EPR of a novel high-spin component in activated hydrogenase from *Desulfovibrio vulgaris* (Hildenborough)

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The EPR of reoxidized hydrogenase from *Desulfovibrio vulgaris* (H.) has been reinvestigated. In contrast to other workers [(1984) Proc. Natl. Acad. Sci. USA 81, 3728–3732] we find the axial signal with $g = 2.06$; 2.01 to be only a minor component of concentration 0.03 spin/mol. In the spectrum of fully active reoxidized enzyme this signal is overshadowed by a rhombic signal (0.1 spin/mol) with $g = 2.11$; 2.05; 2.00 reminiscent of the only signal found for other oxidized bidirectional hydrogenases. In addition, a novel signal has been detected with $g_{eff} = 5.0$ which, under the assumptions that $S = 2$ and $|\Delta m_s| = 2$, quantitates to roughly one spin/mol. Ethylene glycol affects the relative intensity of the different signals. It is suggested that O_2 sensitization parallels a spin-state transition of an iron-sulfur cluster.

Hydrogenase (Desulfovibrio vulgaris) *ESR* *Iron-sulfur cluster*

1. INTRODUCTION

Hydrogenase catalyzes the reversible oxidation of molecular hydrogen to protons in the presence of suitable electron donors/acceptors [1]. The functional classification of bidirectional hydrogenase vs H_2-uptake enzymes roughly coincides with the classification in iron/sulfur containing vs nickel-iron/sulfur containing enzymes, with one exception of a nickel-free uptake hydrogenase [2]. Bidirectional hydrogenases have been purified to homogeneity from the fermentative anaerobes *Clostridium pasteurianum* [2,3] and *Megasphaera elsdenii* [4] and from the sulfate-reducing anaerobe *Desulfovibrio vulgaris*, strain Hildenborough [5,6]. Although these three proteins appear to differ in subunit composition [3,4,7] and tolerance towards molecular oxygen [3–5], they share the structural property of approx. 12 Fe and approx. 12 acid-labile S^{2-} [3–5] which are thought to be arranged in three [4Fe–4S] 'cubane' clusters as indicated by cluster extrusion experiments on the *C. pasteurianum* and *D. vulgaris* enzyme [8,9].

The NH_2-terminal part of the larger subunit from the *D. vulgaris* (H.) enzyme, containing 8 Cys residues, shows striking sequence homology with the bacterial 8Fe/S ferredoxins [7], which strongly suggests that eight of the Fe/S ions are part of two electron-transferring $[4Fe–4S]^{(2+;1+)}$ clusters. EPR and Mössbauer data appear to be consistent with this interpretation [6,10–12].

Recently, several lines of evidence have evolved to indicate that a third cluster – which, by exclusion of the two electron-transferring cubanes, is the most likely site to be involved in H_2 evolution – is atypical in its structure. The remainder of the heavy-chain sequence of the *D. vulgaris* hydrogenase contains 10 Cys residues but shows no significant homology with any known sequence of Fe/S proteins [7]. Anomalous EPR [6], ENDOR [12], and Mössbauer [6,12] spectral properties have been reported for the oxidized proteins. A magnetic circular dichroism (MCD) signal has been observed from a high-spin species in the oxidized enzymes from *M. elsdenii* [13] and *D. vulgaris* [14] which appears to be EPR silent.

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Here we report on two new characteristics of the EPR of *D. vulgaris* (H.) hydrogenase. The active reoxidized enzyme exhibits a sharp, rhombic signal very similar to the enzymes from *C. pasteurianum* and *M. elsdenii*. Furthermore, we have also found a novel signal with $g_{eff} = 5$ that we tentatively ascribe to an $S = 2$ system.

2. MATERIALS AND METHODS

D. vulgaris (strain Hildenborough NCIB 8303) was grown and its hydrogenase was assayed and purified by standard techniques [5].

Ar was scrubbed of O_2 by heated BASF catalyst R 3-11 followed by a light-reduced deazaflavin-EDTA-methyl viologen solution [15]. H_2 was scrubbed of O_2 over heated copper hydride.

