Characterization of the partially reduced cyanide-inhibited derivative of cytochrome *c* oxidase by optical, electron-paramagnetic-resonance and magnetic-circular-dichroism spectroscopy

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Optical, e.p.r. and near-infrared low-temperature m.c.d. (magnetic-circular-dichroism) spectroscopy were used to characterize the partially reduced cyanide-inhibited derivative of cytochrome *c* oxidase produced by anaerobic reductive titration with dithionite. The reductions of cytochrome a^{3+} and Cu_a^{2+} were followed by observation of the e.p.r. signals at $g = 3.03$, 2.21 and 1.5 and at $g = 2.18$, 2.03 and 1.99 . As reduction proceeds new e.p.r. signals ($g = 3.58$ and 1.56) appear that quantify to give one haem per enzyme unit when a small excess of dithionite has been titrated in. The e.p.r. signal of the Cu_a^{2+} titrates in parallel with the disappearance of the band at 820nm in the optical absorption spectrum. The near-infrared m.c.d. spectrum shows the presence of the low-spin ferric haem, a^{3+} , in the oxidized state of the enzyme, as a well-resolved positive peak at 1650nm . As reduction proceeds this band is replaced by one at 1550nm due to haem $a_3^{3+}\text{-CN}$ in the partially reduced state. Hence as haem $a_3^{3+}\text{-CN}$ becomes e.p.r.-detectable it also shows a near-infrared m.c.d. spectrum characteristic of a low-spin ferric haem. It is concluded that the partially reduced state of cyanide-inhibited cytochrome *c* oxidase contains $a^{2+} \cdot \text{Cu}_a^+ \cdot a_3^{3+}\text{-CN} \cdot \text{Cu}_{a_3}^+$.

In the oxidized state of cytochrome *c* oxidase cytochrome a_3 will bind cyanide provided that the enzyme and inhibitor are equilibrated for at least 24 h (Van Buuren *et al.*, 1972). It is not known whether cyanide also interacts with the copper associated with cytochrome a_3 , although there have been suggestions that it may do so. Addition of reducing agents such as sodium dithionite, *NNN'**N'*-tetramethylphenylenediamine, ferrocytochrome *c* or NADH and phenazine methosulphate to the cyanide-inhibited form leads to the formation of a partially reduced state. There is considerable confusion in the literature about the nature of this partially reduced form, in particular, which of the four components of the enzyme are reduced.

Nicholls & Hildebrandt (1978) performed aerobic reductions of cyanide-inhibited cytochrome *c* oxidase both with ferrous cytochrome *c* and with *NNN'**N'*-tetramethylphenylenediamine. In both cases, it was concluded, only two electrons enter the

enzyme, reducing cytochrome *a* and one cupric ion, Cu_a^{2+} , to give a partially reduced state that they termed the 'half-reduced' species, written $a^{2+} \cdot \text{Cu}_a^+ \cdot a_3^{3+}\text{-HCN} \cdot \text{Cu}_{a_3}^{2+}$. These conclusions were based on observations of the optical spectrum of the enzyme between 605 and 630nm . No measurement of e.p.r. was undertaken.

In their study Van Gelder & Beinert (1969) performed an anaerobic reduction of the cyanide-inhibited enzyme with NADH in the presence of phenazine methosulphate and quantified all the e.p.r.-detectable components. They also observed the changes in the haem components optically at 605 and 630nm . They reported that the reduction of the e.p.r.-detectable components, namely cytochrome *a* and Cu_a^{2+} , proceeds linearly with reducing equivalents added. They also claim that all e.p.r. signals have vanished when between 1.0 and 1.5 equivalents/haem group have been added, and that no signals other than those present in the oxidized enzyme ever appear. Consequently they state that two of the four electron acceptors per enzyme unit

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

are not reduced in the presence of cyanide and that these two components are cytochrome a_3 and Cu_a^{2+} . This is in agreement with the conclusions of Nicholls & Hildebrandt (1978).

However, DerVartanian *et al.* (1974) reported that, in the presence of dithionite, cyanide-inhibited cytochrome oxidase shows a new e.p.r. signal with $g = 3.58$, which appears to replace the $g = 3.03$ signals of the oxidized enzyme due to cytochrome a_3^{3+} . A signal at $g = 3.58$ also appears transiently by reacting cyanide with the rapidly reacting form of the enzyme that is produced by anaerobic oxidation, with chemical reagents, of completely reduced cytochrome c oxidase (Shaw *et al.*, 1978). The conclusion has been drawn that the $g = 3.58$ signal is due to low-spin cytochrome a_3^{3+} -CN.

