The optical properties of Cu_A in bovine cytochrome *c* oxidase determined by low-temperature magnetic-circular-dichroism spectroscopy

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1. The visible–near-i.r.-region m.c.d. (magnetic-circular-dichroism) spectrum recorded at low temperature in the range 450–900 nm is reported for oxidized resting mammalian cytochrome *c* oxidase. 2. M.c.d. magnetization curves determined at different wavelengths reveal the presence of two paramagnetic species. Curves at 576, 613 and 640 nm fit well to those expected for an *x,y*-polarized haem transition with *g* values of 3.03, 2.21 and 1.45, i.e. cytochrome *a*³⁺. The m.c.d. features at 515, 785 and 817 nm magnetize as a *S* = ½ paramagnet with average *g* values close to 2, and simulated m.c.d. magnetization curves obtained by using the observed *g* values of Cu_A²⁺, i.e. 2.18, 2.03 and 1.99, fit well to the experimental observations. The form of the m.c.d. magnetization curve at 466 nm is curious, but it can be explained if Cu_A²⁺ and cytochrome *a*³⁺ contribute with oppositely signed bands at this wavelength. 3. By comparing the m.c.d. spectrum of the enzyme with that of extracted haem *a*–bisimidazole complex it has been possible to deconvolute the m.c.d. spectrum of Cu_A²⁺, which shows transitions throughout the spectral region from 450 to 950 nm. 4. The m.c.d.-spectral properties of Cu_A²⁺ were compared with those of a well-defined type I blue copper centre in azurin isolated from *Pseudomonas aeruginosa*. The absolute intensities of the m.c.d. signals at equal fields and temperatures for Cu_A²⁺ are 10–20-fold greater than those for azurin. 5. The optical spectrum of Cu_A²⁺ strongly suggests an assignment as a d⁹ ion rather than Cu(I) bound to a thiyl radical.

The optical and magnetic properties of cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) were intensively studied in order to characterize the nature of the four metal centres in the enzyme (Wikström *et al.*, 1981). However, the presence of two haem groups of the *a* type and two copper ions has created ambiguities in deconvoluting the contributions of individual centres both to the optical spectrum (Lemberg, 1969) and to the magnetic susceptibility (Tweedle *et al.*, 1978). Disagreement has consequently arisen in the interpretation of the experimental results (Malmström, 1979). Therefore there is value in the use of techniques that can separate unambiguously the contribution of each centre to a given spectroscopic signal. We have been analysing the low-temperature m.c.d. spectra of cytochrome *c* oxidase and several of its derivatives (Thomson *et al.*, 1977; Eglinton

et al., 1980; Johnson *et al.*, 1981; Thomson *et al.*, 1982). This technique has a number of useful advantages when used for the study of proteins containing several different metal centres. For example, the wavelength range accessible is 200–2500 nm, well beyond that usefully available for the measurement of the absorption spectra of the metal centres, which is confined to the wavelength range 300–1200 nm because of the absorption of the protein itself in regions beyond this. We have located a number of new electronic transitions in the region between 700 and 2000 nm that are usefully diagnostic of the nature of the haem centres (Eglinton *et al.*, 1980; Thomson *et al.*, 1982). The second aspect of help is the fact that the field- and temperature-dependence of the m.c.d. spectra enables magnetization curves for individual centres to be determined from which ground-state *g*-factors can, in favourable cases, be derived. Therefore it is possible to assign features in the m.c.d. spectrum to

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

species that give e.p.r. signals or that are paramagnetic but e.p.r.-silent.

Previously we have reported analyses of the low-temperature m.c.d. spectra of bovine cytochrome *c* oxidase in two wavelength regions. In the wavelength range 300–450 nm, the Soret-band region, the m.c.d. is dominated by the contributions from the haem groups only (Thomson *et al.*, 1977). By the use of several derivatives it was possible to obtain the magnetization properties of each haem group and to reach conclusions about the magnetic state of haem a_3 in its oxidized cyanide-inhibited form (Johnson *et al.*, 1981). The other region studied was that between 700 and 2000 nm. The m.c.d. spectra were measured both at room temperature (Eglinton *et al.*, 1980) and down to 1.5 K (Thomson *et al.*, 1982). Three of the four metal centres could be unambiguously resolved in the oxidized cyanide-bound form of the enzyme, namely the e.p.r.-detectable copper, Cu_A , and the haems a^{3+} and a_3^{3+} . The three metal centres give m.c.d. spectra at well-separated wavelengths. Therefore the magnetization properties of each could be determined. Bands due to haem a^{3+} and Cu_A were identified by their m.c.d. magnetization properties, on the basis of given knowledge of their ground-state *g*-factors from e.p.r. spectroscopy.

The visible region of the optical spectrum between 450 and 700 nm is complex because it contains overlapping contributions from more than one metal centre (Vanneste, 1966). We have now made an analysis of the low-temperature m.c.d. spectra of cytochrome *c* oxidase in this wavelength range. By the study of a number of derivatives and by measurement of m.c.d. magnetization properties at different wavelengths, we have arrived at a deconvolution of the spectra into those of the metal components. The most important result of this present study is the identification of features in the m.c.d. spectra that can be assigned to Cu_A , the e.p.r.-detectable copper centre.