Concentrated hydrogenase solutions do not readily equilibrate with the gas phase. Other workers have used 8–48 h incubations to obtain maximal reduction of the enzyme by H_2 [6,14]. Since these conditions in our hands do not lead to full activity, we employed the following quick method to equilibrate the enzyme with H_2 or with Ar. A small magnetic stirring bar is put into an EPR tube containing a frozen solution of enzyme. The tube is hooked up to a vacuum manifold that allows evacuation or pressurizing with Ar or H_2. The tube is 6–8 times flushed with Ar while the sample is being allowed to thaw. When ambient temperature is reached the stirring bar is forced several times to cross the liquid/gas interface and to stir the liquid, by means of 4 other stirring bars alongside the tube. The sample is then evacuated and repressurized with the desired gas phase. This sequence of stirring and flushing is then repeated several times over a total period of 15–20 min, the time required for the EPR spectrum to become invariant. When 0.1 mM solutions of the H_2-reduced enzyme are equilibrated with Ar by this method the result is fully reoxidized preparations as judged by the complete abolition of the complex EPR signal that is characteristic of reduced enzyme [6,9], and by the fact that the subsequent addition of a 5-fold excess of dichlorophenolindophenol (DCIP) has no effect on the EPR signals (see section 3) of the reoxidized enzyme.

EPR measurements were made with a Bruker EPR 200 D spectrometer. The sample temperature was calibrated with a dummy sample containing two 5 kΩ Allen-Bradley carbon resistors just below and above the 1.5 cm measuring area of the TE_{102} cavity.

3. RESULTS AND DISCUSSION

3.1. *EPR signals from reoxidized hydrogenase*

Aerobically isolated hydrogenase from *D. vulgaris* (H.) exhibits a weak EPR signal similar to the signals observed for oxidized [3Fe–*x*S] clusters [9]. Reduction by H_2 and/or dithionite results in a complex signal reminiscent of spectra observed for $[4Fe–4S]^{1+(2+;1+)}$ clusters [6,9]. In addition, a weak, 'axial' (i.e. with two resolved g values) signal is observed which can be differentiated from the complex signal by temperature variation [6]. Upon reoxidation of H_2-reduced enzyme with DCIP Huynh et al. observed complete abolition of the complex signal and a 10-fold increase in intensity of the axial signal with no other signals detected [6].

However, in our hands, reoxidation of *D. vulgaris* hydrogenase leads to distinctly different results (see fig.1). The complex signal is completely abolished but the axial signal does not increase in intensity. Concomitantly, we detect two, hitherto unreported, signals. The axial signal with $g = 2.06$; 2.01 is overshadowed by a 'rhombic' (i.e. three resolved g values) signal with $g = 2.11$; 2.05; 2.00 (fig.1A) which is very similar to a signal observed in the oxidized bidirectional hydrogenase from *C. pasteurianum* [12] and *M. elsdenii* [11]. In these latter two enzymes the rhombic signal represents up to 0.6 spin/mol (assuming $S_{eff} = 1/2$) while in the *D. vulgaris* enzyme we consistently find no more than approx. 0.1 spin/mol.

Why are our results on the EPR of reoxidized enzyme not consistent with those reported by Huynh et al. [6] and Stephens et al. [14]? Our experimental approach is different in two respects. First, the time required to reduce the enzyme with H_2 (or rather, to bring the reaction between resting enzyme and H_2 to equilibrium) is some 2 orders of magnitude less. Second, we have checked the H_2-production activity to be unaltered under all conditions of our experiments, i.e. resting, H_2-reduced, Ar-oxidized, and Ar + DCIP-oxidized enzyme. However, in a few instances we have accidentally or deliberately introduced some air into our samples. Upon reoxidation of these

