Chapter 3

Engineering Hydrogenases for H_2 Production: Bolts and Goals

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Summary

Hydrogenases are efficient biological catalysts of H_2 oxidation and production. Most of them are inhibited by oxygen, and a prerequisite for their use in biotechnological applications under air is to improve their oxygen tolerance. A few bacteria, however, contain hydrogenases that activate H_2 even in the presence of O_2. Intriguingly, molecular, kinetic and spectroscopic

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studies lead to assume that different mechanisms might be responsible for the resistance, depending on the enzyme type. In order to better understand the molecular bases of resistance to O_2 inhibition, this chapter focuses on the hydrogenases and their reaction with O_2 and examines the different strategies to lead to engineer kinetically efficient hydrogenases operating under aerobic conditions.

I. Introduction

Climate change, along with the rapid depletion of oil and gas reserves, prompt the world to turn to a search for a clean energy sources to provide the energy necessary for present and projected human activities. A variety of possible fuel sources are being examined at present. Among these, dihydrogen (H_2) has been identified as a clean and renewable energy carrier and is found to be one potential alternative to fossil fuel energy and has drawn a worldwide attention as a future energy source (Mason 2007). Interestingly, the pioneering notion of a "Dihydrogen Energy System" drew inspiration from the French science-fiction novel "The mysterious Island" by Jules Vernes (1874), where the idea of using H_2 as an energy carrier first appeared. However, over 90 % of the production of H_2 remains based upon steam reforming of hydrocarbons and coal gasification, which starts from fossil fuels and requires high temperature and pressure conditions. So, a sustainable, renewable supply of H_2 to power this economy is required. Alternative methods of H_2 generation include electrolysis of water and biological means. The development of new biotechnological processes, designed to meet the future energy demand, may take advantage of microbes that have been using H_2 from very early in the evolution of life (Perez-Arellano et al. 1998; Andersson and Kurland 1999). Many organisms including some *Bacteria*, *Archaea* and unicellular eukaryotes have an active H_2 metabolism, utilizing the cleavage of H_2 to gain energy, or H_2 production to release reducing power (Casalot and Rousset 2001). It has been estimated that these microorganisms produce or consume more than 200 million tons of H_2 per year (Richardson and Stewart 1990). These processes, carried out by hydrogenase occur via the inter-conversions between the molecular hydrogen and two protons plus two electrons ($H_2 \leftrightarrow 2H^+ + 2e^-$).

In this sense, an outlook in the production of hydrogen from water and light energy would be to use photosynthetic microorganisms, such as cyanobacteria and green algae (Antal et al. 2011; Carrieri et al. 2011). Green algae have many areas of potential improvements that often overlap with those of cyanobacteria and are currently recognized as better photobiological hydrogen producers from a demonstrated solar efficiency standpoint (Ghirardi et al. 2009). In principle, there is absorption of light in the form of photons by the photosystem which are going generate a strong oxidant that can oxidize water into protons, electrons/reducing equivalents and O_2. Thereafter, the electrons reduce protons to form H_2, carried out by hydrogenases (Fig. 3.1). In this sense, photosynthetic production of H_2 using water as a source of electrons and sunlight as the source of energy, driving proton reduction, is the most desirable process. Both water and light are available to an almost unlimited

Abbreviations: DFT – Density function theory; ENDOR – Electron nuclear double resonance; EPR – Electron paramagnetic resonance; EXAFS – Extended X-Ray absorption fine structure; Fd – Ferredoxin; FHL – Formate hydrogen lyase; FNR – Ferredoxin NADPH reductase; FTIR – Fourier Transform InfraRed spectroscopy; Hmd – H_2-forming methylenetetrahydromethanopterin dehydrogenase; MBH – Membrane-bound hydrogenase; PFV – Protein film voltammetry; RH – Regulatory hydrogenase; SH – Soluble hydrogenase; SHE – Standard hydrogen electrode; WT – Wild type;

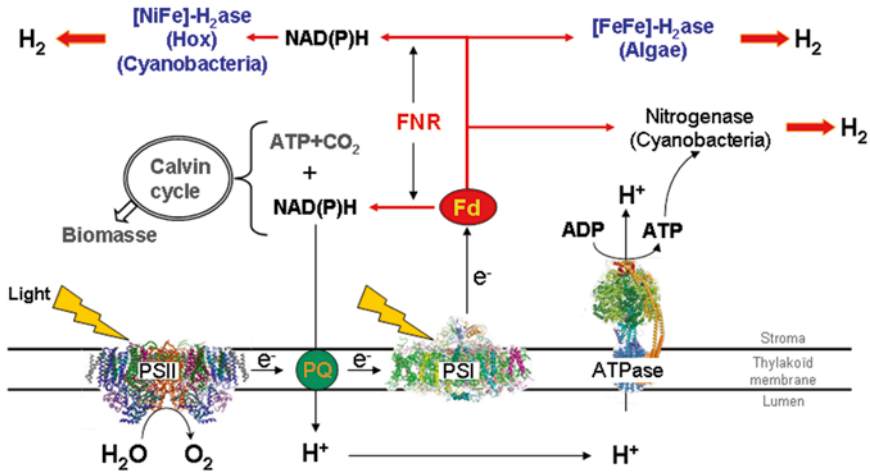

Fig. 3.1. H_2 production by cyanobacteria and algae. Water (*bold*) is oxidized to O_2 by photosystem II (*PSII*) and electrons are transferred to photosystem I (*PSI*) via the plastoquinone pool (*PQ*). Photosystem I transfers electrons to ferredoxin (*Fd*) which can donate electrons to [FeFe]-hydrogenase (H_2ase) in algae or to nitrogenase (N_2ase) in some cyanobacteria. Fd electrons can also be transferred to $NAD(P)^+$ by a ferredoxins NAD reductase (*FNR*). NAD(P)H can donate electrons to [NiFe]-hydrogenase in cyanobacteria.

extent and in addition to H_2 only O_2 is formed, whereas greenhouse gases are avoided. Moreover, photosynthetic production of H_2 is potentially very efficient in terms of energy conservation, since 10 % of the incident light energy can theoretically be recovered into H_2 (Prince and Kheshgi 2005). For example, an average sun-light flux of 46 Mwh/ha/day can be converted, with a 10 % yield, in 1,650 m^3 of H_2 per hectare per day, which represents 145 TOE (ton of oil equivalent) per hectare per year.

In addition to being used to the in vivo H_2 production, hydrogenases are known to be used in a variety of biotechnological applications including biofuel cells, biosensors, prevention against microbial-induced corrosion, and the generation and regeneration of NAD(P) cofactors. In the case of biofuel cell, the nature of the hydrogenases has inspired researchers worldwide to use them as biocatalysts predominantly to replace platinum electrode in hydrogen fuel cells (Fig. 3.2). Indeed, platinum is limited in availability and very expensive and therefore the use of hydrogenases would be good candidates to replace this precious metal in fuel cells. Moreover the hydrogenase-coated electrode confers greater fuel specificity and turnover rates than the platinum and it could be used to as an alternative to allow operation of biofuel cells at neutral pH and ambient temperatures, which are the conditions much more favorable for the handling of fuel cells (Ikeda and Kano 2001; Morozov et al. 2002).

Most applications of H_2-oxidizing and H_2-producing catalysts require them to function in air. For example, an O_2-stable H_2-production catalyst is an essential component for the photobiological production of H_2, a device that uses solar energy to split water into H_2 and O_2. Similarly, use of hydrogenases in biofuel cells requires them to remain active in the presence of O_2 since biofuel cells must work necessarily with O_2 (Figs. 3.1 and 3.2). However, the major barrier of developing an economically viable systems, is the oxygen sensitivity of the vast majority of hydrogenases because their active site react strongly with O_2 (Cournac et al. 2004; Léger et al. 2004; Oh et al. 2011).

At present, structure function relationship studies in hydrogenases have mainly remained in the basic research realm, aimed

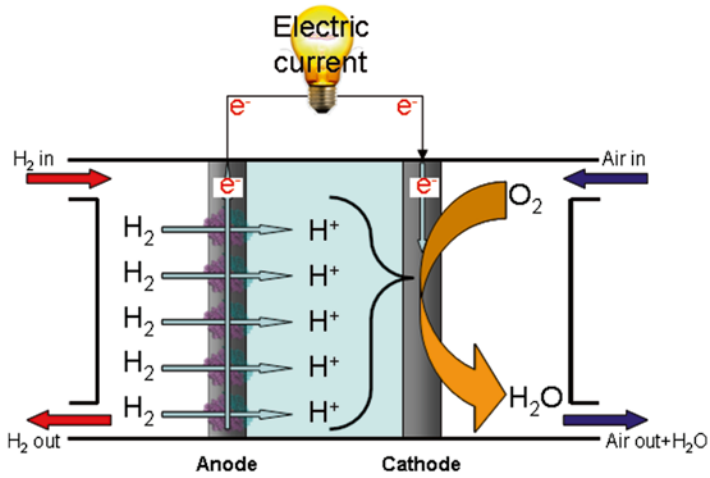

Fig. 3.2. Working principle of biofuel cells using [NiFe]-hydrogenases at the anode.

at understanding the enzyme catalytic mechanism (De Lacey et al. 2005). In order to obtain a sufficient level of enzyme efficiency and robustness for technological purposes, hydrogenases must be functionally optimized by improving efficiency H_2-oxidation or production while improving O_2 tolerance. These challenges can be approached through genetic engineering by two different strategies: at the cellular level, by metabolic engineering, it is possible to create favorable conditions to improve the H_2-production by deletion of hydrogen-uptake system (Liang et al. 2009), in inserting of non native hydrogenases more efficient and tolerant to O_2 (Wells et al. 2011) or to simply avoid O_2 exposure (Kruse et al. 2005; Rupprecht et al. 2006; Henstra et al. 2007). At the enzyme level, by protein engineering, the goal will be to improve hydrogenases so that they can outcompete other enzymes for substrate utilization, use of a thermodynamically more favorable substrate or become more O_2-tolerant. In this chapter, we will review hydrogenase structure-function relationship studies in which new properties of modified enzymes might serve as an inspiration source for rational optimization of hydrogenases for biotechnological processes.

In the first part, we will describe the different hydrogenases, and then we focus on these which are naturally O_2-tolerants. Thereafter, we will process the engineering approaches in three parts: (1) Improving H_2 production by heterologous expression of non-native hydrogenases or their overexpression. (2) Improving the electron transfer by increasing the substrate specificity or redirect redox intermediates. (3) Engineering protein to improve the tolerance of the hydrogenases to O_2.

II. Classification and Physiological Properties of Hydrogenases

Hydrogenases are metalloproteins which are involved in the metabolic machinery of a wide variety of microorganisms by catalyzing the reversible heterolytic splitting of dihydrogen according to the elementary reaction: $H_2 <==> H^- + H^+ <==> 2H^+ + 2e^-$.

Since 1931, when hydrogenases were described by Stephenson and Stickland, extensive research has been conducted in this area. Biochemical, spectroscopic, phylogenetic studies made possible to separate three groups of hydrogenases on the basis of the metal content of their active site: [Fe]-, [FeFe]- and [NiFe]-hydrogenase (Vignais et al. 2001; Cournac et al. 2004).

