Magnetic circular dichroism of DCPIP-oxidised *Desulfovibrio vulgaris* hydrogenase

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Ni-free hydrogenases commonly exhibit a $g_{av} > 2$ EPR spectrum in their most oxidised level This spectrum has been linked with the active site and generally attributed to an oxidised HIPIP-type, $[4Fe\text{-}4S]^{3+}$ cluster We report the low temperature magnetic circular dichroism (MCD) spectrum of 2,6-dichlorophenolindophenol (DCPIP)-oxidised *Desulfovibrio vulgaris* hydrogenase (DvH_2ase), which exhibits an axial $g_{av} > 2$ EPR spectrum Paramagnetic MCD is observed, which however is shown to arise from an EPR-silent, paramagnetic species with $S > 1/2$ No evidence of paramagnetic MCD arising from the $g_{av} > 2$ EPR-detectable species is obtained We conclude that (1) DCPIP-oxidised DvH_2ase does not contain either a $[4Fe\text{-}4S]^{3+}$ or an oxidised 3Fe cluster, (ii) an EPR-silent species with MCD characteristics somewhat similar to those of the oxidised 'P' clusters of the dye-oxidized iron-molybdenum protein of nitrogenase is present in DCPIP-oxidised DvH_2ase, (iii) the MCD and EPR of the $g_{av} > 2$ species exhibit characteristics in common with those of the $g_{av} > 2$ species produced by $Fe(CN)_6^{3-}$ oxidation of the $[4Fe\text{-}4S]^{2+}$ cluster of the 7Fe ferredoxin I of *Azotobacter vinelandii* [(7Fe)FdI] We suggest that the $g_{av} > 2$ EPR signal of this and other hydrogenases arises from a species chemically analogous to that observed in (7Fe)FdI

Magnetic circular dichroism *2,6-Dichlorophenolindophenol* Desulfovibrio gigas *Hydrogenase*

1. INTRODUCTION

Hydrogenases form a heterogeneous group of metalloenzymes. Some contain Ni, others do not. Of those free of Ni, the most thoroughly characterized is the first of two known hydrogenases of *Clostridium pasteurianum* (CpH_2aseI) [1,2]. Analysis yields ~12 Fe and ~12 S (S = inorganic sulfide) per monomeric molecule [3,4] and 3 [4Fe-4S] clusters per molecule have been extruded [4]. EPR shows the redox behavior of CpH_2aseI to be multi-phasic [5]. When fully reduced a complex EPR spectrum is observed, which has been attributed to interacting $[4Fe\text{-}4S]^{1+}$ clusters. Oxidation from this level causes progressive changes in EPR leading finally to a simple, sharp, rhombic spectrum with $g = 2.005$, 2.046 and 2.099, which has been assigned to an oxidised HIPIP-type, $[4Fe\text{-}4S]^{3+}$ cluster on the basis of its possessing $g_{av} > 2$.

Other Ni-free hydrogenases share at least some of the behavior of CpH_2aseI. In particular, the exhibition of a $g_{av} > 2$, 'oxidised HIPIP' EPR spectrum has been identified as a common feature of hydrogenases, and it has therefore been suggested that this spectrum is directly associated with the active site, presumed universal, of hydrogenase [1].

It has become clear very recently that the existence of EPR with $g_{av} > 2$ in an Fe-S protein does not guarantee the presence of a $[4Fe\text{-}4S]^{3+}$ cluster. Clusters containing 3Fe generally exhibit nearly isotropic signals with $g \sim 2.01$ [6]. In addition, an

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anisotropic, $g_{av} > 2$ EPR spectrum is exhibited by the $Fe(CN)_6^{3-}$ oxidation product of the $[4e\text{-}4S]^{2+}$ cluster of the 7Fe *A. vinelandii* ferredoxin I, (7Fe)FdI [7]. The nature of this species, henceforth named [4Fe-4S]′, is not yet proven; it is definitely neither a $[4Fe\text{-}4S]^{3+}$ nor a 3Fe cluster. We have proposed that the EPR originates in a disulfide radical, dissociated from the Fe-S cluster [7]. [4Fe-4S]′ exhibits two noteworthy properties: (i) its low temperature magnetic circular dichroism (MCD) is very small (being undetectable in the presence of the [3Fe-3S] cluster of (7Fe)FdI), a property not shared with any other paramagnetic Fe-S cluster studied to date; (ii) its EPR is observable at quite high temperatures.

