# Electron spin–echo spectroscopy of the [Fe]-hydrogenase from Desulfovibrio desulfuricans, strain ATCC 7757

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# Electron Spin–Echo Spectroscopy of the [Fe]-Hydrogenase from *Desulfovibrio desulfuricans*, Strain ATCC 7757

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Hydrogenase from the sulfate-reducing bacterium *Desulfovibrio desulfuricans*, strain ATCC 7757, contains three iron–sulfur clusters: two [4Fe–4S] 'F' clusters and one 'H' cluster, believed to represent the active site for reaction with hydrogen. Electron spin–echo envelope modulation (ESEEM) in pulsed electron paramagnetic resonance (EPR) was applied to these clusters, in their paramagnetic oxidation states. Three-pulse, stimulated-echo spectra of the oxidized H-cluster showed modulations indicative of cancellation of the nuclear hyperfine and Zeeman interactions, permitting estimation of the quadrupole coupling. The value for the quadrupole coupling constant is unusually high. These data are compared with previous results from the hydrogenase of *Clostridium pasteurianum*, and show that the nitrogen coordination is conserved between these distantly related species. Spectra of samples exchanged into $^2H_2O$ showed deep nuclear modulations for the reduced F clusters, and weaker modulations for the oxidized H clusters. This is evidence for exchangeable protons in close proximity to Fe–S clusters.

Hydrogenases are enzymes, produced by micro-organisms, which catalyse the production or consumption of molecular hydrogen. While the majority of hydrogenases that have been isolated contain iron–sulfur clusters and nickel, there is a group, known as the [Fe]-hydrogenases, which contain iron as the only metallic element. These enzymes all contain various numbers of conventional [4Fe–4S] clusters which act as secondary electron carriers. However, the active site in the [Fe]-hydrogenases, which interacts with hydrogen, appears to be a specific type of iron–sulfur cluster known as the H-cluster. This cluster has been shown, by chemical analysis and Mössbauer spectroscopy, to contain *ca.* six iron atoms, of two different types, but its structure is unknown.[1–3] In the oxidized, active form of the enzyme, the H-cluster yields a unique rhombic EPR signal with *g*-factors between 2.0 and 2.10.[4,5] Comparison of the invariant residues in the amino acid sequences suggest that some of the ligands to the H cluster are cysteine sulfurs.[6,7]

[Fe]-hydrogenases have been isolated from two main groups of bacteria. These are the fermentative bacteria such as *Clostridium pasteurianum*, from which two different hydrogenases, I and II, have been isolated;[1,2] and the sulfate-reducing bacteria, typified by *Desulfovibrio vulgaris*, strain Hildenborough.[3,8] The enzymes differ in their subunit composition, iron content and size. In the case of *C. pasteurianum* hydrogenase I, there is evidence from spin–echo spectroscopy that two nitrogen ligands are also coordinated to the cluster.[9]

The [Fe]-hydrogenase of the sulfate-reducing bacterium *D. desulfuricans*, strain ATCC 7757, has recently been demonstrated to be similar to that of *D. vulgaris*, Hildenborough.[10] In the present paper, we present investigations of the coordination environment of the iron–sulfur clusters, by ESEEM spectroscopy. This pulsed EPR technique has been shown to be valuable for the detection of weak hyperfine and quadrupole couplings to metal centres in proteins.[11,12] Measurements were made on $^{14}N$ of potential ligands to the iron–sulfur clusters, and exchangeable deuterons which, in the case of the H cluster, might act as the substrate for hydrogen production.

