Some Magnetic Properties of *Pseudomonas* Cytochrome Oxidase

By TERENCE A. WALSH,* MICHAEL K. JOHNSON,† COLIN GREENWOOD,* DONALD BARBER,* JON P. SPRINGALL† and ANDREW J. THOMSON†

*School of Biological Sciences and †School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

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The magnetic properties of the haem groups of *Pseudomonas* cytochrome oxidase and its cyanide-bound derivatives were studied in both the oxidized and reduced states by means of m.c.d. (magnetic circular dichroism) at low temperatures. In addition, the oxidized forms of the enzyme were also investigated by e.p.r. (electron-paramagnetic-resonance) spectroscopy, and a parallel study, using both e.p.r. and m.c.d., was made on *Pseudomonas* cytochrome *c*-551 to aid spectral assignments. For ascorbate-reduced *Pseudomonas* cytochrome oxidase, the temperature-independence of those features in the m.c.d. spectrum corresponding to the haem *c*, and the temperature-dependence of those signals corresponding to the haem *d*₁, showed the former to be low-spin and the latter to be high-spin (*s* = 2). However, addition of cyanide to the reduced enzyme gave a form of the protein that was completely low-spin. The e.p.r. and m.c.d. spectra of oxidized *Pseudomonas* cytochrome oxidase and its cyanide derivative were consistent with the haem *c* and *d*₁ components being low-spin in both cases. *Pseudomonas* cytochrome *c*-551 was found to be low-spin in both its oxidized and reduced redox states.

Pseudomonas cytochrome oxidase (EC 1.9.3.2) is a terminal electron carrier in the bacterium *Pseudomonas aeruginosa*. The enzyme can accept electrons from either of two donors present in the organism, namely *Pseudomonas* cytochrome *c*-551 and a copper protein, *Pseudomonas* azurin. Although *Pseudomonas* cytochrome oxidase can accomplish the four-electron reduction of oxygen to water, its physiological function appears to be the single-electron reduction of NO₂⁻ to NO, and it is produced in appreciable amounts only when the bacterium is grown in the presence of NO₃⁻ (Yamanaka *et al.*, 1963). The protein consists of two subunits each of which contains two types of haem group (Kuronen *et al.*, 1975), one a haem *c*, and the other a haem that has been classified as *d*₁ (Lemberg & Barrett, 1973). Ligand-binding studies have shown that in its reduced form *Pseudomonas* cytochrome oxidase can complex CO, CN⁻ and NO [Parr *et al.* (1975), Barber *et al.* (1978) and Shimada & Oriei (1975) respectively] at the haem *d*₁, but only NO is known to bind to the haem *c* in this redox state. In its oxidized form the enzyme can bind CN⁻ and NO to both the *c* and *d*₁ haems (Barber *et al.*, 1978; Shimada & Oriei, 1975), and recent work (T. A. Walsh, D. Barber & C. Greenwood, unpublished work) has shown that similar behaviour is also observed with imidazole and azide. Very little is known about the electronic states of the haems in the protein.

As part of a programme investigating the mech-

anism of the oxidoreductase activity of *Pseudomonas* cytochrome oxidase (Parr *et al.*, 1977; Greenwood *et al.*, 1978), the present paper reports an investigation of the magnetic properties of the enzyme in its fully oxidized and fully reduced forms. This has been done by using the complementary techniques of m.c.d. and e.p.r. spectroscopy. Previous workers (Oriei *et al.*, 1977; Vickery *et al.*, 1978) have published the room-temperature m.c.d. spectra of *Pseudomonas* cytochrome oxidase, but reached no conclusions about the magnetic states of haem *d*₁. By contrast, the low-temperature e.p.r. studies of Gudat *et al.* (1973) indicated that the haem *c* and *d*₁ components were low-spin in the oxidized protein, the haem *c* giving rise to signals at *g* = 2.93, 2.31 and 1.4 and the haem *d*₁ to signals at *g* = 2.45 and 1.71. However, Gudat *et al.* (1973) also found that on addition of KCN to oxidized *Pseudomonas* cytochrome oxidase the signals assigned to the haem *d*₁ were abolished. This is a surprising result and no explanation was put forward by those authors. We have therefore re-examined the e.p.r. spectra of both unbound and cyanide-bound forms of the oxidized enzyme and report our preliminary results here.

