phosphate buffer (pH 7.5) for 1 h at 25 °C. Slides then were removed, washed with 10 mL of water, mounted on the ellipsometer, and allowed to dry before their thickness was measured. At least three measurements were taken on each slide and averaged; the data reported in Table II represent the average increase in thickness of five slides.

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Detection and Characterization of Exchangeable Protons Bound to the Hydrogen-Activation Nickel Site of *Desulfovibrio gigas* Hydrogenase: A ^1H and ^2H Q-Band ENDOR Study

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Abstract: This paper presents a Q-band ENDOR study of the nickel site of the as-isolated (Ni-A), H_2-reduced (Ni-C), and reoxidized (Ni-A/Ni-B) states of *Desulfovibrio gigas* hydrogenase. Through proton and deuteron ENDOR measurements we detect and characterize the possible products of heterolytic cleavage of H_2, namely two distinct types of exchangeable protons, bound to the Ni-C site. One proton, H(1), has a hyperfine coupling, $A^H(1) = 16.8$ MHz and appears to interact directly with Ni-C. The other proton, H(2), has $A^H(2) \approx 4.4$ MHz and could be associated with H_2O or OH^- bound to nickel. We discuss possible binding modes for H(1) and H(2). One type of exchangeable deuteron(s), D(2), associated with the Ni-C center remains associated with the Ni-B center after oxidation of the Ni-C. In addition we confirm that the Ni-A site is inaccessible to solvent protons.

Introduction

The hydrogenase from *Desulfovibrio* (*D.*) *gigas* is composed of two subunits (26 and 63 kDa) and contains one nickel center, one [3Fe-4S], and two [4Fe-4S] clusters.[1,2] It is currently believed that the nickel center is the substrate binding site. As isolated, the enzyme is catalytically inactive, and the nickel center exhibits an intense EPR signal, termed Ni-signal A ($g = 2.31$, 2.26, and 2.02), and a weak signal, termed Ni-signal B ($g = 2.33$, 2.16, and 2.02). Both of these are assigned to a formally trivalent, Ni(III) species, with the odd electron in the d_{z^2} orbital. Upon reduction by hydrogen, both signals disappear and a new nickel signal, termed Ni-signal C ($g = 2.19$, 2.14, and 2.02), is observed. The g values of the Ni-signal C ($g_x \neq g_y > g_z \approx 2$) suggest that its unpaired electron also is associated with the d_{z^2} orbital of the nickel. However, whether the oxidation state of Ni-C is Ni(I) or Ni(III) is still in debate.[3] Upon reoxidation, both Ni-signals A and B appear again with the relative intensity of the Ni-signal B increased.

In correlation with studies of catalytic activity, it has been concluded that the Ni-signals A and B represent inactive states of enzyme,[4,5] and, in fact, some Ni-containing hydrogenases do not show these signals.[6] The Ni-signal C, on the other hand, is observed in all the Ni-containing hydrogenases and is considered to represent a key intermediate in the catalytic cycle.[5b,7] In support of these views, a recent electron spin echo study[8] has shown that the nickel site is inaccessible to solvent protons in the Ni-A state but is accessible in the Ni-C state. However, characterization of the exchangeable protons associated with Ni-C was not possible.

Kinetic studies[9,10] suggest that hydrogen activation by hydrogenase involves heterolytic cleavage of H_2, with the possible formation of a metal hydride species as an intermediate state. There has been no direct evidence for a metal hydride, but the results of photolyzing the reduced enzyme led to the proposal that the Ni-signal C represents such a species.[11,12]

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This report presnts a Q-band ENDOR study of the as-isolated (Ni-A), H_2-reduced (Ni-C), and reoxidized (Ni-A/Ni-B) states of *D. gigas* hydrogenase. We detect and describe two distinct types of exchangeable protons bound to the Ni-C site and show that at least one of these remains associated with Ni-B. In addition, we confirm that the Ni-A site is inaccessible to solvent protons.

Materials and Methods

The as-purified and reduced *D. gigas* hydrogenase samples were prepared as previously described.[2,5] In particular, reduction of enzyme in $H_2O(D_2O)$ buffers employed $H_2(D_2)$ gas. The samples in the Ni-B state were prepared by exposing the reduced samples to air for about 1.5 h. The same samples exposed in air for an additional 24 h showed no change on the EPR signal. The reoxidation procedure was carried out over ice.

