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A pulsed EPR study of redox-dependent hyperfine interactions for the nickel centre of *Desulfovibrio gigas* hydrogenase

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The nickel centre of hydrogenase from *Desulfovibrio gigas* was studied by electron spin echo envelope modulation (ESEEM) spectroscopy in the oxidized, unready (Ni-A) and H_2-reduced active (Ni-C) states, both in H_2O and ^2H_2O solutions. Fourier transforms of the 3-pulse ESEEM, taken at 8.7 GHz, for Ni-A and Ni-C in H_2O contained similar peaks with narrow linewidths at frequencies of 0.4, 1.2 and 1.6 MHz, and a broader peak centred at 4.5 MHz. At 11.6 GHz, the low frequency components showed small field-dependent shifts, while the high frequency component was shifted to 5.1 MHz. These results are consistent with the presence of ^{14}N, possibly from imidazole, coupled to the nickel centre. In ^2H_2O, Ni-A was shown to be inaccessible for exchange with solvent deuterons. In contrast, Ni-C was accessible to solvent exchange, with a deuterium population being in close proximity to the metal ion. Thus, the nickel environment of the active protein is different from that in the oxidized or unready state. On illumination of Ni-C, although EPR changes are seen, ^{14}N coupling remains, and for the ^2H_2O sample, deuterium coupling is also retained.

Hydrogenase; EPR spectroscopy; Electron spin echo envelope modulation; Nickel; Hyperfine interaction

1. INTRODUCTION

Hydrogenases are enzymes, widely distributed in nature, which catalyze the interconversion of protons and H_2. They also catalyze hydrogen-isotope exchange between H_2 and H_2O. The hydrogenase from *Desulfovibrio gigas* contains a nickel centre [1,2], and in addition, one [3Fe-4S] cluster and two [4Fe-4S] clusters per molecule [3].

D. gigas hydrogenase shows characteristic nickel EPR signals dependent upon the redox state of the protein. The oxidized enzyme, as isolated, is in an 'unready state', and is unreactive towards H_2 [4]. It is EPR active, having a signal arising from nickel, designated as Ni-A (g = 2.32, 2.23, 2.01) [5]. After prolonged reductive activation, the EPR spectrum changes and gives the Ni-C signal (g = 2.19, 2.16, 2.01) [6]. Ni-C has been proposed as an intermediate in the reaction cycle [5,7]. The pH dependence of the midpoint potentials for the protein suggests that reduction of the Ni-A to the Ni-C state involves the addition of two electrons and 2–3 protons to the enzyme [8]. An unusual characteristic of the Ni-C state is that it is light-sensitive; at temperatures below 150 K it is converted by light to another species with a significantly different EPR spectrum (g = 2.35, 2.13, 2.045) [9], designated Ni-C* [2]. On annealing the enzyme by raising the temperature above 150 K, the spectrum reverts to the Ni-C form.

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Abbreviations: ESEEM, electron spin echo envelope modulation; EPR, electron paramagnetic resonance; shf, superhyperfine; nqi, nuclear quadrupolar interaction

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Significantly, the rates of photolysis of Ni-C [9] and annealing of Ni-C* show a strong kinetic isotope effect, being about 5-fold slower in ^2H_2O than in ^1H_2O (Cammack, R. and Chapman, A., unpublished).

The electron spin echo envelope modulation (ESEEM) technique of pulsed EPR spectroscopy provides a sensitive method to detect weak hyperfine interactions between paramagnetic metal centres and nuclei such as ^{14}N or ^2H [10]. Previously, the technique has been applied to the nickel-containing hydrogenases of *Methanobacterium thermoautotrophicum* [11] and *Thiocapsa roseopersicina* [12]. ESEEM data for the Ni centres of both of these enzymes showed similar low-frequency modulations indicative of weakly coupled ^{14}N. In this paper we describe the ESEEM spectra arising from nickel in the Ni-A and Ni-C states of *D. gigas* hydrogenase. We address the question of accessibility of these metal centres to solvent by comparing the ESEEM spectra collected for enzyme samples in aqueous buffer with spectra obtained in D_2O buffer. It was found that the nickel centre in the unready enzyme is inaccessible to solvent deuterium exchange, while it becomes accessible to deuterium during activation.

2. MATERIALS AND METHODS

2.1. *Preparation of hydrogenase samples*

Growth of *D. gigas* and subsequent hydrogenase purification were as described previously [13]. Enzyme samples were exchanged by ultrafiltration using an Amicon Centricon-30 membrane into 100 mM Hepes buffer, pH 7.8, with either ^1H_2O or ^2H_2O as solvent, and concentrated to 0.3–0.5 mM; the total time of exchange was at least 4 h. Enzyme samples used for ESEEM study were prepared directly in quartz tubes of 3 mm internal diameter.