Although no e.p.r. signals can be detected from dithionite-reduced *Pseudomonas* cytochrome oxidase (Gudat *et al.*, 1973), the technique of low-temperature m.c.d. spectroscopy is sensitive to the spin states of ferrous haems and has been used, for example, to assign the magnetic states of the ferrous haems in mammalian cytochrome oxidase (Thomson *et al.*, 1977). This sensitivity arises because a diamagnetic haem,

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

such as the low-spin ferrous form, gives a temperature-independent m.c.d. spectrum, whereas high-spin ferrous haems ($s = 2$) give strongly temperature-dependent m.c.d. signals with characteristic band shapes and saturation properties. Low-temperature m.c.d. studies therefore provided a solid basis for investigating the magnetic properties of reduced *Pseudomonas* cytochrome oxidase.

Materials and Methods

All chemicals were of analytical-reagent grade and were obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K., except for ascorbic acid (disodium salt) from Sigma (London) Chemical Co., Poole, Dorset BH17 7NH, U.K., and sodium dithionite, which was a gift from Hardman and Holden, Miles Platting, Manchester, U.K. O₂-free nitrogen gas was supplied by British Oxygen Co., London S.W.19, U.K., and was dispensed from the cylinder and stored in a glass vessel over an alkaline solution of anthraquinonesulphonate.

KCN solutions were freshly prepared in phosphate buffer and the pH adjusted to 7.0 with HCl. *Pseudomonas* cytochrome oxidase and *Pseudomonas* c-551 were isolated and purified from cells of *Pseudomonas aeruginosa* (N.C.T.C. 6750) as described by Parr *et al.* (1976). The ratios $A_{410}^{ox}/A_{280}^{ox}$ and $A_{640}^{ox}/A_{520}^{ox}$ for oxidized *Pseudomonas* cytochrome oxidase were 1.18–1.2 and 1.15–1.2 respectively and concentrations of the enzyme solutions were obtained by using an absorption coefficient of $A_{410}^{ox} = 288\,000 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (M. C. Silvestrini, A. Colosimo, M. Brunori & C. Greenwood, unpublished work). The purity ratio $(A_{551}^{red} - A_{570}^{red})/A_{280}$ for *Pseudomonas* c-551 was 1.14 and concentrations of these protein solutions were obtained by using an absorption coefficient of $A_{551}^{red} = 28.3 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Horio *et al.*, 1960). The extraction of the haem d_1 component of *Pseudomonas* cytochrome oxidase was carried out by using the acid/acetone method of Yamanaka & Okunuki (1963). This procedure yields a red-coloured precipitate, the apoprotein, which contains only the haem c chromophore. After the final washing of the precipitate to remove residual acetone, the protein was resuspended in 2–3 ml of 0.1 M-potassium phosphate buffer, pH 7.0, and redissolved by addition of two or three drops of 2M-KOH. Although previous reports (Horio *et al.*, 1961) have noted the insolubility of the apoprotein at neutral pH, the samples of this material prepared in the present studies would not remain fully dissolved at pH values less than 10. For this reason, work on the apoprotein was performed at pH 10.

Spectrophotometry was carried out with a Cary 118C spectrophotometer at room temperature. Samples of reduced *Pseudomonas* cytochrome oxidase were prepared by the addition of a large (approx. 500-fold) excess of sodium ascorbate to anaerobic enzyme solutions under N₂. Under these conditions, reduc-

tion is complete within 1 min. Samples of *Pseudomonas* c-551 were reduced with sodium dithionite.

