A study of the magnetic properties of haem a_3 in cytochrome c oxidase by using magnetic-circular-dichroism spectroscopy

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(Received 15 July 1980/Accepted 20 October 1980)

M.c.d. (magnetic-circular-dichroism) spectroscopy was used to study the magnetization properties of the haem centres in cytochrome c oxidase with magnetic fields of between 0 and 5.3 T over the temperature range 1.5–200 K. The oxidized, oxidized cyanide and partially reduced cyanide forms of the enzyme were studied. In the oxidized state only cytochrome a^{3+} is detectable by m.c.d. spectroscopy, and its magnetization characteristics show it to be a low-spin ferric haem. In the partially reduced cyanide form of the enzyme cytochrome a is in the diamagnetic low-spin ferrous form, whereas cytochrome a_3 -CN is e.p.r.-detectable and gives an m.c.d.-magnetization curve typical of a low-spin ferric haem. In the oxidized cyanide form of the enzyme both cytochrome a and cytochrome a_3 -CN are detectable by m.c.d. spectroscopy, although only cytochrome a gives an e.p.r. signal. The magnetization characteristics of haem a_3 -CN show clearly that its ground state is an electronic doublet and that another state, probably a spin singlet, lies $>10\text{ cm}^{-1}$ above this. These features are well accounted for by an electronic state of spin $S = 1$ with a predominantly axial distortion, which leaves the doublet, $M_S = \pm 1$, as the ground state and the component $M_S = 0$ as the excited state. This state would not give an e.p.r. signal. Such an electronic state could arise either from a ferromagnetic coupling between haem a_3^{3+} -CN and the cupric ion, Cu_{a_2} , or from a haem in the Fe(IV) state.

Cytochrome c oxidase is an enzyme containing four redox centres (Muijsers *et al.*, 1972), two haem a groups and two copper ions. The haems are labelled a_3 and a to indicate that only one, namely haem a_3 , is able to react with the substrate O_2 and inhibitors such as CO, CN^- and azide. E.p.r. spectroscopy has been used extensively to investigate the oxidized forms of the metal centres. In the fully oxidized state a low-spin haem signal, with $g = 3.03$, 2.21 and 1.45, is found to correspond to one haem only and is assigned to haem a^{3+} . The other haem, a_3 , is not apparent in the e.p.r. spectrum. Also, only 0.7–0.8 of a cupric ion is accounted for by the signal in the region of $g = 2$ (Hartzell & Beinert, 1974; Aasa *et al.*, 1976). Similar results were obtained for the cyanide-inhibited form of the oxidized enzyme (Johnson *et al.*, 1981). The absence of e.p.r. signals from oxidized cytochrome a_3 and the second cupric ion, denoted Cu_{a_2} , led to the sug-

gestion that the two centres are interacting in an antiferromagnetically coupled pair (Van Gelder & Beinert, 1969). Recently an alternative explanation has been advanced suggesting that haem a_3 adopts the oxidation state Fe(IV) in the oxidized state of the protein, with Cu_{a_2} remaining in the diamagnetic cuprous state (Seiter & Angelos, 1980). Several proposals have been put forward about the structure of the centres, most of which envisage a ligand bridging the haem and the copper (Babcock *et al.*, 1976; Caughey, 1971). Attempts have been made to synthesize models of a magnetically coupled pair of metal ions (Kolks & Lippard, 1977; Landrum *et al.*, 1978).

However, there is a lack of techniques with which to probe the metal centres haem a_3 and Cu_{a_2} . For example, the centres do not give e.p.r. signals. Mössbauer spectra of sufficiently good quality to enable the magnetic properties to be studied require ^{57}Fe -enriched material, not a practicable undertaking for bovine heart cytochrome c oxidase. Until recently magnetic-susceptibility data were available

Abbreviation used: m.c.d., magnetic circular dichroism.

only over the limited temperature range 77–300 K (Tsudzuki & Okunuki, 1971; Falk *et al.*, 1977). Now more extensive measurements have been made over the temperature range 7–200 K (Tweedle *et al.*, 1978) and between 1.5 and 4.2 K (Moss *et al.*, 1978). However, susceptibility measurements are not ideal for the task, since they determine the sum of the magnetic properties of all the centres in the protein. Various subtraction procedures are required to obtain the magnetic properties of individual centres. Paramagnetic impurities may pose an especially difficult problem, particularly for high-molecular-weight proteins that have not been crystallized. Clearly it is desirable to have another technique that can check on the validity of conclusions based solely on susceptibility data.

M.c.d. spectroscopy is uniquely fitted for this task since it provides an optical probe of paramagnetism enabling the magnetic properties of a metal ion to be investigated via its electronic absorption bands. In an earlier study (Thomson *et al.*, 1976) we examined the m.c.d. spectra of a set of derivatives of cytochrome *c* oxidase in order to probe the spin states of the haem centres and as a preliminary investigation of the magnetic properties of haem a_3 and Cu_{a_3} . However, we were able to make measurements only over the temperature range 12–300 K with the apparatus then available. It became clear that it is necessary to conduct studies over a wider range of temperatures, especially down to lower values. This we have now done with the aid of a split-coil superconducting solenoid, which gives a field range 0–5.3 T and good control of the sample temperature in the range 1.5–300 K. We have previously conducted an investigation of the magnetization properties of the magnetically well-defined haemoproteins oxidized cytochrome *c* and met-myoglobin cyanide, with spin $S = \frac{1}{2}$, and also of reduced cytochrome *c* oxidase, which contains one high-spin ferrous haem, namely a_2^{2+} , with spin $S = 2$ (Thomson & Johnson, 1980). These examples illustrate how it is possible to use m.c.d. magnetization curves to distinguish between a magnetic ground state that is an isolated doublet and a state that has zero-field levels thermally accessible over the temperature range of the experiment. Furthermore it is possible in favourable cases to make estimates of ground-state *g*-factors. M.c.d. measurements do not appear to have been used in this way before. The general utility of the technique when thus exploited will be apparent from the present study.

