Variable-Temperature Magnetic-Circular-Dichroism Spectra of Cytochrome *c* Oxidase and its Derivatives

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A detailed study of the effect of temperature on the m.c.d. (magnetic circular dichroism) spectra of cytochrome *c* oxidase and some of its derivatives was undertaken to characterize the spin states of haem *a* and a_3 . The fully reduced enzyme contains haem a_3^{2+} in its high-spin form and haem a^{2+} in the low-spin state. This conclusion is reached by comparing the spectrum with that of the mixed-valence CO derivatives and its photolysis product. The cyanide derivative of the fully reduced enzyme contains both haem *a* and a_3 in the low-spin ferrous form. The m.c.d. spectra of the fully oxidized derivatives are consistent with the presence of one low-spin ferric haem group, assigned to *a*, which remains unaltered in the presence of ligands. Haem a_3 is high spin in the resting enzyme and the fluoride derivatives, and low spin in the cyanide form. The partially reduced formate and cyanide derivatives have temperature-dependent m.c.d. spectra due to the presence of high- and low-spin haem a_3^{3+} respectively. Haem *a* is low-spin ferrous in both. A comparison of the magnitude of the temperature-dependence of haem a_3^{3+} in the fully oxidized and partially reduced forms shows a marked difference which is tentatively ascribed to the presence of anti-ferromagnetic coupling in the fully oxidized form of the enzyme, and to its absence from the partially reduced derivatives, owing to the reduction of both Cu^{2+} ions.

Mammalian cytochrome *c* oxidase contains four metal centres, consisting of two haem *a* groups and two copper atoms, which act in a concerted fashion to transfer electrons to oxygen. Since Keilin & Hartree (1939) first described the haem centres in terms of *a* and a_3 , assigning to a_3 the role of the oxygen-binding site, the nature of the interaction between the metal sites has been discussed at length (Tiesjema *et al.*, 1974; Nicholls & Peterson, 1974). Various techniques have been used in an attempt to probe these interactions, but the most powerful one to date has been that of e.p.r.† spectroscopy, in spite of the fact that signals are only detectable from some of the oxidized components of the enzyme. In the fully oxidized protein the signal with a component at $g = 3.0$ is assigned to only one of the haem groups, low-spin a^{3+} . No evidence for the other haem group, a_3^{3+} , is apparent. Likewise only one copper atom is accounted for by the signal in the region of $g = 2$ (Aasa *et al.*, 1976). The lack of e.p.r. signals from a_3^{3+} and the second Cu^{2+} ion has led naturally to the suggestion that these two groups are anti-ferromagnetically coupled (Van Gelder & Beinert, 1969). A theoretical treatment of this interaction has appeared (Griffith, 1971).

† Abbreviations: e.p.r., electron paramagnetic resonance; m.c.d., magnetic circular dichroism.

Somewhat surprisingly, there have been no attempts to use other techniques to probe this postulated coupling. The magnetic-susceptibility studies so far made have only been taken over a limited temperature range, 77–300 K, and no conclusive evidence for the interaction is seen in these measurements (Tsudzuki & Okunuki, 1971). Indeed, since this measurement responds to the average magnetic properties of all the centres, it may well be relatively insensitive to an interaction between half the centres. In contrast with this, the technique of m.c.d. spectroscopy offers a method of probing the magnetic properties of individual centres via their absorption spectra. The technique has not yet been widely used in this manner for the study of metalloproteins, although we have demonstrated that the anti-ferromagnetic coupling in iron-sulphur clusters containing pairs of iron atoms can be probed in this way (Thomson *et al.*, 1977).

It has been realized for a number of years (Stephens & Buckingham, 1966) that the m.c.d. spectra of paramagnetic ions will invariably give temperature-dependent signals via the so-called C terms. It can be shown that, provided $kT \gg g\beta B$, where β is the Bohr magneton, k the Boltzmann constant, g the electronic g -factor and B the applied field, the m.c.d. signal will vary linearly as a function of $1/kT$, giving a typical

Curie-law dependence. The magnitude of the C term under a given absorption band depends on both the ground-state g -factor and the nature of the excited state to which the electronic transition leads. Thus, if either or both the ground and excited magnetic properties are modified by, say, anti-ferromagnetic coupling, this will be reflected in the C term. However, to uncover such a phenomenon it is necessary to extend the study over the widest possible temperature range, and it is especially important to obtain data down to 10 K and preferably below.

