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Review

Native and mutant nickel–iron hydrogenases: Unravelling structure and function

Antonio L. de Lacey [a], Víctor M. Fernández [a,*], Marc Rousset [b]

[a] *Biocatalysis, Instituto de Catálisis, C.S.I.C., C/ Marie Curie s-n, Cantoblanco, 28049 Madrid, Spain*
[b] *Unité de Bioénergetique et Ingénierie des Proteins, Institute de Biologie Structurale et Microbiologie, CNRS, 31 Chemin Joseph Aiguier, 13402 Marseille Cedex 20, France*

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Abstract

In this review, an appraisal of the progress in understanding of the NiFe-hydrogenases catalytic mechanism is presented. The article is focussed on NiFe-hydrogenases from *Desulfovibrio* genus, as these are among the best-characterized H_2-splitting enzymes. The X-ray structures, catalytic reactivity, spectroscopic properties and computer modeling data of the different forms of this class of hydrogenase are analyzed. In addition, the contribution of site-directed mutagenesis studies of *Desulfovibrio fructosovorans* species to structure–function relationships is reviewed.

Keywords: [NiFe] hydrogenase; Nickel; Iron; EPR; FTIR; H_2 biocatalysis; DFT

1. Introduction

The use of hydrogen as a primary energy vector is recognized as providing the best long-term solution for achieving a clean and sustainable energy economy, when generated from water utilising photovoltaic, wind power or other renewable power sources. Hydrogen, if it is to become an important energy source for future human society, is as a matter of fact, one of the major energy sources in the microbial world, in which hydrogen metabolism is widespread. It occurs in both anaerobic and aerobic biotopes, and involves micro-organisms belonging to the kingdoms of *Eubacteria*, *Achaeabacteria* and lower *Eucaryotes*. This property is facilitated by the pres-

* Corresponding author. Tel.: +34 91585 4807; fax: +34 91585 4760.
E-mail address: vmfernandez@icp.csic.es (V.M. Fernández).

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ence of specific enzymes called hydrogenases, which catalyse the reversible heterolytic cleavage of molecular hydrogen according to the reaction:

$$H_2 \rightleftharpoons H^- + H^+ \rightleftharpoons 2H + 2e^-$$

The huge amount of research devoted to the enzymology of hydrogenase is directed towards understanding the catalytic mechanism at work during the splitting of the hydrogen molecule. The expected developments will promote an improved and sustainable hydrogen-production technology as well as new types of catalysts inspired from the hydrogenase active site [1].

From the very beginning the presence of iron among the components of hydrogenases was described [2]. In fact, in the meeting *Hydrogenases: their catalytic activity, structure and function* held in Göttingen in 1978, the presence of iron–sulfur clusters in these enzymes was credited in all the communications dealing with structural aspects of hydrogenases [3]. Notwithstanding, in that meeting the presence of a transition metal other than iron was anticipated in order to explain the inhibition of the hydrogenase activity by CO and the properties of new EPR signals, which were incompatible with that of the well-characterized [Fe–S] redox clusters [4]. At the time, this discovery was not correlated with an earlier observation that indicated that nickel was required for the autotrophic growth of "Knallgas" bacteria [5], for the synthesis of hydrogenase [6] and for the activity of *Nocardia opaca* hydrogenase, which was found to be stimulated by this metal [7]. However, in 1980, Lancaster made a sound assignment of the EPR signal detected in soluble and in membrane-bound fractions, extracted from a methanogenic bacteria to nickel(III) [8]. Later studies by Albracht et al. on a purified hydrogenase from methanogenic bacteria grown in the presence of ^{61}Ni (a nickel nuclear isotope with spin 3/2) showed a clearly resolved nuclear hyperfine structure on the EPR lines at 2.23 and 2.01, which is a fingerprint of Ni atom bound to the hydrogenase molecule [9].

Different laboratories almost simultaneously submitted three reports on the redox-sensitivity of Ni in hydrogenases in the beginning of 1982 [9–11]. All three groups used EPR spectroscopy to obtain complementary information about the enzyme and this, in itself, illustrates how useful this technique has been in the characterization of this type of hydrogenase. The work of Albracht et al. [9] proved by isotope substitution that the EPR spectrum observed in a membrane preparation of *Methanobacterium bryantii* was due to Ni; incubation under hydrogen suppressed the EPR signal which allowed the authors to suggest that the nickel centre was the site of interaction of H_2 with the enzyme. LeGall et al. [10] reported a similar EPR signal for *Desulfovibrio gigas* enzyme, which was also redox sensitive and was assigned to the 0.64 g nickel atom found in the purified enzyme. In turn, Cammack et al. [11] also detected the Ni-EPR signal in samples of purified *D. gigas* enzyme, and carried out redox titrations at different pH values and discussed the possibility of nickel being involved in the catalytic cycle. EPR has also been very useful in the study of hydrogenases because the redox status of the iron–sulfur clusters can be monitored by this technique [12].

After these studies, the identification of Ni as a component of known hydrogenases became the rule rather than the exception [13–15]. Almost simultaneously with the characterization of NiFe-hydrogenases, nickel was also identified as a component of the active site of CO-dehydrogenase of acetogens [16,17].

The discovery of Ni as a component of redox enzymes prompted a boom of scientific research on the spectroscopic properties of this transition metal, where for the last 20 years a key objective has been to correlate the spectroscopic characteristics of the enzyme and the functionality of the hydrogenases [1,18]. The significant events on this Ni "race" came from redox titration of the rhombic Ni-EPR signals carried out by the. Cammack et al. [19], Moura et al. [20] and Albracht [21] groups. The previously known activation process of NiFe-hydrogenases by anaerobic reductive incubation could be correlated with changes of the EPR Ni-signal and a three state model was proposed to explain the evolution of spectroscopic and catalytic properties of NiFe-hydrogenases when subjected to activation–inactivation by reduction/oxidation cycles by Fernandez et al. [22].

Two independently carried out studies on the infrared spectra of hydrogenases from *Allochromatium vinosum* [23,24], and from *D. gigas* [25,26], gave new impetus to the effort to unravel the active site architecture of Ni-hydrogenases. The initial goal of the FTIR studies had been to detect the binding of CO, a strong competitive inhibitor of hydrogenases [27], as this molecule when coordinated to transition metals gives intense FTIR bands in the 2100–1800 cm^{-1} region [28]. A completely unexpected result was that several FTIR bands, in this frequency range were detected in the spectrum of the hydrogenase even before treatment with CO and these bands were proven to be due to intrinsic groups of the enzyme [23]. Moreover, the set of band-frequencies was found to shift in concert with the reducing/oxidizing treatment of the samples either by gas exchange with H_2, N_2 or O_2, or electrochemically in thin layer electrochemical cell, suggesting their origin came from diatomic ligands coordinated to the active center [24,26]. Further work by Albracht's group showed that hydrogenases from different sources, either Fe or Ni–Fe-type, presented sets of bands in the frequency range 1900–2000 cm^{-1} [29].