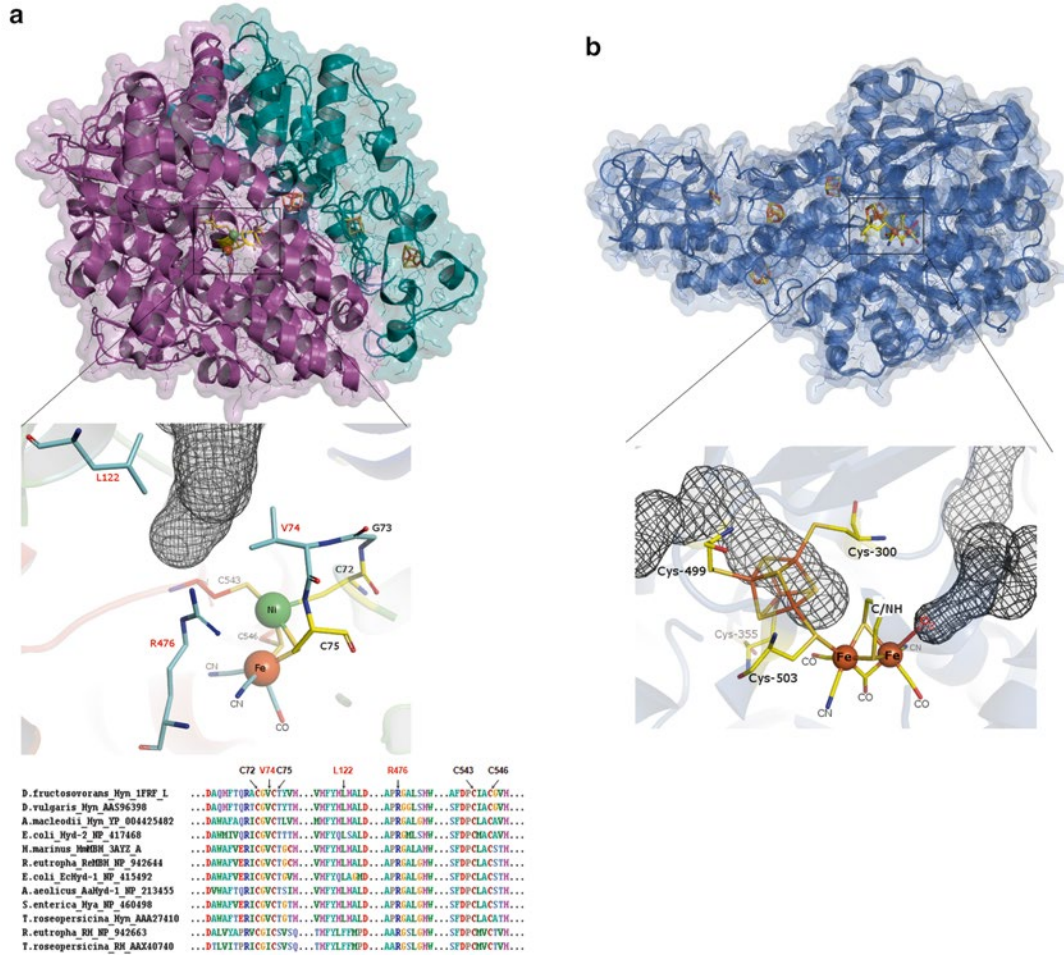

Fig. 3.3. Structure of [NiFe]-hydrogenase, [FeFe]-hydrogenase and [Fe]-hydrogenase. Panel (**a**) shows a structural model of the periplamsic [NiFe]-hydrogenase from *D. fructosovorans* (1YQW). The active site is buried in the large subunit (*purple*) whereas the small subunit contains three consecutive iron-sulfur clusters (*light blue*). Panel (**b**) shows the structure of [FeFe]-hydrogenase of *C. pasteurianum* (CpI: 3C8Y). All cofactors (active site and iron-sulfur) are localized in the same subunit (*blue*). The PyMOL Molecular Graphics System was used for visualization. The *lower part* of the figure shows the catalytic centers of three hydrogenases. The extremity of hydrophobic channels for gas diffusion were computed using Caver2.0 and appear as gray meshes for the panels **a** and **b**. Panel (**a**) residues, component the entrance gate of gases, are indicated in *red* and the multiple sequence alignment (ClustalW) of some [NiFe]-hydrogenase large subunits, is presented.

A. Generalities

The three classes of hydrogenases are evolutionarily unrelated but share similar nonprotein ligand assemblies at their active site that are not observed elsewhere in biology. They all contain a complex active-site cofactor that consists of at least one Fe atom coordinated by varying numbers of cysteine-S ligands and biologically unique carbon monoxide (CO) and in most cases additional cyanides (CN) ligands (Fig. 3.3). Therefore, this structure $Fe(CO)_2$ or Fe(CO-CN) likely represents the minimal cofactor making hydrogenase activity possible. These metal cofactors are synthesized in a coordinated post-translational process that involves up to nine hydrogenase-specific auxiliary proteins

(Böck et al. 2006; Lenz et al. 2010; Mulder et al. 2011). In the bimetallic hydrogenases the active-site cofactor is electronically coupled to FeS clusters that direct the electrons from the active site to the protein surface or from an external electron donor to the active site. Moreover, analysis of crystals structure from all three classes of hydrogenases revealed a network of hydrophobic cavities and channels (Fig. 3.3), or packing defects that form pathways connecting the active site to the surface of the enzyme (Volbeda et al. 1995; Montet et al. 1997; Nicolet et al. 1999, 2002; Fontecilla-Camps et al. 2007; Hiromoto et al. 2009; Mulder et al. 2011; Hong and Pachter 2012; Nicolet and Fontecilla-Camps 2012). Molecular dynamics simulations and xenon mapping of [NiFe]-hydrogenases show that these channels facilitate the diffusion of H_2 between the bulk of solvent and the active site (Montet et al. 1997; Cohen et al. 2005; Fontecilla-Camps et al. 2007; Leroux et al. 2008; Liebgott et al. 2010; Topin et al. 2012). In addition, the pathways can facilitate diffusion of small gas molecules such as CO and O_2 to access the active site, which in the case of CO leads to a reversible inhibition and in the case of O_2, to complete but non destructive inhibition.

The redox chemistry of hydrogenases is rich and involves many intermediate states, as beside catalyzing H_2 oxidation/production, they can also interact with gaseous molecules (i.e. CO, O_2) and become inhibited. It is thus important to gain an understanding into how hydrogenases catalyze H_2 production and oxidation, to determine the mechanisms by which they are inactivated under oxidizing conditions and how they may become re-activated. A combination of spectroscopic and electrochemical methods has provided structural knowledge on the oxidised and reduced forms of the enzyme, namely electron paramagnetic resonance (EPR) spectroscopy, Fourier transform infrared (FTIR) spectroscopy and protein film voltammetry (PFV).

EPR is a spectroscopic technique widely used to study the hydrogenase. This technique allows the detection of chemical species that have unpaired electrons designated as paramagnetic. A large number of molecules contain such paramagnetic atoms such as nickel at the active site, or the iron atoms of the iron-sulfur centers in [NiFe]-hydrogenases. Upon reduction or oxidation, the metal atoms of the prosthetic groups go through several redox states and in some of these states, they become paramagnetic and can unambiguously be identified by their corresponding EPR spectrum. Magnetic coupling between these atoms also provide information of the active or inactive states.

Another powerful method for monitoring reactions at the active sites of hydrogenases is infrared spectroscopy. This is an unusual technique to use for studying enzymes, but for hydrogenases it exploits the fact that CN^- and (particularly) CO are strong infrared-active vibrational oscillators and their stretching frequencies appear in a spectral window where the rest of the protein and water do not absorb.

Beside spectroscopic studies that provide thermodynamic information at equilibrium states, kinetic parameters have equally been studied using protein film voltametry (PFV), where the hydrogenase is adsorbed to an electrode and its activity directly measured by electron transfer through the electrode under oxidizing or reducing potentials during gas exposure (Inhibitors: CO, O_2 or substrate: H_2) (Vincent et al. 2007; Armstrong et al. 2009).

B. Classification

1. [Fe]-Hydrogenase (EC 1.12.98.2)

This type of enzyme was found only in a small group of methanogenic Archaea and has been described for the first time in *Methanothermobacter thermoautotrophicum* (Zirngibl et al. 1992). This enzyme catalyzes CO_2 reduction to methane using H_2 (Vignais and Billoud 2007). Based on the metal content of their active site, although in the past they were considered as metal-free hydrogenases, they have been recently designated as

"iron-sulfur-cluster free hydrogenase" or simply [Fe]-dihydrogenases (Armstrong and Albracht 2005). This enzyme is also known as H_2-forming Methylenetetrahydromethanopterin Dehydrogenase (Hmd) (Corr and Murphy 2011). The structure of the active site and functional models have been reported in 2008 (Shima 2008; Hiromoto et al. 2009). This hydrogenase differs from the others hydrogenases not only by the primary and tertiary structures but also by the fact that the iron, required for enzyme activity is not redox active. Furthermore, the hydrogenase activity is rapidly lost under aerobic conditions and in presence of light (Lyon et al. 2004) which would make the isolation and the characterization very difficult.

2. [FeFe]-Hydrogenase (EC 1.12.7.2)

[FeFe]-hydrogenase have been found mainly in Gram positives and in eukaryotes, as well as in few anaerobic Gram negative (Atta and Meyer 2000; Horner et al. 2000, 2002). It should be emphasized that these are the only type of hydrogenase that is found in eukaryotes but that is absent in the *Archaea* domain (Cournac et al. 2004). In this sense, the hydrogenases present in eukaryotic microorganisms (Green algae) are [FeFe]-hydrogenases only. Generally, [FeFe]-hydrogenases are usually involved in H_2 production but they were also reported to function as an uptake hydrogenase. Indeed, the location of hydrogenases in the bacterial cell reflects the enzyme's function (Nicolet et al. 2000). For instance, the periplasmic *Desulfovibrio desulfuricans* [FeFe]-hydrogenase (DdH) is involved in dihydrogen uptake. Protons resulting from this dihydrogen oxidation create a gradient across the membrane that is thought to be coupled to ATP synthesis in the cytoplasm. *Clostridium pasteurianum* [FeFe]-hydrogenase I (CpI) is a cytoplasmic enzyme that accepts electrons from ferredoxin and generates dihydrogen with protons as electron acceptors.

The only [FeFe]-hydrogenase structures from anaerobic soil bacterium *Cl. pasteurianum* (CpI) (Peters et al. 1998) (pdb code 1FEH and 3C8Y) and sulfate reducing bacterium *D. desulfuricans* (DdH) (Nicolet et al. 1999) (pdb code 1HFE) revealed an unique active site metal cluster, termed as the H-cluster, where catalysis takes place. The H-cluster is composed of a binuclear $[2Fe]_H$ center bound to a $[4Fe\text{-}4S]_H$ subcluster by a bridging cystein, the [4Fe-4S] center is attached to the protein by four cystein ligands. The $[2Fe]_H$ center is coordinated by five diatomic CN^- and CO ligands, as well as a non protein dithiomethylamine ligand (Fig. 3.3b). Molecular masses of [FeFe]-hydrogenases can vary from 45 to 130 kDa according to the number of subunits. [FeFe]-hydrogenases are mainly monomeric and contains only one catalytic subunit, but they often comprise additional domains, which accommodate FeS clusters. For example in the [FeFe]-hydrogenase I from *Cl. pasteurianum* (CpI), three accessory [4Fe4S] clusters and one [2Fe2S] cluster are believed to transfer electrons between the electron donor or acceptor at the protein surface and the active site at the center of the protein (Nicolet et al. 2002; Nicolet and Fontecilla-Camps 2012). Contrarily, the simplest characterized [FeFe]-hydrogenases are observed in the green algae, including *Chlamydomonas reinhardtii*, *Chlorella fusca*, and *Scenedesmus obliquus*, which express enzymes consisting of only the H-cluster without FeS-cluster domains (Florin et al. 2001; Horner et al. 2002; Forestier et al. 2003). These proteins have been exploited more recently for biochemical and spectroscopic characterization because they lack the additional FeS clusters observed in most native [FeFe]-hydrogenases that may complicate the direct examination of the H-cluster (Kamp et al. 2008; Silakov et al. 2009; Stripp et al. 2009; Mulder et al. 2011).

Even though [FeFe]-hydrogenases may appear as the best suited for hydrogen production purposes, enzyme engineering studies for these enzymes are still poorly developed because of their great sensitivity to oxidative damage, which makes any biochemical characterization very uncertain.

a. Reaction with O_2 and CO

In most [FeFe]-hydrogenases, oxygen inhibits the enzyme, possibly by binding to the open coordination site on the distal Fe of the $[2Fe]_H$ center, then form a reactive oxygen species that destroys the $[4Fe4S]_H$ subcluster. This distal Fe would be equally the hydrogen binding site and also the site of reversible CO binding and inhibition (Stripp et al. 2009). The chemical nature of the oxygen species bound to the H-cluster after the exposure to O_2 is not known, but density function theory (DFT) calculations on inactivated states of the H-cluster have proposed a Fe^{II}-Fe^{II} oxidation state for the $[2Fe]_H$ center, with a possible OH group terminally bound to the distal Fe (Liu and Hu 2002).

Oxygen inactivation has been studied using protein film electrochemistry and it was shown that the rate of inhibition of [FeFe]-hydrogenases would be limited by two steps: (1) the diffusion of oxygen through the protein to the active site pocket, and (2) the binding of oxygen to the $[2Fe]_H$ subcluster (Armstrong et al. 2009).

Interestingly, the level of O_2 inhibition varies among [FeFe]-hydrogenases, with I_{50} values ranging from less than a few seconds for *Chlamydomonas reinhardtii* enzymes, to several minutes for the clostridial enzymes (Böck et al. 2006; Baffert et al. 2008). Algal hydrogenases, which lack the additional accessory cluster domain found in bacterial enzymes, are typically more sensitive to O_2 inhibition than are the enzymes isolated from bacteria. However, it is clear that this elevated sensitivity is not solely due to the lack of the N-terminal accessory cluster (Böck et al. 2006; Stripp et al. 2009).

