Characterization of the Low-Temperature Intermediates of the Reaction of Fully Reduced Soluble Cytochrome Oxidase with Oxygen by Electron-Paramagnetic-Resonance and Optical Spectroscopy

G. Marius CLORE,* Lars-Erik ANDRÉASSON,† Bo KARLSSON,† Roland AASA† and Bo G MÄLSTRÖM†
*Department of Biochemistry, University College London, Gower Street, London WC1E 6BT, U.K., and †Department of Biochemistry and Biophysics, Chalmers Institute of Technology, University of Göteborg, S-41296 Göteborg, Sweden

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The reaction of fully reduced soluble bovine heart cytochrome oxidase with O\textsubscript{2} at 173 K was investigated by low-temperature optical and e.p.r. spectroscopy, and the kinetics of the reaction were analysed by non-linear optimization techniques. The only e.p.r. signals seen during the course of the reaction are those attributable to low-spin cytochrome \(a^{3+}\) and \(Cu^{2+}_{A}\). Quantitative analysis of e.p.r. signals shows that, at the end point of the reaction at 173 K, nearly 100% of \(Cu^{2+}_{A}\) is in the cupric state but only about 40% of cytochrome \(a\) is in the ferric low-spin state. The optical spectra recorded at this stage of the reaction show incomplete oxidation of haem and the absence of a 655 nm absorption band. The only reaction scheme that accounts for both the e.p.r. and optical data is a four-intermediate mechanism involving a branching pathway. The reaction is initiated when fully reduced cytochrome oxidase reacts with \(O_2\) to form intermediate I. This is then converted to either intermediate IIA or intermediate IIB. Of these, intermediate IIB is a stable end product at 173 K, but intermediate IIA is converted into intermediate III, which is the stable state at 173 K in this branch of the mechanism. The kinetic analysis of the e.p.r. data allows the unambiguous assignments of the valence states of cytochrome \(a\) and \(Cu^{2+}_{A}\) in the intermediates. Intermediate I contains cytochrome \(a^{2+}\) and \(Cu^{2+}_{A}\), intermediate IIA contains low-spin cytochrome \(a^{3+}\) and \(Cu^{+}_{A}\), intermediate IIB contains cytochrome \(a^{2+}\) and \(Cu^{2+}_{A}\), and intermediate III contains low-spin cytochrome \(a^{3+}\) and \(Cu^{2+}_{A}\). The electronic state of the \(O_2\)-binding \(Cu^{2+}_{A}\) couple during the reoxidation of cytochrome oxidase is discussed in terms of an integrated structure containing \(Cu^{2+}_{B}\), cytochrome \(a^{3+}\) and \(O_2\).

Cytochrome oxidase (ferrocytochrome \(c\)-oxygen oxidoreductase, EC 1.9.3.1) catalyses the terminal reaction, the four-equivalent reduction of molecular \(O_2\) to water, in the respiratory electron-transport chain of all higher organisms. The minimum functioning unit of the mammalian cytochrome oxidase complex is thought to contain four metal centres consisting of two \(A\)-type haems differing only in the nature of their axial ligands, cytochromes \(a\) and \(a_3\), and two copper atoms (Malmström, 1973). From a combination of e.p.r. (Aasa et al., 1976a), m.c.d. (Babcock et al., 1976, 1978; Thomson et al., 1976, 1977), n.m.r. (Falk et al., 1977) and static-magnetic-susceptibility (Moss et al., 1978; Tweedle et al., 1978) studies it has been shown that cytochrome \(a\) is low-spin and magnetically isolated in both the fully reduced and fully oxidized enzyme and detectable by e.p.r. in the ferric state; one copper atom, termed \(Cu^{+}_{A}\), is magnetically isolated and detectable by e.p.r in the cupric state; cytochrome \(a_3\) is high-spin in both the fully reduced and fully oxidized enzyme and anti-ferromagnetically coupled to the other copper atom, termed \(Cu^{2+}_{B}\), forming a spin-coupled dimer of \(S=2\) and \(J\) (exchange coupling constant) \(\ll -200\text{ cm}^{-1}\) in the fully oxidized enzyme; in the fully oxidized enzyme neither cytochrome \(a^{3+}\) nor \(Cu^{2+}_{B}\) is detectable by e.p.r. However, on partial reduction high-spin e.p.r. signals at about \(g=6\) attributable to cytochrome \(a^{3+}\) are seen. No e.p.r. signals attributable to \(Cu^{2+}_{B}\) have as yet been observed in any state of...
the enzyme (Aasa et al., 1976a; Shaw et al., 1978a; Clore et al., 1980).

