Nature and Electronic Structure of the Ni-X Dinuclear Center of *Desulfovibrio gigas* Hydrogenase. Implications for the Enzymatic Mechanism†

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ABSTRACT: The recent determination of the X-ray crystal structure of *Desulfovibrio gigas* hydrogenase has revealed that the active site is a Ni-X dinuclear center [Volbeda, A., Charon, M. H., Piras, C., Hatchikian, E. C., Frey, M., & Fontecilla-Camps, J. C. (1995) *Nature 373*, 580−587]. This unexpected result calls for a re-examination of the magnetic and redox properties that have been attributed previously to a mononuclear Ni center. We have used a combination of dosimetric and electron paramagnetic resonance (EPR) techniques to investigate the nature and the electronic structure of the Ni-X center in the redox forms of *D. gigas* hydrogenase giving EPR signals. The metal atom X was first shown to be Fe by accurate metal content analyses. Next, by determining the EPR characteristics of a polycrystal powder, it was shown that the redox form of the enzyme studied in the X-ray crystal experiments was essentially Ni-A. The temperature dependence of the Ni-A, Ni-B, Ni-C, and Ni-L EPR signals was studied over a large temperature range. No deviation from Curie's law could be detected, which places strong constraints upon the magnitude of the possible magnetic interactions between the Ni and Fe centers. When these results and the other available magnetic data are analyzed in the light of the crystal structure, it is concluded that the Fe center is diamagnetic in all the redox states of the enzyme. On the basis of these results, a mechanistic scheme consistent with a large body of experimental data can be proposed for Ni-containing hydrogenases.

Hydrogenases are enzymes involved in the production and consumption of molecular hydrogen by microorganisms. Many hydrogenases contain stoichiometric amounts of nickel and display electron paramagnetic resonance (EPR) spectra which have been ascribed to a mononuclear nickel center on the basis of the *g* values, the spin-lattice relaxation properties, and the appearance of a hyperfine structure corresponding to an $I = 3/2$ nuclear spin when the protein is enriched with ^{61}Ni (Albracht, 1994). Since the shape of this spectrum depends on the redox state of the enzyme but varies little from one species to another, it has been used to define different redox forms of the enzyme: Ni-A (or unready), Ni-B (or ready), Ni-C (or active), and Ni-L obtained by illumination at low temperature of a sample giving a Ni-C signal. In samples where the [4Fe-4S] centers present in the enzyme are paramagnetic, the Ni-C and Ni-L signals are replaced at very low temperature by the so-called split Ni-C and split Ni-L signals, respectively, which reflects the existence of intercenter magnetic interactions (Guigliarelli et al., 1995; Bertrand et al., 1996; Dole et al., 1996). All these forms and others which are EPR silent have been also characterized by infrared (IR) spectroscopy in the enzymes from *Chromatium vinosum* (Bagley et al., 1995) and *Desulfovibrio gigas* (Volbeda et al., 1996), and the redox equilibria between these different forms have been described (Cammack et al., 1987; Teixeira et al., 1989; Barondeau et al., 1994; Roberts & Lindahl, 1994). The Ni center is considered as being the active site of the enzyme, and several schemes involving valence changes of the Ni ion have been proposed to describe the catalytic mechanism (Van der Zwaan et al., 1985; Fernandez et al., 1986; Teixeira et al., 1989; Albracht, 1994). However, this EPR-based taxonomy is not fully satisfying for at least two reasons: first, spin quantitation of the different signals gives consistently sub-stoichiometric values (Cammack et al., 1982; Albracht et al., 1982, 1983; Moura et al., 1982; Franco et al., 1993), an important issue that has not yet been solved; second, no significant electron density change is detected at the Ni site of these enzymes by X-ray absorption spectroscopy when as much as three electrons are accepted by the active site between the fully oxidized and fully reduced states (Bagyinka et al., 1993; Eidness et al., 1988). New pieces of information relevant to these two points have been brought by the recent determination of the X-ray crystal structure at 2.85 Å resolution of the hydrogenase of *D. gigas* (Volbeda et al., 1995). This heterodimeric protein contains a Ni center and three iron-sulfur centers, two $[4Fe\text{-}4S]^{2+,1+}$ clusters, and one $[3Fe\text{-}4S]^{1+,0}$ cluster, which are believed to be involved in the electron transfer between the Ni center and the physiological partners of the enzyme. All these metal centers have been well characterized spectroscopically and potentiometrically (Cammack et al., 1987; Teixeira et al., 1987, 1989). The X-ray study of the as prepared form of the enzyme has confirmed the nature of the three iron-sulfur centers and has shown that they are distributed in the small subunit according to a quasi linear arrangement pointing toward the Ni center, which is located in the large subunit (Volbeda et al., 1995). However, an unexpected result was that the Ni center is in

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fact a Ni-X dinuclear center bridged by two cysteine thiolates, in which the Ni ion is terminaly coordinated by two other cysteine thiolates and the metal ion X is coordinated by three nonprotein ligands of unknown nature. The two metals might be bridged by a fourth nonprotein ligand, possibly an oxygenic species. The nature of X could not be unambiguously determined, but the results of anomalous dispersion experiments together with preliminary data obtained by metal content analysis lead to propose Fe as the best candidate (Volbeda et al., 1995). The presence of a metal ion bridged to Ni raises the possibility that Ni-X may be a spin-coupled dinuclear center in at least some redox forms of the enzyme. Spin-coupled systems are characterized by the existence of a ladder of energy levels corresponding to different spin states whose values are determined by the interacting spins. In the redox states of *D. gigas* hydrogenase giving EPR signals, several of these spin states might be populated at the temperature at which the intensity measurements are conducted, which would explain why substoichiometric numbers are obtained by spin quantitations. Furthermore, the metal ion X may play the role of a redox center, which could explain why no valence change is detected at the nickel ion by X-ray absorption studies. Thus, a detailed study of the magnetic structure of the Ni-X dinuclear center appears as a prerequisite for understanding the signification of the EPR signals given by this enzyme and for clarifying the role of X.

