A study of one of the iron–sulphur clusters in oxidized hydrogenase from *Megasphaera elsdenii* by magnetic-circular-dichroism spectroscopy

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The m.c.d. spectrum of the oxidized state of hydrogenase from *Megasphaera elsdenii* has been measured at liquid-helium temperatures. This oxidation state of the enzyme displays a characteristic rhombic e.p.r. signal with g -values of 2.101, 2.052 and 2.005 assigned previously to a $[4\text{Fe}-4\text{S}]^{3+}$ cluster as in oxidized HiPIP (high-potential iron–sulphur protein) [Van Dijk, Grande, Mayhew & Veeger (1980) *Eur. J. Biochem.* **107**, 251–261]. The low-temperature m.c.d. spectrum shows no features attributable to an oxidized four-iron cluster of the HiPIP type, but does reveal broad, positive peaks at 460 and 730 nm, which magnetize in a manner untypical of a spin $S = \frac{1}{2}$ cluster with g -values close to 2. The m.c.d. spectrum is most closely similar to that of dye-oxidized P-clusters known in the enzyme nitrogenase. It is therefore proposed that the rhombic e.p.r. spectrum at a g -value close to 2 arises from an m.c.d.-silent radical species that may be related chemically to the cysteine persulphide species, $\text{RS}-\text{S}^{\cdot}$, recently found in the hexacyanoferrate-oxidized seven-iron ferredoxin of *Azotobacter vinelandii* [Morgan, Stephens, Devlin, Stout, Melis & Burgess (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1931–1935].

The dehydrogenase enzyme isolated from the obligate anaerobic bacteria *Clostridium pasteurianum* (Chen & Mortenson, 1974) and *Megasphaera elsdenii* (Van Dijk *et al.*, 1979) belong to a distinct class among the wide range of hydrogenases investigated (Adams *et al.*, 1981). They both contain approx. 12Fe and 12S atoms in a single polypeptide subunit of M_r 60000 and 50000 respectively, display a very high specific activity towards hydrogen evolution in an assay with reduced Methyl Viologen, are rapidly inactivated by oxygen and are devoid of nickel. Recently a comparison has been drawn between these two hydrogenases and a periplasmic enzyme from *Desulphovibrio vulgaris* (Hildenborough) that possesses a similar M_r and metal-ion composition (Grande *et al.*, 1983; Huynh *et al.*, 1984). A variety of studies, including e.p.r. spectroscopy, have led to the conclusion that the enzymes from *Cl. pasteurianum* (Chen *et al.*, 1976) and *M. elsdenii* (Van Dijk *et al.*, 1980) contain three $[4\text{Fe}-4\text{S}]$ clusters, one of the HiPIP type, $[4\text{Fe}-4\text{S}]^{3+/2+}$, and

the other two of the bacterial-ferredoxin type, $[4\text{Fe}-4\text{S}]^{2+/1+}$. The oxidized enzyme of *M. elsdenii* gives a rhombic e.p.r. signal with an average g -value above 2 ($g_z = 2.101$, $g_y = 2.052$ and $g_x = 2.005$) which persists up to 70 K (Van Dijk *et al.*, 1980). This is called the 'HiPIP' signal, although the lineshape and temperature-dependence are not typical of true HiPIP signals (Cammack, 1973; Antanaitis & Moss, 1975). We have shown that the low-temperature m.c.d. spectra of oxidized *Chromatium vinosum* HiPIP cluster is very distinctive, with many positive and negative bands, and is relatively intense (Johnson *et al.*, 1981a). Thus the low-temperature m.c.d. spectrum of the oxidized state of hydrogenase should enable a verification to be made of the presence of an oxidized HiPIP cluster. We report here such a study of the oxidized enzyme of the hydrogenase from *M. elsdenii*.

Experimental

Hydrogenase was purified in the presence of 2.3 mM-sodium dithionite as reported previously (Van Dijk *et al.*, 1979). Chemical analyses for

Abbreviation used: HiPIP, high-potential iron–sulphur protein.

non-haem iron and acid-labile sulphide gave 11.7 ± 1.3 mol of Fe $\cdot$ mol $^{-1}$ and 11.5 ± 0.9 mol of S $^{2-} \cdot$ mol $^{-1}$. An oxidized sample of the enzyme was prepared by transfer into 50 mM-phosphate buffer, pH 5.5, in the presence of 2 mM-Na $_2$ S $_2$ O $_4$. The enzyme concentration was 45 μ M before addition of the glassing agent, ethanediol, as determined by the A_{400} of an air-oxidized sample (Van Dijk *et al.*, 1979). Ethanediol was added to give a final dilution of 50% (v/v). This leads to a reduction in hydrogen-production activity to 25% of its maximal value, but causes no change in the hydrogen-oxidation activity (Van Dijk *et al.*, 1979).