We have therefore begun a study of the m.c.d. spectra of cytochrome *c* oxidase and its derivatives over a wide temperature range in a search for direct evidence of the exchange coupling between the Cu^{2+} ion and haem a_3^{3+} . However, it has been necessary first to make a survey of the m.c.d. spectra in order to establish the spin states of the haems and to assign spectra to the two haem components. This we have attempted to do by examination of a number of derivatives. A preliminary report has appeared (Thomson *et al.*, 1976). A parallel study with this latter aim has been carried out (Vickery *et al.*, 1976; Babcock *et al.*, 1976). In the present paper we establish the characteristics of the temperature-dependence of the m.c.d. spectra of the haem *a* groups, identifying thereby the spin states.

Experimental

Cytochrome *c* oxidase was prepared from ox heart essentially by the method of Yonetani (1960). To ensure the complete removal of adventitiously bound copper, EDTA ($10\ \mu\text{M}$) 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, which contained 1% Tween 80, and was stored in small ampoules at liquid- N_2 temperature. All preparations used had a ratio $A_{444}(\text{reduced})/A_{424}(\text{reduced})$ of 2.3 or better (Gibson *et al.*, 1965), and the concentrations of oxidase samples were established by using the extinction coefficients given by Yonetani (1961). The cyanide and fluoride derivatives were prepared by mixing the protein with the required amount of a neutralized solution of the ligand and left for 24 h to reach equilibrium (Van Buuren *et al.*, 1972). The CO complex was prepared by incubating the reduced protein under 0.1 MPa (1 atm) of CO. The mixed-valence species was prepared by incubation of the oxidized protein under 0.1 MPa (1 atm) of CO (Greenwood *et al.*, 1974). The mixed-valence species of the cyanide complex of cytochrome oxidase ($a^2+a_3^{3+}\text{CN}$) was prepared by reducing the pre-incubated enzyme ($130\ \mu\text{M}$ -oxidase + $100\ \text{mM}$ -KCN) with a small excess of solid sodium dithionite. The sample was then rapidly (approx. 2 min) frozen to

prevent the formation of any fully reduced material. The mixed-valence formate complex was prepared by reduction of the preincubated formate complex ($130\ \mu\text{M}$ -oxidase + $300\ \text{mM}$ -sodium formate) with $100\ \text{mM}$ -ascorbate/ $300\ \mu\text{M}$ -NNN'-tetramethylenephénylenediamine (Nicholls, 1976). For the formate mixed-valence complex, it is imperative to freeze the sample rapidly ($<1\ \text{min}$), as it has been shown by experiments in this laboratory (T. Brittain & C. Greenwood, unpublished work) that the mixed-valence formate complex is in fact in steady-state equilibrium. Reduction by excess of dithionite removes O_2 from solution and rapidly leads to the fully reduced species. But the use of ascorbate/NNN'-tetramethylenephénylenediamine, with no diffusion of O_2 from the air-space above the sample, the mixed-valence species is stable for approx. 3 min. The reduced protein was normally prepared by the addition of a small excess of solid sodium dithionite to the resting oxidized enzyme.

CO - and O_2 -free N_2 used in these experiments was dispensed from cylinders and stored, before use, in glass vessels over an alkaline solution of dithionite-reduced anthraquinonesulphonate. All chemicals were of AnalaR grade and were obtained from BDH Chemicals (Poole, Dorset, U.K.) except for Tween 80, which was obtained from Sigma (London) Chemical Co. (Kingston-upon-Thames, Surrey, U.K.).

Absorption spectrophotometry was performed by using a Cary 118C instrument with 1 cm path-length cells. M.c.d. spectra were recorded with a Cary 61 dichrograph fitted with a superconducting solenoid capable of generating a magnetic field of 5.1 T. All spectra were recorded at a scan speed of 1 nm/s. Room-temperature spectra were recorded in a 1 cm path-length cell at 20°C . The samples used for recording low-temperature spectra were saturated with sucrose and placed in specially constructed cells of 0.8 mm path length before being rapidly cooled to below 100 K in a flow cryostat similar in design to that of Briat *et al.* (1972). The temperature of the sample block was monitored with an Au/Fe thermocouple and varied by controlling the rate of cold helium-gas flow.