In the present work, a combination of dosimetric and spectroscopic techniques is used to elucidate the electronic structure of the Ni-X center in the redox forms of *D. gigas* hydrogenase giving EPR signals. The nature of X was first determined unambiguously by accurate metal content analyses. Next, the redox form of the enzyme studied in the X-ray crystal experiments was determined by comparing the EPR spectra given by a polycrystal powder to those given by a frozen solution. Lastly, the spin-coupled or spin-uncoupled nature of the Ni-X dinuclear center was investigated in different redox states of the enzyme by studying the temperature dependence of the Ni-A, Ni-B, Ni-C, and Ni-L EPR signals over a large temperature range. The results are analyzed to deduce the spin states of the Ni and X centers in these forms and the mechanistic implications of these findings are discussed.

MATERIALS AND METHODS

Purification and Metal Content of Hydrogenase. Eight batches of *D. gigas* (NCIMB 9332) (numbered from 1 to 8) were grown as reported previously (LeGall et al., 1965). Hydrogenase was purified from each batch under aerobic conditions as described (Cammack et al., 1994). The enzyme from each batch exhibited an absorbance ratio $A_{400}/A_{280} = 0.26$ and was homogeneous by visual inspection of polyacrylamide electrophoretic gels.

Hydrogenase activity for H_2 oxidation was determined after activation of the enzyme as described (Cammack et al., 1994), and protein concentrations were routinely measured by the Lowry method (Lowry, 1951) during the purification. The metal content of the protein was determined by inductively coupled plasma emission spectroscopy using a Jobin-Yvon model JY 38 apparatus. Concentrations of the pure enzyme were estimated by quantitative amino acid analysis.

EPR Samples. The Ni-A sample used in the temperature-dependent experiments contained 150 μL of a 150 μM solution of as prepared enzyme. The Ni-C form was directly prepared in an EPR tube by incubation under hydrogen atmosphere at 20 °C overnight. The Ni-L sample was obtained by illuminating during 10 min a Ni-C sample cooled in liquid nitrogen (150 W white light at 10 cm). Full conversion was achieved by rotating slowly the EPR tube during illumination. Only the Ni-L_2 form, as defined in Medina et al. (1996), was studied in the present work. In order to obtain a substantial amount of the Ni-B form, it was necessary to reoxidize a fully reduced sample by 5 μL of a 10 mM thionin solution under strict anaerobic conditions. The polycrystal sample containing a powder of needle shape pseudohexagonal crystals of the as prepared protein was provided by Dr. Juan Fontecilla-Camps, IBS, Grenoble.

EPR Spectroscopy. X-band EPR spectra were recorded on Bruker ESP300 and ESP300 E spectrometers equipped with an Air-Products Helitran gas flow system. Before and after the recording of each spectrum, the temperature was measured with a calibrated thermocouple (chromel vs Au/0.07% Fe) placed in an EPR tube partially filled with water. For spin quantitations, the second integral value of the signal was calibrated against that given by a 1 mM $CuSO_4$ standard recorded at the same temperature. X-band parallel-mode EPR experiments were performed by using a Bruker ER4116DM dual mode cavity.

Numerical Simulations. The Ni-A EPR spectrum of *D. gigas* hydrogenase was simulated by assuming that the spectral broadening was due to *g*-strain effects. The distribution of the **g** tensor around a mean tensor $\mathbf{g_0}$ was described by a three-dimensional tensor **p** whose principal elements are random variables p_i characterized by their standard deviations σ_i. A good simulation was achieved by assuming that $\mathbf{g_0}$ and **p** are collinear and by taking the random variables to be fully positively correlated. The *g* values and line width parameters deduced from this simulation were used to calculate numerically the spectrum given by a magnetically coupled Ni-Fe system in which the total electronic spin $S = 1/2$ interacts magnetically with the nuclear spin $I = 1/2$ of the ^{57}Fe nucleus. The position and the transition probability of the EPR lines were obtained by full diagonalization of the spin Hamiltonian $H = \beta B\mathbf{g_0}S + K_2S\mathbf{A_2}I$, in which $\mathbf{A_2}$ and K_2 are the local hyperfine tensor and the spin projection factor of the iron site, respectively. The different values of K_2 corresponding to the spin-coupling schemes of the Ni and Fe centers leading to a $S = 1/2$ ground state were used in the calculation. The numerical simulations were performed on a Risc 6000 IBM workstation.

RESULTS

Molar Extinction Coefficient and Metal Analyses of Hydrogenase. Accurate measurement of metals present in metalloenzymes depends on the precision of protein concentration estimations. Different estimates lead to different values for the metal content. Estimation of protein concentrations of *D. gigas* hydrogenase samples using the Lowry method gave results that were higher than those obtained by quantitative amino acid analysis. These differences may arise because the colorimetric methods, to some extent, depend on amino acid composition. Differences in protein concentrations as estimated by colorimetric methods and quantitative amino acid analyses have been observed previously in iron-only hydrogenases (Hagen et al., 1986; Hatchikian et al., 1992).

