Electronic Structure of the H Cluster in [Fe]-Hydrogenases

Codrina V. Popescu and Eckard Münck*

Contribution from the Department of Chemistry, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213

Received April 19, 1999

Abstract: [Fe]-Hydrogenase II isolated from *C. pasteurianum* contains 14 Fe which are distributed among the so-called H cluster (the catalytic center) and two [4Fe-4S] clusters. Insights gained from Mössbauer studies of M-[4Fe-4S]$^{2+}$ cluster assemblies (M is a paramagnetic center) in sulfite reductase and carbon monoxide dehydrogenase have suggested that the H cluster contains a [4Fe-4S]$^{2+}$ cluster covalently linked to a smaller Fe-containing cluster. Recent X-ray studies of two [Fe]-hydrogenases, combined with the results of FTIR studies, have revealed that the H cluster contains a novel binuclear Fe cluster, [2Fe]$_H$, that is linked by a cysteinyl sulfur to a [4Fe-4S] cluster; [2Fe]$_H$ was found to have CO, CN$^-$, and thiolate ligands. The analysis of the Mössbauer spectra of Hydrogenase II in the oxidized, reduced, and the CO-inhibited states has enabled us to assign the ^{57}Fe magnetic hyperfine tensors observed by ENDOR and Mössbauer spectroscopy to the two subclusters. Thus, A$_I$ = +25.3 MHz and A$_{II}$ = −28.4 MHz of H$_{ox}$-CO can be assigned to the two delocalized pairs of [4Fe − 4S]$_H^{2+}$. In our coupling model these A-values result for j ≈ 100 cm^{-1} where j describes the exchange interaction between [4Fe − 4S]$_H^{2+}$ and [2Fe]$_H$. The 18 MHz A-value of H$_{ox}$ obtained by ENDOR must result from one Fe site of [2Fe]$_H$, while the 7.5 MHz ENDOR A-value seems to be associated with [4Fe-4S]$_H$. Analysis of the Mössbauer spectra of H$_{red}$ shows that the 4Fe cluster is in the 2+ state and that [2Fe]$_H$ contains presumably two low-spin Fe^{II} sites with $\Delta E_Q \approx 0.85$ mm/s and $\delta \approx 0.08$ mm/s. The observation that the [4Fe-4S] cluster is in the 2+ state in H$_{ox}$, H$_{ox}$-CO, and H$_{red}$ suggests that the [2Fe]$_H$ subcluster is in the mixed-valent Fe^{III}Fe^{II} state in H$_{ox}$ and H$_{ox}$-CO. Given the environment of strong-field ligands in [2Fe]$_H$, the Fe^{III} site must have low-spin configuration. While such an assignment is compatible with the EPR g-values, low-spin Fe^{III} sites with g ≈ 2 commonly exhibit very anisotropic ^{57}Fe A-tensors (due to spin-dipolar interactions) and thus the isotropic A-values of H$_{ox}$ and H$_{ox}$-CO observed by ENDOR are difficult to explain. This point is discussed in some detail.

Introduction

Hydrogenases catalyze the reversible activation of molecular hydrogen according to the reaction H$_2$ ⇌ 2H$^+$ + 2e$^-$. Two major classes of metalloproteins have been found to mediate this reaction, namely [Ni-Fe]-hydrogenases and [Fe]-hydrogenases. Besides the catalytic center (a bimetallic Ni−Fe center in the [Ni-Fe]-enzymes and the so-called H cluster in the [Fe]-hydrogenases), both classes of enzymes contain additional iron−sulfur clusters which serve to transfer electrons to and from the catalytic center. For instance, the bidirectional [Fe]-hydrogenase I from *Clostridium pasteurianum* (*Cp*) contains three [4Fe-4S] clusters and one [2Fe-2S] cluster, while the uptake [Fe]-hydrogenase II from the same organism contains two [4Fe-4S] clusters in addition to the H cluster. The H cluster has been studied with a variety of spectroscopic techniques that include EPR, ENDOR, MCD, Resonance Raman, and Mössbauer spectroscopy.[1] These studies indicate that the H clusters of proteins from different organisms are essentially the same. A comprehensive review summarizing the biochemical and spectroscopic properties of [Fe]-hydrogenases has been published by Adams.[1] That review includes also an extensive discussion of *Cp* hydrogenase II, the subject of the present paper.

In 1987 Rusnak and co-workers reported a Mössbauer study of *Cp* hydrogenase II.[2] Their analysis, based on the assumption

that the enzyme contains 7−8 Fe atoms, led to the conclusion that the H cluster comprises three Fe atoms and that a second cluster was of the [4Fe-4S] type but existed in two forms, called F and F′. In 1989 Adams, Eccleston, and Howard[3] determined the molecular mass of hydrogenase II by quantitative amino acid analysis and found that the protein contains 13.8 ± 0.2 Fe atoms and 11.2 ± 0.4 sulfides. These conclusions, applied to the results of the Mössbauer study, implied that the H cluster has six Fe sites and that F and F′ are two distinct [4Fe-4S] clusters.

The H cluster of hydrogenase II has been studied in three states. In the oxidized state, H$_{ox}$, the cluster exhibits an $S = 1/2$ EPR signal with g-values at g = 2.078, 2.027, and 1.999.[4−6] This signal, without significant broadening, can be observed up to 100 K and was was found to represent 1.00 ± 0.06 spins/molecule.[3] ENDOR studies in Hoffman's laboratory[7,8] revealed two *isotropic* ^{57}Fe resonances corresponding to magnetic hyperfine coupling constants A = 18 (ranging from 18 to 19

(1) Adams, M. W. W. *Biochim. Biophys. Acta* **1990**, *1020*, 115–145.

(2) Rusnak, F. M.; Adams, M. W. W.; Mortenson, L. E.; Münck, E. *J. Biol. Chem.* **1987**, *262*, 38–41.

(3) Adams, M. W. W.; Eccleston, E.; Howard, J. B. *Proc. Natl. Acad. Sci.* **1989**, *86*, 4932–4936.

(4) Adams, M. W. W.; Mortenson, L. E. *J. Biol. Chem.* **1984**, *259*, 7045–7055.

(5) Adams, M. W. W. *J. Biol. Chem.* **1987**, *262*, 15054–15061.

(6) Zambrano, I. C.; Kowal, A. T.; Mortenson, L. E.; Adams, M. W. W.; Johnson, M. K. *J. Biol. Chem.* **1989**, *264*, 20974–20984.

(7) Telser, J.; Benecky, M. J.; Adams, M. W. W.; Mortenson, L. E.; Hoffman, B. M. *J. Biol. Chem.* 1987, *262*, 6589–6594.

* Author to whom correspondence should be addressed. Phone: 412-268-5058. FAX: 412-268-1061. E-mail: em40@andrew.cmu.edu.

MHz as the observing field is varied over the EPR spectrum) and 7 MHz (ranging from 6 to 8 MHz). When oxidized *Cp* hydrogenase II is incubated with CO, the state H_{ox}-CO results.[5] This state exhibits an $S = 1/2$ EPR signal, quantitated to 0.95 ± 0.04 spins/molecule,[3] with *g*-values at 2.032, 2.017, and 1.988.[1] ^{57}Fe ENDOR showed a nearly isotropic resonance corresponding to $A = 9-10$ MHz.[7] Mössbauer studies[2] of H_{ox}-CO revealed two additional ^{57}Fe *A*-values at $A_I = +26.8$ MHz and $A_{II} = -30$ MHz. The F and F′ clusters of oxidized hydrogenase II, in the presence and absence of CO, are in the diamagnetic $[4Fe-4S]^{2+}$ state.

In dithionite-reduced hydrogenase II the F and F′ clusters are both in the paramagnetic $[4Fe-4S]^{+}$ states.[2,4,6] Mössbauer studies have revealed that the H cluster of the reduced hydrogenase, H_{red}, has a diamagnetic electronic ground state.[2] In this state of the H cluster, a spectral component accounting for ca. 28% of the total Fe in the sample, i.e., 4 Fe with the revised molecular mass and Fe content, contributes a doublet with quadrupole splitting $\Delta E_Q \approx 1.4$ mm/s and isomer shift $\delta = 0.43$ mm/s. These parameters are consistent with the presence of a $[4Fe-4S]^{2+}$ cluster. A minor component ($\approx$14% of Fe) of H_{red} was assigned to a doublet with $\Delta E_Q = 0.49$ mm/s and $\delta = 0.26$ mm/s; we propose a new assignment for this component below.