Recent low-temperature kinetic studies carried out by multi-channel spectroscopy at eight wavelength pairs covering the Soret, α-band and near-i.r. regions, and analysed by sophisticated non-linear numerical integration and optimization techniques, have demonstrated the sequential formation of three intermediates in the reaction of fully reduced membrane-bound cytochrome oxidase with O₂ (Clore & Chance, 1978a, 1979; Clore, 1979):

\[ \text{E} + \text{O}_2 \xrightarrow{k_{1}} \text{I} \xrightarrow{k_{2}} \text{II} \xrightarrow{k_{3}} \text{III} \]  

(1)

[The notation is that of Clore & Chance (1978a, 1979); intermediates I and III are equivalent to compounds A₁ and B described by Chance et al. (1975, 1978).] The nature of these intermediates is still unknown, and thus far the assignments of valence state to the metal centres have been largely based on optical data (Chance et al., 1975; Chance & Leigh, 1977; Clore & Chance, 1978a, 1979).

Optical difference spectra of intermediates I and III minus fully reduced cytochrome oxidase have been obtained in the α-band and near-i.r. regions by means of a low-temperature freeze-trapping technique (Chance et al., 1975, 1978; Chance & Leigh, 1977). However, these difference spectra were obtained at only two time points in the reaction and are not pure in that they represent a mixture of intermediates in which the predominant components are intermediates I and III respectively (Clore & Chance, 1978a, 1979).

Preliminary studies by Chance et al. (1975) indicated that both low-spin ferric haem and Cu₅²⁺ e.p.r. signals could be detected in a trapped sample of intermediate III at approx. 50% of the intensity seen in fully oxidized cytochrome oxidase. However, interpretation of these observations is difficult for a number of reasons. (1) Mitochondrial samples containing small amounts of cytochrome oxidase (<10 μM) were used, so that quantitative measurement of the signals is extremely difficult and large errors are likely owing to very poor signal-to-noise ratios, which also lead to the necessary use of saturating powers. In addition, there is considerable overlap of the e.p.r. signals of other components of the respiratory chain. (2) The e.p.r. spectra were not analysed quantitatively by double integration taking into account the transition probability for field-swept spectra in accord with the result obtained by Aasa & Vannård (1975). (3) As in the case of the optical spectra discussed above, it is highly likely that the sample these authors prepared represented a mixture of intermediates rather than a pure preparation of intermediate III.

A particularly useful feature of e.p.r. is that it can be used for the quantitative determination of the concentration of a paramagnetic species through comparison with a standard of known e.p.r. properties such as copper perchlorate. This is because it is easy to calculate the transition probability. Thus, in order to obtain further insight into the nature of the low-temperature intermediates in the reaction of fully reduced cytochrome oxidase with O₂, we have undertaken a detailed quantitative study of the kinetics of the e.p.r. changes occurring during the reaction and correlated these with the optical changes over the entire 500–700 nm spectral range.