The Ni-A species was present in the enzyme as prepared. To obtain the Ni-C species, the hydrogenase was first activated by saturating the solution with hydrogen gas, according to the method of Fernandez et al. [14], then incubated under hydrogen at room temperature for 4 h. The redox potential of concentrated enzyme samples was measured in EPR tubes with a platinum wire loop and a calomel reference electrode connected via a saturated KCl/agar bridge. The potential was adjusted to −280 mV by titrating under a nitrogen atmosphere with dithionite and ferricyanide, at pH 6.2, in the presence of mediators as described elsewhere [15]. The redox state of the nickel centre was monitored by continuous-wave EPR spectroscopy at low temperatures [14].

Illumination of samples was performed using a 300 W projector and a concave reflector to focus the light on the EPR sample tubes, which were immersed in liquid nitrogen in a quartz finger dewar.

2.2. *Method of pulsed EPR spectroscopy*

The pulsed EPR spectrometer used for these studies is described elsewhere [16]. The cavity used for the ESEEM experiments was a folded stripline, which could accommodate frozen samples in conventional 3 mm internal diameter quartz EPR tubes [17,18]. ESEEM data were collected at 1.8 K using the stimulated echo technique ($90°-\tau-90°-T-90°$) with values of τ chosen to suppress modulations arising from weakly coupled protons [19]. Spectra were obtained by Fourier transformation using a modified version of the dead time reconstruction method of Mims [20]. Spectra at higher microwave frequencies were obtained by thawing the samples and transferring them from the EPR tubes to a stripline transmission cavity [21]. In the case of the Ni-C samples this was done in an anaerobic chamber under a N_2 atmosphere.

3. RESULTS

3.1. *ESEEM spectra of Ni-A and Ni-C*

Fig.1 (top) shows the ESEEM spectrum of Ni-A in ^1H_2O, recorded at $g = 2.23$, corresponding to the maximum in the EPR absorption envelope. It shows three sharp lines at 0.4, 1.2 and 1.6 MHz; there is also a broader feature centred at 4.5 MHz with maxima at 4.2 and 4.8 MHz. These four spectral lines are characteristic of ^{14}N hyperfine interactions, where the isotropic portion of the electron-nuclear hyperfine coupling, A_{iso}, is approximately equal to twice the ^{14}N nuclear Larmor frequency. Under these conditions, the ^{14}N superhyperfine splittings for one of the electron spin manifolds will be almost completely determined by the nuclear quadrupole interaction (nqi). Because this interaction is independent of field strength and direction, we observe three sharp lines in the ESEEM spectrum where the two lower frequency components add to give the frequency of the third [22,23]. The broad peak at 4.5 MHz originates from the electron spin manifold where the hf and nuclear Zeeman terms are additive. The complex lineshape of the component is a function of the anisotropy in the shf tensor, the relative orientations of the shf and nqi tensors with respect to the g tensor, and the effective g value of the ESEEM measurement.

An ESEEM spectrum of Ni-C in ^1H_2O recorded at $g = 2.16$ is shown in fig.2 (top). Modulations that give rise to this spectrum were only observed within the range of magnetic field settings of the Ni-C EPR spectrum, indicating that they were not due to iron-sulphur clusters or other nickel species such as Ni-A. The peak positions of the four principal modulation components (0.4, 1.2, 1.6 and

