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Coordination Chemistry Reviews 249 (2005) 1677–1690
COORDINATION CHEMISTRY REVIEWS
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Review

H/D exchange reactions and mechanistic aspects of the hydrogenases

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Received 16 June 2004; accepted 11 January 2005
Available online 14 March 2005

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Abstract

In the presence of D_2 gas (in a D_2/H_2O system), hydrogenases catalyze the proton-deuterium (H/D) exchange reaction, which results in the formation of HD and H_2, measurable by mass spectrometry at mass peaks *m/e* 3 and 2, respectively. This technique can be used to study the activity and function of hydrogenases both in vitro, with isolated enzymes, and in vivo, with whole cells. It allows to follow the reductive activation of oxidized [NiFe]-hydrogenases and to identify physiological activators such as NADH and NADPH, of bidirectional hydrogenases. Besides, since the H/D exchange reaction involves proton exchange with the solvent, it has been used to monitor the effects of site-directed mutagenesis of amino acid residues postulated to belong to proton channels.

Keywords: Membrane-inlet mass spectrometry; H/D exchange reaction; [NiFe]-hydrogenase activation

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doi:10.1016/j.ccr.2005.01.026

1. Introduction

Hydrogenases are metalloproteins containing Fe/S clusters and a dinuclear metallic center, consisting of a Ni and an Fe atom liganded by cysteine residues in the [NiFe]-hydrogenases [1,2], or of two Fe atoms also including sulfur ligands in the [FeFe]-hydrogenases [3,4] (for recent reviews see [5–13]). The [NiFe]- and [FeFe]-hydrogenases belong to two phylogenetically distinct classes of hydrogenases [8].

The catalytic core of [NiFe]-hydrogenases consists of an αβ 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. Additional subunits are present in many of these enzymes. Phylogenetic analyses have shown that the two subunits evolved conjointly [8]. Crystal structures of heterodimeric *Desulfovibrio* [NiFe]-hydrogenases have shown that the two subunits interact extensively through a large contact surface [1,2,14–17]. The bimetallic NiFe center is deeply buried in the large subunit; it is coordinated to the protein by four cysteines. 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 hydrogenase. The [Fe_4-S_4] cluster that is proximal to the active site (within 14 Å) is "essential" to H_2 activation [1,18]. Later studies using infrared spectroscopy revealed the presence of three non-protein ligands, 1 CO and 2 CN^- bound to the Fe atom [14,19,20]. Hydrophobic channels linking the active site to the surface of the molecule have been identified and suggested to facilitate gas access to the buried active site [18,21]. Chains of hydrophilic amino acid residues have been proposed to participate to proton channels [1,10]. The [NiFe]-hydrogenases, widespread in the Archaea and Bacteria domains of life, have been the hydrogenases most extensively studied physiologically, biochemically and genetically. It is to note that the available crystallographic structures and catalytic mechanisms were established with enzymes belonging only to the first sub-group of [NiFe]-hydrogenases (classified into four sub-groups) [8]. Future work may uncover the role of specific protein environments of the active site.

Unlike [NiFe]-hydrogenases composed of at least two subunits, many [FeFe]-hydrogenases are monomeric and consist of the catalytic subunit only (although dimeric, trimeric and tetrameric enzymes are also known) [8]. The smallest [FeFe]-hydrogenases (ca 45–48 kDa) have been found in green algae [22,23]. The catalytic subunit of [FeFe]-hydrogenases varies considerably in size; besides the conserved domain of ca 350 residues containing the active site (H-cluster) [24], it often comprises additional domains which accommodate Fe-S clusters. The H-cluster of the [FeFe]-hydrogenase from *Clostridium pasteurianum* [3] and from *Desulfovibrio desulfuricans* ATCC 7757 [4] consists of a binuclear iron subsite, [Fe_2S_3], bound to a [Fe_4-S_4] cluster by a bridging cysteine sulfur and attached to the protein by a cysteine ligand. Non-protein diatomic ligands, CO and CN^-, are attached to both Fe atoms [3–5,7,11]. The Fe atoms also share two bridging sulfur ligands of a small five-atom molecule, possibly a di(thiomethyl)amine molecule, HN–(CH_2–S^-)$_2$ [11]. The Fe atom distal to the [Fe_4-S_4] cluster (Fe2) has a vacant coordination site which is occupied by carbon monoxide, a competitive inhibitor, in the CO-inhibited form of the enzyme [25–27]; it is therefore thought to be the position where dihydrogen/hydride bind during enzyme turnover. A single hydrophobic channel that runs from the molecular surface to the active site and points at Fe2 is present in the [FeFe]-hydrogenase from *D. desulfuricans* ATCC 7757 and in the hydrogenase I from *C. pasteurianum* [3,4,11]. Similarly to [NiFe]-hydrogenases, a plausible proton pathway has been proposed for [FeFe]-hydrogenases [3,11].

Hydrogenases catalyze the interconversion of molecular hydrogen into protons and electrons according to the reversible reaction:

$$H_2 \leftrightarrow 2H^+ + 2e^- \quad (1)$$

Most if not all known hydrogenases can catalyze the reaction in either direction in vitro, although they are usually committed to catalyze either H_2 uptake or H_2 evolution in vivo, depending on the demands of the host organism. The use of hydrogen isotopes (deuterium, tritium) enables detection of splitting of the hydrogen molecule by hydrogenase. Isotope exchange by hydrogenase was observed first in 1934 with whole cells of *Escherichia coli* (*E. coli*) [28], in 1943 in cells of *Proteus vulgaris* [29], then in crude extracts [30] and in partially purified enzymes [31]. The H^+–D_2, or D^+–H_2, (H/D) exchange reaction is part of the reversible activity of hydrogenase and provides an intrinsic measure of this activity. It has been used to study the mechanism of enzyme action (see below). Krasna and Rittenberg [32,33] studied the isotope exchange and *para*-hydrogen (*para*-H_2) to *ortho*-hydrogen (*ortho*-H_2) (spin nuclear isomers) conversion reaction in *P. vulgaris* cells. They concluded that hydrogenase catalyzes heterolytic splitting of hydrogen with formation of an intermediate enzyme hydride, according to the following equation:

$$E + H_2 \leftrightarrow E : H^- + H^+ \quad (2)$$

where E is the hydride binding site of hydrogenase, the released proton is supposed to be transiently bound to a proton accepting site. If D_2 gas is used, the splitting of the D_2 molecule results in the formation of a deuteron (D^+) and a deuteride (D^-):

$$D_2 + E \leftrightarrow E : D^- + D^+ \quad (3)$$

In the absence of an electron donor or acceptor, the back reaction, in the presence of excess protons from the solvent, leads to the formation of HD:

$$E–D^- + H^+ \leftrightarrow E + HD \quad (4)$$

Formation of HD reflects the reversibility of Eq. (3). Overall, there is no electron transfer. Electron acceptors, if present,

compete with H^+ for the hydride intermediate so that the exchange reaction is lowered and may even be abolished. The kinetics of the isotope exchange reaction and of the conversion reaction were later reinvestigated using purified hydrogenase preparations from *C. pasteurianum* and *P. vulgaris* [34], from *Desulfovibrio vulgaris* Miyazaki [35] and from *Desulfovibrio vulgaris*, strain Hildenborough [36]. The kinetics of hydrogenase from *C. pasteurianum* and *P. vulgaris* were determined by measuring the HD and D_2 produced when the enzyme catalyzed the exchange between H_2 and 100% D_2O [34]. The ratio of D_2 to HD production depends on the enzyme concentration because the HD produced by the exchange of H_2 can react with another enzyme molecule to give D_2 before it escapes to the gas phase. At very low enzyme concentrations, the ratio D_2/HD is independent of enzyme concentration. The experimental data were consistent with the cleavage of dihydrogen into a proton and an enzyme hydride that can exchange with the solvent. It was possible to interpret the data in terms of a hydride that does not exchange, if it is assumed that there is a shell of water molecules around the active site of the enzyme, which prevents HD diffusion away from the enzyme molecule. This so-called "cage-effect" results in the molecule of HD reacting a second time with the enzyme and thus, forming D_2. The cage-effect would be present at infinitely dilute enzyme concentration since it involves only the interaction of the HD with a single enzyme molecule.