ENDOR spectra were recorded on a modified Varian 35 GHz (Q-band) spectrometer. The instrumentation is similar to the 9.5 GHz (X-band) ENDOR spectrometer,[13] except that a Varian E110 microwave bridge is interfaced to the E109 EPR console. Experiments were performed with a silvered TE_{011} sample cavity held at about 2 K in a Janis Corp. liquid helium immersion dewar; mutually perpendicular field-modulation and radio-frequency coils run through the cavity.

ENDOR is performed at a fixed applied field H_0 and is manifested by changes in the EPR signal intensity that result from nuclear transitions induced by a swept radio-frequency field.[14] The ENDOR transition frequency for a nucleus, J, of spin I is given by the equation

$$\nu_{\pm} = |\nu_J \pm A^J/2 + 3P^J(2m - 1)| \quad (1)$$

where $-I + 1 \leq m \leq I$, A^J and P^J are the orientation dependent hyperfine and quadruple coupling constants, and $\nu_J = g_J\beta_n H_0/h$ is the nuclear Larmor frequency. For protons ($I = 1/2$) in biological systems, $\nu_H > A^H/2$, the ENDOR spectrum is a hyperfine-split doublet centered about the free-proton Larmor frequency ν_H (42.6 MHz at $H_0 = 10$ KG) and split by A^H. For the deuteron ENDOR ($I = 1$) described here, $\nu_D > A^D/2 \gg P^D$, eq 1 describes a pattern that consists of a hyperfine-split doublet centered at ν_D, with $\nu_{\pm}$ further potentially split by the quadruple term. The magnetic parameters of exchangeable proton and deuteron nuclei are related by fundamental nuclear properties

$$A^H/A^D = \nu_H/\nu_D = g_H/g_D = 6.514 \quad (2)$$

and thus the magnetic parameters obtained from the resonances for one isotopic species can be used to predict those of the other. Deuteron ENDOR signals cannot easily be seen at X-band because for a $g \approx 2$ EPR signal the deuteron ENDOR pattern would extend from ca. 0.5–3.5 MHz, and it is difficult to obtain ENDOR signals at such low radio frequencies. However, at Q-band, the deuteron ENDOR pattern is centered at $\nu_D \approx 8$ MHz and extends from ca. 6.5 to 9.5 MHz. Resonances in this range are readily detected. All of the deuteron ENDOR spectra shown here have been taken at Q-band microwave frequency.

Results and Discussion

Ni-A State. The 2 K EPR spectrum of the as-isolated *D. gigas* hydrogenase taken at Q-band microwave frequency (35 GHz) in dispersion mode under conditions of rapid passage shows both Ni-signal A and an intense signal at $g = 2.02$ from the oxidized [3Fe-4S] cluster. Ni-signal B is not visible from the EPR spectrum. The proton ENDOR spectrum obtained at $g = 2.31$ from the Ni-signal A (Figure 1A) exhibits numerous doublets centered at the proton Larmor frequency ($\nu_H = 46.8$ MHz at $H_0 = 11$ KG), each corresponding to a set of protons with a distinct hyperfine coupling, A^H; the line positions are given by $\delta\nu_{\pm}{}^H = (\nu_{\pm} - \nu_H) = \pm A^H/2$. One striking feature of the spectrum, the enhanced intensity of the $\delta\nu_+{}^H$ half of the spectrum, reflects details of the spin relaxation at Q-band microwave frequency and is of no importance here, except that it leads us to focus on the $\delta\nu_+{}^H$ features. The proton hyperfine couplings obtained from this spectrum of the as-isolated Ni-A range from $A^H \approx 0$ to $A^H = 12.8$ MHz. The weak feature at $\delta\nu_+{}^H = +7.5$ MHz represents the proton associated with an unresolvably small contribution to the EPR signal from the Ni-B center and will be discussed later.