The assignment of $g_{av} > 2$ EPR signals in Fe-S proteins to $[4Fe\text{-}4S]^{3+}$ clusters can thus be questioned if definitive supporting data is not present. Specifically, since very little additional evidence exists that the $g_{av} > 2$ EPR signals of hydrogenases originate in $[4Fe\text{-}4S]^{3+}$ clusters, this traditional view must be reexamined. We report here a preliminary study of *D. vulgaris* (Hildenborough) hydrogenase (DvH_2ase) in an oxidation level exhibiting $g_{av} > 2$ EPR using low temperature MCD. The metal content, EPR and Mossbauer spectra of this DvH_2ase, purified to high activity, were reported recently [8]. Per molecular mass of 50 kDa, 11 ± 1 Fe was obtained and Ni was absent. EPR spectra of H_2-reduced and 2,6-dichlorophenolindophenol (DCPIP)-oxidised states are similar to those of CpH_2aseI in its most reduced and oxidised levels respectively. The DCPIP-oxidised DvH_2ase EPR, which is easily observed at 77 K, differs in detail from that of oxidised CpH_2aseI with regard to axial/rhombic character. The DvH_2ase EPR is axial ($g_{\parallel} = 2.06$, $g_{\perp} = 2.00$); CpH_2aseI EPR is rhombic. Mossbauer spectroscopy identified the presence of a paramagnetic species and of diamagnetic, $[4Fe\text{-}4S]^{2+}$ clusters in the DCPIP-oxidised state; the background of the diamagnetic clusters precluded a detailed analysis of the spectrum of the paramagnetic species.

2. EXPERIMENTAL

H_2ase was isolated from *D. vulgaris* (Hildenborough, NCIB 8303) as described previously [8]. Reduction by H_2 was accomplished after deaeration in a N_2-flushed O_2-free glove box (Vacuum Atmospheres, $O_2 \leq 0.2$ ppm) by incubation under O_2-scrubbed H_2. Oxidation by DCPIP (sodium salt, Sigma, grade I) and Sephadex G-25 (Sigma) chromatography, using the technique described by Penefsky [9], were carried out in the glove box. Deaerated 50 mM Tris buffer (pH 7.6) was used throughout. Absorption and CD spectroscopy at room temperature were carried out in small-volume quartz cells (Optical Cell Co.), loaded in the glove box into sealed holders with quartz windows, using Cary 17 and JASCO J500-C spectrometers. EPR spectra were obtained on samples frozen in liquid nitrogen using a Varian E-12 spectrometer and an Oxford Instruments ESR-9 flow cryostat. For MCD spectroscopy, protein solutions were diluted 1:1 with deaerated glycerol (Mallinckrodt, AR), placed in an ~1 mm cell with quartz windows and immediately frozen in liquid nitrogen. MCD spectra were obtained using a modified JASCO J500C spectrometer and an Oxford Instruments 3 T SM5 superconducting magnet system [10].

3. RESULTS AND DISCUSSION

DvH_2ase, as isolated and ~0.6 mM, was reduced by H_2 for ~8 h. The visible-near-UV absorption and CD spectra change markedly on reduction (the changes in absorption are qualitatively similar to those occurring on reduction of *C. pasteurianum* ferredoxin [11]) and completion of H_2 reduction was recognized by the absence of further change in absorption and CD on continued incubation. The EPR of the reduced H_2ase at 10 K was essentially identical to that reported earlier [8]. Following removal of excess H_2 by pumping and flushing with N_2, DCPIP was added to give [DCPIP]:[H_2ase] ~ 13:1. After mixing for approximately 1 min, the dye was removed on Sephadex G-25. The protein was then diluted 1:1 by glycerol and frozen in an MCD cell. Comparison of the intensity of the $g_{av} > 2$ EPR at 77 K given by aliquots removed during preparation of the MCD sample showed that the H_2ase concentration in the MCD sample was ~0.1 mM.

