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Near-infrared magnetic and natural circular dichroism of cytochrome *c* oxidase

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A detailed study is presented of the room-temperature absorption, natural and magnetic circular-dichroism (c.d. and m.c.d.) spectra of cytochrome *c* oxidase and a number of its derivatives in the wavelength range 700–1900nm. The spectra of the reduced enzyme show a strong negative c.d. band peaking at 1100nm arising from low-spin ferrous haem *a* and a positive m.c.d. peak at 780nm assigned to high-spin ferrous haem a_3. Addition of cyanide ion doubles the intensity of the low-spin ferrous haem c.d. band and abolishes the 780nm m.c.d. peak because haem a_3 is switched to the low-spin ferrous state. In the reduced carbonmonoxy derivative the haem a_3^{2+}–CO group shows no c.d. or m.c.d. bands at wavelengths longer than 700nm. A comparison of the m.c.d. spectra of the oxidized and cyanide-bound oxidized forms enables bands characteristic of the high-spin ferric form of haem a_3^{3+} to be identified between 700 and 1300nm. At wavelengths longer than 1300nm a broad positive m.c.d. spectrum, peaking at 1600nm, is observed. By comparison with the m.c.d. spectrum of an extracted haem *a*–bis-imidazole complex this m.c.d. peak is assigned to one low-spin ferric haem, namely haem a^{3+}. On binding of cyanide to the oxidized form of the enzyme a new, weak, m.c.d. signal appears, which is assigned to the low-spin ferric haem a_3^{3+}–CN species. A reductive titration, with sodium dithionite, of the cyanide-bound form of the enzyme leads to a partially reduced state in which low-spin haem a^{2+} is detected by means of an intense negative c.d. peak at 1100nm and low-spin ferric haem a_3^{3+}–CN gives a sharp positive m.c.d. peak at 1550nm. The c.d. and m.c.d. characteristics of the 830nm absorption band in oxidized cytochrome *c* oxidase are not typical of type 1 blue cupric centres.

Cytochrome *c* oxidase contains two haems of the *a* type and two copper ions. The haems, although identical when extracted from the protein, are functionally and spectroscopically distinct in the enzyme. One haem, termed haem a_3, will bind added ligands such as CO and CN^- in the reduced state and F^-, CN^- and azide in the oxidized form. This haem is the site of dioxygen reduction. The other haem, called haem *a*, will not react with added ligands. A major obstacle to the understanding of the structures of the four metal centres in this enzyme arises from the fact that the electronic absorption spectra of the two haems overlap one another in the spectral region 300–650nm (Wikström *et al.*, 1976). Very little is known about the nature of the copper centres because their electronic transitions tend to be obscured by the haem components. Attempts have been made to disentangle the relative contributions of each haem to the absorption spectrum at particular wavelengths by the use of difference spectroscopy and by taking advantage of the different reactivities of the two haems towards ligands. However, controversy has continually arisen over the correct interpretation of this type of study (Malmström, 1973; Van Buuren *et al.*, 1972*a*). More recently investigations of the kinetic properties of the electronic absorption bands between 600 and 1000nm have led to suggestions about the relative contributions of the different centres to the absorption in this region (Wilson *et*

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

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al., 1975). There is a need to make a thorough assignment of the electronic absorption bands to particular centres, so that the kinetic properties of each can be defined and indeed conclusions drawn about the electronic states of the centres under various defined conditions.

We have shown that magnetic circular dichroism (m.c.d.) spectroscopy can be a useful probe of cytochrome *c* oxidase, especially when the enzyme is studied at low temperatures (Thomson *et al.*, 1977). This is because some of the paramagnetic states of the haems, namely the high-spin ferrous ($S = 2$) and the low-spin ferric ($S = \frac{1}{2}$) states, at low temperatures (4.2 K) give intense m.c.d. signals that effectively swamp the contributions from other centres. However, there is another way in which m.c.d. spectroscopy can be employed to study the electronic properties of the metal centres. This involves measurement of the electronic spectra in the near-infrared spectral region between 650 and 2000 nm. The technique of natural circular dichroism (c.d.) is also useful in this region.

It has been shown that the near-infrared spectra of haems (Cheng *et al.*, 1973; Rawlings *et al.*, 1977) and of oxidized copper proteins (Dooley *et al.*, 1979; Dawson *et al.*, 1979) reveal bands due to electronic transitions associated with the metal ion. The transitions can be one of two types, either metal localized *d–d* or charge-transfer involving electronic transitions between the metal ion and its surrounding ligands. Therefore these transitions involve the metal ion in a very direct way, and as a consequence they are especially sensitive to the oxidation state and spin state of the metal ion in addition to providing information about the nature and the geometry of the surrounding ligands.

Although the potential value of measuring electronic spectra in this region has been realized for a long time (Day *et al.*, 1967), there have been experimental difficulties. The main problem is due to the presence, at wavelengths longer than 1200 nm, of vibrational absorption bands arising both from the solvent water and the vibrational overtones of the protein itself. By using ^2H_2O as a solvent and by deuterating as many as possible of the exchangeable protons in the protein, it is possible to diminish the absorption background due to vibrational transitions. Even so, there remains considerable ambiguity in deciding whether a particular band observed is due to a vibrational overtone transition or to an electronic transition. Because electronic bands are invariably much broader than the vibrational bands, the electronic transitions form a broad background underlying the vibrational peaks and are often effectively obscured. The advantage of the complementary techniques of c.d. and m.c.d. spectroscopy is that the circular dichroism, natural or magnetic, for vibrational transitions is weaker by a factor of at least 10^3 than that of electronic transitions (Nafie *et al.*, 1976). Hence c.d. and m.c.d. signals due to electronic transitions are readily detected even in the presence of the overlying vibrational transitions. There is a limit to the wavelength range currently accessible. This is set by the onset of the intense vibrational absorption of the protein. For a 5 mm path this limit is effectively 2000 nm.

In the present paper we report for the first time a study of the c.d. and m.c.d. spectra of cytochrome *c* oxidase in the spectral region 700–1900 nm in order to locate new low-energy electronic transitions and to attempt to assign them variously to each of the appropriate four metal centres. By studying a range of derivatives and oxidation levels of the protein we have been able to assign all the new observed bands to the two haems and to a copper centre.

Experimental

All non-deuterated chemicals were of analytical-reagent grade, except $Na_2S_2O_4$, which was laboratory grade, and were obtained from BDH Chemicals, Poole, Dorset, U.K. ^2H_2O and ^2HCl were obtained from Fluorochem, Glossop, Derbyshire, U.K. O_2-free N_2 was supplied by the British Oxygen Co., London S.W.19, U.K., and was further purified for anaerobic work by passage over a reduced B.A.S.F. copper catalyst [B.A.S.F. (U.K.), Cheadle Hulme, Cheshire, U.K.] at 140°C and through a dithionite wash bottle.

Cytochrome oxidase was prepared by a modification of the method of Yonetani (1960) in which 1 μM-EDTA was incorporated as a chelating agent in the last fractionation steps to ensure removal of adventitiously bound copper, which is inactive in electron transfer (Lemberg, 1969). Solutions of cytochrome oxidase prepared as above were 'deuterated' by using the following procedure. The samples were centrifuged at 200000 *g* for 17 h to give a hard pellet of enzyme. The resulting pellets were redissolved in the minimum possible amount of 0.1 M-phosphate buffer, pD 7.4, prepared from the water-free salts Na_2HPO_4 and KH_2PO_4 dissolved in ^2H_2O containing 1% Tween 80. For each sample this deuteration procedure was repeated in order to ensure the maximum possible replacement of protons by deuterons. Finally each sample of solution was centrifuged at 3000 *g* for 15 min to remove any undissolved material. This was discarded. All these operations were carried out at 4°C. The resulting solutions of enzyme in ^2H_2O showed identical spectral absorption characteristics in the range 350–900 nm to the starting solutions in H_2O. Furthermore, the deuteration procedure did not affect the specific activities of the samples, which had turnover numbers in the range 80–200 s^{-1} (electrons/s per cytochrome aa_3) when assayed in an

H_2O solution of 0.1% L-α-phosphatidylcholine (type II; Sigma Chemical Co., Poole, Dorset, U.K.) at 30°C. These values of specific activity compare well with those reported by other authors (Nicholls & Chance, 1974).

