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Characterization of *D. desulfuricans* (ATCC 27774) [NiFe] hydrogenase EPR and redox properties of the native and the dihydrogen reacted states

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Redox intermediates of *D. desulfuricans* ATCC 27774 [NiFe] hydrogenase were generated under dihydrogen. Detailed redox titrations, coupled to EPR measurements, give access to the mid-point redox potentials of the iron-sulfur centers and of the Nickel-B signal that represents the ready form of the enzyme. The interaction between the dihydrogen molecule and the nickel centre was probed by the observation of an isotopic effect on the EPR signals detected in turnover conditions, by comparison of the H_2O/H_2 and D_2O/D_2-reacted samples.

Introduction

Hydrogenases catalyse the reversible oxidation of the dihydrogen molecule, which is important in the anaerobic metabolism of both chemotrophic and phototrophic bacteria. Structurally distinct groups of hydrogenases have been identified based on the number and composition of their redox centers. The hydrogenases isolated from sulfate reducing bacteria of the genus *Desulfovibrio* can be classified as [iron-sulfur]-only, nickel-[iron-sulfur]- and nickel-selenium-[iron-sulfur]-containing hydrogenases [1]. The structure of the active sites of bacterial hydrogenases remain unsolved and unexpected features have been revealed (see Refs. 2, 47 and references therein). [Fe] hydrogenases are the least common, lack the nickel site and contain [4Fe-4S] clusters and an iron cluster of unique structure. [NiFe] hydrogenases are the most common and contain two [4Fe-4S] clusters, a nickel site and a [3Fe-4S] cluster. [NiFeSe] hydrogenases lack the [3Fe-4S] center and have selenium (as a selenocysteine) coordinated to the nickel site.

Hydrogenases are found in the periplasm, the membrane and the cytoplasmic spaces. A given bacterial species may have only one or several different hydrogenases. Study of the correlation between location, structural class and physiological function of hydrogenases is underway. Much effort has been devoted to the purification of [NiFe] hydrogenases and the characterization of their metal centers by biochemical, genetic, spectroscopic and crystallographic techniques. In the last years, we gave special emphasis to the following nickel containing hydrogenases: *Desulfovibrio gigas* NCIB 9334 [NiFe] hydrogenase [3–5], *D. desulfuricans* ATCC 27774 [NiFe] hydrogenase [6], *D. baculatus* DSM1734 [NiFeSe] hydrogenase [7–10], *D. salexigens* [NiFeSe] hydrogenase [11] and *Thermodesulfobacterium mobile* DSM 1276 [NiFe] hydrogenase [12].

D. gigas hydrogenase has been used as a prototype of the [NiFe] hydrogenases and detailed data on active site composition, redox and catalytic properties were obtained by EPR and Mössbauer [3–5], MCD [13], EXAFS [14] and mass spectrometric [15,16] studies. Extension to thermophiles is of great interest, in particular to the hydrogen metabolism and with respect to anaerobic bacterial corrosion [12]. The utilization of an organism such as *D. desulfuricans* ATCC 27774 enables the study of hydrogen metabolism in a situation where the respiratory substrate (sulfate) is replaced by nitrate. The hydrogenase isolated from this organism belongs to the [NiFe] group [6]. It is a complex enzyme

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containing one nickel center, one [3Fe-4S] and two [4Fe-4S] clusters. This is the same configuration that can be found in *D. gigas* hydrogenase [3].

Several characteristic Ni EPR signals have been detected for the Ni-containing enzymes. Nickel-A signals in hydrogenases have been related to the unready state of the enzyme and to the reversible inactivation of hydrogenases. Minor amounts of other Ni signals (Nickel-B) are observed in the oxidized state and can be enhanced by recycling the enzyme in the absence of oxygen [5]. The increase in the amount of Nickel-B has been pointed out to represent an enzyme state that is easy to activate and does not require deoxygenation steps but just a reductive one [5,17]. This latter species is still scarcely characterized. Another relevant species is associated with the Ni-C signal, observed in intermediate oxidation states of the enzyme. Nickel-C is generated from Nickel-B, by a two electron reduction step. The reduction potentials are pH dependent [2,47]. The state has been described in different isoelectronic formulations: Ni(I).$2H^+$, Ni(III).H_2 or Ni(III).H^- [2b]. The fact that the signal is light-sensitive and that the rate of photolysis shows a D/H isotopic effect indicates its direct participation in the reaction cycle [2,47].