The highly purified *D. vulgaris* Miyazaki hydrogenase (H_2:ferricytochrome c_3 oxidoreductase) was shown to catalyze the conversion of *para*-H_2 to normal H_2 over D_2O as well as the isotope exchange reaction in the H_2–D_2O system and it was concluded that the two hydrogen atoms of the enzyme-bound hydrogen molecule can exchange with hydrogen ions of the medium, but at different exchange rates [35]. Both isotope exchange and *para*- to *ortho*-H_2 conversion in D_2O were demonstrated to occur with *D. vulgaris* Hildenborough [NiFe]-hydrogenase whereas with a [NiFeSe]-hydrogenase, which had an exchange activity equivalent to that of the former, practically no *para–ortho* conversion could be observed in D_2O [36].

2. Assays of hydrogenase activity

The methods used to assay hydrogenase activity are based on the enzyme ability to catalyze H_2 evolution and H_2 oxidation, interconversion of *para*- and *ortho*-H_2, and deuterium or tritium exchange reactions with H^+ (in the absence of electron donors or acceptors). Oxidation of H_2 can be associated with the reduction of a dye, measurable by spectrophotometry. Production or consumption of H_2 can be measured amperometrically, using a Clark-type electrode, manometrically or by gas chromatography with a thermal-conductivity detector. Isotope exchange, using tritium gas or tritiated water can be measured by radioactive counting. Exchange with deuterium can be detected by mass spectrometry. These different assay methods have been described in the Methods in Enzymology series [37,38].

2.1. Para-hydrogen - ortho-hydrogen conversion

The *para*-H_2–*ortho*-H_2 conversion depends only upon the reversal of reaction (2). At ambient temperature, normal hydrogen is a mixture of two isomers, namely 25% of *para*-H_2 with antiparallel nuclear spin and 75% of *ortho*-H_2 with parallel nuclear spin. A sample enriched in *para*-H_2 is stable (in the absence of oxygen) for an extended period. In the presence of hydrogenase, the *para*-H_2 molecule is cleaved and its atoms bind to the active site of the enzyme. The hydrogen formed by the reverse reaction has the composition of normal hydrogen, the "*para*" character has been lost during the process. The *para–ortho* conversion is in some way a partial reaction of the isotope exchange reaction, which includes in addition an exchange of the hydrogen atoms with the protons (deuterons) of the solvent.

A method to prepare a mixture of 50% *para*-H_2 and 50% *ortho*-H_2 has been described [39]. *Para*-H_2 and *ortho*-H_2 are separated by gas chromatography on an alumina column immersed in liquid nitrogen with a thermal-conductivity detector [37]. The measurements are normally done in parallel with those of isotope exchange and H_2, HD and D_2 are measured by mass spectrometry [32–36,40,41].

2.2. Isotope exchange: proton–tritium exchange activity

Hydrogenase catalyzes the exchange reaction between hydrogen and tritiated (or deuterated) water [37,42], in the absence of additional electron acceptors or donors. The proton–tritium exchange activity has been used to study the three states of *Desulfovibrio gigas* hydrogenase, designated "active", "unready" and "ready", and the effect of inhibitors on the catalytic sites [43,44]. Tritium gas, used in the exchange reaction, was prepared by the addition of lithium metal to tritiated water. Only the "active" enzyme (obtained by prolonged incubation under hydrogen) catalyzed the isotope exchange reaction, while the "unready" state (in the as-isolated enzyme) and the "ready" state (produced by treatment of the "active" state with argon) were completely inactive.

2.3. Proton–deuterium (H/D) exchange measurements

The use of the H^+–deuterium exchange reaction (H/D) to determine hydrogenase activity involves the mass spectrometric analysis of masses *m*/*e* 2 (H_2), *m*/*e* 3 (HD) and *m*/*e* 4 (D_2). Although the isotope exchange reaction has been measured from the gas phase of a vial uncompletely filled with the enzyme (or particles) solution (suspension) [45,46], the method described here is based on the use of a membrane-inlet mass spectrometer [47]. The direct determination of dissolved gases allows the use of D_2 gas in ordinary water

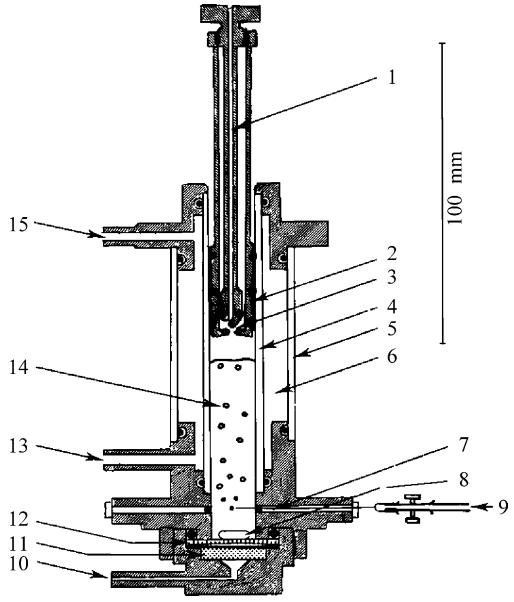

Fig. 1. Reaction chamber for on-line mass spectrometry. (1) Plunger central canal (sparging gas evacuation); (2) plunger fitted with three O-rings; (3) needle stopcock (vessel tightening); (4) inner glass tubing (diameter 15 mm); (5) outer glass tubing (diameter, 36 mm); (6) thermostated jacket; (7) canal fitted with a septum plug (gas or reagent introduction); (8) magnetic bar; (9) gas inlet equipped with a stopcock and a hypodermic needle; (10) vacuum line (towards the cooling trap and the mass spectrometer ion source); (11) fritted-steel disk supporting the Teflon membrane; (12) perforated disk protecting the Teflon membrane; (13) water inlet (top thermostated jacket); (14) liquid medium; (15) water outlet (reproduced from [47] with permission).

equally well as H_2 in D_2O. The system D_2–H_2O is easier to handle than the system H_2–D_2O, which requires lyophilization of the biological material and of buffers to eliminate all H_2O molecules before resuspension in D_2O. Fig. 1 shows the type of reaction vessel used by the group of Cadarache (France) in the 1980s [48–54].

The reaction vessel consisted of a section of glass tubing fitted to a porous fritted-steel disc covered with a Teflon membrane, set by O-rings between upper and lower stainless steel blocks. The inner volume of the reaction vessel (up to 15 ml) was adjustable by means of a hollow plunger. Now, the reaction chamber, of the Hansatech electrode-type, is smaller and can contain only 1.5 ml of medium [55–61]. The liquid inside the vessel is continuously stirred by a magnetic bar and can be sparged with gases by means of a hypodermic needle introduced through the plunger. When the reaction vessel is closed, there is no gas phase above the liquid. Dissolved gases diffuse according to their concentration through the Teflon membrane to a vacuum line directly connected to the ion source of a mass spectrometer. After passing through a cold trap where water vapor is condensed, the gases are admitted into the ion source, where they are ionized and analyzed. The spectrometer sequentially scans the abundance of the different gases (H_2, D_2, HD, O_2) by automatically adjusting the magnet current to the corresponding mass peaks (m/e = 2, 4, 3, 32, respectively). Measuring one mass peak takes 0.5 s. Mass peaks are continuously recorded during the experiment. The electrical signal collected by the spectrometer is directly proportional to the gas concentration in the chamber, the proportionality coefficient varying from one mass to the other according to the ionization properties of the corresponding gas. The factors to convert peak height of m/e 2 and 4 into H_2 and D_2 concentration are determined by preparing a calibration chart for each of the gases, using different concentrations of the gases and correcting for the temperature-dependent solubility of hydrogen isotopes in water [62]. For HD, the factor is taken as the mean of those two values. The results are also corrected for the loss of gas through the mass spectrometer. This loss is a first-order function of the gas partial pressure. It is determined in uninoculated medium (or buffer), sparged under the same conditions as the culture (or cell suspension, or enzyme solution) with the different gases used. In a recent work, the consumption of gases by the mass spectrometer showed first-order kinetics with time constants around 0.09 min^{-1} for H_2, 0.08 min^{-1} for D_2 and 0.024 min^{-1} for O_2 [61]. The use of D_2 in the D_2–H_2O system, in which D_2 disappears and is quantitatively replaced by HD and H_2, allows determination of the in vivo activity of hydrogenase. In earlier reports, the H^+–D_2 exchange reaction was evaluated from the initial rates of HD and/or H_2 formation. A mathematical treatment modeling the H/D exchange reaction catalyzed by hydrogenase in the presence of various relative concentrations of D_2 and of H_2-consuming or H_2-producing processes was recently developed [61]. The result of such calculations applied to the H/D exchange reaction catalyzed by the [NiFe]-hydrogenase from *D. fructosovorans* (in the absence of electron donors and acceptors) is shown in Fig. 2. The treatment allows monitoring of changes in hydrogenase activity over time and permits one to evaluate whether the measured concentration changes of the hydrogen species are simply related to the H/D exchange reaction or are accompanied by H_2 production or uptake.