The nature of Cu_A is unusual and puzzling. The e.p.r. properties are atypical of copper proteins. The *g*-values are close to 2.0 (Beinert *et al.*, 1962), and the hyperfine coupling constants are largely isotropic with a maximum splitting about half that seen in the blue or type 1 copper proteins (Froncisz *et al.*, 1979; Hoffman *et al.*, 1980). The signal also exhibits fast spin relaxation, which prevents its detection above 150 K (Froncisz *et al.*, 1979). It has been pointed out that the e.p.r. signal is similar to that of a thiyl radical ($\text{RS}^\bullet$) co-ordinated to Cu(I) (Peisach & Blumberg, 1974). Electron-nuclear-double-resonance studies of the bovine heart enzyme (Hoffman *et al.*, 1980) and the yeast cytochrome *c* oxidase isotopically substituted with ^{15}N histidine and ^2H cysteine (Stevens *et al.*, 1982) reach rather contradictory conclusions. The former study con-

cludes that the assignment of the e.p.r. signal to a thiyl π -radical is not consistent with the data, and therefore it favours identification of the centre as a Cu(II) ion in a unique environment possibly co-ordinated to more than one cysteinyl thiol group. The latter study, by contrast, concludes that a substantial spin density is delocalized on to a cysteine sulphur atom to give a site formally $\text{Cu(I)}-\text{S}^\bullet$. The isotopically substituted proteins give strong evidence for at least one cysteine and one histidine residue as ligands to Cu_A (Stevens *et al.*, 1982).

Experimental

$^2\text{H}_2\text{O}$ was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. Ethanediol, HCl, P_2O_5 , KH_2PO_4 , KCN and sodium dodecyl sulphate were from BDH Chemicals, Poole, Dorset, U.K. Imidazole and Tween 80 (polyoxyethylene sorbitan mono-oleate) were from Sigma Chemical Co., Poole, Dorset, U.K. Na_2HPO_4 was from Fisons, Loughborough, Leics., U.K. Deuterated ethanediol was prepared by diluting ethanediol into an equal volume of $^2\text{H}_2\text{O}$. Excess $^2\text{H}_2\text{O}$ was removed by standing the solution over P_2O_5 for 1 month.

Cytochrome *c* oxidase was prepared by a modified version of the method of Yonetani as described by Thomson *et al.* (1981). The preparation of deuterated samples for i.r. measurements was done as described by Eglinton *et al.* (1980). The cyanide-bound derivative was made by incubating the oxidized resting enzyme with a 50-fold excess of cyanide. The enzyme was held as a stock solution of $110\ \mu\text{M}$ in protein with a 50-fold molar excess of KCN at 4°C . Each sample was prepared by withdrawing a portion of stock solution and adding to it an equivalent amount of cold (4°C) ethanediol slowly, with stirring. The mixture was then placed in the m.c.d. cell and loaded into the superconducting magnet.

Haem A was extracted from the purified oxidase by the method of Takemori & King (1965). The bisimidazole derivative was made by adding a 1000-fold excess of imidazole from a 10 M neutralized solution to a solution of haem A in 0.1 M-phosphate buffer, pH 7.4, containing 1.0 mM-sodium dodecyl sulphate. The concentration was determined by absorbance spectroscopy by using an absorption coefficient of $13\ \text{mm}^{-1}\cdot\text{cm}^{-1}$ at 592 nm (Brittain *et al.*, 1978).

Azurin from *Pseudomonas aeruginosa* was prepared by the procedure of Parr *et al.* (1976). In order to remove small amounts of contaminating cytochrome c_{511} , which interferes with the m.c.d. measurements, the azurin was electrofocused.

Visible-region m.c.d. spectra were measured with a JASCO J-500D spectropolarimeter. In the near-i.r.

region a micrograph constructed in this laboratory, and described by Eglinton *et al.* (1980), was used. Magnetic fields up to 5 T were supplied by an Oxford Instruments SM-4 superconducting solenoid. The temperature was regulated between 1.5 and 4.2 K by controlling the vapour pressure of He gas in the sample space. Temperatures over 4.2 K were maintained by an Oxford Instruments DTC-2 temperature-controller. Two carbon-calibrated glass resistors (Cryogenic Calibrations) mounted above and below the sample were used to measure the temperature.

Absorbance spectra were run on a Cary 17D or a Pye–Unicam SP.8-200 spectrophotometer.

Results

Resting oxidized cytochrome *c* oxidase

The m.c.d. spectrum of resting oxidized cytochrome *c* oxidase between 450 and 950 nm at temperatures of 1.5, 4.2 and 20.0 K is shown in Fig.