We report here an extensive series of measurements on oxidized cytochrome *c* oxidase, its cyanide form and the partially reduced cyanide derivative. Magnetization curves of all three species were determined. This illustrates how m.c.d. spectroscopy provides a sensitive method of following changes in magnetic properties of metal centres as

the oxidation level is altered. These results lead us to conclusions about the nature of the electronic ground state of haem a_3 -CN that are in disagreement with the conclusions drawn from susceptibility data (Tweedle *et al.*, 1978).

Materials and methods

All chemicals used were of analytical-reagent grade, except $\text{Na}_2\text{S}_2\text{O}_4$, which was laboratory grade, and were obtained from BDH Chemicals, Poole, Dorset, U.K. Tween 80 was obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. O_2 -free N_2 gas was supplied by the British Oxygen Co., London S.W.19, U.K.

Cytochrome *c* oxidase was prepared from bovine heart essentially by the method of Yonetani (1960). To minimize the quantity of adventitiously bound copper, EDTA (10 mM) was included in the buffer solution used in the final fractionation steps. The final product was dissolved in 0.1 M-potassium phosphate buffer, pH 7.4, containing 1% Tween 80. All preparations used had an $A_{444}(\text{reduced})/A_{424}(\text{reduced})$ ratio of 2.3 or better (Gibson *et al.*, 1965). The concentrations of the resulting oxidized resting cytochrome *c* oxidase samples were established per enzyme functional unit by using $\Delta\epsilon_{603}(\text{reduced} - \text{oxidized}) - \Delta\epsilon_{630}(\text{reduced} - \text{oxidized}) = 27 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (Nicholls, 1978). The oxidized cyanide derivative was prepared by mixing the protein with a 100-fold stoichiometric excess of neutralized KCN solution and incubating the mixture for 24 h at 4°C (Van Buuren *et al.*, 1972). All enzyme solutions were diluted with an equal volume of ethanediol before use for m.c.d. experiments, to enable glasses of good optical quality to be formed on freezing. The activities of enzyme preparations in ethanediol have been checked to ensure that no damage is produced by addition of the agent or by freezing. Furthermore the presence of ethanediol did not alter the e.p.r. spectra of either the oxidized or the oxidized cyanide-inhibited forms of cytochrome *c* oxidase.

Absorption spectra were recorded with a Cary 14 spectrophotometer, and m.c.d. spectra were measured with either a Cary 61 dichrograph or a JASCO J-500D dichrograph fitted with an SM4 superconducting solenoid (Oxford Instruments). Samples consist of a solution held between a pair of silica plates separated by a thin (1–2 mm-thick) rubber gasket. The path-length of each such cell is measured with a micrometer. The temperature of the sample is monitored by two carbon–glass resistors (Lake Shore Cryogenics), which are placed one immediately below and the other just above the sample cell. This arrangement facilitates measurement of the temperature gradient along the sample block, this being subsequently minimized. This is

particularly important for experiments above 4.2 K, when the sample is cooled by cold helium gas rather than being immersed in a bath of liquid helium. Further details of temperature and magnetic-field calibration are given elsewhere (Thomson & Johnson, 1980).

The e.p.r. spectra of samples were also measured to check that the correct oxidation level had been achieved. The spectra were recorded on a Varian E-3 spectrometer fitted with an ESR-9 flow cryostat (Oxford Instruments).

The titration of the oxidized cyanide derivative of cytochrome c oxidase against dithionite was performed under anaerobic conditions by using O_2 -free N_2 , further purified by passage over a reduced B.A.S.F. copper catalyst [B.A.S.F. (U.K.), Cheadle Hulme, Cheshire, U.K.] at $140^\circ C$ and through a dithionite wash bottle. Dithionite was dispensed from a strongly buffered (0.5 M-potassium phosphate buffer, pH 7.4) stock solution by using gas-tight Hamilton syringes and added to the enzyme solution. A sample of the enzyme solution was subsequently placed in specially constructed anaerobic m.c.d. cells of measured path-length by using a gas-tight Hamilton syringe. After room-temperature absorption spectra had been recorded the samples were rapidly frozen by immersion in liquid helium.

Results

A detailed study was made of the field- and temperature-dependence of the m.c.d. spectra of three forms of cytochrome c oxidase, namely the oxidized state and the cyanide forms of the oxidized and partially reduced states. The spectra were recorded between 300 and 500 nm at fields between 0 and 5.3 T and at temperatures between 1.5 and 200 K. For each sample over 50 spectra were measured.

A representative set of m.c.d. spectra is shown in Fig. 1. They belong to the oxidized form of cytochrome c oxidase and were measured at 1.94 K and at five magnetic fields between 1 and 5.3 T. The spectra all cross the origin at 426 nm and have main negative and positive peaks at 433 nm and 420 nm respectively. The forms of the spectra are identical for both the oxidized state and the oxidized cyanide state of the enzyme. The crossing point and the positive and negative peaks are unchanged. However, the magnitudes of the signals are very different for these two forms of the enzyme.