3. The enzymatic mechanism for [NiFe]-hydrogenases

3.1. Activation/deactivation

Several redox intermediates generated in [NiFe]-hydrogenases under hydrogen have been detected by various biophysical approaches (electron paramagnetic resonance (EPR) and Mössbauer spectroscopy, Fourier transform infrared (FTIR) spectroscopy coupled to potentiometric measurements (cf reviews [10,63–66]). Possible structures of these forms have been predicted by theoretical studies with density functional theory [67–72]; reviewed in refs. [73–77]. In the oxidized aerobic state most [NiFe]-hydrogenases are inactive. The enzyme has to be activated by reduction (H_2, dithionite) to become catalytically competent [78,79].

Three different EPR-active states have been identified through the paramagnetic properties of the nickel. As isolated, the oxidized inactive forms are characterized by two

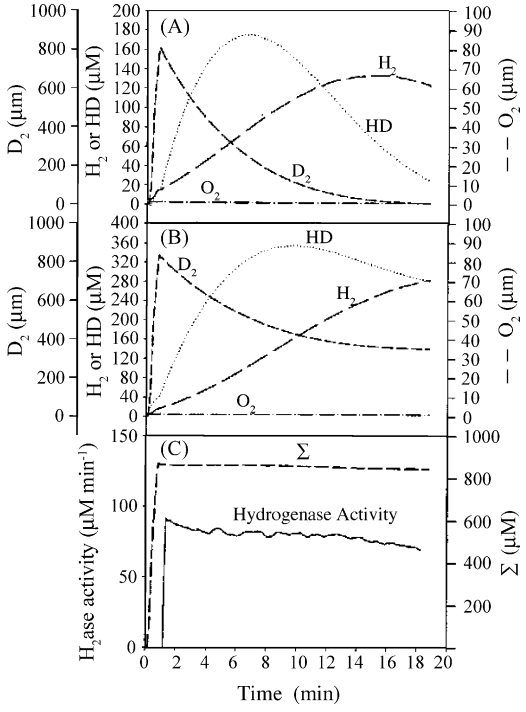

Fig. 2. H_2 and HD production in exchange with D_2 uptake catalyzed by *Desulfovibrio fructosovorans* [NiFe]-hydrogenase. (A) Actual concentrations of the hydrogen species present in the vessel (D_2 concentration reaches zero). (B) Gas concentration changes corrected for consumption by the apparatus (shown is the equivalence between D_2 uptake and H_2 plus HD production). (C) Hydrogenase activity, calculated as $1/\tau$ ($2d[H_2]/dt + d[HD]/dt$), where τ is the isotopic ratio of D in hydrogen ($\tau = ([D_2] + 1/2\ [HD])/\Sigma$). Σ, the total concentration of hydrogen species ($\Sigma = [D_2] + [HD] + [H_2]$) remains constant since only the exchange reaction between hydrogen isotopes and protons of the solvent is taking place here (reproduced from [61] with permission).

Ni-EPR signals (in various ratios, the Ni-A (Ni_u-A signal in Fig. 3, *unready* to catalyze H_2 activation, i.e. the splitting of the H–H bond); this state requires a long activation-period (a deoxygenation step and a reductive step in the presence of H_2 or a reductant), and the Ni-B (Ni_r-B signal in Fig. 3, *ready*, for rapidly converted to the active state by reductants) (Ni-B is obtained by reoxydation of the active enzyme in the absence of O_2)) [80–83]. The third Ni-EPR signal, named Ni-C (Ni_a-C in Fig. 3), characterizes an *active* state of the enzyme [80,83,84]. When illuminated at low temperature, it releases a hydrogen species and gives the EPR spectrum termed Ni-L [85].

The X-ray structure of [NiFe]-hydrogenases revealed the presence of electron density for a bridging monoatomic ligand, labeled X, between the Ni and Fe atoms in the oxidized form of *D. gigas* hydrogenase [14] and *D. vulgaris* Miyazaki F enzyme [2] and no bridging ligand in the reduced forms of the [NiFe]-hydrogenases from *D. vulgaris* Miyazaki F [15] and the [NiFeSe]-hydrogenase from *Desulfomicrobium norvegicum* (formerly *Desulfomicrobium baculatum*) [16]. The bridging ligand has been assigned to an oxygen species for the *D. gigas* [14] and the *D. fructosovorans* [90] [NiFe]-hydrogenase and to a sulfur species (S^{2-}/HS^-) in *D. vulgaris* Miyazaki [2] and *D. desulfuricans* ATCC 27774 [17] enzymes. The exact identity of the oxygen species remains unclear, O^{2-}, OH^- or H_2O are possible candidates. To determine the nature and chemical characteristics of the Ni–X–Fe bridging ligand in *D. gigas* hydrogenase, Carepo et al. [97] performed ^{17}O ENDOR measurements on the Ni-A form of the enzyme, exchanged into $H_2{}^{17}O$, on the active Ni-C prepared by H_2-reduction of Ni-A in $H_2{}^{17}O$, and also on Ni-A formed by reoxidation of Ni-C in $H_2{}^{17}O$. In the native state of the protein (Ni-A), the bridging ligand did not exchange with the $H_2{}^{17}O$ solvent. But after a reduction/reoxidation cycle (Ni-A → Ni-C → Ni-A), an ^{17}O label was introduced at the active site, in the Ni-A state, as seen by ENDOR. The ^{17}O signal was lost upon reductive activation to the Ni-C state; reoxidation to Ni-A led to the reappearance of the signal. Thus, the bridging ligand X in the Ni-A state of *D. gigas* hydrogenase is indeed an oxygenic (a μ-oxo, or μ-hydroxo, or μ-aqueo) species. These experiments further indicated that this bridge is present in Ni-B. DFT calculations indicate that both oxidized states have a μ-hydroxo bridge between the two metal atoms [72]. EPR studies of *Allochromatium vinosum* (formerly *Chromatium vinosum*) oxidized with ^{17}O-enriched O_2 had already shown that an oxygen species must be present close to nickel in both the Ni-A and Ni-B states [98]. In a recent study, it was concluded that the Ni-B state holds a hydroxide ligand bound in an end-on position to Ni(III) [95].