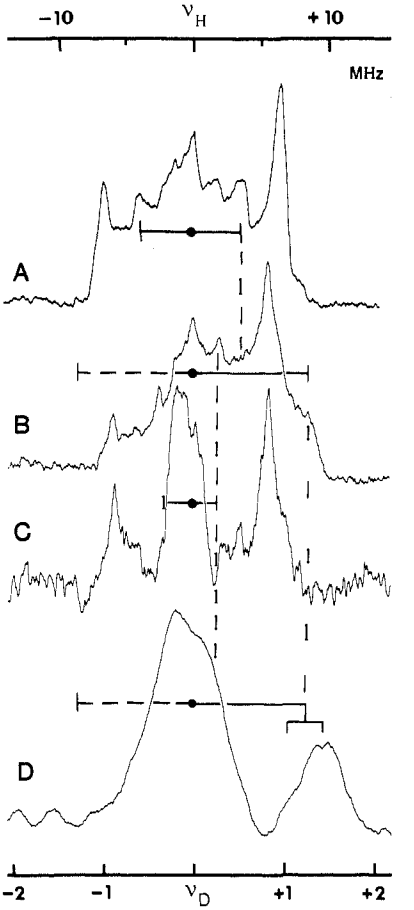

Figure 1. Proton (A–C) and deuteron (d) ENDOR spectra for Ni-A and Ni-C of *D. gigas* hydrogense. The proton ENDOR spectra are presented as $\delta\nu^H = (\nu - \nu_H)$ (top scale; $\nu_H \approx 49$ MHz) and the deuteron ENDOR spectrum as $\delta\nu^D = (\nu - \nu_D)$ (bottom scale; $\nu_D \approx 7.5$ MHz). The frequency axes are scaled by the ratio of the nuclear g factors ($g_H/g_D = 6.514$), and thus features for the two nuclei can be directly compared. The spectra are as follows: proton ENDOR spectra (A) taken from the as-isolated Ni-A (D_2O) sample at $g = 2.31$; (B) from the Ni-C (H_2/H_2O) sample at $g = 2.19$; (C) from the Ni-C (D_2/D_2O) sample at $g = 2.19$; (D) and deuteron ENDOR taken from the Ni-C (D_2/D_2O) sample at $g = 2.19$. Conditions: buffer 0.1 M Tris/HCl, pH 7.0; protein concentration ≈ 1 mM; $T = 2$ K; microwave power ≈ 35 mW; $H_0 = 10\,880$ G (A), 11 500 G (B–D); 100 kHz field modulation, 1.6 G (A–C), 2.5 G (D); rf scan rate, 1 MHz/s (A), 1.5 MHz/s (B and C), 2 MHz/s (D); scans, 140 (A), 1500 (B and C), 300 (D).

Proton ENDOR spectra from the as-isolated Ni-A sample prepared in D_2O (data not shown) are identical with that prepared in H_2O. Furthermore, no deuteron ENDOR signal (see below) is introduced by exchanging the enzyme into D_2O. Thus we conclude that the Ni-A site is inaccessible to H/D exchange.

Ni-C State. The Ni-C EPR signal of a H_2-reduced sample is weaker than that of Ni-A because it corresponds to an intermediate state in the catalytic cycle that attains a maximal intensity of ≈0.4 spins/mol. X-band EPR measurements at 30 K (data not shown) disclose that H/D exchange decreases the line width by ≈1 (FWHM), 2.5 (p–p), and 3 (FWHM) G at $g = 2.19$, 2.14, and 2.01, respectively. A more pronounced narrowing upon H/D exchange, ≈5 G at g_{mid}, was reported for the Ni-hydrogenase from *Chromatium vinosum*.[12] In anticipation of results below, we note here that under the assumption that there is one exchangeable proton associated with the Ni-C site, the line-narrowing can be simulated with a hyperfine tensor of $A^H \approx [15, 22, 25]$ MHz, whereas the tensor will be $A^H \approx [11.5, 16.5, 19.5]$ MHz for two equivalent protons. The simulation was performed with program QPOW[22] as modified to run on a PC.

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The proton ENDOR pattern obtained from Ni-C at $g = 2.19$ (Figure 1B) is generally similar to that from Ni-A (Figure 1A). The most obvious difference is the appearance of a new feature at $\delta\nu_+^H = 8.4$ MHz that represents a doublet signal from proton(s) with $A^H = 16.8$ MHz; from this doublet the asymmetry in intensity is so great that the $\delta\nu_-^H$, partner is not visualized. In addition, at least one proton doublet ($\delta\nu_\pm^H \approx \pm 4$ MHz, $A^H \approx 8$ MHz) seen in the Ni-A spectrum (Figure 1A) is not detected. Figure 1C is the proton ENDOR spectrum taken from the Ni-C state of D_2-reduced enzyme in D_2O solvent. The signal at $\delta\nu_+^H = 8.4$ MHz clearly has been eliminated. In addition, there is a loss of signal intensity at $\delta\nu_\pm^H \approx \pm 2.2$ MHz. Thus, the difference of proton ENDOR spectra upon H/D exchange discloses two types of exchangeable protons, H(1) and H(2), associated with the Ni-C site, with hyperfine couplings of $A^H(1) = 16.8$ and $A^H(2) \approx 4.4$ MHz.

