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Characterization of the Intermediates in the Reaction of Mixed-Valence-State Soluble Cytochrome Oxidase with Oxygen at Low Temperatures by Optical and Electron-Paramagnetic-Resonance Spectroscopy

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The reaction of soluble mixed-valence-state ($a^{3+}Cu_A^{2+} \cdot Cu_B^{+}a_3^{2+}$) cytochrome oxidase with O_2 at low temperature was studied by optical and e.p.r. spectroscopy. The existence of three intermediates [Clore & Chance (1978) *Biochem. J.* **173**, 799–810] was confirmed. From the e.p.r. data it is clear that cytochrome *a* and Cu_A remain in the low-spin ferric and cupric states respectively throughout the reaction. No e.p.r. signals attributable to cytochrome a_3 or Cu_B were seen in the intermediates. The difference spectra (intermediates minus unliganded mixed-valence-state cytochrome oxidase) and absolute spectra of the three intermediates were obtained. The chemical nature of the three intermediates is discussed in terms of their spectroscopic properties. A catalytic cycle for cytochrome oxidase is proposed.

The minimum functioning unit of mammalian cytochrome oxidase (ferrocytochrome *c*–oxygen oxidoreductase, EC 1.9.3.1) is thought to consist of two A-type haems, cytochromes *a* and a_3, differing only in the nature of their axial ligands, and two copper atoms, Cu_A and Cu_B (Malmström, 1973). Cu_A and cytochrome *a* are magnetically isolated and detectable by e.p.r.; Cu_B and cytochrome a_3 are anti-ferromagnetically coupled and undetectable by e.p.r. in fully oxidized cytochrome oxidase (Aasa *et al.*, 1976; Babcock *et al.*, 1976; Thomson *et al.*, 1977; Moss *et al.*, 1978; Tweedle *et al.*, 1978). The e.p.r. signals exhibited by cytochrome *a* at $g = 3$, 2.2 and 1.45 (Aasa *et al.*, 1976) are characteristic of bis-imidazole low-spin ferric haem complexes (Peisach *et al.*, 1973; Babcock *et al.*, 1979). On partial anaerobic reduction of oxidized cytochrome oxidase, high-spin ferric haem signals around $g = 6$, attributable to high-spin cytochrome a_3^{3+}, are seen (Hartzell & Beinert, 1974; Aasa *et al.*, 1976).

The mixed-valence-state cytochrome oxidase–CO complex ($a^{3+}Cu_A^{2+} \cdot Cu_B^{+}a_3^{2+} \cdot CO$) formed by the addition of excess ferricyanide to the fully reduced cytochrome oxidase–CO complex has been characterized by e.p.r. (Leigh *et al.*, 1974; Wever *et al.*, 1974), near-i.r. optical spectroscopy and X-ray-absorption edge spectroscopy (Powers *et al.*, 1979). The use of this mixed-valence-state species, in which cytochrome a_3 and Cu_B are in the ferrous and cuprous states respectively, and cytochrome *a* and Cu_A in the ferric and cupric states respectively, allows one to obtain further insight into the reaction of cytochrome oxidase with O_2 and the interactions of its four metal centres.

In previous papers (Clore & Chance, 1978*b*; Clore, 1979) we examined the kinetics of the mixed valence-state membrane-bound cytochrome oxidase–O_2 reaction at 173K at six wavelength pairs (444–463, 590–630, 604–630, 608–630, 740–940 and 830–940nm). The only mechanism that was found to satisfy the triple criteria developed by Clore & Chance (1978*a*) governing the choice of models for complex kinetic data, namely an S.D. within the standard error of the data, a random distribution of residuals and good determination of the optimized parameters, was a three-intermediate sequential mechanism stated as:

$$E_M + O_2 \underset{k_{-1}}{\overset{k_{-1}}{\rightleftharpoons}} I_M \underset{k_{-2}}{\overset{k_{-2}}{\rightleftharpoons}} II_M \underset{k_{-3}}{\overset{k_{-3}}{\rightleftharpoons}} III_M \quad (1)$$

where E_M is unliganded mixed-valence-state cytochrome oxidase produced by flash photolysis of the mixed-valence-state cytochrome oxidase–CO complex. [The notation is that of Clore & Chance (1978*b*); intermediates I_M and III_M are probably equivalent to compounds A_2 and C respectively

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described by Chance *et al.* (1975*a*).] Although optical difference spectra (intermediate species minus species E_M) have been obtained both in the visible (Chance *et al.*, 1975*a*) and near-i.r. (Chance & Leigh, 1977) regions in which intermediates I_M and III_M are probably the predominant species, no spectra have been obtained illustrating the time course of the reaction. Further, no quantitative e.p.r. data have as yet been obtained and no e.p.r. spectra have as yet been published. It should be noted that a 'new' e.p.r. signal at $g = 2.17$ in intermediate III_M has been reported and attributed to Cu_B^{2+} (Chance *et al.*, 1975*b*), but neither e.p.r. spectra nor experimental details were presented, so that evaluation of this signal has not been possible.

In the work described in the present paper we examined the temporal changes occurring in the reaction of mixed-valence-state soluble cytochrome oxidase with O_2 both by optical spectroscopy in the visible region (500–700nm) and by detailed quantitative e.p.r. spectroscopy in order to obtain further insight into the nature of the intermediates formed during this reaction.

Experimental

Enzyme and chemicals

Cytochrome oxidase was prepared from bovine heart mitochondria by the method of Van Buuren (1972), with a final dialysis step to remove all cholate and $(NH_4)_2SO_4$. The characteristics of our preparation are identical with those given in the preceding paper (Clore *et al.*, 1980).

The concentration of cytochrome oxidase, expressed in terms of a functional unit containing two haems and two copper atoms, was calculated from $\varepsilon^{605}_{red.-ox.} = 24.0\,mM^{-1}\cdot cm^{-1}$ (Van Gelder, 1963).

Sample preparation

The preparation of the mixed-valence-state cytochrome oxidase–CO complex was identical with that of fully reduced cytochrome oxidase–CO complex described in the preceding paper (Clore *et al.*, 1980) except that $K_3Fe(CN)_6$ (final concn. 0.8mM) was added at 250K 30s before the addition of the oxygenated ethylene glycol solution (Chance *et al.*, 1975*a*; Clore & Chance, 1978*b*). This concentration of $K_3Fe(CN)_6$ was chosen to ensure complete formation of the mixed-valence-state cytochrome oxidase–CO complex, as judged from the amount of copper and low-spin ferric haem detected by e.p.r. in the unphotolysed samples (the same amount as in the resting oxidized enzyme), but kept as low as possible so as to minimize interference of the ferricyanide e.p.r. signals with those of the oxidase.

Photodissociation of the mixed-valence-state cytochrome oxidase–CO complex was carried out at 77K by using 10–20 flashes from a 10J xenon-flash lamp (model 610B; Photochemical Research Associates, London, Ont., Canada) with a pulse width of 3μs, to ensure 100% photolysis (Clore *et al.*, 1980). The reaction with O_2 was initiated by warming the photolysed samples to 173K in a liquid-N_2-cooled isopentane bath for a given time and stopped by cooling the e.p.r. tubes at 77K in liquid N_2 (Clore *et al.*, 1980). This procedure was repeated and the optical and e.p.r. spectra were recorded after each warming and cooling cycle.