3. [NiFe]-Hydrogenase (EC 1.12.2.1)

The most numerous and best studied class of hydrogenases have been the [NiFe]-hydrogenases. This type of enzyme was found in Bacteria and Archaea domains. The core enzyme consists of two subunits; the large subunit is approximately 60 kDa and houses the Ni-Fe-active site, whereas the small subunit, of approximately 35 kDa, which can be of variable size and harbors typically three iron-sulfur clusters (Fig. 3.3a): a distal [4Fe-4S] cluster at the surface of the protein and furthest from the active site; a medial [3Fe-4S] cluster; and a proximal Fe-S cluster, with variable properties, the closest to the active site. The large and small subunit exhibit sequence homologies to subunits of NADH:ubiquinone oxidoreductase (Complex I) (Volbeda et al. 2012). In certain enzymes, additional subunits enable the interaction of these clusters with physiological electron carriers such as quinones, pyridine nucleotides (NAD(P)H), ferredoxins, and cytochromes (Cournac et al. 2004). Crystal structure analysis of heterodimeric [NiFe]-hydrogenases from *Desulfovibrio* species (Volbeda et al. 1995, 2002; Higuchi et al. 1997; Matias et al. 2001) (Fig. 3.3a), and photosynthetic bacterium *Allochromatium vinosum* (Ogata et al. 2010), revealed that the Ni-Fe cofactor is deeply buried in the large subunit. The Ni is coordinated to the protein via four thiol groups from conserved cysteine residues; two of these are bridging ligands that coordinate both Fe and Ni (Volbeda et al. 1995, 2002; Higuchi et al. 1997; Matias et al. 2001). Fourier Transform Infrared spectroscopy revealed that the Fe coordination sphere also possesses three diatomic ligands: one CO and two CN molecules (Volbeda et al. 1996; Pierik et al. 1999). The sixth iron coordination position is assumed to be occupied by a bridging hydride between iron and nickel (Pardo et al. 2006; De Lacey et al. 2007). Hydrophobic cavities which channel the gas substrate between the protein surface and the active site (Fig. 3.3a) (Montet et al. 1997; Volbeda et al. 2002; Teixeira et al. 2006) as well as a proton-conducting channel (Léger et al. 2004) were identified inside the hydrogenase.

a. Classification

Based on their primary protein sequences the [NiFe]-hydrogenases have been categorized into four different groups (Table 3.1): Group

Table 3.1. Classification of [NiFe]-hydrogenases and physiological activities (Modified from Vignais and Billoud 2007).

Group	Function	Physiological activity	Microorganisms
I	Membrane-bound H_2 uptake hydrogenases	Energy conservation	*D. fructosovorans, A. aeolicus, R. eutropha* H16, *E. coli*
IIa	"Cyanobacterial" uptake hydrogenases	Energy conservation	*Anabaena variabilis, Nostoc* sp. PCC7120, *A. aeolicus*
IIb	Regulatory hydrogenase	H_2-sensing components in genetic regulation of hydrogenase expression	*R. eutropha, Thiocapsa roseopersicina*
IIIa	F420-reducing hydrogenases	Energy conservation	Methanogens only (*Methanothermobacter marburgensis*)
IIIb	NADP-reducing	Energy conservation/ Fermentation	*Thiobacillus denitrificans, Pyrococcus furiosus, Thermococcus kodakarensis*
IIIc	F420-non reducing	Energy conservation	*Geobacter sulfureducens, Geobacter metallidurans, Methanococcus voltae*
IIId	Bidirectional NAD(P)-reducing	Energy conservation Redox poising	*T. roseopersicina, Synechocystis* sp. PCC6308, *R. eutropha* H16, *D. fructosovorans*
IV	Membrane bound H_2 evolving hydrogenases	Energy conserving, membrane associated H_2 evolving hydrogenase	*P. furiosus, T. kodakarensis, Methanosarcina barkeri, E. coli*

I, H_2-uptake enzymes, localized in the bacterial or archaeal cell membrane, are primarily involved in H_2 oxidation; Group II, (a) cyanobacterial uptake [NiFe]-hydrogenases, whose location is cytoplasmic and which are involved in N_2 fixation and (b) H_2 regulatory hydrogenases or hydrogenase sensors, which detect the presence of H_2 in the environment and trigger a cascade of regulation controlling the synthesis of hydrogenases; Group III, cytoplasmic bidirectional enzymes, these water soluble multi-protein complexes are dependent on NAD(P)H or $NAD(P)^+$ as cofactors; Group IV, energy conserving H_2-evolving hydrogenases, these membrane-associated hydrogenases generate H_2 from reduced ferredoxin with the concomitant generation/utilization of an ion gradient (Vignais and Billoud 2007).

b. Reaction with O_2 and CO

It can be stated, as a general rule, that hydrogenases of either type are inhibited by O_2, but individual sensitivities can vary in a wide extent. Thus, [NiFe]-hydrogenases are considered to be more robust than [FeFe]-hydrogenases because they can be totally reactivated after inhibition by O_2. In the case of [NiFe]-hydrogenases, O_2 has also been shown to oxidize directly the bimetallic active site (van der Zwaan et al. 1990) but the difference with [FeFe]-hydrogenases is that [NiFe]-hydrogenases are not damaged by O_2 as they can be reactivated by reduction. X-ray diffraction studies showed that the main structural difference between oxidized and reduced states of the active site is that oxygen species bridge the metals in the oxidized state (Garcin et al. 1999; Carepo et al. 2002; Volbeda et al. 2005). Therefore, reductions of the oxidized states are triggered by the removal of the bridging oxygen species, which allows H_2 to bind to the active site and catalytic turnover. Studies of EPR revealed that oxidized enzyme may exist under two different states which have been called Ni-A and Ni-B (Fig. 3.4). Another very important feature, that differentiates Ni-A and Ni-B is their reactivation kinetics (Cammack et al. 1986). Ni-B is called the '*ready*' state because it quickly becomes active upon reduction, while Ni-A is called the '*unready*' state because it needs a long

period of incubation under reducing conditions before becoming active. This is illustrated by PFV experiments conducted on the *Allochromatium vinosum* [NiFe]-hydrogenase. After inactivation by O_2, the enzyme is reactivated by reduction at low potential under H_2 (Lamle et al. 2004). For example at -208 mV (SHE) and pH 6, the reactivation process occurs in two phases: a fast (instantaneous) phase corresponding to the reactivation of the Ni-B state and a slower phase (several hundreds of seconds) assigned to Ni-A. FTIR-spectroelectrochemical studies of different hydrogenases indicated that one-electron reduction of Ni-A and Ni-B leads to two different states: Ni-A leads to the Ni-SU state, and Ni-B leads to Ni-SI (De Lacey et al. 2007). The enzyme in the Ni-SI state is active, whereas the Ni-SU state is still inactive (De Lacey et al. 2007). The rate-limiting step of the reactivation process is the a gradual and spontaneous conversion of the Ni-SU to the active Ni-SI state (Lamle et al. 2005) (Fig. 3.4).

As regards the structures in either oxidized state, this stir up still a matter of debate. Indeed, X-ray diffraction, (Volbeda et al. 1995, 2002) EPR (van der Zwaan et al. 1990), ENDOR (Carepo et al. 2002; van Gastel et al. 2006) and EXAFS (Davidson et al. 2000) data indicate that in both states an oxygen species is bridging nickel and iron atoms (De Lacey et al. 2007; Pandelia et al. 2010b). Most of the studies agree with the presence of a hydroxide in the Ni-B state (Black et al. 1994; Davidson et al. 2000; Stein et al. 2001; Stadler et al. 2002; Volbeda et al. 2005) whereas the nature of the oxygen species in Ni-A is more controversial. Indeed, either oxo (Carepo et al. 2002), hydroxo (Davidson et al. 2000; Stein et al. 2001; Stadler et al. 2002; Pandelia et al. 2010b) or peroxo (Lamle et al. 2005; Volbeda et al. 2005) species have been proposed. However, the "peroxo" hypothesis was not ruled out in recent DFT and spectroscopic studies (van Gastel et al. 2008; Pandelia et al. 2010b) but rather a hydroxo species. Moreover, an ENDOR study of a sample of [NiFe]-hydrogenase aerobically oxidized in $H_2{}^{17}O$, demonstrated that the bridging [NiFe]- ligand in Ni-A originates from the solvent water (Carepo et al. 2002). Thus, this will mean that the debate continues on the question of the Ni-A structure with the aim to understand differences between Ni-A and Ni-B states (Fig. 3.4).

Most [NiFe]-hydrogenases are inhibited by CO in a competitive manner (Teixeira et al. 1987; Léger et al. 2004). It reaches the active site using the same gas channel as H_2 and O_2 (Fig. 3.3a) (Liebgott et al. 2010). It binds weakly to the Ni ion at the active site (Albracht 1994; Stadler et al. 2002; De Lacey et al. 2007; Lubitz et al. 2007), only after reductive activation of the enzyme to the Ni-SI forms, presumably when the bridging oxygen species had been removed from the active site. No binding of CO occurs when the enzyme is in the inactive states Ni-A, Ni-B, and Ni-SU. In addition, CO-inhibition blocks electron and proton transfer at the active site, although reduction at the proximal [4Fe-4S] cluster is detected (Stadler et al. 2002). The kinetics of CO inhibition has been studied by PFV experiments and the kinetics of CO binding was fast, about 10^8 s^{-1}/M. Thus, it has been shown that diffusion was the rate-limiting step of CO-inhibition. As a result, this inhibitor has been used to probe gas diffusion in hydrogenases (Leroux et al. 2008; Liebgott et al. 2010).

c. Specific Characteristics of the Naturally Occurring O_2-Tolerant [NiFe]-Hydrogenases

[NiFe] hydrogenases are considered to be more robust than [FeFe] hydrogenases because they can be totally reactivated after inhibition by O_2. Moreover, there are even a few examples in nature of relatively O_2-tolerant [NiFe] hydrogenases. Thus, the [NiFe] hydrogenase can be classified as either 'standard O_2-sensitive' or 'O_2-tolerant' based on their ability to function in the presence of O_2. O_2-tolerance defines one hydrogenase that retains some activity in the presence of O_2. The level of residual activity can vary depending on the enzyme, but it

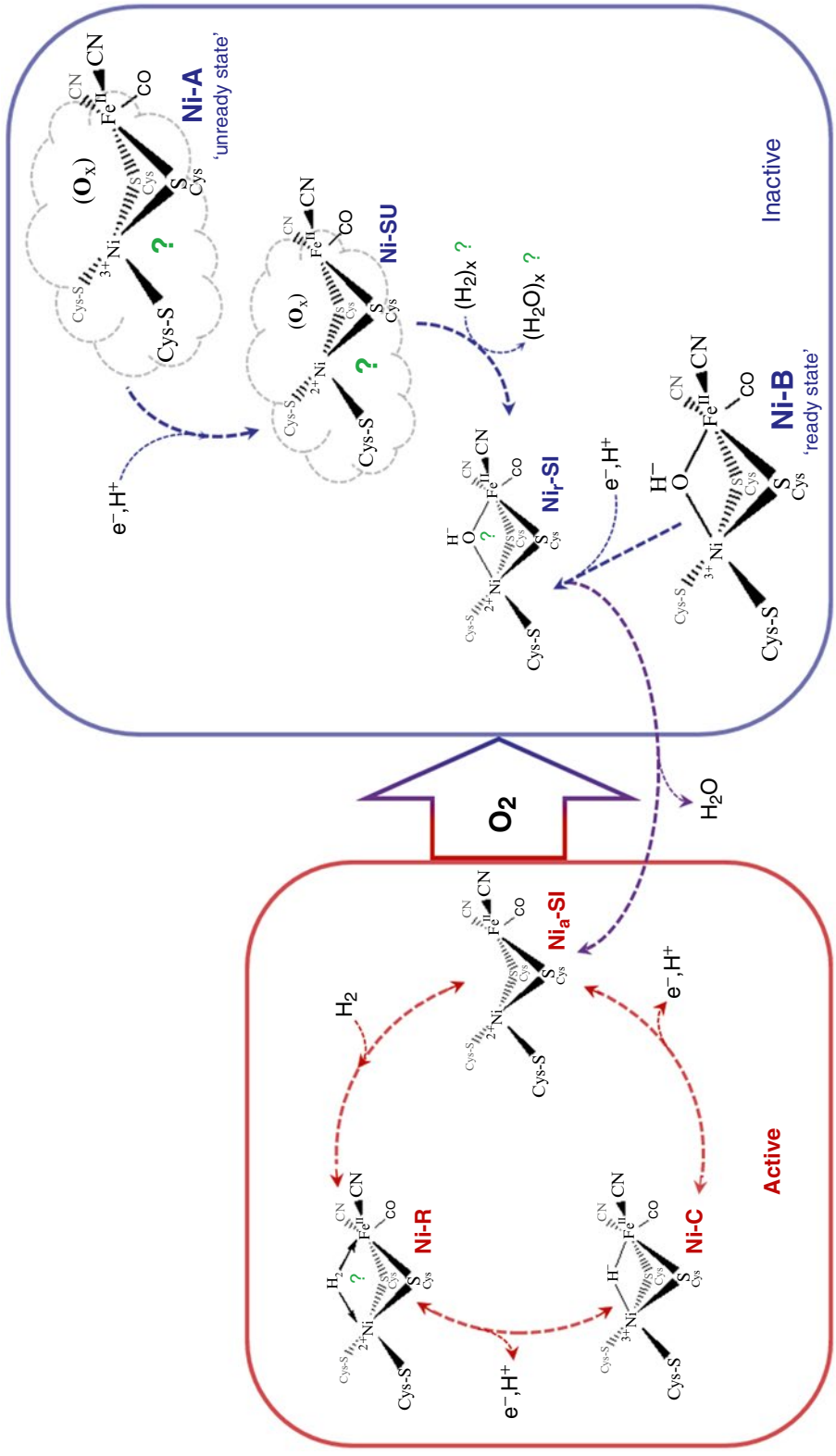

Fig. 3.4. Proposed reaction scheme for the standard O_2 sensitive [NiFe]-hydrogenase from *D. fructosovorans* based on electrochemical titrations, including both active and inactive intermediates. The nomenclature is based on that initially adopted to designate the EPR paramagnetic states (Ni-A, NiB, NiC) and the "Ni-S" designates an EPR silent state. In Ni-A and Ni-SU states, the binding oxygenic species presented by (O_x) has not been conclusively determined. There are multiple forms of Ni-C and Ni-R and these have been suggested to differ in terms of the redox state of the nearest (proximal) iron–sulfur cluster and/or protonation of the cysteinyl sulfur atoms.

should be remembered that trace amounts (a few μM) of O_2 readily inhibit standard O_2-sensitive [NiFe] hydrogenases.