The presence of two types of exchangeable protons in the Ni-C site is confirmed by the observation that H/D exchange introduces deuteron ENDOR signals that are centered at the deuteron Larmor frequency ($\nu_D = 7.5$ MHz at $H_0 = 11.5$ KG). Unlike proton ENDOR, where the signal from exchangeable proton(s) is poorly resolved because of overlapping resonances from many nonexchangeable protons, there is no interference in the corresponding deuteron ENDOR measurements because they show only the signal from deuteron(s) that replaces proton(s) upon H/D exchange. As shown in Figure 1D, the features in the deuteron ENDOR pattern match those of the two sets of protons lost from the proton upon deuteration, if the frequency axes are scaled by the ratio of the nuclear g factors ($g_H/g_D = 6.514$). In particular, the broad deuteron ENDOR feature centered at ν_D corresponds, through eqs 1 and 2, to exchangeable proton(s) with $A^H(2) \leq 4.4$ MHz, and the feature centered at $\delta\nu_+^D = (\nu - \nu_D) \approx 1.2$ MHz corresponds to the $\delta\nu_+^H$ peak of the exchangeable proton with $A^H(1) = 16.8$ MHz but with an additional deuteron quadrupole splitting $3P \approx 0.4$ MHz.

Given the enzymatic function of this enzyme, it is plausible to suggest that proton doublets $A^H(1)$ and $A^H(2)$ represent one exchangeable proton each. Alternatively, $A^H(1)$ could represent "H_2" itself, namely a signal associated with two equivalent protons, whereas $A^H(2)$ could be associated with, say, trapped solvent species. Because we are not able to use ENDOR to determine the full hyperfine tensor of H(1), it is not possible to use the line-narrowing upon H/D exchange to distinguish whether there are one or two of these protons; in either case the values of $A^H(1)$ determined by ENDOR is consistent with the simulation of EPR line-narrowing upon H/D exchange (see above). The number of weakly coupled protons, H(2), cannot be determined because they have a negligible effect on the EPR line width; redox titrations indicate the number could be one or two.[11]

Ni-B State. Two samples containing Ni-C, one prepared by D_2 reduction in D_2O buffer and the other by H_2 reduction in H_2O, were reoxidized in air with the goal of preparing and examining the Ni-B state. However, the EPR signal of each showed Ni-signals A and B with roughly equal intensites (data not shown). We attempted to obtain a signal from the Ni-B alone by setting the field to $g > 2.32$, the maximum g value for the Ni-signal A.

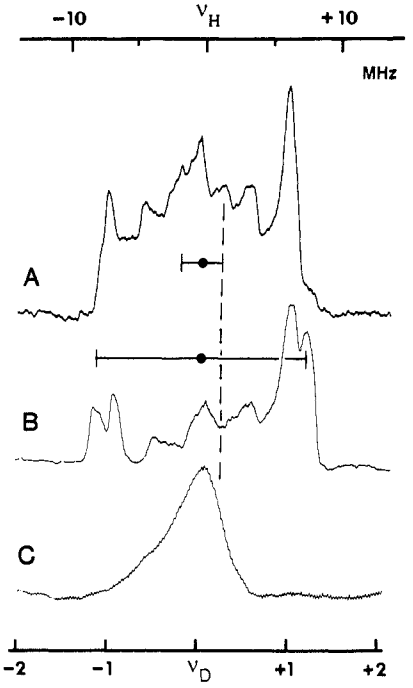

Figure 2. Proton (A and B) and deuteron (C) ENDOR spectra for Ni-A and Ni-B or *D. gigas* hydrogenase. The spectra are presented and scaled as in Figure 1 and are as follows: proton ENDOR spectra (A) taken from the as-isolated Ni-A (D_2O) sample at $g = 2.31$; (B) from the Ni-A/Ni-B (D_2O/D_2O) sample at $g = 2.34$; and (C) deuteron ENDOR spectrum taken from the Ni-A/Ni-B (D_2/D_2O) sample at $g = 2.18$. Conditions: $H_0 = 10\,880$ G (A), 10 850 G (B), 11 600 G (C); 100 kHz field modulation, 1.6 G (A–C); rf scan rate, 1 MHz/s (A–C); scans, 140 (A), 350 (B), 300 (C). Other conditions are the same as Figure 1.