On the face of it these reports point to the existence of two forms of the partially reduced cyanide derivative dependent on the reducing agent used to produce it. The experiments of Nicholls & Hildebrandt (1978) and Van Gelder & Beinert (1969) were interpreted by saying that the partially reduced cyanide species contains both haem a_3 and Cu_a in the oxidized state, interacting magnetically so that neither is e.p.r.-detectable. However, the experiments of DerVartanian *et al.* (1974) and Shaw *et al.* (1978) showed that cytochrome a_3^{3+} -CN became e.p.r.-detectable on partial reduction with dithionite. This would require the proposed magnetic interaction with Cu_a to be broken either because the copper centre has been reduced or because the magnetic interaction has been modified. DerVartanian *et al.* (1974) did not report whether new copper signals became e.p.r.-detectable in their partially reduced derivative. Shaw *et al.* (1978) failed to detect any new copper signals when the $g = 3.58$ signal of haem a_3^{3+} -CN appeared and expressed themselves puzzled. More recently Seiter & Angelos (1980) have proposed an alternative model for cytochrome c oxidase, involving cytochrome a_3^{4+} and Cu_a^{+} in the oxidized enzyme, which does not require magnetic coupling between cytochrome a_3 and Cu_a to explain the lack of e.p.r. signals from these components.

In the present paper we re-examine the nature of the partially reduced cyanide derivative of cytochrome c oxidase by using the techniques of e.p.r., optical and m.c.d. spectroscopy to identify the oxidation states of the various components. Our aims are twofold. First, we wish to show that the partially reduced cyanide derivative has the same composition, as judged by its e.p.r. spectrum, regardless of the nature of the reducing agent used to produce it. Secondly, we seek to determine which of the four metal centres are reduced in the partially reduced cyanide form. In the preceding paper (Thomson *et al.*, 1981) we compared m.c.d. magnetization curves for the oxidized, oxidized cyanide and partially reduced cyanide states of the enzyme.

To facilitate interpretation of that investigation it is important to characterize the nature of the partially reduced cyanide state.

Materials and methods

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

The preparation of oxidized cytochrome c oxidase and the cyanide-inhibited derivative is described in the preceding paper (Thomson *et al.*, 1981). The low-temperature near-infrared m.c.d. measurements were performed with 'deuterated' samples of cytochrome c oxidase that were prepared as described in Eglinton *et al.* (1980). To obtain glasses of optical quality on freezing 'deuterated' cytochrome c oxidase samples for m.c.d., 50% (by vol.) of deuterated ethanediol was added. The concentrations of oxidized cytochrome c oxidase samples were established per enzyme functional unit by using $\Delta\epsilon_{603}$ -(reduced-oxidized) - $\Delta\epsilon_{630}$ -(reduced-oxidized) = $27\text{ mm}^{-1}\cdot\text{cm}^{-1}$ (Nicholls, 1978). Samples of oxidized horse heart cytochrome c and metmyoglobin cyanide were prepared from freeze-dried cytochrome c and myoglobin (BDH Chemicals) respectively dissolved in 0.1 M-potassium phosphate buffer, pH 7.4. The solutions were oxidized with a few grains of $\text{K}_3\text{Fe}(\text{CN})_6$, excess of which was subsequently removed by passage down a Sephadex G-25 column. An approximately 100-fold stoichiometric excess of neutralized KCN solution was added to oxidized myoglobin to obtain metmyoglobin cyanide. The concentrations of the resulting oxidized horse heart cytochrome c and metmyoglobin cyanide solutions were established by using the published absorption coefficients (Margoliash & Schejter, 1966; Smith & Williams, 1970).

E.p.r. spectra were recorded by using a Varian E-3 spectrometer fitted with an ESR-9 flow cryostat (Oxford Instruments), which enabled the sample temperature to be varied over the range 6–300 K. E.p.r. g -values are quoted to an accuracy of ± 0.01 and the instrument was calibrated with respect to 1,1-diphenyl-2-picrylhydrazyl. Near-infrared m.c.d. spectra were recorded at a magnetic field of 5 T by using an SM4 split-coil superconducting magnet (Oxford Instruments) fitted into a near-infrared dichrograph. A description of this instrument, which was constructed in this laboratory, has been published elsewhere (Eglinton *et al.*, 1980). The m.c.d. spectra were recorded at 4.2 K by having the sample

immersed in boiling liquid helium. Absorption spectrophotometry at room temperature was performed by using either a Cary 14 or a Pye–Unicam SP.8-200 instrument.