During the past decade we have studied in our laboratory the electronic structures of $[4Fe-4S]^{2+}$ cubanes as well as those of M-$[4Fe-4S]^{2+}$ assemblies where M is a paramagnetic metal; M is an Fe^{3+}-siroheme in oxidized *E. coli* sulfite reductase[9] and M = Ni^{+} for the A_{red}-CO state of carbon monoxide dehydrogenase (CODH).[10] The covalent link (a bridging cysteinyl sulfur for sulfite reductase[11]) in the M-$[4Fe-4S]^{2+}$ chromophores establishes an exchange pathway between M and one Fe site of the cubanoid. This exchange interaction, described by the coupling constant *j*, mixes an excited cluster state with $S_{cube} = 1$ into the $S_{cube} = 0$ ground state. By this mechanism the four Fe sites of the cube acquire paramagnetism which leads to the observation of magnetic hyperfine structure in the Mössbauer spectra.[9,12] The ground state spin of the coupled assembly is the same as that of M, namely $S = 5/2$ for oxidized sulfite reductase and $S = 1/2$ for A_{red}-CO.

The spin structure of $[4Fe-4S]^{2+}$ clusters reflects an interplay of Heisenberg−Dirac−van Vleck exchange, double exchange, and vibronic interactions. Theoretical and experimental studies have provided compelling evidence that strong double exchange gives rise to two valence-delocalized $Fe^{2.5+}$-$Fe^{2.5+}$ pairs, each containing two equivalent Fe sites.[12−15] The presence of such pairs is reflected in the Mössbauer and ENDOR spectra of the

M-$[4Fe-4S]^{2+}$ assemblies. Thus, the data indicate two pairs of Fe sites, one with positive components of the magnetic hyperfine tensor and one with negative components. Theoretical and experimental work on the coupled assemblies of sulfite reductase[9,12] and carbon monoxide dehydrogenase[10] have shown that the **A**-tensors of the two pairs are proportional to j/Δ where Δ is the energy gap between the $S_{cube} = 1$ and 0 states of the $[4Fe-4S]^{2+}$ cluster. (A graph showing the magnetic hyperfine interactions of the two pairs as a function of j/Δ is shown below in Figure 4.) During the course of our work on CODH we realized that the Mössbauer data of H_{ox} and H_{ox}-CO, together with the revised Fe content and our recent understanding of coupled metal−cluster assemblies, provided strong evidence "that the H cluster is an assembly consisting of a moiety comprised of one or two Fe atoms exchange-coupled to a 4Fe-4S cluster" (Note Added in Proof of ref 10).

Substantial progress in the research of the hydrogenases was achieved when FTIR studies revealed that the active site clusters of [Ni−Fe]- and [Fe]-hydrogenases contained CO and CN^- ligands.[16] These exciting new developments were accompanied by the first X-ray structure of the [Ni-Fe]-hydrogenase from *Desulfovibrio gigas*.[17] This structure revealed a bimetallic Ni−Fe site with three diatomic ligands bound to the Fe; FTIR studies suggested that these diatomic ligands were CO and CN^-. Very recently, an FTIR study[18] and the X-ray structures of two [Fe]-hydrogenases were reported. The structures of the *Cp* hydrogenase I by Peters and co-workers[19] and that of [Fe]-hydrogenase from *Desulfovibrio desulfuricans* by Nicolet et al.[20] show that the H cluster in both enzymes consists of a 2Fe-subcluster linked by a cysteine thiolate to a $[4Fe-4S]$ cluster. (In this paper we will denote the 2Fe-subcluster as $[2Fe]_H$ and the proximal cubane as $[4Fe-4S]_H$.) Moreover, the structures in conjunction with the FTIR results show that each iron of $[2Fe]_H$ is coordinated by one CO and one CN^- ligands and that the two Fe sites are bridged by two sulfur ligands, furnished by 1,3-propanedithiol according to ref 20. The *D. desulfuricans* enzyme contains the H cluster and two additional $[4Fe-4S]$ clusters and thus has the same cluster composition as *Cp* hydrogenase II. A schematic structure of the H cluster as deduced from the crystallographic studies is shown in Figure 1.

In the following we will assemble the spectroscopic evidence that suggested to us that the H cluster contains a $[4Fe-4S]$ subcluster. Moreover, after reexamination[21] of the data of Rusnak et al. in the light of the evidence that the Fe sites of the $[2Fe]_H$ cluster contain the strong field ligands carbon monoxide and cyanide, we have identified these sites in the spectra of H_{red}. Furthermore, we will show that the 18 MHz ENDOR resonance of H_{ox} cannot result from the $[4Fe-4S]_H$ cluster and that the electronic structure of $[2Fe]_H$ in the oxidized state cannot be delocalized. Nicolet et al.[20] have argued that the 4Fe-4S

(8) (a) Wang, G.; Benecky, M. J.; Huynh, B. H.; Cline, J. F.; Adams, M. W. W.; Mortenson, L. E.; Hoffman, B. M.; Münck, E. *J. Biol. Chem.* **1984**, *259*, 14328-14331. (b) Telser, J.; Benecky, M. J.; Adams, M. W. W.; Mortenson, L. E.**;** Hoffman, B. M. *J. Biol. Chem.* **1986**, *261*, 13536−13541.

(9) (a) Christner, J. A.; Münck, E.; Janick, P. A.; Siegel, W. M. *J. Biol. Chem.* **1981**, *256*, 2098−2101. (b) Christner, J. A.; Münck, E.; Janick, P. A.; Siegel, L. M. *J. Biol. Chem.* **1983**, *255*, 11147−11156.

(10) Xia, J.; Hu, Z.; Popescu, C. V.; Lindahl, P. A.; Münck, E. *J. Am. Chem. Soc.* **1997**, *119*, 8301−8312.

(11) Crane, B. R.; Siegel, L. M.; Getzoff, E. D. *Science* **1995**, *270*, 59−67.

(12) Bominaar, E. L.; Hu, Z.; Münck, E.; Girerd, J.-J.; Borshch, S. *J. Am. Chem. Soc.* **1995**, *117*, 6976−6989.

(13) (a) Noodleman, L.; Peng, C. Y.; Case, D. A.; Mouesca, J.-M. *Coord. Chem. Rev.* **1995**, *144*, 199−244. (b) Mouesca, J.-M.; Chen, J. L.; Noodleman, L.; Bashford, D.; Case, D. A. *J. Am. Chem. Soc.* **1994**, *116*, 11898−11914.

(14) Bertini, I.; Ciurli, S.; Luchinat, C. *Struct. Bonding* **1995**, *83*, 1−53 and references therein.

(15) Beinert, H.; Holm, R., H.; Münck, E**.** *Science* **1998**, *277*, 653−659.

(16) (a) Bagley, K. A.; Duin, E. C.; Roseboom, W.; Albracht, S. P. J.; Woodruff, W. H. *Biochemistry* **1995**, *34*, 5527−5535. (b) Happe, R. P.; Roseboom, W.; Pierik, A. J.; Albracht, S. P. J.; Bagley, K. A. *Nature* **1997**, *385*, 126. (c) De Lacey, A. L *J. Am. Chem. Soc.* **1997**, *119*, 7181−7189.

(17) (a) Volbeda, A.; Charon, M.-E.; Piras, E.; Hatchikian, E. C.; Frey, M.; Fontecilla-Camps, J.-C. *Nature* **1995**, *373*, 580−587. (b) Volbeda A.; Garcin, E.; Piras, C.; De Lacey, A. L.; Fernandez, V. M.; Hatchikian, E. C.; Frey, M.; Fontecilla-Camps, J.-C. *J. Am. Chem. Soc.* **1996**, *118*, 12989−12996.

(18) Pierik, A. J.; Hulstein, M.; Hagen, W. R.; Albracht, S. P. J. *Eur. J. Biochem.* **1998**, *258*, 572−578.

(19) Peters, J. W.; Lanzilotta, W. N.; Lemon, B. J.; Seefeldt, L. C. *Science* **1998**, *282*, 1853−1858.

(20) Nicolet, Y.; Piras, C.; Legrand, P.; Hatchikian. C. E.; Fontecilla-Camps, J.-C. *Structure* **1999**, *7*, 13−23.