The oxidized cyanide-bound cytochrome oxidase was prepared by adding a measured volume of 0.2 M-KCN solution in ^2H_2O (neutralized to pD 7.0 by using ^2HCl) to a known volume of the oxidized enzyme solution. This solution was then left for at least 24 h at 4°C until the Soret-band and α-band absorption of the sample indicated that all the enzyme present was cyanide-bound. The final concentration of cyanide in the sample was at least 20 times that of the enzyme.

The oxidized fluoride-bound enzyme sample was prepared by adding solid NaF to a solution of enzyme to give a final concentration of 500 mM-F^-. The sample was then left for 24 h at room temperature to ensure complete ligand binding.

The reduced enzyme was prepared by adding a few milligrams of solid $Na_2S_2O_4$ to the solution, which was then left in a stoppered cell for 30 min before use. The reduced enzyme–CO complex was formed by evacuating a sample of reduced enzyme in an anaerobic cell and then introducing CO to a pressure of 101 kPa. The reduced cyanide-bound enzyme was prepared anaerobically by adding a small known volume of 0.2 M-KCN solution in ^2H_2O (neutralized to pD 7.0 with ^2HCl) to a known volume of the reduced enzyme. The final concentration of cyanide was at least 20 times that of the enzyme solution.

A review of the literature reveals considerable variations between absorption coefficients quoted for different preparations of cytochrome oxidase and between the ratios of the Soret, α and '830 nm' bands of these preparations. One possible reason for these discrepancies is the different degrees of wavelength-dependent scatter present in different solutions of enzyme. The effect of this is to cause an apparent increase in the absorption of the solution, the effect being more marked at the blue end of the spectrum. To minimize the errors due to scatter, all the derivatives used in the present study were standardized for concentration in the following manner. A small amount of the oxidized enzyme used to prepare each derivative was retained and treated in the same way as the experimental sample except that no ligand was added (where necessary distilled water was added to effect equivalent dilutions). For each sample retained the α-band absorption was measured for the oxidized and the reduced form. These spectra were then used to calculate the concentration of sample from ΔA_{605} (reduced − oxidized) − ΔA_{630}(reduced − oxidized) = 27 $mM^{-1} \cdot cm^{-1}$ (Nicholls, 1978).

For the '830 nm' band an additional reason for the wide variations in the quoted absorption coefficients may arise from the fact that this absorption falls close to the extremes of wavelength-sensitivities of both photomultiplier and PbS detectors and hence is prone to errors from stray light. The Cary 14 spectrophotometer used in these studies has been shown to give identical spectra throughout this detector cross-over region in the presence and in the absence of additional stray-light filters.

The titrations of oxidized resting and cyanide-bound derivatives of cytochrome oxidase against dithionite were performed anaerobically. Equal portions of dithionite were dispensed from a stock solution (nominally 3 μM) into specially constructed anaerobic m.c.d. cells of measured path lengths (approx. 5 mm) with a gas-tight Hamilton syringe. The stock solution was prepared by weighing out dithionite into a side arm and dissolving it in 0.5 M-phosphate buffer, made up in ^2H_2O, that had been degassed in the main body of the vessel. During the time required to complete the titration (approx. 4 h) the stock dithionite solution underwent some oxidation, and therefore the volumes of dithionite added could not be quantified rigorously in terms of reducing equivalents. However, by recording absorption spectra before and after measurement of c.d. and m.c.d. spectra, it was established that no oxidation occurred in the sample cell during the spectrophotometric measurements for all the titration points.

Haem *a* was prepared by the method of Takemori & King (1965). The bis-imidazole derivative was prepared by dissolving haem *a* in 0.1 M-sodium phosphate buffer, pD 7.0, containing 1 μM-sodium dodecyl sulphate and 50 mM-imidazole. The absorption coefficient of this derivative was re-determined, during the present work, by atomic-absorption spectroscopy. The values of $\Delta\varepsilon$ given in Fig. 3 are calculated on the basis of this determination.

Absorption spectra were measured with a Cary 14 spectrophotometer. C.d. and m.c.d. spectra were obtained with an instrument constructed in this laboratory and outfitted with a superconducting solenoid having a maximum field of 5.5 T in a 25 mm-diameter room-temperature bore. This dichrograph consists of a 600 W tungsten/halogen lamp and a monochromator (Hilger and Watts model D330: focal length 300 mm) fitted with a grating blazed at either 1.0 or 2.0 μm with 600 and 300 lines/mm respectively. The monochromatic radiation from the exit slit is linearly polarized with a prism and then passed through a photoelastic modulator (Morvue, fused silica element) resonating at 50 kHz. An image of the grating is formed at the centre of the solenoid and then further focused with fused silica lenses on to a large-area (25 mm-diameter) InSb detector (Spectronics). The latter is housed in a Dewar vessel cooled to 77 K. The light

from the source is chopped mechanically at 120 Hz before it enters the monochromator.

The detection circuitry employs an automatic gain control unit (designed and built in this laboratory by Mr. A. J. Strike). Working in conjunction with a phase-sensitive detector (Ortholoc 9502) tuned to 120 Hz, the gain-control circuit holds the signal output of the InSb detector to a constant value. The 50 kHz signal is detected by a second phase-sensitive detector (Ortholoc 9502) tuned to this frequency. The output from this is fed to a chart recorder. The use of the AGC unit provides a very wide dynamic range to the instrument ($1:10^6$). As a result it has proved possible to use the InSb cell as a detector from 700 nm to beyond 2000 nm. This avoids the troublesome necessity of having to switch detectors from a photoconductive device to a photomultiplier tube below 1000 nm.

The instrument is calibrated by using a birefringent plate and a piece of infrared polaroid, type HR, by the method of Osborne *et al.* (1973) as modified by Nafie *et al.* (1976). The amplitude of the modulator is set by hand at certain fixed wavelengths during the scan. The monochromator slits are set at 3 mm, giving a spectral bandwidth for the grating of 600 lines/mm of 15 nm and for the grating with 300 lines/mm of 30 nm.

Absorption spectra are expressed in terms of the molar absorption coefficient, ε. M.c.d. and c.d. spectra are plotted in terms of $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$, where ε_L and ε_R are respectively the molar absorption coefficients for left and right circulalarly polarized light. Values of $\Delta\varepsilon$ for m.c.d. are normalized to 1 T. In both instances values of $\Delta\varepsilon$ are expressed per mol of enzyme.

Results and assignment of spectra

The results are presented in three separate sets comprising, first, the spectra of the reduced enzyme and its cyanide-bound and CO-bound forms, secondly, the spectra of an extracted haem *a* bis-imidazole derivative in the oxidized state, the spectra of the oxidized form of the enzyme and its fluoride and cyanide derivatives, and, finally, titrations of the oxidized enzyme and the oxidized cyanide-bound enzyme with $Na_2S_2O_4$. The aim is the assignment of all the absorption, c.d. and m.c.d. bands to individual metal centres in the enzyme. For reasons of clarity the arguments leading to such assignments are given immediately after the presentation of each set of results.

The assignment of the spectra of the reduced enzyme and its derivatives is straightforward, mainly because the copper centres possess no electronic absorption bands in the near-infrared spectral region. The reduced copper ion, with the filled-shell $3d^{10}$ configuration, gives rise to no *d–d* transitions and is unlikely to exhibit low-energy charge-transfer bands. By contrast, the spectra of the oxidized enzyme and its derivatives present a number of assignment difficulties, since all four metal centres are expected to have low-energy absorption bands due to electronic transitions. For this reason, we have examined the spectra of oxidized extracted haem *a*, a number of enzyme derivatives and also the behaviour of the near-infrared bands when the derivatives of the oxidized enzyme are reduced by $Na_2S_2O_4$.

