REDOX PROPERTIES OF THE ESR-DETECTABLE NICKEL IN HYDROGENASE FROM *DESULFOVIBRIO GIGAS*

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1. Introduction

Hydrogenases (EC 1.12) from numerous bacterial and algal sources are known to be iron–sulphur proteins [1]. A number of them either contain nickel [2] or require nickel for activity [3–6]. An electron-spin resonance (ESR) signal, with rhombic symmetry, has been observed in membranes of *Methanobacterium bryantii* [7]. The signal was tentatively assigned as low-spin Ni(III) because its g-values are all above $g = 2$, indicating a d^7 system, and there is no hyperfine structure [7]. This assignment was confirmed in a nickel-containing hydrogenase isolated from *M. thermoautotrophicum* [8] by substitution with ^{61}Ni and observing the expected hyperfine splitting in the spectrum. The ESR spectrum therefore provides a spectroscopic probe for the properties and function of nickel in hydrogenase.

Hydrogenase of *Desulfovibrio gigas* contains 12 atoms of iron and 12 of acid-labile sulphide in a molecule of M_r 89 500 [9]. We have now detected ~1 nickel/atom enzyme molecule. The ESR spectrum of the oxidized protein contains an intense narrow signal at $g = 2.02$ due to an oxidized iron–sulphur cluster [10]. We have now detected a rhombic signal which is very similar to that from nickel in *M. thermoautotrophicum* hydrogenase. Though broader, this signal is of significant intensity. By using redox titrations we have measured the midpoint reduction potential, E_m of the ESR-detectable nickel, to be −145 mV at pH 7.2. This is higher than the H^+/H_2 couple but much lower than the E_m-values normally found for Ni(III) complexes. Furthermore, the E_m is pH-dependent, indicating that proton transfer takes place during reduction. The Ni(III) signal indicates no superhyperfine splittings due to interaction with nitrogenous ligands or exchangeable protons. Power saturation studies indicate that the ESR-detectable nickel is distant from the ESR-detectable iron–sulphur cluster. The possibility that the nickel may be involved in the reaction cycle of the enzyme is considered.

2. Materials and methods

Hydrogenase was isolated from *D. gigas* cells by a modification of the method in [9] but with particular precautions to exclude oxygen. The buffers used were degassed and all fractions were kept under argon atmosphere. The enzyme had spec. act. 200 μmol H_2 evolved . min^{-1} . mg $protein^{-1}$ and was stored in liquid nitrogen before use. Optical absorption spectra showed negligible contamination by haem. Analysis for nickel was done on a Unicam model SP 1900 atomic absorption spectrometer. Solutions of $Ni(NO_3)_2$ and $Ni(ClO_4)_2$ were used as standards. As a check for accidental contamination during purification, the buffers used were found to contain negligible nickel; also cytochrome c_3 purified in the same preparation with hydrogenase was found to contain <0.02 g atom Ni/mol protein.

ESR spectra were recorded on a Varian E4 spectrometer with an Oxford Instruments helium-flow cryostat. Signal intensities were determined by double integration with correction for baseline, and compared with a Cu-EDTA standard. Redox potentials were measured by titration of the protein with $Na_2S_2O_4$ or $K_3Fe(CN)_6$ in the presence of dye mediators and a platinum electrode [11], and withdrawing samples for ESR spectroscopy.

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3. Results

3.1. *Determination of the nickel content of* D. gigas *hydrogenase*

Five different samples of hydrogenase were analyzed for nickel content. The mean value obtained was 0.95 ± 0.02 g atoms/mol. This is consistent with a content of 1 atom/enzyme molecule.

3.2. *ESR spectra of oxidized hydrogenase*

At <30 K the spectrum of the enzyme (fig.1) was dominated by a narrow signal at $g = 2.02$, which indicates a [4 Fe–4 S]$^{3+}$ or [3 Fe–3 S]$^{3+}$ cluster. At higher temperatures this disappeared and a rhombic spectrum remained to lower field. This spectrum at 110 K is shown in fig.1 at a greatly increased gain.

Although its amplitude is small at 23 K relative to the $g = 2.02$ signal, its intensity is significant, because:

(i) The unpaired spin on the nickel was relaxing relatively slowly at low temperature, so that the signal was saturated. The signal could in fact be detected at up to 220 K before relaxation broadening was observed;

(ii) The rhombic spectrum is considerably broader than the $g = 2.02$ signal.

Integrated intensities of the ESR signals, recorded under non-saturating conditions, were measured on a 149 μM enzyme sample. The values obtained were 68 μM [spin] for the nickel signal and 88 μM for the $g = 2.02$ signal. Hence not all the nickel in the enzyme preparation was ESR-detectable.