The fact that a large number of microbes are able to use H_2 as the sole energy source in the course of aerobic respiration indicates that some [NiFe] hydrogenases afford a specific protection against detrimental effects of O_2 (Tremblay and Lovley 2012). This aerobic H_2 oxidation occurs in phylogenetically diverse groups of prokaryotes such as the nitrogen-fixing bacterium *Bradyrhizobium japonicum* (Kaneko et al. 2002), the photosynthetic proteobacterium *Rhodobacter capsulatus* (Strnad et al. 2010), the hyperthermophilic bacterium *Aquifex aeolicus* (Deckert et al. 1998) and the well-studied proteobacterium *Ralstonia eutropha* H16 that contains even three indigenous O_2-tolerant hydrogenases (Schwartz et al. 2003; Pohlmann et al. 2006; Lenz et al. 2010; Bürstel et al. 2011).

R. eutropha H16 is a bacterium that lives in soil and water and it is one of organisms able to grow chemolithoautotrophically using hydrogen as the sole energy source and dioxygen as terminal electron acceptor. This is called the Knallgas (detonating gas) reaction. The three distinct O_2-tolerant [NiFe] hydrogenases, that each serve unique physiological roles, are: a bidirectional cytoplasmic Soluble Hydrogenase (*Re*SH) able to generate reducing equivalents by reducing NAD^+ at the expense of hydrogen (Group III), a Regulatory Hydrogenase (*Re*RH) which acts in a signal transduction cascade to control transcription of hydrogenase genes (Group II) and a Membrane-Bound Hydrogenase (*Re*MBH) coupled to the respiratory chain (Group I) (Burgdorf et al. 2005). Interestingly, these O_2-tolerant hydrogenases are usually less active than standard O_2-sensitive enzymes. Their H_2 oxidation activities are reduced by a factor of about 5 for the *Re*SH (Ludwig et al. 2009a), about 50 for the *Re*MBH and about 500 for the *Re*RH (Vignais and Billoud 2007). The H_2 production activities are usually considerably weak, especially because of the strong inhibitory effect of H_2 (Goldet et al. 2008). However, these O_2-tolerant enzymes represent precious inspiration sources for the study of the molecular bases of O_2 inhibition.

Three strategies seem to have been developed by *R. eutropha* H16 to allow its [NiFe] hydrogenases to be catalytically active in the presence of dioxygen.

In the case of the SH (Group III), the O_2 resistance was assumed to be due to the presence of extra CN ligands at the active site (Happe et al. 2000) that might be incorporated by a specific maturation protein HypX (Bleijlevens et al. 2004). Deletion of *hypX* led to a lower O_2 resistance of the *Re*SH enzyme (Bleijlevens et al. 2004) while the activities of *Re*MBH (Buhrke and Friedrich 1998) and of the regulatory hydrogenase (*Re*RH) remained unaffected (Buhrke et al. 2001). However, the presence of a Ni-Bound cyanide under native conditions has been recently ruled out (Horch et al. 2010). In this context, the supply of low-potential electrons from the oxidation of NAD(P)H appears to play a major role in preserving catalytic activity under aerobic conditions in vivo. However, despite considerable efforts and promising insights (Horch et al. 2012), the structural and mechanistic basis for this property has still to be resolved. Bidirectional cytoplasmic soluble hydrogenases are of particular interest for biotechnological applications as they are suited for light driven hydrogen production in vivo (Prince and Kheshgi 2005) and the regeneration of NAD(P)H in biocatalytic processes (Okura et al. 1990; Ratzka et al. 2011). Such applications are particularly promising as some of these enzymes are oxygen tolerant in contrast to most other hydrogenases (Happe et al. 2000; Horch et al. 2012).

The *Re*RH and related enzymes adopted apparently another strategy consisting in reducing the gas channel size at the level of the interface with the active site cavity. At the end of the hydrophobic channel, near the active site, two hydrophobic residues, usually valine and leucine that are conserved in O_2-sensitive hydrogenases, are replaced by larger residues, respectively isoleucine and phenylalanine, in the O_2-tolerant hydrogen-sensors

(Volbeda et al. 2002) (Fig. 3.3a). It has therefore been suggested that increasing the bulk of residues occupying these two positions may act like a molecular sieve, reducing the channel diameter at that point, thereby preventing efficient dioxygen access to the active site. This hypothesis was supported by two experiments in which the bulky amino-acids from *Re*RH were substituted by valine and leucine. In both cases, determination of the inactivation kinetics in the presence of dioxygen revealed that the mutated enzymes were inactivated after prolonged incubation and required a reductive activation to reach the maximum activity (Burgdorf et al. 2005; Duche et al. 2005). Even though the mutated enzymes became more sensitive to dioxygen than the wild-type, it should be noted that they retain a significant level of activity after prolonged dioxygen exposure, and therefore still belong to the O_2-tolerant group of hydrogenases.

Membrane bound hydrogenases of the group I represent the best studied group of O_2-tolerant hydrogenases and has recently gained extensive attention, due to their potential biotechnological importance. Thus, the O_2 tolerance of MBH (group I) has been recently discovered through the hydrogenase crystal structures of *R. eutropha* H16 (*Re*MBH, pdb accession number 3RGW) (Fritsch et al. 2011), *E. coli* (*Ec*Hyd-1, pdb 3UQY, 3USC and 3USE) (Volbeda et al. 2012), and *Hydrogenovibrio marinus* (*Hm*MBH, pdb 3AYX, 3AYY, 3AYZ) (Shomura et al. 2011) and spectroscopic data obtained from hydrogenases of *A. aeolicus* (*Aa*Hyd-1) (Pandelia et al. 2011) and *Re*MBH (Fritsch et al. 2011; Goris et al. 2011; Lukey et al. 2011). Therefore, the recent crystallographic structures from three members of the Group I revealed that there are no significant differences between the [NiFe] catalytic centres of standard or O_2-tolerance hydrogenases. The presence of the usual nickel signatures detected by EPR indicates that the chemistry at the active site is identical to that catalyzed by standard hydrogenases.

Remarkably, there are two additional cysteine residues in the close vicinity of the proximal FeS cluster that are absent in O_2-sensitive standard [NiFe] hydrogenases which lead to the construction of a unique proximal [4Fe-3S] cluster with in-total six coordinating cysteine residues (Fig. 3.5). These two additional cysteines are fully conserved in the O_2-tolerant hydrogenase (Pandelia et al. 2010a). The sulfur atom of an extra cysteine replaces one of the inorganic sulfides and thus becomes an intrinsic cluster ligand, whereas the second cysteine terminally coordinates one of the Fe atoms (Fig. 3.5). This particular structure studied by EPR in its reduced or oxidized form in *Hm*MBH, *Aa*Hyd-1 and *Re*MBH, indicate that the [4Fe-3S] cluster is stable in three oxidation states. These redox-dependent structural changes promoted by the surplus of cysteine coordination, give the potential to the proximal cluster to theoretically deliver two electrons for the O_2-reduction instead of one (Goris et al. 2011; Lukey et al. 2011). Thus when O_2-tolerant [NiFe]-hydrogenases are attacked by O_2, they might fully reduce O_2 to water, thereby avoiding the production of reactive oxygen species that would damage or block the active site. As a result, it is currently assumed that the electron deficiency during O_2 attack might be responsible for the formation of the Ni-A inactive form ('unready' state) (Ogata et al. 2009, 2010). O_2-tolerant hydrogenases would escape the 'unready' state by forming only the 'ready' conformation (Ni-B) (in which O_2 has been fully reduced), which reactivates very easily to re-join the catalytic cycle (Armstrong et al. 2009; Lenz et al. 2010). However, in the case of *Aa*Hyd-1, a weak Ni-A signal has been reported to appear after O_2 exposure (Guiral et al. 2006), while only Ni-B was detected in a recent study (Pandelia et al. 2010b). The *Ec*Hyd-1 exhibits a Ni-A signal upon aerobic isolation but this signal is then barely detectable when the enzyme is exposed to O_2 after activation (Lukey et al. 2010).

In PFV experiments, the activity of the *Re*MBH and *Aa*Hyd-1 recover extremely

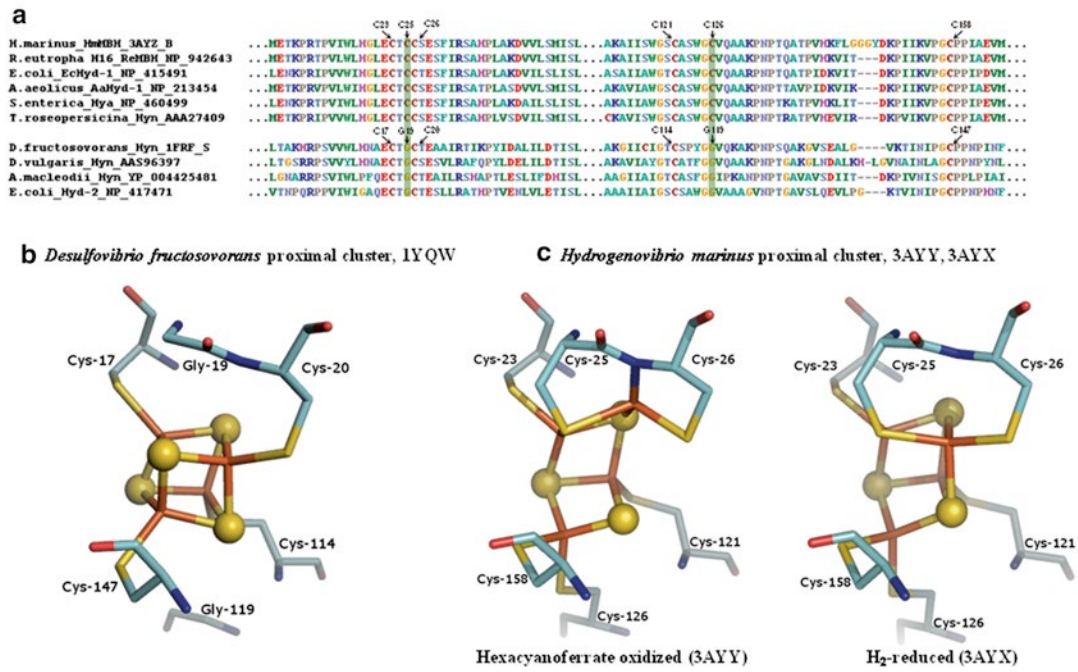

Fig. 3.5. Multiple sequence alignment and structural comparison of the proximal clusters between O_2-sensitive and O_2-tolerant hydrogenases. Panel (**a**) shows the multiple sequence alignment comparing the cysteines which ligate the proximal cluster in O_2-sensitive [NiFe]-hydrogenases and O_2-tolerant enzymes. Panels (**b**) and (**c**) shows the structural comparison between the proximal FeS clusters of *D. fructosovorans* (O_2-sensitive hydrogenase, 1YQW) and *H. marinus* (O_2-tolerant hydrogenase, 3AYY and 3AYX). Panel (**c**) Details of the structural changes associated with 'super-oxidation' of the proximal cluster in membrane bound O_2-tolerant [NiFe]-hydrogenases (Adapted from Parkin and Sargent 2012).

fast after O_2 exposure (Armstrong et al. 2009; Pandelia et al. 2010b). The O_2-tolerance properties of these enzymes are therefore likely to be due to a fast reactivation rate, as shown in recent electrochemical studies (Armstrong et al. 2009; Liebgott et al. 2010; Pandelia et al. 2010b) and by the lack of the Ni-A signal after aerobic inactivation.

The exploitation of these hydrogenases and their molecular determinants is a major challenge for a broad range of biotechnological applications. Indeed, microorganisms harboring optimized hydrogenases may play a major role in H_2 generation for fuels. Bio-fuel cells and biosensors also represent an important potential application of these hydrogenases as immobilized enzymes. At last, these hydrogenases might also allow searches in the metabolic engineering in the aim to improve the H_2 production or in protein engineering to mimic molecular determinants responsible to the tolerance towards of O_2.