The visible-near-UV MCD spectrum of this DCPIP-oxidised H_2ase at 3 T and temperatures ranging from ~1.6 to ~16 K is shown in fig.1. The MCD increases with decreasing T, without significant change in shape, and is predominantly

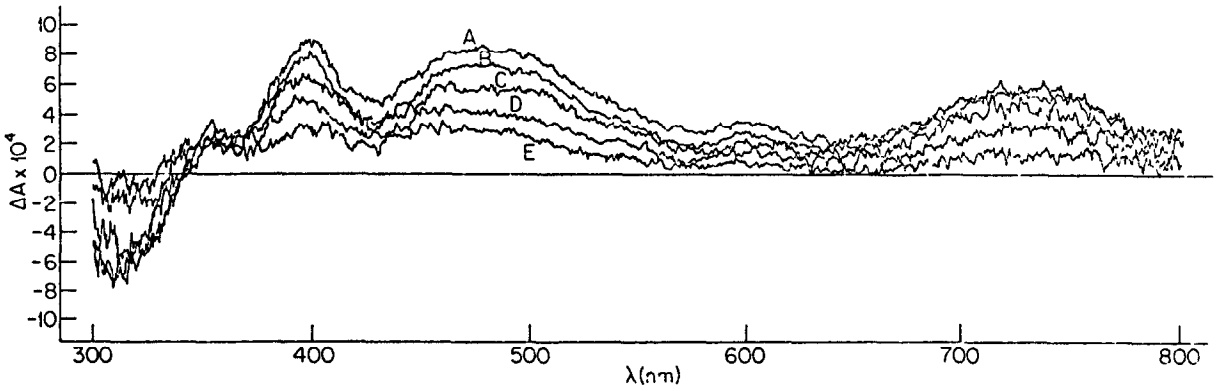

Fig.1 MCD of DCPIP-oxidised DvH_2ase Temperatures were. A, 1.68 K, B, 2 00 K; C, 4.00 K; D, 8.00 K; E, 15 7 K. Magnetic field, +3 00 T; cell pathlength, 0.89 mm; spectral bandwidth, 2 nm; time constant, 2 s Using [DvH_2ase] = 0.1 mM, $\Delta A = 10^{-3}$ corresponds to $\Delta\epsilon = 1.1 \times 10^2$

paramagnetic in character. The dependence of the MCD on magnetic field at two wavelengths and at ~1.6 and ~4.0 K is shown in fig.2. Within experimental error the initial slopes of the MCD at a given wavelength, when plotted vs $\beta H/2kT$, are identical at ~1.6 and ~4.0 K. However, at higher fields the field-dependence differs at the two temperatures. This shows that the ground state exhibits zero-field splitting, and therefore has effective spin $S > 1/2$. At ~1.6 K, the MCD field-dependence exhibits much more rapid saturation than predicted for a state with isotropic $g \sim 2.0$ (see fig.2). The intercept (I) of the initial, linear MCD slope and the line giving the saturation limit [12] occurs at $\beta H/2kT \ll 0.5$. Without correction for diamagnetic MCD, $I = 0.28$ and 0.24 at 397 nm and 730 nm, respectively. Correction for diamagnetic MCD by extrapolation to $1/T = 0$ changes these numbers to $I = 0.23$ and 0.27, respectively. In principle, the ground state g values can be extracted from the field dependence of the MCD. However, when the ground state effective spin is unknown this cannot be carried out uniquely and is not attempted here. The consistency in shape of the MCD over the temperature range studied, and the similarity and regularity of the field-dependence at 397 and 730 nm makes it extremely unlikely that the MCD contains major contributions from two paramagnetic species with very different g values and spectra.

The intensity of the MCD (as gauged by $\Delta\epsilon$ at a given temperature and magnetic field) is typical of paramagnetic Fe-S clusters, showing that it originates in a species present in approximately stoichiometric concentration. The shape and field-dependence of the MCD spectrum are completely different from those of oxidised *Chromatium vinosum* HIPIP [13] and of proteins containing oxidised 3Fe clusters, such as (7Fe)FdI [7,14], *Desulfovibrio gigas* ferredoxin II [15] and beef heart aconitase [14,16] and the observed MCD therefore does not originate in either $[4Fe\text{-}4S]^{3+}$ or oxidised 3Fe clusters. Further, the field-dependence of the MCD at 1.6 K shows that the ground state of the MCD-active species has at least one g value much greater than 2. It is therefore not the state responsible for the EPR with $g_{\|} = 2.06$, $g_{\perp} = 2.00$. As discussed above, the MCD-active species must have $S > 1/2$ and at least one excited level above the ground state. It then follows that the EPR-active state is either an excited level of the MCD-active species, or the ground state of a different species.