D. desulfuricans ATCC 27774 hydrogenase represents a unique situation where the Nickel-B signal is the predominant one in the as isolated state. The enzyme had been previously isolated from ^{61}Ni, ^{56}Fe and ^{57}Fe-enriched media and characterized in terms of molecular weight and spectroscopic properties (EPR and Mössbauer) of the active sites. Isotopic substitution of ^{61}Ni proved that the Nickel-B signal obtained for the native state, was indeed due to the nickel. The Mössbauer data on the native state of the enzyme revealed the presence of a [3Fe-4S] cluster that could contain oxygen or nitrogen ligands, and of two [4Fe-4S]$^{2+}$ clusters [6]. In the present paper we describe detailed EPR studies on the redox properties of the Nickel-B, Nickel-C and [3Fe-4S] redox species of *D. sulfuricans* ATCC 27774 hydrogenase.

Materials and Methods

D. desulfuricans ATCC 27774 cells were grown in a medium described by Liu and Peck [18] containing nitrate rather than sulfate as a terminal electron acceptor. The crude extract was prepared and the hydrogenase samples were purified as previously described [4]. Hydrogenase activity was determined by the H_2 evolution assay using methylviologen as the electron donor [19]. Hydrogen was determined by means of a Varian 4600 gas chromatograph. Nickel and iron were determined by plasma emission spectroscopy using the Jarrell-Ash model 750 Atomcomp. Protein concentration was determined using an $\epsilon_{400} = 48\,000\ M^{-1}\,cm^{-1}$ as for *D. gigas* hydrogenase which has a similar chromophore content [20]. Purity of the hydrogenase was established by SDS-PAGE [21]. EPR spectra were recorded on a Brüker ESP-300 spectrometer, equipped with an Oxford Instruments continuous flow cryostat. Oxidation reduction titration was carried out using the materials and procedures as described in Ref. 3. The redox potentials were measured using a platinum and a saturated calomel standard electrodes and were quoted relative to the standard hydrogen electrode. The D_2/D_2O sample was prepared on a 100 mM Tris-HCl buffer (pH 7.6) and exchanged with D_2O (Sigma, 99.8%) in an Amicon centricon with a cut off of 30 kDa. This sample was reduced under D_2 flow (Union Carbide, chemically pure) directly into the EPR tube.

Results and Discussion

Due to the structural resemblance between *D. gigas* and *D. desulfuricans* ATCC 27774 [NiFe] hydrogenases, the formalism used in this analysis and discussion of results follows closely our previous work on the former enzyme [3].

In the native state, as prepared, *D. desulfuricans* ATCC 27774 [NiFe] hydrogenase is described as containing four non-interacting redox centers [6]. Its EPR spectrum at low temperature indicates the presence of a slow relaxing rhombic Ni(III) species with g values at 2.33, 2.16 and 2.02. This signal can be observed at temperatures up to 100 K without measurable broadening (Fig. 1C)) and accounts for 0.8 spin per molecule. Minor components are detected, better seen at the g_2 spectral region, indicating some heterogeneity at the nickel site. The observed signal can be classified as