Optical spectra

Optical spectra were recorded in 3mm-internal-diameter quartz e.p.r. tubes with a Johnson Research Foundation DBS-2 dual-wavelength spectrophotometer as described in the preceding paper (Clore *et al.*, 1980). The fixed reference wavelength used was 575nm. All spectra were recorded with a bandwidth of 5nm at a rate of 2.56nm/s and a time constant of 1s.

E.p.r. spectra

E.p.r. spectra at 9GHz were recorded at 77K (in liquid N_2) with a Varian E-3 spectrometer and at temperatures between 5 and 80K in a Varian E-9 spectrometer. Temperatures between 5 and 80K were maintained by using an ESR-9 continuous-flow helium cryostat (Oxford Instruments). The microwave frequency was measured with a Hewlett–Packard 5245L electronic counter and a Hewlett–Packard 5257A transfer oscillator. The e.p.r. spectra were digitized by using an automatic x–y reader coupled to a minicomputer (Nova 3; Data General Corporation).

All integrations were carried out taking into account the transition probability for field-swept spectra (Aasa & Vänngård, 1975). Integration of the copper and low-spin haem signals were carried out as described in the preceding paper (Clore *et al.*, 1980).

Integration of the high-spin haem signals was carried out by the truncated second integral method of Aasa *et al.* (1976). This method is based on the fact that the double integral of the $g = 6$ peak is independent of linewidth and rhombic splitting within certain limits. With the microwave frequency in the range 9.1–9.5GHz, the double integral of the experimental first derivative spectrum with the lower integration limit taken below the low-field end of the spectrum, and the upper limit at a field corresponding to an apparent g value of 4.67, yields 47–49% of the total double integral. This is valid if the following conditions are satisfied: (i) linewidths at half height less than 6mT; (ii) $g_z = 2.0$; (iii) $(g_x + g_y)/2 = 5.85$–5.95; (iv) rhombic splittings $(g_x - g_y) < 1.0$; the $g = 6$ signals we observe fall within these limits. It should

also be noted that the signal amplitude at the upper limit equals 350–450 times the double integral up to the same limit multiplied by the square of the integration interval in units of tesla. This relation can be used to check the way the baseline has been drawn. The zero-field splitting D was taken to be 10.5 cm^{-1} (Aasa *et al.*, 1976). All e.p.r. spectra used for integration were recorded under non-saturating conditions.

Results

Optical spectra

Typical difference spectra (reaction sample minus unliganded mixed-valence-state cytochrome oxidase, species E_M) illustrating the time course of the mixed-valence-state cytochrome oxidase–O_2 reaction at 173 and 193 K are shown in Fig. 1. Three distinct optical species may be distinguished. The first species, intermediate I_M, seen in the spectrum taken 20 s after initiating the reaction at 173 K, is characterized by a peak at 592 nm, a shoulder at 605 nm and a trough at 614 nm in the α-band region, and a peak at 549 nm and troughs at 527 and 570 nm in the β-band region. The difference spectrum of intermediate I_M is qualitatively similar to that of the mixed-valence-state cytochrome oxidase–CO complex, but there are significant differences in the positions of the peaks and troughs in the α-band and β-band regions (see Fig. 1 and Table 1). It should also be noted that, whereas the enzyme–CO complex is photolysable, intermediate I_M is not at the flash intensities employed (namely 10 J xenon flash with a 3 μs pulse width). This is entirely consistent with the phenomenon of lower quantum yield of O_2 compounds compared with CO compounds of haem

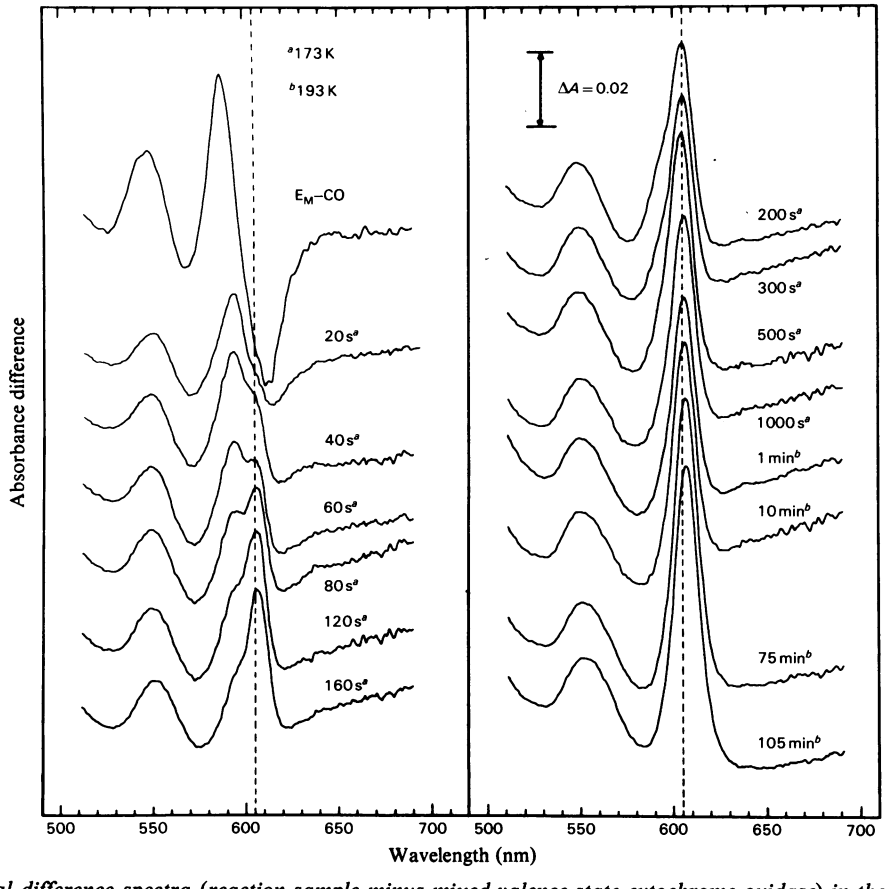

Fig. 1. *Optical difference spectra (reaction sample minus mixed-valence-state cytochrome oxidase) in the visible region obtained at successive times in the reaction of mixed-valence-state cytochrome oxidase with O_2 at 173 and 193 K*

A difference spectrum of the mixed-valence-state cytochrome oxidase–CO complex minus mixed-valence-state cytochrome oxidase is also shown for comparison. It should be noted that the spectra at 193 K (*b*) were obtained after the 1000 s spectrum at 173 K (*a*). The spectra were recorded at 77 K. Experimental conditions are: 10 μM-cytochrome oxidase, 1.6 μM-phenazine methosulphate, 0.3 mM-NADH, 50 mM-sodium phosphate buffer, pH 7.4, 30% (v/v) ethylene glycol, 0.8 mM-$K_3Fe(CN)_6$, 0.8 mM-CO and 0.67 mM-O_2.