Cyanide derivatives of *Pseudomonas* cytochrome oxidase were prepared by mixing the protein with the appropriate amount of 0.5M-KCN, pH 7.0. In the case of the oxidized enzyme–cyanide complex used for e.p.r., the final KCN concentration was 100mM. Similarly, the final KCN concentrations in the samples for m.c.d. were, after sucrose saturation, 76mM for the oxidized enzyme–cyanide complex and 34mM for the reduced enzyme–cyanide complex. These concentrations of KCN were chosen to ensure complete saturation of protein with ligand. The haem c of the oxidized enzyme has a relatively low affinity for cyanide (Barber *et al.*, 1978), hence relatively high concentrations of ligand are necessary for its complete saturation. Absorption spectra of samples run before m.c.d. and e.p.r. measurements were similar to those observed by Barber *et al.* (1978) and therefore did not indicate any adverse effects due to high KCN concentrations.

All m.c.d. samples were left overnight at 4°C in the presence of excess sucrose to ensure saturation and were then placed in specially constructed 0.8 mm path-length cells by a syringe, the transfer being performed in a stream of N₂ under a 'hood' for reduced samples. E.p.r. samples were placed directly into the appropriate tubes.

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. The samples used for recording low-temperature spectra were saturated with sucrose and placed in specially constructed silica cells of 0.8 mm path-length before being rapidly cooled to below 100 K in a gas-flow cryostat. The temperature of the gas flow was monitored with an Au/Fe thermocouple and varied by controlling the rate of cold-helium-gas flow. Spectra were measured in the presence and the absence of a magnetic field and the two curves were subtracted to remove the natural c.d. spectrum. Low-temperature glasses cause some depolarization of the circularly polarized light beam owing to thermal strains. This depolarization is assessed by measuring the c.d. of an optically active sample placed between the frozen sample and the photomultiplier tube. The measured values of the m.c.d. are corrected for the measured degree of depolarization.

M.c.d. spectra are expressed as $\Delta\epsilon/B$, where $\Delta\epsilon$ ($\text{litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) = $\epsilon_L - \epsilon_R$, ϵ_L and ϵ_R being molar absorption coefficients for left and right circularly polarized light respectively; B is the magnetic field in T; ϵ ($\text{litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) is the molar absorption coefficient. Values of ϵ for solutions of *Pseudomonas* cytochrome oxidase are expressed/mol of dimer throughout.

E.p.r. spectra were measured on a Varian E-3 e.p.r. spectrometer fitted with an Oxford Instrument ESR-9

continuous-flow cryostat and DTC 2 temperature controller, enabling the sample temperature to be varied over the range 4.2–300 K.

It has been shown (Williams-Smith *et al.*, 1977) that, on freezing solutions of buffers to 77 K, a substantial apparent pH change can occur. For the buffer used in the present work, namely potassium phosphate, a pH decrease of 1.4 ± 0.7 might take place, although the protein concentrations used (~ 4 mg/ml) would be expected to decrease the magnitude of these changes somewhat.

Results and Discussion

Reduced *Pseudomonas* cytochrome oxidase

The m.c.d. and absorption spectra of ascorbate-reduced *Pseudomonas* cytochrome oxidase are shown in Fig. 1. The absorption spectrum has been recorded only at room temperature (295 K), but the m.c.d. spectrum has been measured at four different temperatures between 16 and 95 K, and is composed of both temperature-dependent and temperature-independent regions. By contrast, the m.c.d. spectra of reduced

Fig. 1. Room-temperature absorption spectrum (a) and low-temperature m.c.d. spectra (b) of ascorbate-reduced *Pseudomonas* cytochrome oxidase

The m.c.d. spectra were run at temperatures of 16 K (—), 27 K (---), 53 K (●—●) and 95 K (—○—) on a sample of enzyme (in 0.04 M-potassium phosphate buffer, pH 7.0) that had been saturated with sucrose to give a final protein concentration of 29 μ M.

Fig. 2. Room-temperature absorption spectrum (a) and low-temperature m.c.d. spectra (b) of dithionite-reduced *Pseudomonas* cytochrome c-551

The m.c.d. spectra were run at temperatures of 16 K (—) and 37.5 K (●—●) on a sample of protein (in 0.04 M-potassium phosphate buffer, pH 7.0) that had been saturated with sucrose to give a final sample concentration of 60 μM .