**Experimental**

**Enzyme and chemicals**

Cytochrome oxidase was prepared from bovine heart mitochondria by the method of Van Buuren (1972), with an extra dialysis step to remove all cholate and (NH₄)₂SO₄. The purified enzyme was stored in liquid N₂ in 0.1 M-potassium phosphate buffer, pH 7.4, containing 0.5% Tween 80. The molecular activity of the enzyme at infinite cytochrome c (Sigma horse heart type VI) concentration determined by the method of Vanneste et al. (1974) was 25–50 s⁻¹ in 0.1 M-potassium phosphate buffer, pH 7.4, containing 0.5% Tween 80. The ratios \( A^{\text{red}}_{460} / A^{\text{ox}}_{455} \) and \( A^{\text{red}}_{444} / A^{\text{ox}}_{444} \) were 5.2 and 2.5 respectively, indicating an optically clear solution with very little non-reducible haem a (Van Buuren, 1972).

The concentration of cytochrome oxidase is expressed in terms of a functional unit containing two haem groups and calculated from \( e_{\text{ox}}^{\text{red}} = 24.0 \text{mM}^{-1} \text{cm}^{-1} \) (Van Gelder, 1963). E.p.r. measurements under non-saturating conditions at 77 K showed that extraneous copper correspond to <10% of the total e.p.r.-detectable copper content. The amounts of haem and intrinsic copper detectable by e.p.r. were respectively 1.0 and 0.92 mol/mol of cytochrome oxidase (see below for quantitative interpretation of e.p.r. signals), which are approximately the same as those found by other workers (Hartzell & Beinert, 1974; Aasa et al., 1976a). It has been shown that a number of preparations contain extraneous reducing equivalents, so that the oxidized enzyme may undergo autoreduction in the presence of CO (Greenwood et al., 1974) or by the action of high-intensity monochromatic light from a laser beam (Adar & Yonetani, 1978). A measure of the purity of our enzyme is that under the conditions described by Greenwood et al. (1974) the oxidized enzyme failed to autoreduce to the mixed-valence state–CO complex in an atmosphere of CO at 0.1 MPa (1 atm) in the absence of O₂ over a period of 24 h as monitored by optical spectroscopy. This indicates that, if extraneous reducing equivalents are present in our preparation, their concentration must be very small.

All chemicals used were analytical grade.
Sample preparation

For the optical studies, 15 μM-cytochrome oxidase was dissolved in a medium containing 30% (v/v) ethylene glycol, 50 mM-sodium phosphate buffer, pH 7.4, and 2.4 μM-phenazine methosulphate. For the e.p.r. studies, the concentrations of cytochrome oxidase and phenazine methosulphate were 160 and 4 μM respectively. It should be noted that the maintenance of pH at low temperatures depends on the protein itself (Williams-Smith et al., 1977). The solutions were made anaerobic by successive evacuation and flushing at least ten times with N₂ (<0.0001% impurities) that had been passed through three columns in series containing acidic solutions of vanadium sulphate (Meites & Meites, 1948). Then 0.45 mm- and 0.9 mm-NADH in the case of the optical and e.p.r. samples respectively were added and the reduction was allowed to proceed for a minimum of 1 h under a continuous N₂ stream. The N₂ stream was then replaced by a CO stream at 0.1 MPa for a further 1 h with occasional agitation to ensure full anaerobiosis and CO saturation. The concentration of CO in the CO-saturated samples was 1.2 mm.

A sample (200 μl) of the reduced CO-saturated enzyme solution was then injected anaerobically into a 3 mm-inner-diameter quartz e.p.r. tube previously de-aerated with CO and cooled at 250 K. A solution (100 μl) of O₂ in 30% (v/v) ethylene glycol in 50 mM-sodium phosphate buffer, pH 7.4 (containing 2 mM-O₂ at 250 K; Clore & Chance, 1978a), was introduced in the dark at 250 K, the two solutions were rapidly mixed (<5 s) and the e.p.r. tube was then transferred to a solid CO₂/ethanol bath at 195 K, resulting in the formation of a homogeneous powder sample. This procedure prevents ligand exchange between O₂ and the fully reduced cytochrome oxidase–CO complex (Chance et al., 1975; Clore & Chance, 1978a). The e.p.r. tubes were then stored in the dark in liquid N₂ until used.