1. The absorption spectrum is also given. M.c.d. magnetization curves were measured at the wavelengths indicated by vertical arrows in Fig. 1(b), corresponding to all the major features in the spectrum. The curves fall into three groups (Figs. 2–4); those monitored at wavelengths of 515, 785 and 817 nm are similar to one another, with all points lying on a smooth curve. This type of curve can be characterized by an intercept value, *I* (Thomson & Johnson, 1980), on the abscissa, indicated by arrows in Figs. 2–4. The values of *I* are given in Table 1. The curves measured at wavelengths of 576 and 640 nm comprise a second group characterized by a steeper initial slope and hence lower *I* values and with considerable deviation of points from the curve at higher temperature (20 K). The curve measured at 466 nm is different from both of the previous kinds, with a very shallow initial slope.

The m.c.d. spectra of the oxidized form of cytochrome *c* oxidase were measured on four

Fig. 1. Absorption spectrum at 1.5 K and m.c.d. spectra at 1.5, 4.2 and 20.0 K of oxidized resting cytochrome *c* oxidase (a) Absorption spectrum at 1.5 K of oxidized resting cytochrome *c* oxidase. Enzyme in 0.1 M-sodium phosphate buffer, pH 7.4, containing 0.5% Tween diluted 1:1 (v/v) with ethanediol to give a final protein concentration of 385 μ M. The pathlength was 0.126 cm. (b) M.c.d. spectra of oxidized resting cytochrome *c* oxidase. The field was 5.1 T. Temperatures were 1.5 K (---), 4.2 K (—) and 20.0 K (.....). The sample concentration was as for (a).

Fig. 2. *M.c.d. magnetization curves of oxidized resting cytochrome c oxidase measured at 515, 785 and 817 nm*
 The results were obtained with the sample described in the legend to Fig. 1. M.c.d. intensity was measured at the temperatures 1.5 K (○), 2.0 K (□), 4.2 K (●) and 20 K (△) at fields of 0.15, 0.29, 0.44, 0.59, 0.74, 0.88, 1.02, 1.16, 1.27, 1.42, 2.06, 2.66, 3.09, 4.10 and 5.1 T. β is in Bohr magnetons, k is Boltzman's constant, B is magnetic flux density (in T) and T is absolute temperature.

separate samples and found to be reproducible in form and with $\Delta\epsilon$ values within $\pm 10\%$.

The assignment of the features in the spectrum

can be made from the I values of the magnetization curves (Table 1) and by fitting these curves to those calculated from e.p.r. parameters (for details see

Fig. 3. *M.c.d. magnetization curves of oxidized resting cytochrome *c* oxidase measured at 576 and 640 nm*
The sample temperature and field details are exactly those given in the legend to Fig. 2.

Fig. 4. *M.c.d. magnetization curve of oxidized resting cytochrome *c* oxidase measured at 466 nm*
The sample, temperature and field details are the same as those given in the legend to Fig. 2.

Thomson & Johnson, 1980). The peaks at 576 and 640 nm have magnetization curves at 1.5 and 4.2 K that fit well to those expected of an *x,y*-polarized haem transition with the *g*-values of cytochrome a_3^{3+} , i.e. $g = 3.03, 2.21$ and 1.45 (Van Gelder & Beinert, 1969). The deviation of points in the data collected at 20 K from the line is not expected if this region contains a contribution only from haem a_3^{3+} , since it has a Kramer's doublet $S = \frac{1}{2}$ ground state. It is possible that another species is contributing to the m.c.d. spectrum at those wavelengths at higher temperatures only, and a possible candidate for this is the coupled binuclear centre $a_3^{3+}-\text{Cu}_B^{2+}$.

The m.c.d. peaks at 515, 785 and 817 nm magnetize as an $S = \frac{1}{2}$ paramagnet with average *g*-values close to 2 (Table 1). Simulated m.c.d. magnetization curves obtained by using the observed *g*-values of Cu_A^{2+} , i.e. $g = 2.18, 2.03$ and 1.99 (Aasa *et al.*, 1976; Greenaway *et al.*, 1977), fit well to the experimental spectra. Thus we conclude that the m.c.d. features at these wavelengths and at 4.2 K are due entirely to Cu_A^{2+} .

This leaves the peak at 466 nm, with an unusual m.c.d. magnetization property, to be assigned. This is only possible after comparison of the m.c.d. spectra of the enzyme with that of extracted haem *a*-bisimidazole complex (Eglinton *et al.*, 1983b). There is considerable evidence to suggest

that cytochrome *a* is a bis-histidine-co-ordinated haem *a* group (Peisach, 1978; Blumberg & Peisach, 1979; Babcock *et al.*, 1979; Eglinton *et al.*, 1980; Thomson *et al.*, 1982). Its spectroscopic properties are successfully modelled by extracted haem *a*-bisimidazole complex. A comparison of the energies of the charge-transfer bands in the near-i.r. spectral region of cytochrome a^{3+} and the extracted haem *a*-bisimidazole complex leave little doubt of the validity of the model (Eglinton *et al.*, 1983*b*). Therefore a comparison was made (Fig. 5) of the m.c.d. spectra, at 4.2 K and 5.1 T, between the visible-region m.c.d. spectra of resting oxidized

cytochrome *c* oxidase and haem *a*-bisimidazole complex. The m.c.d. difference spectrum is also given in Fig. 5.