Table 1: Iron and Nickel Content, Spin Quantitation, and Catalytic Activity of Hydrogenase from Eight Batches of *D. gigas*

	metal estimation (atom/mol)		spin quantitation(spin/mol)			
preparation	Ni	Fe	3Fe signal	Ni signal (Ni-B amount)	Ni/3Fe	activity (units/mg)
1	0.91	12.5	0.96	0.56	0.58	424
2	1.05	11.9	0.83	0.47	0.57	565
3	1	12	0.71	0.49 (10%)	0.69	426
4	1.05	12.1	0.87	0.68	0.78	500
5	1.03	12	0.94	0.55 (6%)	0.58	480
6	1.06	12.1	nd[a]	nd	nd	483
7	0.98	11.9	nd	nd	nd	467
8	1	12.4	nd	nd	nd	478
average	1.01	12.1	0.86	0.55	0.64	478

[a] nd, not determined.

In the present study, a molar extinction coefficient of 53 500 mM^{-1} cm^{-1} at 400 nm was obtained for *D. gigas* hydrogenase, based on protein concentration estimated by amino acid analysis (average value of three different preparations). This value is higher than the previously reported values which were based on protein concentrations measured by colorimetric methods (Hatchikian et al., 1978; Barondeau et al., 1994). Iron and nickel contents of eight different *D. gigas* hydrogenase preparations were measured on the basis of protein concentrations estimated from the molar extinction coefficient reported above. The data show average values of 12.1 ± 0.3 iron atoms and 1.01 ± 0.05 nickel atoms per molecule. (Table 1). The average specific activity of these hydrogenase preparations for H_2 oxidation was found to be close to 480 units/mg when measured with the activated enzyme.

The discovery of a second metal ion bridged to Ni by Cys 68 and Cys 533 in the active site of *D. gigas* hydrogenase by crystallographic analysis (Volbeda et al., 1995) prompted us to analyze the metal content of the protein to determine the nature of this additional metal ion. In addition to Fe, potential candidates include Co, Mn, V, and Cr, which are strong anomalous scatterers (Volbeda et al., 1995) and have all been found to occur in proteins. Purified preparations of *D. gigas* hydrogenase were analyzed for these metals as well as for the presence of copper, previously reported to occur in various Ni-containing hydrogenases (Adams et al., 1986; Albracht, 1994). The metal contents found by plasma emission spectroscopy indicated that *D. gigas* hydrogenase does not contain Mn, V, and Cr, but small amounts (0.18 atom/mol) of cobalt and traces (0.01 atom/mol) of copper were detected. The cobalt content of the enzyme did not increase when the cells were grown on excess of cobalt (100 μM), indicating that it can be regarded as adventitious contamination.

Finally, taking into account that 11 iron atoms are involved in the formation of the three iron-sulfur centers (two [4Fe-4S] and one [3Fe-4S]) of *D. gigas* hydrogenase, our results on the metal content of hydrogenase, and notably the estimate of 12 iron atoms/mol, are indicative of the presence of one iron atom in the active site of the enzyme.

EPR Spectroscopy. In order to correlate the EPR spectral properties with the structural data given by the X-ray crystal study, it was first necessary to determine the redox form of the enzyme in the crystal. In that aim, the EPR spectra given at 15 and 77 K by a sample containing a polycrystal powder were compared to those given at the same temperatures by a solution of proteins prepared in the Ni-A form (Figure 1). It appears that the two samples give very similar signals: the signal given by the $[3Fe\text{-}4S]^{1+}$ cluster is clearly visible

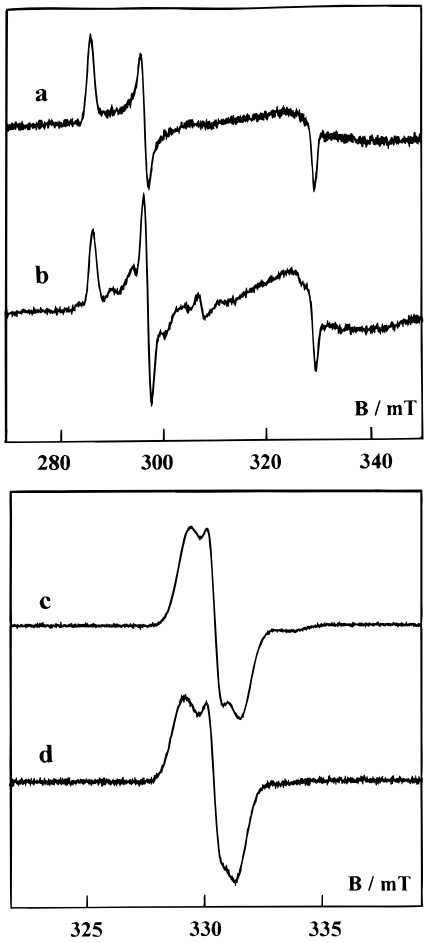

FIGURE 1: EPR spectra of *D. gigas* hydrogenase in solution and polycrystal samples. (a, c) solution sample, (b, d) polycrystal sample. Experimental conditions: temperature, (a, b) 77 K, (c, d) 15 K; microwave frequency, 9.264 GHz; microwave power, (a, b) 10 mW, (c, d) 0.04 mW; modulation amplitude, (a, b) 1 mT, (c, d) 0.1 mT.