The fully reduced cytochrome oxidase–CO complex was photolyzed at 77 K (a temperature at which neither O₂ nor CO react with the oxidase; Chance et al., 1975; Clore & Chance, 1978a,b; Denis & Clore, 1979) by using 10–20 flashes from a 10 J xenon-flash lamp (model 610 B; Photochemical Research Associates, London, Ont., Canada), with a pulse width of 3 μs, to ensure 100% photolysis (as determined when no further absorbance changes could be produced). The reaction with O₂ was activated by placing the e.p.r. tube for a given time in isopentane equilibrated at 173 K and then stopped by replacing the e.p.r. tube back into liquid N₂ at 77 K. The time taken for the temperature of the sample to equilibrate first with the isopentane at 173 K and then with liquid N₂ was determined by placing a copper/constantan thermocouple in an e.p.r. tube containing water and found to be about 2 s. This process was then repeated to obtain spectra at successive time points in the reaction with the same sample. The temperature of the isopentane was maintained at 173 K by equilibrating isopentane contained inside a plastic jacket through which N₂ gas was bubbled with liquid N₂ outside. By regulating the N₂ gas flow the temperature could be maintained within ±0.5°C. Finally, to obtain a control solution of fully oxidized enzyme from the same sample, the sample was warmed to 273 K, additional O₂ stirred in to remove excess reductant, and the mixture left for 30 s and then frozen at 77 K.

The key features and advantages of this technique are as follows: (1) it allows one to obtain optical and e.p.r. spectra under identical conditions so that a direct comparison between the two sets of data can be made; (2) as the optical spectra are recorded at 77 K there are no time limits on the acquisition of the data, so that high-quality spectra with narrow bandwidths and long scanning times may be obtained while still retaining sufficient time resolution to allow kinetic analysis of the data.

The temperature of 173 K was chosen because we found that at this temperature the reaction proceeded at a rate optimal for the time resolution of our technique (i.e. a minimum of 10 s between successive time points).

Optical spectra

Optical spectra were recorded with a Johnson Foundation DBS-2 dual-wavelength spectrophotometer (Chance & Graham, 1971) equipped with two 250 mm-focal-length Bausch and Lomb monochromators (600 lines/mm). The reference and measuring beams were interlaced by a mechanical light-chopper, operating at 50 Hz. The 100 Hz signal was amplified, peak-detected and phase-demodulated from phase-adjustable gates derived from the line frequency. The transmitted light was monitored with a multi-alkali photomultiplier for the 350–700 nm range (EMI 9592 B). The electrical output from the photomultiplier was coupled to an A/D converter and an 8-bit 1024-address digital memory (Varian C-1204) in which the characteristics of the baseline (i.e. reference spectrum plus instrumental characteristics) were stored and from which corrective signals to the measuring wavelengths were read out, the stored baseline being subtracted from the incoming data. The baseline (e.g. fully reduced cytochrome oxidase) was obtained by measuring the difference in absorption between the measuring beam and the reference beam fixed at a single wavelength in terms of the dynode voltage necessary to give a null output of the phase-sensitive demodulator over the spectral range. The fixed reference wavelength employed here was 575 nm in the visible region (500–700 nm).
The e.p.r. tubes were held by clips on a 'cold finger' sample holder maintained at 77K by liquid N\textsubscript{2}, and a stream of dry N\textsubscript{2} was used to prevent condensation on the surface of the dewar and photomultiplier.