It was not until 1995 when the first X-ray structure of a hydrogenase was resolved [30]. The structure of *D. gigas* enzyme revealed that three iron–sulfur clusters are aligned from the protein surface to the active site, which was shown to be an heterobinuclear nickel center anchored to the protein by cysteine residues. Later on, direct crystallographic evidence was presented identifying Fe as a second metal ion component of the active site of this hydrogenase [25]. In addition, three ligands associated with the Fe atom were detected in the difference density maps that were assumed to be responsible for the aforementioned FTIR features of the enzyme [25]. Outstanding work from Albracht's laboratory allowed

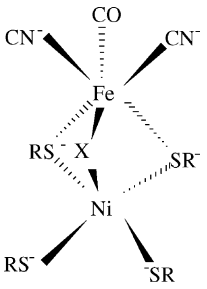

Fig. 1. Scheme of the active site of standard NiFe-hydrogenases. The X ligand is an oxygen species in the oxidized states and a hydrogen species in the reduced species.

the definitive assignment of the IR bands to one CO ligand and two CN ligands of the active site iron atom [31,32]. Fig. 1 shows the active site structure as derived from these observations.

2. The redox states of the active site

The redox chemistry of NiFe-hydrogenases is very rich. Their active sites are able to acquire several stable electronic structures, either catalytically active or inactive, in a quite narrow window of redox potentials. This functional property is probably due to the thiolate (good π-donors), CN^- (good σ-donors) and CO (good π-acceptors) coordination, which can act as an electronic buffer.

The introduction of FTIR spectroscopy for hydrogenase characterization has been a great advantage, as all redox states of the active site can be detected by this technique and the enzyme is amenable to examination by in situ spectroelectrochemistry. A significant breakthrough was the isolation of hydrogenase crystals in different redox states in order to ascertain the structural changes in the active site upon oxidation/reduction. Other spectroscopic techniques such as EPR, EXAFS, ENDOR, Mossbauer and ultraviolet–visible light spectroscopy have given valuable information on the structural and electronic aspects of the active site. Finally, the application of density functional theory (DFT) calculations for modeling the structural and electronic configurations of the hydrogenase active site has helped to fill the gap between the experimental results and the complete picture of the *real thing* [1].

Two types of EPR signals are detected for the oxidized active site of "standard"[1] NiFe-hydrogenases: Ni-A (*g*-values for *D. gigas*: 2.31, 2.23, 2.01) and Ni-B (*g*-values for *D. gigas*: 2.33, 2.16, 2.01). A third EPR signal, named Ni-C (*g*-values for *D. gigas*: 2.19, 2.16, 2.01) appears gradually upon reduction with H_2 or chemical reductants [33–35]. Fernandez et al. [35] showed that the Ni-C state corresponds to a catalytically *active* state, whereas the Ni-A and Ni-B states were inactive. The difference between the two later ones is that Ni-A is an *unready* state that needs a long period of incubation under reducing conditions before becoming *active*, whereas Ni-B is a *ready* state that becomes *active* quickly upon reduction [35]. Redox titrations of these EPR signals showed that (a) one-electron reduction of the Ni-A and Ni-B states gave EPR-silent states, (b) Ni-C is two electrons more reduced than Ni-B, (c) one-electron reduction of Ni-C results in another EPR-silent state named as Ni-R and (d) all these redox equilibria are pH-dependent, thus protons as well as electrons are involved in these processes [36–38].

As already indicated, FTIR allowed the redox titration of all states of the active site by spectroelectrochemistry [26], not only the paramagnetic ones. Fig. 2 shows that the redox titrations of *D. gigas* hydrogenase followed by FTIR, which reflects changes on the electron density of the active site Fe, roughly overlap with the redox titrations by EPR, which reflect changes in the electronic configuration of the Ni atom. Therefore, both metals of the active site interact electronically, that is, the binuclear complex behaves as a sole entity from the electronic point of view. Fig. 3 shows a scheme with the redox and acid–base equilibria that correlate the different states of the active site. FTIR spectroscopy indicated that the EPR-silent states obtained from reduction of Ni-A and Ni-B are different, and were named Ni-SU (*silent-unready*) and Ni-SI (*silent*) respectively. There is a kinetic barrier for the conversion of Ni-SU to Ni-SI, Ni-C or Ni-R, and the process

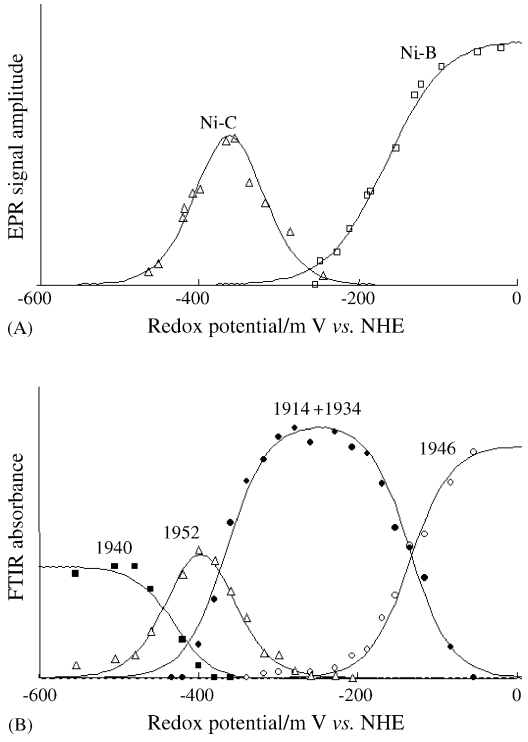

Fig. 2. Redox titrations of the active site of *Desulfovibrio gigas* hydrogenase monitored by: (A) EPR (data from [11,37]); (B) FTIR (data from [26]). Figure kindly provided by Prof. R. Cammack.

[1] *Note:* The term "standard" includes the most studied hydrogenases, which shares similar spectroscopic and kinetic features such as those from *D. gigas*, *D. fructosovorans*, *D. vulgaris* of the *Desulfovibrio genus*,and others from *Chromatium vinosum*, *Thiocapsa roseopercina*, etc.).