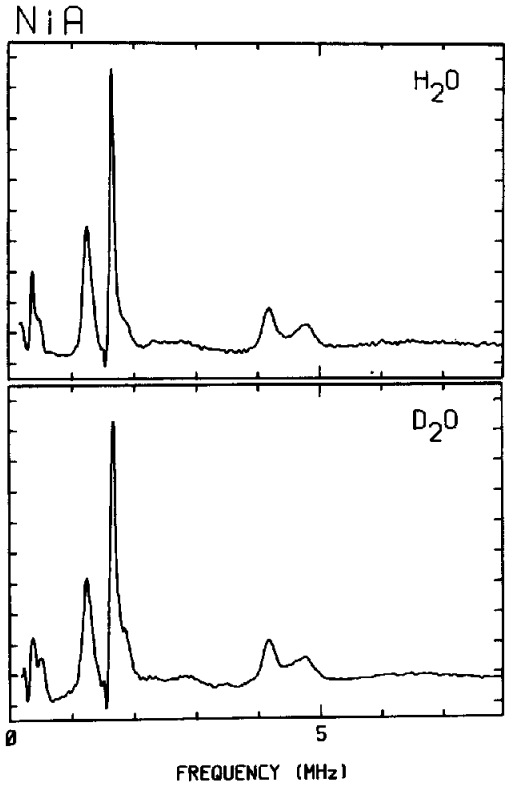

Fig.1. ESEEM spectra for Ni-A in (top) ^1H_2O and (bottom) ^2H_2O buffer. The measurement conditions were: microwave frequency, 8.7 GHz; magnetic field strength, 2790 G; microwave pulse power, 50 W (20 ns full width at half maximum); sample temperature, 1.8 K; and τ value, 252 ns.

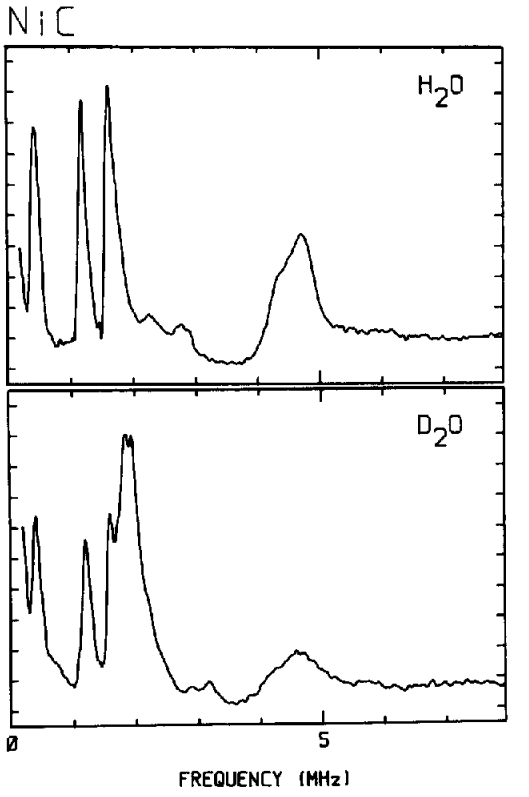

Fig.2. ESEEM spectra for Ni-C in (top) ^1H_2O and (bottom) ^2H_2O buffer. Measurement conditions were identical to those given for fig.1 except for magnetic field strength, 2900 G; and τ value, 243 ns. Vertical scales for both spectra are different. As a consequence, ^{14}N components in the bottom spectrum appear reduced in amplitude.

4.5 MHz) are essentially unchanged from those found for Ni-A at $g = 2.23$ (fig.1). Thus, the coupling between Ni and the ^{14}N nucleus giving rise to the observed ESEEM has not been greatly altered. However, the relative amplitudes of the components and the lineshape of the 4.5 MHz peaks are completely different. Because of the large changes that occur for the Ni g tensor in going from the unready, Ni-A state to the reduced, Ni-C form [14], these amplitude and lineshape differences are not surprising. It should be noted that the relative amplitudes of the low frequency, narrow lines are primarily a function of the orientation of the nqi tensor with respect to the g tensor and the field position, or effective g value for the measurement.

3.2. *Deuterium exchange*

A comparison of the ESEEM spectra of the Ni-A centre in H_2O and in ^2H_2O shows no appreciable difference (fig.1). In contrast the [3Fe-4S] cluster, detected at $g = 2.01$, showed significant coupling with exchangeable deuterium as evidenced from the appearance of a broad spectral line at the Larmor frequency of ^2H (not shown).

Conversion of Ni-A to Ni-C by redox titration in ^2H_2O resulted in the appearance of an additional deep modulation component in the time echo decay envelope. The Fourier transform then contained a spectral line at 1.9 MHz, corresponding to the ^2H Larmor frequency (fig.2, bottom). When the Ni-C sample in ^2H_2O was studied at 11.6 GHz the observed shift for the 1.9 MHz peak to 2.5 MHz supports the assignment of this line to interactions with ^2H nuclei. The same ^2H hyperfine contributions were observed in Ni-C samples produced by reduction of hydrogenase in ^2H_2O with

^2H gas. The large amplitude and broad linewidth of the ^2H ESEEM component for Ni-C are a result of a large anisotropic contribution to the Ni-deuterium hyperfine coupling, indicative of an inner coordination sphere contribution [24].

