B. japonicum*	Van Soom *et al*., 1997; Durmowicz and Maier, 1997.
	HoxA	*R. eutropha*	Zimmer *et al*., 1995.

japonicum and *hoxKGZ* in *R. eutropha*). The *H_2 -specific regulatory system* (see Table 1 for relevant references) comprises:

a) an *H_2-sensor* (also called regulatory H_2ase, RH) encoded by the *hupUV* genes in *B. japonicum* and *R. capsulatus* and by the homologous *hoxBC* genes in *R. eutropha*. This H_2-sensing H_2ase is the H_2 sensor for a two-component regulatory system, which controls the transcription of structural H_2ase operons.

b) a *two-component regulatory system* which consists of a *protein histidine kinase*, termed HupT in *R. capsulatus* and in *B. japonicum* and HoxJ in *R. eutropha*, and a *response regulator*, an NtrC-like transcription factor, termed HupR or HoxA .

In *R. eutropha*, the four genes, *hoxA*, *hoxB*, *hoxC* and *hoxJ* are expressed as an operon from the constitutive weak promoter located upstream from *hoxA*. However, under H_2ase-derepressing conditions, *hoxA* can be transcribed from the strong promoter of the membrane-bound H_2ase structural genes (p*MBH*), thus leading to an enhanced synthesis of the H_2-responding cascade proteins (Schwartz *et al*., 1999). On the contrary, in *R. capsulatus*, *hupR* and *hupT* genes are expressed independently. The *hupR* gene is located within the *hyp* genes and is transcribed constitutively with them from a promoter located in the *hypA* gene (Colbeau A., unpublished). The level of the HupR protein is constant (Dischert *et al*., 1999). In *B. japonicum*, the expression of HoxA is positively autoregulated (Van Soom *et al*., 1999).

In *R. capsulatus*, the *hupT* gene is expressed with the *hupU* and *hupV* genes from a promoter localized just upstream from *hupT* and is regulated by the presence or absence of organic carbon (Elsen *et al*., 1996). *HupT* and *hupUV* mutants exhibit high H_2ase activities, even in absence of H_2, indicating that this system negatively controls H_2ase gene expression (Elsen *et al*., 1993, 1996). The HupT histidine kinase autophosphorylates in the presence of [γ–^{32}P] -ATP (Elsen *et al*., 1997). In the presence of HupT~P, there is transfer of the phosphate group to a conserved aspartate residue of the response regulator HupR. Footprinting experiments and *in vivo* experiments with plasmid-borne *hupS::lacZ* fusions have demonstrated that the transcriptional activator HupR binds to the *hupS* promoter and protects a palindromic sequence TTG-N_5-CAA localized at nt -162/-152 from the transcription start site (Toussaint *et al*., 1997; Dischert *et al*., 1999).

An exception concerning the functioning of the two-component HupT/HupR and HoxJ/HoxA regulatory systems is that the response regulator (HupR or HoxA) activates the transcription of H_2ase genes in the non phosphorylated form (Dischert *et al*., 1999; Lenz and Friedrich, 1998; Van Soom *et al*., 1999), in contrast to what is observed with NtrC in enteric bacteria, which activates transcription in the phosphorylated form. This was demonstrated in *R. capsulatus* by replacing the HupR phosphorylation site (D_{54}) with various amino acids or by deleting it using site-directed mutagenesis. Strains expressing mutated *hupR* genes showed high hydrogenase activities even in the absence of H_2 (Dischert *et al*., 1999). Although HupR binds to an enhancer site of the *hupS* promoter and activates the promoter in concert with the global regulator called "integration host factor" or IHF, the *R. capsulatus hupSL* genes are transcribed by a σ^{70}-linked RNA polymerase (Dischert *et al*., 1999). This is also an exceptional case; usually enhancer-binding transcription factors activate σ^{54}-dependent RNA polymerase, as is the case for H_2ase genes in *R. eutropha* (Römermann *et al*., 1989), *T. roseopersicina* (Colbeau *et al*., 1994) and *B. japonicum* (Black and Maier, 1995). *R. leguminosarum* contains an inactive pseudo-*hoxA* gene; the expression of H_2ase occurs only during symbiotic growth (Brito *et al*., 1997). By using native acrylamide gel electrophoresis, direct interaction between HupUV/HoxBC and HupT/HoxJ proteins has been demonstrated for *R. capsulatus* (Elsen *et al*., 2003) and *R. eutropha* (Buhrke *et al*., 2001). This interaction does not depend on the phosphorylation status of HupT (Elsen *et al*., 2003). The molecular mechanism of H_2 signal transduction is not yet totally elucidated. Although in *R. capsulatus, R. eutropha* and *B. japonicum*, the regulatory cascade is similar, *R. capsulatus* Hup(UV)$^-$ mutants have high hydrogenase activity while *R. eutropha* and *B. japonicum* mutants defective in the H_2 sensor have no H_2ase activity.