III. Maturation of Hydrogenases: Specific and Complex Process

In metal-containing enzymes, complex active sites generally require specific machineries for their synthesis and assembly. Indeed, assembly of the active hydrogenase involves sophisticated biological processes, such as careful co-ordination of cofactor biosynthesis and insertion, subunit recruitment, and protein target processes (Vignais et al. 2001; Paschos et al. 2002; Böck et al. 2006).

A. [FeFe]-Hydrogenase Maturation: Protein Machinery

In order to be catalytically active after its synthesis, the [FeFe]-hydrogenase polypeptide encoded by the *hydA* gene has to incorporate the H-cluster and, when required, accessory [Fe-S] clusters. This post-translational process is extraordinarily complex as it involves a number of difficult reactions including: (i) the synthesis of CO, CN and the dithiolate bridging ligand; (ii) the assembly of the di-iron active site subcluster; (iii) its incorporation into the enzyme already containing the [4Fe-4S] component of the H-cluster and (iv) the assembly and transfer of the accessory FeS clusters.

The [FeFe]-hydrogenase maturation protein machinery was initially discovered in the eukaryotic green alga *Chlamydomonas reinhardtii* incapable of H_2 production (Posewitz et al. 2004). The disruption of either the *hydEF* or *hydG* (Hyd machinery) gene resulted in a mutant that proved to be unable to produce hydrogen, even though full-length hydrogenase accumulated. Genes encoding for HydE, HydF, and HydG are present in all organisms capable of synthesizing an active [FeFe]-hydrogenase (HydA) (Meyer 2007). Thus, it can be concluded that HydEF and HydG provide the minimal protein machinery necessary for the synthesis and assembly of the H-cluster. Whether other proteins are required for an optimal maturation process has not been demonstrated so far. Moreover, several reports of heterologous expression of active [FeFe]-hydrogenases have demonstrated that the Hyd machinery from one organism can be successfully used for the maturation of an enzyme from another. For example, expression of an active HydA1 enzyme from *C. reinhardtii* or *Scenedesmus obliquus* (green algae) has been shown to be possible using *Clostridium acetobutycicum*, another [FeFe]-hydrogenase synthesizing organism, as the expression host (Girbal et al. 2005). Further evidence for the lack of selectivity of the Hyd machinery came from the observation that co-expression of HydE, HydF and HydG from the bacterium *Cl. acetobutylicum* with various algal and bacterial [FeFe]-hydrogenases in *E. coli* resulted in purified enzymes with specific activities that were not very different from those of their counterparts from native sources (Böck et al. 2006). Finally, the bacterium *Shewanella oneidensis* proved to be an efficient system for the expression and maturation of HydA1 from *C. reinhardtii* (Sybirna et al. 2008).

B. [NiFe]-Hydrogenase Maturation: Protein Machinery

At present, cloning [NiFe]-hydrogenases is still very difficult and the progresses realised recently remain very limited (Burgdorf et al. 2005; Ludwig et al. 2009b). Even though [NiFe]-hydrogenase operons are highly conserved and exhibit a high degree of similarity, each maturation system is specific to the corresponding structural subunits, probably because of tight protein-protein interactions occurring during processing (Leach et al. 2007). Indeed, the complex architecture of the active site of [NiFe]-hydrogenases with their diatomic ligand (CN and CO) requires a specific and complex maturation system. There are two main groups of genes responsible for maturation, which are differentiated by their resultant phenotypes. The first group of genes is mainly located on the same transcription unit as the structural genes. Disruption of this group of genes specifically impairs the processing or activity of the hydrogenase encoded *in cis* in the operon, without affecting the maturation of other hydrogenases. The maturation processes mediated by the products of this family of accessory genes cannot be complemented *in trans* by homologous genes from the other hydrogenase operons, regardless of the degree of similarity (Sauter et al. 1992; Menon et al. 1994; Bernhard et al. 1996). This specific barrier is one of the key reasons for the failure of the active hydrogenase production in heterologous hosts. The second group is another set of the *hyp* ('p' for pleiotropic) genes which encode proteins, involved in the insertion of Ni, Fe, CO and CN into the active site (Jacobi et al. 1992; Maier et al. 1996; Wolf et al. 1998; Böck

et al. 2006; Mulder et al. 2011; Petkun et al. 2011). Mutations of these genes affect the synthesis and activity of all the hydrogenase isoenzymes. However, the functions of this set of genes can be complemented *in trans* by heterologous genes (Chaudhuri and Krasna 1990).

C. O_2-Tolerant [NiFe]-Hydrogenase Maturation: Protein Machinery

It would be particularly fruitful to take advantage of the properties of the O_2-tolerant hydrogenases by cloning their corresponding genes into organisms of biotechnological interests. Although [NiFe]-hydrogenase exhibit reversible inhibition by oxygen, the sensitivity of hydrogen production in presence of O_2 is a multifaceted problem, since hydrogenase transcription, and likely maturation and assembly, might be also inhibited by exposure to atmospheric oxygen (Soboh et al. 2012). However, among O_2-tolerant hydrogenases, some are synthesized solely under aerobic conditions (Lukey et al. 2011; Tremblay and Lovley 2012). *E. coli* could be one of the most informative model systems for understanding the biosynthesis of O_2-tolerant enzymes because the bacterium produces both O_2-tolerant MBH (Hyd-1) and standard O_2-sensitive (Hyd-2) hydrogenases. Among the three types of O_2-tolerant hydrogenases, only the MBH family appears to require specific maturation proteins, necessary for the synthesis of the unique [4Fe-3S] proximal cluster (Figs. 3.5 and 3.6). Hyd-1 is produced from an operon of six genes, *hyaABCDEF*, where HyaA is the small subunit, HyaB is the large subunit, HyaC is a cytochrome that anchors the [NiFe]-hydrogenase to the membrane and the HyaD is the specific protease required for large subunit maturation-terminal processing. There are therefore two extra genes *hyaE* and *hyaF* that are not required for the assembly of standard O_2-sensitive hydrogenases (Hyd-2 and others) and are apparently only involved in the assembly of O_2-tolerant respiratory enzymes (Schubert et al. 2007).

In *R. eutropha* H16 the HyaE homolog is HoxO, which has been shown to interact with the small subunit during biosynthesis and is essential for MBH activity in that organism (Schubert et al. 2007) (Fig. 3.6). In *Rhizobium leguminosarum* the HyaE homolog, HupG, was shown to be only required for small subunit maturation under aerobic conditions (Manyani et al. 2005), and in *E. coli*, which only expresses Hyd-1 under anaerobic conditions, the *hyaE* gene was dispensable for Hyd-1 biosynthesis (Dubini and Sargent 2003). Similarly, the HyaF (HoxQ in *R. eutropha* H16 and HupH in *R. leguminosarum*) is also absolutely required for MBH activity. HyaF interacts with HyaE to form a complex together with the small subunit during assembly (Schubert et al. 2007). Genetic two-hybrid studies suggested that HyaE interacted strongly with HyaA, the small subunit, (Dubini and Sargent 2003). It is possible, therefore, that HyaE-like proteins have a role to play in assembly of the [4Fe-3S] cluster. Shomura et al. (2011) suggested that additional negative charges around the proximal [4Fe-3S] cluster in the final structure may be important for its stabilization. Interestingly, HyaE has a perfect thioredoxin-like fold (Parish et al. 2008), except that in the HyaE protein, acid residues (two aspartates and two glutamates) are found at the same position as the redox-active cysteines, originally present in true thioredoxins (Parish et al. 2008). Thus, it is possible that this negatively charged region mediates protein-protein interactions with the small subunit, though it can also be considered that it might be involved in protecting the proximal cluster until the large subunit has docked correctly with its small subunit partner.

IV. Enzyme and Metabolic Engineering to Improve H_2 Production

Genetic modifications of hydrogen metabolism or hydrogenases, can be very promising strategies to achieve an efficient H_2 production system or to improve hydrogenases as biocatalysts. During the last 5 years, many reviews dealt with this topic and summarized the scientific and technological hurdle encountered (Ghirardi et al. 2007; Germer et al. 2009; Brentner et al.

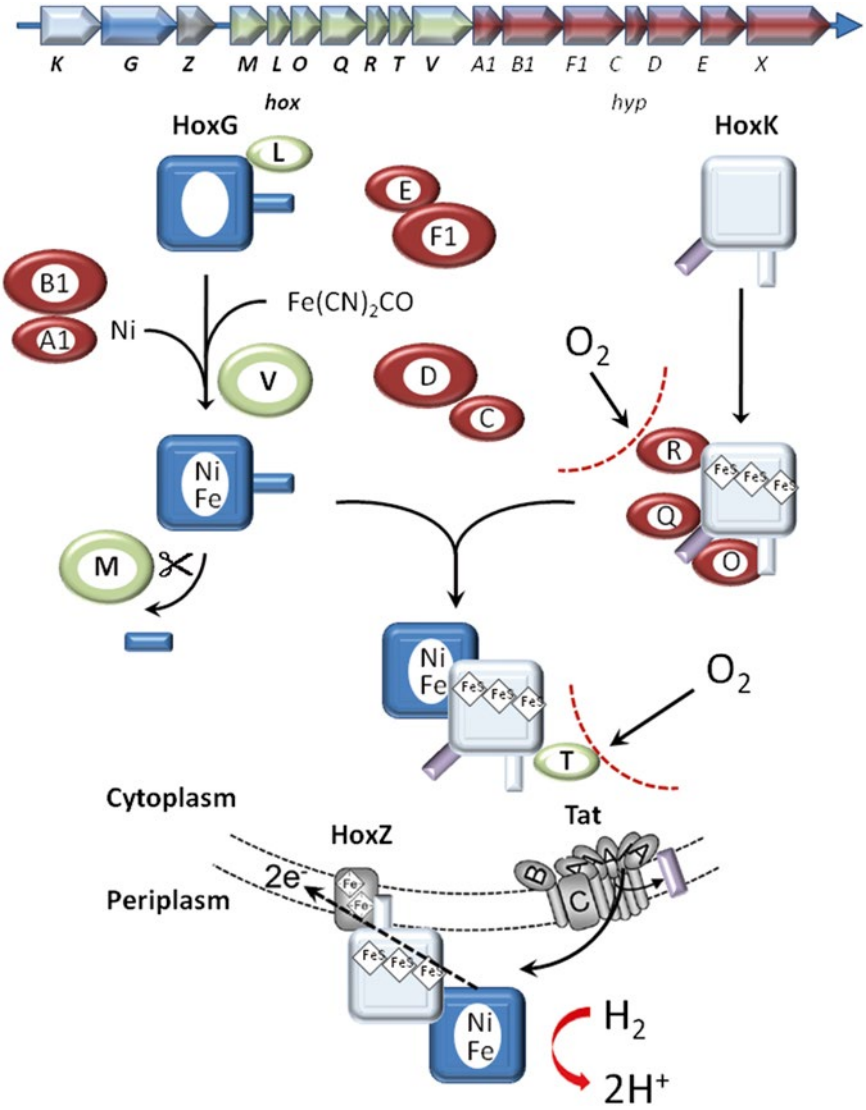

Fig. 3.6. Schematic maturation process of the [NiFe] hydrogenase. The *R. Eutropha* MBH gene cluster is presented at the *top. hoxK* encodes the small subunit and *hoxG* encodes the large subunit. The genes in *red* and *green* encode the accessory proteins, the role of which in the assembly of the active is presented (Adapted from Fritsch et al. 2011).

2010; McKinlay and Harwood 2010; Abo-Hashesh et al. 2011; Hallenbeck et al. 2012). In this section, we will discuss the different processes of engineering used to generate significant improvement in the production of hydrogen or hydrogenases, it will be divided in three points: (A) the heterologous expression and overexpression of interesting hydrogenases; (B) enhancing the efficiency of H_2 production by redirecting the flow of reducing equivalents toward hydrogenases, and (C) increasing the O_2 tolerance of hydrogenase by enzyme engineering.

A. Heterologous-Expression and Overexpression of Hydrogenases

In order to characterize their structure-function properties in greater detail, and to use hydrogenases for biotechnological applications, reliable methods for rapid, high-yield expression and purification are required. Owing to genetic manipulations, the purification of recombinant hydrogenases is greatly facilitated by the use of affinity tags, such as His or StrepII-tag, inserted at the N- or C-terminus (Kim et al. 2012). One approach that has been adopted in order to enhance the amount of enzymes or promote the H_2 production, is the engineering of a stable hydrogenase either through its production in a heterologous host or through overexpression (English et al. 2009; Abo-Hashesh et al. 2011). The interest of heterologous expression systems is not to be demonstrated but is still limited to [FeFe] hydrogenases (Böck et al. 2006; Nicolet and Fontecilla-Camps 2012).