The hydrogenase activation process (the conversion of Ni_u-A to Ni_r-S in Fig. 3) has been linked to the removal of the additional bridging ligand at the active site [87,15]. Upon reductive activation, the ligand, depicted as a hydroxo species in Fig. 3, leaves by protonation to water [99] and the Ni ion is reduced from Ni(III) to Ni(II) to yield the EPR-silent intermediate Ni-S. Ni-S occurs in two states in equilibrium, the Ni_r-S (ready) state (also called SI_{1914} or SI_I) and the Ni_a-S (active) state (also called SI_{1934} or SI_{II}) [87,92,100] (Fig. 3). The Sγ-atom of Cys536 of the *D. desulfuricans* ATCC 27774 [NiFe]-hydrogenase was suggested to be able to interact with the bridging ligand and to assist in the removal and insertion of the bridging atom during enzyme activation and inactivation, respectively [17]. In addition, in the *D. desulfuricans* enzyme purified and crystallized aerobically, modification of the proximal [Fe_4-S_4] cluster, in the small subunit, to [$Fe_4S_3O_3$] was observed [17]. As pointed out by the authors, if the modification is not an artifact due to exposure of the protein to oxygen for an extended time period, the activation process of [NiFe]-hydrogenase may be more complex than initially thought.

3.2. Catalytic cycle

The enzyme in the Ni_a-S (active) state can bind H_2 [86]. The catalytic cycle then progresses with the formation of the EPR-silent Ni-R, where dihydrogen is suggested to be bound side-on at the iron [67,71] in Fig. 3. Ni-R corresponds

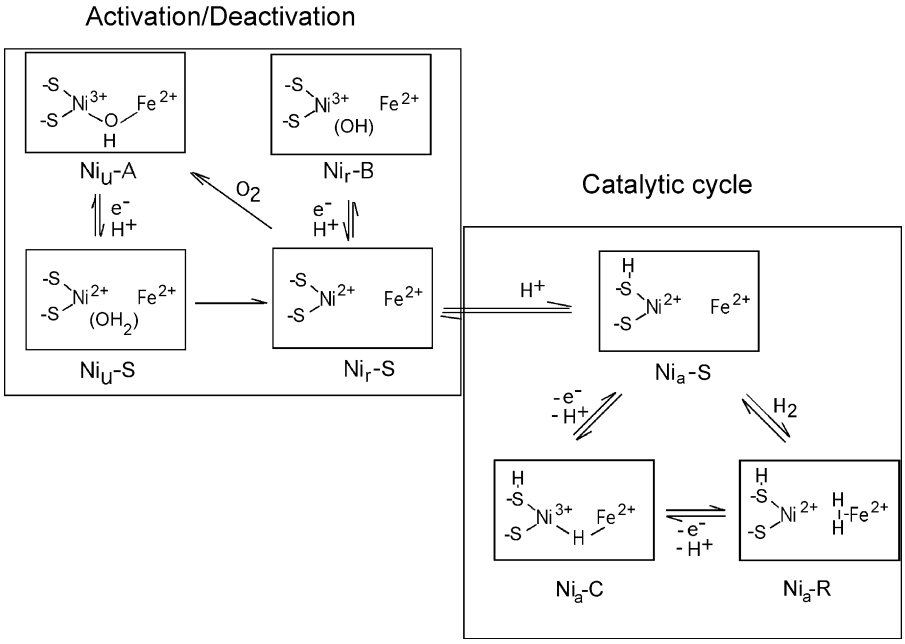

Fig. 3. Scheme illustrating the oxidation states of the bimetallic center of [NiFe]-hydrogenases during the activation/deactivation process and the catalytic cycle. The scheme shows the spectroscopically characterized states and coordination of the NiFe active site. The Ni-A, Ni-B and Ni-C states produce EPR signals, the Ni-S and Ni-R (fully reduced) states are EPR silent. The subscript "u" stands for unready, "r" for ready and "a" for active. 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, kinetics measurements [14,84,86–89] and from the crystal structures of oxidized and reduced [15,16] forms of *Desulfovibrio* [NiFe]-hydrogenases. The EPR and IR values found for *Chromatium vinosum* [NiFe]-hydrogenase, similar to those of *D. gigas* enzyme, are summarized in ref. [65]. 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 [66,89,91]. 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 [88,92]. Ni-C is represented with a bridging hydride ligand coordinated between the Ni and Fe atoms [67–71,93,94] (adapted from [67,71,89,95,96]) (reproduced from [13] with permission).

to the fully reduced protein [91]. Protonation of the Sγ-atom of Cys530 in *D. gigas* enzyme [100], of Cys543 in *D. fructosovorans* enzyme [10], of Cys536 in *D. desulfuricans* enzyme [17] and the corresponding cysteine in *Thiocapsa roseopersicina* [NiFe]-hydrogenase [55], or seleno-cysteine in *Dm. norvegicum* (*Dm. baculatum*) NiFeSe]-hydrogenase [16], has been suggested to assist in the heterolytic cleavage of H_2 by acting as the proton-accepting base in catalysis. Ni-R is in thermodynamic equilibrium with the EPR-active Ni-C state. The Ni-C state is commonly attributed to a Ni(III) oxidation state [101], recent reviews [65,75–77]. Ni-C state is shown in Fig. 3 with a hydride in a bridging position between the Ni and Fe, which are formally in the Ni(III) and Fe(II) oxidation states. The Ni–Fe distance of ∼2.5 Å in the reduced state [15,16] is suitable to accommodate a hydride; however, see [65]. The proposal of a hydride bridge is further supported by density functional calculations of the Ni-C state [67–73,93] and by electron nuclear double resonance (ENDOR) and electron spin-echo envelope modulation (ESEEM) spectroscopy of the regulatory hydrogenase (RH) from *Ralstonia eutropha* [94].

The redox transition between Ni-R and Ni-C states was shown to involve one electron and one proton [84,102,103]. EPR [101] and ^{57}Fe ENDOR experiments [104] led to the conclusion that the iron ion remains diamagnetic, likely in an Fe(II) low-spin state, in all the enzyme forms. This was confirmed by quantum chemical calculations [75,76,105]. Therefore, the redox process is not centered on the Fe ion.

By a further one electron oxidation of the NiFe center, Ni-C yields Ni-S (Ni(II)) and another proton (Fig. 3). The proximal [Fe_4-S_4] cluster (at a redox potential of −340 mV at pH 8 [106] receives the electrons from the NiFe center and transfers them one at a time to the distal [Fe_4-S_4] cluster, close to the surface of the molecule, and able to transfer the electrons also one at a time to external acceptor (or donor). Only the Ni-C/Ni-R and the proximal $[Fe_4\text{-}S_4]^{2+/+}$ redox couples are in thermodynamic equilibrium with H_2 [86,88,89]. The role of the [Fe_3-S_4] cluster located between the two [Fe_4-S_4] clusters and having a redox potential approximately 300 mV more positive than the proximal and distal [Fe_4-S_4] clusters is unclear. The distances between these redox clusters are adequate for fast electron transfer through the protein [107] and the electron transfer between the [Fe_3-S_4] center and the [Fe_4-S_4] clusters appears to be fast enough to be non-limiting in both the H_2 uptake and H_2 evolution reactions [106,108]. Fig. 3 combines current views of hydrogenase activation and catalysis deduced from potentiometric titrations, kinetics measurements, IR spectroelectrochemical titrations, density functional theory (DFT) calculations carried out with *Desulfovibrio* and *Chromatium vinosum* hydrogenases and

from the crystal structures of oxidized and reduced forms of [NiFe]-hydrogenases. Differences exist between the proposed models, in particular concerning the location of hydrogen atoms not visible by crystallography. Methods like DFT test models where species like H_2, H^- and H^+ are explicitly placed at the active site of hydrogenase; however, up to now, they generally do not take into account the protein environment (see reviews [65,75–77]). The midpoint potentials values at pH 7 for the Ni-S/Ni-C and the Ni-C/Ni-R couples were determined by EPR to be −270 and −390 mV, respectively [84] and by infrared (IR) spectroscopy coupled to electrochemistry to be −340 and −405 mV, respectively [87]. A strong electronic interaction between the two metal centers at the active site was deduced from the similarity between the titrations of the redox states of *D. gigas* hydrogenase based on stretching frequencies of the diatomic ligands to the active site Fe (CO and CN) [87], and the stoichiometric reductive titrations of EPR nickel signals [91]. Therefore, the letters S, R and C, which initially designated a redox state of the Ni atom are commonly used now to designate a redox state of the enzyme.