There is no contribution from cytochrome a^{3+} to the low-temperature m.c.d. spectrum between 700 and 950 nm. This supports the evidence of the magnetization curves that this region is dominated by the contribution from Cu_A^{2+} (Thomson *et al.*, 1982). Between 570 and 650 nm the spectrum is almost totally dominated by that of cytochrome a^{3+} . The difference spectrum indicates a small negative m.c.d. trough at 590 nm. However, the magnitude of this trough is critically dependent both on the relative intensities of the haem *a*-bisimidazole spectrum and the oxidase spectrum, and also on the wavelength of the peaks. There are inevitably uncertainties in the absolute determination of values of samples that are in glassy solvents. Furthermore, there could be slight changes between the peak positions of the haem *a*-bisimidazole model and the haem in the enzyme. We have no means of allowing for these. Hence we regard the small negative trough at 590 nm in the m.c.d. difference spectrum with some caution.

The peak at 515 nm in the m.c.d. spectrum of the enzyme should contain very little contribution from that of cytochrome a^{3+} . Thus the excellent-quality m.c.d. magnetization curve indicating only a Cu^{2+} contribution at this wavelength is accounted for. Likewise the curious form of the m.c.d. magnetization curve at 466 nm receives an explanation from Fig. 5. Cytochrome a^{3+} is expected to contribute significantly to the m.c.d. spectrum. The difference spectrum indicates the presence of a negative m.c.d. trough at 466 nm. If this trough is taken to be due to Cu_A^{2+} , then the observed m.c.d. magnetization curve can be simulated satisfactorily (Fig. 6). The contributions to the magnetization

Table 1. Experimental intercept values, $I^{\text{exp.}}$, of m.c.d. magnetization curves as a function of wavelength (λ), and calculated intercept values, $I^{\text{calc.}}$, determined from *g*-values observed by e.p.r.

$I^{\text{calc.}}$ was determined from formulae given by Thomson & Johnson (1980) as follows:

$$g_{\perp} = \sqrt{(g_x^2 + g_y^2)} / \sqrt{2}$$

$$\sigma = g_{\parallel} / g_{\perp}$$

$$\frac{1}{D} = \frac{\cosh \alpha}{\sinh^3 \alpha} \left(\frac{\sinh 2\alpha}{4} - \frac{\alpha}{2} \right)$$

$$I^{\text{calc.}} = 3/D \cdot g_{\parallel}$$

Wavelength λ (nm)	$I^{\text{exp.}}$	$I^{\text{calc.}}$
515	0.45	0.47*
785	0.45 ± 0.0101	
817	0.46	
576	0.37	0.39†
640	0.37	

* Parameters used are observed *g*-values of Cu_A^{2+} , $g = 2.18, 2.03$ and 1.99 , and $\sigma = 1.08$ and $D = 2.91$.

† Parameters used are observed *g*-values of cytochrome a^{3+} , $g = 3.03, 2.21$ and 1.45 , and $\sigma = 1.62$ and $D = 2.52$.

Fig. 5. M.c.d. spectrum of Cu_A in oxidized cytochrome *c* oxidase

The difference spectrum (.....) was generated by taking the difference between the m.c.d. spectrum of the oxidized, resting enzyme (—) and the bisimidazole adduct of isolated haem *a* (O—O). The spectrum of resting, oxidized cytochrome *c* oxidase is reproduced from Fig. 1(b). The haem *a*-bisimidazole sample contained 380 μM -haem *a* in 0.1 M-sodium phosphate buffer, pH 7.4, containing 1 mM-sodium dodecyl sulphate and 400 mM-imidazole in a 1:1 (v/v) mixture with ethanediol. The temperature was 4.2 K and the field 5.1 T.

Fig. 6. Analysis of the 466 nm magnetization curve of oxidized resting cytochrome *c* oxidase

The magnetization curves expected of cytochrome *a* (—) and Cu_A (.....) were obtained by using the spectral contributions determined from Fig. 5. The result of adding these two together is shown (-----) and compared with the measured values at 466 nm (●).

curves at 466 nm from cytochrome a^{3+} and Cu_A^{2+} are plotted. The difference spectra of Fig. 5 indicate that the two chromophores contribute with oppositely signed bands. The resultant curve fits the experimental data well.

Cyanide-inhibited oxidized cytochrome *c* oxidase

In the preceding subsection we showed that all the features of the low-temperature m.c.d. spectrum of resting oxidized cytochrome *c* oxidase can be assigned to only two components, namely cytochrome a^{3+} and Cu_A^{2+} . No features are apparent that can be assigned to cytochrome a_3^{3+} or Cu_B^{2+} . In order to check this conclusion, we investigated the low temperature m.c.d. spectrum between 450 and 950 nm of the cyanide-inhibited form of the oxidized enzyme. We have previously shown that when cyanide is bound to cytochrome a_3^{3+} it contributes to the m.c.d. spectrum in the Soret region (Thomson *et al.*, 1977, 1981; Johnson *et al.*, 1981) and in the near-i.r. region at 1946 nm (Thomson *et al.*, 1982). The m.c.d. magnetization properties of cytochrome a_3^{3+} in the oxidized form of the enzyme are quite distinct from those of cytochrome a^{3+} and Cu_B^{2+}

(Thomson *et al.*, 1981, 1982). They correspond to a ground state with a spin $S = 1$ arising from a ferromagnetically coupled pair $a_3^{3+}\text{--CN--Cu}_B^{2+}$. Therefore we expect to observe a contribution from cytochrome $a_3^{3+}\text{--CN}$ and possibly also from its coupled copper partner in the visible-region m.c.d. spectrum of the cyanide-inhibited enzyme.