Combined optical and e.p.r. titrations of the oxidized cyanide-inhibited enzyme with dithionite were performed under anaerobic conditions by using O₂-free N₂ further purified by passage over a reduced B.A.S.F. copper catalyst [B.A.S.F. (U.K.), Cheadle Hulme, Cheshire, U.K.] at 140°C and through a dithionite wash bottle. A parallel titration against horse heart cytochrome *c* was used to calibrate the number of reducing equivalents added to the enzyme. Dithionite was dispensed from a strongly buffered (0.5 M-potassium phosphate buffer, pH 7.4) stock solution by using gas-tight Hamilton syringes, and equal volumes were added to both the degassed enzyme and oxidized cytochrome *c* solutions. The enzyme solution was subsequently sampled by using a gas-tight Hamilton syringe for e.p.r. and absorption measurements and the cytochrome *c* for absorption only. The e.p.r. tubes and absorption cells were fitted with vaccine caps and purged with O₂-free N₂ before use.

The optical titrations of oxidized cyanide-inhibited cytochrome *c* oxidase against NADH were performed in an absorption cell in the presence of catalytic amounts of phenazine methosulphate by a similar anaerobic technique to that described above. The concentration of the NADH solution in 0.1 M-potassium phosphate buffer, pH 7.5, was obtained at both the start and end of the titration by using $\epsilon_{340} = 6.2 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (Horecker & Kornberg, 1948).

The stepwise anaerobic reduction of 'deuterated' samples of oxidized cyanide-inhibited cytochrome *c* oxidase by dithionite was performed as described in Eglinton *et al.* (1980). The amounts of dithionite added were not quantified in terms of reducing equivalents. Samples were taken at intervals and placed in specially constructed anaerobic m.c.d. cells of measured path-length by using gas-tight Hamilton syringes.

Cu²⁺ and haem e.p.r. signals were quantified by the methods of Aasa & Vänngård (1976), and numerical double integration was performed by the method of Wyard (1965). For haem signals only the low-field 'absorption-shaped' resonance was used for quantification, since in the oxidized states of cytochrome *c* oxidase the central 'derivative-shaped' resonance is obscured by the Cu²⁺ signal. For haem quantifications metmyoglobin cyanide ($\approx 1 \text{ mM}$) was used as the standard and the e.p.r. spectra were recorded at 2 mW microwave power and 30 K. The concentration of the metmyoglobin cyanide was assessed spectrophotometrically. For quantification of the copper signal, 1 mM-CuEDTA was used as standard and the e.p.r. spectra were recorded at 2 mW microwave power and 70 K. To increase the

accuracy of such e.p.r. quantifications the same e.p.r. tube, filled to the same level, was used for all samples and standards to eliminate errors arising from variations in e.p.r.-tube radii and differences in the temperature gradient along the tube.

Results

Optical spectra

Fig. 1 shows the room-temperature absorption spectrum of the cyanide derivative of cytochrome *c* oxidase plus the spectra resulting from anaerobic titration with dithionite. Three spectral regions were studied, namely those containing the Soret band, the α -region and the 820 nm band. The course of reduction is seen by the splitting of the Soret band, the increase in the intensity of the α -band and the decrease in the 820 nm band. The changes in the α -band and 820 nm-band region for an analogous but separate titration are plotted in Fig. 4(b) against reducing equivalents of dithionite added per enzyme functional unit for comparison with changes in the e.p.r. spectrum. The dithionite solution was calibrated against cytochrome *c* in a parallel titration. The absorbance changes plotted are $A_{605} - A_{620}$ for the α -band and $A_{820} - A_{720}$ for the long-wavelength band. Initially the former changes linearly with added dithionite, whereas there is a pronounced lag in the reduction of the 820 nm band.