proteins (Gibson & Ainsworth, 1957). In the α-band region, the difference spectrum of intermediate I_M we describe for the soluble cytochrome oxidase is similar to that of compound A_2 described by Chance *et al.* (1975*a*) for the membrane-bound cytochrome oxidase except that the peak and trough of the latter are blue-shifted by 2nm. The formation of the second species, intermediate II_M, from intermediate I_M can be seen in the spectra taken from 40 to 200s after initiation of the reaction at 173K, and is characterized by the progressive formation of a peak at 605nm in the α-band region, a 1nm red-shift in the 549nm β-peak of intermediate I_M to 550nm, and shifts in the 527 and 570nm β-band troughs of intermediate I_M respectively to 529 and 575nm (see Table 1). Intermediate II_M appears to be fairly stable at 173K, at least up to 1000s after initiation of the reaction with O_2. If the sample is then warmed to 193K, intermediate II_M is slowly converted over a period of approx. 50min into a third species, intermediate III_M, which is characterized by a 607nm peak (of slightly greater intensity than the corresponding 605nm peak of intermediate II_M) in the α-band region, and a 552nm peak and 530 and 582nm troughs in the β-band region. In the α-band region, the difference spectrum of intermediate III_M we describe for the soluble cytochrome oxidase is similar to that of compound C described by Chance *et al.* (1975*a*). It should be noted that in the membrane-bound cytochrome oxidase intermediate III_M is present in over 75% of its maximum concentration at 1000s and is fully formed within 3000s at 173K, at which time more than 99% of the cytochrome oxidase is in the form of intermediate III_M (Clore & Chance, 1978*b*). Therefore the rate constant for the conversion of intermediate II_M into intermediate III_M at 173K must be smaller than that for the membrane-bound cytochrome oxidase by a factor of at least 2. Neither intermediate II_M nor intermediate III_M is photolysable at the flash intensities used. The characteristics of the optical difference spectra of intermediates I_M, II_M and II_M and of the mixed-valence-state cytochrome oxidase–CO complex minus unliganded mixed-valence-state cytochrome oxidase (species E_M) are summarized in Table 1.

Table 1. *Absorption maxima and minima of the difference spectra of intermediates I_M, II_M and III_M and the mixed-valence-state cytochrome oxidase–CO complex minus mixed-valence-state cytochrome oxidase (species E_M) in the visible region*

The reference species, E_M, is produced by flash photolysis of the mixed-valence-state cytochrome oxidase–CO complex. Intermediates I_M, II_M and III_M were trapped 20s at 173K, 500s at 173K and 1h at 193K respectively after initiation of the mixed-valence-state cytochrome oxidase–O_2 reaction. Abbreviations: p, peak; s, shoulder; t, trough.

Species	β-Band region			α-Band region		
Intermediate I_M	527 (t)	549 (p)	570 (t)	592 (p)	605 (s)	614 (t)
Intermediate II_M	529 (t)	550 (p)	575 (t)		605 (p)	
Intermediate III_M	530 (t)	552 (p)	582 (t)		607 (p)	
Mixed-valence-state cytochrome oxidase–CO complex ($Cu_A^{2+}a^{3+}\cdot Cu_B^{+}a_3^{2+}\cdot CO$)	525 (t)	547 (p)	566 (t)	586 (p)		610 (t)

Table 2. *Characteristics of the absolute spectra in the visible region of mixed-valence-state cytochrome oxidase (species E_M), the mixed-valence-state cytochrome oxidase–CO complex, intermediates I_M, II_M and III_M, fully reduced and fully oxidized cytochrome oxidase and the fully reduced cytochrome oxidase–CO complex*

The various species were produced as described in Table 1. Abbreviations: p, peak; s, shoulder.

Species	β-Band region		α-Band region		
Mixed-valence-state cytochrome oxidase (E_M)	520 (s)			600 (p)	
Mixed-valence-state cytochrome oxidase–CO complex	545 (p)		588 (p)	604 (s)	
Intermediate I_M	546 (p)		595 (p)	604 (s)	
Intermediate II_M	550 (p)			603 (p)	
Intermediate III_M	551 (p)			605 (p)	
Fully reduced cytochrome oxidase	550 (p)	562 (p)		605 (p)	
Fully reduced cytochrome oxidase–CO complex	550 (p)		591 (s)	604 (p)	
Fully oxidized cytochrome oxidase	545 (s)		598 (p)		655 (p)

The absolute spectra of all molecular species observed are shown in Fig. 2 and their properties summarized in Table 2. The striking feature of the absolute spectra of intermediates II_M and III_M is the large absorption band in the 600–610nm region, which is about half the intensity of that of fully reduced cytochrome oxidase. The absolute spectra of intermediates II_M and III_M are qualitatively similar to the 'oxygenated' form obtained by Greenwood *et al.* (1974) on mixing the mixed-valence-state cytochrome oxidase with O_2 at room temperature. A further feature of note is the complete absence from the spectra of the mixed-valence-state cytochrome oxidase, the mixed-valence-state cytochrome oxidase–CO complex and intermediates I_M, II_M and III_M of the 655nm band characteristic of fully oxidized cytochrome oxidase (Beinert *et al.*, 1976) and thought to be due to antiferromagnetic coupling between high-spin cytochrome a_3^{3+} and Cu_B^{2+} (Palmer *et al.*, 1976).

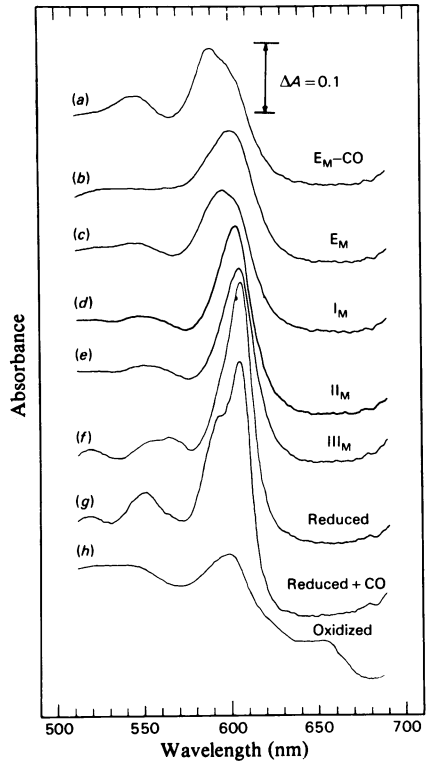

Fig. 2. *Absolute spectra in the visible region of the mixed-valence-state cytochrome oxidase–CO complex, mixed-valence-state cytochrome oxidase, intermediates I_M, II_M and III_M, fully reduced cytochrome oxidase, the fully reduced cytochrome oxidase–CO complex and fully oxidized cytochrome oxidase*

Mixed-valence-state cytochrome oxidase was obtained by flash photolysis of the mixed-valence-state cytochrome oxidase–CO complex at 77K. Intermediates I_M, II_M and III_M were trapped 20s at 173K, 500s at 173K and 1h at 193K respectively after initiation of the mixed-valence-state cytochrome oxidase–CO reaction, and spectra were recorded at 77K. Experimental conditions: (*a*), (*b*), (*c*), (*d*) and (*e*): as in Fig. 1; (*f*) 107μM-cytochrome oxidase, 1.6μM-phenazine methosulphate, 0.3mM-NADH, 50mM-potassium phosphate buffer, pH7.4, and 30% (v/v) ethylene glycol: (*g*) as in (*f*) plus 1.2mM-CO; (*h*) 107μM-cytochrome oxidase, 50mM-potassium phosphate buffer, pH7.4, and 30% (v/v) ethylene glycol.