1. [FeFe]-Hydrogenase

In recent years, several groups have developed different strategies for the expression of recombinant [FeFe]-hydrogenases (English et al. 2009) (Table 3.2). These include using as hosts organisms bacteria expressing naturally native [FeFe]-hydrogenase (Girbal et al. 2005; Sybirna et al. 2008), or using *E. coli* for heterologous expression in a more common way. At present, in order to obtain large amounts of [FeFe]-hydrogenases for biochemical and biophysical studies, the studies are focused on the high yield heterologous production mainly in *E. coli* (Kuchenreuther et al. 2010; Yacoby et al. 2012). On the other hand, the overexpression of [FeFe]-hydrogenase is also an important factor for the improvement of H_2 production. Indeed, the most efficient hydrogen-producing enzymes are [FeFe]-hydrogenases, which can have an activity about 10–100 times higher than that of [NiFe]-hydrogenases. As a result, overexpressing (HydA) in *Cl. paraputrificum* M-21 (Morimoto et al. 2005) improved the H_2 yield from 1.4 to 2.4 mol H_2 per mol of glucose. Jo et al. (2009) also reported that *Cl. tyrobutyricum* JM1 showed an improved H_2 yield (1.8 mol H_2/mol glucose) compared to the parental strain (1.2 mol H_2/mol glucose), when HydA was overexpressed. Regarding metabolic engineering, the inactivation of *ack*, which encodes acetate kinase of *Cl. tyrobutyricum* (Liu et al. 2006) increased the H_2 production yield by 1.5-fold compared to the wild-type strain.

2. [NiFe]-Hydrogenase

The main interests in producing [NiFe]-hydrogenases in heterologous hosts are to improve hydrogen production by focusing on (a) the heterologous expression of bidirectional [NiFe]-hydrogenases of the group III and (b) to take advantage of the O_2 tolerance properties of some [NiFe]-hydrogenases (Carrieri et al. 2011) (Table 3.2). However, as discussed above, the development of heterologous expression systems for the biosynthesis and molecular engineering of [NiFe]-hydrogenases is challenging due to the complexity and the high specificity of the maturation process. There are several documented examples of non-functional heterologous expression, which have only recently been reported for a limited number of organisms. For example, heterologous expression of [NiFe]-hydrogenases from *Rhodococcus opacus*, *Desulfovibrio vulgaris*, and *Synechocystis* sp. PCC6803 all resulted in the production of non-functional hydrogenases (Voordouw et al. 1987; Grzeszik et al. 1997; Maeda et al. 2007). For this reason, expression and purification of [NiFe]-hydrogenase for structural and in vitro studies are most often accomplished through the development of plasmid -based expression in homologous strains or closely related species as expression hosts (Rousset et al. 1998; Burgdorf et al. 2005; Ludwig et al. 2009b).

E. coli is an ideal microorganism commonly used in genetic engineering due to its well-characterized genome, well known

Table 3.2. Expression of [FeFe]- and [NiFe]-hydrogenases in different systems and their activities.

[FeFe]-hydrogenase genes	Expression host	H_2 evolution activity (µmol H_2.min^{-1}.mg^{-1})	Bibliography
Chlamydomonas reinhardtii	*Escherichia coli*	0.4	Posewitz et al. (2004)
Clostridium acetobutylicum	*C. acetobutylicum*	10	Girbal et al. (2005)
Chlamydomonas reinhardtii	*C. acetobutylicum*	760	Girbal et al. (2005)
Scenedesmus obliquus	*C. acetobutylicum*	633	Girbal et al. (2005)
Clostridium paraputificum M-21	*C. paraputrificum*	1.7 fold more	Morimoto et al. (2005)
Clostridium acetobutylicum	*E. coli*	75.2	King et al. (2006)
Chlamydomonas reinhardtii	*S. oneidensis*	700	Sybirna et al. (2008)
Clostridium tyrobutyricum	*C. tyrobutyricum*	1.7 fold more	Jo et al. (2009)
Enterobacter aerogenes	*Enterobacter aerogenes*	1.95 fold more	Zhao et al. (2010)
Chlamydomonas reinhardtii	*E. coli*	641	Kuchenreuther et al. (2010)
Clostridium pasteurianum	*E. coli*	1,087	Kuchenreuther et al. (2010)
Chlamydomonas reinhardtii	*E. coli*	1,000	Yacoby et al. (2012)

[NiFe]-hydrogenase genes	Expression host	H_2 uptake/evolution activity, MV-linked (µmol H_2.min^{-1}.mg^{-1})	Bibliography
Desulfovibrio gigas (*Group I*)	*D. fructosovorans*	Nd	Rousset et al. (1998)
Desulfovibrio fructosovorans (*Group I*)	*D. fructosovorans*	700 (up.)	Rousset et al. (1998)
Rhodococcus opacus (*Group III*)	*Ralstonia eutropha*	7.8 (up.)	Porthun et al. (2002)
Ralstonia eutropha (*Group II*)	*E. coli*	Nd	Posewitz et al. (2004)
Ralstonia eutropha (*Group II*)	*Ralstonia eutropha*	1.8 (up.)	Buhrke et al. (2005)
Ralstonia eutropha (*Group I*)	*Pseudomonas stutzeri*	19 (up.)	Lenz et al. (2005)
Synechocystis sp. PCC6803	*E. coli*	Nd	Maeda et al. (2007)
Pyrococcus furiosus (*Group III*)	*E. coli*	89 (evol.)	Sun et al. (2010)
Hydrogenovibrio marinus (*Group I*)	*E. coli*	0.06 (evol.)	Kim et al. (2011)
Alteromonas macleodii (*Group I*)	*Synechococcus elongatus*	~1.10^{-6} (evol.)	Weyman et al. (2011)
Thiocapsa roseopersicina (*Group I*)	*Synechococcus elongatus*	~1.10^{-6} (evol.)	Weyman et al. (2011)
Alteromonas macleodii (*Group I*)	*E. coli*	~4.10^{-3} (evol.)	Weyman et al. (2011)
Thiocapsa roseopersicina (*Group I*)	*E. coli*	~4.10^{-4} (evol.)	Weyman et al. (2011)
Klebsiella oxytoca (*Group IV*)	*K. oxytoca*	11 (evol.)	Bai et al. (2012)

metabolism, and its ability to utilize a wide range of carbon sources including hexoses and pentoses. In addition, *E. coli* has the potential advantages over at least some other microorganisms to exhibit a rapid growth, has simple nutritional requirements and also harbors four [NiFe]-hydrogenases. Two hydrogenases, one O_2-tolerant hydrogenase (Hyd-1) and one standard O_2-sensitive hydrogenase (Hyd-2) encoded by the *hya* and *hyb* operons respectively, are involved in periplasmic hydrogen uptake. Two others, hydrogenase 3 and 4, are part of cytoplasmically oriented hydrogenase complexes. Hydrogenase 3, encoded by the *hyc* operon, produces hydrogen from formate as a part of the formate hydrogen lyase complex (FHL-1), which is active in hydrogen production during mixed-acid fermentation at acidic pHs (Vignais et al. 2001). Hydrogenase 4, encoded by the *hyf* operon, appears to be cryptic under normal circumstances (Self et al. 2004).

Thus, *E. coli* has a set of specific maturation proteins, which might be used for the maturation of heterologous enzymes (Porthun et al. 2002; Maroti et al. 2003, 2009). Although *E. coli* is perhaps the most useful organism as a target for metabolic engineering, the lack of any NADH-dependent hydrogenases (Group III) is one major hurdle for the engineering of hydrogen metabolism in this organism. Indeed, these bidirectional cytoplasmic hydrogenases function reversibly in their physiological setting, coupling hydrogen uptake or emission, to oxidation and reduction of cellular coenzymes, such as NAD(P)H (Cournac et al. 2004). So, in *E. coli*, NAD(P)H generated through carbon metabolism cannot be used directly for H_2 production (Schmitz et al. 2002). Therefore, expressing a heterologous NAD(P)H-dependent hydrogenase is one of the main goals for increasing the maximum H_2 yield in *E. coli*.

a. Heterologous Expression of Bidirectional [NiFe]-Hydrogenases (Group III) in *E. coli*

The [NiFe]-hydrogenase of the cyanobacterium *Synechocystis* sp. PCC6803 is a well-studied representative of the bidirectional [NiFe]-hydrogenases from Group III. Therefore, the maturation process has been reconstituted to allow functional expression of this hydrogenase in *E. coli* (Maeda et al. 2007; Wells et al. 2011; Zheng et al. 2012). As a result, the introduction of the bidirectional [NiFe]-hydrogenase of *Synechocystis* sp. PCC6803 has altered the whole metabolism for hydrogen production in *E. coli*. Firstly, the hydrogen productivity was enhanced up to 41-fold in comparison with *Synechocystis* sp. PCC6803 (Maeda et al. 2007). Secondly the expression of this hydrogenase also showed a distinct H_2 production pathway than the one initially presents in *E. coli* (Wells et al. 2011). Finally, this heterologous expression has suppressed the transcription of native uptake [NiFe]-hydrogenases (Hya and Hyb) (Maeda et al. 2007; Zheng et al. 2012), increasing significantly the production of H_2. Similarly, the successful expression in *E. coli* of a recombinant cytoplasmic, NADP-dependent hydrogenase from *Pyrococcus furiosus*, an anaerobic hyperthermophile increased significantly the production of H_2. Remarkably, the native *E. coli* maturation machinery was able to generate a functional hydrogenase when transformed with only the genes encoding the hydrogenase structural subunits and the C-terminal protease (Sun et al. 2010).

b. Heterologous Expression of O_2-Tolerant [NiFe]-Hydrogenase (Group I)

The biotechnological goal of algal and cyanobacterial hydrogen production is to divert the reducing equivalents away from normal growth functions and to redirect them toward hydrogenases. All the enzymatic components for hydrogen production from water splitting and sunlight are present in cyanobacteria. As discussed above, hydrogenases are inactivated by molecular oxygen, which represent a major technological hurdle for hydrogen production from cyanobacteria at high solar efficiencies. Thus, strategies for overcoming this barrier include heterologous expression of a more oxygen-tolerant hydrogenase in cyanobacteria. In this connection, a system of heterologous expression has been developed to express O_2-tolerant [NiFe]-H_2ase belonging to group I from the bacteria *Alteromonas macleodii* and *Thiocapsa roseopersicina* (Vargas et al. 2011) in the cyanobacterium *Synechococcus elongatus* (Maroti et al. 2009); (Vargas et al. 2011). The cloned enzymes were active, indicating that it is possible to express hydrogenases in the cyanobacteria.

Recently, Kim et al. (2011) have succeeded in performing a heterologous expression of oxygen-tolerant *Hydrogenovibrio marinus* [NiFe]-hydrogenase in *E. coli*. Interestingly, recombinant *H. marinus* [NiFe]-hydrogenase produced of sevenfold to ninefold more hydrogen than did *E. coli* [NiFe]-hydrogenase (Hyd-1) in a gaseous environment containing 5–10 % (v/v) oxygen. Likewise, the same team (Kim et al. 2012)

has improved the H_2 production (1.3 fold more) of this recombinant *H. marinus* [NiFe]-hydrogenase in co-expressing it with the proteorhodopsin under light conditions.

B. Substrate Selectivity, Competition and Linking

When expressed in vivo, hydrogenases interact with electron carriers which are generally at the junction of numerous redox reactions (respiration, CO_2 fixation, assimilation, etc.). A key point is that these reactions that appear as competitors for biotechnological purposes are often essential for cell survival or development. This explains in part the difficulty and the slow progress in biohydrogen research. One proposed research direction, to overcome this kind of limitation, would be to increase the amount of specific redox partners of hydrogenases. In this sense, a recent study has showed that the overexpression of ferredoxin-NADPH-reductase (FNR) coupled to that of ferredoxin (Fd) and a [FeFe]-hydrogenase, increased the H_2 production when NADPH was added in the medium (Weyman et al. 2011).