2) O_2 regulation

The synthesis of most H_2ases is negatively regulated by O_2. Optimal synthesis of H_2ases requires either strict anaerobiosis or microaerobiosis and O_2 regulation involves different pathways and regulators.

When growing in symbiosis, Rhizobia form bacteroids in the roots of legumes. Under these growth conditions, expression of H_2ase is included in a complex network

coordinating the process of N_2 fixation. The sensing of low O_2 concentration involves global regulatory proteins homologous to the *E. coli* Fnr protein, but various pathways are used in Rhizobia. The *E.coli* anaerobic global regulator Fnr (for fumarate nitrate reduction) (Spiro and Guest, 1990) is a cytoplasmic O_2-responsive regulator with a sensory and a regulatory DNA-binding domain. Fnr regulates transcription initiation in response to oxygen starvation. It activates the transcription of genes involved in anaerobic respiratory pathways while it represses the expression of genes involved in aerobic energy generation (Iuchi and Lin, 1993). Upstream regions of Fnr-regulated genes are characterized by Fnr consensus sequences of dyad symmetry, TTGAT-N_4-ATCAA, to which the protein binds as a dimer. Fnr activity depends on the presence of a $[4Fe\text{-}4S]^{2+}$ cluster, which, in the presence of O_2, is converted rapidly to a more O_2-stable $[2Fe\text{-}2S]^{2+}$ cluster; the monomeric $[2Fe\text{-}2S]^{2+}$-containing Fnr is inactive (Kiley and Beinert, 1999). It is the O_2-lability of the $[4Fe\text{-}4S]^{2+}$ cluster which makes of Fnr an O_2 sensor (Bates *et al*., 2000; Beinert and Kiley, 1999). The Fnr protein binds and activates in anaerobiosis the *hyp* operon in *E. coli* and thus affects indirectly H_2ase synthesis. In Rhizobia, Fnr homologues, which regulate H_2ase synthesis, are either Fnr-like (such as FixK1 in *B. japonicum* or FnrN in *R. leguminosarum*) or FixK-like (such as FixK2 in *B. japonicum*). FixK-like proteins lack the N-terminal region (of Fnr) for binding of the [4Fe-4S] cluster. The main difference between Fnr-like and FixK-like would be at the level of the redox control. Indeed, FixK-like proteins, which lack the redox sensitive cysteines, are activated by an associated O_2-sensitive two-component system, FixLJ (Guttiérez *et al*., 1997).