Pseudomonas cytochrome *c*-551 at 16 and 37.5 K (Fig. 2) are identical, within experimental error, over the entire spectral range and thus indicate that in this case the haem *c* is diamagnetic, being low-spin ferrous.

Comparison of Figs. 1 and 2 shows that the haem *c* in ascorbate-reduced *Pseudomonas* cytochrome oxidase contributed to the sharp bands between 500 and 550 nm that are temperature-independent. Therefore the haem *c* is diamagnetic in the reduced enzyme. However, the m.c.d. peaks between 550 and 700 nm are strongly temperature-dependent. Since the haem *c* m.c.d. spectrum is devoid of bands in this region, these peaks belong to the haem *d*₁. Hence the haem *d*₁ is paramagnetic in ascorbate-reduced *Pseudomonas* cytochrome oxidase.

The Soret region of the spectrum confirms these conclusions, but is more complex because the bands from the haems *c* and *d*₁ overlap. The peaks in the oxidase m.c.d. spectrum (Fig. 1) marked with arrows at 413 and 424 nm clearly belong to the spectrum of the haem *c* component; comparison with Fig. 2 makes this apparent. They are underlain, however, by a broad temperature-dependent negative peak that must belong to the haem *d*₁. Similarly, the negative peak at 450 nm and the positive peak at 467 nm must also be assigned to the haem *d*₁. Thus the m.c.d. spectrum is dominated throughout the Soret region, at low temperature, by the signals from the haem *d*₁. This contrasts with the absorption spectrum in which the sharp strong peak at 416 nm arises from the haem *c*, the weaker band at 457 nm arising from the haem *d*₁. The m.c.d. spectrum shows that the Soret region of the haem *d*₁ spectrum is very broad, running from about 390 to 490 nm.

The form of the m.c.d. spectrum of haem *d*₁ in the Soret region is similar to that of deoxymyoglobin (Springall *et al.*, 1976) and reduced mammalian cytochrome *c* oxidase (Thomson *et al.*, 1977), except that the bands of the haem *d*₁ are spread over a wide region of the spectrum. This is consistent with the spectrum of extracted haem *d*₁ derivatives, which show a broad split Soret band in the reduced state (Yamanaka & Okunuki, 1963; T. A. Walsh, D. Barber & C. Greenwood, unpublished work). It has been suggested that haem *d*₁ is a chlorin-type haem, but one in which the conjugation of the tetrapyrrole ring is modified by the introduction of hydroxy groups (Lemberg & Barrett, 1973). If so, the resulting lower symmetry of haem *d*₁, compared with a protoporphyrin ring, may lead to a splitting of its Soret band. The similarity of the Soret-region m.c.d. spectrum of haem *d*₁ to that of deoxymyoglobin shows that the haem *d*₁ has the high-spin (*s* = 2) ferrous form.

The absorption and m.c.d. spectra of the cyanide-bound form of ascorbate-reduced *Pseudomonas* cytochrome oxidase are shown in Fig. 3. The cyanide concentration was chosen to ensure complete binding of haem *d*₁; the haem *c* does not appear to bind

cyanide in the reduced state (Barber *et al.*, 1978). The m.c.d. spectra were measured at various temperatures between 9 and 40.5 K, and it is clear that both haems *c* and *d*₁ are diamagnetic. The visible region between 500 and 550 nm, corresponding to the transitions of haem *c*, have temperature-independent m.c.d. transitions and the form and heights of the peaks are almost identical with those in Fig. 1. Thus the haem *c* spectrum has been very little perturbed by the binding of cyanide to the haem *d*₁. The m.c.d. peaks corresponding to the haem *d*₁ between 550 and 650 nm are also independent of temperature, showing that the haem *d*₁ has switched to the low-spin ferrous form on binding cyanide.