Another strategy to favor H_2 production would be to carry out an expression of synthetic or chimerical enzymes based on native hydrogenase fused to the electron transfer subunit (Kontur et al. 2012). In the case of algal [FeFe]-hydrogenases for instance, the redox partner is Fd (Fig. 3.1), which is also involved in photosynthetic carbon fixation via NADPH production by FNR. Indeed, under the anaerobic conditions that support hydrogen production, there is a significant loss of photosynthetic electrons toward NADPH production supplied by ferredoxin:$NADP^+$ oxidoreductase (FNR). No algal [FeFe]-hydrogenase structure is available yet, but Horner et al. (2002) modeled algal hydrogenase structure and charge distribution, and identified a set of amino-acids likely to participate in electrostatic interaction with algal Fd. Mutagenesis experiments conducted at the (putative) Fd binding sites in hydrogenase (and also at the Fd binding site in FNR) could be a way to modify relative affinities of these enzymes for their substrate and ultimately tune these affinities for an optimal ratio between photosynthetic and hydrogen-producing capabilities. Some results illustrated the feasibility of utilizing directly attached redox partners for H_2 production in vivo (Agapakis et al. 2010). Indeed, it has been reported that H_2 production via Fd-dependent hydrogenase can be improved by manipulating the interaction between hydrogenase and Fd via protein surface engineering in *E. coli*. Some chimerical enzymatic complexes have been expressed in *E. coli*, in which a ferredoxin and heterologous [FeFe] hydrogenase were either immobilized in a modular protein scaffold or directly attached to each other via an amino acid linker. H_2 production from cells containing these complexes showed 3-fold and 4.4-fold increases in H_2 production, respectively, over cells containing separate versions of the same proteins (Agapakis et al. 2010). However, yields from these complexes were relatively low (<0.1 mol H_2/mol glucose). More recently, a complex consisting of the Fd fused to the [FeFe]-hydrogenase HydA from *C. reinhardtii* (green algae), was shown to improve the light-dependent H_2 production in vitro when incubated with purified PSI (Yacoby et al. 2011). This algal Fd-HydA fusion prevents the competition between FNR and HydA that both exhibit affinity with Fd. Moreover, Fd-HydA fusion improved HydA function in several respects. First, the specific activities were up to sixfold higher than for the native HydA. Second, the fusion successfully insulates its internal Fd electrons, because only 10 % of the electrons are lost with external competitors such as FNR. Third, the fusion was able to overcome the limitation caused by FNR, as more than 60 % of photosynthetic electrons were diverted to hydrogen production, compared to less than 10 % for nonfused HydA (Yacoby et al. 2011).

Another tempting approach to favor H_2 production would be to tightly connect a specific electron carrier or a photosystem with hydrogenase, making the electrons flow directly from the photosystem to the

hydrogenase or from the hydrogenase to the electron acceptor, avoiding competition with the bulk of electron carriers. In a study, Ihara et al. (2006) engineered a 'hard-wired' protein complex consisting of a hydrogenase and a photosystem. They designed an artificial fusion protein composed of the membrane-bound [NiFe]-hydrogenase from *R. eutropha* H16 and the peripheral photosystem I (PSI) subunit PsaE (involved in the docking of Fd to the PSI) of the cyanobacterium *Thermosynechococcus elongatus*. The resulting hydrogenase-PsaE fusion protein when associated with PsaE-free PSI spontaneously formed a complex which showed light-driven hydrogen production at a rate of 0.58 µmol H_2/mg chlorophyll/h. The complex retained accessibility to the native electron acceptor Fd, which is necessary for autotrophic growth of these cells. But unfortunately, the activity was totally suppressed in the presence of the physiological PSI partners, Fd and FNR. In an attempt to establish a H_2 photoproduction system in which the activity is not interrupted by Fd and FNR, the same group introduced a chimeric protein of PsaE and cytochrome c3 (cytc3) from *D. vulgaris* into the cyanobacterium *Synechocystis* sp. PCC6803 (Ihara et al. 2006). The covalent adduct of cytc3 and PsaE assembled with PsaE-free PSI and formed a complex which was still able to reduce Fd for photosynthesis (approximately 20 % of the original activity). Interestingly, this complex was able to drive hydrogen production when coupled with hydrogenase from *D. vulgaris* even in the presence of Fd and FNR, although the rate was limited (around 0.30 µmol H_2/mg chlorophyll/h). These results suggest, however, that this type of complex may eventually be modified to produce H_2 in vivo. More recently, *Cl. acetobutylicum* [FeFe]-hydrogenase was fused with *Synechococcus* sp. PCC 7002 PSI via a 1,6-hexanedithiol molecular wire (Lubner et al. 2010). However, while this complex also catalyzed light-dependent H_2 production in vitro, the abiotic nature of the wire make the system difficult to implement in vivo. Another system in which a fused PSI-[NiFe]-hydrogenase complex was immobilized on a gold electrode (Krassen et al. 2009) produced H_2 at the equivalent of 3 mmol H_2/mg chlorophyll/h (Bürstel et al. 2011), it then was suggested that this system is analogous to the in vivo situation where membrane-bound PSI receives electrons from the photosynthetic electron transport chain (Bürstel et al. 2011).

C. Protein Engineering to Improve the O_2-Tolerance of Hydrogenases

To develop a viable H_2 technology, hydrogenases should work in presence of O_2. Indeed, the direct biological photoproduction of hydrogen at the expense of water oxidation will unavoidably lead to a certain exposure to O_2. Similarly, the bio-fuel cells operate necessarily with O_2 to realize the oxidation of H_2 by hydrogenases. Thus, improving hydrogenase oxygen resistance is then a major challenge for a broad range of biotechnological applications such as hydrogen photoproduction, bio-fuel cells, and biosensors. As said above, there are currently several projects in progress with the single objective of identifying and expressing natural O_2-tolerant [NiFe]-hydrogenases in model host organisms in order to over-express the hydrogenases of interest or to improve H_2 production.

Another strategy is to carry out molecular engineering studies directly on the hydrogenases by directed mutagenesis for instance. Significant interest surrounds molecular engineering studies aimed at achieving hydrogenases with low levels of sensitivity to O_2 (Bürstel et al. 2011), and standard O_2-sensitive [NiFe]-hydrogenases are often the target of these studies because they are reversibly inhibited by oxygen unlike [FeFe]-hydrogenases. One major difficulty, however, lies in the complexity of the maturation process associated with the low production of native [NiFe]-hydrogenases. One of the most studied is the periplasmic O_2-sensitive [NiFe]-hydrogenase from *Desulfovibrio fructosovorans* (Rousset et al. 1998). This enzyme is soluble, highly produced, genetically

accessible and it can be crystallized without much difficulties. All the mutants of interests were fully characterized, at the structural, spectroscopic and kinetic levels. Studies of this enzyme, at the molecular level, enabled to determine the mechanisms of electron transfer (Dementin et al. 2006, 2011), of proton transfer (Léger et al. 2004) and to increase the tolerance towards O_2 (Dementin et al. 2009; Liebgott et al. 2010; Dementin et al. 2011). Research strategies to increase the tolerance towards oxygen have been primarily inspired by some key features observed in the O_2-tolerant hydrogenase from *R. eutropha* H16 (Bleijlevens et al. 2004; Burgdorf et al. 2005; Fritsch et al. 2011). Thus, two general strategies have been followed: (1) slowing down the oxygen diffusion along the gas channel and (2) changing the reactivity of oxygen with the active site.

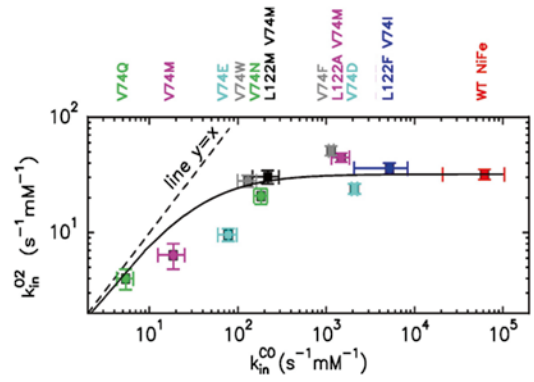

Fig. 3.7. Rate of inhibition by O_2 ($k_{in}^{O_2}$) plotted against the rate of binding of CO (k_{in}^{CO}). The *dashed line* depicts $y = x$ for which O_2-inhibition rate would be a linear function of diffusion rate; the *plain line* is the best fit to equation: $1/k_{in}^{O_2} = 1/k_{in}^{CO} + 1/k_{in}^{O_2}$ max, with $k_{in}^{O_2}$ max = 32 s^{-1} $mM(O_2)^{-1}$. *Error bars* represent either the deviation from the average of three to five independent determinations or the estimated error introduced from the extrapolation to 40 °C (Adapted from Liebgott et al. 2010).

1. Slowing Down of the Oxygen Diffusion Along the Gas Channel

Firstly, it is important to understand how the structure of the tunnel in hydrogenases determines the diffusion rate and possibly the selectivity of the enzymes with respect to substrates and inhibitors of similar sizes. From a multiscale simulation approach, associated with the comparison between the sequences and biochemical properties of homologous [NiFe] hydrogenases, it has been proposed that diffusion in these enzymes is controlled by two gates, which guard the entrance of the active site (Fig. 3.3a), and may determine the accessibility of the active site and therefore the resistance to O_2. One is located between residues 74 and 476 and the other between residues 74 and 122 (Fig. 3.3a) (Volbeda and Fontecilla-Camps 2004; Kim et al. 2012; Topin et al. 2012) (We use *D. fructosovorans* amino acids numbering throughout). This hypothesis has been erratically supported by the results of mutagenesis studies. Both *R. eutropha* H16 and *R. capsulatus* RH (regulatory hydrogenase) become oxygen sensitive when the two conserved bulky amino-acids, Phe122 and Ile74, are replaced with smaller residues, Leu and Val respectively, which are conserved in "standard," O_2-sensitive [NiFe] hydrogenases, such as those from *D. fructosovorans* and *A. vinosum* (Burgdorf et al. 2005; Duche et al. 2005).

Conversely, inspired by the RH, substitutions of the Val74 and Leu122 with isoleucine and phenylalanine, respectively, were carried out in the [NiFe]-hydrogenase from *D. fructosovorans* (Fig. 3.7). Surprisingly, these substitutions did not improve O_2 tolerance and did not significantly modify the catalytic properties of the enzyme under anoxic conditions (Dementin et al. 2009). Consequently, the residue bulkiness at these positions was not the only parameter affecting O_2 tolerance. Indeed, the existence of two control points in different locations might explain why the reduction in the experimental diffusion rate does not simply correlate with the width of the main gas channel. Moreover, the orientation or chemical nature of the side chain are also crucial to slow down the diffusion rate and the inhibition rate by O_2 (Leroux et al. 2008).

a. Slowing Down of the Diffusion Rate

In order to explore the respective roles of the bulk, hydrophobicity, charge and polarity, a number of single and double mutants at positions 122 and/or 74 have been constructed and purified. Val74 was replaced with aspartate (D), asparagine (N), tryptophan (W), glutamate (E), glutamine (Q), isoleucine (I), and phenylalanine (F) while Leu122 was replaced with phenylalanine, alanine (A) and methionine (M) (Liebgott et al. 2010). The mutants were screened using PFV to estimate different parameters such as the rate of inhibition by CO ($k_{in}CO$; the limiting step is the diffusion rate) and the rate of O_2-inhibition ($k_{in}O_2$; the limiting step is the reaction rate) (Leroux et al. 2008; Liebgott et al. 2010) (Fig. 3.7).

The effect of the mutations that keeps the side chain hydrophobic indicates a simple correlation between bulk and diffusion rate. Compared to the wild-type (WT), the diffusion rate is reduced by about two orders of magnitude for the V74F and about three orders of magnitude for the V74W. The increase of the molecular volume (calculated according to Ref. Häckel et al. 1999) of the amino-acid side chains lining the channel has a strong influence on the diffusion rate. In this set of experiments, the effect observed is only due the steric hindrance that shrivels the tunnel (Fig. 3.7).

In the case of polar amino-acids, two levels of impact were observed on diffusion, involving both bulk and charge. The influence of changing the size and polarity of the residue at position 74 was analyzed by comparing the V74E, V74D, V74Q and V74N mutants two at a time. Increasing the length of the side chain by one carbon (V74D to V74E, or V74N to V74Q) slows the diffusion rate about 30-fold. The magnitude of this effect lies within the same range as that observed with hydrophobic residues. Nevertheless, charge also matters as the substitution V74 to D74 slows the diffusion by a factor 40, while the molecular volume of aspartate is 40 % smaller than that of valine (Häckel et al. 1999). The polarity has an even stronger impact as the substitution V74 to Q74 reduces the diffusion by about four orders of magnitude, while the molecular volumes of these two amino acids are quite similar (Häckel et al. 1999). Within the polar amino acids, replacing a carboxylic acid with an amide, keeping the Van der Waals volume constant (V74E to V74Q, or V74D to V74N), slows diffusion by a factor of about 12. The two contributions, size and polarity, are independent of each other, and therefore the combination of the two (V74D to V74Q) decreases the rate of diffusion by more than two orders of magnitude. The electrostatic interaction of the amino acid side chains lining the tunnel is therefore very unfavourable to CO diffusion (Fig. 3.7). This could be due to a direct interaction of the polar group with the gas molecule or the Arg476 residue (residue strictly conserved within [NiFe]-hydrogenases, which lead to complications in actual experimental mutations), or to the stabilization of a water molecule that would be part of the barrier to ligand entry, as observed in certain myoglobin mutants (Nienhaus et al. 2003).

b. Slowing Down of the Inhibition Rate by O_2

In [NiFe] hydrogenase, the rate of inhibition by CO is about four orders of magnitude faster than the rate of aerobic inhibition (3.10^4 s^{-1}/M) (Léger et al. 2004; Liebgott et al. 2010). Considering that CO and O_2 diffuse within the protein at about the same rate, this observation implies that the rate of inhibition by O_2 is limited by the reaction at the active site. Mutations such as V74N or V74W decrease the rate of intramolecular diffusion by blocking the tunnel, but this has no effect on the overall reaction with O_2 because the diffusion process does not limit the inhibition rate (Fig. 3.7). However, other mutations decrease the rate of diffusion in such a large extent (three orders of magnitude for V74E or four orders of magnitude for V74Q) that this step becomes limiting because is slower than the reaction of O_2 at the active site, thus decreasing the overall rate of inhibition by O_2.