Several modifications and precautions are required to obtain high-quality optical spectra from samples in e.p.r. tubes. (1) Because e.p.r. tubes are cylindrical it is necessary to use a very narrow beam of light focused at the centre of the tube to ensure that the beam is normal to the incident surface of the tube and to minimize light-scattering. (2) Because powder samples are used, a considerable amount of light is lost by scattering unless care is taken to reflect the scattered light back on to the photomultiplier. This was achieved by placing the tube in the focus of a parabolic reflector and resulted in a several-fold (3–5-fold) improvement in the signal-to-noise ratio. (3) Because our technique involves repeated warming and cooling in the 77–173K range, it is necessary to ensure that the crystalline state of the sample remains optically identical throughout. It was found that samples prepared as glasses [prepared by using 50\% (v/v) ethylene glycol and slow cooling], despite their better optical properties initially (i.e., decreased light-scattering and increased transmittance), tended to crack and become opaque after two or three warming and cooling cycles. The powder samples, however, retained identical scattering properties, as checked by using samples of fully oxidized cytochrome oxidase in 30\% (v/v) ethylene glycol. (4) E.p.r. tubes are not optically identical at all points over their circumference. Consequently, if spectral distortions are to be avoided, the e.p.r. tube must always be placed in the same position as it was in when the baseline spectrum was recorded. To this end, the e.p.r. tubes were marked so that the correct position could be easily ascertained. When different e.p.r. tubes are used for the stored reference spectrum and the other spectra [e.g., when ‘absolute’ spectra are recorded with the spectrum of frozen 30\% (v/v) ethylene glycol being used as the reference], it is necessary to determine the position of the second tube that is optically identical with that of the first tube used to record the reference spectrum. This is achieved by recording a reference spectrum of frozen 30\% (v/v) ethylene glycol (which freezes as a homogeneous white powder when frozen initially at 195K in a solid CO\textsubscript{2}/ethanol bath), and then rotating the second e.p.r. tube, also containing frozen 30\% (v/v) ethylene glycol, until a position is reached at which the difference spectrum is flat and horizontal. The position is then marked on the tube.

The quality of the spectra obtained by using the above technique is illustrated in Fig. 1, where the spectra of reduced cytochrome c recorded in e.p.r. tubes at 77K as a glass with a bandwidth of 1nm and as a powder with a bandwidth of 5nm are compared with a room-temperature (298K) spectrum obtained in a 1cm-optical-pathlength cell with a bandwidth of 0.2nm. Despite the fact that the bandwidth used for the powder sample at 77K is 25 times that used for the solution at 298K, the resolution of the absorption bands is as good in the powder spectrum owing to their considerable narrowing at low temperatures (cf. glass spectrum).

All low-temperature optical spectra recorded on cytochrome oxidase were obtained with a bandwidth of 5nm at a scanning 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\textsubscript{2}) with a Varian E-3 spectrometer and at temperatures between 5 and 80K in a Varian E-9

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**Fig. 1. Absolute spectra of reduced cytochrome c solution frozen as a glass (a) and as a powder (b) recorded at 77K, together with a spectrum recorded at room temperature (298K) (c)**

Reduction of cytochrome c was carried out by the addition of trace amounts of Na\textsubscript{2}S\textsubscript{2}O\textsubscript{4}. Samples (a) and (b) were recorded in e.p.r. tubes (3mm internal diameter) with bandwidths of 1 and 5nm respectively by using a Johnson Foundation DBS-2 spectrophotometer as described in the Experimental section. Sample (c) was recorded in a flat 10mm-pathlength cell with a bandwidth of 0.2nm in a Beckman Acta M IV spectrophotometer. The concentrations of cytochrome c were (a) 16\textmu{}M, (b) 1\textmu{}M and (c) 16\textmu{}M, as calculated from $e_{\text{red}}^{\text{cyto}} = 21.1 \text{mm}^{-1}\text{cm}^{-1}$ (Margoliash, 1957). Note the large enhancement in absorption of the bands in sample (b) due to scattering (Butler & Norris, 1960).
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 extent of photolysis of the concentrated samples used for e.p.r. was checked by using glass samples prepared with 50% (v/v) ethylene glycol. Identical results were obtained with powder samples containing 30% (v/v) ethylene glycol.