The binding of CN^- ion to the oxidized form of the enzyme is very slow (Antonini *et al.*, 1971; Van Buuren *et al.*, 1972). We have therefore observed the course of binding by measuring the absorption and m.c.d. spectra over a 24 h period. The absorption spectra at 1.5 K were measured and the m.c.d. spectra were taken at 4.2 K and 5.1 T. The absorption and m.c.d. spectra in the Soret region (Fig. 7) show the changes expected as CN^- binds. The visible-region m.c.d. spectrum is also changed (Fig. 8). Clearly, throughout the wavelength range 450–650 nm considerable changes in intensity are apparent as new m.c.d.-detectable components appear. The overall form of the m.c.d. spectra is not substantially altered. The temperature-dependence of the m.c.d. spectrum of the fully cyanide-bound form is given in Fig. 9.

The m.c.d. difference spectrum between the oxidized and cyanide-inhibited enzyme (Fig. 10) reveals the contribution of cytochrome a_3^{3+} brought about by the binding of CN^- . The magnetization properties of this contribution are known from the m.c.d. magnetization curve of the band at 1946 nm that is assigned to the pair $a_3^{3+}\text{--CN--Cu}_B^{2+}$ (Thomson *et al.*, 1982). This curve has an unusually steep initial slope, giving an I value of 0.286 ± 0.004 , compared with an I value for cytochrome a^{3+} of 0.400. Thus a mixture of a contribution from the former species will cause the m.c.d. magnetization curves to steepen, and to give I values between the extremes of 0.286 and 0.4 for overlapping contributions from cytochrome a_3^{3+} and cytochrome a^{3+} and between 0.286 and 0.5 for mixtures of cytochrome a_3^{3+} and Cu_A^{2+} . In keeping with those conclusions, the magnetization curves measured at 785 and 825 nm give I values of 0.48, close to those for the resting oxidized oxidase. Therefore we conclude that only the chromophore, Cu_A^{2+} , is contributing to the m.c.d. spectrum of the oxidized resting and the cyanide-bound cytochrome *c* oxidase between 650 and 950 nm. At 510 nm the I value is 0.425, corresponding to an admixture of the Cu_A^{2+} and $a_3^{3+}\text{--CN}$. At 578 nm the I value is 0.363, as expected for contributions from cytochrome a_3^{3+} and cytochrome a^{3+} . The important feature of the m.c.d. spectra of the cyanide-inhibited oxidase is the presence of the positive peak at 510 nm. This peak persists in the spectra of both the resting enzyme and the cyanide-bound enzyme, confirming that it arises from Cu_A^{2+} and not from cytochrome a_3^{3+} or its coupled partner Cu_B .

Fig. 7. Absorbance changes in the Soret region and m.c.d. spectra in the Soret region during cyanide binding to oxidized resting cytochrome *c* oxidase

(a) Absorbance changes in the Soret region during the course of cyanide binding to oxidized resting cytochrome *c* oxidase. The enzyme concentration was 55 μ M in a 1:1 (v/v) mixture of 0.1 M-sodium phosphate buffer, pH 7.4, containing 0.5% Tween 80 with ethanediol. The spectra are: —, before cyanide addition; ---, 3 min 45 s after the addition of 10 mM-KCN; - · - · - ·, 2 h 15 min after cyanide addition; · · · · ·, 24 h after cyanide addition. The spectra were measured at 1.5 K. (b) M.c.d. spectra in the Soret region during the course of cyanide binding to oxidized resting cytochrome *c* oxidase. The experimental conditions are the same as those given in (a). The temperature was 4.2 K and the field 5.1 T.

Discussion

Assignment of m.c.d. spectra

The key to the assignment of bands in the m.c.d. spectrum is a knowledge of the ground-state magnetic properties of all the metal centres. This enables the form of the m.c.d. magnetization curve to be calculated and compared with that determined experimentally at a given wavelength. The magnetic properties of the centres in the oxidized resting form of the enzyme are as follows. Cytochrome *a* is a low-spin ferric haem, which is e.p.r.-detectable, with *g*-values of 3.03, 2.21 and 1.45 (Van Gelder & Beinert, 1969). Cu_A also gives e.p.r. signals, with *g*-values of 2.18, 2.03 and 1.99 (Aasa *et al.*, 1976). These two centres are magnetically isolated. The

magnetic properties of cytochrome *a*₃ and Cu_B in the oxidized resting state of bovine heart cytochrome *c* oxidase are less well understood. The e.p.r. spectrum of resting oxidized cytochrome *c* oxidase contains a broad signal at low magnetic fields, the so-called '*g* = 12' resonance (Greenaway *et al.*, 1977). This signal has an intensity and shape that are strongly temperature-dependent. Although there is controversy in the detailed assignment of this resonance (Brudvig *et al.*, 1981; Hagan, 1982), there is agreement in attributing it to a paramagnet with spin *S* = 2. One group assigns the signal to the $\Delta M_s = \pm 2$ transition, i.e. between the *M_s* levels of +1 and -1 (Brudvig *et al.*, 1981). The other worker (Hagan, 1982) concludes, on the basis of parallel-mode cavity e.p.r. experiments, that the transition is

Fig. 8. *M.c.d. spectral changes in the visible region as cyanide binds to oxidized cytochrome *c* oxidase*
The sample and spectral conditions are the same as those indicated in the legend to Fig. 7. The temperature was 4.2 K and the field 5.1 T.