It is apparent from Fig. 1 that the m.c.d. spectra are not linearly dependent on magnetic field at 1.94 K. In Fig. 2 the data for the oxidized and oxidized cyanide forms of the enzyme have been plotted as $\Delta\epsilon_p$, the distance between the positive and

Fig. 1. M.c.d. spectra for oxidized resting cytochrome c oxidase at 1.94 K and magnetic fields of 1T (.....), 2T (-.-.-), 3T (-•-•-), 4T (----) and 5T (—)

The sample was prepared in 0.1 M-phosphate buffer containing 1% Tween 80 and diluted with an equal volume of ethanediol to give a final concentration of $17.5 \mu M$. The cell path-length was 1.24 mm.

negative peaks at 420 nm and 433 nm, against $\beta B/2kT$. Such plots display all the data points taken for a given derivative and represent magnetization curves for the two forms. For both the derivatives a total of 61 data points have been measured. For the oxidized cyanide form of the enzyme measurements were taken at the following magnetic fields and temperatures; at each of the temperatures 1.53, 1.98 and 4.22 K with fields of 0, 0.15, 0.29, 0.45, 0.59, 0.74, 0.88, 1.03, 1.16, 1.29, 1.42, 2.06, 2.66, 3.24, 3.81, 4.39 and 5.10 T, and at temperatures of 26.2, 35.8, 45.8, 55.5, 66.5, 76.5, 101.0, 126.0, 152.0 and 198.0 K at a single field of 5.1 T. For the oxidized resting form of the enzyme measurements were made at each of the temperatures 1.56, 1.92 and 4.22 K with the same fields between 0 and 5.1 T as given above, and at temperatures of 25.0, 34.5, 45.0, 55.0, 65.0, 75.5, 98.0, 125.0, 149.0 and 192.0 K at a single field of 5.1 T.

The difference between the two sets of data points is plotted in Fig. 3. When data points for the two forms of the enzyme were not available at identical temperatures, interpolation between plots of $\Delta\epsilon_p$ against $1/T$ at the same magnetic field was used to obtain a point to plot in Fig. 3.

Also contained in Fig. 3 are the data for the partially reduced cyanide form of the enzyme. This species was produced by the anaerobic reduction of the oxidized cyanide form with dithionite. The

Fig. 2. M.c.d. magnetization curves (i.e. plots of peak to trough for the Soret-band m.c.d., $\Delta\epsilon_p$, versus $\beta B/2kT$) for the oxidized resting (▲) and oxidized cyanide (●) derivatives of cytochrome *c* oxidase

The inset shows an expansion of the region at low $\beta B/2kT$ values. The ○ symbols show the form of the theoretical m.c.d. magnetization plot obtained by using the g -values for haem a^{3+} (i.e. $g_z = 3.03$, $g_y = 2.21$, $g_x = 1.45$), assuming that the transition is completely x - y -polarized (Thomson & Johnson, 1980). The theoretical curves have been normalized to give to the same $\Delta\epsilon_p$ value as the plot of oxidized cytochrome *c* oxidase magnetization at $\beta B/2kT = 1$. See the text for field and temperature ranges.

course of reduction was monitored by observation of the e.p.r. and absorption spectra as well as by measurement of m.c.d. magnetization curves at various intermediate stages of reduction. In the e.p.r. experiments the gradual abolition of the $g = 3.03$ signal, due to cytochrome a^{3+} , is observed concomitantly with the development of a new low-spin ferric haem signal at $g = 3.58$, due to cytochrome a_3^{3+} -CN (Johnson *et al.*, 1981). In the latter set of experiments the magnetization curves show a steady fall from the upper curve (Fig. 2) until the partially reduced state is reached. At this point all trace of the e.p.r. signal due to cytochrome a^{3+} was abolished and the absorption spectrum showed the double-peaked Soret band characteristic of the partially reduced cyanide-inhibited state (Johnson *et al.*, 1981). The resulting magnetization curve for the partially reduced cyanide-inhibited state is plotted in Fig. 3. Measurements were made at temperatures of

Fig. 3. M.c.d. magnetization curve (i.e. plots of peak to trough for the Soret-band m.c.d., $\Delta\epsilon_p$, versus $\beta B/2kT$) for the partially reduced cyanide derivative of cytochrome *c* oxidase (●)

The ▲ symbols represent the difference as a function of $\beta B/2kT$ between the magnetization curves of the oxidized cyanide and oxidized resting cytochrome *c* oxidase derivatives, as shown in Fig. 2. The inset shows an expansion of the region at low $\beta B/2kT$ values. See the text for field and temperature ranges.

1.54, 1.98 and 4.22 K with the 17 different fields used to study the oxidized cyanide form, and at temperatures of 25.0, 34.5, 45.0, 55.0, 65.0, 75.5, 98.0, 125.0, 149.0 and 192.0 K and a single field of 5.1 T. The forms of the m.c.d. spectra at low temperature are identical with those shown in Fig. 1, with the wavelengths of the crossing points and the negative and positive peaks being identical. At temperatures above about 20 K a new positive peak at 450 nm becomes visible as the intensity of the major peak decreases. This is due to the presence of the diamagnetic low-spin ferrous form of cytochrome a^{2+} .