E.p.r. spectra

E.p.r. spectra of the molecular species observed are shown in Figs. 3 and 4. The only e.p.r. signals seen are those attributable to low-spin cytochrome a^{3+} ($g = 3$, 2.2 and 1.45), Cu_A^{2+} ($g = 2.18$, 2.02 and 1.99) and high-spin ferric haem (around $g = 6$). The concentrations of low-spin cytochrome a^{3+}, rhombic high-spin ferric haem and e.p.r.-detectable copper in these species are given in Table 3.

No new e.p.r. signals are seen with intermediates I_M, II_M and III_M. Although Chance *et al.* (1975*b*) have reported a 'new' signal at $g = 2.17$ attributed to Cu_B^{2+} in intermediate III_M, no e.p.r. spectrum of this signal has ever been published and no experimental details (method of preparation of intermediate III_M, conditions of e.p.r. spectroscopy etc.) were provided. We therefore conclude that the $g = 2.17$ signal was in all probability an artifact.

A small axial high-spin ferric haem signal at $g = 6$ of constant intensity is seen in the mixed-valence-state cytochrome oxidase–CO complex, unliganded mixed-valence-state cytochrome oxidase (species E_M), intermediates I_M, II_M and III_M and fully oxidized cytochrome oxidase; in the resting fully oxidized enzyme this signal represents less than 0.01mol of high-spin ferric haem/mol of cytochrome oxidase and probably represents haem in denatured molecules (Hartzell & Beinert, 1974).

After flash photolysis of the mixed-valence-state cytochrome oxidase–CO complex at 77K, yielding unliganded mixed-valence-state cytochrome oxidase (species E_M), rhombic high-spin ferric haem signals at $g_x = 6.55$ and $g_y = 5.28$ appear (Fig. 3). These correspond to 0.06mol of haem/mol of cytochrome oxidase, a result consistent with the findings obtained by other workers (Wever *et al.*, 1974; Leigh & Wilson, 1972; Leigh *et al.*, 1974; Beinert & Shaw, 1977). The rhombicity (R) as a percentage of the total difference between a completely tetragonal and a completely rhombic field is given by (Peisach *et al.*, 1971):

$$R = [(g_x - g_y)/16] \times 100\%$$

The values of g_x, g_y and R found in unliganded mixed-valence-state cytochrome oxidase (E_M) are

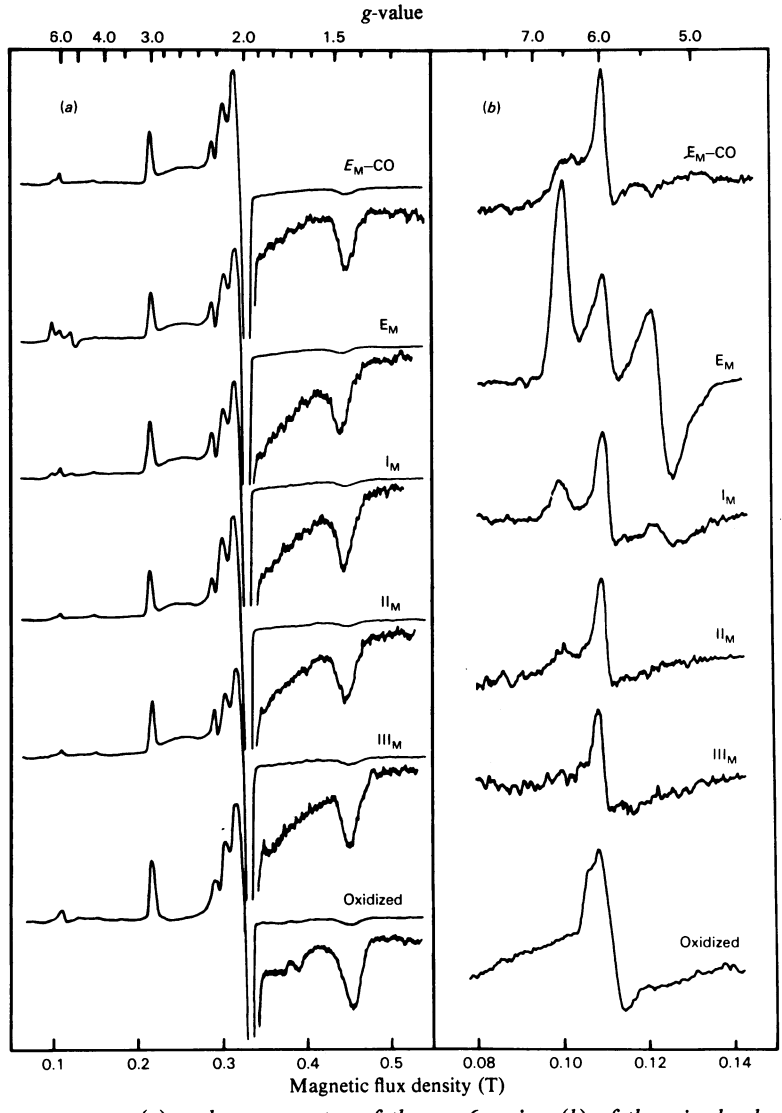

Fig. 3. *Wide-field e.p.r. spectra (a) and e.p.r. spectra of the g = 6 region (b) of the mixed-valence-state cytochrome oxidase–CO complex, mixed-valence-state cytochrome oxidase, intermediates I_M, II_M and III_M and fully oxidized cytochrome oxidase*

Mixed-valence-state cytochrome oxidase was obtained by flash photolysis of the mixed-valence-state cytochrome oxidase–CO complex at 77K. Intermediates I_M, II_M and III_M were trapped 20s at 173K, 500s at 173K and 1h at 193K respectively after initiation of the mixed-valence-state cytochrome oxidase–O_2 reaction. Conditions of e.p.r. spectroscopy: (*a*) microwave power = 2mW, microwave frequency = 9.127GHz, modulation amplitude = 2mT, temperature = 10K, scanning rate = 500mT/min, time constant = 0.1s; the insets from 0.325 to 0.525T are recorded at 10-fold higher gain with a scanning rate of 250mT/min and a time constant of 0.3s; (*b*) as in (*a*) but gain is 10-fold higher, scanning rate = 50mT/min, and time constant = 0.3s. Experimental conditions: for all samples except the oxidized enzyme, 107μM-cytochrome oxidase, 2.67μM-phenazine methosulphate, 0.6mM-NADH, 50mM-sodium phosphate buffer, pH 7.4, 50% (v/v) ethylene glycol, 0.8mM-$K_3Fe(CN)_6$, 0.8mM-CO and 0.67mM-O_2; for the oxidized enzyme: 107μM-cytochrome oxidase, 50mM-sodium phosphate buffer, pH 7.4, and 50% (v/v) ethylene glycol.

compared with those found in partially reduced cytochrome oxidase produced by partial anaerobic reduction of fully oxidized cytochrome oxidase in Table 4. The percentage rhombicity is a sensitive measure of the direct effects of protein environments on the haem and varies from protein to protein, and among different high-spin compounds of the same protein (Peisach *et al.*, 1972).