Reduced enzyme and some derivatives

The absorption, c.d. and m.c.d. spectra of the reduced form of the protein and the reduced cyanide-bound derivative are shown in Fig. 1. Although the absorption spectra contain a rising background from 1300 to 1900 mm, with pronounced peaks superimposed at 1425 and 1700 nm, the dichroism spectra are devoid of any bands at wavelengths longer than about 1300 nm. It has been noticed that the absorption spectra in the region

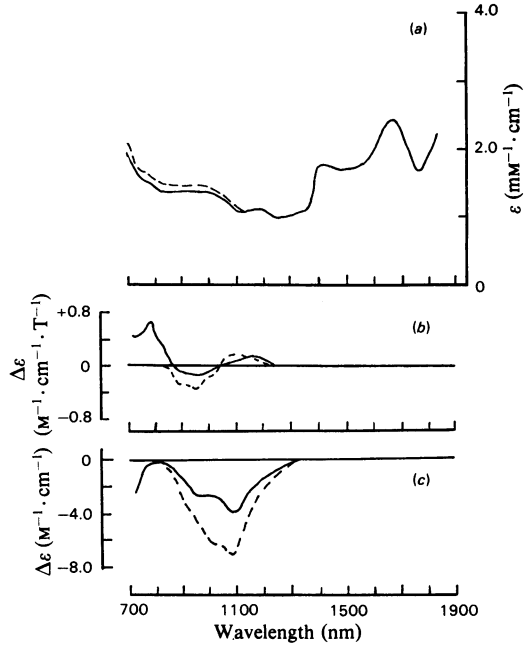

Fig. 1. *Absorption (a), m.c.d. (b) and c.d. (c) spectra of reduced derivatives of cytochrome c oxidase at room temperature*

The path length was 5.3 mm. Samples were in sodium phosphate buffer in ^2H_2O, pD 7.0, containing 1% Tween 80. ——, Reduced enzyme, concentration 0.856 mM; – – – –, reduced cyanide derivative, concentration 0.775 mM. All concentrations throughout are expressed per mol of enzyme.

1300–1900nm vary slightly in intensity and form from sample to sample, depending on the protein batch and the deuteration procedure. However, only one representative plot is shown in each of the Figures in order to illustrate typical absorbance values. The absence of any circular dichroism, either natural or magnetic, between 1300 and 1900nm shows unequivocally that the absorption in this region is due to protein amino acid residues that have been incompletely deuterated, and also possibly to small amounts of residual H_2O. It is known from n.m.r. studies that there are regions in proteins that remain inaccessible to solvent, and that residues in these regions do not exchange their labile protons with ^2H_2O even after the proteins have been soaked for many weeks (Hill & Smith, 1979). We have noticed that cytochrome *c* oxidase seems especially difficult to exchange with ^2H_2O compared with water-soluble proteins such as myoglobin and iron–sulphur proteins. This may be due to a number of factors, including the micellar nature of aqueous suspensions of the enzyme, its high molecular weight and, indeed, the fact that the protein normally functions in a membrane environment.

Fig. 1 shows that the reduced state of cytochrome *c* oxidase gives a c.d. spectrum between 800 and 1300nm that appears as a slightly structured negative band. The m.c.d. spectrum shows features attributable to the same electronic transition, with a negative band at 950nm, followed by a positive peak at 1100nm, crossing the origin at the centre of the c.d. band. Both the c.d. and m.c.d. signals in this region almost exactly double in intensity on addition of cyanide to the reduced form of the enzyme. In addition, the relatively sharp positive band at 770nm in the m.c.d. spectrum of the reduced enzyme disappears on addition of cyanide.

It has been shown previously by low-temperature m.c.d. studies of the Soret-band spectral region that in the reduced form the enzyme contains one haem, haem a_3, in the high-spin ferrous form and the other, haem *a*, in the low-spin ferrous state. Addition of cyanide to haem a_3 converts it into the low-spin ferrous state, so that both haems are diamagnetic in the cyanide-bound form of the enzyme (Thomson *et al.*, 1977). This result has been confirmed by a low-temperature susceptibility study (Tweedle *et al.*, 1978).

The c.d. bands between 800 and 1300nm in the reduced enzyme can therefore be assigned to the low-spin ferrous form of haem *a*. The fact that these bands double in magnitude on addition of cyanide shows that cyanide-bound haem a_3 in the reduced state has a c.d. spectrum in this region of energy identical with that of low-spin ferrous haem *a*. The m.c.d. spectrum in this energy region is also clearly due to the same spin state. Thus we conclude that the c.d. and m.c.d. spectra of low-spin ferrous haem *a* and cyanide-bound haem a_3 are quite similar from 800 to 1300nm.

The positive m.c.d. peak at 770nm in the spectrum of the reduced enzyme that disappears on addition of cyanide can be assigned to the high-spin ferrous state of haem a_3. A very similar positive m.c.d. band has also been detected in the spectrum of deoxymyoglobin and deoxyhaemoglobin at 780nm (Nozawa *et al.*, 1976; Eaton *et al.*, 1978). Both of these proteins contain the high-spin ferrous haem.

The absorption, c.d. and m.c.d. spectra of the CO derivative of the reduced oxidase are shown in Fig. 2. No bands at wavelengths longer than 1300nm have been detected in the dichroism spectra. Comparison of Figs. 1 and 2 shows that in the region 800–1300nm the c.d. and m.c.d. spectra of the CO derivative are identical in form and magnitude with those of the reduced form of the enzyme. Moreover, there is no band at 770nm in the m.c.d. spectrum of the CO derivative. Therefore we conclude that there is no high-spin ferrous haem a_3 in this derivative, but only one low-spin ferrous haem contributes dichroism bands in the region 800–1300nm. Since previous m.c.d. studies in the Soret-band region

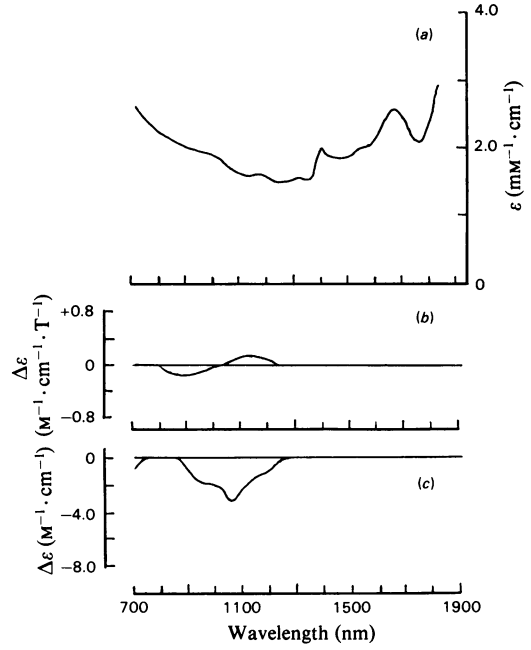

Fig. 2. *Absorption (a), m.c.d. (b) and c.d. (c) spectra of CO derivative of reduced cytochrome c oxidase at room temperature*

The path length was 5.31mm. Samples were in 0.1M-sodium/potassium phosphate buffer in ^2H_2O, pD 7.0, containing 1% Tween 80. The concentration was 0.665mM.

showed that the CO form contains both haem *a* and haem a_3 in the low-spin ferrous state (Thomson *et al.*, 1977; Babcock *et al.*, 1976) we conclude that the haem a_3–CO centre has no electronic states at wavelengths longer than 700nm.