E.p.r. spectra

The e.p.r. spectrum of the fully oxidized cyanide-inhibited form of cytochrome *c* oxidase at 10 K is very similar to that reported for the oxidized enzyme (Aasa *et al.*, 1976; Greenaway *et al.*, 1977), with signals in the cyanide-inhibited enzyme due to cytochrome a^{3+} at $g = 3.03$, 2.21 and 1.45 and due to Cu²⁺ at $g = 2.18$, 2.03 and 1.99. Fig. 2(a) shows the e.p.r. spectrum at 10 K of the partially reduced cyanide derivative of cytochrome *c* oxidase obtained by addition of excess of solid dithionite to an anaerobic sample of the oxidized cyanide derivative. The absorption-shaped resonance at $g = 3.58$ and the broad derivative-shaped resonance centred at $g \approx 1.56$ are components of the same signal. This was demonstrated by a study of the temperature- and power-dependence of the signal amplitude over the ranges 6–70 K and 1–60 mW respectively. Their form suggests they are components of a rhombic signal. However, we have been unable to distinguish clearly a third component at higher magnetic fields. Possibly it is too broad to be detectable. The resonance at $g = 3.58$ has a shape that is temperature-dependent, consisting of a peak at $g = 3.60$ and a shoulder at $g = 3.56$ at 6 K, but that broadens to give a symmetrical absorption shape peaked at $g = 3.58$ at 30 K (see Fig. 2b). The degree

Fig. 1. Anaerobic titration of oxidized cyanide-inhibited cytochrome *c* oxidase, monitored by the room-temperature absorption spectrum

The titration was performed in a dual-path-length absorption cell with a path-length of 1 cm used in the region 500–900 nm and of 0.46 cm in the region 350–500 nm. The enzyme concentration was 30 μ M. The accumulative volume of dithionite solution added for each spectrum was 0 μ l (—), 70 μ l; (---), 140 μ l (— · —), 190 μ l (·····) and 240 μ l (----).

Fig. 2. E.p.r. spectrum at 10 K of partially reduced cyanide-inhibited cytochrome *c* oxidase obtained by addition of excess of solid dithionite to an anaerobic sample of the oxidized cyanide-inhibited enzyme

(a) The enzyme concentration was 800 μ M. The spectrometer settings were: 5 mW microwave power, 0.63 mT modulation amplitude and 9.165 GHz microwave frequency. (b) Temperature-dependence of the $g = 3.58$ resonance under the same conditions as (a) except 2 mW microwave power.

of asymmetry of the low-temperature shape varies slightly between different enzyme preparations. This may reflect an inhomogeneity in the enzyme preparation.

Apart from the signal of unknown origin ($g =$

1.94), the other sharp signals in the region of $g \approx 2$ can be assigned to so-called 'inactive' copper on the basis of the temperature- and power-dependence of their intensities, and by comparison with the reported e.p.r. parameters (Greenaway *et al.*, 1977).

'Inactive' copper is invariably present to various degrees in all cytochrome *c* oxidase preparations (Beinert *et al.*, 1962; Beinert & Palmer, 1964; Greenaway *et al.*, 1977).

The e.p.r. spectrum of the partially reduced cyanide derivative, prepared by the addition of a stoichiometric excess of NADH in the presence of catalytic amounts of phenazine methosulphate to the oxidized cyanide form of the enzyme, was identical with that shown in Fig. 2(a), except that the free-radical signal centred at $g = 1.94$ is replaced by another centred at $g = 2.00$. The e.p.r. spectrum resulting from an aerobic reduction with a stoichiometric excess of *NNN'**N'*-tetramethylphenylenediamine consisted of signals at $g = 3.58$ and 3.03 as well as some of the Cu^{2+} signal associated with active copper. This species is therefore intermediate between the oxidized cyanide and partially reduced cyanide forms of the enzyme.

In order to correlate the appearance of the $g = 3.58$ signal with changes in the optical spectrum,

anaerobic titrations of the oxidized cyanide enzyme against dithionite were monitored by absorption and e.p.r. spectroscopy. The dithionite solution was calibrated by parallel titration against cytochrome *c*. The changes in the e.p.r. spectrum during the reduction for both the Cu^{2+} signals and the low-field haem components are shown in Figs. 3(a) and 3(b) respectively. The signals at $g = 3.58$ and 3.03 and the cupric signals were quantified and plotted, in terms of component per enzyme functional unit against the number of reducing equivalents per enzyme functional unit added (Fig. 4a). Fig. 4(a) shows clearly as reduction proceeds the loss of both the $g = 3.03$ and active copper signals and the growth of the $g = 3.58$ signal. The sum of the total haem detectable by e.p.r. spectroscopy during reduction is also plotted in Fig. 4(a). It is apparent that the $g = 3.03$ signal is lost faster than the $g = 3.58$ signal appears, suggesting that they do not belong to interconverting species.