Integration of e.p.r. spectra

When the magnetic field is swept rather than the frequency, the total intensity of an e.p.r. powder spectrum is proportional to the average intensity factor $g_p^{av}$ given by the approximate equation:

$$g_p^{av} = \frac{2}{3} \left\{ \frac{g_x^2 + g_y^2 + g_z^2}{3} \right\}$$

$$+ \frac{1}{3} \left( g_x + g_y + g_z \right)$$

which gives the correct value for $g_p^{av}$ within 1.5% in the range $1 < g_x/g_y < 8$ and within 0.7% for $1 < g_x/g_y < 2$, taking $g$-values in the order $g_x < g_y < g_z$ (Aasa & Vännegård, 1975).

The double integral of an experimental first derivative spectrum divided by $g_p^{av}$ gives a quantity that is proportional to the number of spins in the sample, so that the concentration ($c_s$) of a paramagnetic species is given by:

$$c_s = \frac{I_s}{g_p^{av}, r} \frac{g_p^{av}, s}{I_r} c_r$$

where $I_s$ and $I_r$ are the total intensities of the e.p.r. spectra of the paramagnetic sample and reference standard (e.g. copper perchlorate) respectively, $g_p^{av}, s$ and $g_p^{av}, r$ are the average intensity factors of the paramagnetic sample and reference standard respectively and $c_r$ is the concentration of the reference standard.

In the case of the Cu$_A^{2+}$ e.p.r. signal ($g = 2.18$, 2.03, 1.99), the entire spectrum can be integrated because at the powers used the intensity of the partially overlapping signal at $g = 2.21$ due to low-spin ferric haem is negligible. However, in the case of the low-spin ferric haem signal ($g = 3.03$, 2.21 and 1.45) this cannot be done owing (1) to overlap of the $g = 2.21$ peak with the Cu$_A^{2+}$ signals and (2) to the very small amplitude and large linewidth of the $g = 1.45$ peak making quantitative measurement difficult. However, in the case of $S' = 1/2$ systems, the area (i.e. first integral) under an 'absorption' peak in the first derivative experimental spectrum is proportional to the total intensity provided that this peak is sufficiently far away from the other two peaks. This is the case for the $g = 3.03$ peak of the low-spin ferric haem spectrum. The concentration of low-spin ferric haem species, $c_s$, is then obtained from the relation:

$$c_s = c_r \frac{T_{obs}, \Delta g^{av}, r}{T} \frac{g_p^{av}, s}{I_r} \frac{\Delta g}{\Delta^2}$$

where $c_r$ is the concentration of the Cu standard, $g_p^{av}, r$ is the average intensity factor of the Cu standard given by eqn. (2), $I_r$ is the double integral of the first derivative spectrum of the Cu standard determined from the relation $I_r = \sum y_f^2$ with the amplitudes $y_f^2$ in the first derivative spectrum taken at points with equidistant spacing $\Delta r$, $T_{obs}$ is the observed area under the isolated $g_s$ peak by $T_{obs} = \sum y_f^2$ where $y_f^2$ values are the amplitudes observed at points with equidistant spacing $\Delta s$, and $T$ is the calculated area under the isolated $g_s$ peak for a spectrum with no hyperfine structure given by the relation:

$$T = \frac{\mu_B}{h \nu} \frac{g_x^2 + g_y^2}{2(1 - \rho_3)(1 - \rho_3)}$$

where $\rho_3 = g_3/g_2^2$, $\mu_B$ is the Bohr magneton, $h$ is Planck's constant and $\nu$ is the microwave frequency.

All e.p.r. spectra used for integration were recorded under non-saturating conditions. The e.p.r. spectra were digitized by using an automatic x-y reader coupled to a minicomputer (Nova 3; Data General Corporation). Double integration of the Cu$_A^{2+}$ signal was performed with a step length of 50μT and single integration of the isolated $g = 3.03$ peak of low-spin ferric haem with a step length of 25μT.

Kinetic analysis of the data

The optical spectra recorded at successive time points were digitized by using an automatic computer-controlled x-y reader coupled to the minicomputer, and the data at selected wavelength pairs were used for kinetic analysis together with the total integrated intensities of the Cu$_A^{2+}$ and low-spin ferric haem e.p.r. signals. The overall standard error of the data determined by the method of Clore & Chance (1978a) and given by the weighted mean of the standard errors of the individual progress curves was $2.5 \pm 0.2\%$.