The Soret region of the m.c.d. spectrum in Fig. 3 is dominated by the peaks of the haem *c* at 412 and 424 nm; there are no clear features corresponding to the haem *d*₁ discernible. We have found that very small amounts of oxidized *Pseudomonas* cytochrome oxidase (less than 1% contamination) can distort the m.c.d. spectrum of the reduced enzyme-cyanide complex in the Soret region. This is because at low temperatures the Soret-band m.c.d. of oxidized haem *c* is extremely intense relative to that of reduced haem *c* (see Figs. 2 and 5), and therefore very small amounts of any oxidized protein can be detected readily. It is possible that the small inflexion at 418 nm and the small increase in intensity at 412 nm with temperature in the m.c.d. spectrum of Fig. 3 arise from a slight oxidation of the haem *c* component of the sample.

In summary, the results presented above indicate that, in the reduced state, *Pseudomonas* cytochrome oxidase contains low-spin haem *c* and high-spin haem *d*₁. However, on binding cyanide the haem *d*₁ becomes low-spin, the haem *c* remaining unchanged. This behaviour is similar to that of reduced mammalian cytochrome *c* oxidase, where the ligand-binding haem component *a*₃ is high-spin when unbound but low-spin when complexed with cyanide, and where the haem *a* component is low-spin in both cases. Similarly, there is a parallel with the corresponding forms of the oxygen-carrying pigments myoglobin and haemoglobin, and it is tempting to suppose that the haem *d*₁ is five-co-ordinate when in the high-spin ferrous form.

Oxidized Pseudomonas cytochrome oxidase

The absorption and m.c.d. spectra of oxidized *Pseudomonas* cytochrome oxidase are given in Fig. 4. The variable-temperature m.c.d. spectra are rich in detail, all the peaks having strongly temperature-dependent intensities. The corresponding spectra of oxidized *Pseudomonas* cytochrome *c*-551 have also been measured and are shown in Fig. 5. The latter protein contains the ferric form of haem *c* fully in its low-spin state. This has been verified by measurement of the e.p.r. spectrum of *Pseudomonas* cytochrome *c*-551 at 10 K (results not shown), which showed *g* factors at 3.1 and 2.2, but no high-spin signal at *g* ~ 6.

Fig. 3. Room-temperature absorption spectrum (a) and low-temperature m.c.d. spectra (b) of the ascorbate-reduced *Pseudomonas* cytochrome oxidase-cyanide complex

The m.c.d. spectra were run at temperatures of 9 K (—), 14 K (---) and 27 K (●—●) in the region 350–490 nm, and 15.5 K (—) and 40.5 K (●—●) in the region 480–700 nm, on a sample of enzyme (in 0.04 M-potassium phosphate buffer, pH 7.0) that had been saturated with sucrose to give a final protein concentration of 50 μ M.

Comparison of Figs. 4 and 5 allows the assignment of the intense positive and negative m.c.d. peaks at 402 and 413 nm in Fig. 4 to the haem *c* component of *Pseudomonas* cytochrome oxidase, and we therefore conclude that this moiety is low-spin. The Soret-region m.c.d. spectra of a number of low-spin ferric haems such as metmyoglobin-cyanide (Springall *et al.*, 1976) and the haem *a* of oxidized mammalian cytochrome *c* oxidase (Thomson *et al.*, 1977) have this characteristic form.

The wavelength range 600–700 nm also shows m.c.d. peaks that are strongly temperature-dependent, and, since these bands lie beneath the haem *d*₁ absorption peak centred at 640 nm, we deduce that the haem *d*₁ is paramagnetic. The strength of the temperature-dependence suggests that the haem *d*₁ is low-spin, as high-spin ferric haems have m.c.d. spectra that are only weakly temperature-dependent (Springall *et al.*, 1976). This conclusion is substantiated by the e.p.r. spectrum of oxidized *Pseudomonas* cytochrome

Fig. 4. Room-temperature absorption spectrum (a) and low-temperature m.c.d. spectra (b) of oxidized *Pseudomonas* cytochrome oxidase

The m.c.d. spectra were run at temperatures of 16 K (—), 34 K (---), 85 K (●—●) and 293 K (-·-·-) on a sample of enzyme (in 0.04M-potassium phosphate buffer, pH 7.0) that had been saturated with sucrose to give a final protein concentration of 29 μ M.