Both Figs. 2 and 3 contain insets in which the data points determined at low magnetic fields and at high temperatures are plotted. It is important to be able to check whether the data points at all temperatures and fields lie on a single smooth curve. It can be seen that there is some scattering of points, which is more apparent in the expanded scale of the inset in Fig. 3. This prompted a more detailed investigation at higher temperatures.

Fig. 4. *M.c.d. magnetization curves at constant magnetic field (i.e. plots of peak to trough for the Soret-band m.c.d., $\Delta\epsilon_p$ versus $1/T$) for the oxidized resting cytochrome oxidase and the oxidized cyanide and partially reduced cyanide derivatives of cytochrome c oxidase*

(a) Plots of $\Delta\epsilon_p$ (peak to trough for the Soret-band m.c.d.) against $1/T$, over the temperature range 6–200 K and at a constant magnetic field of 5.1 T, for the oxidized cyanide (●), oxidized resting (○) and partially reduced cyanide (▲) derivatives of cytochrome c oxidase. The broken lines indicate the initial (high-temperature) slopes of these plots. (b) Difference between the $\Delta\epsilon_p$ against $1/T$ curves of the oxidized cyanide and oxidized resting derivatives of cytochrome c oxidase (shown in a) as a function of $1/T$. The broken line indicates the initial (high-temperature) slope of this plot.

Figs. 4(a) and (b) plot the m.c.d. magnitudes, measured as $\Delta\epsilon_p$, at a constant magnetic field of 5.1 T versus $1/T$, the absolute temperature over the range 6–200 K. Fig. 4(a) gives the data for the oxidized form, the oxidized cyanide form and the partially reduced cyanide state of cytochrome c oxidase. The broken lines represent the slopes for the high-temperature data points. It can be seen that the curves for both the oxidized enzyme and the partially reduced cyanide state are linear above 20 K. However, the oxidized cyanide form gives a curve that bends upwards away from the linear line defined by the high-temperature data points. The difference between the curves for the oxidized and the oxidized cyanide forms is plotted in Fig. 4(b), which shows even more clearly the non-linear form of the curve below 40 K. It should be noted that there is no reason to expect necessarily that the curves of Figs. 4(a) and 4(b) should pass through the origin. If they do so this implies that the magnitudes

of the m.c.d. diamagnetic signals are small compared with paramagnetic m.c.d. signals. In fact the high-temperature data points fit well to lines passing through the origin. The straight lines in Figs. 4(a) and 4(b) were not constrained to pass through the origins.

There have been reports (Adar & Yonetani, 1978; Salmeen *et al.*, 1978) that the fully oxidized forms of cytochrome c oxidase are photoreduced by irradiation in the Soret-band spectral region with laser light. This phenomenon has been observed in samples held at temperatures between 77 and 300 K. We have checked that the samples used in the m.c.d. experiments reported in the present paper were not being photoreduced by the monochromatic light in the dichrograph used for measurement of m.c.d. spectra. Two checks were made. First, there was no time-dependence with any of the m.c.d. spectra. This would have been expected if photoreduction of one of the haem centres were taking place. Secondly, the

e.p.r. spectra did not change when the sample was irradiated within the cavity with light from a monochromator. A thermopile was used to check that the monochromator was producing at least as many photons as there were present in the light-beam in the dichrograph used.

Discussion

We have chosen to probe the magnetic properties of the haem groups in cytochrome *c* oxidase by measurement of the m.c.d. spectra as a function of magnetic field and temperature over as wide a range of these variables as possible. Measurements were confined to the Soret region of the spectrum for several reasons. First, the bands in the absorption spectrum in this region are unambiguously assigned to haem, and furthermore all known haem groups have intense absorption bands in this spectral region. Secondly, all haemoproteins so far studied show absorption in the Soret region that is polarized in the plane of the haem ring. Polarized single-crystal spectra of simple proteins such as metmyoglobin derivatives and horse heart cytochrome *c* show this to be so (Eaton & Hochstrasser, 1967, 1968). If the polarizations of the optical transitions used to probe the ground state are known, then the number of parameters required in the interpretation of the m.c.d. data is correspondingly decreased.

We discuss the results obtained for each of the derivatives in turn.

Oxidized cytochrome *c* oxidase

There is abundant evidence that, in this state, the enzyme contains one low-spin ferric haem, namely cytochrome a^{3+} . For example, the e.p.r. spectrum shows signals at $g = 3.03$, 2.21 and 1.45, which integrate to one haem group per functional unit of enzyme (Hartzell & Beinert, 1974; Aasa *et al.*, 1976). The near-infrared m.c.d. spectrum shows a band at 1550 nm characteristic of low-spin ferric haem and having the same magnitude as a model low-spin haem *a* derivative (Eglinton *et al.*, 1980). The m.c.d. spectra in the Soret region show bands that are similar in form to those of low-spin ferric haem *a* (Thomson *et al.*, 1977; Brittain *et al.*, 1976). The magnetization curve of this species (Fig. 2) is a continuous function of B/T and saturates at high fields and low temperature. This is behaviour identical with that of metmyoglobin cyanide derivative and oxidized horse heart cytochrome *c* (Thomson & Johnson, 1980) and is required of a simple $S = \frac{1}{2}$ ground state. Moreover, Fig. 4 shows that the m.c.d. becomes linearly dependent on $1/T$ above 20 K at a field of 5.1 T. In other words, the paramagnet being observed exhibits Curie-Law behaviour above 20 K. This shows that this paramagnet has no electronic states thermally excited

over the temperature range of the experiments. These characteristics point to the conclusion that the m.c.d. spectrum in the Soret region of the oxidized enzyme is dominated by the contribution from cytochrome a^{3+} only.