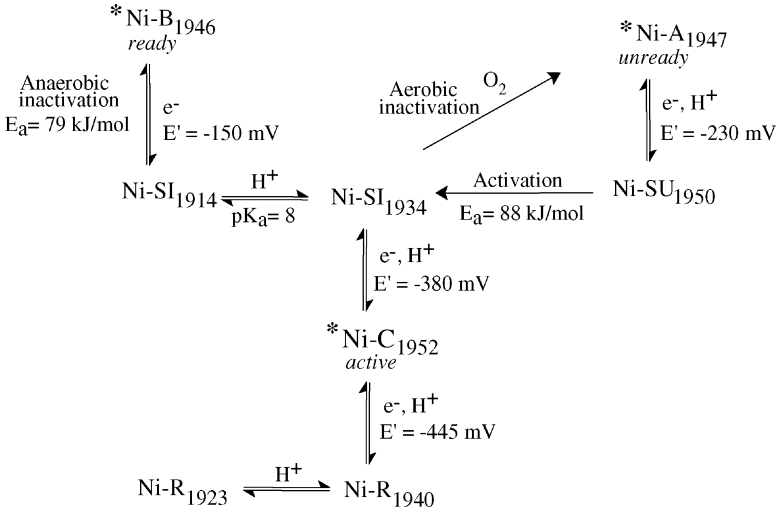

Fig. 3. Scheme of the different redox states of standard NiFe-hydrogenases detected by FTIR-spectroelectrochemistry. The E', E_a and pK_a values are from [22,26]. The subscript of each state corresponds to the frequency of the CO band of the FTIR spectrum. The EPR-visible states are marked with an asterisk.

is irreversible in the absence of O_2. This observation is also in agreement with EPR results in which the disappearance of the Ni-A signal upon reduction is fast, whereas evolution of the Ni-C signal is a slow temperature-dependent process [35]. Two different sub-forms are detected for Ni-SI, which differ by one proton [26]. The same occurs for Ni-R [39,40]. A kinetic barrier is also detected for the anaerobic oxidation of Ni-SI to Ni-B, which has been detected by both EPR [41] and FTIR [26,40].

The redox titrations have given a picture of the different states of the NiFe-hydrogenases' active site, the electron and proton balance of the equilibria that correlate them, and the kinetic barriers of some of the steps. However, we may wonder at this stage what are the structural and electronic differences between all these states? X-ray diffraction data have shown that the main structural difference between oxidized and reduced NiFe-hydrogenase crystals is the disappearance of an oxygen species bridging the two metal atoms of the active site [42,43]. Unfortunately, X-ray data cannot detect H atoms, thus it can neither give details of the protonation level of the active site nor the presence of H_2 or H^- ligands in the reduced states. However, other spectroscopic techniques have given some clues about hydrogen species ligation and about the oxidation states of the metals. The iron atom of the active site is low-spin Fe(II) in all states as observed from ENDOR and EPR spectroscopy [44,45], which is in agreement with its coordination by the strong-field and π-accepting CO and CN^- ligands. Moreover, the frequency shifts observed in FTIR of the active site upon change of redox state are considerably smaller than observed in model complexes for Fe(II)/Fe(III) transition [46]. Therefore, changes of formal oxidation state in the active site should take place in the Ni atom, as expected from the redox titrations of the Ni-EPR signals. The EPR spectra of the Ni-A and Ni-B states are typical of a Ni(III) ion [21,47]. Nickel-K edge XAS spectra are consistent with the formal oxidation state of the Ni metal oscillating from Ni(III) in all EPR active states to Ni(II) in the EPR silent states [48]. L-edge XAS experiments [49] and DFT calculations [50] favor a high-spin configuration for the later.

The structural difference between the oxidized Ni-A and Ni-B states is still a matter of debate. X-ray diffraction [30,51], EPR [52], ENDOR [53] and EXAFS [54] data indicate that in both states an oxygen species is bridging both active site metals. DFT calculations based on EPR data have favored a hydroxide bridge for Ni-B [55,56], and the Ni-O distance measured by EXAFS for that state is more in agreement with a bridging hydroxide [54]. The nature of the oxygen species in Ni-A is more controversial, either oxo [55] or hydroxo [56,57] species have been proposed from DFT calculations. EXAFS studies have suggested that the oxygen species in Ni-A is also hydroxo and it does not come out of the active site upon reduction to NI-SU [54]. From the pH-dependence of the formal redox potential of this equilibrium and from the fact that the vibrational frequencies of the FTIR bands hardly shift, which indicates that the electron density on the active site seldom changes, it seems reasonable to assume that one proton enters the active site coupled to the electron. Theoretical calculations have proposed that either this proton goes to the oxygen ligand [57,58] or to a terminal cysteine ligand [56]. However, very recent work has led to the idea that Ni-A may have a peroxide as a ligand, or alternatively, a hydroxide and one of the cysteines oxidized to sulfoxide [59]. Concerning the Ni-SI state, from EXAFS experiments, it is suggested that the oxygen species has left the active site, thus a vacant bridging position is favored by several authors [53–55,60] Alternatively, a hydride has been proposed as a bridging ligand in Ni-SI [61]. A Ni(III) formal oxidation state with a H^- ligand bridging both metals of the active center are the most probable features of the Ni-C state. EPR experiments, two decades earlier, showed that photodissociation of a hydrogen species was observed for

Ni-C [34,36]. In the last years, DFT calculations [60,62,63], and, more recently, ENDOR studies [64] and single crystal EPR studies [65] lead to a Ni(III) with a bridging hydride assignment. The photodissociated state of Ni-C is named as Ni-L and is generally considered to be a Ni(I) state with the H^+ gone to another location of the active site [66,67]. There is much less experimental evidence of the nature of the hydrogen species bound to the Ni-R state. From DFT calculations, either a terminal H_2 bound to the Fe [57] or a bridging H^- [60,61,68] has been proposed. FTIR spectroscopy has not been able to detect a band due to a metal–H^- bond in any state, although a bridging coordination would decrease the intensity of this band and shift it to frequency values where the solvent interference is very high [28].

3. The catalytic function

Crystallographic structures, spectroscopic data and redox titrations give us a picture of the different redox states of the active site, but in order to obtain structure/function relationships in NiFe-hydrogenases it is important to combine these studies with kinetic characterization. Several tools have shown their utility for kinetic studies, like quantitative determination of H_2-production/uptake with redox dyes, mass spectrometry for measuring H^+/D^+ isotopic exchange activity, enzyme immobilization on electrodes for evaluating the catalytic properties by electrochemical methods, inhibition studies, stopped flow analysis, theoretical calculations and site-directed mutagenesis. The comparison and careful discussion of the data obtained from the different techniques is helping to unveil the catalytic mechanism and the activation/inactivation processes of NiFe-hydrogenases.