In all cases the natural circular dichroism observed in the absence of an applied magnetic field was subtracted from the m.c.d. spectrum. Samples used to obtain m.c.d. spectra had a maximal absorption, in the spectral region of interest, of 1.0 ± 0.1 , to optimize the quality of the signal obtained.

To extract m.c.d. parameters from variable-temperature spectra, one of two approaches can be used. Either a curve-fitting procedure can be adopted, the so-called rigid-shift assumption, or a moments analysis can be applied. It has been shown that the latter method is the most powerful and rigorous when broad bands are involved (Henry *et al.*, 1965). It is

the moments of m.c.d. spectra that are most directly related to the molecular parameters of interest and should therefore be used when attempting to compare the m.c.d. spectra of different derivatives to ascertain whether they contain identical haems with the same spin and oxidation states.

The definition of moments that we have used here are those of Stephens *et al.* (1971) and are used as follows. First the absorption spectrum, plotted in molar extinction coefficient per haem a group against frequency, ν , in cm^{-1} , is used to define ν^0 , the frequency about which m.c.d. moments are to be taken.

$$\langle \epsilon \rangle_n = \int (\epsilon/\nu)(\nu - \nu^0)^n d\nu$$

where n is the order of the moment. $\langle \epsilon \rangle_1$ is set equal to zero, so that:

$$\nu^0 = \int \epsilon d\nu / \langle \epsilon \rangle_0$$

and $\langle \epsilon \rangle_0 = 1.089 \times 10^2 D_0$, where D_0 is the dipole strength.

Then the m.c.d. spectrum, plotted in differential molar extinction coefficient per unit magnetic field (T) against frequency, ν (cm^{-1}), is analysed as follows:

$$\langle \Delta \epsilon \rangle_n = \int (\Delta \epsilon/\nu)(\nu - \nu^0)^n d\nu$$

The first moment is strongly temperature-dependent when the m.c.d. spectra have the form seen in the Soret region of the spectrum. Further, it is the temperature-dependence of the moment which contains the molecular parameter related to the ground-state g -factor and the excited-state spin-orbit coupling constants. We therefore define a further quantity ΔS , related to the slope of the plot of the first moment against $1/T$ as follows:

$$\Delta S = \frac{\langle \Delta \epsilon \rangle}{D_0} \frac{1}{1/T}$$

When measuring the moments of derivatives containing two haems in different oxidation states the value of ν^0 used is taken from the spectrum of a derivative containing both haems in the same oxidation state. This is necessary because the Soret band of ferrous haem *a* is at a very different energy from that of ferric haem *a*.

Results and Discussion

Our preliminary studies of the m.c.d. spectrum of cytochrome oxidase (Thomson *et al.*, 1976) have shown the presence of anomalous features in the α -band region of the spectrum. Because of this we have confined our attention in this study to the Soret-band region. It seems clear that this region contains only one electronic transition and is therefore more amenable to a moments analysis and to a comparison

with other typical haemoproteins (Briat *et al.*, 1972; Vickery *et al.*, 1976; Springall *et al.*, 1976).

Reduced cytochrome oxidase

Since all four metal centres are reduced, there can be no complicating features in the temperature-dependent m.c.d. spectra due to anti-ferromagnetic coupling with Cu^{2+} ions. This oxidation state there-

Fig. 1. Room-temperature absorption spectrum (upper) and m.c.d. spectra (lower) of reduced cytochrome *c* oxidase (cyanide form), at 40 K (—), and 100 K (---).

The path length for absorption spectra was 10 mm and for m.c.d. spectra it was 0.8 mm in Figs. 1–6, 9 and 10. In Figs. 1–3, 5–7, 9 and 10 $\Delta \epsilon (\text{litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}) = \epsilon_L - \epsilon_R$, where ϵ_L , ϵ_R are molar extinction coefficients for left and right circularly polarized light respectively. B is the magnetic field (T). ϵ ($\text{litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) is molar extinction coefficient. λ (nm) is wavelength.