It has been previously suggested that the addition

Fig. 3. Anaerobic titration of oxidized cyanide-inhibited cytochrome *c* oxidase against dithionite, monitored by low-temperature e.p.r. spectroscopy

The enzyme concentration was $222 \mu\text{M}$. The numbers of reducing equivalents of dithionite added per enzyme functional unit were: 1, 0; 2, 0.5; 3, 1.1; 4, 1.8; 5, 2.6; 6, 3.5; 7, 4.8; 8, 5.5. (a) shows the Cu^{2+} signal at 70 K and (b) shows the low-field haem signal at 30 K. The spectrometer settings for both sets of spectra were: 2 mW microwave power, 0.63 mT modulation amplitude and 9.24 GHz microwave frequency. The receiver gain settings were increased by a factor of 2 for spectra 7 and 8 and by a factor of 1.67 for spectrum 6 in (a).

Fig. 4. Anaerobic titration of oxidized cyanide-inhibited cytochrome *c* oxidase against dithionite, monitored by parallel e.p.r. and optical spectroscopy

The abscissa corresponds to the number of reducing equivalents of dithionite added per enzyme functional unit. The dithionite was calibrated by parallel titration against oxidized cytochrome *c*. (a) shows the results of quantifying the $g = 3.03$ (○), $g = 3.58$ (●) and active Cu^{2+} (Δ) e.p.r. signals. The quantification of the $g = 3.58$ signal was made by using $g_z = 3.58$, $g_y = 1.56$ and $g_x = 1.00$. The broken line (▲) indicates the sum of total haem (i.e. $g = 3.03$ plus $g = 3.58$) detectable by e.p.r. during the course of the titration. (b) shows the corresponding changes in the absorption of the α -band (□) [plotted as $\Delta(A_{605}-A_{620})$] and 820 nm band (■) [plotted as $\Delta(A_{820}-A_{720})$].

of excess cyanide to oxidized cytochrome *c* oxidase results in the reduction of inactive copper and the formation of a Cu^+ -cyanide complex (Beinert *et al.*, 1962). Fig. 5 compares the copper e.p.r. signals for samples of oxidized and oxidized cyanide cytochrome *c* oxidase at the same concentration. The e.p.r. conditions used, i.e. 2 mW microwave power and 70 K, were chosen to be non-saturating for both active and inactive copper. Clearly the features attributed to inactive copper (Greenaway *et al.*, 1977) are lost on addition of excess cyanide. Thus the quantification of the copper signal in the oxidized cyanide form of the enzyme corresponds almost entirely to active copper. The inactive copper, which is both cyanide- and dithionite-irreducible (see Figs. 2a and 3a), is always found to correspond to less than 0.03 Cu^{2+} per enzyme functional unit. By

Fig. 5. E.p.r. spectra at 70 K of (a) oxidized cytochrome *c* oxidase and (b) oxidized cyanide-inhibited cytochrome *c* oxidase

In both cases the enzyme concentration was $145 \mu\text{M}$. The spectrometer settings were identical for both spectra, i.e. 2 mW microwave power, 0.63 mT modulation amplitude and 9.25 GHz microwave frequency.

quantifying the e.p.r. signals such as those in Fig. 5, the enzyme preparations used in the present work have been shown to contain between 20 and 40% inactive copper. This compares favourably with the values of 25% reported by Aasa *et al.* (1976) and values between 20 and 40% estimated by Greenaway *et al.* (1977).

The method of generating the oxidized cyanide derivative used in the present work, i.e. incubation with cyanide for 24 h at 4°C , invariably gives samples that have undergone slight autoredox. This is evidenced by the small $g = 3.58$ signal in the e.p.r. of the material freshly prepared. Allowing for this, the e.p.r. integrations of the $g = 3.03$ and the Cu^{2+} signals on four separate samples of the oxidized cyanide derivative accounted for between 0.9 and 1.1 haem groups and 0.7 and 0.8 active Cu^{2+} per enzyme functional unit. These results are in good agreement with e.p.r. quantifications of the oxidized resting cytochrome *c* oxidase (Aasa *et al.*, 1976). Accurate quantification of the $g = 3.58$ signal is problematical, since the g -value of the high-field resonance is not known. However, by using $g_z = 3.58$, $g_y = 1.56$ and $g_x = 1.0 \pm 0.5$, quantification of this band at the conclusion of a titration with dithionite accounts for between 0.9 and 1.1 haem groups per enzyme functional unit. In contrast, we have found that the total haem recovered by integration is consistently low, between 0.45 and 0.65 haem group per enzyme functional unit, if excess of solid dithionite is added to an anaerobic solution of the cyanide form of oxidized cytochrome *c* oxidase to generate the partially reduced state in one step.