(21) To our knowledge, *Cp* Hydrogenase II preparations are currently not available in any laboratory.
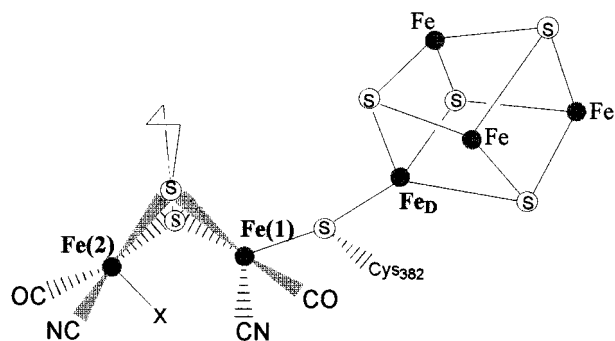
Figure 1. Schematic representation of the H cluster according to the crystal structure *D. desulfuricans* Fe-hydrogenase[20] (the structure of ref 19 shows Fe(2) with a coordinated H_2O and X is replaced by a bridging CO).
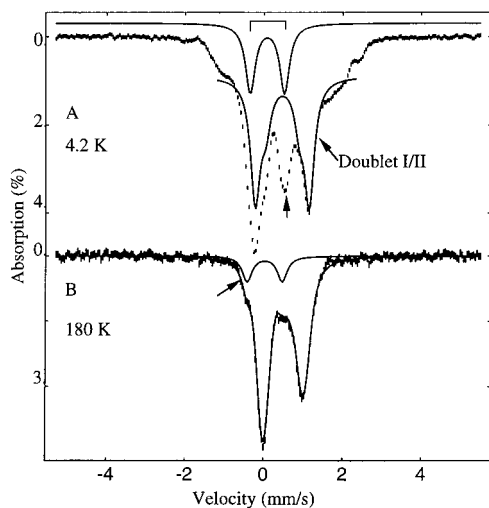
Figure 2. Mössbauer spectra of the reduced *Cp* hydrogenase II recorded in 0.05 T applied field, at 4.2 K (A) and 180 K (B). The spectrum shown in part A (hatch marks) was obtained by subtracting the contribution of F_{red} from the raw data. The 180 K spectrum B contains the contribution of all the clusters (the solid line through the data is a least-squares fit). The major doublet feature in A, designated doublet I/II, belongs to $[4Fe-4S]_H^{2+}$. The minor doublet (12%, solid line marked by the bracket) is assigned to the two Fe sites of $[2Fe]_H$.

cluster of H_{ox}, H_{ox}-CO, and H_{red} is in the 1+ oxidation state. We will show here that the cluster, in all three states, is in the 2+ state. Finally, we will elaborate on the very unusual magnetic hyperfine interactions observed in H_{ox} and H_{ox}-CO.

Results

Mössbauer Results for H_{red}. Figure 2 shows two Mössbauer spectra of the dithionite-reduced *Cp* hydrogenase II. In this state both the F and F′ clusters are in the reduced 1+ state and both clusters contribute at 4.2 K Mössbauer spectra exhibiting paramagnetic hyperfine structure. As pointed out previously, the EPR and Mössbauer spectra of the reduced F cluster behave as those of typical $[4Fe-4S]^{1+}$ clusters.[2] Therefore, we have simulated its Mössbauer spectrum (see also ref 2) and subtracted it from the experimental spectrum recorded at 4.2 K. Thus, the spectrum in Figure 2A contains only the contributions of F'_{red} and H_{red}.

The 4.2 K spectrum in Figure 2A contains two quadrupole doublets which, as previously shown,[2] belong to diamagnetic species. Because F′ is paramagnetic ($S = 1/2$ ground state), the diamagnetic features in this spectrum must represent H_{red}. The major doublet feature (solid line, doublet I/II) consists of two

unresolved doublets, namely one with $\Delta E_Q \approx 1.35$ mm/s and $\delta \approx 0.47$ mm/s (ca. 21% of the total iron, representing 3 Fe) and a second one with $\Delta E_Q \approx 0.85$ mm/s and $\delta \approx 0.47$ mm/s ($\approx$7% of the total iron, i.e., 1 Fe). These parameters are characteristic for $[4Fe-4S]^{2+}$ clusters (a site ratio of 3:1 has been observed for a variety of such clusters).

Rusnak et al.[2] identified a second doublet with $\Delta E_Q = 0.49$ mm/s and $\delta = 0.26$ mm/s, having its high-energy line at 0.52 mm/s (arrow in Figure 2A). The low-energy line of this doublet was thought to give rise to the shoulder at 0.01 mm/s. In the following we will argue that this doublet, with a reassigned low-energy line, results from $[2Fe]_H$.

In our recent studies of the [Ni-Fe]-hydrogenase from *C. vinosum* we have been able to identify by Mössbauer spectroscopy a spectral component with $\delta = 0.06$ mm/s and $\Delta E_Q = 1.44$ mm/s.[22] We have shown that this component is associated with the Fe site of the bimetallic [Ni-Fe] cluster and that it contributes a shoulder at the low-energy side of the high-temperature spectra. The small value of the isomer shift reflects a low-spin site, most probably Fe^{II}. FTIR spectroscopy[16] of the *C. vinosum* [Ni-Fe]-hydrogenase has demonstrated that the Fe site of the Ni-Fe cluster is coordinated by one CO and two CN^- ligands, and X-ray crystallography, in combination with the FTIR results, of the analogous *D. gigas* [Ni-Fe]-hydrogenase has revealed a $[Fe(CO)(CN)_2(S-Cys)_2]$ site.[17] Undoubtedly, this type of coordination environment must be responsible for the small isomeric shift (and for the low-spin configuration) of the spectral component identified in the Mössbauer spectra of the *C. vinosum* enzyme. The recent FTIR results reported for the [Fe]-hydrogenase from *D. vulgaris*[18] and X-ray structures of the two [Fe]-hydrogenases[19,20] prompted us to search for a low-spin ferrous site in the Mössbauer spectra of *Cp* hydrogenase II. The 180 K spectrum of Figure 2B exhibits a shoulder (arrow) at −0.47 mm/s. This shoulder was not explicitly mentioned by Rusnak et al.,[2] who considered it as a possible component due to F or F′. In view of the recent crystallographic data, the FTIR, and the Mössbauer results, it is reasonable to pair this shoulder with the line at +0.52 mm/s in the spectrum of Figure 2A (arrow), and assign the resulting doublet to $[2Fe]_H$. To pair the shoulder in the 180 K spectrum with the 0.52 mm/s line at 4.2 K, we corrected for a ca. 0.06 mm/s second-order Doppler shift between the spectra recorded at 4.2 and 180 K. The sites of $[2Fe]_H$ in H_{red} are most probably low-spin Fe^{II},[23] and thus we may assume that the quadrupole splitting is independent of temperature. Taking these considerations into account and using spectral simulations to fit the doublet best into the spectra of Figure 2, we obtained $\Delta E_Q \approx 0.87$ mm/s and $\delta \approx 0.08$ mm/s at 4.2 K. We have estimated the amount of Fe represented by this species by fitting the intensity of the shoulder in the 180 K spectrum. The fits (Figure 2B) suggest that this doublet represents $\approx$12% of total Fe, i.e., approximately 1.7 Fe,

(22) Natarajan, R.; Münck, E.; Albracht, S. P. J. Manuscript in preparation.