Fig. 2. Room-temperature absorption spectrum (upper) and m.c.d. spectra (lower) of reduced cytochrome *c* oxidase at 30 K (-----), 46 K (—) and 73 K (-.-.-)

Fig. 3. Room-temperature absorption spectrum (upper) and m.c.d. spectra (lower) of 'mixed-valence' CO derivatives of cytochrome *c* oxidase at 24 K (-----), 41.5 K (—) and 65 K (-.-.-)

fore provides the simplest starting point for an analysis.

Fig. 1 shows the spectrum of the reduced enzyme after incubation with cyanide. At room temperature a positive peak is found at 450 nm and two negative peaks are seen at 437 and 415 nm respectively. On cooling the sample there is a sharpening of the spectra which leads to the appearance of a new feature at 410 nm between 100 and 293 K. But the close similarity of the spectra taken at 40 and 100 K shows unequivocally that both haems are diamagnetic. The similarity of the room-temperature spectra of cyanide and carbon monoxide derivatives indicates that the latter also contains two low-spin ferrous haems (Thomson *et al.*, 1976).

The reduced oxidase without added ligand gives a spectrum which is characterized by a positive peak at 446 nm and a negative peak at 436 nm, as well as two negative shoulders at 416 and 378 nm, which become well resolved at lower temperatures. The

crossover point in the m.c.d. spectrum is located at 441 nm, in other words 3 nm shifted towards the blue from the absorption maximum. By contrast with the cyanide derivative, a strongly temperature-dependent spectrum is observed (Fig. 2). In the Soret region the spectrum is remarkably similar in form to that of deoxymyoglobin (Springall *et al.*, 1976). This is evidence that at least one haem is high-spin ferrous. A comparison of the first moments as a function of temperature for these two haemoproteins shows that both have the same sign but different magnitudes. Thus it is clear that deoxymyoglobin is not an entirely appropriate model for reduced cytochrome oxidase and that the relative magnitude of the two moments cannot be used as an indicator of the number of high-spin ferrous haems in oxidase.

Therefore we have used a different method of answering this question.

In the presence of CO, fully oxidized cytochrome oxidase undergoes reduction, haem a_3 being reduced and trapped as the CO complex (Greenwood *et al.*, 1974). Thus this derivative, the so-called mixed-valence species, contains reduced cytochrome a_3 -CO and oxidized cytochrome *a*. The m.c.d. spectrum at room temperature is in good agreement with this description (Brittain *et al.*, 1976). However, on attempting to measure the low-temperature m.c.d. spectrum of the mixed-valence species the CO is photolysed off cytochrome a_3^{2+} , and an m.c.d. spectrum typical of high-spin ferrous haem is seen (Fig. 3). Haem *a* is not contributing to the spectrum in the way expected for a low-spin ferric haem. It has been argued (Brittain *et al.*, 1976) that this is because haem *a* has switched to the high-spin ferric form for which there is some e.p.r. evidence (Wilson *et al.*, 1976).

Comparison of the magnitudes of the m.c.d. signals at equal temperature for the reduced oxidase and photolysed mixed-valence CO derivative shows them to be remarkably similar. If the latter contains only one high-spin ferrous haem, this implies that the former derivative also possesses only a single high-spin ferrous haem. This conclusion has also been reached by G. Palmer (personal communication) by examination of a different set of derivatives.

Oxidized cytochrome oxidase

The analysis of the m.c.d. spectrum of the oxidized derivatives may be complicated by the presence of anti-ferromagnetic coupling between ferric cytochrome a_3 and a Cu^{2+} ion. Therefore we compare the m.c.d. spectra of a number of derivatives of cytochrome oxidase with the spectra of derivatives of metmyoglobin, using as wide a temperature range as is currently available to us, to see how far there are similarities between the two types of haem centres. Again the spectra are compared only in the Soret region of the spectrum.

The m.c.d. spectra of a number of metmyoglobin derivatives have been measured and the first moments of the Soret band plotted against $1/T$. The results are given in Fig. 4: the slope of the first moment is strongly dependent on the nature of the axial ligand. The cyanide and fluoride derivatives serve to establish the characteristics of the haems, which are, respectively, totally low spin and almost completely high spin. The oxidized fully low-spin haem has in this case a strongly positive first moment, which is linear over the temperature range studied. By contrast, the first moment of the high-spin oxidized haem is negative and only weakly dependent on temperature; moreover, the line is non-linear.