In *B. japonicum,* O_2 signal transduction is organized along two regulatory cascades involving the activators FixK2 and NifA (Sciotti *et al*. 2003). The transcription of H_2ase structural genes is activated by DNA binding of FixK2 whose expression depends on the FixL/FixJ two component regulatory system (Nellen-Anthamatten *et al*., 1998) (Figure 6). The transmembrane sensor FixL has a heme binding domain which belongs to the PAS superfamily (Taylor and Zhulin, 1999) and directly senses O_2. Association of O_2 to FixL induces a conformational change (Gilles-Gonzales *et al*., 1991; Gong *et al*., 2000; Miyatake *et al*., 2000; Dunham *et al*., 2003), which activates the C terminal kinase domain and triggers autophosphorylation from ATP and phosphoryl transfer to FixJ. At reduced levels of O_2, expression of the *fixK2* gene is stimulated; its product, FixK2, activates the *hyp* and *hup* genes (Durmowicz and Maier, 1998) and also the *rpoN* gene encoding a sigma 54 factor (Nellen-Anthamatten *et al*., 1998). This provides a connection with nitrogenase synthesis since expression of the *nifA* gene, itself regulated by the global two-component system RegSR (homologous to RegBA, see below) (Bauer *et al*., 1998; Emmerich *et al*., 1999), uses a σ^{54}-dependent RNA polymerase, and explains why FixK2 and FixJ mutants are devoid of H_2ase activity and are Nif^-. The H_2ase promoter presents two potential binding sites for FixK2: TTGA-C–GATCAA-G. In *B. japonicum*, besides the H_2ase genes, FixK2 regulates the expression of the *fixNOQP* operon encoding a terminal oxidase with a high affinity for O_2, and several genes involved in nitrate metabolism in response to low oxygen concentrations.

As in *B. japonicum*, H_2ase transcription in *R. leguminosarum* is co-regulated with that of nitrogenase. It is controlled by two global regulators NifA and FnrN in response to low oxygen in the nodules. However, in this bacterium, NifA activates directly H_2ase expression by binding to an upstream activating sequence (UAS) of the promoter region of the structural *hupSL* genes. This promoter also binds the global regulator IHF and is transcribed by a σ^{54}-RNA polymerase (Brito *et al*., 1997). Moreover, the Fnr-like protein, FnrN, indirectly activates H_2ase expression by binding to the σ^{70}-dependent promoter of *hyp* genes (Hernando *et al*., 1995), which are expressed in vegetative cells. There are two copies of *fnrN* genes, both possessing two anaeroboxes, TTGAT-N_4-ATCAA (Colombo, *et al*., 2000) and expressed from a σ^{54}-RNA polymerase (Clark *et al*., 2001). The distal site is involved in activation of the promoter and the proximal site, which overlaps the -10 sequence, has a repressing effect, so that accumulation of the Fnr protein is limited. Only double mutants form ineffective nodules lacking both H_2ase and nitrogenase. In this bacterium, H_2ase expression has been obtained independently from nitrogenase expression by replacing the NifA-dependent *hupSL* promoter by the FnrN-dependent *fixN* promoter. Under these conditions, high levels of H_2ase were obtained in free-living bacteria as well as in bacteroids (Brito *et al*., 2002).

The two [FeFe]-H_2ases (HydA1 and HydA2) of the green alga *Chlamydomonas reinhardtii* are both expressed upon anaerobic induction, however, the intervening regulator is not known. Their expression is further modulated by changes in growth conditions, such as the re-addition of O_2 or the presence of acetate (Forestier *et al*., 2003).

3) Redox regulation

E. coli can encode four H_2ases expressed only in anaerobiosis. H_2ase-1 and -2 (encoded by the *hya* and *hyb* operons, respectively) are H_2-uptake enzymes. H_2ase-1 is generally assumed to recycle H_2 produced by H_2ase-3. H_2ase-2 participates in the H_2-dependent reduction of fumarate and H_2ase-3 evolves H_2, being active under fermentative conditions (growth on glucose and formate) or during anaerobic respiration (growth on glycerol and fumarate). These H_2ases are regulated differently.