3.3. *The midpoint reduction potential of ESR-detectable Ni(III)*

E_m for the Ni(III) was determined from graphs of the $g = 2.24$ signal amplitude against applied redox potential (fig.2). The values are consistent with a 1-e$^-$ reduction process. The titrations showed good reversibility during reduction and reoxidation. The intensity of the fully-oxidized Ni(III) signal decreased by 10% or less during each titration, and there was a corresponding decrease in activity. The E_m-values were pH-dependent, changing by –60 mV/pH unit (fig.2).

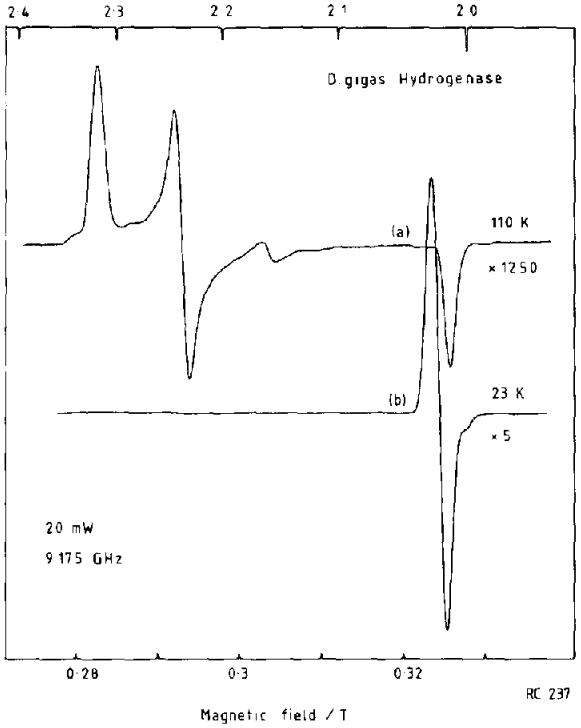

Fig.1. ESR spectra of *D. gigas* hydrogenase, as prepared: (a) recorded at 110 K, gain setting 1250; (b) recorded at 23 K, gain setting 5. Spectra were recorded on a Varian E-4 spectrometer with the following instrument settings: microwave power, 20 mW; frequency, 9.175 GHz; modulation amplitude, 1 mT.

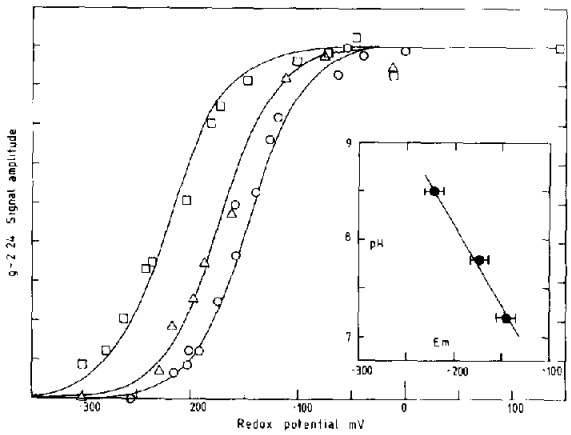

Fig.2. Redox titration of the $g = 2.24$ signal due to Ni: (○) titration done in 0.1 M MOPS buffer (pH 7.2); (△) in 0.1 M Tris–HCl, 0.1 M glycine (pH 7.8); (□) in 0.1 M Tris–HCl, 0.1 M glycine (pH 8.5). The mediators (all 80 μM) were: methyl viologen, benzyl viologen, diquat, safranine T, phenosafranin, anthraquinone-2-sulphonate, pyocyanin, indigodisulphonate, indigotrisulphonate, and methylene blue. Samples were adjusted to various potentials with small additions of 0.1 M $Na_2S_2O_4$ or 0.2 M $K_3Fe(CN)_6$. After equilibration for 5 min, samples were taken anaerobically with a syringe and frozen. ESR signal amplitudes were measured as for fig.1. Curves are calculated from Nernst equation, assuming $n = 1$.

This indicates that reduction involves addition of a proton as well as an electron (see [11]).

E_m for the $g = 2.02$ signal was measured to be -35 ± 10 mV, independent of pH. This reasonably agrees with the estimate of -30 mV in [10].

3.4. *Interaction with exchangeable protons*

The water in a sample of hydrogenase was exchanged with D_2O by drying down under N_2 gas and resuspending 3 times in D_2O. No decrease in linewidth was observed. This makes it unlikely that Ni(III) has an exchangeable proton in its coordination sphere, which would cause hyperfine splitting or broadening.

3.5. *Microwave power saturation*

As a test to see if the nickel centre is close to the Fe–S centre with the $g = 2.02$ signal, the microwave power saturation was measured. The presence of a nearby rapidly-relaxing species will usually cause an enhancement of the spin-lattice relaxation [12]. The power saturation of the nickel centre was measured in redox samples in which the $g = 2.02$ signal was maximum ($E^{o'} = +270$ mV) and in which it was 95% reduced ($E^{o'} = -96$ mV). A difference in saturation behaviour between these samples would be a clear indication of an interaction between the centres. However, it was not observed. At 31 K both samples gave a power for half-saturation of the nickel centre, $P_{1/2} = 6$ mW (fig.3). This is *prima facie* evidence that the 2 centres are no different protein molecules or distantly situated within the same protein molecule.