Fig. 4. Plot of m.c.d. first moments against $1/T$ for metmyoglobin derivatives

▲, Cyanide derivative; ●, azide derivative; ■, thiocyanate derivative; ○, hydroxide derivative; △, aquo derivative; ▲, fluoride derivative.

The m.c.d. spectra of the mixed-spin derivatives of metmyoglobin are dominated by the contribution from the low-spin component. Thus even the aquo species, containing only about 20% of the low-spin form, has a positive first moment linear over the higher temperature range.

It has been suggested that the magnitude of the m.c.d. signal at room temperature is linearly related to the percentage of the low-spin component (Vickery *et al.*, 1976), but Fig. 4 shows that this is not necessarily valid, because the slopes of the various derivatives are not identical. Thus comparison of the ratios of the m.c.d. signals at 77 K, say, will give a different value from the ratio at 300 K. A quantitative estimation of the position of the high- $\rightleftharpoons$ low-spin equilibrium is only possible if all haem derivatives were to possess the same value of the first moment when totally low spin. Our data enable this point to be checked by normalizing the first moments of metmyoglobin derivatives to 100% low spin (Table 1); there is considerable variation in the first moment. This is not unexpected, since the first moment depends on ground-state *g*-factors and on spin-orbit coupling in the excited state of the haem Soret band. The latter depends in turn on the extent of metal-haem electronic mixing.

Table 1. Analysis of temperature-dependence of first moment of m.c.d. in Soret-band region for a number of metmyoglobin derivatives

Percentages are estimated from magnetic-susceptibility data (Smith & Williams, 1968). The metmyoglobin derivatives were produced as described by Springall *et al.* (1976).

Axial ligand	Percentage of low-spin form	$10^{-3} \times \Delta S$ normalized to 100% low spin
CN ⁻	100	8.84
N ₃ ⁻	83	4.83
OH ⁻	35	7.18
SCN ⁻	18	17.71
H ₂ O	11	8.05

Fig. 5. Room-temperature absorption spectrum (upper) and m.c.d. spectra (lower) of oxidized cytochrome *c* oxidase at 10.5 K (-----), 25 K (—) and 46.5 K (-.-.-)

We conclude from this study that the swing of a haem spin state from high to low spin or vice versa can be detected by observation of the change in magnitude

Fig. 6. Room-temperature absorption spectrum (upper) and m.c.d. spectra (lower) of oxidized cytochrome *c* oxidase (cyanide form) at 13 K (-----), 22.5 K (—) and 48.5 K (-.-.-)

of the first moment of the Soret-band m.c.d. spectrum. Therefore we have measured the temperature-dependence of the Soret-band m.c.d. spectrum of oxidized cytochrome oxidase and two of its derivatives namely the cyanide and fluoride forms. The spectra are shown in Figs. 5–7 and the first moments are plotted against $1/T$ in Fig. 8. The spectrum of the oxidized form is characterized by positive extrema at 421.5 nm with a shoulder at 400 nm, and negative extrema at 433 and 377 nm. The crossover in the m.c.d. spectra occurs at $426 \text{ nm} \pm 0.5 \text{ nm}$, markedly red-shifted from the absorption maximum, which is located at 420 nm.

Addition of cyanide to the oxidized enzyme causes little change in the position of the extrema noted

Fig. 7. Room-temperature absorption spectrum (upper) and m.c.d. spectra (lower) of oxidized cytochrome *c* oxidase (fluoride form) at 11 K (---), 20 K (—) and 41 K (····).

above for the oxidized enzyme, but results in a large increase in signal intensity at room temperature (the observed signal doubles on addition of cyanide); however, the variable-temperature studies show that at low temperatures an even more marked increase in intensity of the cyanide form as compared with the unliganded species is observed. On addition of fluoride to the oxidized enzyme, as with the cyanide complex, little change in the location of the peak is observed. However, in contrast with the cyanide complex, where the crossover in the m.c.d. and the peak location in the absorption spectrum are virtually coincident, the crossover in the fluoride spectrum shows a red shift of 7 nm from the absorption maximum at 418 nm.