All buffers and solutions were thoroughly degassed before use and all operations were carried out in a recirculating glove box (Faircrest Engineering, Croydon CR0 2XX, Surrey, U.K.) with an oxygen level of less than 1 p.p.m. Samples for m.c.d. were loaded into sandwich cells comprising a thin rubber gasket separating two silica windows by using a fine hypodermic syringe. E.p.r. samples were taken at the same time as the m.c.d. samples. The sample was frozen to 4.2 K.

E.p.r. spectra were measured with a ER-200D Bruker spectrometer fitted with a liquid-helium flow cryostat (ESR-900; Oxford Instruments, Oxford, U.K.). M.c.d. measurements were made with a JASCO J-500D spectropolarimeter and a superconducting magnet SM-4 (Oxford Instruments) with a maximum field of 5 T. M.c.d. spectra are expressed in units of $\Delta\epsilon = \epsilon_L - \epsilon_R$ where ϵ_L and ϵ_R are the molar absorption coefficients for the absorption of left and right circularly polarized light respectively.

Results and discussion

The m.c.d. spectra at 4.2 and 10 K and a magnetic field of 5 T of the oxidized state of the hydrogenase from *M. elsdenii* are shown in Fig. 1(a). The spectrum at 1.5 K and 5 T is virtually superimposable on the 4.2 K one. The e.p.r. spectrum of the same sample (Fig. 1b) shows the expected signals at $g = 2.09$, 2.04 and 2.00 with minor features underlying them. Typical spin integrations of this signal in this enzyme are 0.6 spins $\cdot$ mol $^{-1}$ (Van Dijk *et al.*, 1980). The m.c.d. spectrum of Fig. 1(a) extends throughout the visible region and has a temperature-dependent intensity. This latter fact shows that the species giving rise to the m.c.d. spectrum is paramagnetic. Since optical transitions in the visible region can only be associated with a transition-metal centre, we conclude that the m.c.d. signal belongs to a paramagnetic iron-sulphur cluster. Information about the electronic ground state of this centre can be obtained from the magnetic-field-dependence of the m.c.d. spectrum at low temperature. Fig. 2

Fig. 1. (a) M.c.d. and (b) e.p.r. spectra of oxidized *M. elsdenii* hydrogenase

(a) The sample was at a concentration of 45 μ M in 50 mM-phosphate buffer (pH 5.5)/2 mM-sodium dithionite before dilution 1:1 with ethanediol. The magnetic field was 5 T. Temperature: —, 4.2 K; ---, 10 K. (b) Temperature, 11.8 K; power, 2.01 mW; modulation amplitude, 0.2 mT; microwave frequency, 9.4 GHz. The sample was the same as that in (a).

Fig. 2. M.c.d. magnetization curve of the 730 nm m.c.d. band of oxidized *M. elsdenii* hydrogenase measured at 1.57 K as a function of magnetic field between 0 and 5 T (---●---)

The continuous line represents a simulated m.c.d. magnetization curve for a paramagnet with a spin $S = \frac{1}{2}$ and an isotropic g -value of 2.00 (Thomson & Johnson, 1980). The arrows on the abscissa represent the intercept values, I , of the experimental and simulated curves, being 0.25 and 0.5 respectively. β is the Bohr magneton, B is the magnetic field, k is Boltzman's constant and T is the absolute temperature.

gives a plot of the magnetic-field-dependence at 1.5 K of the intensity of the m.c.d. spectrum at 730 nm. This constitutes a m.c.d. magnetization curve (Thomson & Johnson, 1980). Similar-shaped curves are obtained at the wavelengths of the other major peaks in Fig. 1. The shape of the magnetization curve that will arise from a paramagnet with an isolated spin- $\frac{1}{2}$ ground state with g -values close to 2.0 is readily calculated (Thomson & Johnson, 1980). It is plotted in Fig. 2 for comparison with the experimentally determined curve. A convenient index of the steepness of an m.c.d. magnetization is provided by the intercept value, I , the ratio of the slope of the curve at low fields in the linear region to that of the asymptotic value. The I values for the experimental and the $S = \frac{1}{2}$, $g \approx 2.0$ curves are indicated in Fig. 2 by the arrows on the abscissa. For $S = \frac{1}{2}$, $g \approx 2.0$ the I value is 0.5 (Thomson & Johnson, 1980). For a paramagnet with a spin $S > \frac{1}{2}$ or g -values of much greater than 2.0, a steeper magnetization curve with a lower I value is obtained (Thomson & Johnson, 1980). The I value of the magnetization curve of the m.c.d. spectrum of oxidized *M. elsdenii* hydrogenase is 0.25. Therefore we conclude first that the m.c.d. spectrum is dominated by the contribution from a paramagnet with g -values quite different from those observed in the e.p.r. spectrum, namely $g = 2.09, 3.04$ and 2.00 . The m.c.d. magnetization curve of a paramagnet with these latter g -values will be very similar to the simulated curve shown in Fig. 2 with $I \approx 0.5$. Since there is no e.p.r. signal at 9.2 K in the spectrum of oxidized *M. elsdenii* hydrogenase other than those around $g \approx 2.0$, we conclude that the paramagnet giving rise to the m.c.d. signals is not e.p.r.-detectable at this temperature. This fact, coupled with the evidence of the low I value, suggests that the m.c.d. spectrum arises either from an even-spin paramagnet with $S > \frac{1}{2}$ and a negative zero-field splitting parameter such that an e.p.r.-silent Kramers doublet ($M_S \geq \pm \frac{3}{2}$) is lowest in energy. Further studies of the magnetization curves at higher temperature are required before more specific conclusions can be drawn.