(23) The electronic properties of the ligand environment provided by $[2Fe]_H$ are largely unexplored. While there is little doubt that the iron sites of $[2Fe]_H$ are low spin, isomer shift data for this type of environment are lacking, and therefore the oxidation state of the $[2Fe]_H$ cluster in H_{ox} and H_{red} is not established by Mössbauer spectroscopy. It seems reasonable to us to assume that the diamagnetic state of $[2Fe]_H$ observed for H_{red} reflects a low-spin $Fe^{II}Fe^{II}$ pair. However, recent studies by M. Y. Darensbourg and co-workers show that Fe^IFe^I forms are possible for complexes such as $[(\mu-SCH_2CH_2CH_2S)Fe_2(CO)_6]$ and its derivatives, which bear distinct similarities to the diiron organometallic unit of the H cluster.[24] In the following we assume that $[2Fe]_H$ attains the $Fe^{II}Fe^{III}$ and $Fe^{II}Fe^{III}$ oxidation states. However, this choice is not demanded by available data. Much of our discussion regarding the intriguing electronic properties of $[2Fe]_H$ can be applied, with perhaps nonessential modifications, to an Fe^IFe^I/Fe^IFe^{II} redox couple.
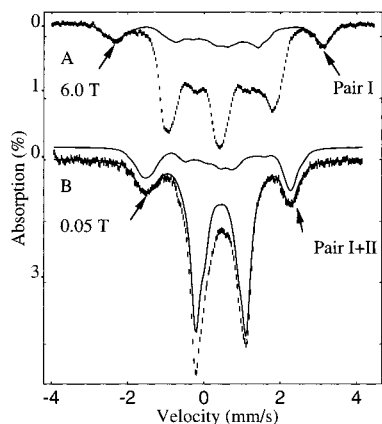
Figure 3. Mössbauer spectra (4.2 K) of the oxidized hydrogenase reacted with CO, recorded in 6.0 T (A) and 0.05 T (B); these are the spectra of Rusnak et al. The solid line in part A (drawn to represent 14% of the total Fe in sample, i.e., 2 Fe) is a simulation of both delocalized pairs of the $[4Fe-4S]_H^{2+}$ cluster (the outer features, indicated by the arrows, are due to Pair I, which has $A > 0$). The solid line above the data in part B represents the contribution of both pairs of the $[4Fe-4S]_H^{2+}$ cluster (28% of total Fe in the sample); the solid line drawn through the data in B is a simulation that includes also the contribution of F_{ox} and F'_{ox}, but not that of $[2Fe]_H$. The theoretical curves for H_{ox}-CO were generated using the ΔE_Q and δ of H_{red} indicated in the text and the A-values [Pair I (A_x, A_y, A_z)], [Pair II (A_x, A_y, A_z)]: [(25.6, 25.9, 22.0) MHz], [(−26.3, −34.2, −26.1) MHz].

suggesting that both Fe of the $[2Fe]_H$ contribute to the $\Delta E_Q \approx 0.87$ mm/s doublet.[25]

Analysis of the Mössbauer Spectra of H_{ox}-CO and H_{ox}. Figure 3 shows the 4.2 K spectra of the CO-bound oxidized *Cp* hydrogenase II, recorded by Rusnak et al. in applied fields of 6.0 (A) and 0.05 T (B). These authors have identified two paramagnetic components (called I and II in ref 2), each accounting for 13−14% of the total Fe in the sample. We believe that components I and II belong to $[4Fe-4S]_H$ and that this subcluster is in the $2+$ state, for the following reasons. First, the H cluster is the only paramagnetic species in this state of the enzyme and therefore components I and II belong to the H cluster. Second, each of these components represents one pair of irons (14%), together accounting for ≈28% of the total iron in the sample, i.e., one $[4Fe-4S]$ cluster. Third, we have paid particular attention to the isomer shifts of components I and II and we found that both have $\delta = 0.44 \pm 0.02$ mm/s. Within the experimental uncertainties, this is the same isomer shift as that of doublet I/II in H_{red}. Thus, the $[4Fe-4S]_H$ cluster is in the $2+$ oxidation state in both H_{ox}-CO and H_{red}. A fourth argument is based on a theoretical model that readily reconciles the apparent contradiction between the evidence that $[4Fe-4S]_H$ is in the $2+$ state (a state well documented to be diamagnetic for isolated clusters) and the observed hyperfine interactions associated with this subcluster. Rusnak et al. simulated the magnetic spectra of components I and II by assuming that $\Delta E_Q = 1.0$ mm/s. Since we have now good evidence that the oxidation state of $[4Fe-4S]_H$ does not change between H_{red} and

Figure 4. ^{57}Fe hyperfine coupling constants of the two delocalized pairs of the $[4Fe-4S]^{2+}$ cluster in a $[4Fe-4S]^{2+}$-[M] assembly, plotted as a function of j/Δ (the theory is described in ref 10); j describes the exchange coupling between [M] and the $[4Fe-4S]^{2+}$ cluster and Δ is the energy gap between the cluster ground state and the first excited state with $S_{cube} = 1$. The diamond and triangle correspond to A_I and A_{II} of H_{ox}-CO, respectively. The solid circle represents $A_I = A_{II}$ of H_{ox} (see text).

H_{ox}-CO, it is reasonable to assume that the ΔE_Q values in the CO-inhibited state are similar to those observed for H_{red}. Because the overall splittings of components I and II depend on the magnetic hyperfine tensor, **A**, as well as on the parameters describing the quadrupole splitting (ΔE_Q and η), we have simulated the spectra of these components with various values of ΔE_Q and η to assess their effect on the magnitude of the A-values. Taking the isotropic part of **A** (see caption of Figure 3) we obtained from the simulations $A_I = +(25.3 \pm 1)$ MHz and $A_{II} = -(28.3 \pm 1.5)$ MHz. Because we used here slightly larger values for ΔE_Q, our A_{iso} values are about 5% smaller than those of Rusnak et al. ($A_I = + 26.8$ MHz, $A_{II} = − 30$ MHz).

Figure 4 shows a plot of the A-values of the two valence delocalized pairs of a $[4Fe-4S]$ cluster that is exchange-coupled to a paramagnetic center M with $S = 1/2$ via a bridging ligand. The exchange coupling is described by $j\mathbf{S}_M \cdot \mathbf{S}_D$, where $\mathbf{S}_D$ is the local spin of the Fe_D site of $[4Fe-4S]_H$ that is linked to M (Figure 1). The details of the theory have been described elsewhere;[10,12] we follow here the nomenclature of Xia et al. Two comments are in order here. First, the A-values depend on the spin expectation values $\langle \mathbf{S}_i \rangle$, where i = A, B, C, D are the labels of the four sites of the cubane. Because of the strong double exchange interactions within the $[4Fe-4S]^{2+}$ cluster, $\langle \mathbf{S}_A \rangle = \langle \mathbf{S}_B \rangle$ and $\langle \mathbf{S}_C \rangle = \langle \mathbf{S}_D \rangle$ and $\langle \mathbf{S}_A \rangle = \langle \mathbf{S}_B \rangle \approx -\langle \mathbf{S}_C \rangle = -\langle \mathbf{S}_D \rangle$.[12] Second, the amplitudes of the two curves are given by the product $A_i = 2\langle \mathbf{S}_i \rangle a_i$, where a_i is the intrinsic coupling constant of the Fe site i. For a_i for a valence-delocalized pair we have employed the same value as in ref 10, namely −22 MHz (see also Table 5 of ref 26). The theoretical model predicts that the pair with $A < 0$ has A-values with larger magnitudes than the pair with $A > 0$. The diamond and triangle symbols in Figure 4 mark the experimental A-values of pairs I and II, respectively. Note that both signs and magnitudes agree well with the theoretical curve for $j/\Delta \approx 0.44$. The above arguments provide a strong case for assigning the spectral components I and II to the delocalized pairs of a $[4Fe-4S]^{2+}$ cluster that is coupled to a moiety with $S = 1/2$.

The ENDOR data of Telser et al.[7] have revealed for H_{ox}-CO an isotropic resonance corresponding to $\mathbf{A}_3 = 9.5$ MHz. In the following, we will reserve for the A-values of the $[4Fe-4S]_H$

(24) Lyon, E. J.; Georgakaki, I. P.; Reibenspies, J. H.; Darensbourg, M. Y. Manuscript submitted for publication.

(25) That we obtain 1.7 Fe rather than 2 Fe may have several reasons. First, within our experimental uncertainties, 1.7 Fe is not significantly different from 2 Fe. Second, this site may have a smaller recoilless fraction at 180 K than the sites of the three $[4Fe-4S]^{2+}$ clusters. Zambrano et al.[6] have suggested, on the basis of EPR studies, that the reduction of some of the clusters may not be quantitative. This does not seem to apply for the data of Rusnak et al.[2] Thus, the analysis of Rusnak et al. resulted in fractions corresponding almost exactly to the cluster distribution which is now established.

(26) Mouesca, J.-M.; Noodleman, L.; Case, D. A.; Lamotte, B. *Inorg. Chem.* **1995**, *34*, 4347−4359.