at 15 K while the Ni-A signal is more easily observed at 77 K where the $[3Fe\text{-}4S]^{1+}$ signal is almost broadened beyond detection. This near identity of the *g* values and relaxation properties indicates that the structure of the two paramagnetic centers is the same when the enzyme is in the crystal and in solution. However, it is worth noting that the 77 K spectrum of the polycrystal sample displays extra features showing some heterogeneity. The main part of these features can be attributed to a minor Ni-B species whose contribution to the total integrated intensity amounts to 10−15%. Spin quantitation measurements showed that the total Ni signal intensity corresponds to 0.7 spin per molecule for the solution sample and that the ratio of the integrated intensity of the Ni and $[3Fe\text{-}4S]^{1+}$ spectra is equal to 0.7 and 0.5 in solution

and in the crystal, respectively. Thus, the amount of EPR detectable nickel appears to be substoichiometric in the crystal as well as in solution. This deficit, which is consistently observed in Ni-containing hydrogenases, may be due to a departure of the Ni-A signal intensity from Curie's law. To test this possibility, we have carefully studied the temperature dependence of the Ni-A spectral intensity over the largest possible temperature range. For temperatures less than 100 K, the intensity measurements were complicated by the overlap of the Ni-A and $[3Fe\text{-}4S]^{1+}$ signals. Since the line width of the different spectral features did not change in this temperature range, the intensity variations could be followed by monitoring the amplitude variations. For temperatures higher than 100 K, a relaxation broadening was observed and it was necessary to integrate the spectra. The experiments were restricted to temperatures less than 240 K, since at higher temperatures the sample began to thaw, which changed the conditions of signal detection and precluded any quantitative study. The results are reported in Figure 2a, which shows that no significant deviation from Curie's law is detected in the range 10−240 K.

In order to get information about the magnetic structure of the active site in the other redox forms of the enzyme giving EPR signals, the temperature dependence of the Ni-B, Ni-C, and Ni-L signals was also studied. In the case of the Ni-C and Ni-L signals, part of the spectrum was in the split state at low temperature and accurate intensity measurements were precluded by the overlap of the contribution arising from the reduced $[4Fe\text{-}4S]^{1+}$ clusters, which limited somewhat the temperature range of the study. Moreover, the study of the Ni-L signal was limited to temperatures lower than 120 K since at higher temperature the Ni-L → Ni-C conversion took place. The results are reported in Figure 2.

If we consider that the Ni center is a dinuclear Ni-Fe cluster, the $S = 1/2$ ground states observed by EPR could arise from an exchange interaction $-2JS_1S_2$ between the Ni and Fe ions, characterized by the spin S_1 and S_2, respectively. In such a situation, even a moderate J value would lead to a ladder of energy levels characterized by different values of the total spin $S = S_1 + S_2$. We have quoted in Table 2 the different coupling schemes leading to a $S = 1/2$ ground state, for the valencies Ni(I), Ni(II), and Ni(III) of the nickel ion, and Fe(0), Fe(I), Fe(II), and Fe(III) of the iron ion. For all these schemes, the closest excited level is a $S = 3/2$ state separated from the doublet by $|3J|$. Let us examine whether such coupling schemes are consistent with the temperature dependence of the Ni-A signal intensity represented in Figure 2a. The absence of any deviation from Curie's law within experimental errors in the range 10−240 K may signify that, either the exchange coupling is very strong so that only the $S = 1/2$ spin state is significantly populated in the whole temperature range, or the exchange interaction is so weak that the different spin states are occupied according to their spin degeneracy. Whatever the coupling scheme (Table 2), the former hypothesis leads to $|J|$ values larger than 200 cm^{-1}, whereas the latter gives $|J|$ smaller than 1 cm^{-1} (Figure 2a). Such low values of $|J|$ can be excluded since in this case values smaller than 0.33 spin per molecule would be expected for the $S = 1/2$ spin state population, which is clearly in disagreement with the Ni-A EPR signal intensity usually observed (Table 1). The same analysis holds for the temperature dependence of the other Ni EPR signals (Figure 2, panels b−d).

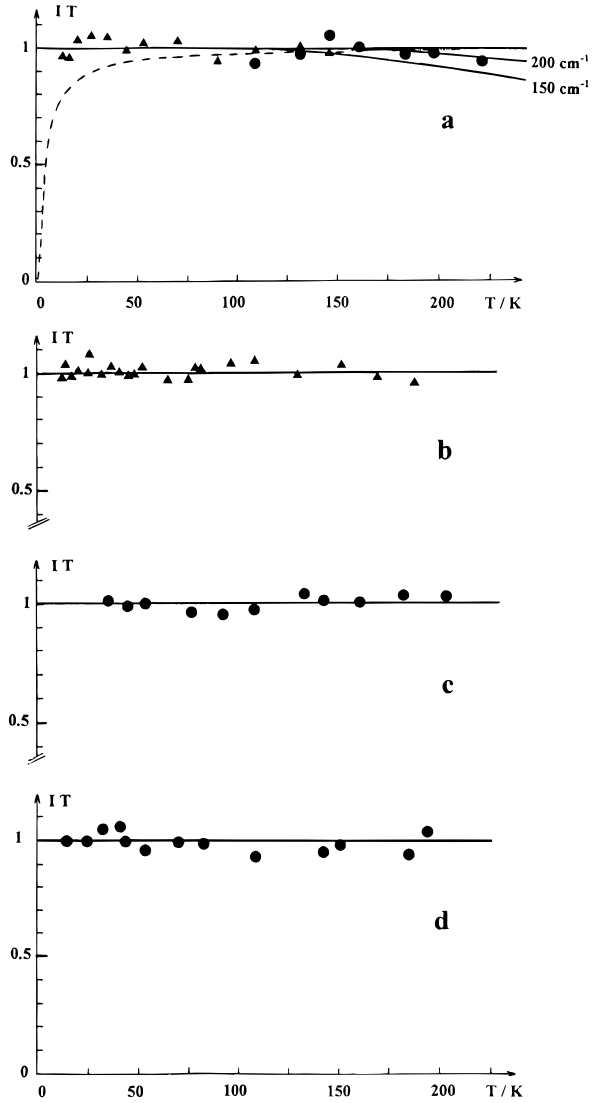

FIGURE 2: Temperature dependence of the intensity of the Ni EPR signals in *D. gigas* hydrogenase. The normalized values of the product IT were obtained by double integration of the EPR signal (full circles) or measurements of the g_Y peak amplitude (triangles). (a) Ni-A signal, (b) Ni-B signal, (c) Ni-C signal, (d) Ni-L signal. Horizontal lines represent Curie's law. In panel a, the solid lines represent the variations of the IT product calculated by taking $S_1 = 3/2$, $S_2 = 2$, $S = 1/2$ and $|J| = 150$ or 200 cm^{-1}. The dashed line represents the variation of the IT product calculated for a ferromagnetic coupling with $J = 1$ cm^{-1}. In this case, for the sake of clarity, the maximum value of the product IT was normalized to unity.