3.1. Activation and inactivation processes

Standard NiFe-hydrogenases when oxidized are not catalytically competent. In this case, as mentioned before, we can differentiate between *unready* enzyme, which has its active site in the Ni-A state, and *ready* enzyme, which has its active site in the Ni-B state. Both states are Ni(III) and have an oxygen species bridging the metals, which is not present in the reduced states. Therefore, it must be the presence of this species that impedes H_2-activation at the active site. In fact, FTIR experiments show that carbon monoxide, which is a competitive inhibitor of hydrogenases, does not bind to the active site in the states that have an oxygen ligand (Ni-A, Ni-B and Ni-SU) [39]. When aerobically isolated, NiFe-hydrogenases are mostly in the *unready* state and need a long period, about 4 h at room temperature, under reducing conditions to become fully active. The rate of this process was first followed by incubating the enzyme under H_2 or other reductants, and measuring the H_2-uptake activity at different incubation times. Lissolo et al. [69] showed that the activation process followed first-order kinetics and was independent of the H_2 partial pressure. Fernandez et al. [22] measured the rate of the activation process for *D. gigas* hydrogenase at different temperatures and obtained a $\Delta E_a = 88\,kJ\,mol^{-1}$ for this kinetic barrier. An FTIR-spectroelectrochemical study showed that during the activation process Ni-A was first reduced to the Ni-SU state and then this state gradually converted to the Ni-SI, NI-C or Ni-R states depending on the applied redox potential, this conversion being the rate-limiting step [26]. In the same work, it was reported that the rate of Ni-SU conversion was independent of the redox potential or pH. These results have been confirmed by the activation of hydrogenases immobilized on electrodes, in which both thermodynamics and kinetics are electrochemically controlled [70,71]. From the experimental results it has been proposed that the rate-limiting step of the activation of *unready* hydrogenase is the removal of the oxygen species from the active site [26,39,42,54]. However, the activation of *ready* hydrogenase is fast upon reduction [22,26,41,72,73], although it also has a bridging oxygen ligand [52,54]. The rate of this process has shown to be redox potential dependent [72]. A key question is how this different kinetic behaviour of *ready* and *unready* hydrogenase can be explained on the basis of the similar structures of Ni-A and Ni-B? One hypothesis is that the different reactivity of the two oxidized states is not due to structural differences in the active site but to a different conformational rearrangement of the protein environment of the active site that allows faster accommodation of a water molecule coming out of the active site in the case of *ready* enzyme than in the case of *unready* enzyme [56]. However, this study does not exclude that the oxygen ligand ejected during activation is different in *unready* and *ready* enzyme. In fact, the X-ray structure, which supports this DFT study [30], was obtained from crystals of *D. gigas* hydrogenase mostly in the *unready* state, but with important contributions of *ready* and EPR-silent state. Another hypothesis for explaining the slower activation rate of Ni-A is that its oxygen species is different to Ni-B. In very recent work, it was proposed that the activation of Ni-A may involve the removal from the active site of a hydrogen peroxide molecule, which is larger than the water molecule coming out of the active site upon activation of Ni-B [59].

Inactivation of *active* enzyme can be accomplished by aerobic or anaerobic oxidation. Anaerobic activation is caused by oxidation via the iron–sulfur clusters with oxidants other than O_2 [22,70,74] or directly at an electrode surface [72]. Electrochemical experiments have shown that anaerobic inactivation is a slow process, with a rate very dependent on temperature [26,41] and independent of the redox potential [70,72]. Therefore, it was proposed that the rate-limiting step was a chemical step and not an electrochemical one [70,72]. FTIR-spectroelectrochemical results have shown that this process corresponds to the oxidation of Ni-SI to Ni-B [26] and that there is a primary kinetic solvent isotope effect, which indicates that in the rate-limiting step, a solvent-exchangeable group is transferred [75]. Taking in account all these experimental data and the most probable structure of Ni-B [54,56,68], it can be concluded that the rate-limiting step of

anaerobic oxidation is the introduction of a hydroxo ligand into the bimetallic active site.

Aerobic oxidation of NiFe-hydrogenases is more difficult to interpret, as depending on the oxygen concentration, pH, and the temperature, different ratios of Ni-A/Ni-B states are obtained [21,25,76,77]. O_2 can oxidize directly the bimetallic active site by diffusion through the hydrophobic channel that connects the protein surface to the active site, which explains the hyperfine structure due to a ^{17}O nucleus observed in the EPR spectra of Ni-A and Ni-B obtained by oxidation of active enzyme with $^{17}O_2$ [52], or indirectly via the iron–sulfur clusters. The latter alternative should be equivalent to anaerobic inactivation and should lead exclusively to Ni-B. FTIR-stopped flow experiments indicate that oxidation of completely reduced hydrogenase with excess O_2 leads quickly to Ni-B, which indicates that direct oxidation takes place in the active site because oxidation via the iron–sulfur clusters is very slow [73]. In the same work, the authors observed that with smaller concentrations of O_2 partial oxidation of the active site takes place first, probably via the iron–sulfur clusters, and then Ni-A starts to appear, presumably due to reaction of O_2 directly at the active site. These results were interpreted in the sense that reaction of O_2 with a fully reduced active site causes its four-electron reduction to H_2O and formation of Ni-B, whereas reaction with a partially oxidized active site causes its two-electron reduction to H_2O_2 and formation of Ni-A [73].

3.2. The catalytic cycle

Although the conversion of molecular hydrogen into two electrons and two protons, and vice versa, is formally a simple chemical reaction, its mechanism is rather complicated and the details of the catalytic cycle of hydrogenases are still a matter of debate [78–84]. First of all the H_2 substrate has to reach the active site where it will be oxidized by the bimetallic center. Hydrogen isotope exchange experiments indicate that the H_2 cleavage reaction is heterolytic, thus a hydride and a proton are formed in the first step [85–87]. In a second step, the two electrons of the hydride are extracted and a second proton is formed. Subsequently, the two electrons have to be transported, via an intra-molecular electron transfer chain, from the active site to the redox partner of the hydrogenase (a redox protein or NAD^+ (P) in vivo, a redox dye or an electrode in vitro). The two protons have also to be transferred to the protein environment. These steps are reversed in the case of H_2-production activity. These processes are illustrated in Fig. 4.