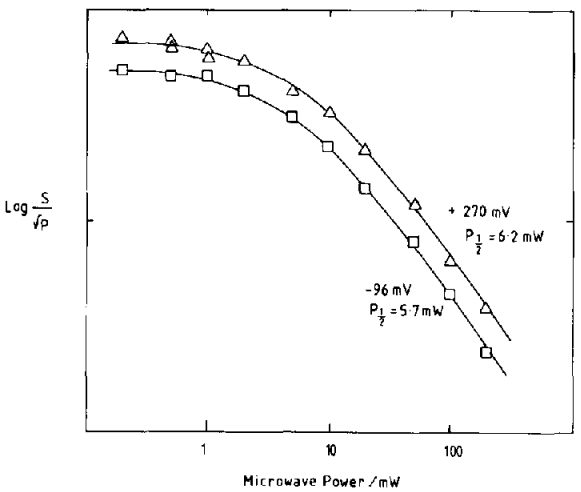

Fig.3. Microwave power saturation curves for the nickel signal. Samples were taken from a redox titration at pH 7.2: (△) sample at +270 mV ($g = 2.02$ signal fully developed); (□) sample at -96 mV ($g = 2.02$ 95% reduced). Spectra were measured at 31 K. The theoretical curves are calculated for $P_{1/2}$-values shown, and inhomogeneity parameter, $b = 1.65$ [12].

4. Discussion

The redox titration data indicate that:

(i) Reduction of Ni(III) to Ni(II) is a 1-e^- process;

(ii) Ni(III) does not appear to interact with an exchangeable proton, but on reduction a proton is added, either to the Ni or one of its ligands;

(iii) The E_m-value for Ni(III) is considerably more negative than that found in nickel–peptide complexes [13,14] though it is still ~280 mV more positive than the H^+/H_2 couple. Therefore if it is to be proposed that this nickel is involved in hydrogen production, the reaction must involve the addition of a second electron at a considerably more negative potential.

The quantitative measurements (fig.3) indicate that only a fraction (46%) of the nickel was present as ESR-detectable Ni(III). Therefore, although this nickel clearly has some unusual properties which make it a possible candidate for the active site of the enzyme, these data do not exclude the possibility that the ESR-undetectable nickel or an iron–sulphur cluster (see [1]) may be the site of hydrogen activation.

Acknowledgements

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Note added in proof

The redox-active nickel ESR signal in hydrogenase from *D. gigas* has also been observed by Drs A. V. Xavier, J. J. G. Mousa, I. Moura and J. Leball (1982) Biochem. Biophys. Res. Commun. in press.

References

[1] Adams, M. W. W., Mortenson, L. E. and Chen, J.-S. (1981) Biochim. Biophys. Acta 594, 105–176.
[2] Graf, E.-G. and Thauer, R. K. (1981) FEBS Lett. 136, 165–169.
[3] Aggag, M. and Schlegel, H. G. (1974) Arch. Microbiol. 100, 25–39.
[4] Friedrich, B., Heine, E., Fink, A and Friedrich, C. G. (1981) J. Bacteriol. 145, 1144–1149.
[5] Takakuwa, S. and Wall, J. D. (1981) FEMS Microbiol. Lett. 12, 359–363.
[6] Partridge, C. D. P. and Yates, M. G. (1982) Biochem. J., in press.
[7] Lancaster, J. R. (1980) FEBS Lett. 115, 285–288.
[8] Albracht, S. P. J., Graf, E.-G. and Thauer, R. K. (1982) FEBS Lett. 140, 311 -313.
[9] Hatchikian, E. C., Bruschi, M. and Le Gall, J. (1978) Biochem. Biophys. Res. Commun. 82, 451–461.
[10] Cammack, R., Lalla-Maharajh, W. V. and Schneider, K. (1982) in: Electron Transport and oxygen utilization (Ho, C. ed) vol. 2, pp. 411–415, Elsevier Biomedical, Amsterdam, New York.
[11] Dutton, P. L. (1978) Methods Enzymol. 54, 411–434.
[12] Rupp, H., Rao, K. K., Hall, D. O. and Cammack, R. (1978) Biochim. Biophys. Acta 537, 255–269.
[13] Bossu, F. P. and Margerum, D. W. (1977) Inorgan. Chem. 16, 1210–1214.
[14] Sakurai, T., Hongo, J.-I., Nakahara, A. and Nakao, Y. (1980) Inorgan. Chim. Acta 46, 205–210.