In an attempt to detect possible integer spin states arising from the Ni-Fe pair, hydrogenase samples poised in the Ni-A, Ni-SI, Ni-C, and Ni-R redox states were investigated by parallel-mode EPR. In these experiments, the magnetic field of the microwave is set parallel to the static magnetic field, which enables the $|\Delta m| = 0$ transitions within non-Kramers' doublets to be detected (Hagen, 1992). Except for the well-known $S = 2$ $[3Fe\text{-}4S]^0$ signal (Teixeira et al., 1989), no other signal was observed between 5 and 670 mT on the parallel-mode spectra given by these samples in the temperature range 10−200 K.

DISCUSSION AND CONCLUSION

The detailed metal content analyses presented in this work demonstrate conclusively that the only transition metal atoms present in *D. gigas* hydrogenase are one Ni and 12 Fe atoms.

Table 2: Spin Coupling Schemes in a Ni-Fe Ion Pair Giving a Total Spin $S = 1/2$[a]

		$Ni^{III}(3d^7)$			
	S_2	$S_1 = 3/2$	$S_1 = 1/2$	$Ni^{II}(3d^8)$ $S_1 = 1$	$Ni^{I}(3d^9)$ $S_1 = 1/2$
$Fe^{III}(3d^5)$	1/2			$S = 1/2, 3/2$ $K_2 = -1/3$	
$Fe^{II}(3d^6)$	2	$S = 1/2, ..., 7/2$ $K_2 = 2$			
$Fe^{I}(3d^7)$	3/2			$S = 1/2, ..., 5/2$ $K_2 = 5/3$	
$Fe^{I}(3d^7)$	1/2			$S = 1/2, 3/2$ $K_2 = -1/3$	
$Fe^{0}(3d^6)$	1	$S = 1/2, ..., 5/2$ $K_2 = -2/3$	$S = 1/2, 3/2$ $K_2 = 4/3$		$S = 1/2, 3/2$ $K_2 = 4/3$

[a] K_2 is the spin projection factor for the spin S_2 in the state defined by S_1, S_2, $S = 1/2$.

Since 11 Fe atoms are mobilized into the [3Fe-4S] cluster and the two [4Fe-4S] clusters, the dinuclear cluster revealed by the X-ray crystal study is necessarily a Ni-Fe center. The same conclusion was drawn independently from a recent X-ray crystal study performed at different wavelengths on the same protein (Volbeda et al., 1996).

The discovery of a Ni-Fe dinuclear center at the active site of the *D. gigas* enzyme calls for a re-examination of the magnetic properties that have been attributed previously to a mononuclear Ni center with $S = 1/2$. Our study of the temperature dependence of the Ni EPR signals shows that the hypothesis of $S = 1/2$ spin states arising from an exchange coupled Ni-Fe pair would required $|J|$ values larger than 200 cm^{-1} or smaller than 1 cm^{-1}. Weak values of $|J|$ are not consistent with the Ni signal intensity measurements (Table 1) and can also be excluded on the basis of low-temperature MCD experiments carried out on the Ni-containing hydrogenases from *Methanobacterium thermoautotrophicum* (Johnson et al., 1985) and *D. gigas* (Johnson et al., 1986), which have shown that the magnetization of the Ni-A species is only due to an $S = 1/2$ spin state. Concerning the high $|J|$ values, an examination of the copious literature dealing with magnetostructural relationships (Bencini & Gatteschi, 1990; Kahn, 1993) shows that such strong antiferromagnetic interactions occur in dinuclear clusters only when some general conditions are satisfied. First, the two interacting ions must be identical and the complex must be symmetrical. For example, in the case of [2Fe-2S] clusters, the $|J|$ value which is about 150−200 cm^{-1} when both Fe are ferric, is reduced 2-fold when one Fe becomes ferrous (Palmer et al., 1971; Anderson et al., 1975; Peterson et al., 1980; Gayda et al., 1976, 1981; Bertrand et al., 1980), and for complexes based on the Cu(II)$(NNN)_2$ Cu(II) bridging unit, $|J|$ is larger than 500 cm^{-1} when the complex possess a mirror plane and almost vanishes when the symmetry is broken (Kahn, 1993). Second, the overlap of the magnetic orbitals of the two metal centers, which is very sensitive to the nature and the geometry of the bridging structure, must be optimized so as to maximize the antiferromagnetic interaction. In the case of $[2Fe\text{-}2S]^{2+}$ clusters, the replacement of the bridging sulfide by thiolate ligands decreases the magnitude of $|J|$ from about 150−200 to 50 cm^{-1} (Herskowitz et al., 1975). None of these favorable conditions are met in the case of the Ni-Fe center of *D. gigas* hydrogenase: the two metal ions are different, the structure is highly asymmetric, and the nonplanar Ni(μ-Scys)$_2$ Fe structure does not appear suited for mediating strong antiferromagnetic exchange interactions. Even if the bridging putative oxygenic species was a μ-oxo, its efficiency would be probably greatly reduced due to its nonsymmetrical bonding to the Ni and Fe ions (Volbeda et al., 1996). From this analysis, we conclude that a strongly antiferromagnetically coupled Ni-Fe center is not expected in the *D. gigas* enzyme. This is supported by the low value $J = -12$ cm^{-1} measured in a Ni(II) ($S_1 = 1$)−Fe(III) ($S_2 = 5/2$) complex bridged by a phenolate oxygen (Holman et al., 1990).