Extracted haem a

The m.c.d. spectrum of oxidized haem *a* bis-imidazole derivative in ^2H_2O and sodium dodecyl sulphate shown in Fig. 3 has a positive signal rising in intensity from 1000nm to a shoulder at 1350nm followed by a positive peak at 1550nm. This derivative has been shown by low-temperature m.c.d. studies of the Soret-band and visible spectral regions to be a low-spin ferric haem (Brittain *et al.*, 1978). E.p.r. measurements also confirm this (Babcock *et al.*, 1979). The form of the near-infrared m.c.d. spectrum is similar to that of other low-spin ferric haems as studied in proteins such as met-myoglobin imidazole derivative (Nozawa *et al.*, 1976), methaemoglobin imidazole derivative (Rawlings *et al.*, 1977) and oxidized cytochrome *c* (Rawlings *et al.*, 1977). However, the energies of the m.c.d. peaks are sensitive to the nature of the axial ligands that co-ordinate the iron atom of the haem. Fig. 3 therefore gives the m.c.d. characteristics of low-spin ferric haem *a* in the near infrared when co-ordinated by two imidazole groups.

Oxidized enzyme and its derivatives

The absorption and m.c.d. spectra of the oxidized and oxidized cyanide-bound forms of cytochrome *c* oxidase are compared in Fig. 4. The c.d. spectra are not shown, as the signals are weak and are not detected at the same instrumental sensitivity settings required to measure the m.c.d. spectra in an applied field of 5T. Hence the c.d. signals are nowhere greater than $\Delta\varepsilon$ 0.1 $M^{-1}\cdot cm^{-1}$ in any of the fully oxidized derivatives of the enzyme.

The absorption spectrum of the oxidized enzyme shows a prominent peak at 850nm followed by a broad shoulder between 1000 and 1350nm. At longer wavelengths the absorption spectrum is similar to that of the reduced derivatives. Hence this latter region is dominated by the vibrational overtone absorption of the protein and residual solvent water. However, the m.c.d. spectrum shows the presence of electronic states extending through from 700 to 1900nm, the long-wavelength limit of these studies. The most prominent features are the strong negative band at 870nm and the positive peak at 1600nm.

The absorption spectrum of the cyanide derivative still shows the pronounced peak at 830nm, although decreased in intensity compared with that of the oxidized form. The broad shoulder to longer wavelength in the spectrum of the oxidized enzyme is largely lost. The m.c.d. spectra of the cyanide and oxidized forms are very similar. The negative band at 870nm is weaker in intensity in the cyanide derivative, but in the region 1250–1900nm there is a small but definite increase in the strength of the positive m.c.d. signal. The only new spectral feature to appear is the positive intensity at 1900nm in the m.c.d. spectrum.

The absorption and m.c.d. spectra of the fluoride derivative of the oxidized enzyme are given in Fig. 5. The absorption spectrum shows the prominent peak at 840nm, although the shoulder on the long-wavelength side of this band is much decreased in

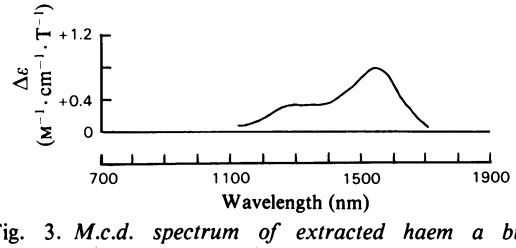

Fig. 3. *M.c.d. spectrum of extracted haem a bis-imidazole complex*

The haem *a* was dissolved in 0.1M-sodium phosphate buffer, pD7.4, containing 1μM-sodium dodecyl sulphate and 50mM-imidazole. The concentration of haem *a* was 0.19mM. The path length was 10mm.

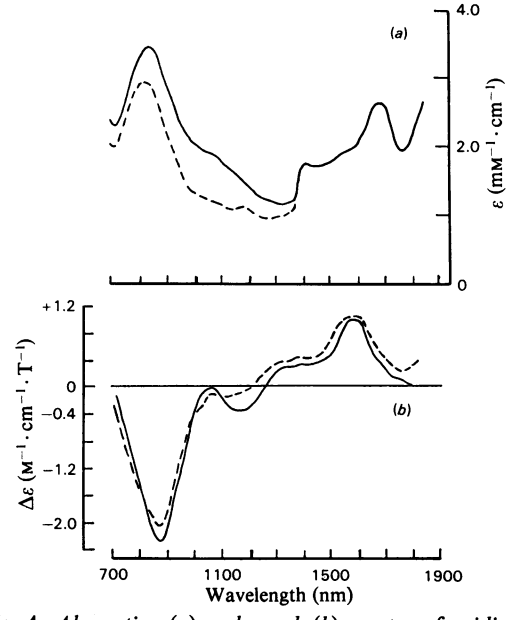

Fig. 4. *Absorption (a) and m.c.d. (b) spectra of oxidized cytochrome c oxidase derivatives at room temperature*

The path length was 5.0mm. ——, Oxidized enzyme, concentration 0.711mM; – – – –, cyanide derivative, concentration 0.711mM.

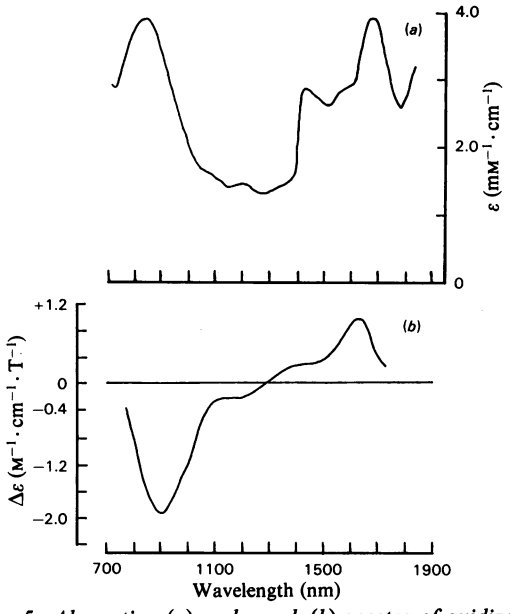

Fig. 5. *Absorption (a) and m.c.d. (b) spectra of oxidized fluoride derivative of cytochrome c oxidase at room temperature*

The path length was 5.0mm. Samples were in 0.1 M-sodium/potassium phosphate buffer in ^2H_2O, pD 7.0, containing 1% Tween 80. The concentration was 0.466 mM.

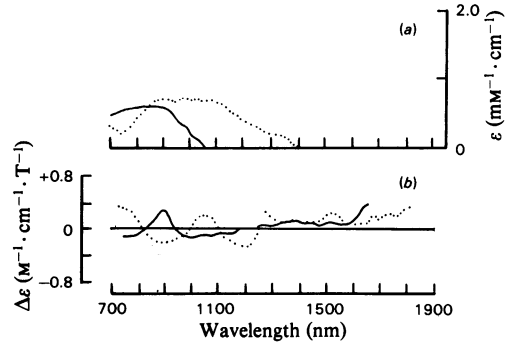

Fig. 6. *Difference spectra of oxidized cytochrome c oxidase derivatives derived from Figs. 4 and 5*

(*a*) Absorption difference spectrum; (*b*) m.c.d. difference spectrum. ····, Oxidized enzyme minus cyanide derivative; ——, fluoride derivative minus cyanide derivative.

intensity compared with that in the spectrum of the oxidized enzyme. The m.c.d. spectrum shows the same form as that of the oxidized enzyme but with the small negative trough at 1200nm much decreased.

To obtain the absorption and m.c.d. spectra of the components that alter on addition of cyanide to the oxidized enzyme and the fluoride derivative, difference spectra have been computed and are given in Fig. 6. The difference absorption spectrum of the oxidized minus cyanide-bound forms shows a broad flat-topped band between 700 and 1200nm. Similarly the difference absorption spectrum of the oxidized fluoride minus the cyanide-bound form gives a broad band of similar shape between 700 and 950nm. We conclude that addition of cyanide either to the oxidized enzyme or to its fluoride form causes the disappearance of a species with a broad absorption band in the region 700–1200nm with an absorption coefficient 700 $M^{-1}\cdot cm^{-1}$. The m.c.d. difference spectra in the same wavelength range reveal the m.c.d. spectra of the species that possess this broad absorption band. The spectra show alternating negative and positive signs from long to short wavelengths over the range 1300–700nm. At wavelengths longer than 1200nm the m.c.d. difference spectra (Fig. 6) show a positive signal steadily rising up to 1850nm. In the present study we are unable to report m.c.d. spectra with confidence at wavelengths longer than about 1900nm because the solvent, ^2H_2O, has an intense overtone absorption that rises sharply beyond 1900nm. To make measurements within the region of this intense absorption it is necessary to use a path length of 1mm or less. This in turn requires either that the protein concentration is raised above the concentration of millimolar or that instrumental sensitivity is increased.