## Experimental

The hydrogenase was purified using a modified version of the scheme of Glick *et al.*,[13] as previously described.[10] EPR samples were prepared in 100 mmol $dm^{-3}$ *N*-(2-hydroxyethyl)-piperazine-*N'*-(2-ethanesulfonate) buffer (pH 8.0). For the enzyme in $^1H_2O$, fully reduced samples were made by reduction under $^1H_2$ gas. The samples in $^2H_2O$ were prepared by diluting oxidized enzyme with buffer made with $^2H_2O$, and then concentrating 10-fold in a centrifugal ultrafiltration cell (Amicon Centricon 30), and re-diluting in $^2H_2O$ buffer, three times. The enzyme was reduced with a small excess of sodium dithionite in the presence of 5 μmol $dm^{-3}$ methyl viologen. Partially oxidized samples showing the H-cluster signal were made by allowing the enzyme, after an activation period, to auto-oxidize by stirring under nitrogen or argon gas.

Continuous-wave EPR spectra were recorded on a Bruker ESP300 X-band spectrometer, equipped with a TE102 resonator, with an Oxford Instruments ESR900 liquid helium flow cryostat. Pulsed EPR measurements were made on a Bruker ESP380 spectrometer, equipped with a variable *Q* dielectric resonator, in an Oxford Instruments CF935 immersion cryostat. ESEEM data were collected at 4 K using either the 2-pulse (90°–τ–180°–echo) or the three-pulse stimulated echo (90°–τ–90°–T–90°–echo) procedures. For the latter method, values of τ used were calculated for maximal suppression of modulations due to weakly coupled protons. The data collected were linear phase corrected and the exponential decay subtracted, before Fourier transformation using the Bruker pulsed spectra manipulation routines.

## Results

Continuous-wave, first-derivative EPR spectra of *D. desulfuricans* [Fe]-hydrogenase samples used for ESEEM are shown in Fig. 1, together with their integrated forms, which correspond to the absorption-mode signal as observed in pulsed EPR. Fully reduced samples of the hydrogenase showed an EPR signal, with a complex lineshape, typical of coupled [4Fe–4S] (F) clusters [Fig. 1(*a*)] as previously described.[10,14] Partially oxidized samples showed a rhombic EPR signal, with *g*-factors 2.10, 2.04, 2.00 as shown in Fig. 1(*c*) and (*d*).

ESEEM experiments were carried out on both fully reduced (F cluster) and partially oxidized (H cluster) samples in both $^1H_2O$ and $^2H_2O$. ESEEM spectra were recorded over a range of *g* factors but, to avoid spectral overlap, the spectra presented here were recorded at $g = 1.97$ for the F-

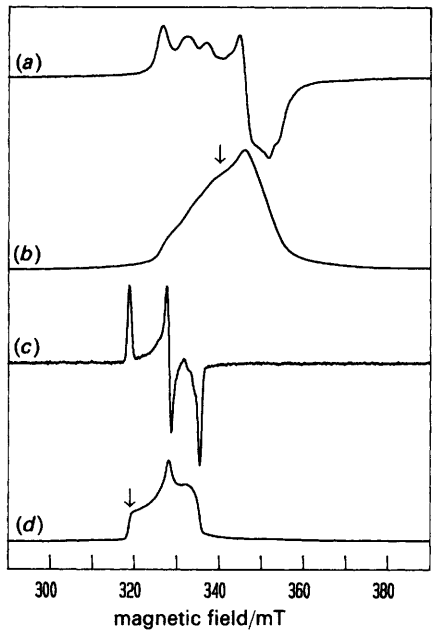


**Fig. 1** X-Band c.w. EPR spectra of *D. desulfuricans* ATCC 7757 hydrogenase: (*a*) fully reduced; (*b*) integral of (*a*); (*c*) partially oxidized; (*d*) integral of (*c*). Measurement conditions: microwave frequency, 9.37 GHz; modulation amplitude, 1 mT; modulation frequency, 100 MHz; temperature, 15 K; microwave power (*a*) 20 mW, (*b*) 0.02 mW. Arrows indicate the *g* factors used for ESEEM measurements.