Hydrogenase that had been reduced in ^2H_2O was reoxidized with oxygen to give the Ni-A species. The ESEEM spectra then showed a broad line at 1.9 MHz attributable to a ^2H hyperfine interaction in addition to those ^{14}N components observed in Ni-A prior to reduction in ^2H_2O. Moreover, on transferring the reoxidized protein into ^1H_2O buffer, the ^2H line remained, showing that the ^2H bound to nickel would not exchange completely with water. The amplitude of this ^2H line was approximately half that of the sharp ^{14}N component at 1.6 MHz.

3.3. *Effects of illumination*

The effect of light on the Ni-C species is illustrated in fig.3 (top). The spectrum shows some changes in the relative intensities of the ^{14}N hyperfine lines, which are most probably caused by changes in the g tensor of the Ni. Ni-C samples in ^2H_2O that had been illuminated still showed deep deuterium modulation (fig.3, bottom).

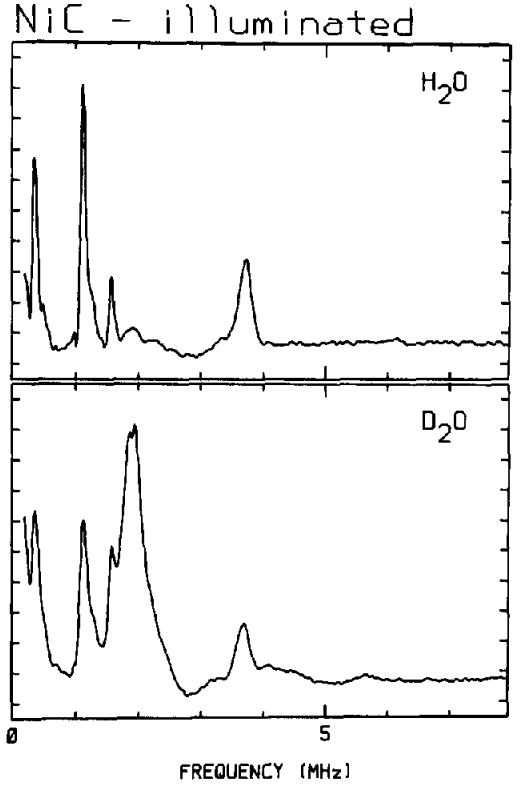

Fig.3. ESEEM spectra for Ni-C after illumination in (top) ^1H_2O and (bottom) ^2H_2O buffer. Measurement conditions were identical to those given for fig.2.

4. DISCUSSION

The ^{14}N ESEEM spectra for the Ni centre of *D. gigas* hydrogenase are similar to those observed in the nickel-hydrogenases of *M. thermoautotrophicum* (deazaflavin-reducing) [11] and *T. roseopersicina* [12], and confirm the similarities between these enzymes as suggested by their EPR spectra. The ^{14}N shf coupling for these enzymes is indicative of ^{14}N in close proximity to the nickel centres in all of them. Previous EXAFS studies have demonstrated sulphur coordination to the metal [25,26]. It was implied [26] that lighter atom coordination could be masked by the predominance of sulphur scattering in the data, making complete structural assignment difficult. Further, from the magnitude of nuclear quadrupole parameters for both Ni-A and Ni-C as determined from the ESEEM, it is suggested that electron nuclear interaction that is observed arises from a ^{14}N nucleus that has a relatively small electric field gradient associated with it ($e^2qQ \simeq 1.9$ MHz). Such an environment is consistent with that found for the remote nitrogen of a strongly coupled histidyl imidazole ligand or the directly coordinated nitrogen of a weakly coupled imidazole [27]; however, it is inconsistent with the environment typically found for tri-coordinate ^{14}N, where a lone pair of electrons is present in the non-hybridized p-orbital. Moreover, since the ^{14}N lines in the spectrum are sharp and no combination frequencies [23] are observed, this suggests that only a single ^{14}N is coupled to the electron spin of nickel.

The apparent inaccessibility of the nickel site in the oxidized Ni-A enzyme to exchangeable deuterium ions, as detected by ESEEM, is perhaps unexpected for a centre which has been implicated in the reduction of solvent protons to water. However, it is consistent with other observations on this state. The lack of activity of the oxidized enzyme in hydrogen isotope exchange assays [28,29], and hydrogen-uptake activity with high-potential acceptors [4] indicate that the oxidized

nickel site is unreactive with hydrogen gas. The apparent accessibility of the Ni-C site to solvent protons is consistent with catalytic function towards hydrogen.