The absorption difference spectra (Fig. 6) show clearly the disappearance of a broad band in the range 700–1200nm when cyanide becomes bound to haem a_3 in the oxidized enzyme or when it replaces fluoride ion bound to haem a_3. The form of the m.c.d. spectrum of the species that disappears is also clearly seen in the m.c.d. difference spectra. This species has the characteristics of a high-spin ferric haem. However, the near-infrared absorption and m.c.d. spectra of the high-spin ferric forms of the following haemoproteins have been studied: haemoglobin–F, aquo-haemoglobin (Stephens *et al.*, 1976), myoglobin–F and aquo-myoglobin (Nozawa *et al.*, 1976). In all cases a broad absorption band in the range 700–1100nm is seen, and the m.c.d. spectrum is a characteristic *A*-term shape, with a negative lobe at longer wavelength, followed by a positive peak, and with the aquo species these are followed by two further bands, first negative then positive. Hence the m.c.d. difference spectra of Fig. 6 resemble the m.c.d. spectra of well-characterized high-spin ferric haemoproteins. Furthermore, it has been demonstrated that the near-infrared m.c.d. bands of a series of high-spin ferric haemoglobin derivatives move to shorter wavelength as the sixth axial ligand is changed in the order H_2O, HCO_2^-,

$CH_3CO_2^-$, F^- (Rawlings *et al.*, 1977). The spectra in Fig. 6 similarly show that the m.c.d. bands of the high-spin fluoro form of haem a_3 are at shorter wavelength than those of the oxidized form of the enzyme. The m.c.d. spectrum of the high-spin haem a_3 in the oxidized form of the enzyme is very similar to that of aquo-haemoglobin. However, it is premature to draw conclusions from this comparison about the possible identity of the sixth axial ligand of haem a_3 in cytochrome oxidase. Nevertheless, these data provide spectroscopic evidence that can give a guide to the identity of this ligand.

Overlaying these bands arising from high-spin haem a_3^{3+} is the intense peak between 840 and 850nm in the absorption spectrum that gives a strong negative m.c.d. band at 870nm. This band has no counterpart in the reported spectra of any haemoproteins other than cytochrome *c* oxidase. It has been assigned variously to a haem centre and to a copper centre (Tsudsuki & Wilson, 1971; Van Gelder *et al.*, 1973). The assignment of this band is considered again in the Discussion section of the present paper.

Turning to the spectral region between 1300 and 1900nm, comparison of the m.c.d. spectra in Figs. 4 and 5 with that of extracted haem *a* (Fig. 3) suggests that the m.c.d. peak at 1600nm in the spectrum of the oxidized enzyme and the fluoride-bound form is due solely to a low-spin ferric haem. Comparison of the intensities, which are expressed per mol of enzyme in the case of the enzyme, support the argument that the enzyme contains one low-spin ferric haem, namely haem a^{3+}. However, the m.c.d. spectrum of the cyanide-bound form of the enzyme between 1300 and 1900nm (Fig. 4) is quite unexpected. Our interpretation of the spectral region 700–1300nm suggests that addition of cyanide abolishes bands due to a high-spin ferric haem, implying that haem a_3^{3+} has become cyanide-bound and hence low-spin. Therefore this haem is expected to appear as a typical low-spin ferric haem in the m.c.d. spectrum between 1300 and 1900nm. But only a small increase in intensity is seen in the m.c.d. spectrum in this spectral region (Fig. 4). The m.c.d. difference spectra (Fig. 6) indicate that the new species generated by the addition of cyanide ion to haem a_3 has a broad positive m.c.d. signal increasing in magnitude across the whole of the region 1300–1900nm and possibly extending beyond 1900nm, the long-wavelength limit of our experiments. We conclude that in the fully oxidized form of the enzyme the species haem a_3^{3+}–CN has an m.c.d. spectrum in the range 1300–1900nm unique among the m.c.d. spectra of low-spin ferric haemoproteins.

Reductive titrations of cytochrome oxidase and cyanide-bound enzyme

Anaerobic reduction with $Na_2S_2O_4$ of the oxidized enzyme provides a useful check that the signals assigned to different components are behaving in the expected manner. The absorption, c.d. and m.c.d. spectra of the enzyme after successive additions of $Na_2S_2O_4$ show the abolition of the 830nm band and the shoulder between 1000 and 1300nm (Fig. 7). Little change is apparent in the region of the protein and solvent vibrational overtone absorption, as expected. The c.d. spectra show the gradual appearance, as reduction proceeds, of signals at 720nm and also between 900 and 1300nm, which are attributable to the reduced form of haem *a*. The m.c.d. spectra show the gradual disappearance of all the signals due to the oxidized components and the appearance both of a strong signal at 760nm, attributed to high-spin ferrous haem a_3, and also of the sigmoid curve centred at 1000nm due to low-spin ferrous haem *a*. Hence it is feasible to follow separately the disappearance of each of the

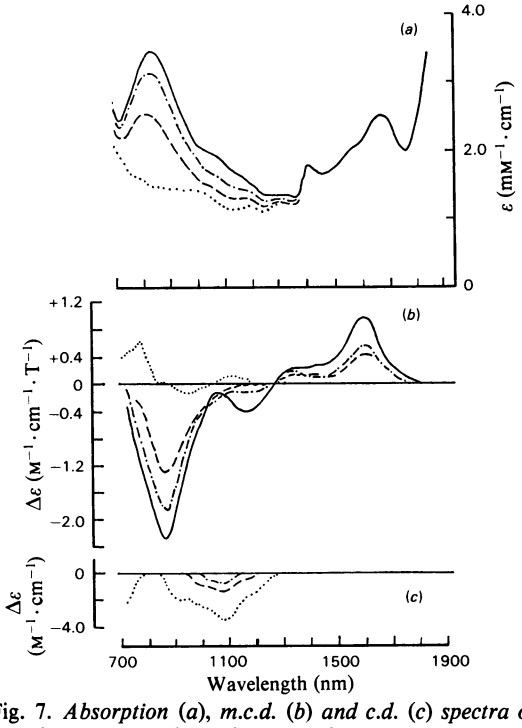

Fig. 7. *Absorption (a), m.c.d. (b) and c.d. (c) spectra of cytochrome c oxidase during reductive titration with* $Na_2S_2O_4$

The path length was 5.18mm. Samples were in 0.1M-sodium/potassium phosphate buffer in ^2H_2O, pD 7.0, containing 1% Tween 80. The concentration of protein was 0.551mM, and that of $Na_2S_2O_4$ was nominally 3μM. ——, Fully oxidized; –·–·–·, after addition of 80μl of $Na_2S_2O_4$; – – –, after addition of 120μl of $Na_2S_2O_4$; ····, after addition of 220μl of $Na_2S_2O_4$.

oxidized haems and their appearance in their reduced states. The dichroism bands of each are quite distinct and well separated in the c.d. and m.c.d. spectra.