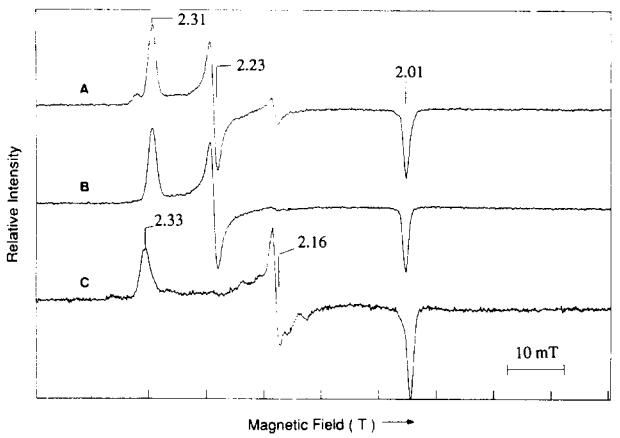

Fig. 1. Comparison of native EPR spectra of [NiFe] hydrogenases exhibiting different nickel signals in the native (as prepared) state. (A) *D. gigas* hydrogenase (Nickel-A: $g_1 = 2.31$, $g_2 = 2.23$, $g_3 = 2.01$ and Nickel-B: $g_1 = 2.33$, $g_2 = 2.16$, $g_3 = 2.01$) (B) *D. gigas* hydrogenase (Nickel-A) (C) *D. desulfuricans* ATCC 27774 hydrogenase (Nickel-B) Experimental conditions: temperature, 100 K; microwave frequency, 9.42 GHz, microwave power, 2 mW; modulation frequency, 100 kHz; modulation amplitude, 1 mT and receiver gain, $5 \cdot 10^5$.

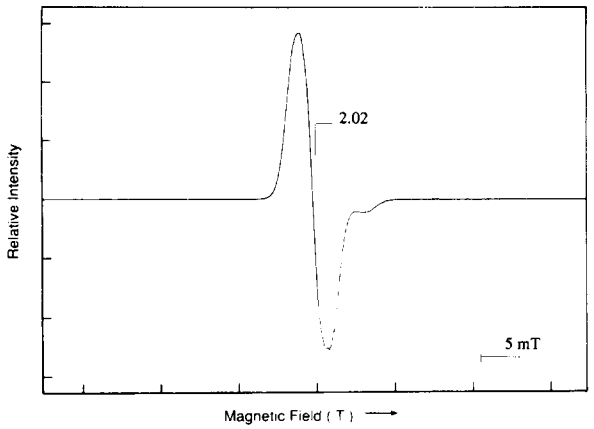

Fig. 2. EPR spectrum showing the isotropic signal display by the oxidized [3Fe-4S] cluster from the native hydrogenase from *D. desulfuricans* ATCC 27774. Experimental conditions: Temperature, 10 K; microwave frequency, 9.42 GHz, microwave power, 2 mW; modulation frequency, 100 kHz; modulation amplitude, 1 mT; receiver gain, $5 \cdot 10^5$.

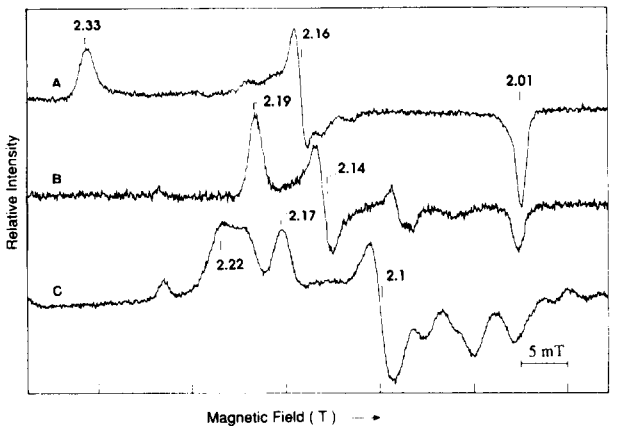

Fig. 3. Nickel EPR active species of *D. desulfuricans* ATCC 27774 hydrogenase (A) Native species at high temperature (T = 100 K) (B) Dihydrogen reacted species (Nickel-C) at 100 K, showing the slow relaxing $g = 2.19$-type signal (C) H_2-reacted species (Nickel-C) at low temperature (4.2 K) showing the fast relaxing $g = 2.22$-type signal. Other experimental conditions as in Fig. 1.

Nickel-B, since it has the same values as the minor species present in the native state of *D. gigas* hydrogenase (Fig. 1A), postulated to represent a deoxygenated (ready) form of the enzyme [5,22]. Nickel-A (unready form) is the dominant species in native *D. gigas* hydrogenase (Fig. 1B).