The first moment of the spectrum of the cyanide derivative has a strongly positive slope when plotted against $1/T$ (Fig. 8), suggesting that at least one low-spin ferric haem is present. The question arises, as with the reduced enzyme, does this correspond to one or two haems? We have previously

Fig. 8. Plot of m.c.d. first moments against $1/T$ for cytochrome *c* oxidase derivatives

○, Partially reduced oxidase (cyanide form); ▲, oxidized cytochrome *c* oxidase (cyanide form); ●, oxidized cytochrome *c* oxidase (fluoride form); ■, oxidized cytochrome *c* oxidase; △, partially reduced oxidase (formate form).

shown that absolute magnitude is not necessarily a good guide without precise knowledge of a suitable model. Therefore we resort again to the use of derivatives and partially reduced species.

The first moment of the oxidized enzyme and the fluoride form have a much weaker temperature-dependence than that of the cyanide derivative. This suggests that on removal of cyanide or on addition of fluoride the amount of low-spin haem has sharply decreased. The e.p.r. spectra show the presence of a low-spin ferric haem, presumed to be cytochrome *a*, which corresponds to roughly 50% of the total haem and whose properties are not markedly affected by addition of cyanide (Van Gelder & Beinert, 1969).

Therefore we conclude that the m.c.d. spectra of both the fluoride form and the oxidized enzyme are dominated by the contribution of low-spin haem a^{3+} . On addition of cyanide ion the additional contribution must come from haem a_3^{3+} which has become low spin. Since the haem a_3^{3+} appears to be m.c.d. silent in the fluoride and resting forms of the enzyme, we conclude that it is high spin. In our preliminary account (Thomson *et al.*, 1976) we attempted to estimate the ratio of high-spin ferric cytochrome *a*₃ in the resting and fluoride forms of the enzyme on the basis of room-temperature data. As we have discussed above, this is likely to be an unreliable technique, and indeed we now see in the temperature-dependence data that the slopes of the first moments of those two

derivatives are almost identical, certainly within the experimental uncertainty of the points plotted. Thus we cannot find a difference between the amount of high-spin ferric cytochrome a_3 in the resting and fluoride forms of the enzyme.

On the basis of the present m.c.d. and earlier e.p.r. studies we conclude that there are two low-spin haems in the cyanide derivative and one low-spin haem a^{3+} and one high-spin haem a_3^{3+} in the resting oxidized enzyme, on the assumption that the absolute magnitudes of the first moment change, on altering spin states, in the same manner as do those of the metmyoglobin derivatives.

Partially reduced derivatives of cytochrome oxidase

The exact form of the temperature-dependence of the m.c.d. signals must depend on ground-state g -factors as well as excited-state properties. In the preceding section the working assumption was made that the first moments of oxidized cytochrome

oxidase will change, on altering spin state, in the same qualitative way as do the first moments of the haem Soret band in metmyoglobin derivatives. Since the presence of anti-ferromagnetic coupling between centres may alter totally ground-state g -factors, it is necessary to examine the validity of this assumption. This we do with the aid of cytochrome oxidase derivatives which contain haem a_3 in the oxidized form and haem a in the reduced form (Nicholls, 1976). By the use of the formate and cyanide mixed-valence derivatives, the spectra of the high- and low-spin forms of haem a_3 alone can be obtained in the ferric oxidation state, since haem a is thought to remain in the low-spin ferrous form (Nicholls, 1976).

Fig. 9 shows the m.c.d. spectrum of the partially reduced cytochrome oxidase formate derivative as a function of temperature. There is a simple derivative curve crossing the origin at 443 nm which is temperature-independent. This can be compared with the

Fig. 9. Room-temperature absorption spectrum (upper) and m.c.d. spectra (lower) of partially reduced cytochrome c oxidase (formate form) at 11.5 K (----), 31 K (—) and 54 K (-.-).

Fig. 10. Room-temperature absorption spectrum (upper) and m.c.d. spectra (lower) of partially reduced cytochrome c oxidase (cyanide form) at 16 K (----), 32 K (—) and 80 K (-.-).

spectrum of the reduced cytochrome oxidase cyanide derivative (Fig. 1). Clearly the formate derivative contains low-spin haem *a* in the ferrous state. The weak features with peaks and troughs at 424, 415 and approx. 405 nm in Fig. 9 must be due to high-spin ferric haem *a*₃. Qualitatively the spectrum is similar to that of metmyoglobin fluoride (Springall *et al.*, 1976), although the temperature-dependence is much more marked below 80 K. Thus the m.c.d. spectrum provides striking confirmation of the conclusions of Nicholls (1976).