cluster signal, and $g = 2.098$ for the H-cluster signal, as indicated in Fig. 1. For the F clusters in $^1H_2O$, two-pulse measurements revealed only modulations due to matrix protons. Three pulse ESEEM measurements were also carried out, using a $\tau$ value of 136 ns to suppress proton modulations. In these spectra the echo envelopes were featureless and the Fourier transforms showed no frequencies that could be attributed to other coupled nuclei such as $^{14}N$ (data not shown). A similar absence of nitrogen modulations has been noted for the [4Fe–4S] clusters of ferredoxins.[15,16] This is in contrast to [2Fe–2S] clusters in proteins, in which hyperfine interactions with peptide $^{14}N$ nuclei are easily detectable.[15–17]

When the protein was transferred to $^2H_2O$, ESEEM spectra of the F clusters showed deep modulations. The Fourier transform was dominated by a peak at the Larmor frequency of the $^2H$ nucleus (Fig. 2). Similar deep deuterium modulations have been seen in two-pulse ESEEM spectra of [4Fe–4S] ferredoxins in $^2H_2O$.[15,16]

ESEEM spectra of the H-cluster, recorded at a magnetic field corresponding to the low-field extremum of the EPR spectrum, are shown in Fig. 3 and 4. At this position, the spectra derive from a subset of the protein molecules have a unique orientation relative to the applied magnetic field.[18]

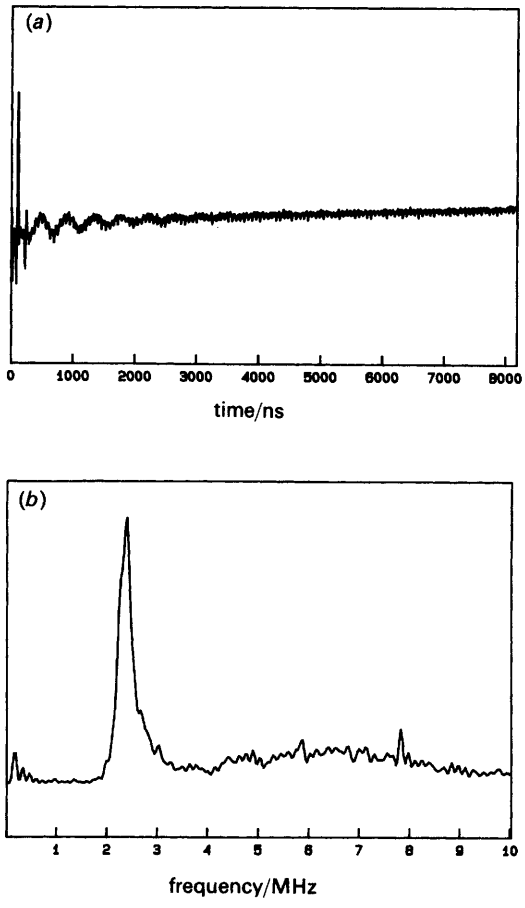


**Fig. 2** X-Band three-pulse ESEEM spectrum of a fully reduced sample of *D. desulfuricans* hydrogenase in $^2H_2O$: (*a*) echo envelope; (*b*) frequency domain obtained by Fourier transform of (*a*). Measurement conditions: microwave frequency, 9.759 GHz; magnetic field, 353.7 mT; $\tau$ value, 136 ns; pulse width, 16 ns; shot repetition time, 20 ms; temperature, 3.8 K.

For the H-cluster in $^1H_2O$, a complex pattern of modulations was seen in the echo envelope [Fig. 3(*a*)]. The Fourier transform showed several sharp lines [Fig. 3(*b*); Table 1]. These exceptionally narrow lines are characteristic of an electron spin manifold in which the hyperfine interaction approximately cancels the nuclear Zeeman term.[11] In this condition, the modulation frequencies correspond to NQR frequencies $\nu_0$, $\nu_-$ and $\nu_+$. From these, the nuclear quadrupole couping $e^2qQ/h$ and the asymmetry parameter $\eta$ can be estimated (Table 1).