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REFERENCES

[1] Cammack, R., Hall, D.O. and Rao, K.K. (1985) in: Microbial Gas Metabolism (Poole, R.K. and Dow, C. eds) pp.209–213, Academic Press, New York.
[2] Cammack, R., Fernandez, V.M. and Schneider, K. (1988) in: Bioinorganic Chemistry of Nickel (Lancaster, J.R. jr ed.) pp.167–189, VCH Publishers, Deerfield Beach, Florida.
[3] Teixeira, M., Moura, I., Xavier, A.V., DerVartanian, D.V., LeGall, J., Peck, H.D., Huynh, B.H. and Moura, J.J.G. (1983) Eur. J. Biochem. 130, 481–484.
[4] Fernandez, V.M., Hatchikian, E.C. and Cammack, R. (1985) Biochim. Biophys. Acta 832, 69–79.
[5] Teixeira, M., Moura, I., Xavier, A.V., Huynh, B.H., DerVartanian, D.V., Peck, H.D. jr, LeGall, J. and Moura, J.J.G. (1985) J. Biol. Chem. 260, 8942–8950.
[6] LeGall, J., Ljungdahl, P.O., Moura, I., Peck, H.D., Xavier, A., Moura, J.J.G., Teixeira, M., Huynh, B.H. and DerVartanian, D.V. (1982) Biochem. Biophys. Res. Commun. 106, 610–616.
[7] Cammack, R., Patil, D. and Fernandez, V.M. (1985) Biochem. Soc. Trans. 13, 572–578.
[8] Cammack, R., Patil, D.S., Hatchikian, E.C. and Fernandez, V.M. (1987) Biochim. Biophys. Acta 912, 98–109.
[9] Van der Zwaan, J.W., Albracht, S.P.J., Fontijn, R.D. and Slater, E.C. (1985) FEBS Lett. 179, 271–277.
[10] Mims, W.B. and Peisach, J. (1980) in: Biological Applications of Magnetic Resonance (Shulman, R.G. ed.) pp.221–269, Academic Press, New York.
[11] Tan, S.L., Fox, J.A., Kojima, N., Walsh, C.T. and Orme-Johnson, W.H. (1984) J. Am. Chem. Soc. 106, 3064–3066.
[12] Cammack, R., Kovacs, K., McCracken, J. and Peisach, J. (1989) Eur. J. Biochem., submitted.
[13] Hatchikian, E.C., Bruschi, M. and LeGall, J. (1978) Biochem. Biophys. Res. Commun. 82, 451–461.
[14] Fernandez, V.M., Hatchikian, E.C., Patil, D.S. and Cammack, R. (1986) Biochim. Biophys. Acta 883, 145–154.
[15] Chapman, A., Cammack, R., Weiner, J.H., McCracken, J. and Peisach, J. (1988) Biochim. Biophys. Acta, in press.
[16] McCracken, J., Peisach, J. and Dooley, D.M. (1987) J. Am. Chem. Soc. 109, 4064–4072.
[17] Lin, C.P., Bowman, M.K. and Norris, J.R. (1985) J. Magn. Reson. 65, 369–374.
[18] Britt, R.D. and Klein, M.P. (1987) J. Magn. Reson. 74, 535–540.
[19] Mims, W.B. (1972) Phys. Rev. B: Solid State 5, 2409–2419.
[20] Mims, W.B. (1984) J. Magn. Reson. 59, 291–306.
[21] Mims, W.B. (1974) Rev. Sci. Instrum. 45, 1583–1591.
[22] Mims, W.B. and Peisach, J. (1978) J. Chem. Phys. 69, 4921–4930.
[23] McCracken, J., Pember, S., Benkovic, S.J., Villafranca, J.J., Miller, R.J. and Peisach, J. (1988) J. Am. Chem. Soc. 110, 1069–1074.
[24] Mims, W.B., Davis, J.L. and Peisach, J. (1984) Biophys. J. 45, 755–766.
[25] Scott, R.A., Wallin, S.A., Czechowski, M., DerVartanian, D.V., LeGall, J., Peck, H.D., jr and Moura, I. (1984) J. Am. Chem. Soc. 106, 6864–6865.
[26] Lindahl, P.A., Kojima, N., Hausinger, R.P., Fox, J.A., Teo, B.K., Walsh, C.T. and Orme-Johnson, W.H. (1984) J. Am. Chem. Soc. 106, 3062–3064.
[27] Ashby, C.I.H., Cheng, C.P. and Brown, T.L. (1978) J. Am. Chem. Soc. 100, 6057–6063.
[28] Berlier, Y.M., Fauque, G., Lespinat, P.A. and LeGall, J. (1982) FEBS Lett. 140, 185–188.
[29] Hallahan, D.L., Fernandez, V.M., Hatchikian, E.C. and Cammack, R. (1986) Biochim. Biophys. Acta 874, 72–75.