3.3. Proton channels

Concerning proton transport pathways to the molecular surface, several routes have been proposed for [NiFe]-hydrogenases and most probably several pathways may be used during catalysis. One of the cysteine ligands to the nickel atom (Cys543 in *D. fructosovorans*, Cys530 in *D. gigas*, Cys536 in *D. desulfuricans* and the corresponding Cys in *T. roseopersicina* or SeCys in *Dm. norvegicum*) is generally considered to be the proton acceptor group after heterolytic cleavage of H_2 in the active site. This hypothesis is supported by data obtained by chemical modification of the cysteines [55], by X-ray diffraction [1,10,16,17,90,109], by X-ray absorption spectroscopy (XAS) [64], by EPR spectroscopy [94,100], ENDOR spectroscopy [110], model complexes chemistry (e.g. [111,112]), and theoretical calculations [68–70,73]. A glutamic acid residue, strictly conserved in all hydrogenase structures, (Glu24 in *D. desulfuricans* [NiFe]-hydrogenase and the corresponding Glu18 in *Dm. norvegicum* [NiFeSe]-hydrogenase) was suggested to be involved in proton transfer between the active site and the molecular surface along with the Sγ-atom of Cys536 (*D. desulfuricans)* [17] or the Se-atom of Se-Cys492 [16]. In the *D. desulfuricans* enzyme, close to Glu 24, on the other side of Cys536, three other strictly conserved residues are present, Arg469, Asp534 and His117 and a water molecule is found within hydrogen bonding distance between one of the carboxyl oxygen atoms of Asp534 (ca 2.7 Å) and one of the carboxyl oxygen atoms of Glu24 (ca 3.0 Å). Matias et al. [17] suggested that this water molecule, conserved in all the hydrogenase structures analyzed, except in the *D. gigas* enzyme, could mediate a proton transfer between Glu24 and Asp534. Thus, Cys536 and residues Glu24 and Asp534 and the water molecule could participate to a proton transfer chain towards the surface of the protein molecule.

Site-directed mutants targeted to those types of amino acids combined to isotopic hydrogen reactions are powerful tools to verify or establish proton transport pathways as is discussed in the accompanying review [113]. The involvement of the homologous glutamic residue (Glu25 in *D. fructosovorans*) in proton transfer between the active site and the protein surface during the catalytic cycle was demonstrated by the use of hydrogenase mutants targeted to Glu25 [41]. Replacement of that glutamic residue by a glutamine did not modify the spectroscopic (FTIR and EPR) properties of the enzyme but abolished the catalytic (H/D exchange) activity except the *para*-H_2 to *ortho*-H_2 conversion. The *para*-H_2/*ortho*-H_2 conversion only involves heterolytic splitting and recombination of molecular hydrogen but no proton transport. On the other hand, proton transfer through the protein is compulsory for the H/D exchange reaction. Thus, with the E25Q mutant, in which the fast proton transfer between the active site and the protein environment is impaired, glutamate25 is demonstrated to be essential for proton transfer during the catalytic cycle [41]. Similarly, in the *R. eutropha* soluble [NiFe]-hydrogenase, a mutant similar to E25Q has been shown to loose its H_2 uptake and H/D activities [114], reviewed in [113]. In a former work on *R. eutropha* (by then named *Alcaligenes eutrophus*), a mutant of the same hydrogenase where Arg40 was replaced by a leucine exhibited a normal level of H/D exchange activity but a complete lack of H_2:acceptor oxidoreductase activity [115]. This result indicates that Arg40 is rather involved in electron transfer and that electron and proton transfer are not necessarily coupled.

4. Applications of the H^+/D_2 exchange reaction

4.1. Hydrogenase activation

The H/D exchange reaction was used more than twenty years ago to monitor hydrogenase activation of *Alcaligenes eutrophus* (*Ralstonia eutropha*) [116] and *Desulfovibrio* [NiFe]-hydrogenases [51]. It was concluded that the process involves two successive steps: (a) a slow non-reductive step probably consisting in the removal of oxygen from the active center, or rather the dissociation of an enzyme–oxygen combination [30] and (b) a fast reductive step linked to the reduction of the enzyme by H_2 or a reductant (e.g. dithionite). These two steps are illustrated in Fig. 4 for the activation of *D. fructosovorans* [NiFe]-hydrogenase, which had been stored at +4 °C under air. The enzyme did not catalyze the H/D exchange reaction in the medium made totally anaerobic by addition of O_2 scavengers (glucose + glucose oxidase + catalase) until it was activated by reduced methyl viologen. The H/D exchange reaction then started immediately with initial rates of 195 μmol HD produced min^{-1} (mg protein)$^{-1}$ and

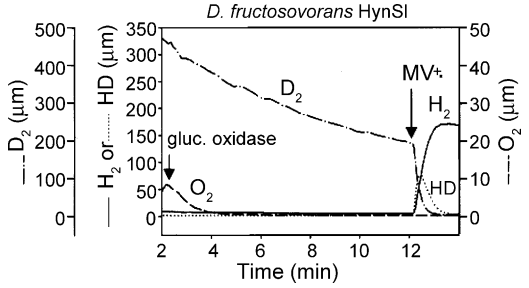

Fig. 4. Activation by reduced methyl viologen (MV^+) and H/D exchange activity of *D. fructosovorans* [NiFe]-hydrogenase stored for 1 month at +4 °C under air. The buffer (20 mM K phosphate, 0.1 M KCl, pH 7.0, 1.5 ml) was first introduced in the reaction chamber and gased with D_2. The chamber was closed, the medium was made anaerobic by addition of catalase (500 U), glucose (5 mM) and glucose oxidase (40 U), the hydrogenase (2.5 μg), and MV^+ (0.16 mM) were then added as shown in the figure (reproduced from [59] with permission).

150 μmol H_2 produced min^{-1} (mg protein)$^{-1}$ at an initial D_2 concentration of 200 μM (Fig. 4).

Anaerobic activation of the bidirectional NAD(P)-dependent multimeric hydrogenase from *Synechocystis* PCC 6308, by the reduced nucleotides, NADH or NADPH, in the presence of D_2, was also demonstrated by the H/D exchange method (Fig. 5).

In this case, while HD appeared immediately after NAD(P)H addition, a lag, in the range of minute(s), was observed before H_2 appeared in the medium. Although D_2 has been reported to inhibit H_2 evolution in *Azotobacter vinelandii* [46], this delay is not observed when a fully activated enzyme is used. These data raise the question whether in the inactivated form of the *Synechocystis* enzyme only the NiFe center is modified or whether conformational changes capable of modifying proton channels can also occur. This may be more specific to this type of enzyme since the homologous NAD^+-reducing hydrogenase from *R. eutropha* has been reported to have unusual properties due to the presence of two additional CN-ligands to the NiFe center [117,118] (so that the active site remains EPR silent under all redox conditions tested) and the NAD-linked hydrogenase of *Nocardia opaca* was shown to be unstable and to dissociate into two heterodimers [119].

4.2. The catalytic cycle

The H/D exchange provides an ideal assay for determining the activity of the enzyme active site alone even in systems as complex as whole microorganisms. It was even used to demonstrate the activity of hydrogenase in the dry state [120]. The authors measured the amount of HD and D_2 found in the gas phase of a vessel containing a powder of deuterated *D. vulgaris* Miyazaki hydrogenase (0.8 nmol) in contact with H_2 gas (12.3 μmol). After 35 days, the molar fraction of HD and D_2 were found to be 6.9 and 0.2%, respectively. The total amount of deuterium atoms liberated into the gas phase (after a couple of months) was found to be 1.53 μmol, a value which corresponds to 140 D atoms liberated per hydrogenase molecule [120].