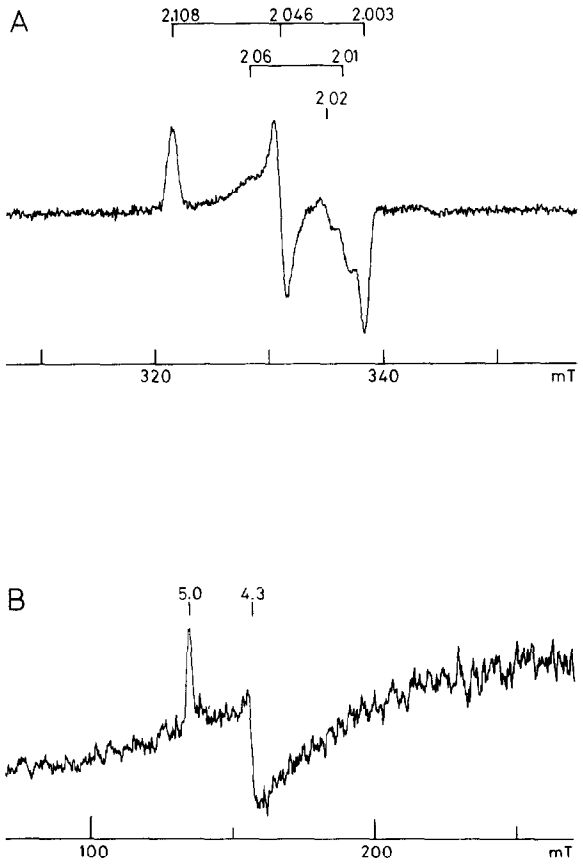

Fig.1. EPR of fully active reoxidized hydrogenase from *D. vulgaris* (H.). The enzyme was H_2-reduced and subsequently reoxidized under Ar as described in the text. (A) Region around the free electron g value exhibiting a rhombic signal, a minor axial signal, and possibly a trace amount of the signal found with resting enzyme. (B) Low-field region with a novel high-spin signal at $g = 5.0$ and the $g = 4.3$ signal possibly from a trace of adventitious iron. EPR conditions: microwave frequency, 9.48 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.8 mT; microwave power, 0.51 μW (A) or 200 mW (B); temperature, 8.6 K.

samples we observed the axial signal in the absence of the rhombic signal (not shown), concomitantly with a 90–100% decrease in activity. We therefore propose that the absence of the rhombic signal in the reoxidized enzyme is a monitor for incomplete anaerobicity and thus for inactivation by molecular oxygen. We note that this conclusion does not necessarily imply that the axial signal represents an inactive species. Prolonged contact of reoxidized samples with air ultimately abolishes also the axial signal.

In addition to the rhombic and axial signals we observe at low field a weak, novel signal with the unusual $g_{eff} = \sim 5.0$ (fig.1B). This 'high-spin' signal cannot be saturated with 200 mW microwave power at a temperature of 6 K. We have observed the high-spin signal in 7 different batches of purified *D. vulgaris* hydrogenase and we found its intensity to be proportional to the protein concentration within the experimental error. There is also a weak signal with $g_{eff} = 4.3$ but its intensity varies by a factor of 2–3 for the 7 batches. Also, the ratio of intensities of the $g = 5.0$ and the $g = 4.3$ signal varies with temperature and with microwave power level. Under the conditions of fig.1B the $g = 4.3$ line is approximately saturated to half its intensity. The $g = 4.3$ line has been observed in many biological preparations and is thought to represent adventitiously bound ferric iron. Quantitation under non-saturating conditions gives significantly less than 0.01 Fe^{3+}/mol, assuming isotropic g_{eff}, $S = 5/2$, $|D| \ll kT$. We recall here our recent finding of weak signals in the $g = 4$–6 region of the spectrum from Fe-proteins

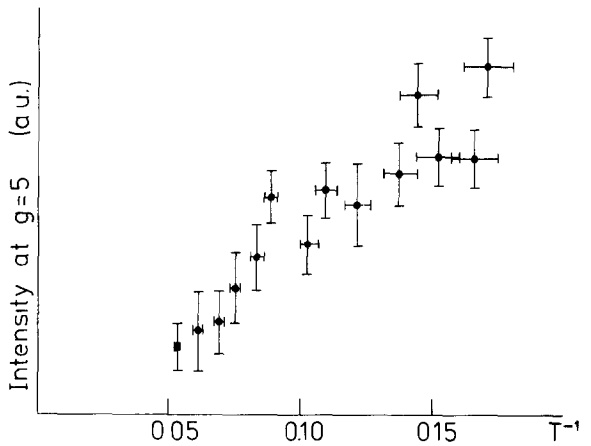

Fig.2. Dependence of the intensity of the high-spin resonance at $g = 5.0$ on the reciprocal temperature. An effective $S = 1/2$ system would give a straight line through the origin (Curie's law). The relation observed indicates a forbidden transition within a thermally fairly isolated doublet. EPR conditions: microwave frequency, 9.41–9.47 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.8 mT; microwave power, 200 mW; temperature, 5.9–19 K.

of nitrogenase, which were shown to represent near-stoichiometric amounts of an $S = 3/2$ system, while their amplitude was comparable to the $g = 4.3$ line from contaminating traces of Fe [16].