The technique of numerical integration of simultaneous non-linear stiff ordinary differential equations and non-linear optimization were exactly as described previously (Clore & Chance, 1978a,b,c, 1979) and performed on an IBM-360 computer (University College London Computer Centre).

Our choice of models is based on the criteria developed by Clore & Chance (1978a,b,c, 1979), which consist of the following triple requirement: an
within the standard error of the data, good
determination of the optimized parameters (as
measured by the standard deviation of the natural
logarithm of the optimized parameter, s.d.\textsubscript{m}) and a
random distribution of residuals. Thus, for a given
set of data, it must be emphasized that, although
there may be many models with an s.d. within the
standard error of the data, models with too many
degrees of freedom will fail such an analysis because
of underdetermination, whereas models with too few
degrees of freedom will fail such an analysis as a
result of the introduction of systematic errors in the
distribution of residuals.

The crude computed changes in absorbance (for
the optical data) or intensity (for the e.p.r. data)
\((W_i)\) in units of concentration are given by:

\[
W_i(t) = \sum_i F_i(t)\alpha_i(t)
\]  

where \(F_i(t)\) is the concentration of the \(i\)th inter-
mEDIATE at time \(t\) of a given kinetic scheme obtained
by numerical integration of the coupled simulta-
neous ordinary differential equations representing
that scheme, and \(\alpha_i(t)\) is the relative contribution
of the \(i\)th intermediate to the \(i\)th progress curve. The
\(\alpha_i(t)\) values are defined relative to two reference
species \(x\) and \(z\) by the relation:

\[
\alpha_i(t) = \frac{\alpha_i(t) - \alpha_i(z)}{\alpha_i(x) - \alpha_i(z)}
\]  

where \(\alpha_i(t)\), \(\alpha_i(x)\) and \(\alpha_i(z)\) are molar absorption
coefficients at the \(i\)th wavelength or molar intensity
coefficients at the \(i\)th e.p.r. signal of species \(i\), \(x\) and
\(z\) respectively.

All the absorbance changes have been digitzed
and normalized with respect to the difference in
absorbance between fully reduced minus fully
oxidized cytochrome oxidase \([\alpha_i(\text{red.}) - \alpha_i(\text{ox.})]\), so
that from eqn. (7) the relative contributions of fully
reduced and fully oxidized cytochrome oxidase are
set equal to 1 and 0 respectively. All the e.p.r. intensity
changes have been digitized and normalized with
respect to the difference in intensity between
fully oxidized minus fully reduced cytochrome
oxidase \([\alpha_i(\text{ox.}) - \alpha_i(\text{red.})]\), so that from eqn. (7)
the relative contributions of fully oxidized and fully
reduced cytochrome oxidase are set equal to 1 and 0
respectively. \(W_i(t)\) is normalized by dividing eqn. (6)
by the total concentration of cytochrome oxidase.

Computation of ‘true’ difference and absolute
spectra of the optical species

When the rate constants governing the reaction
have been determined, the ‘true’ difference spectra of
the optical species (i.e. in 100% concentration) can
be obtained by the solution of a set of linear simul-
taneous equations of the form:

\[
\Delta A_i(t) = \sum_{l=1}^{m} \sum_{i=1}^{n} F_i(t) \Delta \epsilon_i(l-r)
\]  

for each wavelength \(i\), where \(\Delta A_i(t)\) is the observed
difference in absorbance at the \(i\)th wavelength
between the reaction and reference samples at time \(t,
F_i(t)\) is the computed concentration of the \(i\)th inter-
mEDIATE determined from the optimized values of the
rate constants, and \(\Delta \epsilon_i(l-r)\) is the molar difference
absorption coefficient at the \(i\)th wavelength between
the \(i\)th optical species and the reference sample
obtained by solution of eqn. (8). The ‘true’ absolute
spectrum of the optical species is then obtained by
adding the true difference spectrum of the optical
species minus the reference sample to the absolute
spectrum of the reference sample (in our case fully
reduced cytochrome oxidase).