The synthesis of H_2ase-1 and -2 depends on the global two-component regulatory system ArcB/ArcA (Iuchi and Lin, 1993). ArcB is a transmembrane sensor protein, the histidine kinase activity of which is stimulated in absence of O_2. Under anaerobic conditions, the ArcB sensor kinase autophosphorylates and then transphosphorylates the global transcriptional regulator ArcA. In chemostat cultures of *E. coli*, ArcA was recently found to exert a major control under conditions of oxygen-restricted growth, and it was concluded that the ArcBA system should be considered a microaerobic redox regulator (Alexeeva *et al*., 2003). Phosphorylation of Arc induces multimerization, a prerequisite for DNA binding (Jeon *et al*., 2001). ArcA~phosphate (ArcA~P) is the active form, which represses target genes of aerobic metabolism and activates genes of anaerobic metabolism. Quinones are redox signals for the Arc system. Oxidized forms of

quinone electron carriers act as direct negative signals and inhibit autophosphorylation of ArcB during aerobiosis, thus providing a link between the electron transport chain and gene expression (Georgellis *et al*, 2001). ArcA activates the anaerobic induction of the *hya* operon (Br∅nsted and Atlung, 1994; Atlung *et al*., 1997; Richard *et al*., 1999). In contrast, it represses the expression of the *hyb* operon during anaerobic growth (Richard *et al*., 1999). Dissimilar responses of *hya* and *hyb* operons to external pH are also mediated by ArcA (King and Przibyla, 1999).

The regulation of H_2ase-1 synthesis by ArcA involves the global regulator AppY (Br∅nsted and Atlung, 1994; Atlung *et al*., 1997). Depending on the growth conditions, the ArcA and AppY proteins act either cooperatively or independently. AppY synthesis is itself repressed in aerobiosis by the two-component DpiAB system (Ingmer *et al*., 1998). Thus, the *hya* operon is controlled by two two-component redox sensitive systems, one active in anaerobiosis (ArcB/ArcA) and the other (AppY/DpiAB) in aerobiosis (Richard *et al*., 1999). The transcriptional regulation of the *hyb* operon coding for H_2ase-2 is less well understood.

Expression of both *hya* and *hyb* operons is repressed in presence of nitrate, repression being mediated by the two-component systems NarL/NarX and NarP/NarQ (Richard *et al*., 1999). Several potential binding sites for these regulators are present on *phya* and *phyb*. This repression can also be indirectly due to ArcA. Indeed, phosphorylation of ArcA by ArcB depends on the respiratory state of the cell. When nitrate is present, ArcA may be more activated than in its absence (Br∅nsted and Atlung, 1994).

Oxidation of H_2 by uptake H_2ase produces electrons which can feed several energy-consuming processes such as carbon dioxide reduction, dinitrogen fixation or ATP synthesis and this integrates the H_2ase in the redox metabolism of the cell. A global two-component signal transduction system, called RegB/RegA in *R. capsulatus*, and PrrB/PrrA in *R. sphaeroides*, which functions by phosphate transfer (Bird *et al*., 1999), is implicated in the redox control of those processes (Joshi and Tabita, 1996) as well as of respiration (Swem *et al*., 2001). Signal transduction by RegB is mediated by a redox-active, highly conserved, cysteine. Exposure of RegB to oxidizing conditions results in the formation of an intermolecular disulfide bond, which converts the kinase from an active dimer into an inactive tetramer state. Disulfide bond formation is metal-dependent (the metal fulfilling a structural role) (Swem *et al*., 2003). The RegA protein has been shown to regulate negatively the expression of H_2ase by binding to *phupS,* the promoter of *R. capsulatus* structural H_2ase genes. By foot-printing experiments, two binding sites for RegA were found, a high affinity site located between the binding sites for the global regulator IHF and for RNA polymerase, and a low affinity site overlapping the binding site for IHF. RegB- and RegA-defective mutant strains, grown either aerobically or anaerobically, have 3 to 5 times more activity than the wild type, either in presence or absence of H_2 (Elsen et al, 2000). Thus, in *R. capsulatus,* global regulation by the RegBA system is superimposed on the H_2-specific regulation.