The spectrum of low-spin haem *a*₃ can be obtained from the m.c.d. of partially reduced cytochrome oxidase cyanide form. Fig. 10 gives the results. A strong temperature-dependence is seen in the peak and trough at 420 and 433 nm. The small positive peak at approx. 450 nm can, by comparison with the spectra in Fig. 1, be assigned to low-spin ferrous haem *a*. The signs of the temperature-dependent spectrum assign it to a low-spin ferric haem *a*, presumably *a*₃.

The temperature-dependences of the first moments for the partially reduced formate and cyanide derivatives are plotted in Fig. 8. The temperature-dependence is due to haem *a*₃³⁺, and as we wish to make a comparison between the temperature-dependence of the m.c.d. spectrum of this haem in the fully oxidized cytochrome oxidase and in partially reduced cytochrome oxidase, it is important to compute the first moment about the same energy in each case. Therefore we have used as the value for this energy the value obtained from the absorption spectrum of the fully oxidized cytochrome oxidase and its cyanide derivative. The slopes of the temperature-dependence plots are given in Table 2.

The values can now be compared to ascertain whether the temperature-dependence of haem *a*₃³⁺ is the same in the fully oxidized and in the partially reduced cytochrome oxidase. Therefore we represent the temperature-dependence of fully oxidized (oxid.) cytochrome oxidase as follows:

$$\Delta S(\text{oxid.}) = \Delta S(\text{l.s. } a_3^{3+}) + \Delta S(\text{h.s. } a_3^{3+})$$

where l.s. denotes a low-spin haem and h.s. a high-spin haem.

Then for the cyanide derivative of the fully oxidized (oxid.) oxidase

$$\Delta S(\text{oxid.} \cdot \text{CN}) = \Delta S(\text{l.s. } a_3^{3+}) + \Delta S(\text{l.s. } a_3^{3+})$$

For the partially reduced derivatives (PR)

$$\Delta S(\text{PR} \cdot \text{HCO}_2\text{H}) = \Delta S(\text{h.s. } a_3^{3+})$$

and

$$\Delta S(\text{PR} \cdot \text{CN}) = \Delta S(\text{l.s. } a_3^{3+})$$

We note that the slopes should be additive. The quantities $[\Delta S(\text{oxid.} \cdot \text{CN}) - \Delta S(\text{oxid.})]$ and $[\Delta S(\text{PR} \cdot \text{CN}) - \Delta S(\text{PR} \cdot \text{HCO}_2\text{H})]$ both give a measure of $[\Delta S(\text{l.s. } a_3^{3+}) - \Delta S(\text{h.s. } a_3^{3+})]$, except that the latter gives this value in the partially reduced enzyme.

Table 2. *Temperature-dependence of the fully oxidized and partially reduced derivatives of cytochrome c oxidase*
See the text for definition of ΔS and ν^0 .

	$10^{-3} \times \Delta S$	$10^{-3} \times \nu^0$ (cm ⁻¹)
Fully oxidized, cyanide	+6.18	23.77
Fully oxidized	+1.45	24.06
Partially reduced, cyanide	+6.71	23.77
Partially reduced, formate	-0.10	24.06

Inserting the values given in Table 2 into these expressions we obtain a value of 4.71×10^3 for the fully oxidized enzyme and 6.81×10^3 for the partially reduced enzyme. Hence there is a difference between the temperature-dependence of haem *a*₃ in the fully oxidized and the partially reduced derivative. It is not therefore strictly valid to subtract the spectra of these derivatives in order to obtain spectra characteristics of individual cyochromes *a*³⁺ and *a*₃³⁺.

It has not been conclusively established whether in the partially reduced formate and cyanide derivatives both Cu²⁺ ions are reduced. There is a report of a new low-spin ferric haem e.p.r. signal on reduction of the cyanide derivative of oxidized cytochrome oxidase (Dervartanian *et al.*, 1974). Presumably this is due to haem *a*₃³⁺ appearing as Cu²⁺ ion is reduced and the coupling is broken. Thus it is possible that in both partially reduced derivatives all components except haem *a*₃ are reduced. This may then account for the difference in the temperature-dependence of haem *a*₃³⁺ between the oxidized and partially reduced enzyme. In this case the spectra in Figs. 9 and 10 show the m.c.d. of high- and low-spin cytochrome *a*₃³⁺ in the uncoupled form.