An observation relevant to this issue is the absence of any effect due the replacement of ^{56}Fe with ^{57}Fe on the X-band Ni-A spectrum of the *D. gigas* enzyme (Moura et al., 1988). This point must be carefully examined by taking into account the magnitude of the expected hyperfine interaction, the EPR line widths, and all the possible spin-coupling schemes. If we note $K_i = \langle S_i \rangle / \langle S \rangle$, the two spin projection factors corresponding to the spin state defined by S_1, S_2, and $S = 1/2$, the effective hyperfine tensor $\mathbf{a_2}$ is related to $\mathbf{A_2}$, the local hyperfine tensor of the iron site, by $\mathbf{a_2} = K_2\mathbf{A_2}$ (Bencini & Gatteschi, 1990). For the spin-coupling schemes giving a $S = 1/2$ state, the K_2 values range from $-1/3$ to 2 (Table 2). For each of these spin-coupling schemes, we have calculated the X-band EPR spectrum expected for a 100% enrichment in ^{57}Fe by using the spectral line widths determined by simulating the experimental ^{56}Fe Ni-A spectrum (Figure 3a), and by considering $\mathbf{A_2}$ as isotropic. It turns out that even with a large value $A_2 = -30$ MHz (Holman et al., 1990), the broadening due to the hyperfine coupling should be hardly detectable for $|K_2|$ values equal to or smaller than 2/3 (Figure 3, panels b and c). Thus, at least three spin-coupling schemes would not lead to any detectable broadening of the Ni-A signal in ^{57}Fe-enriched hydrogenase (Table 2), so that the result of ^{57}Fe enrichment on the Ni-A spectrum of *D. gigas* hydrogenase alone is not sufficient to demonstrate that the Ni-Fe dinuclear center is not a spin-coupled system. Actually, the very existence of a Ni-Fe spin-coupled center is ruled out by our analysis of the Ni-A, Ni-B, Ni-C, and Ni-L EPR signal intensity. An additional support comes from recent ENDOR experiments carried out on ^{57}Fe-enriched samples of *D. gigas* hydrogenase, which have shown that no hyperfine interactions larger than 1 MHz could be detected on the Ni-A and Ni-C EPR signals (Cammack, R., personal communication). Since EPR experiments carried out on ^{61}Ni-enriched samples have shown that the Ni center is paramagnetic in the Ni-A, Ni-B, and Ni-C forms of *D. gigas* hydrogenase (Moura et al., 1982), the spins of the Ni and Fe centers are necessarily $S_1 = 1/2$ and $S_2 = 0$, respectively, in all these forms (Table 3).

Let us now consider the two EPR-silent states obtained by one electron oxidation and reduction of the Ni-C form, called Ni-SI and Ni-R, respectively (Barondeau et al., 1994).

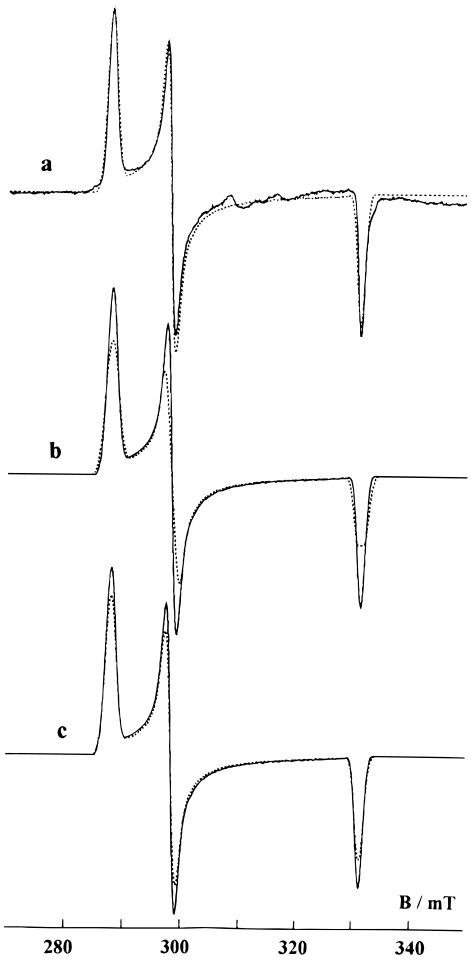

FIGURE 3: Numerical simulations of the ^{57}Fe hyperfine interaction effects on the Ni-A EPR signal. (a) Solid line, experimental EPR spectrum of oxidized *D. gigas* hydrogenase recorded at 100 K; dashed line, EPR spectra calculated with the following parameters: $(g_X, g_Y, g_Z) = (2.012, 2.235, 2.316)$, standard deviations of the g values $(\sigma_X, \sigma_Y, \sigma_Z) = (0.0042, 0.0054, 0.0071)$. (b, c) Solid lines, same calculated spectra as in panel a; dashed lines, calculated spectra expected for a ^{57}Fe-enriched hydrogenase sample by taking $A_2 = -30$ MHz and (b) $|K_2| = 4/3$ or (c) $|K_2| = 2/3$.