Unfortunately the breadth of the EPR signals, in particular "tailing" of the Ni-signal A intensity to low field that is not apparent in the typical absorption-derivative EPR spectrum, prevented complete discrimination, and ENDOR spectra at all fields were superpositions of those from both Ni-A and Ni-B centers. Nonetheless, by comparing the ENDOR spectra of as-isolated Ni-A with those of the reoxidized Ni-A/Ni-B and by taking spectra at several fields, thereby changing the relative EPR intensities of Ni-A and Ni-B, it was possible to discriminate between ENDOR signals from these two centers.

Proton ENDOR spectra were taken at the low-field edge of the EPR signal ($g = 2.34$) for the two samples, Ni-A/Ni-B (D_2/D_2O) (Figure 2B) and Ni-A/Ni-B (H_2/H_2O) (data not shown). The spectra are nearly identical except for a loss of signal intensity at $\delta\nu_\pm^H \approx \pm 2.2$ MHz for the sample prepared in D_2/D_2O. Comparison of these proton ENDOR spectra with that from as-isolated Ni-A shows two obvious differences as follows: (1) The tiny feature at $\delta\nu_+^H = +7.5$ MHz in the Ni-A spectrum (Figure 2A), which corresponds to $A^H = 15$ MHz, becomes much stronger in reoxidized Ni-A/Ni-B, both in the D_2/D_2O (Figure 2B) and H_2/H_2O samples. This must represent a doublet signal from proton(s) associated with the Ni-B center; it cannot represent exchangeable proton(s) incorporated during the reduction, reoxidation cycle because it appears in the Ni-A/Ni-B sample prepared in D_2O. We presume that this proton doublet for the Ni-B corresponds to the strong proton doublet at $\delta\nu_\pm^H = \pm 6.4$ MHz (Figure 2A) in as-isolated Ni-A and that the change in A^H reflects a subtle structural difference between the Ni-B and Ni-A. (2) The signal at $\delta\nu_\pm^H \approx \pm 2.2$ MHz for the Ni-A/Ni-B (D_2/D_2O) sample is reduced in intensity relative to that from as-isolated Ni-A as well as that from the Ni-A/Ni-B (H_2/H_2O) sample. We interpret this to signify that the H/D exchange that occurs in the Ni-C state has led to the trapping of a deuteron in the Ni-A and/or Ni-B site and that at the site of exchange is that represented by the $A^H \approx 4.4$ MHz proton doublet that loses intensity upon D_2 reduction of the Ni-A to form Ni-C (Figure 1C).

The observation of an intense deuteron ENDOR spectrum

(16) For a proton lying in the nodal plane of a $2p_z$ orbital, comparison of exact calculation of the dipolar interaction (Barfield, M. *J. Chem. Phys.* **1970**, *53*, No. 10, 3836–3843) with the point-dipole approximation (eq 4) shows that the latter is quite accurate for $d = 2$ Å and overestimates the coupling for shorter distance; at $d \approx 1.5$ Å the overestimation is $\approx$ 2-fold. The same trends will be true for a proton in the nodal plane of a $3d_{z^2}$ orbital.

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(Figure 2C) from the Ni-A/Ni-B (D_2/D_2O) sample clearly confirms that at least one deuteron that was introduced into Ni-C has remained associated with the Ni-A and/or Ni-B centers after reoxidation of Ni-C. The signal is a single line centered at ν_D = 7.9 MHz (H_0 = 11 600 G), with a line width of about 0.7 MHz. It represents exchangeable deuterons with $A^D \leq 0.7$ MHz, which corresponds to a proton coupling of $A^H \leq 4.6$ MHz. This deuteron ENDOR signal matches the deuteron of Ni-C (Figure 1D) and the proton doublet at $\delta\nu_\pm{}^H \approx \pm 2.2$ MHz that loses intensity in the Ni-A/Ni-B prepared in D_2/D_2O (Figure 2B).