In addition to the nickel EPR detectable species, an EPR active oxidized [3Fe-4S] cluster (Fig. 2) can be observed up to 40 K, associated with an almost isotropic EPR signal centered at $g = 2.02$, with broad features at higher field. This signal is very similar to the one reported for the trinuclear center of *D. gigas* and *D. vulgaris* [NiFe] hydrogenases [7,26]. The signal accounts for approx. 1 spin per molecule.

Upon reaction of the enzyme with the substrate catalytically-relevant species develop. A major EPR detectable nickel species with g-values at 2.19, 2.14 and 2.02 and a minor species at g value 2.28 (Fig. 3 B) are observed at 100 K, reminiscent of Nickel-C and light-induced Nickel-C species observed in *D. gigas* hydrogenase [24]. In addition, a component with a complex interactive pattern with $g_1 = 2.21$ develops at very low temperature. This signal corresponds to a fast relaxing species, since it can only be observed at low temperature with high microwave power. It was suggested to be derived from an interaction between nickel and one of the reduced [4Fe-4S] clusters (Fig. 3C) [3,23,24]. Both signals subsequently disappear upon longer exposure to H_2 gas. The EPR spectrum of the dihydrogen reacted enzyme shows the presence of reduced [4Fe-4S] centers associated with broad signals with g_2 values around 1.94 (see redox titrations below). The temperature dependence of the EPR signals of the H_2-reacted enzyme (Fig. 4) shows that it converts from fast to slow relaxing species as the temperature rises.

The mediated titrations, shown in Fig. 5, indicate the oxidation-reduction properties of the three different types of signals that can be distinguished. These signals originate from: (i), the nickel center: Nickel-B and Nickel-C (appearance and disappearance); (ii), the [3Fe-4S] cluster and (iii), the reduced [4Fe-4S] clusters. Two EPR signals observed at the weak-field region were attributed to the reduced [3Fe-4S] cluster: (i), a signal with a crossover point at g approx. 16, comparable to the $g = 12$ signal previously observed in *D. gigas* hydrogenase [3] and (ii), a broad signal at the very weak-field region (approx. 3 mT), called Fe-S signal-B, also by comparison with the previous results. The reduced [3Fe-4S] center is then detected at low field with

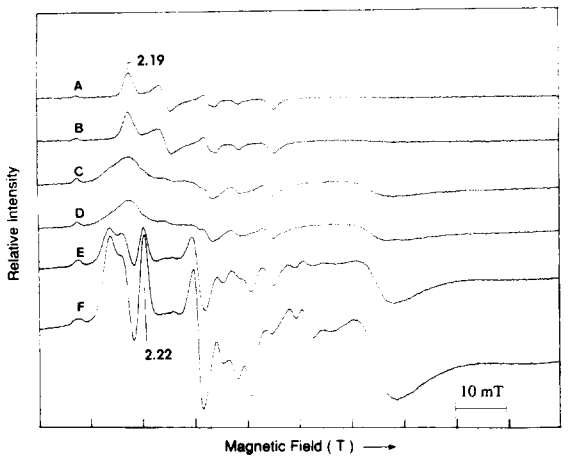

Fig. 4. Temperature dependence of the H_2-reacted Nickel-C species detected in *D. desulfuricans* ATCC 27774 hydrogenase. (A), $T = 14.0$ K; (B), $T = 11.0$ K; (C), $T = 9.6$ K; (D), $T = 8.2$ K; (E), $T = 6.3$ K; (F), $T = 4.2$ K. Experimental conditions: variable temperature; microwave frequency, 9.42 GHz; microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 1 mT and receiver gain, $5 \cdot 10^5$.

a $g = 12$ feature characteristic of the $S = 2$ state and this signal shifts to lower magnetic field values when the 4Fe centers get reduced. The reduced 4Fe centers show unusual properties, as observed for *D. gigas* hydrogenase [3].