Table 3: Proposed Spin States of the Ni and Fe Ions in the Different States of Hydrogenase

hydrogenase state	Ni ion, S_1	Fe ion, S_2
Ni-A	1/2	0
Ni-B	1/2	0
Ni-SI	0	0
Ni-C	1/2	0
Ni-L	1/2	0
Ni-R	0	0

It has been proposed that the diamagnetism of these states may arise from the spin-coupling of the Ni and Fe ions (Fontecilla-Camps, 1996). However, this attractive hypothesis would need strong antiferromagnetic interactions since (i) a careful saturation magnetization study of the *D. baculatus* hydrogenase has demonstrated that the Ni-SI form is diamagnetic up to 100 K (Wang et al., 1992), (ii) variable temperature MCD experiments carried out on the *C. vinosum* enzyme have shown that no magnetization could be attributed to Ni-SI and Ni-R species (Thomson, A. J., & Cheesman, M. R., personal communication), and (iii) no paramagnetic excited level was revealed by the parallel-mode EPR experiments we performed up to 200 K on the Ni-SI and Ni-R states of the *D. gigas* enzyme. As discussed previously,

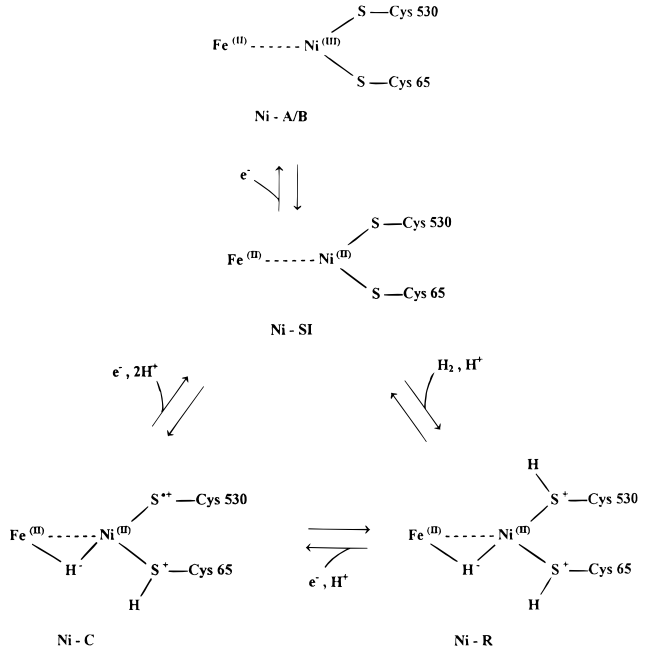

FIGURE 4: Proposed model for the active center structure during the catalytic cycle of NiFe hydrogenases.

large antiferromagnetic interactions are not consistent with the highly distorted structure of the Ni-Fe center and consequently, we favor the hypothesis of the Ni and Fe centers being *separately* diamagnetic in the Ni-SI and Ni-R forms of nickel hydrogenases. This implies that the Fe center remains diamagnetic in all the redox forms of the enzyme (Table 3). Since the active site receives one electron at each stage of the sequence Ni-B, Ni-SI, Ni-C, and Ni-R (Roberts & Lindahl, 1994), it appears very likely that the valency of the Fe ion does not change, a low-spin Fe(II) being the best candidate. This assignment is in agreement with the presence of three diatomic nonprotein ligands (CO or CN^-) in the coordination sphere of the Fe ion, as shown by X-ray crystal structure refinement and infrared spectroscopy (Bagley et al., 1995; Volbeda et al., 1996), a coordination that is expected to put a Fe ion in a low-spin state. Concerning the valency of the Ni ion, the simplest assignment consistent with the spin values of Table 3 is Ni(III) for Ni-A and Ni-B, Ni(II) for Ni-SI, Ni(I) for Ni-C and Ni-L, and Ni(0) or Ni(II)$-H^-$ for Ni-R. This assignment has already been proposed by several authors in the context of a mononuclear Ni center and of a Ni-based redox chemistry (Van der Zwaan et al., 1985; Fernandez et al., 1986; Teixeira et al, 1989; Roberts & Lindahl, 1994; Albracht, 1994). However, it is not consistent with the results of EXAFS experiments carried out on *D. gigas* and other nickel-containing hydrogenases which have shown that the nickel K-edge energy, and hence the electron density at the Ni atom, varies slightly between the Ni-A and the Ni-SI forms but remains essentially constant during the more reducing stages (Eidness et al., 1988; Bagyinka et al., 1993; Gu et al., 1996). These striking results have lead to the proposal of a ligand-based redox chemistry involving sulfur radical cations (Choudhury et al., 1994).

We have then built a mechanistic scheme which is consistent with the data of Table 3 and with the results of EXAFS experiments. In this model (Figure 4), the Ni-SI and Ni-R states contains Ni and Fe ions that are both diamagnetic, the most likely valence states being Ni(II) and Fe(II), respectively. Studies on nickel compounds (Marganian et al., 1995) have shown that the presence of thiolate

or selenolate ligands in the coordination sphere of the Ni ion is essential to the formation of hydride adducts, these ligands acting as a base for helping the heterolytic cleavage of H_2. According to this mechanism, the reaction of H_2 with the Ni-SI species should lead to the protonation of the terminal cysteinate (or selenocysteinate) ligands and to the binding of a hydride on the Ni ion, thus giving a Ni-R species (Figure 4) that is reminiscent of the structure proposed by Roberts and Lindahl (1994). The protonation of the thiolate ligands from Cys 68 and Cys 533 appears unlikely, owing to their bridging position between the Ni and Fe ions.