M.c.d. spectra

We have recently reported the room-temperature c.d. and m.c.d. spectra of cytochrome *c* oxidase derivatives in the near-infrared spectral region (Eglinton *et al.*, 1980). It was shown that the low-spin ferrous forms of cytochrome *a* and cytochrome *a₃* give characteristic natural c.d. bands at 1100nm, whereas low-spin ferric cytochrome *a* shows a positive m.c.d. peak at 1650 nm. In the fully oxidized form of the enzyme cytochrome *a₃*-CN has a broad positive m.c.d. spectrum increasing steadily from 1300 to 1900nm, the long-wavelength limit of our measurements. The form of the spectrum is not typical of low-spin ferric haem. However, in the partially reduced cyanide derivative cytochrome *a₃*-CN is evident in the m.c.d. spectrum as a sharp positive peak at 1550nm, in this case apparently a typical low-spin ferric haem signal. In addition, the natural c.d. spectrum of the partially reduced cyanide form of the enzyme reveals a natural c.d. band at 1100nm characteristic of low-spin ferrous haem. Thus the near-infrared c.d. and m.c.d. spectra of the partially reduced cytochrome oxidase clearly show the presence of one low-spin ferrous cytochrome, assigned to cytochrome *a²⁺*, and one low-spin ferric cytochrome, assigned to cytochrome *a₃³⁺*-CN. The m.c.d. spectrum of the oxidized cyanide form of the enzyme shows a strong negative band at 870nm, the counterpart of the 830nm absorption band, assigned to cupric ion. This band is absent from the m.c.d. spectrum of the partially reduced cyanide derivative.

Here we present near-infrared m.c.d. spectra measured at 4.2 K that confirm and extend the data already reported. These are the first near-infrared m.c.d. spectra at ultra-low temperatures to be published.

Fig. 6. Near-infrared m.c.d. spectra of oxidized (—) and oxidized cyanide-inhibited (---) cytochrome *c* oxidase

Spectra were recorded at 4.2 K and at a magnetic field of 5 T and were corrected for zero-field circular dichroism. The dotted line represents the difference between the two spectra.

Fig. 6 compares the m.c.d. spectra at 4.2 K of two fully oxidized derivatives of the enzyme, the oxidized and the oxidized cyanide-inhibited forms. The m.c.d. spectra are about 70 times more intense at 4.2 K than at room temperature, showing the paramagnetism of all the components contributing to the spectra at low temperature. The spectrum of the oxidized derivative between 1200 and 1700nm contains the contribution from one low-spin ferric haem, namely cytochrome *a*, whereas that of the oxidized cyanide bound form is expected to contain in addition contributions from cytochrome *a₃*-CN. There may also be a contribution from cupric ion to the m.c.d. spectrum in this region, since this ion is known to have *d-d* bands in this energy range (Solomon *et al.*, 1980). However, this is expected to be much weaker than the contribution from low-spin ferric haem. Furthermore, provided that the optical spectrum of the cupric ion is unchanged by addition of CN⁻ ion to the enzyme, then any contribution from cupric ion will not appear in the m.c.d. difference spectrum between the oxidized and the oxidized cyanide forms. The difference spectrum (Fig. 6) therefore gives the form of the m.c.d. spectrum of the cytochrome that has bound cyanide ion, namely cytochrome *a₃*. The spectrum lacks a pronounced positive peak and is not typical of a low-spin ferric haem. However, the difference spectrum has increased by about 70-fold on cooling from room temperature to 4.2 K and, in fact, continues to increase in intensity on cooling further to 1.5 K at 5 T. Thus the component responsible for the difference spectrum in Fig. 6 is paramagnetic.

A stepwise reduction of the cyanide-bound enzyme with dithionite was performed and the m.c.d.

Fig. 7. Near-infrared m.c.d. spectra during the anaerobic reductive titration of oxidized cyanide-inhibited cytochrome *c* oxidase against dithionite.