In the native state, all the nickel is EPR-detectable and is in the Nickel-B form. Hence, we were able to titrate this form directly. The potential determined, at pH 7.6, after a simple Nernst equation fitting, is $E_0' = -170$ mV (Fig. 6 A). This value is pH dependent. Previous attempts to determine this potential had been unsuccessful, except for the case of *C. vinosum* hydrogenase [25], where the Ni(III) ready state (or Nickel-B) and the Ni(III) unready state (or Nickel-A), the major components in the native state of the enzyme, were simultaneously titrated. The assayed preparation had been previously enriched in the Nickel-B form by anaerobic cycling. The midpoint potentials for both forms were claimed to be equal ($E_0' = -115$ mV, at pH 8.0 and 30°C). In the case of *D. gigas* hydrogenase the Nickel-B potential was not determined, but studies of anaerobic cycling of the enzyme suggest a value for the midpoint potential that is slightly lower than the value for the Nickel-A form ($E_0' = -150$ mV vs. NHE at pH 7.0, pH dependent) [5].

Recent results on *D. vulgaris* strain Miyazaki [NiFe] hydrogenase, an enzyme that shows both Nickel-A and Nickel-B species as isolated, give access to the mid-point potential of Nickel-B (-230 ± 10 mV at pH 7.7) [26]. This value is rather negative when compared with the one obtained for *D. desulfuricans* ATCC 27774 [NiFe] hydrogenase in the present study. The appearance and disappearance of the Nickel-C signal display a bell-shaped curve with mid-point potentials of -360 mV and -395 mV, respectively. These results were

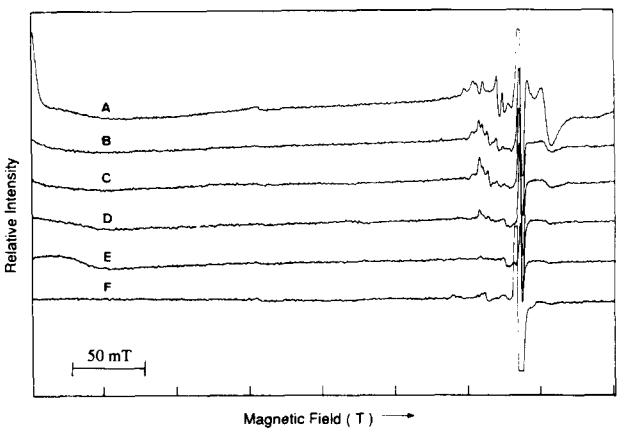

Fig. 5. Selected EPR spectra of *D. desulfuricans* ATCC 27774 hydrogenase obtained during the mediator poised redox titration at pH 7.6. (A), -424 mV; (B), -384 mV; (C), -366 mV; (D), -326 mV; (E), -300 mV; (F), $+376$ mV. Other experimental conditions: temperature, 4.2 K; microwave frequency, 9.42 GHz; microwave power, 2 mW; modulation frequency, 100 kHz; modulation amplitude, 1 mT and receiver gain, $5 \cdot 10^5$.

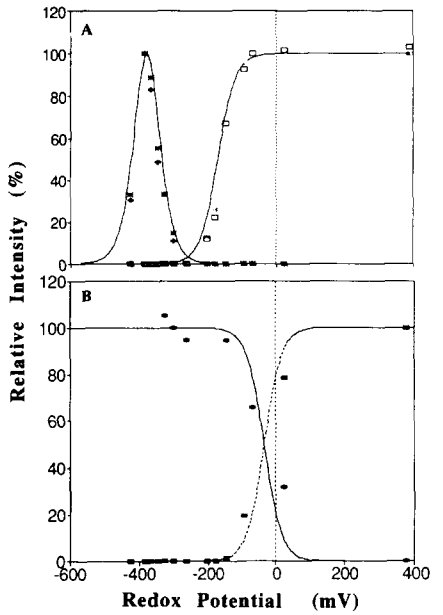

Fig. 6. Plot of the relative intensity of the Nickel-B (□, ■), Nickel-C (+,*) (A) and [3Fe-4S] cluster. (B) EPR signals of *D. desulfuricans* ATCC 27774 hydrogenase as a function of redox potential. The amplitudes of the nickel signal were measured at g values of 2.33 (□) and 2.16 (■) for Nickel-B and at g values of 2.14 (+) and 2.19 (*) for the appearance and disappearance of Nickel-C. For the [3Fe-4S] cluster, the measured intensities originated from the $g = 2.02$ signal for the oxidized cluster and from the Fe-S signal-B for the reduced cluster. The theoretical lines represent Nernst curves with midpoint redox potentials of -170 mV for the Nickel-B, and -360 mV and -395 mV for the appearance and disappearance, respectively, of the Nickel-C signal (assuming consecutive redox processes), and -35 mV for the [3Fe-4S] cluster.