Fig. 9. *Temperature-dependence of the visible-region m.c.d. spectrum of oxidized cyanide-bound cytochrome *c* oxidase*
The sample is the same as that used in the experiment shown in Fig. 7. These spectra were recorded on the final oxidized cyanide-bound species. The spectra were measured at the temperatures 1.5 K (---), 4.2 K (—) and 20 K (.....) at a field 5.1 T.

$\Delta M_s = \pm 4$ transition, between M_s levels of +2 and -2. The paramagnetic species with $S = 2$ could arise either from high-spin cytochrome a_3^{3+} ($S = \frac{3}{2}$) antiferromagnetically coupled to Cu_B^{2+} ($S = \frac{1}{2}$) or from high-spin cytochrome a_3^{4+} ($S = 2$). Magnetic-susceptibility studies on the enzyme by two groups

have led to the conclusion that there is a paramagnet present with a spin $S = 2$ (Tweedle *et al.*, 1978; Moss *et al.*, 1978).

However, it is now clear from experiments with NO as a spin label of the Cu_B^{2+} centre that resting oxidized cytochrome *c* oxidase has preparation-

Fig. 10. *M.c.d. difference spectrum of the cytochrome a_3 -CN $^-$ species*

The difference spectrum (O—O) was obtained by subtracting the spectrum of the oxidized resting enzyme (Δ — Δ) from that of the oxidized cyanide-bound species (—). The oxidized, resting and cyanide-bound samples are those described in the legends to Figs. 1 and 9 respectively. Subtraction was done on the spectra measured at 4.2 K and 5.1 T.

Fig. 11. *Comparison of the m.c.d. spectrum of Cu_A in cytochrome c oxidase with that of azurin from *Pseudomonas aeruginosa**

The Cu_A spectrum was obtained as shown in Fig. 5. The azurin concentration was 6.57 mM in a 1:1 (v/v) deuterated mixture of 0.1 M-phosphate buffer and ethanediol (pH 7.4). Both spectra were obtained at a temperature of 4.2 K and a field of 5.1 T.

dependent e.p.r. properties (Brudvig *et al.*, 1981). The centre that gives rise to the ' $g = 12$ ' signal is unaffected by binding of NO to some of the Cu_B^{2+} centre, whereas a $g = 6$ signal is elicited from cytochrome a_3^{3+} by NO binding. It is clear that there are at least two types of reactive centre, whose relative concentrations are preparation-dependent. One type gives rise to the ' $g = 12$ ' signal and is

unreactive to NO, whereas the other is reactive to NO, which evokes a $g = 6$ signal. These results inevitably place some uncertainty over the interpretation of the magnetic-susceptibility results. These require a knowledge of the concentration of the paramagnetic centre being detected. This uncertainty is heightened by the demonstration with m.c.d. magnetization curves that the magnetic-sus-

Fig. 12. *M.c.d. spectra of oxidized cyanide-inhibited cytochrome *c* oxidase as a function of added dithionite* (a) M.c.d. spectral changes in the visible region when the oxidized cyanide-bound species was reduced with $\text{Na}_2\text{S}_2\text{O}_4$. The oxidase concentration was $80\text{ }\mu\text{M}$ in an equal mixture of 0.1 M-sodium phosphate buffer, pH 7.4, and ethanediol. The dithionite concentrations are as follows: ---, $120\text{ }\mu\text{M}$; —, $200\text{ }\mu\text{M}$; ·····, $400\text{ }\mu\text{M}$; ●—●, $680\text{ }\mu\text{M}$. (b) Comparison between the disappearance of the 790 nm m.c.d. trough and the 830 nm absorbance band during the reductive titration of the oxidized cyanide-bound species. ●, Data taken from (a); —, data taken from a similar experiment by Johnson *et al.* (1981). The dithionite solution was calibrated in equivalents by a parallel titration of oxidized cytochrome *c*.

ceptibility data (Tweedle *et al.*, 1978) on the cyanide-inhibited form of cytochrome *c* oxidase are wrong (Thomson *et al.*, 1981, 1982).

In summary, we conclude that there are likely to be two types of magnetic centre associated with cytochrome a_3 and Cu_B in the resting oxidized enzyme whose properties are preparation-dependent. One type gives rise to the ' $g = 12$ ' e.p.r. signal from a transition between excited levels of an $S = 2$ state. The other is e.p.r.-silent until NO becomes bound to it, when a $g = 6$ high-spin a_3^{3+} signal arises. For our purpose we note that both types of centre should have recognizable m.c.d. magnetization curves distinct from those of the magnetically characterized centres cytochrome a_3^{3+} and Cu_A^{2+} .