Table 1. Assignment of the ^{57}Fe Hyperfine Coupling Constants for the H Cluster

	$[4Fe\text{-}4S]^{2+}_H$	$[2Fe]_H$
H_{ox}	$|A_I| = |A_{II}| = 7.5$ MHz^a	$A_3 = (-)18$ MHz^{b,c}
$H_{ox}\text{-}CO$	$A_I = +25.3$ MHz	$A_3 = (-)9.5$ MHz^{b,c}
	$A_{II} = -28.3$ MHz	

^{a} Suggested assignment based on spectral simulations (this component is not resolved in the Mössbauer spectra). ^{b} A_3 of H_{ox} is associated with a single Fe site. A_3 of H_{ox}-CO could possibly reflect a delocalized Fe^{III}Fe^{II} (or Fe^{II}Fe^I) pair. ^{c} The ENDOR spectra of Telser et al.[7] have provided the magnitude of A_3. The ENDOR data show also that A_3 is isotropic. Because the Mössbauer spectra of $[2Fe]_H$ are essentially masked by the contributions of the [4Fe-4S] clusters, our data provide little information on the **A**-tensor components; however, spectral simulations of a 6.0 T spectrum (not shown) show that the sign of A_3 is negative.
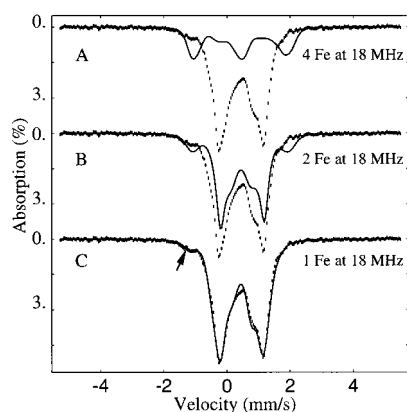
Figure 5. Mössbauer spectra of the oxidized hydrogenase recorded at 4.2 K in a parallel field of 0.05 T. The solid lines are simulations for the following cases: (A) all four Fe sites of $[4Fe\text{-}4S]^{2+}_H$ with $A = 18$ MHz; (B) Pair I of $[4Fe\text{-}4S]^{2+}_H$ with $A_I = 18$ MHz and Pair II with $A_{II} = 6$ MHz and diamagnetic F'_{ox} (the choice of any value of $|A_{II}| < 10$ MHz will not affect the conclusion that a pair with $A = 18$ MHz cannot be accommodated); (C) one Fe site of $[2Fe]_H$ with $A = 18$ MHz, diamagnetic F'_{ox} and $[4Fe\text{-}4S]^{2+}_H$, plus one site of $[2Fe]_H$ with $\Delta E_Q = 1$ mm/s and $\delta = 0.3$ mm/s. The "experimental" spectrum was obtained by subtracting a simulation of F_{ox} from the raw data (the spectrum represents 10 Fe).

cluster the indices I and II and we will denote those assigned to $[2Fe]_H$ as A_3 (see also Table 1). The above discussion shows that $A_3 = 9.5$ MHz must be associated with $[2Fe]_H$. Because the Mössbauer spectrum of a species with $A = 9.5$ MHz has a rather small magnetic splitting, it cannot be resolved from the contributions of the F and F' clusters and therefore it remains unidentified in the Mössbauer spectra.

Telser et al.[7] also have shown that in the oxidized enzyme, H_{ox} exhibits two nearly isotropic ENDOR resonances, corresponding to $A = 18$ and 7.5 MHz. We have pointed out previously that these small A-values would yield Mössbauer spectral features that are essentially masked by the doublets of F_{ox} and F_{ox}'.[2] Nevertheless, using the fact that the H cluster comprises the coupled $[4Fe\text{-}4S]^{2+}$-[2Fe] assembly, we can elicit some information from the Mössbauer spectra. For $j/\Delta \approx 0.22$, our theoretical model (Figure 4) predicts the A-values $A_I = +17.7$ MHz and $A_{II} = -18.8$ MHz. These values are too close to be resolved by ENDOR. The obvious question arises whether the 18 MHz resonance is associated with $[4Fe\text{-}4S]^{2+}_H$.

Figure 5 shows the 4.2 K Mössbauer spectrum of oxidized hydrogenase II.[27] The solid line drawn through the data of Figure 5A is a simulation assuming that both delocalized pairs of $[4Fe\text{-}4S]^{2+}_H$ have A_{iso} values of 18 MHz. This assumption is quite clearly in conflict with the data, because the predicted absorption

at 2 and −1 mm/s Doppler velocity is not supported by the experimental spectrum. In Figure 5B we have explored the possibility that one delocalized pair of $[4Fe\text{-}4S]_H$ has $A = 18$ MHz (because the wings of the quadrupole doublets of F_{ox} and F'_{ox} contribute underneath the outer features of the 18 MHz component, we have included F_{ox} and F'_{ox}, but not $[2Fe]_H$, in this simulation). As can be seen, this assignment is not in agreement with the experimental data either. (This mismatch would be enhanced when the simulation of $[2Fe]_H$ would be included.)

The $[2Fe]_H$ cluster is most likely in the mixed-valence Fe^{II}-Fe^{III} state in H_{ox} and in H_{ox}-CO and given the structural information available, both Fe sites should have low-spin configuration. The question arises whether this mixed-valence pair has localized or delocalized valencies. If we assume that $[2Fe]_H$ is delocalized and that this delocalized pair produces the 18 MHz ENDOR resonance, we have a situation similar to that illustrated in Figure 5B, namely, a pair of Fe sites with 18 MHz cannot be accommodated in the Mössbauer spectra.

The experimental spectrum of Figure 5 displays a shoulder at −1 mm/s (arrow in Figure 5C). This shoulder is the low-energy feature of a magnetic component and it is still present at 50 K, implying that it belongs to a species whose spin relaxes slowly on the Mössbauer time scale. At 120 K, this magnetic component has collapsed into a quadrupole doublet unresolved from those of the three [4Fe-4S] clusters. These observations are consistent with the temperature dependence of the $S = 1/2$ EPR signal of H_{ox}.[1] A simulation assuming that one Fe site of $[2Fe]_H$ contributes the 18 MHz ENDOR resonance is shown in Figure 5C. It can be seen that one Fe site with 18 MHz can produce the low-energy shoulder with the correct intensity. We wish to stress that we have very little additional information about the 18 MHz species. However, its quadrupole splitting and isomer shift can be confined to 0.7 mm/s $< \Delta E_Q < 1.2$ and 0.1 mm/s $< \delta < 0.3$ mm/s. We have no spectral information about the second site of $[2Fe]_H$ in the state H_{ox}. If this site were a low-spin Fe^{II}, it would contribute a quadrupole doublet (in the spectrum of Figure 5) that would be masked by the dominant feature of the F' cluster. Spectral simulations suggest but do not strictly prove that the 7.5 MHz ENDOR resonance results from the two pairs of $[4Fe\text{-}4S]^{2+}_H$. In our theoretical model, this A-value can be explained if the coupling between $[4Fe\text{-}4S]_H$ and the paramagnetic $[2Fe]_H$ is weaker than in H_{ox}-CO, corresponding to $j/\Delta = 0.08$; this j/Δ value would result in $|A| = 7.5$ MHz for both pairs of the cubane as indicated by the full circle in Figure 4.