4) Ni-specific regulation

The *B. japonicum* HupUV proteins were initially suggested to form a possible Ni-sensing regulatory system for transcription of the [NiFe]-H_2ase (*hup*) genes (Black *et al*., 1994). HupUV does indeed regulate the transcription of the *hup* genes (see above) and needs Ni to be active. It is rather HypB, a nickel-binding GTPase necessary for incorporation of Ni into the H_2ase apoprotein that relays the signal to the regulatory proteins (Olson *et al*., 1997; Olson and Maier, 2000). On the other hand, the homologous HoxBC (RH) protein in *R. eutropha*, which contains Ni (Pierik *et al*., 1998), is nearly independent of HypB (Buhrke *et al*., 2001). Also not clear is the mechanism by which Ni regulates H_2ase transcription in *R. leguminosarum* (Brito *et al*., 2000), in *Nostoc* strains (Axelsson and Lindblad, 2002) or how Hmd H_2ase is induced and F_{420}-reducing H_2ase completely repressed in *M. marburgensis*, under nickel limitation (Afting *et al*., 2000).

5) Regulation by metabolites, electron donors or acceptors

a) Formate regulation. Optimal expression of the *hyc* operon, coding for *E. coli* H_2ase-3, requires anaerobiosis, absence of nitrate and acidic pH. All these factors act at the transcriptional level by regulating the level of formate. The *hyc* operon belongs to the formate regulon regulated by the transcriptional regulator FhlA (Rossmann *et al*., 1991). FhlA shares homology with regulators of the NtrC family in its central and C terminal domains but differs in possessing an extended N terminal domain lacking the aspartate residue which is the site of phosphorylation of response regulators. Thus, FhlA is not activated by phosphorylation but by binding an effector molecule, formate. Direct binding of formate by FhlA has the same role as phosphorylation for classical regulators such as NtrC. It promotes a strong and specific binding to specific sequences of DNA. FhlA is a homotetramer, which binds to and activates the *hyc, hyp, fhlF* and *hypF* promoters (Hopper *et al*., 1994; Schlensog *et al*., 1994). Thus, the regulator FhlA controls the expression of the structural and accessory genes of H_2ase. These promoters are of the σ^{54} type and are activated by the global regulator IHF (Hopper *et al*., 1994). Moreover, the formate regulon is regulated negatively by HycA, which either interacts directly with FhlA or prevents FhlA binding to the UAS. The *hyf* operon, which can encode a putative H_2ase-4 in *E. coli*, was found to resemble the *hyc* operon in being induced under anaerobic conditions by formate at low pH and by binding of HyfR, the homologue of FhlA (Skibinski *et al*., 2002).

b) Carbon monoxide induction. In *R. rubrum*, CO was shown to induce the *de novo* synthesis of a H_2ase linking to the CO oxidation pathway (Bonam *et al*., 1989). This synthesis is activated by CooA (CO-oxidation activator), a heme containing transcription factor, member of the cyclic AMP receptor protein, (CAP/CRP)/FNR, superfamily of regulatory proteins (Lanzilotta *et al*., 2000; Youn *et al*., 2004). CooA is unable to bind CO when the Fe heme is oxidized; upon reduction, there is an unusual switch of protein ligands to the six-coordinate heme and the reduced heme is able to bind CO. CO binding stabilizes a conformation of the dimeric protein that allows sequence-specific DNA binding and transcription is activated through

contacts between CooA and RNA polymerase. Thus, the CooA dimer functions both as a redox sensor and as a specific CO sensor (Aono *et al*., 2000; reviewed by Roberts *et al*., 2001; Aono, 2003). Structural models for the heme movement during CO-specific activation of CooA have been proposed, based on mutational and spectroscopic studies (Nakajima *et al*., 2001; Coyle *et al*., 2003; Youn *et al*., 2003).

c) Induction under N limitation. In some N_2-fixing prokaryotes, H_2ase is co-regulated with nitrogenase. The transcription of uptake H_2ases (HupSL) is controlled by the global regulator NifA (see above). The expression of the bidirectional NAD(P)-dependent H_2ase in the cyanobacterium *Gloeocapsa alpicola* CALU 743 *(Synechocystis* PCC 6308) is increased in nitrate-limiting growth conditions (Sheremetieva *et al*., 2002).