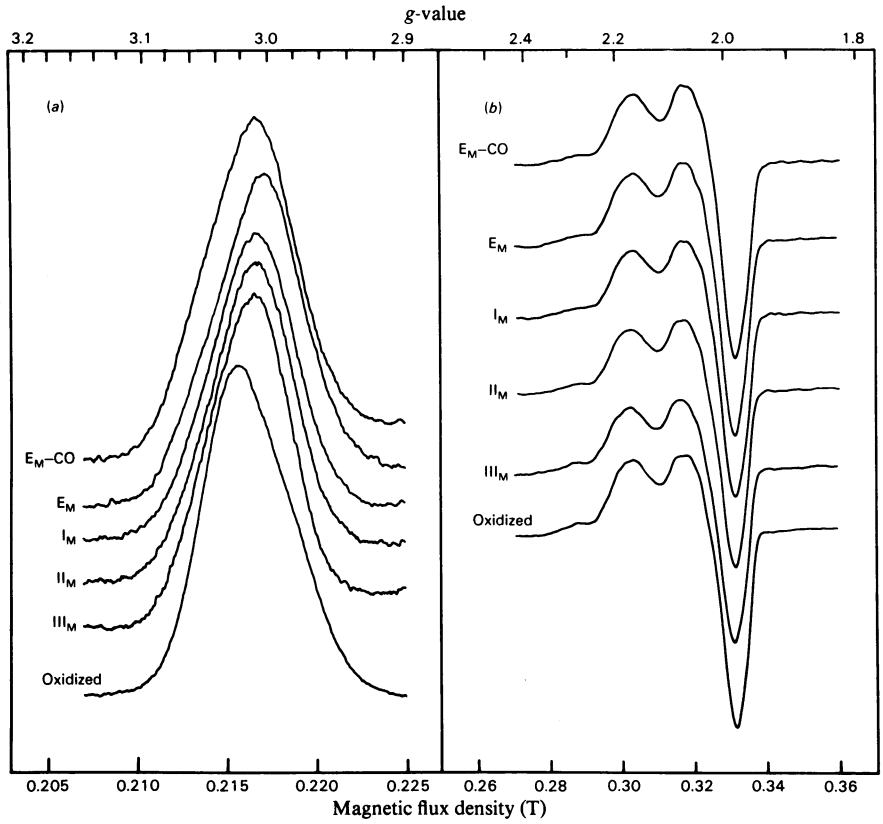

Fig. 4. *E.p.r. spectra of the g = 3 (a) and g = 2 (b) regions of the mixed-valence-state cytochrome oxidase–CO complex, mixed-valence-state cytochrome oxidase, intermediates I_M, II_M and III_M and fully oxidized cytochrome oxidase*

Mixed-valence-state cytochrome oxidase was obtained by flash photolysis of the mixed-valence-state cytochrome oxidase–CO complex at 77K. Intermediates I_M, II_M and III_M were trapped 20s at 173K, 500s at 173K and 1h at 193K respectively after initiation of the mixed-valence-state cytochrome oxidase–O_2 reaction. Conditions of e.p.r. spectroscopy: (*a*) as in Fig. 3(*a*) but gain is 5-fold higher, scanning rate = 10mT/min and time constant = 0.3s; (*b*) microwave power = 20mW, frequency = 9.172GHz, modulation amplitude = 2mT, temperature = 77K, scanning rate = 50mT/min, time constant = 0.3s. Other experimental conditions were as given in Fig. 3 legend.

Table 3. *Concentrations of low-spin cytochrome a^{3+}, rhombic high-spin ferric haem and e.p.r.-detectable cupric copper in the mixed-valence-state cytochrome oxidase–CO complex, mixed-valence-state cytochrome oxidase (species E_M), intermediates I_M, II_M and III_M and fully oxidized cytochrome oxidase*

The various species were produced as described in Table 1.

Species	Low-spin cytochrome a^{3+} (mol/mol of cytochrome oxidase)*	Rhombic high-spin ferric haem (mol/mol of cytochrome oxidase)*	E.p.r.-detectable cupric copper† (mol/mol of cytochrome oxidase)*
Mixed-valence-state cytochrome oxidase–CO complex	1.00	0	1.00
Mixed-valence-state cytochrome oxidase (E_M)	0.93	0.06	1.00
Intermediate I_M	1.00	0.02	0.94
Intermediate II_M	1.01	<0.01	0.93
Intermediate III_M	0.98	0	0.93
Fully oxidized cytochrome oxidase	1.00	<0.01	1.00

* The S.D. is ± 0.03 unit/mol of cytochrome oxidase.

† This includes both Cu_A^{2+} and extraneous copper. The latter accounts for 0.08mol/mol of cytochrome oxidase in our preparation, and probably represents denatured Cu_A^{2+}, as the total concentration of e.p.r.-detectable copper in fully oxidized cytochrome oxidase is always equal to 1mol/mol of cytochrome oxidase in our preparation.

Table 4. *Values of g_x, g_y and percentage rhombicity (R) of rhombic high-spin ferric haem in mixed-valence-state cytochrome oxidase (species E_M) produced by photolysis of the mixed-valence-state cytochrome oxidase–CO complex and in partially reduced cytochrome oxidase produced by partial anaerobic reduction of fully oxidized cytochrome oxidase*

Species	g_x	g_y	R (%)	Type and source of cytochrome oxidase	Reference
Mixed-valence-state cytochrome oxidase (E_M)	6.55	5.28	8.0	Soluble (bovine heart)	Present paper
	6.62	5.29	8.3	Membrane-bound (bovine heart submitochondrial particles)	Wever *et al.* (1974)
	6.60	5.37	7.7	Soluble (bovine heart)	Wever *et al.* (1974)
	6.58	5.40	7.4	Membrane-bound (bovine heart submitochondrial particles)	Leigh & Wilson (1972)
Partially reduced cytochrome oxidase	6.421	5.371	6.6	Soluble (bovine heart)	Aasa *et al.* (1976)
	6.270	5.486	4.9	Soluble (bovine heart)	Aasa *et al.* (1976)

The appearance of rhombic high-spin ferric haem signals on flash photolysis of the mixed-valence-state cytochrome oxidase–CO complex is associated with a decrease in the concentration of low-spin cytochrome a^{3+} from 1.0 to 0.93 mol/mol of cytochrome oxidase. No change, however, in the concentration of e.p.r.-detectable copper is seen. Further, on reaction with O_2, the rhombic high-spin ferric haem signals progressively disappear and the concentration of low-spin cytochrome a^{3+} signal is restored to 1 mol/mol of cytochrome oxidase as the unliganded mixed-valence-state cytochrome oxidase (species E_M) is converted into intermediates I_M, II_M and III_M. Within the errors specified the concentration of [low-spin cytochrome a^{3+} + rhombic high-spin ferric haem] is always equal to 1 mol/mol of cytochrome oxidase in all the species observed (see Table 1).