Having established that the m.c.d. spectrum is dominated by a paramagnetic but e.p.r.-silent iron-sulphur cluster, we turn to the question of the identity of this cluster. The low-temperature m.c.d. spectra of a wide variety of iron-sulphur clusters have been reported by this laboratory (Johnson *et al.*, 1982). The m.c.d. spectrum of the $[4\text{Fe-4S}]^{3+}$ cluster present in oxidized *Chromatium vinosum* HiPIP is intense at low temperature, with many sharp positive and negative features (Johnson *et al.*, 1981a). If the e.p.r. signals at $g = 2.09, 2.04$ and 2.00 in the *M. elsdenii* hydrogenase were due to a $[4\text{Fe-4S}]^{3+}$ cluster at a spin concentration of

$\sim 0.6/\text{mol}$, this would be clearly evident in the low-temperature m.c.d. spectrum. Thus the form of the m.c.d. spectrum points to the absence of a $[4\text{Fe-4S}]^{3+}$ cluster in addition to the evidence from the m.c.d. magnetization curve.

Examples of e.p.r.-silent paramagnetic iron-sulphur clusters are known in the reduced form of three-iron clusters such as in *Desulphovibrio gigas* FdII (Thomson *et al.*, 1981), which have an even-spin ground state, probably with $S = 2$, and the oxidized P-cluster in the thionine-oxidized iron-molybdenum protein of the nitrogenase from *Klebsiella pneumoniae* (Johnson *et al.*, 1981b) and in a mutant (*nifB*⁻) of the same enzyme (Robinson *et al.*, 1984). There is a quite striking similarity between the m.c.d. spectrum of oxidized *M. elsdenii* hydrogenase and that of dye-oxidized P-clusters, although the wavelengths of the major bands are somewhat shifted. The I value of the P-cluster m.c.d. magnetization curve is 0.125 (Johnson *et al.*, 1981b), which is smaller than that of the clusters in hydrogenase. However, this value will be sensitive to zero-field splitting parameters which may be different between the two proteins.

Finally, we turn to consider the likely assignment of the paramagnetic species with g -values of 2.09, 2.04 and 2.00. We have shown that this species gives no intense and detectable m.c.d. spectrum in the visible region and therefore lacks optical transition in this region. The e.p.r. spectrum is detectable up to 70 K. These two properties suggest that the species may be a radical. It may be that there is a parallel with the species formed by hexacyanoferrate oxidation of the $[4\text{Fe-4S}]^{2+}$ cluster in the seven-iron ferredoxin of *A. vinelandii* (Morgan *et al.*, 1984). This cluster, it is proposed, undergoes a three-electron oxidation to generate a cysteine persulphide radical, $\text{RS-S}^{\cdot}$, which is e.p.r.-detectable and an adjacent diamagnetic fragment of the iron-sulphur cluster $[4\text{Fe-4S}]^{2+}$. Although the g -values of this radical and the shape of its e.p.r. spectrum are somewhat different from those of the signal in *M. elsdenii* hydrogenase, it is possible that the latter belongs to a sulphur-radical species possibly stabilized by weak interaction with an iron-sulphur cluster. The midpoint potential of this e.p.r. signal is $-250 (\pm 30) \text{ mV}$ at pH 7.0 (Van Dijk *et al.*, 1980). This is too high to be the centre responsible for hydrogen evolution. However, this centre is very sensitive to oxygen, and it may be that the extreme oxygen-sensitivity of this enzyme arises from susceptibility of this centre to damage by oxygen.

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