By using the method described by Thomson & Johnson (1980) g -factors of a paramagnet can be estimated from an m.c.d. magnetization curve. We show here that this method gives a satisfactory result for the magnetization curve of oxidized cytochrome *c* oxidase (Fig. 2). The curve has two parameters that characterize it, namely the initial slope and the asymptotic limit. The ratio (asymptotic limit/initial slope) is called I , the intercept. The g -factor g is related to I for an x - y -polarized transition by the equation:

$$g = 3/D \cdot I$$

where D is a parameter depending on the anisotropy of the g -tensor, $\sigma = \sqrt{2g_z/(g_x^2 + g_y^2)^{1/2}}$. The relationship between D and σ has been given (Thomson & Johnson, 1980). Table 1 shows the results of such an analysis performed on the magnetization curve of Fig. 2 for two different samples of cytochrome *c* oxidase. The values of g_z determined from the curves are 3.02 ± 0.14 and 3.11 ± 0.10 . These are in reasonable agreement with the value of 3.03 determined by e.p.r. spectroscopy.

The complete magnetization curve can also be calculated with knowledge of the g -factors from e.p.r. spectroscopy and assuming the Soret-band transition is x - y -polarized (Thomson & Johnson, 1980). This curve is plotted in Fig. 2. The experimental curve falls almost exactly on the calculated curve.

These results bear out the contention that the magnetization curve for the oxidized form of cytochrome *c* oxidase is dominated by the contribution from cytochrome a^{3+} . However, this raises the question as to why cytochrome a_3 is m.c.d.-silent in this form of the enzyme. It has previously been suggested that the haem is high-spin ferric in this form (Thomson *et al.*, 1977). Since high-spin ferric haem groups in metmyoglobin and in the partially reduced formate form of cytochrome *c* oxidase have m.c.d. spectra in the Soret region that are weaker than those of low-spin ferric haem groups by about 10-fold at room temperature, and moreover appear to have only weakly temperature-dependent m.c.d. spectra (Springall *et al.*, 1976; Thomson *et al.*, 1977), this explanation is plausible. Recently bands in near-infrared m.c.d. spectra of some cytochrome *c* oxidase derivatives have been assigned to the high-spin ferric form of cytochrome a_3^{3+} (Eglinton *et al.*, 1980). Since then the proposal has been advanced that the cytochrome a_3 in the oxidized form of the enzyme is in the high-spin Fe(IV) state (Seiter & Angelos, 1980). This electronic state does

Table 1. *Estimate of g -factors of oxidized haem groups in various cytochrome c oxidase derivatives by using m.c.d. magnetization curves*

For details see the text. I is the asymptotic limit/initial slope; values are taken from magnetization curves (Figs. 2 and 3). σ is the anisotropy of the ground-state g -factors; $\sigma = g_{||}/g_{\perp}$ for an axial paramagnet and $\sigma = \sqrt{2g_z/(g_x^2 + g_y^2)}$ for a rhombic paramagnet. D is related to σ ; for details see Thomson & Johnson (1980).

Sample		I	g -factors obtained from e.p.r. spectroscopy	σ	D	g -factor estimated from m.c.d. magnetization curve
Haem a , in oxidized enzyme	(i)	0.392 ± 0.018	3.03, 2.21, 1.45	1.62	2.54	$g_z = 3.02 \pm 0.14$
	(ii)	0.380 ± 0.012				$g_z = 3.11 \pm 0.11$
Haem a_3 , in partially reduced cyanide form of enzyme	(i)	0.310 ± 0.015	$3.58, 1.56, 1.0 \pm 0.5\ddagger$	2.73 ± 0.40	2.29 ± 0.05	$g_z = 4.24 \pm 0.30^*$
	(ii)	0.320 ± 0.015				$g_z = 4.11 \pm 0.28$
Haem a_3 , in oxidized cyanide form of enzyme	(i)	0.268 ± 0.016	None detected	∞	2	$g_{ } = 5.6 \pm 0.4, g_{\perp} = 0$
	(ii)	0.275 ± 0.015				$g_{ } = 5.5 \pm 0.3, g_{\perp} = 0$

* Inclusion of a small degree of z -polarization alters this value as follows:

% of z -polarization	g_z
10	3.94 ± 0.30
20	3.64 ± 0.30
30	3.34 ± 0.30

$\ddagger$ Johnson *et al.* (1981).

not appear to have been identified in any haemo-proteins, and hence its m.c.d. characteristics are unknown.

Partially reduced cyanide form of cytochrome c oxidase

We consider this form of the enzyme next because the evidence supports the view that the enzyme possesses a low-spin ferrous haem, namely cytochrome a^{2+} , and a low-spin ferric haem, cytochrome a_3^{3+} -CN. The near-infrared m.c.d. and c.d. spectra identify those species clearly (Eglinton *et al.*, 1980). Cytochrome a^{2+} appears in the c.d. spectrum with a characteristic band centred at 1000 nm. Cytochrome a_3^{3+} -CN is apparent as a low-spin ferric haem with an m.c.d. band at 1550 nm. The latter haem also possesses an e.p.r. spectrum with a readily detectable g_z -factor at 3.58. The two other components of the rhombic signal are, however, only poorly defined (DerVartanian *et al.*, 1974; Johnson *et al.*, 1981).