Discussion

In the preceding section we have provided an extension of the analysis of the Mössbauer spectra of *Cp* hydrogenase II published in 1987 by Rusnak et al.[2] Our present analysis shows that the $[4Fe\text{-}4S]^{2+}_H$ subcluster of the [2Fe]-[4Fe-4S] assembly is in the 2+ state in both the oxidized and the reduced forms of the H cluster. Thus, $[2Fe]_H$ accommodates the electron when H_{ox} is reduced to H_{red}. The assertion that $[4Fe\text{-}4S]_H$ retains the 2+ oxidation state is based on the isomeric shifts of H_{red}, H_{ox}, and H_{ox}-CO. The isomer shift of a [4Fe-4S] cluster is a very

(27) For clarity we have removed the contribution of F_{ox} from the raw data. A representation of F_{ox} was obtained as follows: at a potential of −300 mV the F cluster is in the 1+ state, while the H and F' clusters remain in the oxidized state. By subtracting the spectrum of the oxidized enzyme from that of a sample poised at −300 mV the contributions of H and F' cancel. After simulating the magnetic spectrum of F_{red} and adding it to the F_{ox}−F_{red} difference spectrum, a fairly reliable representation of F_{ox} is obtained.
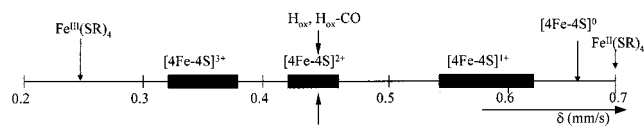
Figure 6. Average isomer shifts, $\delta_{ave} = (\delta_1 + \delta_2 + \delta_3 + \delta_4)/4$, for [4Fe-4S] cubanoid clusters.

good indicator of its core oxidation state.[30] Figure 6 shows the average isomer shift of 4Fe clusters, $\delta_{ave} = (\delta_1 + \delta_2 + \delta_3 + \delta_4)/4$, and its variation among clusters from different proteins in four established oxidation states; for reference we have included also the δ-values of the $Fe^{III}(RS)_4$ and $Fe^{II}(RS)_4$ sites of oxidized and reduced rubredoxin. It can be seen that the δ-values for different cluster oxidation states are well separated. We have shown above that doublet I/II of H_{red} represents 4 Fe atoms and that this doublet has $\delta_{ave} = 0.44 \pm 0.02$ mm/s. This value of the isomer shift unambiguously establishes the 2+ core oxidation state for $[4Fe-4S]_H$.

In the CO-inhibited state, H_{ox}-CO, we obtained for $[4Fe-4S]_H$ an isomeric shift of 0.44 ± 0.02 mm/s, indicating that this cluster remains in the same oxidation state in H_{red}- and H_{ox}-CO and, by extension, in H_{ox}.[31] We have shown above that the magnetic hyperfine interactions observed for $[4Fe-4S]_H$ in the oxidized hydrogenase can be explained by assuming an exchange coupling between a paramagnetic entity and the $[4Fe-4S]_H^{2+}$ cluster. The covalent link implied by the presence of the exchange coupling between $[4Fe-4S]_H^{2+}$ and $[2Fe]_H$ is clearly evident in the X-ray crystallographic structures of *Cp* hydrogenase I[19] and of *D. desulfuricans* [Fe]-hydrogenase.[20] One might argue that the data could be explained by assuming that the $[4Fe-4S]_H$ cluster of H_{ox}-CO is in the 3+ state ($S = 1/2$), while $[2Fe]_H$ remains diamagnetic. This possibility is excluded by the following considerations. First, if $A_I = +25.3$ MHz and $A_{II} = -28.3$ MHz are assigned to the two iron pairs of a $[4Fe-4S]^{3+}$ cluster, the 9.5 MHz A-value would remain unassigned because it is unlikely that it could originate from the low-spin Fe^{II} sites of the diamagnetic $[2Fe]_H$ cluster. Second, the observed isomeric shifts are incompatible with the presence of a $[4Fe-4S]^{3+}$ cluster. Third, $A = 7.5$ MHz, the only value assignable to $[4Fe-4S]_H$, is too small to be associated with a $[4Fe-4S]^{3+}$ cluster; for instance the $[4Fe-4S]^{3+}$ cluster from the high-potential ferredoxin of *C. vinosum* exhibits $A_I \approx +20$ MHz and $A_{II} \approx -30$ MHz.[33] Finally, the potential of the H_{ox}/H_{red} couple, $E^{\circ\prime} = -410$ mV,[1] is too low for a $[4Fe-4S]^{3+/2+}$ pair.

In summary, from our analysis the following picture has emerged. In H_{ox}, H_{ox}-CO, and H_{red}, the $[4Fe-4S]_H$ cluster is in the 2+ oxidation state. The Mössbauer data of H_{red} are consistent with two low-spin ferrous sites in $[2Fe]_H$ and this assignment

(28) The raw data used are from the studies of Rusnak et al.; see also ref 29.

(29) Rusnak, F. M. Ph.D. Thesis, University of Minnesota, 1988.

(30) (a) Christou, G.; Mascharak, P. K.; Armstrong, W. H.; Papaefthimiou, G. C.; Frankel, R. B.; Holm, R. H. *J. Am. Chem. Soc.* **1982**, *104*, 2820−2831. (b) Yoo, S.; Angove, H. C.; Burgess, B. K.; Hendrich, M. P.; Münck, E. *J. Am. Chem. Soc.* **1999**, *121*, 2534.

(31) B. H. Huynh[32] has reported preliminary results for the Fe hydrogenase from *Desulfovibrio vulgaris*. This study focused on the magnetic splittings of an iron−sulfur cluster associated with H_{ox}-CO (the so-called "axial 2.06" state in the *D. vulgaris* nomenclature). The values of the isomer shifts, quoted as 0.50 and 0.58 mm/s (see Table 3 of ref 32), have large uncertainties and are not suitable for a critical comparison with the data of hydrogenase II. The spectra of the *D. vulgaris* enzyme are presently being reanalyzed (B. H. Huynh, personal communication).

(32) Huynh, B. H. *Methods Enzymol.* **1994**, *243*, 523−543

(33) (a) Middleton, P.; Dickson, D. P. E.; Johnson, C. E.; Rush, J. D. *Eur. J. Biochem.* **1980**, *104*, 289−296. (b) Papaeftimiou, V.; Millar, M. M.; Münck, E. *Inorg. Chem.* **1986**, *25*, 3010−3014.

is also consistent with the observation that all six Fe sites of the H cluster are diamagnetic in H_{red}.[23] Thus, oxidation of the H cluster to H_{ox} and H_{ox}-CO is confined to the $[2Fe]_H$ subcluster which attains the $S = 1/2$ $Fe^{II}Fe^{III}$ state.

Nicolet and co-workers[20] have recently proposed that the $[4Fe-4S]_H$ cluster is in the 1+ oxidation state in both H_{ox} and H_{red}. Such an assignment is ruled out by the isomer shifts observed for this cluster in both oxidation states. In particular, the spectra of the reduced enzyme do not contain any diamagnetic spectral component that could possibly be assigned to the 1+ state of the $[4Fe-4S]_H$ subcluster. Moreover, $[4Fe-4S]^{1+}$ clusters have $g_{ave} < 2.0$, while H_{ox} and H_{ox}-CO exhibit $g_{ave} > 2$. Furthermore, the ^{57}Fe A-values of H_{ox} differ substantially from those reported for $[4Fe-4S]^{1+}$ clusters.

We have shown above (Figure 5, A and B) that the 18 MHz ENDOR resonance of H_{ox} cannot belong to the $[4Fe-4S]_H$ cluster, and that it must therefore result from $[2Fe]_H$. Moreover, our analysis suggests that this resonance cannot result from an $Fe^{III}Fe^{II}$ system in which the $S = 1/2$ spin is delocalized over the two Fe sites of $[2Fe]_H$. Thus, the unpaired spin is centered at one Fe site of the $Fe^{III}Fe^{II}$ subcluster. The question arises whether the Fe^{III} site is Fe(1) or Fe(2) of Figure 1. It is noteworthy that the A-value associated with $[2Fe]_H$ changes from 18 to 19 MHz in H_{ox} to 9.5 MHz in H_{ox}-CO. It is possible that the 50% reduction reflects delocalization of the unpaired spin over the two Fe sites of $[2Fe]_H$ in H_{ox}-CO. If this were indeed the case, we would conclude that the Fe^{III} site of H_{ox} is distal to $[4Fe-4S]_H$, arguing as follows. The exchange interactions between the cubane and the distal Fe(2) are mediated through the thiolate bridge, the diamagnetic Fe(1) site, and the two sulfurs of the propanedithiol, and consequently, the coupling is rather weak. (Long-range exchange coupling through an intervening diamagnetic metal has been reported recently by Wieghardt and co-workers.[34]) Upon CO binding to Fe(2), the $[2Fe]_H$ cluster would become delocalized, and the attendant transfer of unpaired spins to Fe(1) would increase the exchange interactions between the cube and the cluster. The idea that CO binds to the distal iron finds support in the X-ray structure of the *D. desulfuricans* enzyme,[20] which indicates an open coordination position at Fe(2). The structure of *Cp* hydrogenase I has been refined with a water ligand coordinated to the distal iron and this ligand could possibly be displaced by CO.[19] Alternatively, one could argue that Fe(1) is the Fe^{III} site and that binding of CO would cause some structural arrangement that would provide for a more efficient exchange pathway to $[4Fe-4S]_H$.