oxidase (Fig. 6a), which agrees well with the one reported by Gudat *et al.* (1973). The features at $g = 4.42$ and 2.08 are most probably due to adventitious iron and copper respectively. Since we have observed that the strong feature at $g = 2.53$ and the weak signal at $g = 1.72$ are not found in the acid/acetone-extracted apoprotein, we confirm their assignment by Gudat *et al.* (1973) to the haem d_1 component. In fact the

apoprotein gave signals at $g = 2.85$ and 2.31, and this therefore indicates that the features at $g = 3.02$ and 2.29 in the spectrum of the holoenzyme (Fig. 6a) arise from the haem c component. We have been unable to detect the weak signal at $g = 1.4$ reported by Gudat *et al.* (1973).

The absence of any signals in the $g \sim 6$ region of the e.p.r. spectrum of *Pseudomonas* cytochrome

Fig. 5. Room-temperature absorption spectrum (a) and low-temperature m.c.d. spectra (b) of oxidized *Pseudomonas* cytochrome c-551

The m.c.d. spectra were run at temperatures of 20 K (—), 39 K (---) and 62.5 K (●—●) in the region 350–450 nm and a protein concentration of $50 \mu\text{M}$ after saturation of a sample in 0.04 M-potassium phosphate buffer, pH 7.0, with sucrose. In the region 440–650 nm, a protein concentration of $150 \mu\text{M}$ was used, and the spectra were run at 17.5 K (—) and 37.5 K (●—●).

oxidase suggests that no high-spin components are present. Therefore, unless high-spin haem d_1 possesses a novel e.p.r. spectrum, both the m.c.d. and e.p.r. spectra show the haems c and d_1 to be low-spin in the unbound form of the oxidized enzyme.

Addition of cyanide to the oxidized form of

Pseudomonas cytochrome oxidase results in binding of the ligand to both the haems c and d_1 . The low-temperature m.c.d. spectrum of this species is shown in Fig. 7, and it may be clearly seen that the Soret-region peaks at 402 and 413 nm point to the continued presence of the low-spin ferric form of haem c in this

Fig. 6. Low-temperature e.p.r. spectra of oxidized *Pseudomonas* cytochrome oxidase and its cyanide complex
 (a) The e.p.r. spectrum of oxidized *Pseudomonas* cytochrome oxidase. The spectrum was run at 10 K on a $63 \mu\text{M}$ -protein sample in 0.04 M-potassium phosphate buffer at pH 7.0. The microwave power was 5.0 mW, field modulation amplitude 0.63 mT, microwave frequency 9.155 GHz and receiver gain 2.5×10^5 . B is the magnetic field strength (expressed in T).
 (b) The e.p.r. spectrum of the cyanide complex of oxidized *Pseudomonas* cytochrome oxidase. The spectrum was run at 10 K on a $70 \mu\text{M}$ -protein sample in 0.04 M-potassium phosphate buffer, pH 7.0, and in the presence of 100 mM-KCN. The microwave power was 20 mW, field modulation amplitude 0.63 mT, microwave frequency 9.150 GHz and receiver gain 2.0×10^5 .

Fig. 7. Room-temperature absorption spectrum (a) and low-temperature m.c.d. spectra (b) of the cyanide complex of oxidized *Pseudomonas cytochrome oxidase*

The m.c.d. spectra in the region 350–450 nm were run at 25 K (—), 43 K (---), 67 K (●—●) and 293 K (—○—○) on a sample of enzyme (in 0.04 M-potassium phosphate buffer, pH 7.0) that had been saturated with sucrose to give a final protein concentration of 32.6 μ M. Over the region 440–700 nm the spectra were run at 16 K (—), 27.5 K (---), 37 K (●—●) and 293 K (—○—○) with a 30 μ M-enzyme sample.