The EPR of the sample whose MCD was studied exhibits the $g_{av} > 2$ EPR signal previously reported [8] and shows no EPR signals at 10 K at g values very different from 2.0 over the range $g = 1.5$–10. The $g_{av} > 2$ EPR signal is easily observable at 77 K. At low temperatures, it saturates very easily, complicating measurement of its intensity below 10 K. Nevertheless, the signal remains observable at 4 K. Since the MCD at ~1.6 and ~4 K is an identical function of $\beta H/2kT$ up to fields greater than the ~0.3 T field used in EPR measurement, at 4 K ex-

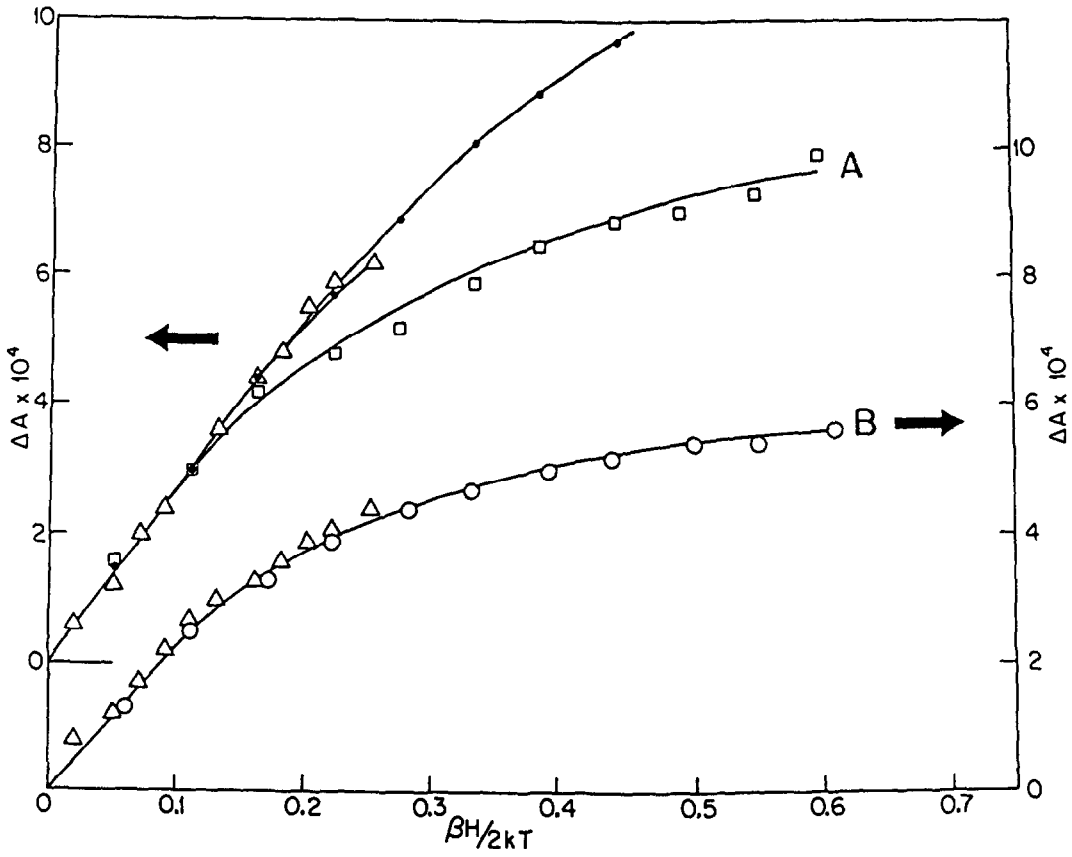

Fig 2. Magnetic field-dependence of the MCD of DCPIP-oxidised DvH_2ase. Wavelengths are: A, 397 nm; B, 730 nm. Temperatures are: ○, 1.63 K; □, 1.64 K; △, 4.00 K. The theoretical magnetic field-dependence for an isotropic $S = 1/2$ ground state [12] with $g = 2.06$ and at 1.64 K is also shown (●—●), normalised to the MCD data at 397 nm and 1.64 K. $\beta H/2kT = 0.336 \times (H/T)$, when the magnetic field, H, and the absolute temperature, T, are in T and K, respectively.

cited levels of the MCD-active species should not be observable by EPR. It follows that the $g_{av} > 2$ EPR signal originates in a species separate from that producing the paramagnetic MCD.