obtained from single Nernst curves and assuming two consecutive redox processes (Fig. 6A). The values determined for Nickel-C redox potentials in *D. gigas* hydrogenase were -310 mV and -380 mV at pH 7.0 [3]. Cammack and co-workers determined these potentials to be -270 mV (-120 mV/pH unit) for the appearance and -390 mV (-60 mV/pH unit) for the disappearance of the Nickel-C signal [24]. Fitting of the progressive reduction of the trinuclear center with a one electron process gives a mid-point potential of -75 mV (pH independent). The mid-point potential of this center is in the same range of those of *D. gigas* and *D. vulgaris* enzymes [3,26,27]. The redox transitions associated with the *D. desulfuricans* ATCC 27774 hydrogenase centers at pH 7.6 are compiled in Table I.

D_2/D_2O and H_2/H_2O reactions were used to probe the substrate binding at the nickel site. A narrowing of 0.5 mT at the g_1 feature associated with the Nickel-C EPR signals was observed (Fig. 7). This effect had been previously observed on *D. gigas* hydrogenase and attributed to a weak interaction with one or more protons from the solvent [27]. In this context it is important to refer to the isotopic effect detected in a light induced transition on the Nickel-C signal of *C. vi-*

TABLE I

Redox transitions associated with D. desulfuricans ATCC 27774 hydrogenase redox centers

EPR signal	Origin	Hypothesized redox transition	Mid-point potential (vs. NHE) (mV)
$g = 2.02$	[3Fe-4S]	+1/0	−75
$g_1 = 2.33$	Nickel-B	Ni(III)/Ni(II)	−170
$g_1 = 2.19$	Nickel-C	Ni(III)/Ni(II)	−360 *
	Ni(III)-H^-		−395 **

* Appearance of the signal.
** Disappearance of the signal.

nosum hydrogenase and in fact, the rate of the photochemical reaction proved to be about 6-times slower in D_2O than in H_2O [28]. This result clearly indicates the involvement of hydride.

These experiments are of great interest from a mechanistic point of view. The demonstration of involvement of a hydride species is important, since the H_2 heterolytic cleavage has been much searched for and put in evidence by the isotopic exchange reactions [29]. In favour of the existence of this intermediate, ENDOR studies on the *D. gigas* hydrogenase Nickel-C form revealed that this site is accessible to solvent protons and/or gaseous dihydrogen, as opposite to Nickel-A which is not accessible. It was also concluded that two different types of exchangeable protons are bound to the former form of nickel: (i), one type characterized by a small hyperfine coupling (A^H approx. 4.4 MHz), which is consistent with the proton being associated with H_2O or OH^- and (ii), the other type with a larger hyperfine coupling (A^H = 16.8 MHz), identified as an in-plane hydride or an X-H proton involved in an agostic interaction with nickel or even as H_2 [30].

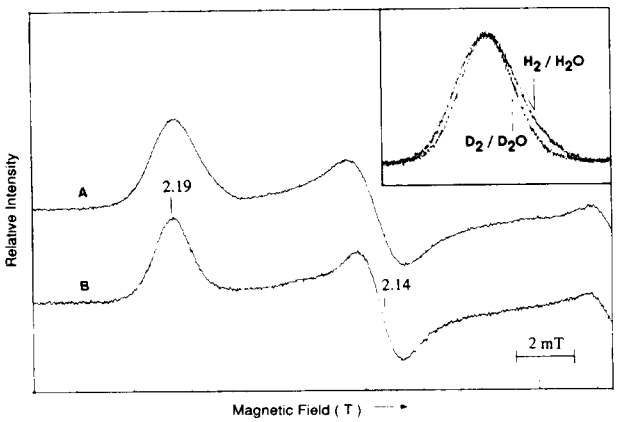

Fig. 7. D/H isotopic effect on Nickel-C EPR signal of *D. desulfuricans* ATCC 27774 hydrogenase. (A) H_2/H_2O-reacted sample and (B) D_2/D_2O-reacted sample. Inset: details on the resonances at low fields. A narrowing of about 0.5 mT of the g_1 feature associated with this signal can be observed for the D_2/D_2O-reacted samples.