The accumulative volume of dithionite solution added for each spectrum was 0 μl (—), 80 μl (---) and 410 μl (.....). Spectra were recorded at 4.2 K and at a magnetic field of 5 T and were corrected for zero-field circular dichroism.

spectra of the partially reduced intermediates were recorded at 4.2 K and 5 T (Fig. 7). A dramatic change takes place as reducing agent is added. The bands in the spectrum become better resolved and sharpen markedly. When excess dithionite is present the m.c.d. spectrum is due only to cytochrome a_3^{3+} -CN in the low-spin ferric state. The remarkable changes in the spectra as reduction proceeds are due to two effects. First, the contribution to the m.c.d. spectrum of low-spin ferric cytochrome a is lost as it is reduced. Secondly, the m.c.d. spectrum of cytochrome a_3 -CN undergoes a change from the poorly resolved broad spectrum (the difference spectrum of Fig. 6) to the well-resolved sharp spectrum of Fig. 7, which is attributed to low-spin cytochrome a_3^{3+} -CN. Several possible reasons for this remarkable change have already been advanced (Eglinton *et al.*, 1980).

Discussion

In establishing the nature of the partially reduced cyanide-inhibited form of cytochrome c oxidase we consider first the haem centres. The reduction plots of Fig. 4 show clearly that cytochrome a^{3+} , measured by the $g = 3.03$ signal, is reduced as dithionite is added to the oxidized cyanide enzyme. The course of reduction of this haem group is also shown, in part at least, by the increase in the absorbance at 605 nm. But we note that the optical signal at 605 nm and the $g = 3.03$ signal do not vary in the same way with added dithionite. It is possible that other chromophores contributing to the 605 nm band are also changing with reduction. The failure of the $g = 3.03$ signal to titrate perfectly parallel with the change in absorbance at 605 nm has also been noted in reductive titrations of the oxidized enzyme in the absence of inhibitor (Babcock *et al.*, 1978). The appearance of low-spin cytochrome a^{2+} as dithionite is added has been observed most clearly in the near-infrared natural c.d. spectrum at 1100 nm (Eglinton *et al.*, 1980).

As reduction of cytochrome a^{3+} proceeds, new e.p.r. signals ($g = 3.58$ and 1.56) appear. The low-field component of this signal in the partially reduced form of the cyanide inhibited enzyme has been reported previously by DerVartanian *et al.* (1974). However, the signal was not quantified and its other components were not reported. A similar signal appears at $g = 3.58$ when a rapidly reacting form of cytochrome oxidase, produced by re-oxidation of the reduced enzyme, is exposed to cyanide (Shaw *et al.*, 1978). Under these conditions quantification accounted for about 50% of one haem group. The present work shows that about one haem group per enzyme unit is obtained by stepwise reduction with dithionite. On the other hand reduction by addition of a single portion of excess of solid dithionite leads to the observation of only about 50%

of this haem group by e.p.r. spectroscopy. We interpret this result as indicating that the partially reduced cyanide-inhibited species, as characterized by a fully developed $g = 3.58$ signal, will slowly undergo some further reduction in the presence of a large excess of dithionite.

The question arises as to the identity of the cytochrome that gives rise to this new signal. The assumption has been made that it is due to the cyanide form of cytochrome a_3^{3+} (DerVartanian *et al.*, 1974). Our evidence bears out this assumption. Fig. 4(a) shows that the total haem observed by integration and addition of the $g = 3.03$ and 3.58 signals varies between 1.0 and 0.8 during the course of the titration. This suggests that the two signals arise from centres that titrate differently as a function of added reductant and therefore do not arise from a single centre.

When other derivatives of cytochrome c oxidase inhibited either by azide or by sulphide are reduced, new low-spin ferric haem signals appear in the e.p.r. spectrum (Erecinska *et al.*, 1979; Van Gelder & Beinert, 1969). Both sets of authors concluded that the new signals were due to the low-spin form of cytochrome a_3^{3+} liganded by azide and sulphide respectively. Van Gelder & Beinert (1969) also performed a reduction titration of the cyanide-inhibited cytochrome c oxidase, but failed to report a low-spin signal at $g = 3.58$ in this case. Indeed, they remarked that no new signals appeared as reduction proceeded. This surprising result was the mainspring for the present work. The near-infrared m.c.d. spectrum also shows the presence of a low-spin ferric haem signal at 1550 nm in the partially reduced cyanide cytochrome c oxidase, both at 4.2 K (Fig. 7) and at room temperature (Eglinton *et al.*, 1980).