Therefore haem a^{2+} is diamagnetic and haem a_3^{3+} -CN is in the paramagnetic low-spin ferric form. The ferric haem a_3^{3+} -CN dominates the m.c.d. spectrum at low temperatures, giving a typical low-spin ferric haem signal similar to that shown in Fig. 1. The magnetization curve constructed from the m.c.d. data (Fig. 3) is a smooth function of B/T showing almost complete saturation when $\beta B/2kT \approx 1$. The m.c.d. intensities obey the Curie Law above 20 K and 5 T. All of these facts point to a ground state for the haem a_3^{3+} -CN in the partially reduced form of the enzyme that is a simple Kramer's doublet, $S = \frac{1}{2}$.

Table 1 shows the intercept values, I , obtained on two separate occasions from magnetization curves analogous to those shown in Fig. 3. The anisotropy, σ , is uncertain, since the e.p.r. spectrum does not show a well-defined signal attributable to g_x (Johnson *et al.*, 1981). By using the limits given in Table 1 the values of g_z estimated are 4.24 ± 0.30 and 4.11 ± 0.28 . These values are rather higher than the value of 3.58 obtained from e.p.r. spectroscopy. The values are changed if the assumption of x - y -polarization of the Soret band is relaxed. For example, allowing for a small percentage of z -polarization in the Soret band the values of $g_{||}$ given in Table 1 can be obtained. A minimum of 10% z -polarization would be required to bring the g -factor estimated from the m.c.d. magnetization curve within the range of the e.p.r. value. No data are available on the polarization of the Soret-band transition in cytochrome c oxidase.

Oxidized cyanide-inhibited form of cytochrome c oxidase

The binding of CN^- ion to the oxidized form of the enzyme more than doubles the intensity of the m.c.d. signal at any given field or temperature. This effect is clearly shown in Figs. 2 and 4(a). However, the form of the m.c.d. signal remains exactly as shown in Fig. 1. The wavelengths neither of the crossing point nor of the positive or negative peak positions change. The e.p.r. spectrum of the enzyme is virtually unaltered when CN^- ion becomes bound (Johnson *et al.*, 1981). It is important to check that

no substantial autoreduction has taken place in the presence of excess CN^- ion, since the binding process is slow, taking about 24 h to reach equilibrium. Autoreduction is clearly visible by the appearance of the $g = 3.58$ signal. The magnetic susceptibility of the enzyme falls significantly also on binding of CN^- ion (Tweedle *et al.*, 1978). The change in the magnetic states of the haem moieties is shown by the m.c.d. magnetization curves. Since the magnetic state of cytochrome a_3^{3+} is unaltered, as judged by its invariant e.p.r. signals, its m.c.d. magnetization curve must also be unchanged. Therefore we can ascribe all the changes in Figs. 2 and 4(a) on addition of cyanide to the magnetic properties of cytochrome $a_3\text{-CN}$, which becomes m.c.d.-detectable. Hence by subtracting the magnetic characteristics of haem a_3^{3+} we are left with the properties of haem $a_3\text{-CN}$ alone in the oxidized form of the enzyme. Note that this procedure is strictly valid only provided that the wavelengths of the m.c.d. peaks and trough remain unchanged, as is the case here.

Figs. 3 and 4(b) therefore give the m.c.d. magnetization properties of the cyanide-bound form of haem a_3 in the oxidized state of the enzyme. At first sight the properties are virtually indistinguishable from those of haem $a_3^{3+}\text{-CN}$ in the partially reduced form of the enzyme (Fig. 3). However, the magnetization curve at 5 T plotted versus $1/T$ (Fig. 4b) shows the unusual magnetic properties of this haem state.

The following are the properties of haem $a_3\text{-CN}$ in the oxidized state of the enzyme that need to be accounted for by the theoretical model of the electronic ground state of this haem. (i) The haem is not e.p.r.-detectable. (ii) The m.c.d. magnetization curve saturates in a form almost identical with that of the e.p.r.-detectable low-spin ferric haem, namely haem $a_3^{3+}\text{-CN}$ in the partially reduced state (Fig. 3). (iii) The haem does not obey the Curie Law at 5 T until the temperature is above ≈ 40 K. Below this temperature the magnetization curve first steepens, before flattening out at very low temperatures (Fig. 4b).

Electronic ground state of haem $a_3\text{-CN}$ in oxidized cytochrome c oxidase

The facts summarized at the end of the preceding section lead us to the following conclusions about the electronic ground state of haem $a_3\text{-CN}$ in the oxidized enzyme. The magnetization curve in Fig. 3 is quite characteristic of an electronic doublet as the lowest state. The fact that the curve approaches an asymptotic limit at low temperatures and high fields confirms that the lowest state is magnetic and cannot