Reduction of the cyanide-bound oxidized enzyme with $Na_2S_2O_4$ under anaerobic conditions leads to the fully reduced cyanide-bound form of the enzyme only if the enzyme is left in the presence both of excess of reducing agent and cyanide ion for several hours (Van Buuren *et al.*, 1972*b*). However, there is a rapid reduction step to form a so-called partially reduced cyanide form of the enzyme. Fig. 8 shows the absorption, c.d. and m.c.d. spectra of a titration of the cyanide-bound oxidized enzyme to the partially reduced state with $Na_2S_2O_4$ solution. The absorption spectra show the gradual loss of the 840nm peak as reduction proceeds. When excess of dithionite is present the absorption spectrum between 700 and 1400nm is rather flat and featureless, although the absorbance is not zero. At the beginning of reduction two relatively intense c.d. bands begin to appear, one at 720nm and the other in the range 850–1300nm. No c.d. bands at wavelengths longer than 1300nm could be detected at the same sensitivity setting.

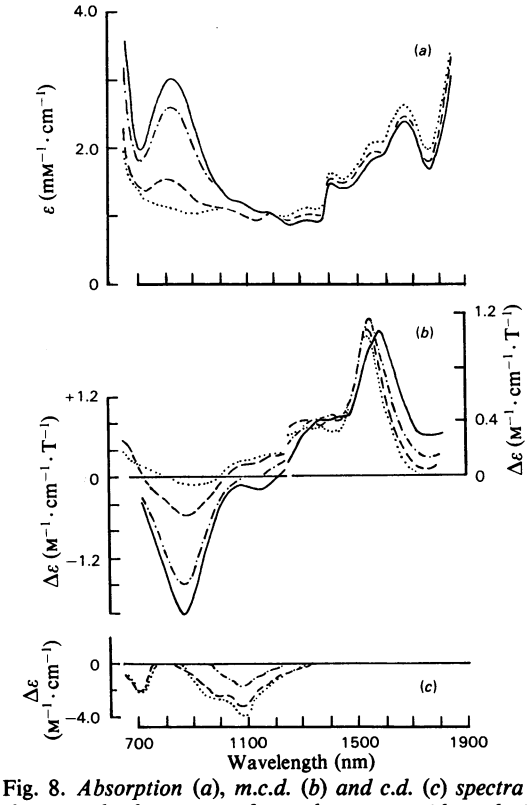

Fig. 8. *Absorption (a), m.c.d. (b) and c.d. (c) spectra of the cyanide derivative of cytochrome c oxidase during reductive titration with* $Na_2S_2O_4$

The path length was 5.31mm. Samples were in 0.1M-sodium/potassium phosphate buffer in ^2H_2O, pD7.0, containing 1% Tween 80. The concentration of protein was 0.429mM, and that of $Na_2S_2O_4$ was nominally 3μM. ——, Oxidized cyanide derivative; –·–·–·, after addition of 40μl of $Na_2S_2O_4$; – – – –, after addition of 70μl of $Na_2S_2O_4$; ····, after addition of 210μl of $Na_2S_2O_4$.

The m.c.d. spectra show the gradual disappearance of the negative peak at 840nm until in the presence of excess of dithionite there remains a negative m.c.d. peak at 900nm crossing to a positive signal at 1000nm. The most interesting changes take place in the m.c.d. spectrum between 1300 and 1800nm. As reduction proceeds the broad peak at 1550nm is replaced by a sharper peak at 1500nm. Subsequent reduction steps sharpen the band further and slightly decrease the intensity of the peak.

The spectra show most clearly the course of reduction of the cyanide-bound form of the enzyme, and make an interesting contrast with the reduction of the unbound oxidized enzyme. As reduction proceeds the species responsible for the 840nm band is reduced. The growth of the natural c.d. signals in the range 850–1300nm indicates the appearance of a low-spin ferrous haem, namely haem a^{2+}. The sigmoid m.c.d. band associated with this haem also appears. The intensity of this c.d. band, even in the presence of excess of dithionite, never exceeds a value of $\Delta\varepsilon = -2.0\,M^{-1}\cdot cm^{-1}$, the value for the fully reduced unbound enzyme. In other words, only one low-spin ferrous haem is formed in the presence of excess of dithionite.

The m.c.d. spectrum at wavelengths longer than 1300nm shows the disappearance of the low-spin ferric haem *a* signal at 1550nm and its replacement by the narrower peak at 1500nm. All m.c.d. absorption at 1800nm and beyond is also abolished by reduction. Since haem *a* appears in the c.d. spectrum of the partially reduced state in the low-spin ferrous form, the m.c.d. at 1500nm must be due to low-spin ferric haem a_3^{3+}–CN.

Therefore we conclude that in the partially reduced cyanide form cytochrome *c* oxidase contains low-spin ferrous haem *a* and low-spin ferric haem a_3^{3+}–CN. Moreover, the m.c.d. spectrum of the latter species is quite typical of low-spin ferric haems, in contrast with the m.c.d. spectrum of this species in the fully oxidized state of the enzyme. Also, the characteristic 840nm band is absent from the absorption, c.d. and m.c.d. spectra of the partially reduced cyanide state.

Discussion

In the preceding section results have been presented showing that both the oxidized and reduced states of cytochrome *c* oxidase contain

electronic states in the near-infrared region between 700 and 1300nm in the latter case and between 700 and 1900nm in the former case. Arguments were presented to show that all of the new bands can be assigned with reasonable confidence to one or more of the metal centres in the protein. It is clear that this spectral region allows observation of the two haems a and a_3 separately, and it is to be hoped that this will allow more detailed studies of the reduction and oxidation characteristics of individual haem centres. However, the spectra contain in addition a great deal of information about the structures of the metal centres. In this discussion we draw attention to some of these more interesting aspects and, where possible, suggest possible interpretation.

Reduced enzyme

The present work has established that low-spin haem a^{2+} and low-spin haem a_3^{2+}–CN have electronic states between 800 and 1300nm that give c.d. bands with relatively high values of the anisotropy factor, defined as $\Delta\varepsilon/\varepsilon$, of the order of 5×10^{-3}. This fact and the low absorption coefficients of the bands (less than $700\,M^{-1}\cdot cm^{-1}$) suggest that these states arise from d–d configurations. In ferrocytochrome c bands between 700 and 1000nm that give rise to c.d. spectra with values of $\Delta\varepsilon/\varepsilon = 2 \times 10^{-3}$ have been assigned to d–d transitions (Eaton & Charney, 1969). There is, however, no previous report of the identification of the d–d bands of a low-spin ferrous haem co-ordinated by cyanide. The fact, established by the present work, that the c.d. bands of haem a_3^{2+}–CN are identical in sign and in energy with those of low-spin haem a^{2+} is noteworthy. This suggests that the two haems, a^{2+} and a_3^{2+}–CN, experience identical ligand fields. There is evidence from e.p.r. studies of the NO-bound form of reduced cytochrome c oxidase that one of the axial ligands of haem a_3^{2+} is histidine. It is therefore possible that in the cyanide-bound reduced state of cytochrome c oxidase haem a^{2+} has two histidine ligands and haem a_3^{2+} has one cyanide and one histidine ligand, thereby generating virtually identical ligand fields at the two haem iron centres (Stevens *et al.*, 1979).

In contrast with this, we have shown that haem a_3^{2+}–CO, in the CO-bound form of cytochrome c oxidase, has no d–d states at wavelengths longer than 700nm. This is not unexpected, since the CO-bound derivatives of reduced haemoglobin and myoglobin likewise have no d–d transitions at wavelengths longer than their α-bands (Eaton *et al.*, 1978; Nozawa *et al.*, 1976). Careful analysis of the c.d. spectrum of CO–haemoglobin reveals that the d–d bands lie in the visible region below the haem α- and β-bands. The strong ligand field of the CO group displaces the d-states to high energy. Therefore it seems likely that the d–d transitions of haem a_3^{2+}–CO must lie at wavelengths shorter than 700nm. A study of the c.d. spectrum in the visible region of reduced cytochrome c oxidase–CO complex may reveal them.