The results presented show that the low-temperature m.c.d. spectrum of resting oxidized cytochrome *c* oxidase can be deconvoluted into contributions from only two of the four metal components, namely cytochrome a_3^{3+} and Cu_A^{2+} . Because these two centres are magnetically isolated $S = \frac{1}{2}$ ions with known g -factors, the m.c.d. magnetization properties are readily and unambiguously

calculated. We have demonstrated that all the features in the m.c.d. spectra have magnetization curves at 4.2 K and below that belong either to cytochrome a_3^{3+} or to Cu_A^{2+} . The deconvolution is confirmed by two other lines of argument. The low-temperature m.c.d. spectrum of the extracted haem a_3^{3+} -bisimidazole complex is used as a model for cytochrome a_3^{3+} . This leads to the same assignment of m.c.d. peaks as do the magnetization properties. Secondly, we measured the visible-region m.c.d. spectrum of the cyanide-inhibited oxidized cytochrome *c* oxidase. This produces an additional contribution to the m.c.d. spectrum from cytochrome a_3^{3+} -CN- Cu_B^{2+} . However, features seen in the spectrum of the resting oxidase persist in the presence of cyanide, again confirming that cytochrome a_3^{3+} and Cu_B^{2+} give no detectable contribution to the visible-region m.c.d. spectra of the resting oxidase.

Two findings of general interest therefore emerge from this study. The first is the m.c.d. spectrum of Cu_A^{2+} . The ability to disentangle the spectrum of Cu_A^{2+} from the haem spectrum represents the first

report of the optical properties of this centre. The second aspect on which we need to comment is the absence of an m.c.d. signal from cytochrome a_3^{3+} and Cu_B^{2+} in the oxidized resting form of the enzyme.

Nature of Cu_A^{2+}

The m.c.d. evidence presented here shows that there are optical transitions associated with the $g = 2.0$ paramagnet throughout the spectral region from 450 to 950 nm. Thus this e.p.r. signal must arise from a metal centre. It is instructive to compare the m.c.d. properties of Cu_A^{2+} with those of a type 1 copper protein of well-defined co-ordination geometry. We therefore investigated the low-temperature m.c.d. spectra of oxidized azurin from *Pseudomonas aeruginosa*. The co-ordination geometry of the copper ion is crystallographically defined in this protein and shown to be two histidine ligands, one cysteine ligand and one methionine ligand in a distorted tetrahedral array (Colman *et al.*, 1978).

A comparison between the m.c.d. spectra of oxidized azurin and that of Cu_A^{2+} in cytochrome *c* oxidase is given in Fig. 11. Most striking is the difference between the absolute intensities of the m.c.d. signals at equal fields and temperatures. Those of Cu_A^{2+} are 10–20 times greater than those of azurin.

The detailed assignments made of the azurin optical spectrum has established that the bands between 550 and 800 nm arise from charge-transfer transitions from cysteinyl thiol (RS^-) to $\text{Cu}(\text{II})$, whereas those between 450 and 550 nm are imidazole-to- $\text{Cu}(\text{II})$ and possibly methionine-sulphur-to- $\text{Cu}(\text{II})$ charge-transfer bands (Solomon *et al.*, 1976, 1980). Clearly, the optical spectrum of Cu_A^{2+} shows intense bands in the same energy regions. We therefore propose that Cu_A^{2+} has both cysteine and histidine groups as ligands.

The intense negative m.c.d. trough at 790 nm in the spectrum of Cu_A^{2+} undoubtedly arises from a cysteinyl-thiol-to- $\text{Cu}(\text{II})$ charge-transfer process. There are no protein side chains as potential ligands that are likely to give charge-transfer bands at such a low energy. It is possible that the high intensity of this band arises because of the presence of more than one cysteinyl group as ligands of Cu_A^{2+} . There are no suitable models available to test this hypothesis at present. However, this agrees with suggestions made on the basis of e.p.r. and electron-nuclear-double-resonance studies that two cysteinyl thiol ligands are present (Greenaway *et al.*, 1977; Hoffman *et al.*, 1980; Stevens *et al.*, 1982).

The bands between 450 and 550 nm could arise from histidine-to- $\text{Cu}(\text{II})$ charge-transfer transitions, as in the case of azurin (Solomon *et al.*, 1980). But we are faced with the necessity of accounting for the

much higher m.c.d. intensity. Without m.c.d. experiments on a wider range of well-defined copper models and proteins we cannot make these assignments definitive. However, it is noteworthy that subunit II of cytochrome *c* oxidase from human, bovine and yeast mitochondria has a conserved sequence homologous with type 1 copper proteins (Steffens & Buse, 1979) except that, in the oxidase sequences, there is an additional conserved cysteine residue in place of proline. Therefore there is the possibility of generating, within subunit II, a copper site with two cysteine residues, at least one histidine residue plus methionine residue.