To determine whether the $\delta\nu_\pm{}^H \approx \pm 2.2$ doublet is associated with Ni-A or Ni-B, we compared the deuteron ENDOR spectra taken at g_{mid} of the Ni-signal A (g = 2.26) and at g_{mid} of Ni-signal B (g = 2.16). The total EPR intensities are roughly equal at these two fields, and both Ni-A and Ni-B contribute at each field; however, the ratio of intensities is Ni-A/Ni-B > 1 at g = 2.26, and the converse is true at g = 2.16. Hence, if the exchangeable proton were associated with the Ni-B site, the deuteron ENDOR signal should be stronger at g = 2.16 than that at g = 2.26 and conversely if it were associated with the Ni-A. We find that the deuteron ENDOR signal at g = 2.16 is two times stronger than that at g = 2.26. This demonstrates that the deuteron ENDOR signal is associated with the Ni-B center; it suggests but does not prove that there is no deuteron signal associated with the Ni-A center.

Assignments. ENDOR measurements reveal two types of exchangeable protons covalently interacting with the Ni-C site. The major chemical distinction to be made is whether a proton interacts directly with nickel, say as a bound hydride, or whether it is bound to an atom X = O, S, or N that binds to Ni, say as an H_xO (H_2O or OH^-) ligand or even whether it is from a neighboring imidazole or amide nitrogen that is H bonded to a coordinating sulfur.

The available data are limited to the ENDOR spectra taken at a few magnetic field positions around the low-field edge of the EPR envelope because of the strong overlap of the Ni-C EPR signal with those of the iron–sulfur centers and because of the low EPR and ENDOR intensities of the Ni-C intermediate. Thus, it has not been possible to determine the full hyperfine tensors of these protonic species, normally the best basis for making an assignment. Nonetheless, reasonable progress in identification of H(1) and H(2) can be made. The argument to be presented requires an orbital assignment for the odd electron on the Ni-C. As noted above, the g values of the Ni-signal C ($g_x \neq g_y > g_z \approx 2$) indicate that its unpaired electron occupies the d_{z^2} orbital of nickel and that the nickel center has octahedral symmetry with a rhombic distortion.[3,15] Consider first the possible assignment as an exchangeable proton of an in-plane H_2O ligand to nickel. The distance between the nickel and proton should be about $r_H \approx 2.5$ Å or longer, in which case the point-dipole approximation to the dipolar interaction is valid[16] and the hyperfine tensor would be axial. Simulations[17] of ENDOR spectra that arise from an axial hyperfine interaction show that the dominant feature in the ENDOR spectra at every field will be a doublet whose hyperfine splitting is determined only by the perpendicular component of the hyperfine tensor, $A_\perp{}^H$

$$\nu_+{}^H - \nu_-{}^H = A_\perp{}^H = |a^H{}_{iso} - T^H/2| \qquad (3)$$

where $a^H{}_{iso}$ is the isotropic component and T^H is the through-space dipolar interaction constant of the proton with the unpaired electron on nickel:

$$T^H = (4/7)g_e\beta_e g_n\beta_n/r^3_{eH} \qquad (4)$$

Taking $r_{eH} \approx 2.6$ Å as in $Cu(H_2O)_6{}^{2+}$, eq 4 gives $T^H \approx 10$ MHz. If we assign the H(1) and H(2) doublets to $A_\perp{}^H$ features, then use of eqs 3 and 4 gives $a_{iso}(H(1)) \approx -12$ or 22 MHz and $a_{iso}(H(2)) \approx 0.5$ MHz, depending on the sign of $A_\perp{}^H$.

$Cu(H_2O)_6{}^{2+}$ provides a reference for an in-plane H_2O coordinated to the metal ion.[18] Here, the unpaired electron occupies a molecular orbital that involves the $d_{x^2-y^2}$ orbital on the copper ion and an sp hybrid on the oxygen. Spin polarization of the O–H bonds by spin on the oxygen gives a small isotopic coupling, $|a^H{}_{iso}(Cu)| \leq 1.15$ MHz; the through-space dipolar interaction is very near to that expected from eq 4, $T^H(Cu) \leq 10.6$ MHz. In the case of H_2O bound in-plane to the Ni-C site in hydrogenase, where the unpaired electron occupies the d_{z^2} orbital, spin density can only pass to the proton by spin polarization of the in-plane Ni–O bond by the d_{z^2} odd electron followed by spin polarization of the O–H bond. This extra polarization step means that $|a_{iso}|$ for the proton Ni-C site must be less than that for $Cu(H_2O)_6{}^{2+}$. The value of $T^H(Ni)$ would be comparable to that for $Cu(H_2O)_6{}^{2+}$, and, thus even without knowing the sign of a_{iso}, we can predict for a H_2O bound in-plane to Ni-C that $|A_\perp{}^H(Ni)| \leq |a_{iso}(Cu)| + T^H(Cu)/2 < 6.5$ MHz, which is much less than $A^H(1) = 16.8$ MHz but consistent with $A^H(2) \approx 4.4$ MHz.