A hydrophobic channel that connects the protein surface with the active site for H_2 transport has been detected by X-ray diffraction studies in *Desulfovibrio fructosovorans* NiFe-hydrogenase [88]. This channel also, most probably, allows the access of the inhibitors CO and O_2 to the active site. As indicated before, the identification of the electron transfer pathway in hydrogenases is quite straightforward from the crystallographic structures. In standard [NiFe] hydrogenases, it is formed by two [4Fe–4S] clusters, one proximal to the active site and the other one exposed to the protein surface (the distal cluster) and one [3Fe–4S] cluster placed between the two [4Fe–4S] clusters [30]. The distances between these redox centers are such that they allow fast tunneling of electrons between the active site and the distal cluster [89]. The crystallographic structure of *D. gigas* hydrogenase indicates that the distal cluster has an exposed histidine ligand surrounded by carboxylic residues [30]. Chemical modification of the glutamic and aspartic residues of this hydrogenase in order to change the polarity of the surroundings of the distal [4Fe–4S] greatly affected its H_2-uptake kinetics: the activity measured with positively charged redox acceptors decreased greatly, whereas it increased with negatively charged ones. This work confirmed that the distal cluster is the redox center that exchanges electrons with the redox partner of the hydrogenase, and that electrostatic interactions between them play an important role in H_2-uptake/production kinetics [90]. This latter conclusion had already been reported for several other hydrogenases [91–93]. Recently, molecular modeling studies concluded that interaction of *D. desulfuricans* NiFe-hydrogenase with its physiological partner, the tetrahaem cytochrome c_3, was favored between the distal [4Fe–4S] of the former and the haem IV of the later [94].

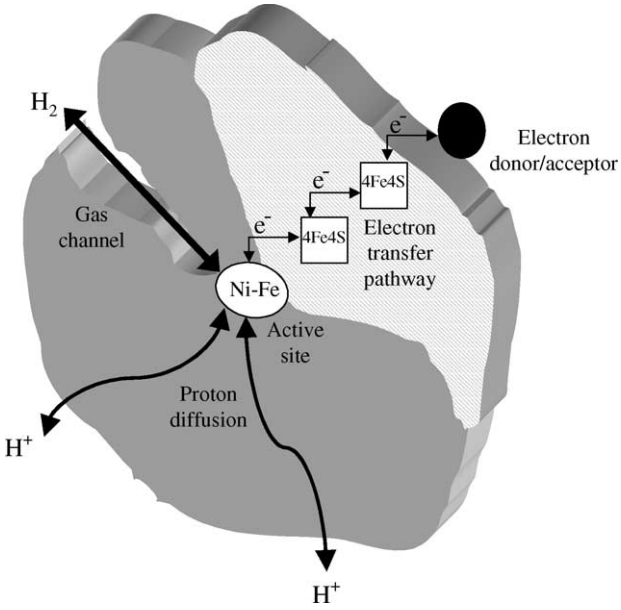

Fig. 4. Scheme of the catalytic function of NiFe-hydrogenases. Only two iron–sulfur clusters are represented for the sake of clarity.

There is much less evidence regarding the proton transport pathway. Protons are transported inside proteins via motions of water molecules and amino acid residues with acid–base properties, like histidine, glutamic acid and aspartic acid [95]. Several routes have been proposed for NiFe-hydrogenases, and probably proton transport during catalysis does not stick to a single one [30,43]. However, there is a general agreement in considering that the proton acceptor group after heterolytic cleavage of H_2 in the active site is one of the terminal cys-

teine ligands of the Ni atom [45,51,60,61,76,80,94,96–99]. Therefore, most probably this cysteine is the starting point, or ending point in the case of H_2-production activity, of the proton transport pathway. A glutamic acid residue conserved in NiFe-hydrogenases (Glu18 in *D. gigas* hydrogenases) is H-bonded to the mentioned terminal cysteine bound to Ni. Thus, it is a potential candidate for the next step of the proton transport pathway [94,96]. De Lacey et al. reported that chemical modification of histidine residues of *D. gigas* hydrogenase in order to change the acid–base properties of their imidazolium rings caused the loss of H_2-uptake and H^+/D^+-exchange activities but not the *para*-H_2/*ortho*-H_2 conversion activity [90]. This result suggests that some histidine residues are involved in proton transfer through the protein.

Several additional questions arise about the catalytic mechanism of hydrogenases: (a) What are the rate-limiting steps? (b) Which of the known redox states of the active site participate in it? (c) How does H_2 cleavage/formation at the bimetallic site take place? (d) Which amino acid residues of the protein have a catalytic role in catalysis?

The first question has been approached by Bertrand et al. [100]. It is clear from the reported kinetic data that the H_2-uptake activity of NiFe-hydrogenases is rate-limited by the reduction of the redox partner of the hydrogenase (either the natural one, cytochrome c_3 or ferredoxin, or an artificial one), as the catalytic turnover depends on its formal redox potential and net charge [22,90,91,93,100,101]. Only when the hydrogenase is directly connected to an electrode, a high overpotential is applied and the temperature is kept low, are the kinetics not controlled by electron transfer from the hydrogenase to the redox acceptor, which in this case is the electrode. Under those conditions there is no pH-dependence of the H_2-uptake activity [102] but there is a small H_2/D_2 isotope effect [71], which suggests that substrate transport to the active site limits the kinetics. Concerning the H_2-production and H^+/D^+-exchange activities, taking into account their pH profiles and the H/D isotope effects, it seems that they share the same limiting process in some hydrogenases, which could be proton transfer to the active site or H_2 formation at the active site [100,103,104]. The kinetic study of the H_2-production activity of *A. vinosum* hydrogenase by direct electrochemistry showed that a protonation step with $pK_a \approx 7$ controls the reaction rate [102]. Which is the site of this protonation step? It could be Cys 530, one of the terminal ligands of the Ni atom, as it has a high temperature factor in the crystallographic structures of reduced hydrogenases indicating high conformational flexibility that could be due to a proton transfer role [43,94]. Moreover, FTIR spectroscopy indicates that in several of the NiFe-hydrogenase redox states, there are two or three subforms with different protonation levels [26,40]. The shifts of the vibrational frequencies upon changing the pH fit quite well with the protonation of a terminal cysteine ligand [63]. In spite of this, it cannot be excluded that the rate-limiting step could take place in another position of the proton transfer pathway.

Another important issue of the catalytic cycle is to determine which redox states of the active site participate during the catalytic turnover. Certainly, the Ni-A, Ni-B and Ni-SU are non-active states [26,35,105]. On the other hand, hydrogenase in the Ni-SI, Ni-C and Ni-R states is fully active in the H^+/D^+-exchange assay [26]. A very recent stopped-flow infrared study has shown that Ni-SI reacts with H_2 to form Ni-R in less than 100–200 ms (which is the timescale limit of the technique) [73]. The same thing happens with Ni-C if the proximal iron–sulfur cluster is oxidized [73]. However, these results do not necessarily mean that these active states are part of the catalytic cycle, as the kinetic assays indicate that during turnover the timescale of one cycle in the active site is less than a few ms [90,106,107]. In any case, the kinetic data obtained by electrochemical methods can be fitted to catalytic mechanisms that involve three redox states correlated by one-electron/one-proton equilibria with redox potentials compatible with those measured for the redox titrations of Ni-SI, Ni-C and Ni-R [102,108].