be, for example, a spin singlet. Furthermore the smooth dependence of the curve on B/T points to a simple doublet state. The absence of an e.p.r. signal makes it unlikely that this doublet is a Kramer's pair, $S = \frac{1}{2}$. The remaining possibilities are therefore a Kramer's pair from a spin state with S greater than $\frac{1}{2}$, say the pair $\pm\frac{3}{2}$ from the state with $S = \frac{3}{2}$ or a pair of levels from an even-electron state that remain degenerate or nearly so in the absence of a magnetic field. Both situations imply the presence of low-lying excited states. The sigmoid nature of the magnetization curve taken at 5 T up to 200 K (Fig. 4b) shows clearly the presence of at least one such state. However, this state does not appreciably perturb the smooth character of the curve of magnetization plotted versus B/T (see Fig. 3). This case is unique in our experience of m.c.d. magnetization curves. For example, the $S = 2$ state of the high-spin ferrous haem generates a set of nested magnetization curves (Thomson & Johnson, 1980). The implication of this is that the low-lying state or states either have no circularly polarized transitions out of them or give rise to circularly polarized transitions that are weak in comparison with the transitions from the ground-state doublet. In this case the only consequence for the m.c.d. properties of the presence of this level is to affect the population difference between the ground-state Zeeman components and hence to disrupt Curie-Law behaviour. If this excited state were a Kramer's pair, then it would be expected to contribute significantly to the m.c.d., since there will be a Boltzman population difference between its two components in an applied magnetic field. However, a spin singlet cannot contribute to the m.c.d. in this way.

These arguments lead us to the view that the most probable electronic ground state of haem $a_3\text{-CN}$ has a spin $S = 1$ with a pronounced axial distortion such that two of its three components with $M_s = \pm 1$ lie lower and the third component, $M_s = 0$, is at higher energy. If the state is virtually a pure orbital singlet and spin triplet then the effective g -factors of the ground-state doublet are $g_{\parallel} = 4.0$, $g_{\perp} = 0.0$. However, note that e.p.r. signals will not be detectable, since $\Delta M_s = \pm 2$ transitions are forbidden. This picture requires that any rhombic distortion is smaller than Zeeman energies, say 1 cm^{-1} . This is not unreasonable, perhaps, in a haem system where the major distortion is likely to be axial in character.

We now present a simple scheme to demonstrate that this model of the ground state of haem $a_3\text{-CN}$ correctly reproduces the essential features of the magnetization curves. The temperature-dependence of the m.c.d. signal, $\Delta\epsilon_p$, is given by:

$$\frac{K \cdot (1 - e^{-W_1/kT})}{(1 + e^{-W_1/kT} + e^{-(W_2 + \frac{1}{2}W_1)/kT})} \quad (1)$$

Fig. 5. Theoretically computed m.c.d. magnetization curves for an electronic ground state $S = 1$ with axial zero-field distortion of various magnitudes for an x - y -polarized transition

(a) Theoretical plots of $\Delta\epsilon_p$, as assessed by eqn. (1), against $1/T$, for $g_{||} = 4$ and $W_2 = 5\text{ cm}^{-1}$ (Δ), 10 cm^{-1} ($\circ$) and 20 cm^{-1} ($\bullet$). The broken line indicates the theoretical slope at infinite temperature. (b) Theoretical plots of $\Delta\epsilon_p$, as assessed by eqn. (1), against $\beta B/2kT$. The values of W_2 are 5 cm^{-1} for (I), 10 cm^{-1} for (II) and 20 cm^{-1} for (III). In all three plots $g_{||} = 4$. The temperatures for which eqn. (1) was evaluated are 2 K ($\blacktriangle$), 3 K ($\blacksquare$), 4 K ($\circ$), 6 K ($\blacktriangledown$), 8 K ($\triangle$), 10 K (∇), 20 K ($\square$) and 50 K ($\bullet$).

where K is a constant that depends on the intensity of the circularly polarized transitions and W_1 is the Zeeman splitting of the ground state for a z -directed magnetic field, B . Thus we have the relationship $W_1 = g_{\parallel} \beta \cdot B$, where β is the Bohr magneton, and W_2 is the energy separation between the $M_s = 0$ and the $M_s = \pm 1$ levels in zero field. Strictly this expression must be averaged over all angles to take account of the fact that the sample is a frozen glass containing all possible orientations of the haem plane to the applied magnetic-field direction. This procedure also will take into account field-induced mixing of the $M_s = \pm 1$ and $M_s = 0$ levels. However, this leads to expressions that are cumbersome and that cannot be solved in closed form, requiring therefore to be evaluated on a computer. However, we wish only to show that our model can account for the salient features of the experiments. We do not claim quantitative validity for the g -factors and zero-field splitting parameters produced by this simplified model.

Fig. 5 shows the form of the function, eqn. (1), evaluated with $g_{\parallel} = 4.0$ and for various values of W_2 , namely 5, 10 and 20 cm^{-1} . Two types of plot are given in Fig. 5. The first (Fig. 5a) is the plot against $1/T$ for a constant field of 5 T. This reproduces the essential characteristic of Fig. 4(b), namely a linear region at high temperatures, when $kT \gg W_2$, with a steepening of the Curie-Law slope at lower temperatures, when $kT \sim W_2$, followed by saturation as $kT < W_2$. The second type of plot (Fig. 5b) is against $\beta B / 2kT$, for it is necessary to demonstrate that the presence of the low-lying excited state $M_s = 0$ does not disrupt this curve. The results show that, as this excited state approaches the ground-state doublet ($W_2 \rightarrow 0$), the curve comes ragged. In order to obtain a curve as smooth as the experimental one (Fig. 3) it is necessary for W_2 to be $> 10 \text{ cm}^{-1}$.