The optical spectrum of Cu_A^{2+} strongly suggests an assignment as a d^9 ion rather than $\text{Cu}(\text{I})$ bound to a thiol radical. Coloured complexes of $\text{Cu}(\text{I})$ are formed with π -unsaturated ligands such as *p*-phenanthroline and *o*-quinoline (Hollebone *et al.*, 1969). In this case the transitions are metal-to-ligand. However, there is a requirement for a π -unsaturated ligand with highly delocalized empty π^* -orbitals. The only possible candidate for such a ligand in a protein is tryptophan. No examples of $\text{Cu}(\text{I})$ -tryptophan complexes appear to have been reported. Therefore we conclude that the optical properties of Cu_A point against an assignment of $\text{Cu}(\text{I})$ coupled to a thiol radical. A wider range of model copper-thiol complexes is clearly required before a full interpretation of the m.c.d. spectrum of Cu_A can be made.

Nature of Cu_B^{2+} cytochrome a_3^{3+}

The second point of general interest to emerge from the present study is the fact that in the oxidized resting state of cytochrome oxidase the low-temperature m.c.d. spectrum contains no features between 350 and 2000 nm that can be assigned either to haem a_3^{3+} or to Cu_B^{2+} . The deconvolution of the room-temperature m.c.d. spectrum into contributions from each of the metal centres is far from unambiguous (Babcock *et al.*, 1976). If cytochrome a_3^{3+} and Cu_B^{2+} are not detectable, then no positive conclusion can be drawn about their magnetic states. However, one may list possible reasons for the lack of m.c.d. signals from those centres. Haem a_3^{3+} , at least, has optical absorption bands in the visible region. Thus the absence of m.c.d. signals is due to their weakness compared with those of Cu_A^{2+} and cytochrome a_3^{3+} . This may be because haem a_3^{3+} is diamagnetic and hence giving rise only to *A* and *B* terms, the *C* term being absent (Vickery, 1978). This seems to be possible only if haem a_3^{3+} were low-spin in the oxidized resting state and strongly antiferromagnetically coupled to Cu_B^{2+} . The only other explanation is the one favoured earlier by ourselves (Thomson *et al.*, 1976, 1977) and others (Babcock *et al.*, 1976). This depends on the fact that the m.c.d. signals of

high-spin ferric haems are at least an order of magnitude weaker than those of low-spin ferric haems in the magnetically isolated state (Thomson *et al.*, 1976). It has been confirmed that at low temperatures high-spin ferric haems still give m.c.d. spectra much weaker than those of low-spin ferric haems (Rots & Zandstra, 1982; Eglinton *et al.*, 1983a). The absolute intensity is a function of the magnitude of spin-orbit coupling within the haem. Therefore magnetic coupling of the haem to an adjacent copper ion is not expected to increase the intensity significantly.

It should be noted that we have searched the near-i.r. m.c.d. spectra of oxidized resting cytochrome *c* oxidase for charge-transfer transitions of haem a_3^{3+} (Eglinton *et al.*, 1980; Thomson *et al.*, 1982). We could identify features in the room-temperature spectrum between 1000 and 1200 nm of the right shape to be the high-spin haem charge-transfer bands. Thus this provides the only positive piece of m.c.d. evidence for the spin-state of cytochrome a_3^{3+} in the oxidized resting form of the enzyme.

One final point concerns the possible nature of Cu_B²⁺. It has been suggested that this is very similar to the copper ion in stellacyanin, a type 1 blue copper protein. We have recently investigated the low-temperature m.c.d. spectrum of stellacyanin (J. Peterson, C. Greenwood, A. J. Thomson, K. W. Penfield & E. Solomon, unpublished work). It is similar in overall intensity to the m.c.d. spectrum of azurin (Fig. 11). If Cu_B²⁺ were to have the same m.c.d. properties as stellacyanin and were to contribute to the m.c.d. spectra of cytochrome *c* oxidase, we would not have been able to detect it because of the relative weakness of the m.c.d. spectra of the type 1 copper centres. Therefore we draw no conclusions about the co-ordination properties of Cu_B²⁺ from the present data. Finally, we comment on the controversy concerning the relative contributions to the optical absorption spectrum between 700 and 900 nm of Cu_A²⁺ and Cu_B²⁺ (Powers *et al.*, 1979; Beinert *et al.*, 1980). Although we have shown in the present work that Cu_A²⁺ totally dominates the low-temperature m.c.d. spectra of the oxidized enzyme between 700 and 900 nm, this does not enable us to state that Cu_A²⁺ must therefore dominate the absorption spectrum in this region. However, the course of a dithionite titration monitored by m.c.d. spectroscopy is presented in Fig. 12(a) and compared with similar results obtained by Johnson *et al.* (1981) in Fig. 12(b). The changes seen at 790 nm (m.c.d.) correspond well to those obtained at 830 nm by using conventional spectrophotometry. During the course of such titrations Cu_B²⁺ is reduced (Johnson *et al.*, 1981), and if it contributed significantly to this spectral region we would expect to see it. This result confirms our

suggestion that the 830 nm band can be assigned almost completely to Cu_A²⁺.

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