d) Sulfur and selenium regulation. The hyperthermophilic archaeon *P. furiosus* can grow on maltose either in the absence of elemental sulfur S^0 (it then produces H_2 as an end product instead of H_2S) or in the presence of S^0. Recently, the effect of S^0 on the levels of gene expression in *P. furiosus* cells grown at 95° C with maltose as the carbon source was investigated with the use of DNA microarrays (Schut *et al*., 2001). Eighteen ORFs that encode subunits associated with the three H_2ases characterized in *P. furiosus* (two cytoplasmic, H_2ases I and II, and one membrane-bound) were found to be strongly downregulated by S^0 (an indication that these H_2ases are probably not directly involved in S^0 reduction). The nature of the enzyme system that reduces S^0 as well as the mechanism by which S^0 affects H_2ase gene expression in *P. furiosus* are still unknown.

A regulation by selenium has been described in *Methanococcus voltae,* endowed with multiple, differently regulated, H_2ases. The Se-free [NiFe]-H_2ases, Vhc and Frc, are produced only upon Se deprivation (Berghöfer and Klein, 1995) from the two *vhc* and *frc* transcription units, linked by a common 453-bp intergenic region subject to negative and positive regulation (Noll *et al*., 1999). A 55-kDa protein binds to 11-bp specific sequences and is responsible for the activation of both transcription units (Müller and Klein, 2001).

Biohydrogen

Molecular hydrogen produced from renewable sources (biomass, water, organic wastes) either biologically or photo-biologically is called "biohydrogen". Biohydrogen can be produced by both types of H_2ases and also by a third enzyme, the nitrogenase enzyme, which functions as an H_2-evolving H_2ase in the absence of N_2 (not covered here). Potential applications of photosynthetic and fermentative microorganisms in the generation of H_2 by direct biophotolysis, indirect biophotolysis, photo-fermentations and dark-fermentations have often been reviewed (Markov *et al*., 1995; Rao and Hall 1996; Appel and Schulz, 1998; Hansel and Lindblad 1998; Das and Veziroglu, 2001; Melis and Happe, 2001; Akkerman *et al*., 2002; Pinto *et al*., 2002; Melis, 2002; Tamagnini *et al*., 2002; Happe *et al*., 2002), and the potentially critical factors identified and discussed (Hallenbeck and Benemann, 2002; Levin *et al*., 2004). Fermentative mesophilic bacteria (such as clostridia) or thermophiles (e. g. *Pyrococcus*) have a real potential (Levin *et al*., 2004). The main problems with dark fermentative processes is that produced H_2 is contaminated by various gases (SH_2, CH_4), which have to be eliminated for use of H_2 in fuel cells. In oxygenic phototrophs (cyanobacteria and green algae), the photosynthetic reactions that produce reductant from water can be coupled to the reduction of H^+ to generate molecular hydrogen at the expense of solar energy, but here O_2 is the contaminant.

In phototrophs, H_2 production is a means to dissipate excess reducing equivalents. In *Scenedesmus obliquus* (Florin *et al*., 2001; Wünschiers *et al*., 2001a) or *C. reinhardtii* (Melis and Happe, 2001; Happe and Kaminsky, 2002) the electrons provided by fermentative metabolism are transferred to PSI in the light via the plastoquinone pool. In turn, PSI reduces a [2Fe-2S] ferredoxin, the physiological electron donor to [FeFe]-H_2ase. From the effect of the uncoupler carbonyl cyanide *p*-trifluoro-methoxyphenylhydrazone (FCCP), Cournac *et al*. (2002) concluded that PS II-independent H_2 production in *C. reinhardtii* is limited by the trans-thylakoidal proton gradient. In cyanobacteria, the soluble NAD(P)-dependent bidirectional [NiFe]-H_2ase is using protons to reoxidize the pyridine nucleotides reduced during dark anaerobic metabolism (Stal and Moezelaar, 1997; Troshina *et al*., 2002). In the cyanobacterium *Synechocystis* PCC 6803, the bidirectional H_2ase produces significant amounts of H_2 in the dark, in anaerobiosis (Schütz *et al*., 2004; Cournac *et al*., 2004), the rate of H_2 production being higher in the presence of fermentative substrates such as glucose. A mutant lacking the NADPH-dehydrogenase complex (NDH-1), impaired in CO_2 uptake and CO_2 fixation was shown to produce H_2 in the light using electrons gained by water photolysis (Cournac *et al*., 2004).