On the basis of our data, we cannot say whether the rhombic high-spin ferric haem signal produced on flash photolysis of the mixed-valence-state cytochrome oxidase–CO complex is due to a change in spin state of cytochrome a^{3+} [as suggested by Leigh *et al.*(1974)] or electron redistribution between cytochrome a_3^{2+} and cytochrome a^{3+} [as suggested by Wever *et al.* (1977) and Beinert & Shaw (1977)]. It is clear, however, from Table 4 that the percentage rhombicity of the unliganded mixed-valence-state cytochrome oxidase (species E_M) is significantly different from that of the two rhombic ferric haem species seen on partial anaerobic reduction of fully oxidized cytochrome oxidase and known to arise from cytochrome a_3^{3+} on the basis of their behaviour towards exogenous ligands (Beinert & Shaw, 1977; Shaw *et al.*, 1978). The observation that the rhombic high-spin ferric haem signals are produced in less than 5 ms (the limit of the time resolution available) on flash photolysis of the mixed-valence-state cytochrome oxidase–CO complex at 5 K (Leigh *et al.*, 1974) indicates that, if a spin-state change occurs, this must involve quantum-mechanical mixing of $S = 1/2$ and $S = 5/2$ states; if electron redistribution between cytochromes a_3^{2+} and a^{3+} occurs, this must involve quantum-mechanical electron tunnelling.

Table 3 shows that, whereas the concentration of e.p.r.-detectable copper in the mixed-valence-state cytochrome oxidase–CO complex, unliganded mixed-valence-state (species E_M) and fully oxidized cytochrome oxidase is 1 mol/mol of cytochrome oxidase, in intermediates I_M, II_M and III_M it is decreased to approx. 0.93 mol/mol of cytochrome oxidase. Given that Cu_A^{2+} is magnetically isolated (Babcock *et al.*, 1976; Thomson *et al.*, 1977; Tweedle *et al.*, 1978), the small decrease in the concentration of e.p.r.-detectable copper in intermediates I_M, II_M and III_M may be due to electron redistribution between Cu_A and the Cu_Ba_3 couple. However, it should be noted that the concentrations of copper quoted include extraneous copper. The latter accounts for 0.08 mol/mol of cytochrome oxidase in our preparation (Clore *et al.*, 1980), and probably represents denatured Cu_A^{2+}, as in our preparation the total concentration of e.p.r.-detectable copper is always found to be 1 mol/mol of cytochrome oxidase (in agreement with the finding obtained by Greenaway *et al.*, 1977). It is therefore also possible that the decrease in the concentration of e.p.r.-detectable copper in intermediates I_M, II_M and III_M may be due to the reduction of extraneous cupric copper.

Significant differences in the linewidths and peak positions of the $g = 3$ 'absorption' peak of low-spin cytochrome a^{3+} are seen (Table 5). The linewidths decrease in the order: E_M > mixed-valence-state cytochrome oxidase–CO complex > I_M = fully oxidized cytochrome oxidase > II_M > III_M. The peak positions of the $g = 3$ 'absorption' peak shift towards

Table 5. *Linewidths and g values of g_z 'absorption' peak of low-spin cytochrome a^{3+} in mixed-valence-state cytochrome oxidase, the mixed-valence-state cytochrome oxidase–CO complex and intermediates I_M, II_M and III_M*

The various species were produced as described in Table 1.

Species	g_z 'absorption' peak of low-spin cytochrome a^{3+}	
	Linewidth (mT)*	g-value†
Mixed-valence-state cytochrome oxidase (E_M)	6.4	3.004
Mixed-valence-state cytochrome oxidase–CO complex	6.2	3.011
Intermediate I_M	6.0	3.009
Intermediate II_M	5.8	3.009
Intermediate III_M	5.6	3.013
Fully oxidized cytochrome oxidase	6.0	3.019

* The S.D. of the linewidths is ±0.1 mT.

† The S.D. of the *g*-values is ±0.002.

higher *g* values in the order: $E_M < I_M = II_M \leqslant$ mixed-valence-state cytochrome oxidase–CO complex $\leqslant III_M <$ fully oxidized cytochrome oxidase.

It should also be noted that, whereas the $g = 3$ 'absorption' peak of the mixed-valence-state cytochrome oxidase–CO complex, mixed-valence-state cytochrome oxidase (species E_M) and intermediates I_M, II_M and III_M are symmetric, the $g = 3$ 'absorption' peak of fully oxidized cytochrome oxidase is slightly skewed.

A detailed interpretation of these differences is not possible on the basis of the presently available data, but they do indicate significant small differences in the environment of the iron atom of low-spin cytochrome a^{3+} in each of the above species.

Discussion

We have confirmed the existence of three intermediates in the reaction of mixed-valence-state cytochrome oxidase with O_2 and characterized them by optical and e.p.r. spectroscopy.

From the e.p.r. spectra the following are clearly established. (1) Cytochrome *a* remains in the low-spin ($S = 1/2$) ferric state in intermediates I_M, II_M and III_M. (2) Cu_A remains in the cupric state in intermediates I_M, II_M and III_M. [Note that, although the concentration of e.p.r.-detectable copper is slightly lower in intermediates I_M, II_M and III_M than in unliganded mixed-valence-state cytochrome oxidase (species E_M), the decrease in concentration is very small, accounting for only 0.07 mol of copper/mol of cytochrome oxidase, and may well be due to the reduction of extraneous cupric copper.] (3) No rhombic high-spin ferric haem e.p.r. signals are present in intermediates I_M, II_M and III_M. [It should be noted that intermediates I_M and II_M cannot be trapped as pure species but only as mixtures of species in which they are the predominant species; the concentrations of rhombic high-spin ferric haem given in Table 3 for intermediates II_M and III_M are due to residual unliganded mixed-valence-state cytochrome oxidase (species E_M) in the trapped samples.] (4) No new e.p.r. signals attributable to Cu_B^{2+} are seen.

The optical difference spectrum of intermediate I_M minus unliganded mixed-valence-state cytochrome oxidase (species E_M) is very similar to the difference spectrum of intermediate I minus unliganded fully reduced cytochrome oxidase (Clore *et al.*, 1980), confirming the findings obtained by Chance *et al.* (1975*a*). These difference spectra can be entirely accounted for by the formation of a cytochrome a_3–O_2 bond, the electronic configuration of which would be approximately the same in both intermediates (Chance *et al.*, 1975*a*; Clore & Chance, 1979). The shoulder around 590 nm in the absolute spectra of the fully reduced and mixed-valence-state cytochrome oxidase–CO complexes and of intermediates I_M and I has been attributed to a $d\pi_{Fe} \rightarrow p\pi^*_{ligand}$ charge-transfer transition (Clore & Chance, 1979). The peak of this absorption band in intermediate I_M lies at 592 nm and is red-shifted 6 nm relative to that of the mixed-valence-state cytochrome oxidase–CO complex at 586 nm (from the difference spectra of these species minus unliganded mixed-valence-state cytochrome oxidase; Fig. 1 and Table 1). This 6 nm red-shift corresponds to a decrease in the energy of the $d\pi_{Fe} \rightarrow p\pi^*_{ligand}$ transition of about 2 kJ/mol, which is to be expected on account of the greater acceptor power of the $p\pi^*$ orbitals (i.e. the greater oxidizing power) of O_2 relative to that of CO (Williams, 1955; Cotton & Wilkinson, 1972). The nature of the cytochrome a_3–O_2 bond in intermediates I_M and I has been discussed at length by Clore & Chance (1979), and on the basis of available data on model compounds is best represented by the configuration $a_3^{2+\delta}$–$O_2^{-\delta}$, in which the charge localized on the iron of cytochrome a_3 is greater than +2.5 ($\delta > 0.5$). No e.p.r. signals would be expected from such a con-