In the D_2–H_2O isotope exchange reaction, generally the formation of both the single exchange product (HD) and the double-exchange product (H_2) are observed. Exceptionally, no HD production was detected for the hydrogenase from *A. vinelandii* [46] and for the H_2-sensing hydrogenase from *R. eutropha* [121]. As recalled in Section 1, the origin of H_2 produced by exchange of D_2 with H_2O as solvent, was already discussed more than forty years ago. The HD molecule formed initially can react with another molecule of hydrogenase to give the double-exchange product (H_2) (this intermolecular reaction of HD, observed at relatively high protein concentrations [45], is diffusion-limited) or react a second time with the active site before escaping into the solvent (this intramolecular reaction, the cage-effect [34,121], is independent of the enzyme concentration) or both the deuterons and the deuteride formed by heterolytic cleavage of D_2 can exchange separately, at different velocity [35,50,55].

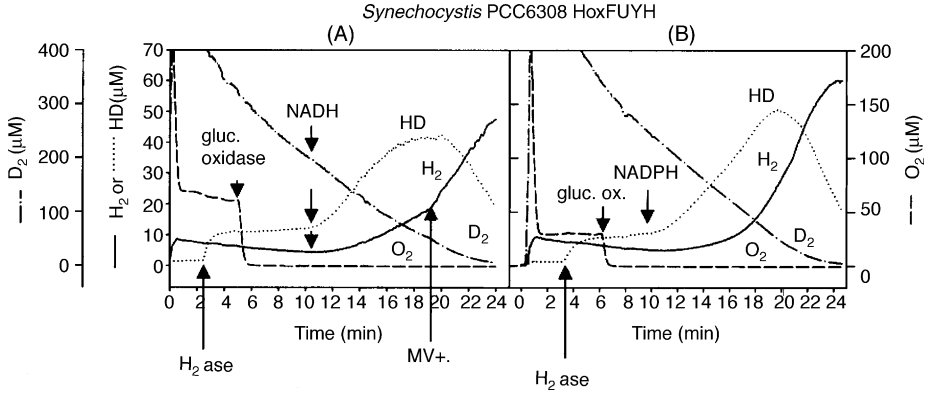

Fig. 5. Anaerobic activation of *Synechocystis* PCC 6308 bidirectional [NiFe]-hydrogenase by reduced nicotinamide nucleotides, measured by the H/D exchange reaction. The enzyme had been stored at +4 °C in 50 mM phosphate buffer, pH 7.0, under argon. In the reaction chamber, MES buffer (50 mM, pH 6.0, 1.5 ml) was saturated with D_2, the chamber was closed, hydrogenase was added, the medium was made anaerobic by addition of catalase + glucose + glucose oxidase (cf legend to Fig. 4 and NADH or NADPH were added as indicated by the arrows. (A) Hydrogenase (0.2 mg), NADH (200 μM), MV^+ (100 μM), (B) hydrogenase (0.4 mg), NADPH (200 μM). The figure shows the actual concentrations of the hydrogen species present in the vessel (reproduced from [59] with permission).

The rates of HD and H_2 formation depend on the environment of the active site, on the nature of the primary proton acceptor at the active site and of the amino acid residues involved in proton channels. This explains the pH-dependence and the isotope effect observed in D_2O of the H/D exchange [46,55,122–124]. The same pH profiles, with optima at acidic pH, have been obtained for H_2 evolution (from reduced methyl viologen) and H/D exchange for *Paracoccus denitrificans* [50], *Bradyrhizobium japonicum* [45], *D. salexigens* [122], *Rhodobacter capsulatus* [58] and *Dm. baculatum* [59,123] hydrogenases, and equal rates of H_2 production by isotope exchange and of H_2 evolution from reduced methyl viologen were reported for the enzymes from *T. roseopersicina* [55] and *A. vinelandii* (where these reactions are characterized by the same activation energies) [46]. These data indicate that the two reactions share the same rate-limiting step in a same reaction pathway. From the isotope effects and pH-dependence it has been concluded that the proton uptake is the rate-limiting step in the H^+/D_2 exchange reaction [108]. On the other hand, for H_2 oxidation, optimal at basic pH values [50,55,59,106,123], it was concluded that the intermolecular electron transfer to the electron acceptor is the rate-limiting step [108].

The pH optima of HD and H_2 formation and the H_2 to HD ratios have been used to distinguish various types of *Desulfovibrio* hydrogenases [122]. In particular, the [NiFeSe]-hydrogenase from *Dm. baculatum* (*D. baculatus*) produced H_2/HD ratios higher than one, while this ratio was found lower than one for non-selenium containing hydrogenases [122,124]. This property was at the time considered to be a specificity of [NiFeSe]-hydrogenases. However, this fact is not linked to the presence of SeCys at the active site since the membrane-bound uptake [NiFe]-hydrogenases from *P. denitrificans* [50], *R. capsulatus* [47] and *B. japonicum* [45] also produce H_2 at a higher rate than HD and the H_2/HD ratio is higher than one in all cases. It more probably depends on the relative exchange velocity (and then on proton transport and accessibility to water molecules) of the second (hydride) hydrogen atom of the dihydrogen molecule.

Measurements of the H/D exchange reaction in whole cells of the photosynthetic bacterium *R. capsulatus* have demonstrated, for the first time, that the regulatory HupUV protein could catalyze H/D exchange and thus bind H_2, a prerequisite for a H_2 detector [56]. This H_2-sensing hydrogenase is the first component of a signal transduction cascade responding to H_2, which also comprises the histidine kinase HupT and the transcriptional regulator HupR [125–128]. A similar system is found in *R. eutropha* [129,130] in which the H_2-sensing regulatory protein is termed HoxBC or RH [121,131,132].

The H/D exchange reaction has also been useful to demonstrate the presence of a fourth hydrogenase in a mutant of *D. fructosovorans* deleted from its three known hydrogenases [133,134].

4.3. Effects of the inhibitors, acetylene and carbon monoxide

The tritium exchange and H_2 oxidizing activities of membrane-bound [NiFe]-hydrogenases from *A. vinelandii*, *Rhizobium japonicum* (*B. japonicum*), *A. eutrophus* (*R. eutropha*) are inhibited by acetylene. Acetylene inhibition is competitive with CO and H_2, suggesting a common binding site (the active site Ni) for all three gases [135], since the [FeFe]-hydrogenase from *D. vulgaris* is not inhibited by acetylene [136]. Acetylene is an active-site-directed, slow-binding, reversible inhibitor of [NiFe]-hydrogenase; it binds tightly to the large subunit but is released from the enzyme during recovery from inhibition [137]. Acetylene inhibition requires catalytically active enzyme [55,138]. With the hydrogenase from *T. roseopersicina*, acetylene was similarly shown to inhibit the H/D exchange reaction, to abolish the Ni-C EPR signal and to protect the enzyme from irreversible inhibition by iodoacetamide [55]. Iodoacetamide inhibition also requires an active form of the enzyme [55]. The active site might be accessible to these inhibitors (acetylene, iodoacetamide) through hydrophobic channels [21]. However, in the [NiFeSe]-hydrogenase of *Dm. baculatum*, either such channels do not allow access of the active site to iodoacetamide [55] and only feably to acetylene [136] or selenocysteine reacts poorly with iodoacetamide.

By the use of acetylene, in whole cells of *R. capsulatus*, the H/D exchange activity of the H_2 uptake hydrogenase (HupSL protein) could be distinguished from that of the H_2-sensor (HupUV). The former is inhibited by acetylene while the latter is not [57,58]. Another application of the specific features of the H/D exchange catalyzed by the H_2-sensing hydrogenase HupUV (insensitivity to O_2 and to acetylene) and by the uptake hydrogenase (inhibition by O_2 and by acetylene) has been the demonstration that, in *R. capsulatus*, the synthesis of both hydrogenases requires at least the accessory proteins HypF [57] and HypD [58]. HypF and HypD have been shown recently to participate (with HypE) in the synthesis and incorporation of the intrinsic CN^- (and also probably CO) ligands to the active site Fe [139], review [13]. Indeed, the presence of those non-protein diatomic ligands in the homologous H_2-sensor from *R. eutropha* has been revealed by FTIR experiments [121,140].