Even if we suppose that Ni-SI, Ni-C and Ni-R do take part in the catalytic cycle it is not evident how H_2 cleavage/formation takes place in the bimetallic center, as there is no general agreement on their structural and electronic nature. Helpful information can be obtained from the chemistry of biomimetic compounds of NiFe-hydrogenases and theoretical calculations. The Ni atom is probably the site where the H_2 binds as a terminal ligand because the gas transport channel of the enzyme ends there [83,88]. Moreover, the crystallographic structure of CO-inhibited hydrogenase shows that the extrinsic CO is terminally bound to the Ni [99]. Alternatively, some DFT studies have led to the supposition that H_2 binds to the Fe atom of the active site because the quantum-calculations indicated that this model was more favorable energetically [109,110]. Other authors favor this hypothesis because of the precedents of H_2 complexes of low-spin d^6 metals [80,111]. However, it must be remembered that the active site is embedded within the protein matrix, which can impose restrictions in the chemistry at the bimetallic center [56,61]. Base-assisted heterolytic cleavage of the H_2 molecule should be the next step of the catalytic cycle. Biomimetic models have shown that this process in Ni(II) complexes lead to metal hydrides [97,98,112–114]. There is general agreement in considering Ni-C as having a bridging hydride between the metals, thus it is a likely intermediate of the catalytic cycle. The most probable candidate for acting as base in this process is the Cys 530 terminal ligand of the Ni atom, as discussed above. A DFT calculation study also supports this hypothesis, as the energy barrier calculated for heterolytic cleavage assisted by a terminal S ligand [110] was very similar to the activation energy measured for the H_2-uptake activity of hydrogenase by direct electrochemistry, $55\,kJ\,mol^{-1}$ [115]. Alternatively, a water molecule bound to the Fe has been proposed to act as base [116]. Ni-C is a Ni(III) state, thus hydride formation causes the release of an electron from the active site to the proximal cluster. This process is probably coupled with the release of

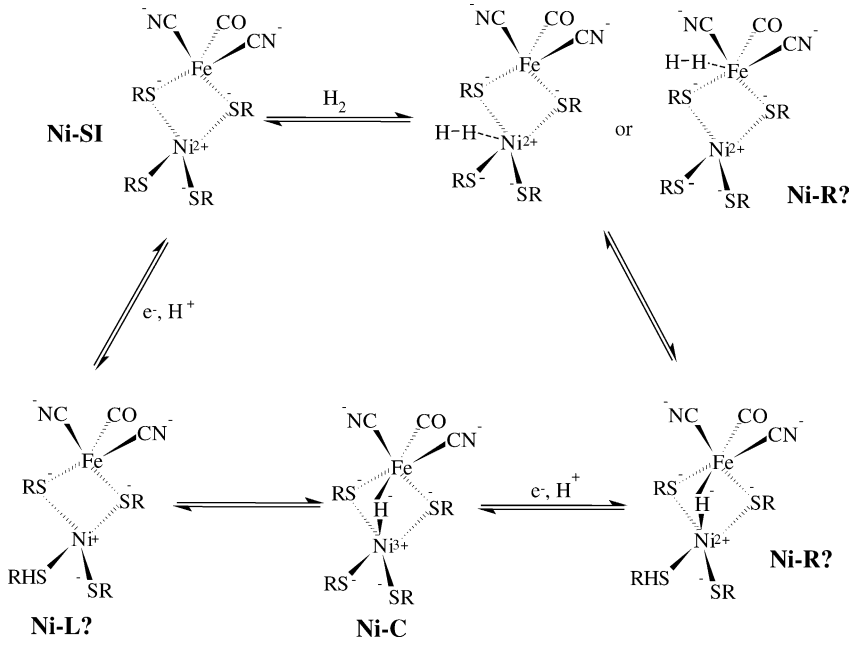

Fig. 5. Possible catalytic pathway at the active site of NiFe-hydrogenases. In some of the states the terminal cysteines may have a mixture of protonation levels to account for the different subforms observed by FTIR [26,39,40] and the kinetic data reported by Leger et al. [102].

the first proton to make it more energetically more favorable. The final step of the catalytic turnover could be the release of a second electron and proton which gives rise to a Ni(II) state with a vacant position (in either of the metals) for the binding of a new H_2 molecule. This is the most generally accepted configuration for the Ni-SI state [54,60,63,82,84]. Nevertheless, a transient of the last step could be a Ni(I) state with a H^+ gone to a cysteine ligand [63,78], which explains the double H^+/D^+ exchange activity of hydrogenases [104]. This transient could resemble the Ni-L state detected upon photodissociation of Ni-C at low temperatures [65,66,67]. Fig. 5 shows of scheme of the catalytic cycle of NiFe-hydrogenases taking in account most of the aspects discussed in this section.

As mentioned above the role of the protein matrix could be very important for modulating the catalytic function of hydrogenases. Site-directed mutagenesis followed by spectroscopic and kinetic characterization of the mutants is a powerful tool for establishing structure/function relationships.

4. Site-directed mutagenesis and mutant characterization

The cellular system based on the exceptional *Desulfovibrio* properties provides a unique tool for the study of the [NiFe] hydrogenase catalytic mechanism, as this is the only cellular system that allows the creation and the expression at high rate of recombinant hydrogenase.

The hydrogenase content of *D. fructosovorans* is exceptional in the sense that it is one of the very few organism possessing both types of hydrogenase, it therefore provides a single cell that carries out a combination of the various functions carried out by hydrogenases in several microorganisms [117–122]. *Desulfovibrio* is for that reason an ideal system for studying the diversity of hydrogen metabolisms. Moreover, this bacterium also exhibits the unusual property of expressing, at high-level, soluble forms of both types of hydrogenases. This is also very important because the complex and very specific maturation machinery of [NiFe] hydrogenases makes heterologous expression and over-expression impossible. *Desulfovibrio* provides therefore one of the rare cellular systems suitable for conducting site-directed mutagenesis studies on [NiFe] hydrogenase.