An alternate possible assignment would be as the proton of an axially coordinated H_2O. To estimate the possible proton hyperfine coupling for such a ligand, we recall that ENDOR from aquomethmyoglobin[19] suggests that the ^{14}N hyperfine coupling constant for an in-plane (ip) nitrogen is roughly the same as that of the axial (ax) nitrogen (L = ^{14}N).

$$A^{ax}(L) \approx A^{ip}(L) \qquad (5)$$

The same is expected to be roughly true if L is the oxygen of an H_2O ligand,[20] which implies that the spin density on the oxygen bonded in-plane to a $d_{x^2-y^2}$ orbital is roughly equal to that for the oxygen bonded out-of-plane to d_{z^2}. This in turn implies that a_{iso} for the protons of an axial H_2O for Ni-C should be comparable to a_{iso} for the in-plane H_2O of $[Cu(H_2O)_6]^{2+}$. As the metal ion-to-proton distances should be comparable, one further expects $T^H(Ni) \approx T^H(Cu) \leq 10.6$ MHz (eq 3). Thus the prediction is $A^H{}_\perp(Ni^{ax}) \approx A^H{}_\perp(Cu^{ip}) \leq 6.5$ MHz. This is again inconsistent with the larger proton hyperfine coupling obtained for H(1) but not for H(2). We conclude that the strongly coupled exchangeable proton signal, H(1), is not assignable either to an in-plane H_2O or an axial H_2O ligand, whereas H(2) quite possibly assigned to coordinated H_2O.

A metal-bound H_xO might also be OH^-. ENDOR studies on aconitase[23] suggest that the solvent OH^- and H_2O bound to the $[4Fe\text{-}4S]^{1+}$ cluster exhibit comparable proton hyperfine coupling constants. One would expect similar behavior for the proton of solvent OH^- and H_2O bound to the Ni-C site. Thus we infer that the strongly coupled exchangeable protons, H(1), is unlikely to be from a H_xO solvent species, but the weakly coupled proton(s), H(2), could be from H_2O or OH^-.

We also do not favor the possibility that H(1) is associated with a Ni-bound cysteinyl sulfur that has been protonated to produce a mercaptan whose sulfur is bound to the Ni-C; it seems unlikely that this type of Ni–S bond could be sufficiently covalent as to lead to the large value of $A^H(1)$. For the same reason, it is unlikely that H(1) is on an amine or peptide nitrogen bound to nickel; in addition neither a nitrogen ENDOR signal nor a nitrogen hyperfine splitting of the EPR signals is observed for the Ni-C site. Similar arguments hold for a H bond to sulfur bound to nickel. In short it does not appear that H(1) can represent an X–H proton, X = O, S, N, where X is bound directly to nickel and the H(1) hyperfine coupling arises from transferred spin density. Instead, we infer that H(1) interacts directly with nickel.

A model often considered for the hydrogenase active site is that of a hydride bound to the Ni-C. One would expect that a hydride axially bound to the nickel would generate a strong crystal field, raising the energy of the d_{z^2} orbital above that of $d_{x^2-y^2}$. This picture is consistent with Ni(I) with an axially bound hydride. However, EPR results have been reported for $[HNi(CN)_4]^{2-}$,[21] which has a hydrogen (hydride) as an axial ligand to a nickel ion with odd electron in the d_{z^2} orbital. The EPR spectra disclosed substantial electron delocalization from the nickel to hydrogen, with an exceptionally large proton hyperfine coupling $A^H \approx 460$ MHz. This is roughly 27 times larger than $A^H(1)$ from *D. gigas* hydrogenase, and so that assignment can be discarded.