A similar modulation pattern has been observed for the H-cluster signal of the [Fe]-hydrogenase of *C. pasteurianum*.[9] Thomann *et al.*[9] carried out a detailed study of this spectrum, by the use of ESEEM at two different frequencies, and of pulsed ENDOR. The latter method has proved to be comple-

**Table 1** ESEEM modulation frequencies for the H-cluster in $^1H_2O$; spectra were recorded as for Fig. 3

| ESEEM conditions | hydrogenase | | |
|---|---|---|---|
| | *D. desulfuricans*[a] | *C. pasteurianum*[b] | |
| magnetic field/mT | 332.4 | 270.6 | 310.1 |
| microwave frequency/GHz | 9.759 | 7.93 | 9.087 |
| *g*-factor | 2.098 | 2.094 | 2.094 |
| NQR frequencies: $\nu_0$, $\nu_-$, $\nu_+$/MHz | 0.84, 3.31, 4.05 | 0.5, 3.39, 3.89 | 0.58, 3.37, 3.95 |
| $(e^2qQ/h)$/MHz | 4.90 | 4.85 | 4.88 |
| $\eta$ | 0.30 | 0.20 | 0.24 |

[a] This work. [b] Ref. 9.

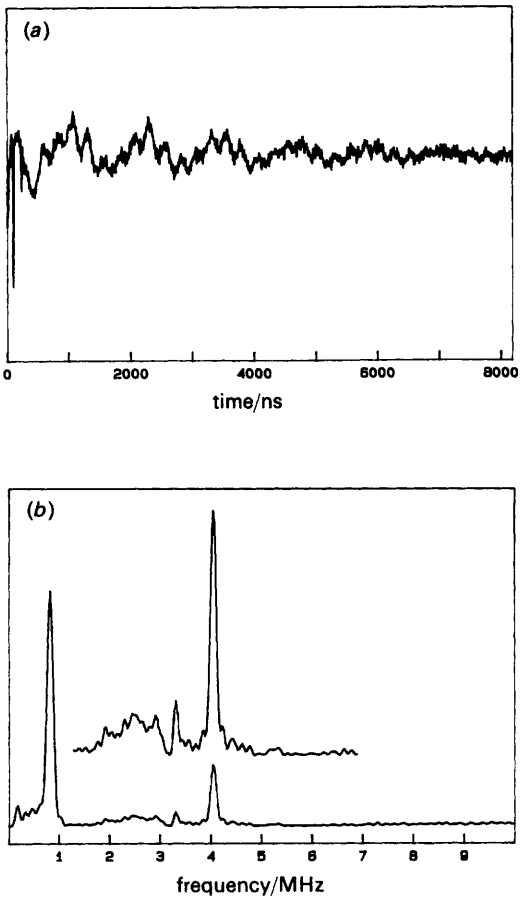


**Fig. 3** X-Band three-pulse ESEEM spectrum of a partially oxidized sample of *D. desulfuricans* hydrogenase in $^1H_2O$: (*a*) echo envelope; (*b*) frequency domain obtained by Fourier transformation of (*a*). Measurement conditions as in Fig. 2 except for: magnetic field, 332.4 mT; temperature, 3.9 K. The inset region is amplified four-fold.

mentary to ESEEM, in that the nuclear frequencies which are of low intensity in ESEEM, are more intense in pulsed ENDOR, and *vice versa*. Their analysis confirmed that the three most intense lines in the ESEEM spectrum correspond to NQR frequencies of one electron spin manifold of a particular $^{14}N$ nucleus [N(1)], coordinated to the H-cluster. The frequencies associated with the other electron spin manifold are $\nu_0$, $\nu_-$, $\nu_+ \approx 2.4$, 2.4, 5.4 MHz. The lines centred at 2.4 MHz are spread over a wide frequency range due to anisotropy of the hyperfine interaction. Similar features are visible in the spectrum of *D. desulfuricans* hydrogenase [Fig. 3(*b*)], on expanded scale.