species of the enzyme. However, the spin state of the haem d_1 cannot be decided with complete certainty from the data in Fig. 7. Although the m.c.d. bands between 600 and 700 nm are due in part to the haem d_1 and seem similar in form to the corresponding bands

of the unbound enzyme in Fig. 4, the binding of cyanide to the haem d_1 moves its absorption peak at 640 nm to 633 nm, thus leading to a greater overlap of the haem d_1 and c spectra. Furthermore, since cyanide-bound ferric haems are known to show a broad

positive m.c.d. peak between 600 and 650 nm that increases when the temperature is lowered (Springall *et al.*, 1976), it would appear that the haem d_1 m.c.d. spectrum of this region in Fig. 7 is overlapped by a rising 'background' due to the haem c . This results in the negative peak at approx. 625 nm in Fig. 7 being lifted and becoming more positive under the influence of these haem c signals, rather than becoming more negative as the temperature is lowered.

No unambiguous assignment of the m.c.d. bands of the oxidized and cyanide-bound oxidized oxidase that lie between 400 and 600 nm (Figs. 4 and 7) can be made. The m.c.d. spectrum of oxidized cytochrome c -551 is shown for comparison in Fig. 5. It resembles remarkably closely the m.c.d. spectrum of the cyanide-bound oxidized oxidase (Fig. 7). Therefore there are no features in the m.c.d. spectrum (Fig. 7) between 400 and 600 nm that can be assigned to haem d_1 . On the other hand the m.c.d. spectrum of oxidized oxidase (Fig. 4) shows marked differences from the spectrum of cytochrome c -551 in the region of 450 nm. Hence these features can be assigned to part of the Soret region of haem d_1 . Presumably these features are lost in the m.c.d. spectrum of the cyanide-bound oxidase (Fig. 7) because the binding of cyanide to the haem d_1 has had an effect on the Soret-band region of the spectrum. The absorption spectrum shows that the haem d_1 Soret band is shifted to higher energy on binding cyanide. This shift may be sufficient to hide the Soret-band m.c.d. peaks of cyanide-bound haem d_1 below the intense Soret-band peaks of cytochrome c . We conclude that the m.c.d. spectrum of the cyanide-bound oxidase is totally dominated by the features corresponding to haem c over the range 350–600 nm.

To confirm the implication of the m.c.d. spectrum that the haem d_1 remains low-spin ferric when bound by cyanide, the e.p.r. spectrum of the oxidized cyanide-bound form of the enzyme was measured (Fig. 6b). This spectrum shows that the peak corresponding to the haem c has shifted from $g = 3.02$ to $g = 3.31$ on binding the ligand. Likewise, the haem d_1 peaks at $g = 2.53$ and $g = 1.72$ have shifted to $g = 2.58$ and $g = 1.61$ respectively; no high-spin $g \sim 6$ signals were observed. This result has been verified a number of times, care being taken to check spectrophotometrically that the cyanide has bound to both haems. The finding is totally at variance with that of Gudat *et al.* (1973), who reported the complete disappearance of the haem d_1 e.p.r. signals on addition of KCN. We have also been able to demonstrate (T. A. Walsh, D. Barber, C. Greenwood, M. K. Johnson, J. P. Springall & A. J. Thomson, unpublished work) that the binding of the azide ion and imidazole to oxidized haem d_1 results in shifts and intensity changes in the $g \sim 2.6$ signal, but it is not lost entirely.

To conclude, the above m.c.d. and e.p.r. spectral data suggest that in the fully oxidized form of *Pseudomonas* cytochrome oxidase the haem c is low-

spin ferric in the absence of added ligands and stays low-spin when the strong field ligand cyanide displaces a protein ligand to give the cyanide complex. The haem d_1 is also in the low-spin form when unbound and apparently remains so when cyanide binds. This is a surprising result, since we have shown that, in the reduced form, the ligand-free haem d_1 is high-spin and probably five-co-ordinate. If such a geometry were maintained in the oxidized form, it seems likely that a high-spin or mixed-spin form of the haem d_1 would result. However, we can find no evidence for the presence of any high-spin haem d_1 in the oxidized enzyme; possibly the oxidized haem picks up a ligand from the protein, or perhaps it is so constrained that it remains low-spin, although five-co-ordinate. At this stage we have no evidence to decide between these possibilities.

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