Regarding the nickel oxidation states referred to in Table I it has been proposed that the Nickel-C state is related to a Ni(I) species. Support of this suggestion comes from redox titrations and the interpretation of the EPR spectra with nickel having H_2 and [^{13}C]CO as ligands [31]. However, EPR spectroscopy cannot distinguish between Ni(I) and Ni(III) [32]; but a more rhombic signal is expected for Ni(III) species than for Ni(I) species, as indicated by the study of various model compounds. Additional support for Nickel-C being a Ni(III) oxidation state came from the study of nickel-substituted rubredoxins [33–35]. This protein binds nickel to cysteinyl sulfur in a distorted tetrahedron. Previous studies of this nickel-substituted protein had concluded that it presented very interesting similarities with hydrogenase: it produced H_2 in the presence of an electron donor and it catalysed the H^+/D_2 exchange reaction (although at very low rates) and its activity showed CO inhibition [33]. Recently, Huang et al. [34] were able to generate EPR signals in the oxidized nickel-substituted *P. furiosus* rubredoxin that have analogous EPR properties to the *D. gigas* hydrogenase Nickel-C intermediate. In the presence of cyanide, as an exogenous ligand, a signal develops that is characteristic of square pyramidal or tetragonally elongated octahedral Ni(III) species (unpaired electron in d_z^2) with an equatorial coordinated cyanide, just as proposed for Nickel-C with a hydride in the place of cyanide. In the absence of cyanide, the EPR properties are very similar to those reported for oxidized Ni(II)-substituted rubredoxin from *D. vulgaris* [35] and correspond to those of the low-temperature photolysis product of Nickel-C in hydrogenases. EPR and Mössbauer studies have demonstrated that the two [4Fe-4S] clusters are diamagnetic in the active state of the enzyme, the so-called 'EPR-silent' state [3,5,6]. Nickel is in the +2 oxidation state (diamagnetic, low spin, $S = 0$), as proved by Multifield Saturation Magnetization measurements [10]. Further reduction of the enzyme generates the Nickel-C species, attributed to Ni(I) [28] or Ni(III) [27,32]. The similarity of this signal in hydrogenases from different organisms suggests that they share a common active site and a similar catalytic mechanism [17]. Based on the available information on the nickel redox cycling, a simplified scheme is then proposed:

$$\text{Ni(II) (EPR-silent)} + H^+ + e^- \leftrightarrow \text{Ni(III)-}H^- \text{ (Nickel-C)} \quad (1)$$

$$\text{Ni(III)-}H^- + H^+ + e^- \leftrightarrow \text{Ni(II)-}H_2 \text{ (EPR-silent)} \quad (2)$$

$$\text{Ni(II)-}H_2 \leftrightarrow \text{Ni(II)} + H_2 \text{ (regeneration of the catalyst)} \quad (3)$$

EXAFS measurements on *D. baculatus* native hydrogenase [9] and *Thiocapsa roseopercisina* hydrogenase in the Nickel-C state [38] indicate a strong resemblance, suggesting that the charge density does not change too

much in the nickel site, thus emphasizing the role of the ligands on the redox process. Several authors have stressed the importance of sulfur coordination to the nickel, as it influences the metal oxidation states. Sulfur coordination is supported by EXAFS data on several hydrogenases that indicates two to four sulfur atoms as direct ligands to the nickel atom [9,36–38]. EPR results show measurable broadening due to ^{33}S in *Wolinella succinogenes* hydrogenase isolated from bacteria grown in enriched media [39]. For the *D. baculatus* [NiFeSe] hydrogenase, EPR lines obtained with enzyme purified from ^{77}Se-enriched media also revealed broadening due to that isotope, confirming a direct coordination of the nickel to that atom [8]. These results were confirmed by Voordouw et al. [40] indicating that, in *D. baculatus* hydrogenase, the amino-acid sequence for the conserved putative nickel-binding region is homologous to the same region in several [NiFe] hydrogenases, except for one of the cysteines that is substituted by a selenocysteine. The direct coordination of this residue to the nickel site, gives further evidence for the importance of the sulfur ligation in the nickel redox chemistry.