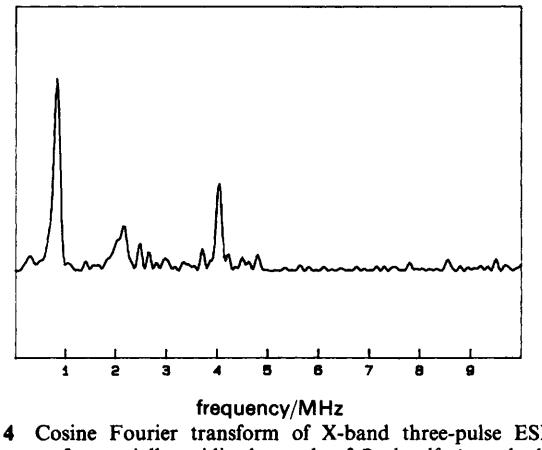


**Fig. 4** Cosine Fourier transform of X-band three-pulse ESEEM spectrum of a partially oxidized sample of *D. desulfuricans* hydrogenase in $^2H_2O$. Measurement conditions as in Fig. 2 except for: magnetic field, 332.3 mT; temperature, 3.7 K.

The spectra of *C. pasteurianum* hydrogenase were simulated by Thomann *et al.*,[9] using a hamiltonian incorporating the hyperfine and quadrupolar tensors. The isotropic hyperfine coupling was estimated to be $|A_{iso}| \approx 1.2$ MHz, indicating a covalent interaction between the nitrogen and the iron. The value of $e^2qQ/h$, 4.85 MHz, is unusually large, and indicates a nitrogen in an unusual state of coordination. The nearest typical value for the types of nitrogen found in proteins, is 3.4 MHz for peptide nitrogens.[19] The values observed in *D. desulfuricans* hydrogenase are consistent with the *C. pasteurianum* data, if corrected for magnetic field (Table 1).

In their analysis of the ESEEM spectra of the *C. pasteurianum* [Fe]-hydrogenase, Thomann *et al.* obtained evidence for a second $^{14}N$ nucleus [N(2)] coupled to the H-cluster. This gave rise to another modulation frequency at 3.68 MHz at 270.6 mT, and 3.87 MHz at 310.1 mT.[9] Additional weak resonance were observed at combination frequencies for the two nitrogens, N(1) and N(2). In our measurements at 332.4 mT, the corresponding frequency would be expected at 4.0 MHz, which would be concealed under the $\nu_+$ line for N(1). No combination frequencies were detected above noise level. Hence, we are not able to determine, from the present data, if a similar nitrogen is coupled to the H-cluster in the *D. desulfuricans* hydrogenase.

Since the substrate of the enzyme is hydrons, it is of interest to look for exchangeable hydrogens in the vicinity of the H-cluster. ESEEM spectra of the H-cluster in hydrogenase samples in $^2H_2O$, are shown in Fig. 4. Nitrogen modulations were observed that were similar to those obtained in $^1H_2O$. In addition a resonance may be seen at 2.16 MHz, corresponding to the deuterium Larmor frequency at this field.

## Conclusions

The ESEEM spectra of the H clusters of the [Fe]-hydrogenases reveal hyperfine couplings to nitrogen nuclei that are not detected by conventional continuous-wave ENDOR spectroscopy.[20] The magnitude of the isotropic hyperfine coupling indicates that the nitrogen is covalently bonded to the cluster, but the quadrupole coupling parameters indicate a very unusual coordination. This nitrogen coupling is conserved in the two distantly related hydrogenases from fermentative and sulfate-reducing bacteria.

In samples prepared in $^2H_2O$, modulations attributed to exchangeable deuterons were observed in the F clusters and the H-cluster. This if the first evidence for hydrogen–deuterium exchange in the oxidized form of the H-cluster, which is believed to be the catalytic site of hydrogen production.

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