Turning now to a consideration of the copper centres, we note that the e.p.r.-detectable cupric centre is reduced as dithionite is added, until as partial reduction is attained the only copper signal remaining is that due to a very small percentage of 'inactive' copper. However, the titration curve (Fig. 4a) clearly shows a lag in the reduction of this centre. This feature was not present in the e.p.r. reduction titration curve reported by Van Gelder & Beinert (1969), who stated that the reduction of the copper centre proceeded linearly with addition of reducing equivalents, in this case being supplied by NADH in the presence of phenazine methosulphate. However, the titration of the 820 nm optical absorption band does show a lag (Figs. 1 and 4b) as dithionite is added. Since the major part of the absorption intensity of the 820 nm band has been attributed to the e.p.r.-detectable cupric centre, it was important to check this observation by Van Gelder & Beinert (1969) and to correct it. In the reductive titration of azide-inhibited oxidized

cytochrome *c* oxidase the cupric e.p.r. signal does show a lag according to Van Gelder & Beinert (1969), although in this case the course of reduction of the 820nm-absorption band was not measured. Hence we conclude that, in the partially reduced cyanide derivative, the copper centre Cu_a is reduced.

The other copper centre, Cu_{a_3} , is not detectable in the e.p.r. spectrum of the partially reduced cyanide derivative. Thus it must be concluded that it is either present as Cu_a^+ or that it is interacting magnetically with cytochrome a_3 -CN. However, the latter possibility is unlikely, since both the near-infrared m.c.d. and e.p.r. spectra of the partially reduced cyanide form indicate the presence of low-spin ferric cytochrome a_3^3+ -CN. Previous evidence for the oxidation state of this centre has rested on the results of quantitative reductive (Nicholls & Hildebrandt, 1978) or re-oxidative titrations (Yong & King, 1972). However, in neither case was e.p.r. spectroscopy used to define the point of partial reduction. In the present work it was found that aerobic reduction of cyanide-inhibited cytochrome *c* oxidase with *NNN'*-tetramethylphenylenediamine, as performed by Nicholls & Hildebrandt (1978), does not lead to complete loss of the $g = 3.03$ signal. In other words, this reducing agent does not drive the enzyme to a well-defined partially reduced state. Nicholls & Hildebrandt (1978) estimate that excess of *NNN'*-tetramethylphenylenediamine will lead to the take-up by the oxidized enzyme of 3 reducing equivalents. But, because they observed no optical changes during this process, they concluded that this reduction is not to a physiologically active site. It can be seen that the partially reduced form as defined by Nicholls & Hildebrandt (1978) does not correspond to the form produced by dithionite and most plausibly corresponds to a distribution of 2 reducing equivalents between three metal centres.

The reduction with dithionite has consistently led to estimates of reducing equivalents added that are in excess of that required. For example, Fig. 4 shows that over 4 reducing equivalents/mol of enzyme are required to reach the partially reduced state. In optical titration with NADH in the presence of phenazine methosulphate we find that between 2.6 and 3.1 reducing equivalents are required to reach the partially reduced state. An overestimate of the number of reducing equivalents required is invariably obtained with dithionite as reductant (Van Gelder & Beinert, 1969), possibly because other adventitious reducible centres are available to dithionite. We have noticed, for example, that adventitious iron signals at $g = 4.3$ are reduced by dithionite.

In conclusion, we can say that the partially reduced cyanide derivative of cytochrome *c* oxidase, as defined by near-infrared m.c.d. and e.p.r. studies, contains $a^{2+} \cdot \text{Cu}_a^+ \cdot a_3^3+ \text{-CN} \cdot \text{Cu}_{a_3}^+$. In the preceding

paper (Thomson *et al.*, 1981) the nature of the electronic ground state of cytochrome a_3 -CN in the oxidized cyanide derivative of the enzyme was discussed at length. It was concluded that the most probable electronic ground state has $S = 1$, which arises either from a low-spin cytochrome a_3^{4+} -CN or from ferromagnetic coupling between a low-spin cytochrome a_3^{3+} -CN and $\text{Cu}_{a_3}^{2+}$. In either case 3 reducing equivalents would be required to obtain the partially reduced cyanide form, since for the low-spin cytochrome a_3^{4+} -CN possibility it is necessary to have Cu_{a_3} in the cuprous state for both the oxidized cyanide and the partially reduced cyanide forms of the enzyme. At present we are unable to decide between these two alternatives, but it is hoped that future m.c.d. studies on Fe^{4+} haem systems, particularly in the near-infrared region, may resolve the question.

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