A state $S = 1$ with a large axial distortion leaves a doublet ± 1 with g -factors $g_{\parallel} = 4.0$ and $g_{\perp} = 0$. We have attempted to estimate the g -factors from the m.c.d. magnetization curve of Fig. 3 by using the method prescribed previously (Thomson & Johnson, 1980). The intercept values, I , from magnetization curves run on two separate samples are given in Table 1. The values were taken from the difference curve as shown in Fig. 3 by using data only at temperatures below 4.2 K, so that the non-linearity of the Curie-Law curve does not give an erroneous value for the initial slope. Assuming that the Soret band is purely x - y -polarized, the value of $g_{\parallel}$ has been estimated (Table 1). A value of $g_{\parallel} \approx 5.5$ is obtained when $g_{\perp} = 0$. If $g_{\perp}$ is allowed to take a small non-zero value, then $g_{\parallel}$ is decreased, and similarly z -polarization will lead to a diminution in this value. Hence an accurate value of $g_{\parallel}$ cannot be quoted. However, it is clear simply by inspecting the intercept values, I , in Table 1 that haem a_3 -CN in

the oxidized enzyme has a substantially higher g -factor than have haem a_3^{3+} -CN in the partially reduced form of the enzyme and haem a^{3+} in the oxidized enzyme. More experiments on a wider range of derivatives such as the azide- and sulphide-inhibited forms are clearly needed now to explore the limits of the ground-state g -factors of the e.p.r.-undetectable haem.

Conclusion

The m.c.d. spectra recorded over a wide range of magnetic fields and temperature point to a ground state of the cyanide-bound form of cytochrome a_3 in oxidized cytochrome c oxidase that has a spin $S = 1$ and a predominantly axial zero-field splitting that leaves a doublet, $M_s = \pm 1$, lower in energy and an excited level, with $M_s = 0$, greater than 10 cm^{-1} higher in energy. Any rhombic field present is probably less than 1 cm^{-1} .

This conclusion is quite at variance with that drawn from magnetic-susceptibility measurements between 7 and 200 K (Tweedle *et al.*, 1978). These authors analysed their data in terms of a ground state with $S = 0$ and a state $S = 1$ at 80 cm^{-1} higher in energy. It was suggested that this set of states arises from antiferromagnetic coupling between the copper ion, Cu_{a_2} , and the haem a_3^{3+} -CN, in the low-spin ferric state. However, the m.c.d. data in the present paper show conclusively that the ground state cannot have $S = 0$, a diamagnetic state. If this were so at 1.5 K, the lowest temperatures employed in the present m.c.d. study, only the $S = 0$ state would be populated, and the m.c.d. signals would vary linearly with applied magnetic field. No saturation of the m.c.d. would be apparent. The experimental findings shown in Figs. 2 and 3 show that saturation of the m.c.d. signal takes place. We can offer no comment on the analysis of the magnetic-susceptibility data of the resting oxidized form of the enzyme (Tweedle *et al.*, 1978; Moss *et al.*, 1978), since, in this form, cytochrome a_3 appears to have a very weak m.c.d. signal.

The final point to be considered is the origin of a state with $S = 1$ that arises as the ground state in haem a_3 -CN in the oxidized form of the enzyme. There appear to be two possibilities. The first is that the haem is in the low-spin ferric state, with $S = \frac{1}{2}$, and is magnetically coupled to Cu_{a_2} , which must be in the cupric state, also with effective spin $S = \frac{1}{2}$. However, the nature of the magnetic interaction would have to be ferromagnetic in character in order to leave a ground state with $S = 1$. This would imply the presence of another state, with $S = 0$ at higher energies. It is possible that this latter state is close to the $M_s = 0$ component arising from the $S = 1$ ground state by the action of an axial field. Our experiments could not rule this out. However,

equally possible is that the $S = 0$ state is well above 200 cm^{-1} , so that the linear portion of the magnetization curve (Fig. 4b) at higher temperature is not disturbed.

We see no way of combining antiferromagnetic coupling and a low-symmetry field in order to arrive at the ground-state electronic properties of cytochrome a_3 -CN found by m.c.d. spectroscopy. The second possibility that must be considered is the recent proposal by Seiter & Angelos (1980) that the cytochrome a_3 is in the Fe(IV) state and low-spin so that it has a ground state $S = 1$. This is a plausible explanation. However, on reviewing the data available on the magnetic properties of Fe(IV) haem groups we can find none that appear to be similar to the electronic state of haem a_3 -CN. Fe(IV) haem groups are known to result from the reaction of H_2O_2 with metmyoglobin and leghaemoglobin, and in Compounds I and II of peroxidases such as horse-radish peroxidase and cytochrome c peroxidase (Dunford & Stillman, 1976). The magnetic data are very sparse on such systems. There are no susceptibility data below 77 K. Mössbauer spectra on the H_2O_2 compounds of metmyoglobin and Japanese-radish peroxidase a (Harami *et al.*, 1977) and on horse-radish peroxidase Compound I (Schulz *et al.*, 1979) have been interpreted in terms of an $S = 1$ ground state with an axial compression leading to a lower level, $M_s = 0$, and a higher doublet, $M_s = \pm 1$, at 12.5 cm^{-1} and 21 cm^{-1} respectively. It is possible that cytochrome a_3 -CN has the Fe(IV) oxidation level with an axial elongation. However, there is no precedent among other haemoproteins studied to date for the ground-state electronic properties suggested for haem a_3 -CN in the oxidized enzyme by the m.c.d. magnetization properties.

M. K. J. and P. E. G. thank the Science Research Council for a Research Fellowship and a Senior Research Assistantship respectively. C. G. and A. J. T. acknowledge receipt of grants from the Science Research Council, The Royal Society and N.A.T.O. We thank Mr. A. Thompson for his skilled technical assistance in preparing cytochrome c oxidase.

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