2. Changing of the Reactivity of Oxygen with the Active Site

Contrary to the *Re*RH, the O_2 tolerance of both MBH and SH is performed by a peculiar reaction of the active site with O_2. In the case of MBH the presence of a unique proximal [4Fe-3S] cluster is thought to be responsible for the tolerance of these hydrogenases towards O_2. The complexity and specificity of the maturation process of this unique [4Fe-3S] cluster will make the realization of variant enzymes, mimicking the capacities of O_2-tolerant [NiFe] Hydrogenases (MBH), more difficult. However, drawing inspiration from *Re*SH various mutations have been carried out. Amino acids like cysteine and methionine have a high affinity for oxygen, which results in a strong reactivity with reactive oxygen species and they are also known to participate in oxidative stress responses and protection in several proteins (Kim et al. 2001; Stadtman et al. 2002; Stadtman 2004, 2006; Reddie and Carroll 2008). With this knowledge in mind, several mutants at position 74 and 122 have been performed to replace valine by methionine or cysteine (Dementin et al. 2009).

a. Introduction of Methionines in the Gas Channel Near the Active Site

In order to decrease the sensitivity of the [NiFe] hydrogenase from *D. fructosovorans*, the effects of replacing Val74 and Leu122 with methionines have been tested. Indeed, methionines placed at the entrance of the active site cavity at positions V74M and L122M may protect the Ni-Fe site from oxidation, either by reacting or at least by interacting with the oxygen species present at the active site under oxidizing conditions (Volbeda et al. 2005). The goal of that substitution was twofold: slowing diffusion and modifying the reactivity with O_2.

At diffusion rate level, the molecular volume of methionine is 30 % larger than that of valine and about the same as leucine (Häckel et al. 1999). The diffusion rate in the case of the V74M-L122M is decreased by more than two orders of magnitude and even three orders of magnitude for the V74M (Fig. 3.7), which goes far beyond the expected effect of the volume increase on the diffusion. The interaction of gases with methionine is therefore stronger than a simple steric obstruction. Indeed, another interesting property of the V74M-L122M mutant was detected by PFV: unlike the WT enzyme, it partly reactivates in the presence of H_2 even under very oxidizing conditions (Dementin et al. 2009) (Fig. 3.8). The V74M mutant also reactivates under H_2 at high potential but to a lesser extent (Dementin et al. 2009). This process is slow and has a small amplitude, but it is significant because under the very oxidizing conditions used in the experiments, one would expect nothing but the inactivation of the enzyme (Vincent et al. 2007; Leroux et al. 2008) (Fig. 3.8).

Crystallographic and spectroscopic studies showed that methionines are not modified in the oxidized enzymes but that they interact with the active site by modifying its environment. FTIR studies determined that the mutants were inactivated more slowly and reactivated more rapidly than the native enzyme. This slower inactivation is attributed to a reduced active site accessibility that is due to partial tunnel obstruction by the mutations (Leroux et al. 2008). However the faster activation necessarily involves a quicker removal of the bound oxygen species. This was assumed to involve methionine that would stabilize the rearrangement of the oxygen species that is necessary to allow its protonation, facilitating its escape from the oxidized enzyme. As a result, the phenotype of the V74M and V74M-L122M mutants is not a consequence of a modification of the structure of the active site, but rather reveals subtle changes in the kinetics of the reaction with O_2.

These different studies clearly showed that the V74M-L122M and V74M mutated hydrogenases became O_2 tolerant, since these mutant hydrogenases continued to operate in the presence of 150 µM of O_2, which is close to the O_2 concentration of

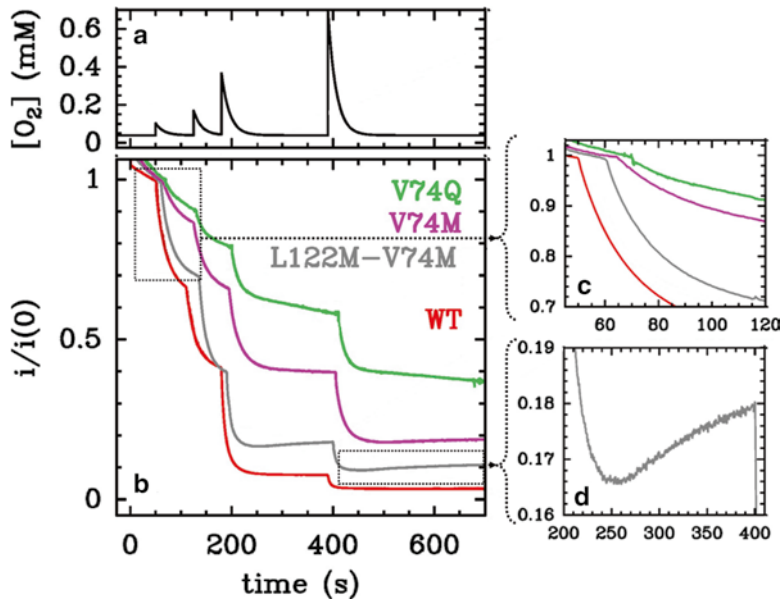

Fig. 3.8. The inhibition by O_2 of *D. fructosovorans* [Ni-Fe]-hydrogenase selected mutants. (**a**) The change in dioxygen concentration plotted against time, reconstructed from the amount of O_2 injected and the time constant of the exponential decay; the latter is calculated from fitting the change in current. (**b**) The plain lines show the change in [Ni-Fe]-hydrogenase activity (current *i* normalized by its value *i*(0) just before the inhibitor is added); (**c**, **d**) Enlarged views of the data in **b**, showing the decreases in current after the first exposure to O_2 (**c**) and the partial reactivation of the L122M-V74M mutant (**d**). E=+200 mV versus SHE, T=40 °C, pH 7, electrode rotation rate w=2 kr.p.m (Adapted from Liebgott et al. 2010).

200 µM in air-equilibrated solutions (Dementin et al. 2009).

b. Introduction of a Cysteine in the Gas Channel Near the Active Site

As described above, the O_2-tolerance of membrane-bound [NiFe]-hydrogenases, results from the fact that they only convert into the Ni-B state under O_2. Moreover, the reactivation rate of this inactive state is greater than that of the same species in O_2-sensitive enzymes. In order to test the reaction of a thiol function with O_2, the valine 74 in the wild type enzyme, has been exchanged with a cysteine (Liebgott et al. 2011). The obtained mutant has showed an activity during several minutes under oxygenated atmosphere. Further FTIR and PFV experiments showed that the inhibition of V74C by oxygen led to the formation of Ni-B state only, unlike the wild-type enzyme (Fig. 3.9). Moreover, the Ni-B state of V74C reactivated 17–25 times faster than the same species in the wild type (Fig. 3.9). Two other point mutations, at the level of valine 74, have been constructed to further address the role of cysteine 74 in modulating oxygen reactivity. The V74S and V74N mutations have showed also a significant increase of the rate of reactivation after inactivation under aerobic conditions (~ 4 and 11 times faster than wild type), although these rates are smaller than that of V74C (Fig. 3.10) Thus, the V74C mutant exhibits similar features typical of the naturally-occurring oxygen-tolerant hydrogenases (MBH). However, in the resistant *Ec*MBH, *Re*MBH and *Aa*MBH enzymes, it has been hypothesized that an additional source of electrons supplied to the active site would be responsible for the conversion into the Ni-B species only. V74C cannot supply electrons but from a combination of biochemical, spectroscopic (EPR, FTIR), X-ray and PFV studies, the authors proposed that V74C accelerates the electron transfer from the FeS clusters to the active site. This may promote (i) complete reduction of O_2 into

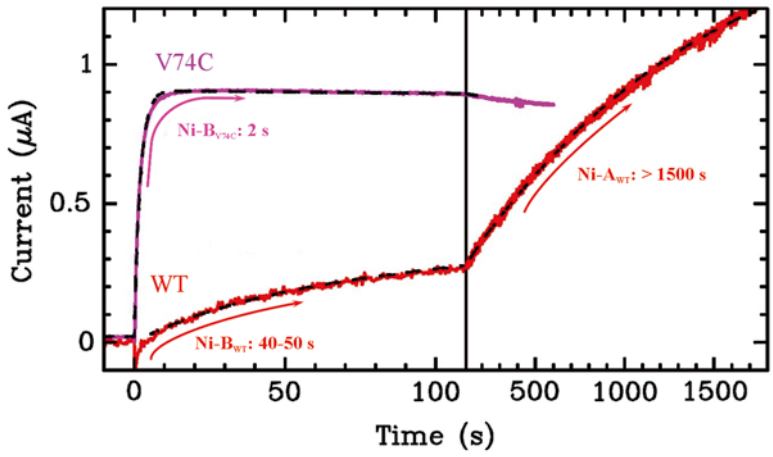

Fig. 3.9. Reactivation of the WT (*red) and V74C (purple) enzymes, at-90* mV, pH 5.5, 1 atm of H_2, after aerobic inactivation at +215 mV, under 1 atm of Argon. The WT reactivates in two phases (NiB and NiA or 'ready' and 'unready' states respectively). In contrast, the reactivation of the V74C mutant is essentially monophasic and has a time constant of reactivation 20 times faster than for WT (Adapted from Liebgott et al. 2011).

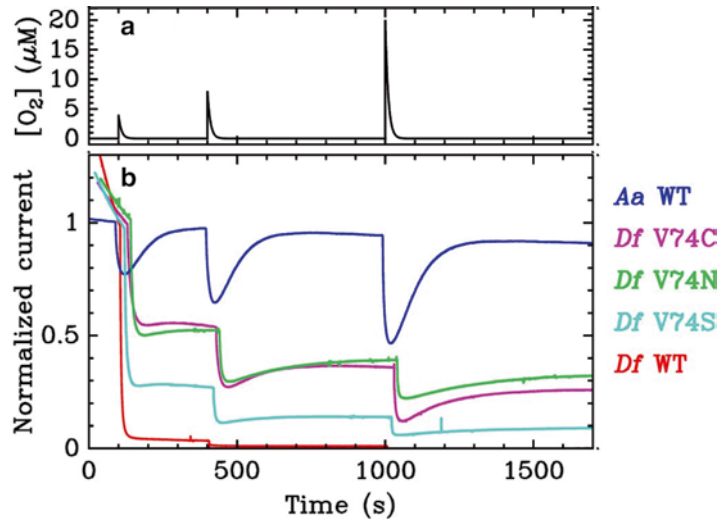

Fig. 3.10. Aerobic inhibition of *D. fructosovorans* WT (*red*), V74C, (*purple*), V74S (*cyan*), and V74N (*green)* and *A. aeolicus* MBH (*dark blue*) hydrogenases adsorbed at a rotating electrode poised at +140 mV, 40 °C, pH 5.5, 1 atm H_2. (**a**) Oxygen concentration in the cell during experiment. The three peaks correspond to injections of 100, 200, and 500 μL of an air-saturated solution (**b**) Effect of O_2 on enzyme's activity. The traces were recorded after a 200 s (WT) or 500 s (V74C) poise at +140 mV (Adapted from Liebgott et al. 2011).

H_2O and OH^-, which favors the formation of the Ni-B state and (ii) fast reactivation of this state (Dementin et al. 2011). Thus, with the V74M and V74M-L122M mutants (Dementin et al. 2009), the V74C mutant provides another evidence that it is possible to improve the oxygen tolerance of hydrogenases, which is a prerequisite for their biotechnological use in air. Interestingly, the V74C mutant was not designed to copy the sequence of the oxygen resistant enzymes, these results are totally unexpected but they suggest new strategies for conceiving oxygen-tolerant enzymes.

The main outcome of these studies is the demonstration that it is possible to induce

O_2-tolerance in a [NiFe] hydrogenase, in such a way that modified enzymes are active in the presence of O_2 at concentrations close to that in air-equilibrated solutions. The fact that the substitutions tested are located in conserved regions makes it possible to engineer [NiFe]-hydrogenases in a wide range of organisms, as heterologous expression of [NiFe]-hydrogenase is still difficult. This achievement opens the way for future development in the field of biological hydrogen production or utilization.

V. Conclusion

Hydrogenase engineering is a very difficult issue not only because of the complexity of these enzymes but also because of the vital processes in which they are involved. However, in spite of the difficulties, research is very active in several countries because of the potential spin offs that might participate in the development of a new hydrogen energy economy. The enzyme bias, substrate specificity and oxygen resistance are the main domains in which some progress have already been made, opening the way towards future applications. But other issues, like heterologous expression of [NiFe]-hydrogenases that would facilitate molecular research and organism engineering or deciphering the catalytic mechanism that would allow the development of biomimetic catalysts, are also the subjects of intense research and will contribute to biohydrogen implementation.

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