One of the main difficulties encountered in the use of H_2ases to produce H_2 is their sensitivity to O_2. One way to circumvent O_2 sensitivity is to maintain a metabolic state with low O_2 production. In *C. reinhardtii* under sulfur deprivation, the photochemical activity of PS II declines, rates of photosynthetic O_2 evolution drop below those of O_2 consumption by respiration and the culture becomes anaerobic; H_2ase synthesis in the chloroplasts is then induced (Ghirardi *et al*., 2000; Melis *et al*., 2000; Winkler *et al*., 2002; Kosourov *et al*., 2002; 2003). However, S deprivation significantly reduces electron transport capacity from water and limits the H_2-production capability of the algal cultures. A careful titration of the supply of S nutrients in the green alga medium might permit the development of a continuous H_2-production process (Zhang and Melis, 2002). The ideal would be to develop a water splitting system that can produce H_2 under aerobic conditions. To that end, it is important to understand the reasons for O_2 sensitivity, since some H_2ases are O_2-tolerant. This is the case for the soluble NAD-dependent H_2ase of *R. eutropha*, the metallocenter of which is modified and contains two additional CN^- ligands (Happe R.P. *et al*., 2000) and for the H_2 sensors (Vignais *et al*., 2002), in which the hydrophobic cavities serving as gas channels are significantly narrower, and then may impede O_2 access to the active site (Volbeda *et al*., 2002). In line with this observation, amino acids close to the metallocenter of the [FeFe]-H_2ase from *C. reinhardtii* have been modified by site-directed mutagenesis to render

the H_2ase O_2-tolerant (Seibert *et al*., 2001). *Rubrivivax gelatinosus* also contains a H_2ase tolerant to O_2. This H_2ase, linked to a CO oxidation pathway, was shown to produce H_2 using electrons from reduced ferredoxin of a cyanobacterial source. If the H_2ase can use the host electron donor, then a cyanobacterium-bacterium hybrid system may be expected to be able to mediate H_2 production from water photolysis (Maness *et al*., 2002).

The [FeFe]-H_2ases are enzymes of high turnover but, besides their high sensitivity to O_2, they are also light sensitive (Chen *et al*., 2002). This may pose an additional problem for their biotechnological use in photosynthetic organisms such as algae.

Conclusions and Perspectives

H_2ases are a structurally and functionally diverse group of enzymes and phylogenetic analysis has led to the identification of several phylogenetically distinct groups and subgroups which form the basis of a coherent system of classification. The large number of H_2ase gene sequences has been augmented by whole genome sequencing, which has revealed the presence of multiple H_2ases in several *Bacteria* and *Archaea*. Post-genomic analysis (transcriptome, proteome, metabolome) has and will be essential to elucidating the metabolic roles of these enzymes and the regulation of their biosynthesis and activity. While the mechanisms of [NiFe]-H_2ases biosynthesis begin to be understood, nothing is known yet on the biosynthesis of [FeFe]-H_2ases. Studies of regulation have essentially been restricted to the uptake [NiFe]-H_2ases of *Proteobacteria*, but have recently been extended to other types of H_2ase and other microorganisms, such as the hyperthermophilic archaeon, *P. furiosus*.