The effect of acetylene is reminiscent of that of carbon monoxide. CO is a competitive inhibitor of [NiFe]-hydrogenases [44,141]. It binds to the enzyme in the Ni_a-C state and the Ni_a-C.CO complex, as Ni-C, dissociates upon illumination to yield the Ni-L EPR spectrum [98]. This was the basis for the suggestion that CO and H_2 bind to the same site on Ni in this state [98]. More recently, CO binding to the [NiFe]-hydrogenases was demonstrated by IR spectroscopy [142] and information on the Ni-S_r.CO complex in *C. vinosum* hydrogenase has been obtained by X-ray absorption spectroscopy; in this complex the CO is bound to Ni as a terminal ligand [100]. A stopped-flow infrared study has shown that the competition between H_2 and CO for binding to the

active site is dependent on the redox state of the nickel ion; it is considerably faster with Ni^{2+} (Ni_a-S and Ni_a-R states) than with Ni^{3+} (Ni_a-C state) [143]. From IR spectroelectrochemical studies of the binding of CO to the active site of *D. fructosovorans* hydrogenase [92] it was concluded that only after reductive activation of hydrogenase can CO bind to the active site; therefore the redox states Ni-A, Ni-B and Ni_u-S do not bind extrinsic CO. The authors proposed that CO inhibition is caused by a weak interaction of extrinsic CO with the Ni atom, blocking electron and proton transfer at the active site.

4.4. H/D exchange and O_2 sensitivity of hydrogenases

Most hydrogenases are sensitive to O_2. They may be reversibly inactivated by O_2, as is the case for [NiFe]-hydrogenases or irreversibly inactivated (most [FeFe]-hydrogenases). The H/D exchange reaction has been used to demonstrate that the $NAD(P)^+$-reducing hydrogenase of the cyanobacterium *Synechocystis* is reversibly inactivated in vivo by O_2 produced photosynthetically in the light and that the activity is recovered in the dark once O_2 has been totally consumed by respiration (Fig. 6).

However, some [NiFe]-hydrogenases are O_2-tolerant. This is the case in particular for the H_2-sensing regulatory hydrogenases (HupUV in *R. capsulatus* [58,59] and HoxBC

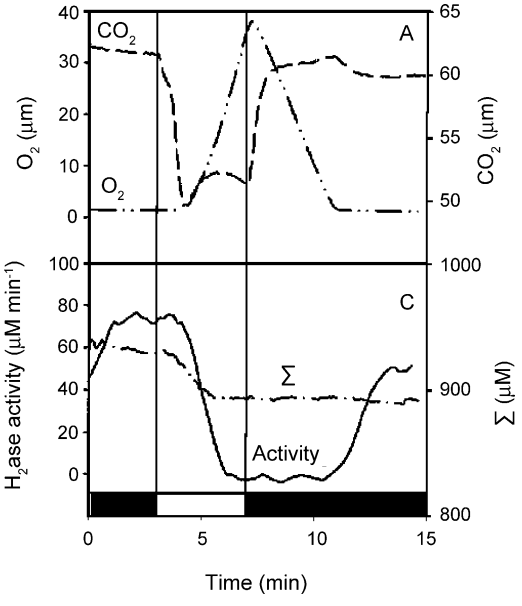

Fig. 6. Mass spectrometric measurements of O_2 and CO_2 exchange (A) and H/D exchange (C) during dark–light–dark transitions in *Synechocystis* PCC 6803 cells adapted to dark anaerobic conditions in the presence of D_2. Hydrogenase activity was calculated as $1/\tau$ ($2d[H_2]/dt + d[HD]/dt$), where τ is the isotopic ratio of D in hydrogen ($\tau = ([D_2] + 1/2\,[HD])/\Sigma$). The total concentration of hydrogen species, Σ ($\Sigma = [D_2] + [HD] + [H_2]$), drops at the beginning of the illumination period when the hydrogenase is still active then remains constant when the enzyme is inactivated by O_2 produced photosynthetically. Hydrogenase activity resumes when all O_2 has been consumed by respiration. Dark periods are represented on the *x* axis by black bars and the light period by a white bar (reproduced from [61] with permission).

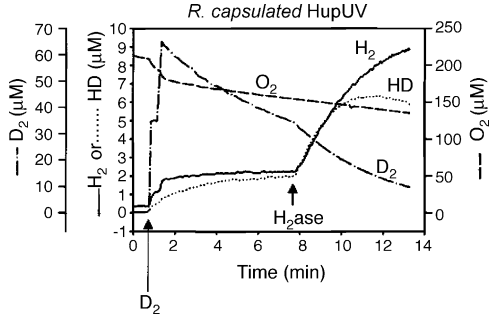

Fig. 7. H/D exchange activity of the H_2-sensing hydrogenase (HupUV) from *R. capsulatus* in the presence of O_2. The reaction chamber was filled with 1.4 ml of air saturated MES buffer (50 mM, pH 6.0) and closed, then 0.1 ml of MES buffer saturated with D_2 and the hydrogenase (5 μl, 6 μg) were introduced by Hamilton seringes at the time indicated. The figure shows the actual concentrations of the hydrogen species present in the vessel (reproduced from [59] with permission).

or RH in *R. eutropha* [118]); they function as H_2-sensors in bacteria growing aerobically. These hydrogenases do not require reductive activation to become active and, accordingly, EPR measurements did not detect a Ni-A or Ni-B state for this type of enzyme [121,140]. Fig. 7 shows that the H_2-sensor from *R. capsulatus* can catalyze H/D exchange in the presence of 160 μM O_2, at variance from the hydrogenase of *D. fructosovorans* (Fig. 2) and of *Synechocystis* (Figs. 5 and 6), which catalyzed H/D exchange only under anaerobic conditions. The reasons for this O_2 insensitivity have not yet been elucidated. A structural reason has been put forward by Volbeda et al. [90] who observed that the putative principal gas channel is significantly narrower than in standard hydrogenases due to the presence of bulkier amino acid residues close to the NiFe center. A partial blocking of the gas channel limiting gas access to the active site may explain the insensitivity to O_2 and also to C_2H_2 and CO, reported for the H_2-sensors. Another reason, also linked to obstruction of the NiFe center, may be an unusual Ni ligand environment as was reported for *R. eutropha* regulatory hydrogenase, RH [144] and for the *R. eutropha* NAD^+-reducing hydrogenase, also highly insensitive to O_2, the metallocenter of which contains two additional CN^- ligands [117], one of them, the Ni-bound cyanide group, is responsible for the O_2 insensitivity of the enzyme [118].

4.5. H/D exchange catalyzed by H_2-forming enzymes others than hydrogenases

4.5.1. H/D exchange catalyzed by nitrogenase

An intrinsic property of nitrogenase is to reduce solvent protons along with N_2 and to produce H_2. The reaction is ATP-dependent and irreversible. As hydrogenases, nitrogenase can also catalyze a H/D exchange reaction but, at variance from hydrogenases, HD formation by nitrogenase requires the presence of N_2 as well as D_2 [145] for it is mediated by a partially reduced dinitrogen intermediate [146,147] such as diazene [146]. With hydrogenases, both of

the D atoms can exchange with H_2O, although the hydride exchanges more slowly than the protonated amino acid [34,35,55]. Tritium labeling experiments on nitrogenase showed that the rate of incorporation of T^+ into the aqueous phase was much slower than the rate of HD formation under the same conditions [146]. Thus, it appears that unlike the hydrogenase-catalyzed reaction, cleavage of D_2 by nitrogenase does not produce readily exchangeable protons.