The high-spin ferrous form of haem a_3 shows a prominent positive m.c.d. peak at 770nm with a peak value $\Delta\varepsilon$ of $0.6\,M^{-1}\cdot cm^{-1}\cdot T^{-1}$. The c.d. associated with this band is largely swamped by the contribution from the low-spin ferrous haem a. Very similar m.c.d. spectra have been reported for deoxy-haemoglobin (Eaton *et al.*, 1978), myoglobin (Nozawa *et al.*, 1976) and reduced cytochrome c' (Rawlings *et al.*, 1977), each displaying a prominent positive peak at 780nm. In addition, two weaker positive m.c.d. peaks are reported in the spectra of the latter group of proteins between 800 and 1000nm. In the present work we have been unable to observe the counterparts of these bands in the spectrum of high-spin haem a_3^{2+} because the transitions from haem a^{2+} obscure them. However, at low temperature the m.c.d. spectrum of the paramagnetic haem a_3 will undoubtedly dominate, and the complete near-infrared m.c.d. spectrum of high-spin haem a_3^{2+} should become visible.

Oxidized enzyme

Our previous studies of the variable-temperature m.c.d. spectra in the Soret-band region of oxidized and partially reduced derivatives of cytochrome c oxidase led to the conclusion that the enzyme, in the native oxidized state, most probably contains high-spin haem a_3^{3+} and low-spin haem a^{3+}. Binding of cyanide to haem a_3^{3+} causes it to become low-spin (Thomson *et al.*, 1976, 1977). In establishing these conclusions the m.c.d. spectra were measured over the temperature range 20–300K, to confirm that m.c.d. spectra of the two haems had the expected temperature-dependence and to establish that antiferromagnetic coupling between haem a_3^{3+} and copper was not modifying the magnitudes of the m.c.d. signals. Near-infrared spectral studies should provide verification of this model, since the high-spin and low-spin states of ferric haems give quite distinctive m.c.d. bands in separate regions of the near infrared. However, as described in the Results and assignment of spectra section, these expectations are not fully realized in cytochrome oxidase.

By using difference m.c.d. spectroscopy we have located electronic states in the region 700–1300nm in both the oxidized enzyme and its fluoride-bound form that have the form of m.c.d. spectrum expected for a high-spin ferric haem. Naturally we assign these to haem a_3^{3+}. Furthermore these bands disappear when cyanide ion is bound to haem a_3^{3+}, confirming our expectation that this haem becomes low-spin. However, haem a_3^{3+}–CN, in the oxidized form of the enzyme, does not give a typical low-spin haem band in the region 1500–1600nm. Haem a^{3+}

is clearly visible as a low-spin ferric haem in the m.c.d. spectra in this region.

To facilitate comparison, Fig. 9 collects together the m.c.d. data in the range 1100–1900nm from all the derivatives studied in the present work that contain at least one low-spin ferric haem. It is obvious that all the derivatives have m.c.d. intensities very similar to that of a model extracted haem *a* bis-imidazole derivative. The second haem is readily accounted for in all the enzyme derivatives except that of the oxidized cyanide-bound one. For example, in the oxidized enzyme there are high-spin ferric haem bands (haem a_3^{3+}) between 700 and 1100nm and typical low-spin haem bands (haem a^{3+}) between 1300 and 1800nm. In the partially reduced cyanide derivative one low-spin ferrous haem (haem a^{2+}) is identified by the natural c.d. bands between 800 and 1200nm, and the other (haem a_3^{3+}–CN) appears as the low-spin ferric form at 1500nm. It is the oxidized cyanide derivative that, at first sight, is abnormal, with only one haem (haem a^{3+}) appearing to give a peak in the m.c.d. at 1550nm. The evidence from the m.c.d. data in the Soret-band region (Thomson *et al.*, 1976, 1977) indicates that both haem a^{3+} and haem a_3^{3+}–CN are low-spin in this derivative. Therefore we are forced to conclude that the haem a_3^{3+}–CN contributes the broad positive m.c.d. signal between 1300 and 1400nm seen most clearly in the m.c.d. difference spectrum (CN-oxidized minus oxidized). If this conclusion is correct then haem a_3^{3+}–CN has a broad featureless m.c.d. spectrum extending from 1300 to beyond 1900nm in the oxidized form of the enzyme, although in the partially reduced state haem a_3^{3+}–CN gives a typical low-spin ferric haem m.c.d. peak.

We have no conclusive explanation for this dramatic change in the near-infrared spectral characteristics of the centre haem a_3^{3+}–CN as the other metal centres in the enzyme are reduced. The typical low-spin ferric haem m.c.d. bands in the region of 1500nm have been assigned to porphyrin-to-ferric charge-transfer transitions (Stephens *et al.*, 1976). It appears as though these band have been spread out across the wavelength range 1300 to beyond 1900nm in the oxidized cyanide-bound derivative of cytochrome *c* oxidase. It may be that the main peak is at lower energy beyond the range of our instrument. This may be due to an unusually distorted environment of the haem a_3^{3+}, to a set of unusual ligands or possibly to an interaction between the haem a_3^{3+} and a copper centre. However, the m.c.d. spectra of Fig. 9 provide a set of 'fingerprint' spectra against which to compare the spectra of synthetic models of the haem a_3^{3+}–CN centre.

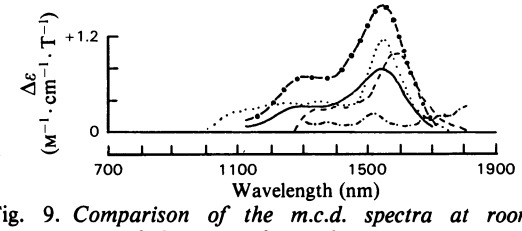

Fig. 9. *Comparison of the m.c.d. spectra at room temperature of low-spin ferric haem a in various environments*

Data are extracted from other Figures in the present paper. ——, Haem *a* bis-imidazole (Fig. 3); –•–•–•, twice the haem *a* spectrum of Fig. 3; – – – –, haem a^{3+} in the oxidized enzyme (Fig. 4). ····, haem a_3^{3+}–CN in the partially reduced cyanide form (Fig. 8); –·–·–·, haem a_3^{3+}–CN in the oxidized enzyme, obtained by difference spectroscopy (Fig. 6).

Origin of the 840nm band

Oxidized derivatives of cytochrome *c* oxidase show a relatively prominent peak in their absorption spectra at about 840nm. In the oxidized enzyme (Fig. 4) the peak maximum is 840nm with ε approx. $3500\,M^{-1}\cdot cm^{-1}$ (expressed per mol of enzyme), and in the cyanide-bound form (Fig. 4), the peak maximum is 820nm with ε approx. $3000\,M^{-1}\cdot cm^{-1}$. The assignment of this band has been problematical. Wharton & Tzagaloff (1964) suggested that the absorption is due, in large part, to cupric ion in the enzyme. The present work has shown clearly that this band is composite in the oxidized form of the enzyme, since bands due to high-spin haem a_3^{3+} run across the spectrum from 700 to 1300nm. However, in the cyanide-bound form there is no evidence for a contribution in this region from haem.

The following arguments suggest strongly that the major part of the intensity of the 840nm band arises from cupric ion, not from haem. (i) The band is not present in the spectrum of extracted haem *a* bis-imidazole derivative (Fig. 3). (ii) The band is not present in the spectrum of partially reduced cyanide-bound enzyme (Fig. 8). (iii) In a reductive titration of the cyanide-bound enzyme the 840nm band titrates quite differently from that of the e.p.r. signal arising from haem a^{3+} but in a manner identical with that of the e.p.r. signal of the cupric ion (M. K. Johnson, D. G. Eglinton, C. Greenwood, P. E. Gooding & A. J. Thomson, unpublished work). Hence the major part of the band intensity can be assigned to cupric ion. In the absence of any knowledge about the reduction characteristics of the e.p.r.-silent cupric centre, it is not possible to determine whether or not this centre contributes to the 840nm band in addition to the e.p.r.-detectable cupric centre.