The existence of multiple H_2ases within a living organism allows the organism to best meet its energy need. The main role of H_2ases is clearly the oxidation of H_2 or the reduction of protons, coupled to energy-conserving electron transfer chain reactions, which allow energy to be obtained either from H_2 or from the oxidation of substrates of lower potential. These energy-conserving reactions are generally restricted to the prokaryotes, but are widely distributed among the bacterial and archaeal domains of life. In the last decade, additional roles have been revealed. Thus, the so-called H_2-sensor H_2ases are involved in regulating the biosynthesis of uptake [NiFe]-H_2ases in response to their substrate, H_2. Other, bidirectional H_2ases may interact with respiratory electron transport chains and act as electron "valves" to control the redox poise of the respiratory chain at the level of the quinone pool. This is essential to ensure the correct functioning of the respiratory chain in the presence of excess reducing equivalents, particularly in photosynthetic microorganisms. An additional finding concerns some H_2ases which were originally thought to play a purely fermentative role, but which are now known to be involved in membrane-linked energy conservation through the generation of a transmembrane protonmotive force.

The current interest in H_2 as an alternative to fossil fuels has led to a resurgence of interest in the biological production of H_2, and research into H_2ases will clearly play a major role in this area. Structural studies of H_2ases will be important in directing protein engineering, e.g. in rendering these enzymes O_2-tolerant. Identification of factors, linked to the protein environment of the active site and indispensable for stability and high efficiency of the enzyme, will contribute to the development of synthetic chemical systems able to mimick the active site metallocenter. Studies of H_2 metabolism and regulation will also be important in engineering microorganisms at the cellular level in order to maximize H_2 production. The isolation of novel H_2-producing organisms will also be a priority. Prokaryotic biodiversity is much greater than previously thought, and whole phylogenetic groupings exist, e.g. the mesophilic *Crenarchaeota*, which have never been cultivated. Given the importance of H_2 metabolism among microorganisms generally, it can be anticipated that many of these so-far uncultivated species will contain H_2ases and that novel types of H_2ase and of H_2 metabolism remain to be discovered.

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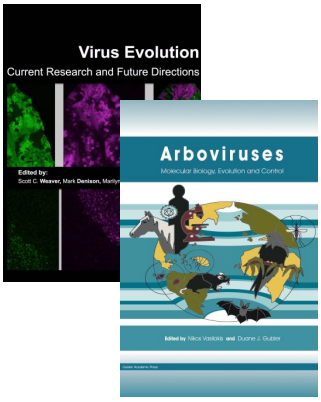

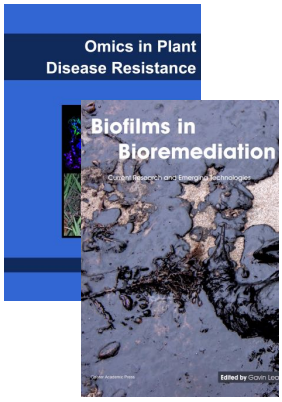

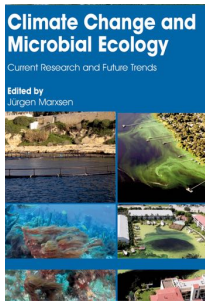

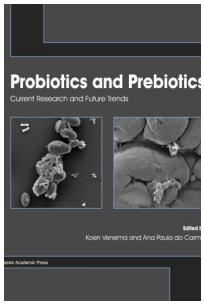

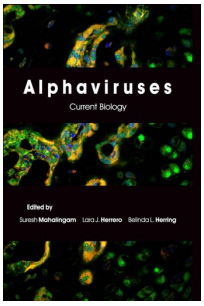

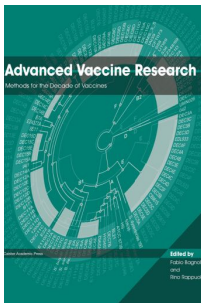

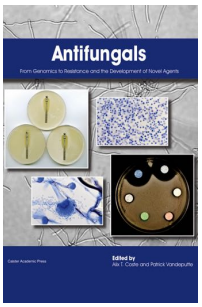

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