The nitrogenase MoFe protein contains the active site metallocluster called FeMo-cofactor. The question whether H_2 inhibition of N_2 fixation and N_2-dependent HD formation are manifestations of the same molecular process has been addressed by the use of mutants. It was shown that substitutions of the His195 (α-195^{His}) residue in the α-subunit, in the FeMo-cofactor environment, yielded mutants unable to catalyze N_2 reduction to NH_3. However, N_2 was bound by the α-195^{Asn} MoFe protein, apparently in the same way as the wild-type enzyme, and inhibited the reduction of both protons and C_2H_2. H_2 was found to inhibit N_2 binding, but the mutant did not produce HD under a D_2/N_2 atmosphere. The authors suggested that initial binding of N_2 at the active site FeMo-cofactor suffers from direct competition with H_2, whereas a reduced form of N_2 may be required for HD formation [147]. Based on quantum calculations, a model for the HD formation reaction catalyzed by nitrogenase has been recently proposed [148]; it involves, as intermediates, both a reduced N_2 species (N_2H_2) and a metal hydride at which D_2 scrambling can occur.

Practically, the specific features of the enzymes permit to distinguish the nitrogenase-mediated H/D exchange from the H/D exchange catalyzed by hydrogenases. Fig. 8 shows that, in the photosynthetic bacterium *R. capsulatus*, where the large amounts of ATP required for the reaction is produced by photophosphorylation, no H/D exchange was observed in the dark, in a mutant deleted from its hydrogenase activities, nor in the presence of ammonium ions, inhibitors of nitrogenase activity [58]. A light-stimulated H/D exchange activity stemming from nitrogenase was also demonstrated in the cyanobacterium *Nostoc punctiforme*, strain ATCC 29133 [149].

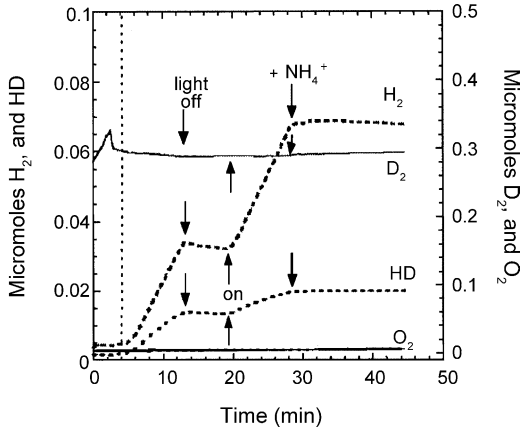

Fig. 8. Nitrogenase-mediated H_2 and HD production, in the presence of D_2 and H_2O, by a hydrogenase-less mutant of *R. capsulatus*. Cells were grown under nitrogenase-derepressing conditions. The culture (1.5 ml, 0.6 mg protein) placed in the reaction chamber was sparged with D_2. At the time indicated by the vertical dotted line, the vessel was closed and the H/D exchange reaction in whole cells was measured under light (light on) or in darkness (light off) and after addition of 10 mM ammonium sulfate, as indicated by arrows. The curves have been corrected for gas consumption by the mass spectrometer (reproduced from [58] with permission).

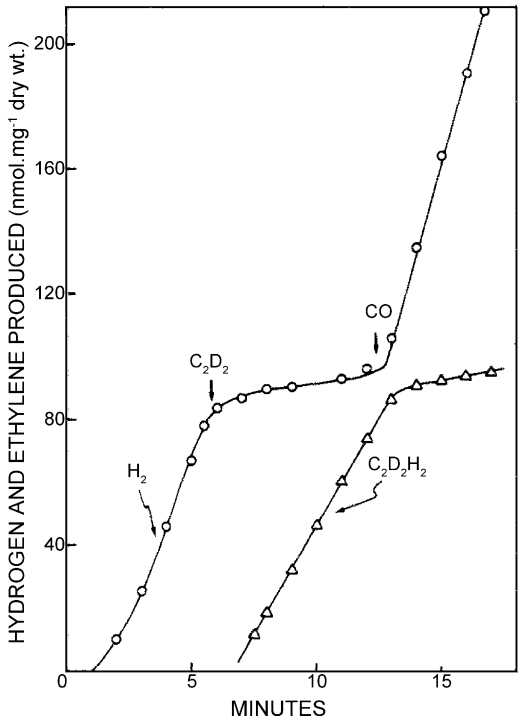

Fig. 9. H_2 production and acetylene reduction by *R. capsulatus* Mo-nitrogenase (reproduced from [47] with permission).

Reduction of acetylene to ethylene constitutes an easy assay of nitrogenase activity. Membrane-inlet mass spectrometry allowed to demonstrate that reduction of acetylene prevents H_2 formation. Fig. 9 further shows that CO can displace acetylene from the active site under turnover conditions [47]. Recently, it was suggested that, in the MoFe protein, the side chain of arginine 196 (α-Arg96) acts as a gatekeeper, moving during turnover in order to permit accessibility of acetylene to a specific [Fe_4-S_4] face of the active site FeMo-cofactor [150].

4.5.2. H/D exchange catalyzed by the iron sulfur cluster free hydrogenase, Hmd

The H_2-forming N^5,N^{10}-methylenetetrahydromethanopterin dehydrogenase (Hmd) found in most methanogenic archaea is another type of enzyme which produces H_2. It catalyzes the reversible conversion of N^5,N^{10}-methylenetetrahydromethanopterin ($CH_2 = H_4MPT$) to N^5,N^{10}-methenyltetrahydromethanopterin ($CH \equiv H_4MPT^+$) and dihydrogen:

$$CH_2 = H_4MPT + H^+ \leftrightarrow CH \equiv H_4MPT^+ + H_2 \qquad (5)$$

The formation of H_2, HD and D_2 by Hmd isolated from *Methanobacterium thermoautotrophicum* strain Marburg (now called *Methanothermobacter marburgensis*) was studied in experiments in which either the methylene group of $CH_2 = H_4MPT$ or water were deuterium labeled [151]. The results indicated that Hmd catalyzes the transfer of a hydrogen, most likely a hydride, from the methylene group of $CH_2 = H_4MPT$ to a proton of water since more than 50% of the dihydrogen formed from $CD_2 = H_4MPT$ in H_2O and from $CH_2 = H_4MPT$ in D_2O was HD. Besides HD, also significant amounts of H_2 (in H_2O) or D_2 (in D_2O) were formed and the formation of HD and H_2 and of HD and D_2 proceeded simultaneously rather than consecutively, an indication that H_2 or D_2 was not formed by exchange of HD with the solvent. Evidence has been presented that HD is not an intermediate in the formation of D_2 and H_2, respectively [152]. Although Hmd is considered to be a novel type of hydrogenase, it does not catalyze the reversible oxidation of H_2 (reaction (1)) and does not catalyze the H/D exchange in the absence of the substrate ($CH \equiv H_4MPT^+$). This type of iron–sulfur free hydrogenase differs from the [NiFe]- and [FeFe]-hydrogenases not only by the primary and tertiary structures but also by the presence of an iron-containing cofactor, sensitive to light, at the active site [153,154]. A mechanism of H_2 activation without metal had been proposed [155] before it was known that the iron in the cofactor has a functional role in the enzyme [153].

4.6. H/D exchange reaction catalyzed by synthetic chemical models

Catalysis of isotopic scrambling in H_2/D_2O or D_2/H_2O mixtures, leading to the formation of HD, is a requirement for functional models of hydrogenase active sites. Such H/D exchange tests have been done for [FeFe]-hydrogenase model complexes [156–158].

Model chemistry has also been used to support the iodoacetamide inhibition of the active site of NiFe hydrogenase. Darensbourg and colleagues [159] have shown that nickel-bound thiolates were reactive with iodoacetamide, giving precedent to the assumption that the cysteine modification agent indeed could make it into the active site and react with the cysteines there.

Acknowledgements

The author gratefully thanks Drs J. Meyer, N.A. Zorin, V.M. Fernandez, and P. Bertrand for critical reading of the manuscript and Dr G. Klein for his kind help for the finishing of the review.

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