This conclusion leads to the prediction that in the partially reduced formate derivatives the e.p.r. signal of high-spin cytochrome *a*₃³⁺ should be detectable.

Note Added in Proof (Received 30 May 1977)

Since the submission of this paper it has been discovered that the negative peak at 377 nm prominent in the m.c.d. spectra of Figs. 5, 6, 7 and 9 is due to a paramagnetic impurity in the cryostat cell windows, and is not present in the protein. This peak does not overlap the protein spectrum and has not been included in the moments analysis. Hence our conclusions are not affected.

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References

- Aasa, R., Albracht, S. P. J., Falk, K. E., Lanne, B. & Vänngård, T. (1976) *Biochim. Biophys. Acta* **422**, 260–272
- Babcock, G. T., Vickery, L. E. & Palmer, G. (1976) *Biophys. J.* **16**, 87a
- Briat, B., Berger, D. & Leliboux, M. (1972) *J. Chem. Phys.* **57**, 5606–5607
- Brittain, T., Springall, J. P., Greenwood, C. & Thomson, A. J. (1976) *Biochem. J.* **159**, 811–813
- Dervartanian, D. V., Lee, I. Y., Slater, E. C. & Van Gelder, B. F. (1974) *Biochim. Biophys. Acta* **347**, 321–327
- Gibson, Q. H., Palmer, G. & Wharton, D. C. (1965) *J. Biol. Chem.* **240**, 915–920
- Greenwood, C., Wilson, M. T. & Brunori, M. (1974) *Biochem. J.* **137**, 205–215
- Griffith, J. S. (1971) *Mol. Phys.* **21**, 141–153
- Henry, C. H., Schnatterly, S. E. & Slichter, L. P. (1965) *Phys. Rev.* **137**, 583–602
- Keilin, D. & Hartree, E. F. (1939) *Proc. R. Soc. London Ser. B* **127**, 167–191
- Nicholls, P. (1976) *Biochim. Biophys. Acta* **430**, 13–29
- Nicholls, P. & Peterson, L. C. (1974) *Biochim. Biophys. Acta* **357**, 462–467
- Smith, D. W. & Williams, R. J. P. (1968) *Biochem. J.* **110**, 297–301
- Springall, J. P., Stillman, M. J. & Thomson, A. J. (1976) *Biochim. Biophys. Acta* **453**, 494–501
- Stephens, P. J. & Buckingham, A. D. (1966) *Annu. Rev. Phys. Chem.* **17**, 399–432
- Stephens, P. J., Mowery, R. L. & Schatz, P. N. (1971) *J. Chem. Phys.* **55**, 224–231
- Thomson, A. J., Brittain, T., Greenwood, C. & Springall, J. P. (1976) *FEBS Lett.* **67**, 94–98
- Thomson, A. J., Cammack, R., Hall, D. O., Rao, K. K., Briat, B., Rivoal, J. C. & Badoz, J. (1977) *Biochim. Biophys. Acta* in the press
- Tiesjema, R. H., Hardy, G. P. M. A. & Van Gelder, B. F. (1974) *Biochim. Biophys. Acta* **357**, 24–33
- Tsudsuki, T. & Okunuki, K. (1971) *J. Biochem. (Tokyo)* **69**, 909–922
- Van Buuren, K. J. H., Nicholls, P. & Van Gelder, B. F. (1972) *Biochim. Biophys. Acta* **256**, 258–276
- Van Gelder, B. F. & Beinert, H. (1969) *Biochim. Biophys. Acta* **189**, 1–24
- Vickery, L. E., Nozawa, T. & Sauer, K. (1976) *J. Am. Chem. Soc.* **98**, 351–357
- Wilson, D. F., Erecinska, M. & Owens, C. S. (1976) *Arch. Biochem. Biophys.* **175**, 160–172
- Yonetani, T. (1960) *J. Biol. Chem.* **235**, 845–852
- Yonetani, T. (1961) *J. Biol. Chem.* **236**, 1680–1688