The present work shows that the 840nm band gives rise to a relatively intense negatively signed m.c.d. band (Δε approx. $2.0\,M^{-1}\cdot cm^{-1}\cdot T^{-1}$) (Figs. 4

and 5). However, the natural c.d. is weak, $\Delta\varepsilon < 0.1\,M^{-1}\cdot cm^{-1}$. Studies of the near-infrared m.c.d. and c.d. spectra of copper proteins are not extensive. However, recent papers (Dooley *et al.*, 1979; Dawson *et al.*, 1979) report data on laccase, caeruloplasmin and a variety of blue (type 1) copper proteins. The c.d. and m.c.d. of bovine superoxide dismutase, which contains type 2 copper, has also been reported (Rotilio *et al.*, 1973). Type 1 copper proteins have absorption bands in the range 500–900nm with absorption coefficients up to $5 \times 10^3\,M^{-1}\cdot cm^{-1}$. The m.c.d. spectra, at room temperature, consist of one or more negative bands (Table I of Dooley *et al.*, 1979) with $\Delta\varepsilon$ values between −0.2 and $-0.4\,M^{-1}\cdot cm^{-1}\cdot T^{-1}$. The $\Delta\varepsilon$ value of $-0.88\,M^{-1}\cdot cm^{-1}\cdot T^{-1}$ at 684nm reported for caeruloplasmin probably arises from the presence of two type 1 cupric centres per mol of enzyme. The natural c.d. spectrum is complex, with several positive and negative bands, but it is invariably intense compared with the m.c.d. signals. For example, values of $\Delta\varepsilon$ range from $3.6\,M^{-1}\cdot cm^{-1}$ for plastocyanin to $3.96\,M^{-1}\cdot cm^{-1}$ for azurin (Solomon *et al.*, 1976). Type 2 copper in superoxide dismutase gives near-infrared bands with ε values up to $250\,M^{-1}\cdot cm^{-1}$ at 680nm, m.c.d. signals of $-0.16\,M^{-1}\cdot cm^{-1}\cdot T^{-1}$ and c.d. signals with $\Delta\varepsilon$ $0.80\,M^{-1}\cdot cm^{-1}$ (Rotilio *et al.*, 1973).

There have been suggestions (Powers *et al.*, 1979) that cytochrome *c* oxidase contains a blue type 1 copper centre and that this is responsible for the 830nm absorption band. Comparison is drawn, by these authors, with stellacyanin, a blue copper protein with absorption in this region. However, the present work shows that the c.d. and m.c.d. characteristics of the 830nm band are quite untypical of type 1 blue copper centres. The m.c.d. is certainly negative in sign, but the intensity of the signal is between 5 and 10 times that of blue copper signals, whereas the c.d. bands are very weak, being less than $0.1\,M^{-1}\cdot cm^{-1}$ compared with values of $3.6–4.0\,M^{-1}\cdot cm^{-1}$ for blue copper. Therefore we conclude that, although the major part of the intensity of the 830nm band in oxidized cytochrome oxidase appears to belong to a cupric centre, it does not have the c.d. or m.c.d. characteristic of a typical type 1 copper centre.

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References

Babcock, G. T., Vickery, L. E. & Palmer, G. (1976) *J. Biol. Chem.* **251**, 7907–7919

Babcock, G. T., Van Steelandt, J., Palmer, G., Vickery, L. E. & Salmeen, I. (1979) in *Cytochrome Oxidase* (King, T. E., Orlii, Y., Chance, B. & Okunuki, K., eds.), pp. 105–115, Elsevier/North-Holland, Amsterdam

Brittain, T., Greenwood, C., Springall, J. P. & Thomson, A. J. (1978) *Biochem. J.* **173**, 411–417

Cheng, J. C., Osborne, G. A., Stephens, P. J. & Eaton, W. A. (1973) *Nature (London)* **241**, 193–194

Dawson, J. H., Dooley, D. M., Clark, R., Stephens, P. J. & Gray, H. B. (1979) *J. Am. Chem. Soc.* **101**, 5046–5053

Day, P., Smith, D. W. & Williams, R. J. P. (1967) *Biochemistry* **6**, 3747–3750

Dooley, D. M., Rawlings, J., Dawson, J. H., Stephens, P. J., Andréasson, L.-E., Malmström, B. G. & Gray, H. B. (1979) *J. Am. Chem. Soc.* **101**, 5038–5046

Eaton, W. A. & Charney, E. (1969) *J. Chem. Phys.* **51**, 4502–4505

Eaton, W. A., Hanson, L. K., Stephens, P. J., Sutherland, J. C. & Dunn, J. B. R. (1978) *J. Am. Chem. Soc.* **100**, 4991–5003

Hill, H. A. O. & Smith, B. E. (1979) *J. Inorg. Biochem.* **11**, 79–93

Lemberg, M. R. (1969) *Physiol. Rev.* **49**, 48–121

Malmström, B. G. (1973) *Q. Rev. Biophys.* **6**, 389–432

Nafie, L. A., Keiderling, T. A. & Stephens, P. J. (1976) *J. Am. Chem. Soc.* **98**, 2715–2723

Nicholls, P. (1978) *Biochem. J.* **175**, 1147–1150

Nicholls, P. & Chance, B. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., ed.), pp. 479–534, Academic Press, New York

Nozawa, T., Yamamoto, T. & Hatano, M. (1976) *Biochim. Biophys. Acta* **427**, 28–37

Osborne, G. A., Cheng, J. C. & Stephens, P. J. (1973) *Rev. Sci. Instrum.* **44**, 10–15

Powers, L., Blumberg, W. E., Chance, B., Barlow, C. H., Leigh, J. S., Smith, J., Yonetani, T., Vik, S. & Peisach, J. (1979) *Biochim. Biophys. Acta* **546**, 520–538

Rawlings, J., Stephens, P. J., Nafie, L. A. & Kamen, M. D. (1977) *Biochemistry* **16**, 1725–1729

Rotilio, G., Calabrese, L. & Coleman, J. E. (1973) *J. Biol. Chem.* **248**, 3855–3859

Solomon, E. I., Hare, J. W. & Gray, H. B. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1389–1393

Stephens, P. J., Sutherland, J. C., Cheng, J. C. & Eaton, W. A. (1976) *Proc. Int. Conf. Excited States in Biological Molecules* (Birks, J. B., ed.), pp. 434–442, John Wiley and Sons, Chichester

Stevens, T. H., Bocian, D. F. & Chan, S. I. (1979) *FEBS Lett.* **97**, 314–316

Takemori, S. & King, T. E. (1965) *J. Biol. Chem.* **240**, 504–513

Thomson, A. J., Brittain, T., Greenwood, C. & Springall, J. P. (1976) *FEBS Lett.* **67**, 94–98

Thomson, A. J., Brittain, T., Greenwood, C. & Springall, J. P. (1977) *Biochem. J.* **165**, 327–336

Tsudsuki, T. & Wilson, D. F. (1971) *Arch. Biochem. Biophys.* **145**, 149–154

Tweedle, M. F., Wilson, L. J., Garcia-Iniguez, L., Babcock, G. T. & Palmer, G. (1978) *J. Biol. Chem.* **253**, 8068–8071

Van Buuren, K. J. H., Zuurendonk, P. F., Van Gelder, B. F. & Muijsers, A. O. (1972*a*) *Biochim. Biophys. Acta* **256**, 243–257

Van Buuren, K. J. H., Nicholls, P. & Van Gelder, B. F. (1972*b*) *Biochim. Biophys. Acta* **256**, 258–265

Van Gelder, B. F., Tiesjema, R. H., Muijsers, A. O., Van Buuren, K. J. H. & Wever, R. J. (1973) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **32**, 1977–1980

Wharton, D. C. & Tzagaloff, A. (1964) *J. Biol. Chem.* **239**, 2036–2041

Wikström, M. K. F., Harmon, H. J., Ingledew, J. W. & Chance, B. (1976) *FEBS Lett.* **65**, 259–276

Wilson, M. T., Greenwood, C., Brunori, M. & Antonini, E. (1975) *Biochem. J.* **147**, 145–153

Yonetani, T. (1960) *J. Biol. Chem.* **235**, 845–852