The redox transition between the Ni-R and Ni-C states was shown to involve one electron and one proton (Cammack et al., 1987; Teixeira et al., 1989; Barondeau et al., 1994). This redox process is not centered on the Fe ion since it is diamagnetic in both states. Moreover, EXAFS studies have shown that the electron density of the Ni atom does not vary between the Ni-R and Ni-C states, which suggests that the redox process occurs on a Ni ligand. The hyperfine interactions with ^{77}Se observed in the Ni-C signal of ^{77}Se-enriched NiFeSe hydrogenase (He et al., 1989; Sorgenfrei et al., 1993) confirm that the magnetic orbital of the Ni-C species is largely chalcogenide in character (Choudury et al., 1994) and show that the cation radical is formed on the Cys 530 terminal ligand (Figure 4). Actually, the spin density is partly delocalized on the Ni ion, as suggested by the absence of strong hyperfine coupling with the nonexchangeable β-CH_2 protons of this Cys ligand (Fan et al., 1991) and by the hyperfine interaction with ^{61}Ni in the Ni-C signal (Moura et al., 1982). However, by comparison with the Ni-A signal, the isotropic component of the hyperfine coupling with ^{61}Ni is approximately 2-fold reduced in the Ni-C signal (Moura et al., 1982; Albracht et al., 1982; Kojima et al., 1983), which indicates that the mesomeric form Ni(III)−S− is less probable than the Ni(II)−$S^{\bullet+}$− form in the Ni-C species. This also explains the weakness of the hyperfine interaction with the exchangeable hydrogen species presumably bound to the Ni ion in the Ni-C state, as observed by EPR (Van der Zwann et al., 1985; Franco et al., 1993; Medina et al., 1996) and ENDOR (Fan et al., 1991; Whitehead et al., 1993) spectroscopies. Such a spin density delocalization is reminiscent of the situation encountered in nitrosyl ferrous heme complexes where the unpaired electron is delocalized on the metal and NO orbitals (Kon, 1968; Mun et al., 1979; Morse et al., 1980).

Recently, we have analyzed the magnetic interactions which develop between the Ni-C species and the proximal $[4Fe\text{-}4S]^{1+}$ cluster in active *D. gigas* hydrogenase (Guigliarelli et al., 1995; Bertrand et al., 1996). The structural parameters deduced from the numerical simulations of the split Ni-C signal were in good agreement with the crystallographic data and with the assumption that the Ni-C species can be considered as a point dipole, the spin density of which is entirely located on the Ni atom (Bertrand et al., 1996). If we consider now that this spin density is actually located on the sulfur atom of Cys 530, the intercenter distance parameter used in the spectral simulations would be increased by 0.03 nm, which is smaller than the precision of the method. In contrast, if the radical cation were located on the sulfur atom of Cys 65, the intercenter distance parameter would be decreased by 0.23 nm since the Ni-S(Cys65) bond points directly toward the proximal [4Fe-4S] cluster. Such a short distance is clearly ruled out by our analysis of the spin−spin interactions (Bertrand et al., 1996).

In the last step of the catalytic cycle, the one electron oxidation of the Ni-C species is accompanied by the release of two protons (Cammack et al., 1987; Teixeira et al., 1989; Roberts & Lindahl, 1994) and restores the Ni-SI species (Figure 4). A subsequent oxidation of the Ni-SI state leads to the Ni-A/B species where the Fe ion is still diamagnetic (Table 3) and then corresponds to a Ni(II) → Ni(III) valence change. This is in agreement with the EXAFS results since the Ni-A → Ni-SI transition is the only one which is accompanied by a Ni K-edge shift (Eidness et al., 1988; Gu et al., 1996). Thus, our model suppresses the apparent discrepancy between the EXAFS results and the known redox properties of NiFe hydrogenases, while taking into account the dinuclear structure of the active site.

In contrast with a recently proposed catalytic scheme (Fontecilla-Camps, 1996), the present model excludes the involvement of the Fe ion in the redox processes. Valence changes of the Fe ion were postulated to account for the shifts of the three IR bands attributed to its diatomic ligands in the different states of the hydrogenases. In fact, the magnitude of the IR band shifts between the Ni-SI, Ni-R, and Ni-C states are about 15 cm^{-1} (Bagley et al., 1995), which is much smaller than the 120 cm^{-1} shift expected for the CO stretching frequency upon a one electron change of the electronic density on a metal atom (Purcell & Kotz, 1980). For the sake of simplicity, the hydride species was represented in Figure 4 as a bridging ligand in both the Ni-R and Ni-C states, but its binding to the Ni-Fe pair could be different in these states. The IR band shifts could actually arise from changes in the coordination sphere of the Fe ion, related to the binding or removal of the hydride species.

The light sensitivity of the Ni-C species at cryogenic temperatures is a common feature of all NiFe hydrogenases. The strong kinetic isotope effect observed in D_2O for the Ni-C → Ni-L photoprocess in *C. vinosum* hydrogenase had led to the initial proposal that a hydrogen species is coordinated to the Ni ion and dissociates upon illumination (Van der Zwann et al., 1985). The strong variations of this kinetic isotope effect among enzymes from different species together with the presence of at least three different Ni-L species (Medina et al., 1996) indicate that the photoreaction of the Ni-C species is a complex process likely involving several rearrangements of the Ni environment. By studying the magnetic interactions between the Ni site and the proximal $[4Fe\text{-}4S]^{1+}$ center, we have recently shown that the photoprocess is accompanied by the cancellation of the exchange interaction between the two centers and that the magnetic direction of the Ni-C and Ni-L species are not greatly different (Dole et al., 1996). The structure proposed for the Ni-C species (Figure 4) is consistent with all these observations. First, the breaking of the exchange pathway between the Ni and [4Fe-4S] centers could arise from the deprotonation of the thiolate of Cys 65. Second, the disappearance of the weak proton hyperfine coupling in the Ni-L species (Whitehead et al., 1993; Medina et al., 1996) would be related to the breaking of the Ni−H^- bond. Lastly, these modifications of the Ni ion environment would be indirectly responsible for structural variations of the sulfur radical cation and of changes of its **g** tensor. Thus, changes of the $3p_Z$ character of the sulfur orbital containing the unpaired electron could explain the small differences observed between the Ni-C and Ni-L magnetic axes (Dole et al., 1996). This attractive interpretation suggests that it could

be possible to trigger separately these different photoevents and experiments are in progress in that aim.

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