Beside the attractive hydrogenase content of *D. fructosovorans*, this bacterium is one of the rare *Desulfovibrio* species able to degrade a sugar, fructose in this case [123], which presents two strong advantages. First, it provides the bacterium with enough energy to reach much higher cell densities than with any other *Desulfovibrio*, making it possible to collect more cells and enzymes with smaller culture volumes. Second, during the oxidation of fructose, there is enough ATP synthesized by substrate-level phosphorylation so that mutations affecting oxidative phosphorylation functions will not be lethal, which might be expected from hydrogenase gene deletions. Finally, in the course of mutagenesis experiments, this bacterium exhibited very high recombination efficiencies, which led to the easy constructions of knock out mutants [124].

4.1. A unique cellular system

In order to turn the special properties of this bacterium to the best account, genetic tools adapted to *Desulfovibrio* have been developed for several years. We first started to explore the roles of the different hydrogenases by making sev-

eral simple and multiple deletion mutants [119,121,124,125]. This strategy did not lead to a precise understanding of the metabolic functions but these deletion mutants provided, however, essential cloning hosts for the expression of recombinant hydrogenases.

A new family of shuttle vectors has been constructed based on the origin of replication of a cryptic plasmid from *Desulfovibrio desulfuricans* (pBG1), and the origin of replication from *E. coli* plasmid (pMB1) [126]. Different antibiotic resistance reporting genes were used in the constructions, making possible the cloning in various mutant backgrounds, and a multi-cloning site in the ω-peptide is also present, which makes possible the blue–white screening. This series of high copy number shuttle vectors are introduced into *Desulfovibrio* by electroporation with an efficiency of 10^6 transformants/μg.

The cloning of the periplasmic [NiFe] hydrogenase structural genes in the related deletion mutant resulted in an expression level identical to that of the wild-type strain, which corresponds to about 1 mg of pure enzyme per litre of culture [127]. Recently, we succeeded in constructing a tagged hydrogenase. The Strep-tagII (IBA GmbH, Göttingen), which consists in an eight amino acid peptide mimicking the interaction of biotin with streptavidin, was introduced in the operon. The resulting recombinant hydrogenase was expressed at the same level as previously described, and was purified in one chromatographic step on a streptavidin column [128]. We therefore have at our disposal a unique cellular system, which makes possible the study and the production of recombinant [NiFe] hydrogenase.

4.2. Kinetic and spectroscopic analysis of mutants

4.2.1. Mutation affecting the electron transfer chain

The first site-directed mutation was conducted with the aim of understanding the role of the high-potential [3Fe–4S] cluster, which is a high-potential centre (−70 mV versus NHE at pH 8) located half-way between the distal and proximal low-potential [4Fe–4S] clusters (−350 and −410 mV versus NHE at pH 8, respectively) [36,129,130]. This apparent potential barrier to electron pathway is quite intriguing and its significance needs to be understood in order to elucidate the intra-molecular electron transfer pathway involved in the catalytic cycle of the enzyme [30]. Proline 238 of *D. fructosovorans* hydrogenase, which is homologous to Proline 239 of *D. gigas* enzyme and occupies the position of a potential additional ligand to the intermediate Fe–S cluster, was replaced by a cysteine residue. The mutation resulted in a functional enzyme, which maintained its structural integrity and exhibited a decrease in midpoint potential of the modified centre from +65 to −250 mV. EPR studies and X-ray crystallographic analysis demonstrated that the [3Fe–4S] cluster had been converted into a [4Fe–4S] centre in the P238C mutant, without significantly altering the spectroscopic and redox properties of the NiFe centre and the two native [4Fe–4S] clusters [127]. It was then rather striking that the decrease by about 300 mV of the midpoint potential of the median cluster in the P238C mutant (−250 mV versus +65 mV), which is kinetically more favorable, had such a small effect on the catalytic activity. A possible explanation for such an effect is that the midpoint potential of the [3Fe–4S] centre, as measured by equilibrium redox titration, is an apparent potential that is higher than the microscopic potential related to the redox states involved in the catalytic cycle of the enzyme as a result of redox cooperativity between the clusters. It is also reasonable to assume, and this does not exclude the previous hypothesis, that electron transfer is not a rate-limiting step of the catalytic reaction. In fact, a subsequent theoretical study concluded that endergonic tunneling is possible in electron transfer chains within enzymes at rates faster than the overall catalytic turnover [89]. Thus, it must be concluded that a high redox potential of the [3Fe–4S] is not an obstacle for fast electron tunneling through the protein. Site-directed mutagenesis on cysteine ligands of the medial cluster of other NiFe-hydrogenases has also been carried out, however the kinetic studies showed that the effect caused by the mutations varied upon the substrate used in the activity assay [131,132]. Thus, an interpretation of these results is not so straightforward.

4.2.2. Mutations affecting the active site cavity

With the goal of determining the role of the protein background on the functionality of the active site, mutants of the amino acids surrounding the diatomic ligands have been created and analysed in *D. fructosovorans* hydrogenases [133]. In two of the mutants, Ala or Cys replaced the residue Ser499, which according to the crystallographic structure may establish a hydrogen bond between its hydroxyl group and one of the CN^- ligands [30]. In another mutant, the target was Pro498, which makes van der Waals contact with the CO and one of the CN^- ligands was replaced by Ala. The fourth mutant had its Val78 residue, which is at van der Waals distance of the CO ligand, replaced by Ser (Fig. 6). From the analysis of the spectroscopic characterization of the mutants it was concluded that CN^- ligands establish hydrogen bonds with serine residues next to the active site (Fig. 6). However, these mutations do not the affect the kinetic properties of *D. fructosovorans* hydrogenase, which excludes a relevant role in catalysis of the Ser499, Pro498 and Val78 amino acids.

Replacement of residues proximate to the Ni–Fe active site has also been accomplished in the soluble hydrogenase from *Ralstonia eutropha*. Exchange of any of the cysteine ligands of the Ni–Fe site (Figs. 1 and 6) blocked a correct assembly of the enzyme and therefore produced catalytically inactive mutants [134]. Thus, these ligands have an essential structural role for the configuration of the active site. The binuclear Fe–Ni complex conforms as a highly stabilized functional entity. Most probably the sulfur bridges and hydrogen bonds through the CN^- ligands ensure correct positioning of the active site in relation to the gas supply channel and for efficient electron transfer via the iron–sulfur clusters chain.