Alternatively, H(1) might be an in-plane hydride directly bonded to the Ni-C (which would favor a Ni(III) formulation[3]). In this case, a_{iso} would arise from $d_{z^2} \rightarrow d_{x^2-y^2}$ spin polarization on nickel and not direct delocalization from d_{z^2} and thus could be much smaller than for an axial hydride. As one possible

analysis, if we take the H(1) hyperfine tensor estimated for a single interacting proton from ENDOR and EPR line-narrowing and assign signs such that $A^H(1) = [+15, -22, -25]$ then $a_{iso} \approx -11$ MHz; the resulting dipolar tensor also appears to be acceptable[16] for the expected Ni-H distance,[25] $d \gtrsim 1.5$ Å. Although we tend to favor this assignment, these data also appear to be consistent with a not previously considered assignment of H(1) to an X-H (X = O, S, or N) moiety where the proton is involved in a direct (agostic) interaction with nickel.[26] Such an interaction is thought to stabilize higher valence states and again is most appropriate for Ni(III). Changing the proposed signs of the hyperfine tensor would give $a_{iso} \approx +11$ MHz, as expected for direct interaction; the dipolar interaction calculated from this or other assignments is reasonable for the expected Ni-H distance of $d \geq 2$ Å. It is straightforward to construct catalytic cycles that involve an intermediate with such a structure. Finally, we note the possibility of direct interaction with H_2. In this case, $d > 1.5$ Å is expected,[27] and formulation as a lower value state, Ni(I), would be most appropriate.

Conclusions

Our present EPR and ENDOR studies permit the following conclusions about the nickel site of *D. gigas* hydrogenase.

(1) The Ni-A site is inaccessible to solvent protons, consistent with the suggestion reached by activity studies that the enzyme in the Ni-A state is inactive.

(2) The Ni-C site is accessible to protons from solvent and/or gaseous hydrogen. This is in agreement with recent electron spin echo measurement[8] and is consistent with the Ni-C site having a catalytic role in H_2 activation. The ENDOR measurements have now characterized the exchangeable hydrogenic species. The Ni-C center exhibits one type of exchangeable proton that has a large hyperfine coupling ($A^H(1) = 16.8$ MHz). Analysis rules out as assignment an H_2O (or OH^-) or as a hydride bound axially to the Ni-C. Three other modes of direct interaction between H(1) and Ni appear to be possible: as an in-plane hydride, as an X-H proton directly interacting with nickel (agostic interaction), and even as H_2. A second type of exchangeable proton is associated with the Ni-C and has a smaller hyperfine coupling ($A^H(2) \approx 4.4$ MHz), which is consistent with the proton being associate with H_2O (or OH^-) bound to the Ni-C.

(3) At least one type of exchangeable deuteron associated with the Ni-C center remains associated with the Ni-B center after oxidation of the Ni-C. The small coupling is consistent with that of H_2O (or OH^-) bound to the Ni-B.

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Correlation of Nuclear Magnetic Deshielding with Compression of Interstitial Atoms in Transition-Metal Clusters

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Abstract: A correlation of NMR shift with compression is demonstrated for interstitial carbide and nitride in transition-metal clusters, from the trigonal prism to the octahedron, square prism and butterfly geometries. The nuclear magnetic shielding decreases with a decrease in the cavity radius, more clearly in simple clusters than in more complicated ones with capping or bridging species. This correlation explains the unusual shift pattern observed for interstitial carbides and nitrides. All cluster carbides are compressed below the normal covalent radius of carbon, and the shieldings are very low, below the normal range (except for carbonium ions). Interstitial nitride is less compressed (or even expanded from its normal radius), and nitride shieldings are much higher, lying within the range observed for organic compounds. This periodicity can be shown to extend to other interstitials, and the correlation is observed in non-metal cages also. Comparable correlations and the factors in nuclear magnetic shielding that are affected by the compression are discussed.

To the many correlations of NMR shift with molecular strain,[1] we now add a correlation with compression for interstitial carbide and nitride in transition-metal clusters. The figures and tables (which give references for individual clusters) show that nuclear shielding decreases with decrease in the cavity radius ($r(I) = r(MI)_{av} - r(MM)_{av}/2$, where the metal M is bonded to the interstitial I). In each figure, the shielding decreases from the trigonal prism to the octahedron-based species, including the square prism and butterfly (in which the interstitial shares all its valence electrons in σ and π bonding with the cluster[2,3]). Outliers are clusters with distorted cavities and (or) capping or bridging species; differences between (fluxional) solution and solid-state

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