figuration, as even in its most extreme form, $a_3^{3+}\cdot O_2^-$, one would expect the unpaired electron on O_2 to be spin-coupled to an unpaired electron on cytochrome a_3^{3+}. The formation of intermediates II_M and III_M is characterized by large increases in absorbance in the 600–610 nm region relative to unliganded mixed-valence-state cytochrome oxidase (species E_M), intermediate I_M and the mixed-valence-state cytochrome oxidase–CO complex (Figs. 1 and 2 and Tables 1 and 2). This is best seen in the difference spectra of intermediates II_M and III_M minus unliganded mixed-valence-state cytochrome oxidase (species E_M), which are characterized by narrow absorption bands ($\Delta\nu_{\frac{1}{2}} = 355$ and $410\,cm^{-1}$ respectively), with peaks at 605 and 607 nm respectively. Given that cytochrome *a* and Cu_A remain in the ferric and cupric states respectively in intermediates II_M and III_M on the basis of the e.p.r. data, these absorption bands must therefore be attributed to a component(s) of the $[Cu_Ba_3O_2]$ unit. Powers *et al.* (1979) have assigned the 607 nm absorption band of intermediate III_M to the σ_S (thiolate)$\rightarrow d_{x^2-y^2}$ charge-transfer transition of type I blue copper proteins. We, however, consider this assignment highly unlikely, as the linewidths at half-height of the 605 and 607 nm absorption bands of intermediates II_M and III_M are very much narrower than those seen in blue copper proteins (e.g. for the 604 nm band of stellacyanin, $\Delta\nu_{\frac{1}{2}} = 3095\,cm^{-1}$, and for the 610 nm band of *Polyporus* laccase, $\Delta\nu_{\frac{1}{2}} = 4705\,cm^{-1}$; Malkin & Malström, 1970). Particularly relevant to the assignment of the valence state of Cu_B in intermediates II_M and III_M are the observations of a 740 nm absorption band in intermediates III_M (Chance & Leigh, 1977) and of significantly greater absorbances at 740 and 830 nm in intermediates II_M and III_M relative to those obtained with intermediate I_M, unliganded mixed-valence-state cytochrome oxidase (species E_M) and the mixed-valence-state cytochrome oxidase–CO complex (Clore & Chance, 1978*b*; Clore, 1979). Also relevant is the finding that the ratios of the absorption coefficients of intermediate II_M to those of intermediate III_M at 740 and 830 nm are 0.93 and 0.92 respectively (Clore & Chance, 1978*b*; Clore, 1979). The assignment of the near-i.r. absorption band of cytochrome oxidase entirely to Cu_A^{2+} and Cu_B^{2+} seems confirmed, and is well founded on reductive titrations of fully oxidized cytochrome oxidase (Wever *et al.*, 1977) and correlations with X-ray-absorption edge data (Powers *et al.*, 1979). The above data lead one to the firm conclusion that Cu_B is in the cupric state in intermediates II_M and III_M (Clore & Chance, 1978*b*). Since no e.p.r. signal attributable to Cu_B^{2+} can be detected, the unpaired electron on Cu_B^{2+} must be spin-coupled to an unpaired electron from another paramagnetic centre.

On the basis of kinetic studies in the Soret, α-band and near-i.r. regions, Clore & Chance (1978*a*) proposed three possible sets of valence-state assignments for the metal centres in intermediates II_M and III_M (Fig. 5).

Scheme 1 (Fig. 5) can be completely excluded, since the e.p.r. data clearly demonstrate that cytochrome *a* is in the low-spin ferric state in intermediate II_M (Fig. 3) [note that even if cytochrome a_3 were in the low-spin ferric state in intermediate II_M, as proposed in Scheme 1, its *g*-values would be significantly different from those of cytochrome *a* ($g = 3$, 2.2 and 1.45), which are characteristic of neutral bisimidazole low-spin ferric haem complexes (Babcock *et al.*, 1979; Peisach *et al.*, 1973)].

Schemes 2 and 3 (Fig. 5) can both account for the e.p.r. data. (1) In both schemes cytochrome *a* and Cu_A remain in the low-spin ferric and cupric states respectively in intermediates II_M and III_M. (2) A paramagnetic centre with which Cu_B^{2+} can undergo spin–spin coupling is provided, so that Cu_B^{2+} remains e.p.r.-undetectable. In the case of intermediates II_M, the paramagnetic centre is provided by an unpaired electron on O_2^- and O_2^{3-} in Schemes 2 and 3 respectively. In the case of intermediate III_M, the paramagnetic centre is provided by the free radical X*, which may be either a protein free radical, as in compound ES of cytochrome *c* peroxidase (Yonetani, 1976), or a porphyrin π-cation radical, as in Compound I of horseradish peroxidase and catalase (Dolphin *et al.*, 1971, 1973).

Scheme 3 (Fig. 5), however, seems highly unlikely on account of two factors. Firstly, the optical spectra of intermediates II_M and III_M (Fig. 2) bear no resemblance to those of other haem proteins containing quadrivalent iron (Coulson *et al.*, 1971; Dolphin *et al.*, 1971, 1973; Felton *et al.*, 1973;

Scheme 1

$$\begin{matrix} a_3^{3+}Cu_B^{2+} \\ a^{2+}Cu_A^{2+} \end{matrix}\cdot O_2^- \rightleftharpoons \begin{matrix} a_3^{2+}Cu_B^{2+} \\ a^{3+}Cu_A^{2+} \end{matrix}\cdot O_2^-$$

Scheme 2

$$\begin{matrix} a_3^{2+}Cu_B^{2+} \\ a^{3+}Cu_A^{2+} \end{matrix}\cdot O_2^- \rightleftharpoons X^*\cdot \begin{matrix} a_3^{2+}Cu_B^{2+} \\ a^{3+}Cu_A^{2+} \end{matrix}\cdot O_2^{2-}$$

Scheme 3

$$\begin{matrix} a_3^{4+}Cu_B^{2+} \\ a^{3+}Cu_A^{2+} \end{matrix}\cdot O_2^{3-} \rightleftharpoons X^*\cdot \begin{matrix} a_3^{4+}Cu_B^{2+} \\ a^{3+}Cu_A^{2+} \end{matrix}\cdot O_2^{4-}$$

II_M III_M

Fig. 5. *Three schemes for the assignments of valence states of the metal centres in intermediates II_M and III_M (Clore & Chance, 1978b)*

X* is a free radical. See the text for further details.

Yonetani, 1976; Schonbaum & Chance, 1976). Secondly, it seems highly improbable that the conversion of intermediate I_M into intermediate II_M would involve the transfer of a minimum of two electrons either simultaneously or in very fast succession from $a_3^{2+\delta}$ and Cu_B^+ to $O_2^{-\delta}$ to form $Cu_B^{2+}a_3^{4+}\cdot O_2^{3-}$, by-passing the potentially quite stable O_2^{2-} state.