3.2. *Characterization of the high-spin species*

The zero-field splittings within the ground manyfold of states of many biological high-spin systems, including Fe/S proteins (cf. [17]), are frequently significantly greater than the energy of the X-band microwave quantum, i.e. $|D| > 0.3$ cm^{-1}. If this condition holds then only one (for half integer spin systems) or zero (for integer spin systems) regular, $|\Delta m_s| = 1$ transition can occur. If the $g = 5$ signal in hydrogenase were part of a $|\Delta m_s| = 1$ spectrum, then the axial zero-field splitting must be negative and the observed transition is within the highest doublet of the spin multiplet in order to account for the low amplitude of the line. Three observations indicate this assignment to be incorrect. First the shape of the signal would imply that $g = 5.0$ is the highest value of the rhombic g tensor. The signal is sufficiently sharp to at least expect the appearance of the second g value, which is not observed, unless it coincides with the resonance from adventitious iron. Second the line is sufficiently sharp to expect some saturation of the signal at 6 K and 200 mW microwave power, which is also not observed. Third, in the absence of lifetime broadening the amplitude of a resonance from a highest doublet of low population should be strongly dependent on the temperature. We have measured the $g = 5$ signal up to a temperature of some 20 K with no observable change in the linewidth. The intensity as a function of the reciprocal temperature is given in fig.2. For an isolated $S_{eff} = 1/2$ system this relation would be a straight line through the origin (i.e. Curie's law). The data only slightly deviate from a straight line indicating some depopulation at higher temperatures (i.e. no extrapolation to the origin).

In view of the previous we conclude that $|\Delta m_s| \neq 1$. Assuming $S = 2$ and $|\Delta m_s| = 2$, comparison of the $g = 5$ line with the line of the $S = 2$ standard $Fe(H_2O)_6^{2+}$, taking 40 mT as the width of this asymmetrical line [18], gives a concentration of 1 spin/mol.

3.3. *Interrelation of EPR-detectable components*

Table 1 summarizes the integrated intensity of the 5 EPR species of *D. vulgaris* hydrogenase for 4 different states of the enzyme.

The initial state is the enzyme 'as isolated'. We call this the resting state since an induction phase is observed in the H_2-uptake activity assay [19]. We have never observed any lag in the onset of H_2-evolution activity which is in line with the view that the reaction mixture supplies the conditions for rapid activation [1] but contrasts observations made with the low-activity, H_2-evolving enzyme from *D. gigas* [20]. The resting state is the only one in which the enzyme is not inactivated by molecular oxygen. Only one signal is observed at $g = \sim 2.02$ which essentially disappears upon reduc-

Table 1

Spin quantitation of EPR signals from *D. vulgaris* (H.) hydrogenase in different redox states

Species	g values	Status[a]			
		Resting	Red.	Ox.	Ox.-glass
Resting	2.02	0.1	0	trace	trace
Complex	~1.93	0	1.7[b]	0	0
Axial	2.06/2.01	0	0.03	0.03	0.04
Rhombic	2.11/2.05/2.00	0	0	0.1	0.02
High-spin	5.0	0	0	~1[c]	~1.1

[a] Red., reduced; ox., oxidized; ox.-glass, oxidized in the presence of 50% (v/v) ethylene glycol

[b] At $E_6' = -440$ mV (i.e. pH = 7.5 and $pH_2 = 1.3$ atm)

[c] Order of magnitude estimated assuming $S = 2$ and $|\Delta m_s| = 2$

tion, although a trace seems to be detectable in the spectrum of reoxidized enzyme (cf. fig.1A).

The spectrum of reduced enzyme has been reported [6,9]. The bulk of this complex signal is ascribed to the two regular, ferredoxin-type cubanes on the basis of its similarity to the spectra of 8Fe ferredoxins [6]. The minor axial component that was described in some detail by Huynh et al. [6], is also present in all our spectra of reduced enzyme (cf. fig.6 in [9]), but it was previously mistaken as a radical signal from methyl viologen [9].

In contrast to what Huynh et al. reported [6], we do not find any increase in intensity of the axial signal upon reoxidation. In this oxidized state we find the two new signals, the rhombic signal and the high-spin signal. In the presence of 50% (v/v) ethylene glycol the intensity of the rhombic signal of reoxidized hydrogenase has decreased to less than a quarter when compared to the situation in the absence of ethylene glycol (table 1). The high-spin signal appears to have increased somewhat but with the present signal-to-noise level this is a tentative observation.

From the combination of 5 signals with 4 states (table 1) it can be seen that no two signals exhibit concerted redox behaviour. Assuming that the two ferredoxin cubanes only contribute to the complex signal, we have 4 different EPR forms of the active site. Three of these are of low integrated intensity and may, therefore, not be related to activity. The high-spin species appears to be present in a significant concentration. Based on the previous experiments we propose the following working hypothesis: in *D. vulgaris* hydrogenase O_2 sensitization as well as activation are brought about by a spin-state transition of an Fe/S cluster from $S = 0$ to $S = 2$. The cluster can exist in a modified conformation giving rise to one or more of the minor signals, the rhombic, the axial and the resting signal.

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