Krüger et al. [41,42] studied different nickel model compounds and pointed out that the two main factors allowing low redox potentials for the Ni(III)/Ni(II) transition are: (i), anionic and polarizable ligands and (ii), a -2 formal charge for the nickel complexes. Cysteine seems to be the most effective natural ligand in the stabilization of a Ni(III) form. Also, from the study of model complexes containing sulfur as direct ligands to the nickel, Kumar et al. [43] have proposed that all the redox chemistry occurs in the sulfur and not in the nickel. In fact, according to these authors, the 'Ni(III)' EPR signal could be interpreted as arising from a Ni(II), tetragonal center with $S = 1$, with the spin in the $d_{x^2-y^2}$ orbital antiferromagnetically coupled to the thiyl radical ($S = 1/2$). The resulting system, with an unpaired electron in the d_{z^2} orbital, is consistent with the observed EPR spectra and the EXAFS data and is more consistent with the known redox chemistry for nickel thiolates. The same authors [44] propose a model for hydrogenase deactivation by oxygen based on cysteine oxidation; their work on model complexes with sulfur as a direct nickel ligand enables them to conclude that nickel thiolate complexes can undergo oxidation to sulfinato complexes under biological conditions (i.e., air oxidation). The conjunction of these and other pieces of information indicate that the reactive site (Ni plus S/Se) is the site for storage of electrons and proton/hydride in order to perform the heterolytic cleavage of the dihydrogen molecule.

Studies on hydrogenase nickel model compounds lead to the proposal of an interesting and plausible mechanism that accounts for the many spectroscopic features collected for [NiFe] hydrogenases using, as a working hypothesis, the direct participation of ligands in the nickel coordination sphere [45]:

$$[\text{Ni(II)-S}]^{2+} + e^- \rightarrow [\text{Ni(II)-S}^{\cdot -}]^{+}$$

$[\text{Ni(II)-S}]^{2+}$-active state EPR silent form (4)

$$[\text{Ni(II)-S}^{\cdot -}]^{+} + H^+ \rightarrow [\text{H-Ni(III)-S}]^{2+}$$

$[\text{H-Ni(III)-S}]^{2+}$-reaction intermediate Nickel-C/Ni hydride (5)

$$[\text{H-Ni(III)-S}]^{2+} + e^- + H^+ \rightarrow [\text{H}_2\text{-Ni(II)-S}]^{2+}$$

$[\text{H}_2\text{-Ni(II)-S}]^{2+}$-reaction intermediate EPR silent form (6)

$$[\text{H}_2\text{-Ni(II)-S}]^{2+} \leftrightarrow [\text{Ni(II)-S}]^{2+} + \text{H}_2 \quad (7)$$

In contrast to this working hypothesis, where the Ni site mostly retains its Ni(II) state throughout the catalytic cycle, recent results on a pentacoordinate Ni(II) complex containing aromatic nitrogen and thiolate ligands [46], readily afford Ni(I) and Ni(III) analogues, leading support to mechanistic proposals involving the alteration of the nickel redox states. Recent Saturation Magnetization measurements on *D. baculatus* [NiFeSe] hydrogenase, suggested pentacoordination for the Ni(II)-'EPR-silent state' [10]. The above-mentioned complex is capable of binding small ligands such as CN and CO in the EPR active states, Ni(I) and Ni(III), and forms a hexacoordinated compound. Both the unligated and ligated complexes yield EPR spectra reminiscent of those observed for the Ni-containing hydrogenases, suggesting that alteration of the coordination sphere through the activation steps and reaction with substrates may be a requirement for making the active site available for binding and processing the dihydrogen molecule.

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