The experimental A-values for $[4Fe-4S]_H^{2+}$ of H_{ox}-CO, $A_I = +25.3$ MHz and $A_{II} = -28.3$ MHz yield $j/\Delta \approx 0.4$, where j measures the exchange interaction between $[2Fe]_H$ and the $[4Fe-4S]_H^{2+}$ cluster and Δ is the energy of the first excited cluster state with $S_{cube} = 1$. For $[4Fe-4S]^{2+}$ clusters, Δ-values of 200 cm^{-1} have been estimated,[12,13b] suggesting that $j \approx 100$ cm^{-1}.[35] Our Mössbauer analysis of H_{ox} suggests that the 7.5 MHz A-value observed by ENDOR belongs to the $[4Fe-4S]_H$ subcluster. In this interpretation H_{ox} would have $j/\Delta \approx 0.1$ (see Figure 4).

We cannot yet explain the isotropic A-values observed by ENDOR for the $[2Fe]_H$ cluster. In the following we wish to elaborate on this point. It seems reasonable to assume that the

(34) Glaser, T.; Beissel, T.; Bill, E.; Weyermüller, T.; Schünemann, V.; Meyer-Klaucke, W.; Trautwein, A. X.; Wieghardt, K. *J. Am. Chem. Soc.* **1999**, *121*, 2193−2208.

(35) The A cluster of CODH in the A_{red}-CO state has a j-value similar to that obtained here for the H_{ox}-CO cluster, suggesting that the Ni^I and the $[4Fe-4S]^{2+}$ cluster of A_{red}-CO are linked also by a cysteinyl sulfur.

[2Fe]$_H$ subcluster contains two low-spin Fe^{II} sites in the diamagnetic state H$_{red}$.[23] A one-electron oxidation of the H cluster would yield an Fe^{III}Fe^{II} subcluster, and the observed $S = 1/2$ spin of H$_{ox}$ (and H$_{ox}$-CO) could then be attributed to a low-spin Fe^{III} site. Kowal, Adams, and Johnson have reported photodissociation experiments on the *Cp* hydrogenase I that support this assignment.[36] When H$_{ox}$-CO ($g = 2.07, 2.01, 2.01$) was illuminated at 8 K, an EPR signal with the g-values of H$_{ox}$ (2.10, 2.04, 2.00) was observed. This observation has been interpreted[36] as evidence that CO binds to an empty coordination site of H$_{ox}$ and that the Fe sites of H$_{ox}$ and H$_{ox}$-CO are isoelectronic (similar experiments have been reported for the [Fe]-hydrogenase from *D. vulgaris*[32]). When H$_{ox}$-CO was illuminated at 30 K, an EPR signal with $g = 2.26, 2.12, 1.89$ was obtained. The $g = 2.26, 2.12, 1.89$ signal has also been observed for *Cp* hydrogenase II.[37] This set of g-values is quite reminiscent of a low-spin Fe^{III} center.[36]

The electronic structure of low-spin Fe^{III} ions in octahedral coordination environments is generally well described by a model proposed by Griffith[38] and extended by Oosterhuis and Lang.[39] In this model the g-values can be used to determine the values Δ/λ and V/λ, where Δ and V are the axial and rhombic ligand field parameters that split the t$_{2g}$ levels and λ is the spin−orbit coupling constant. These parameters in turn, together with two scaling factors (P and κ, see ref 39), determine the ^{57}Fe-magnetic hyperfine tensor in the given complex. The **A**-tensor can be written as the sum of an isotropic contact contribution ($A_{contact} = P\kappa$) and the anisotropic spin-dipolar and orbital contributions. The latter is proportional to ($g_i - 2$), where g_i ($i = x, y, z$) are the principal components of the **g**-tensor. Given that the g-values of H$_{ox}$ and H$_{ox}$-CO are near $g = 2$, the orbital contribution to **A** should be <5 MHz for H$_{ox}$ and <3 MHz for H$_{ox}$-CO. The dipolar term, however, can be considerable for a low-spin site. For instance, a hole in a d$_{yz}$ orbital of the t$_{2g}$ manifold produces A_y(dip) ≈ -36 MHz and A_x(dip) $\approx A_z$(dip) $= +18$ MHz, i.e., the spin dipolar term contributes an anisotropy of about 50−60 MHz.[40]

We have recently studied EPR and Mössbauer spectra of a mononuclear Fe^{III} complex (synthesized in Koch's laboratory) that has a phosphine, three thiolates, one CN$^-$, and one CO as ligands. The EPR properties of this complex ($g = 2.10, 2.06, 2.02$) are close to those of H$_{ox}$ and H$_{ox}$-CO and the ^{13}CO hyperfine interactions are similar to those observed for H$_{ox}$-CO of *Cp* hydrogenase I.[40] The ^{57}Fe magnetic hyperfine tensor has components ranging from $A_z = 10$ MHz to $A_x = -60$ MHz ($A_{iso} = -20$ MHz)[40] which are consistent with those predicted by the Griffith model, the large anisotropies being attributable to the spin-dipolar term. These considerations and the general observations reported for many low-spin ferric complexes suggest that difficulties are expected in attempting to explain the isotropic A-values of H$_{ox}$ and H$_{ox}$-CO with a simple low-spin Fe^{III} model. The coupling of [2Fe]$_H$ to the [4Fe-4S]$^{2+}$ cluster cannot account for the unusual **A**-tensor of the Fe sites of [2Fe]$_H$ of H$_{ox}$ and H$_{ox}$-CO, as indicated by the following considerations.

An exchange Hamiltonian such as $j\mathbf{S}_{Fe(1)}\cdot\mathbf{S}_D$ reflects incipient bonding between Fe(1) and Fe$_D$ (see Figure 1) through interven-

ing ligands such as a bridging thiolate sulfur.[42] The presence of such a bridge is not expected to impose unusual properties on the intrinsic parameters of Fe(1) and Fe$_D$. For instance, in oxidized *E. coli* sulfite reductase, the high-spin Fe^{3+} of the siroheme is linked by a cysteinyl sulfur to one Fe of a [4Fe-4S]$^{2+}$ cluster.[11] Yet as expected from theoretical considerations, the magnetic hyperfine interactions of the siroheme are very much like those observed for typical isolated high-spin Fe^{3+}-porphyrins.[9] Our studies of the Ni$^+$-[4Fe-4S]$^{2+}$ coupled assembly of the A cluster of carbon monoxide dehydrogenase suggest that the g- and A-values of the Ni$^+$ are modified by perhaps 30% relative to an uncoupled Ni$^+$ (see eq 5 in ref 10), and for H$_{ox}$ and H$_{ox}$-CO we would expect that the A-values of [2Fe]$_H$ are scaled toward $g = 2$ not more than 20% by the coupling with the [4Fe-4S]$_H^{2+}$. Moreover, because of the isotropic exchange coupling, all the **A**-tensor components would be similarly reduced. Thus, the isotropy of the A-values reported for [2Fe]$_H$ is not likely to be rationalized by invoking the coupling to the cubane.

To find an explanation for the lack of anisotropies of the magnetic hyperfine tensor **A**$_3$ in H$_{ox}$ and H$_{ox}$-CO, we have considered also the following possibilities. First, could the **A**-tensor of H$_{ox}$ have components of equal magnitudes but opposite sign, for instance $A_{3x} = A_{3y} = -18$ MHz and $A_{3z} = +18$ MHz? The combination of the original experimental ENDOR data at X-band[7] with recent simulations (Dr. J. Telser, personal communication) shows that this case would indeed be difficult to distinguish from $A_{3x} = A_{3y} = A_{3z} = \pm18$ MHz. This situation may appear somewhat contrived, but it cannot be dismissed. Second, for an octahedral low-spin Fe^{III} complex, there are two cases for which the Griffith model yields $|g_x| = |g_y| = |g_z| = 2$. For $\Delta \gg |\lambda|$ the orbital angular momentum is essentially quenched and the ^{57}Fe **A**-tensor displays sizable anisotropies due to the spin-dipolar term. For $|\lambda| \gg |\Delta|, |V|$, the three orbital states are mixed in equal proportion in the wave function of the resulting ground doublet, and the **A**-tensor of this doublet is isotropic, with $A_{iso} \approx -P(8/7 - \kappa/3)$ expected to be ≈ -70 MHz. Neither of these cases fits the isotropic $A = 18$ MHz observed for H$_{ox}$. Third, the spin-dipolar contribution of **A**$_3$ could in principle be reduced by the presence of anisotropic covalency. However, while the Koch model complex mentioned above contains a similar coordination environment as the Fe sites in [2Fe]$_H$, its **A**-tensor exhibits the expected anisotropy. Fourth, we have considered the possibility that the oxidation from H$_{red}$ to H$_{ox}$ occurs by removal of an electron from a sulfur orbital, perhaps centered on the bridging thiols of [2Fe]$_H$. This suggestion is not in conflict with the observed g-values. However, while such a radical could interact with an Fe 4s orbital to produce an isotropic ^{57}Fe A-value, this is expected to be positive, rather than negative. Nevertheless, the presence of a sulfur radical should be tested by studying the EPR spectra of ^{33}S-enriched protein, an expensive but possibly rewarding experiment. On the other hand, the isotropic 18 MHz A-value observed for H$_{ox}$ is similar to $A_{iso} \approx -20$ MHz obtained for the aforementioned model complex, thus arguing against substantial delocalization of d-electron density into a ligand-