Scheme 2 (Fig. 5), on the other hand, can account for the optical data as well as the e.p.r. data. In intermediate II_M, the intense absorption band at 605 nm is attributed to a charge-transfer band arising from interaction between cytochrome a_3^{2+}, Cu_B^{2+} and O_2^-. In intermediate III_M, the intense absorption band at 607 nm is attributed to a charge-transfer band arising from interaction between cytochrome a_3^{2+}, Cu_B^{2+}, O_2^{2-} and the free radical X*.

On the basis of the above evidence we strongly believe the assignments of valence states of the metal centres in intermediates II_M and III_M given in Scheme 2 (Fig. 5) to be the correct ones.

Scheme for the reaction of mixed-valence-state cytochrome oxidase with O_2 at low temperatures

On the basis of the optical and e.p.r. data given in the present paper together with the kinetic data of Clore & Chance (1978*a*), we present the following scheme for the mixed-valence-state cytochrome oxidase–O_2 reaction at low temperatures (Fig. 6).

The first step is an oxidative addition reaction involving the binding and simultaneous reduction of O_2 to the $O_2^{-\delta}$ state and the concomitant oxidation of cytochrome a_3^{2+} to the $a_3^{2+\delta}$ state (i.e. the charges on the iron of cytochrome a_3 and O_2 lie between +2.5 and +3 and between −0.5 and −1 respectively in intermediate I_M). The second step involves an internal oxidation–reduction resulting in the oxidation of Cu_B to the cupric state and the reduction of cytochrome a_3 to the ferrous state. The third step involves a one-electron reduction of O_2^- to the O_2^{2-} state and the production of a free radical X^+. The present data do not allow one to determine whether the free radical X^+ is a protein free radical or a porphyrin π-cation radical. The unpaired electron of Cu_B^{2+} in intermediate II_M and III_M is spin-coupled with the unpaired electron on O_2^- and X^+ respectively, forming an exchange-coupled complex of whole spin ($S = 0$ or 1) that is undetectable by e.p.r.

Relevance of the observed intermediates to the catalytic mechanism of cytochrome oxidase

The results given in the present and the preceding paper (Clore *et al.*, 1980) confirm that three inter-

$$Cu_A^{2+}[Cu_Ba_3]^{3+} + O_2 \rightleftharpoons Cu_A^{2+}[Cu_B^{+}a_3^{2+\delta}\cdot O_2^{-\delta}] \rightleftharpoons Cu_A^{2+}[Cu_B^{2+}a_3^{2+}\cdot O_2^{-}] \rightleftharpoons Cu_A^{2+}[Cu_B^{2+}a_3^{2+}\cdot O_2^{2-}\cdot X^+]$$

E_M I_M II_M III_M

Fig. 6. *Scheme for the reaction of mixed-valence-state cytochrome oxidase with O_2 at low temperature*
To get the correct total charge on the unit in brackets the radical X* (Fig. 5) has been designated X^+; see the text for further details.

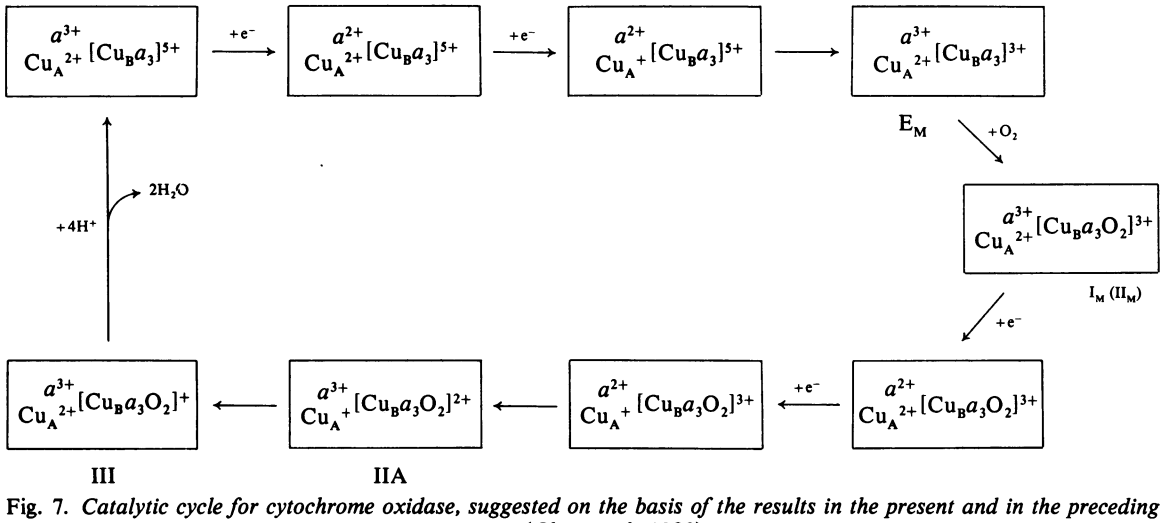

Fig. 7. *Catalytic cycle for cytochrome oxidase, suggested on the basis of the results in the present and in the preceding paper (Clore et al., 1980)*
The intermediates identified have been indicated in the scheme. (We are not implying that all H^+ transfers and formation of water occur in the last step, and they are included only to balance the equations.)

mediates are formed in the reaction of O_2 with the mixed-valence-state cytochrome oxidase and four intermediates with fully reduced cytochrome oxidase. They also make it apparent, however, that optical data provide an inadequate basis for assigning electronic configurations to these intermediates and thus fail to clarify the electron-transfer processes involved in the reduction of the O_2 molecule. For example, it is clear that the intermediates formed from fully reduced cytochrome oxidase involve successive one-electron donation to the $[Cu_B a_3 O_2]^{3+}$ unit from cytochrome a^{2+} and Cu_A^+ respectively (Fig. 8 in Clore *et al.*, 1980), whereas this unit retains the same total charge in the reactions of the mixed-valence-state enzyme, the intermediates involving electron redistributions within this unit only.

It should be noted that the molecular states reached in the reactions of fully reduced cytochrome oxidase as well as of the mixed-valence-state oxidase with O_2 are probably artificial compared with the states occurring during catalytic turnover. Nevertheless we believe that the results of our two papers taken together suggest a plausible electron-transfer scheme for the normal catalytic cycle. The initial reaction with O_2 probably involves not fully reduced cytochrome oxidase molecules but mixed-valence-state molecules formed by the partial reduction of fully oxidized cytochrome oxidase by ferrocytochrome *c* in which the $Cu_B a_3$ unit has received two electrons from ferrocytochrome *c* via cytochrome *a* and Cu_A. Thus the reaction of species E_M with O_2 to form intermediate I_M, and perhaps intermediate II_M (Fig. 6), would represent the first step involving O_2 during turnover. The donation of electrons from ferrocytochrome *c* to intermediate I_M or II_M might, however, be expected to be more rapid than the formation of intermediate III_M, so that intermediates IIA and III in Fig. 8 of Clore *et al.* (1980) are likely states in the overall catalytic reaction. In Fig. 7 we summarize schematically the electron-transfer reactions suggested to be involved in the reduction of O_2 to two molecules of water catalysed by cytochrome oxidase.

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