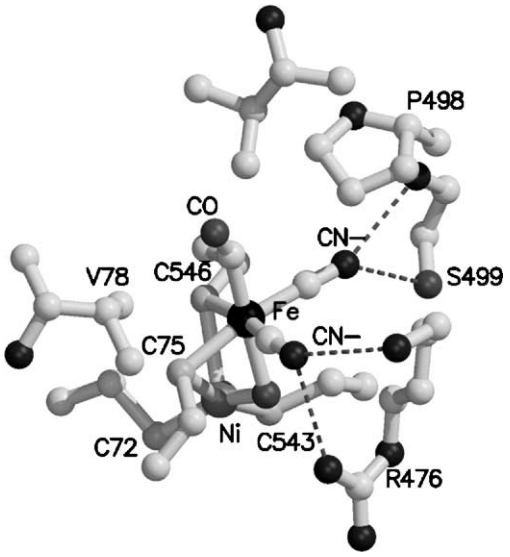

Fig. 6. The active site of *D. fructosovorans* NiFe-hydrogenase and neighbouring amino acid residues that have been exchanged by mutations. The dashed lines indicate putative hydrogen bonds. Coordinates were obtained from the crystal structure at 2.54 Å resolution of *D. gigas* hydrogenase (Brookhaven Protein Data Bank). Reproduced from reference [133] with permission of Springer-Verlag.

4.2.3. Mutation affecting the proton transfer

In order to study the possible proton transfer pathways, the role of the conserved glutamic acid residue (Glu25 in *D. fructosovorans* and Glu18 in *D. gigas* hydrogenases) in proton transfer was investigated. This amino acid was substituted by an aspartate residue (same chemical function but a shorter lateral chain) or a glutamine (same lateral chain length but the carboxylate group is replaced by an amide group) (Fig. 7) [128]. FTIR and EPR studies conducted on the E25Q mutant revealed that this protein has identical spectroscopic properties to those of the native enzyme, both in the oxidized and reduced states and after illumination. These results indicate that the redox properties and the architecture of the mutant active site remained unchanged. However, in a most interesting way, this apparent similarity of the structural and redox properties of the mutant with the native enzyme belies the complete alteration of the catalytic properties. The replacement of Glu25 by Gln abolished the hydrogen uptake, and D_2/H^+-exchange activities, for which proton transfer through the protein was compulsory. The substitution of the glutamate carboxylate (wild-type) by the glutamine amide (mutant) had suppressed the acid–base properties of the residue involved in this link, strongly decreasing the proton transfer rate. The fact that the *para*-H_2/*ortho*-H_2 conversion activity of the mutant was almost not affected suggested that the binuclear centre was fully functional. Besides, this Glu25 residue also seems to have a role during the anaerobic inactivation process as its replacement by glutamine slows down the process six-fold. It has been suggested that it may help the incorporation of the hydroxide ligand of the active site when oxidized to the Ni-B state [75].

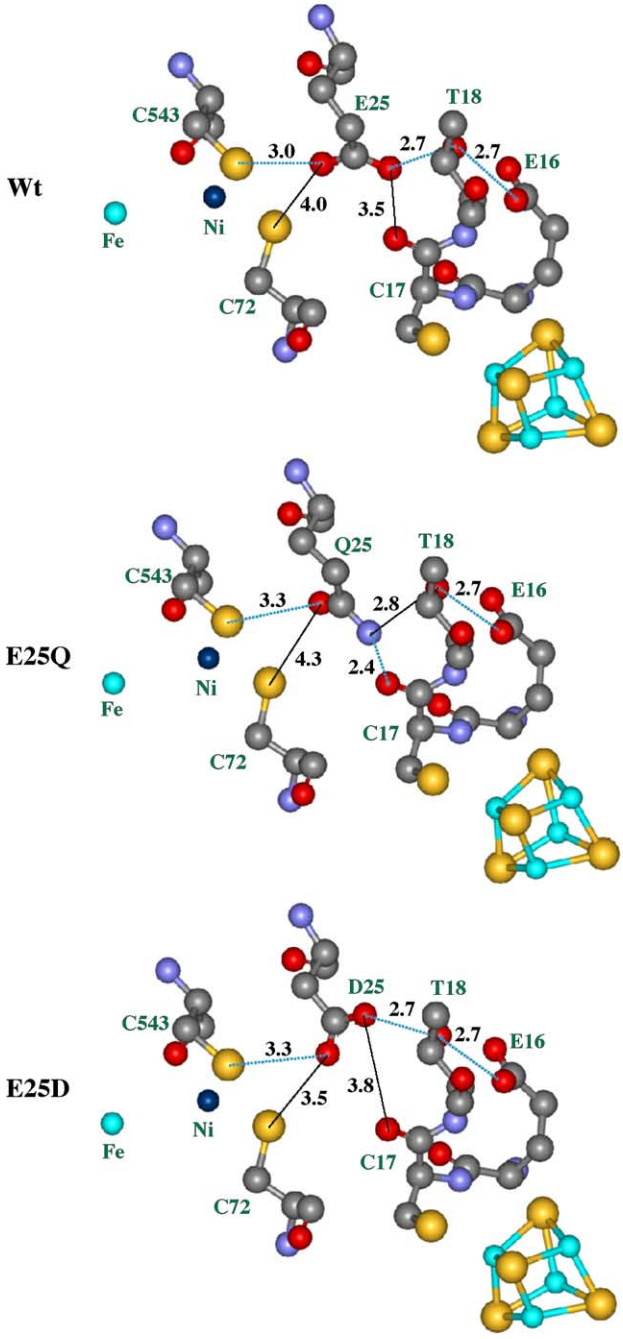

Fig. 7. Crystallographic structure (Wt) [124] and simulations (E25Q and E25D) of the active site of the [NiFe] hydrogenase from *D. fructosovorans*. The substitution simulations and the H-bond calculations were performed using DeepView 3.7. H-bonds are represented as blue dotted lines, and solid lines visualize the measured distances, which are indicated by numbers. Reproduced from reference [128] with permission of the American Society for Biochemistry and Molecular Biology.

Extensive site-directed mutagenesis studies on residues possibly involved on proton transfer have also been done with *Ralstonia eutropha* [134,135]. The most interesting result was achieved with Arg40/Leu mutant, which was completely devoid of H_2-uptake activity but maintained a high level of H^+/D^+-exchange activity. This result suggests that

proton transfer is uncoupled from the flow of electrons [134].

5. Conclusions

In spite of the great effort afforded for the structural and kinetic characterization of NiFe-hydrogenases in the last decade, there is still much to be learned on the catalytic function of these types of enzymes. This knowledge is fundamental in order to progress on technological applications (hydrogen bioproduction and fuel cells). Site-directed mutagenesis is a powerful tool for elucidating structure/function relationships in enzymes and these approaches have yielded important insights into the operation of the NiFe-hydrogenases. It is expected that studies of this sort will play a key role in the ultimate refinement of the catalytic mechanism of this class of hydrogenases.

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