We therefore conclude that, (i) an EPR-silent paramagnetic species is present in DCPIP-oxidised DvH_2ase, and (ii) that the EPR-active, $g_{av} > 2$ species does not contribute measurable MCD. The new species detected could be either even- or odd-electron with either even ($S \geqslant 1$) or odd ($S \geqslant 3/2$) spin. The former is more likely in view of its undetectability by EPR. However, the oxidised 'P' clusters of the dye-oxidised FeMo protein of nitrogenase provide an example of an EPR-undetectable species with odd spin [17]. It is striking, in fact, that of the currently available paramagnetic MCD spectra of Fe-S proteins, while none is identical, the spectrum most closely resembling the DvH_2ase spectrum is that of dye-oxidised Kpl [18]. However, the MCD spectrum of DvH_2ase is not identical to that of oxidised Kpl: band positions and relative intensities differ significantly, and the saturation of the Kpl MCD is appreciably more rapid.

The absence of detectable MCD from the $g_{av} > 2$ species parallels our earlier results on $Fe(CN)_6^{3-}$-oxidised (7Fe)FdI [7]. Although the g values and shape of the EPR spectrum in (7Fe)FdI differ somewhat, the essential characteristics of the spectrum, including its observability at relatively high temperatures, are identical. We therefore propose that the species responsible for the $g_{av} > 2$

signals in the two proteins are chemically identical. Our work on the $Fe(CN)_6^{3-}$ oxidation of (7Fe)FdI has led to a hypothesis that the anisotropic $g_{av} > 2$ EPR signal is associated with a 3-electron oxidation product of the $[4Fe\text{-}4S]^{2+}$ cluster of (7Fe)FdI (as isolated), in which a dissociated cysteinyldisulfide radical is formed. Further work is ongoing to evaluate this model.

Further analysis of our MCD data on DvH_2ase is complicated by the absence to date of detailed studies of its redox behavior. DCPIP oxidation of H_2-reduced DvH_2ase leads to the $g_{av} > 2$ EPR signal. However, the EPR signal is maximally developed only at high [DCPIP]:[DvH_2ase] ratios (~10–20) and even then the EPR signal integrates to only ~0.3 spin/molecule. Further, DCPIP oxidation of oxidised (as isolated) DvH_2ase does not generate the EPR signal; H_2 reduction is required initially. (This accounts for the inability of earlier workers to observe the $g_{av} > 2$ state in DvH_2ase [19]. The reasons for the requirements of a large excess of DCPIP and of prior H_2 reduction and for the low spin yield are currently unknown. In addition, we have shown that, in the presence of a large excess of DCPIP, DvH_2ase is not stable; production of $g_{av} > 2$ EPR is followed quite rapidly by its removal. The MCD-active and EPR-active species could therefore each be associated with either reversible or irreversible oxidation of DvH_2ase. One could be obtained by further oxidation of the other or both could be simultaneously present in the same molecule. Clearly, detailed studies of the redox behavior of DvH_2ase, focussing on stoichiometry and reversibility, are essential.

This work finds no evidence for an oxidised HIPIP, $[4Fe\text{-}4S]^{3+}$ cluster in DCPIP-oxidised DvH_2ase. This assignment of the $g_{av} > 2$ EPR signals observed in other hydrogenases must therefore be questioned. At this time, it seems quite likely that these signals have a common chemical origin and that they bear a significant relationship to the $[4Fe\text{-}4S]'$ species occurring in $Fe(CN)_6^{3-}$-oxidised (7Fe)FdI. If indeed these signals originate in the active site of hydrogenases, the latter would then be accessible to investigation not only directly, but indirectly via (7Fe)FdI. Further studies of DvH_2ase and other hydrogenases, as well as of (7Fe)FdI, will illuminate this hypothesis.

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