(36) Kowal, A. T.; Adams, M. W. W.; Johnson, M. K. *J. Biol. Chem.* **1989**, *264*, 4342−4348.

(37) Unpublished data of Adams, M. W. W. quoted in ref 1.

(38) (a) Griffith, J. S. *Nature* **1957**, *180*, 30−31. (b) Griffith, J. S. *Mol. Phys.* **1971**, *21*, 135−139.

(39) Oosterhuis, W. T.; Lang, G. *Phys. Rev.* **1969**, *178*, 439−456.

(40) Popescu, C. V.; Münck, E.; Hsu, H.; Albracht, S. P. J.; Koch, S. A. Manuscript in preparation.

(41) Pierik and co-workers[18] have proposed, as a working hypothesis that the H cluster comprises one low-spin Fe(III) linked by (two) cysteinyl sulfurs to a [4Fe-4S] cluster. These authors point out that the EPR and FTIR properties of the model complex of S. A. Koch and co-workers support their proposal of a low-spin Fe^{III} site. While the EPR properties of this model complex are reminiscent of those of H$_{ox}$ and H$_{ox}$-CO, its ^{57}Fe **A**-tensor is dramatically different from those of the H cluster in H$_{ox}$ and H$_{ox}$-CO.

(42) For the considerations in this paragraph we assume for simplicity that the unpaired spin resides on Fe(1).

based orbital. Fifth, there is another system that exhibits an as yet unexplained (nearly) isotropic **A**-tensor. We have studied in our laboratory the Mössbauer spectra of a bis-(μ-oxo)-bridged diiron complex, synthesized by Que and co-workers.[43] This complex exhibits a delocalized $Fe^{III}Fe^{IV}$ mixed-valence state. While low-spin ($S = 1/2$) Fe^{III} and Fe^{IV} ($S = 1$) are expected to have intrinsic **A**-tensors with large anisotropies, the valence-delocalized state exhibits a nearly isotropic A-value, $A \approx -7$ MHz). Sixth, if H_{ox} would represent an $Fe^{I}Fe^{II}$ state,[23] the problem of the isotropic 18 MHz A-value is not solved because the unpaired electron is expected to reside in an e_g orbital (localized or delocalized) and it would be expected to produce a substantial spin dipolar term.

In summary, our Mössbauer data on hydrogenase II demonstrate a covalent link between $[2Fe]_H$ and $[4Fe-4S]_H^{2+}$. Analysis of the Mössbauer spectra shows that the $[4Fe-4S]_H$ cluster is in the 2+ state in H_{ox}, H_{ox}-CO, and H_{red}.[44] Hence, when oxidized hydrogenase is exposed to H_2, the $[2Fe]_H$ cluster is reduced from the mixed-valence $Fe^{II}Fe^{III}$ state to the diferrous state. The second electron supplied by H_2 is not stored on the $[4Fe-4S]_H$ cluster, but transferred to one of the F clusters. We have been able to assign the paramagnetic components of H_{ox} and H_{ox}-CO observed by Mössbauer and ENDOR spectroscopy to the subclusters of H. We understand the spectroscopic features introduced by the coupling of the $[4Fe-4S]$ cluster to $[2Fe]_H$ quite well (as expressed in Figure 4), but the lack of anisotropy of the ^{57}Fe magnetic hyperfine interactions of the $[2Fe]_H$ subcluster indicates structural and electronic features which we do not yet fully appreciate.[46] While the electronic structure of the model complex of Koch and co-workers is similar to that of other low-spin Fe^{III} complexes, the electronic structure of the $[2Fe]_H$ cluster might reflect peculiarities associated with the fact that $[2Fe]_H$ is a binuclear cluster rather than a mononuclear complex. Further elucidation of the problem requires the study of suitable model complexes and hydrogenase mutants.

(43) Dong, Y.; Fujii, H.; Hendrich, M. P.; Leising, R., A.; Pan, G.; Randall, C. A.; Wilkinson, E.; Zang, Y.; Que, L.; Fox, B. G.; Kauffmann, K.; Münck, E. *J. Am. Chem. Soc.* **1995**, *117*, 2778−2792.

(44) During the reductive activation of inactive [Fe]-hydrogenase from *Desulfovibrio vulgaris* an EPR signal with $g = 2.06$, 1.96, and 1.89, the so-called rhombic 2.06 signal, is observed. This signal appears at a potential of approximately −50 mV and disappears around −250 mV concomitantly with the appearance of the signal characteristic of H_{ox}.[45] This signal is not observed for the active enzyme.[45b] In light of our present results it appears that the rhombic 2.06 signal originates from $[4Fe-4S]_H^+$ and that reductive activation of the *D. vulgaris* enzyme involves a conformational change that lowers the potential of $[4Fe-4S]_H$ such that the 1+ state is not accessible anymore at potentials above −450 mV.

(45) (a) Patil, D. S.; Moura, J. J. G.; He, S. H.; Teixeira, M.; Prickril, B. C.; DerVartanian, D. V.; Peck, H. D., Jr.; LeGall, J.; Huynh, B.-H. *J. Biol. Chem.* **1988**, *263*, 18732-18738. (b) Pierik, A. P.; Hagen, W. R.; Redeker, J. S.; Wolbert, R. B. G.; Boersma, M.; Verhagen, M. F. J. M.; Grande, H. J.; Veeger, C.; Mutsaers, P. H. A.; Sands, R. H.; Dunham, W. R. *Eur. J. Biochem.* **1992**, *209*, 63−72.

(46) Low-temperature magnetic circular dichroism studies of *Cp* hydrogenase I and II,[6] *D. vulgaris* [Fe]-hydrogenase,[47a] and the [Fe]-hydrogenase from *M. elsdenii*[47b] in the state H_{ox} have yielded magnetization curves that exhibit nesting, a phenomenon indicative of systems with $S > 1/2$. It is not clear to us whether H_{ox} does not exhibit MCD signals that magnetize with spin $S = 1/2$ behavior or whether the nesting indicates unidentified $S > 1/2$ contaminants (or cluster forms) whose contribution is superimposed upon the signals of H_{ox}. For hydrogenase II nesting is barely perceptible (see Figure 11 of ref 6) whereas nesting is pronounced for the *D. vulgaris* and *M. elsdenii* enzymes.[47] Interestingly, the latter two enzymes yield also low-spin concentrations for the EPR signal of H_{ox} (0.3 and 0.6 spins/molecule, respectively), allowing for the possibility that the H cluster could exist in some as yet not recognized (active or inactive) paramagnetic state. Alternatively, as pointed out by the authors of the MCD studies, the $S = 1/2$ state of H_{ox} could yield very weak MCD features and the observed signal might result from some unidentified entity.

(47) (a) Thomson, A. G.; George, S. G.; Richards, A. J. M.; Robinson, A. E.; Grande, H. J.; Veeger, C.; Van Dijk, C. *Biochem. J.* **1985**, *227*, 333−336. (b) Stephens, P. J.; Devlin, F.; Morgan, T. V.; Czechowsky, M.; DerVartanian, D. V.; Peck, H. D., Jr.; LeGall, J. *FEBS Lett.* **1985**, *180*, 24−28.