Results

Optical spectra

Typical optical difference spectra (reaction sam-
ple minus fully reduced cytochrome oxidase) in the
visible region following the reaction of fully reduced
cytochrome oxidase with O$_2$ at 173 K are shown in
Fig. 2. A difference spectrum of fully oxidized minus
fully reduced cytochrome oxidase is also shown for
comparison. Three optically distinct species may be
distinguished. The first is characterized by a peak
around 590 nm and a trough around 610 nm (20 s
spectrum), the second by peaks around 595 and
614 nm and a small trough around 605 nm (60 s to 400 s
spectra), and the third by a small peak around
580 nm and a large trough at 604 nm (500 s to 2000 s
spectra). It should be noted that at most 10% of
the 655 nm band, which is characteristic of fully
oxidized cytochrome oxidase (Beinert et al., 1976)
and thought to be indicative of anti-ferromagnetic
coupling between high-spin cytochrome $a_3^{3+}$ and
Cu$_n^{2+}$ (Palmer et al., 1976), is seen in the spectra
from 500 to 2000 s. It should also be noted that the
difference spectra at 20 s and 2000 s are qualita-
tively similar to those of compounds $A_1$ and $B$
described by Chance et al. (1975) and Denis &
Clore (1979).

The kinetics of the absorbance changes at 590–
630, 604–630 and 614–630 nm are shown in Fig. 5
and are discussed in detail below. It should be noted
that the choice of reference wavelength is in part
arbitrary because, although 630 nm is the isosbestic
point for the spectra of fully reduced and fully
oxidized cytochrome oxidase, it is not necessarily an
isosbestic point for the intermediates. However,
because the absorbance changes in the 625–635 nm
region are very small (<2%) compared with the
absorbance changes at 590, 604 and 614 nm, as determined from the absolute spectra at the different time points, 630 nm can be used as a reference wavelength.

**E.p.r. spectra**

The e.p.r. difference spectra (reaction sample minus fully reduced cytochrome oxidase) at 20, 200 and 2000 s after initiation of the reaction of fully reduced cytochrome oxidase with O\textsubscript{2} at 173 K are shown in Fig. 3. The e.p.r. spectrum of fully reduced cytochrome oxidase and the e.p.r. difference spectrum of fully oxidized minus fully reduced cytochrome oxidase are also shown. The small copper signal around g = 2 in the fully reduced sample is due to non-reducible extraneous copper and amounts to less than 10% of the total detectable copper in the fully oxidized enzyme.

A number of interesting features may be noticed. (1) The only e.p.r. signals detected are those attributable to low-spin cytochrome a\textsuperscript{3+} (g = 3.03, 2.21, 1.45) and Cu\textsubscript{A}\textsuperscript{2+} (g = 2.18, 2.03, 1.99). No high-spin haem signals around g = 6 are seen. No shift in the g-values of low-spin cytochrome a\textsuperscript{3+} or Cu\textsubscript{A}\textsuperscript{2+} can be seen compared with those of fully oxidized cytochrome oxidase. (2) Virtually no change is observed in the 20 s e.p.r. spectrum despite the large optical changes seen in Fig. 2 and attributable to species I. (3) The ratios of the amplitude of the g = 3 'absorption' peak of low-spin cytochrome a\textsuperscript{3+} to the amplitude of the g = 2 Cu\textsubscript{A}\textsuperscript{2+} signal are much smaller at 200 and 2000 s than in fully oxidized cytochrome oxidase. At 2000 s the total intensities of low-spin cytochrome a\textsuperscript{3+} and Cu\textsubscript{A}\textsuperscript{2+} are 40 and 90% respectively of those seen in fully oxidized cytochrome oxidase. (4) The lineshape of the Cu\textsubscript{A}\textsuperscript{2+} signal remains unchanged throughout the course of the reaction. Further, the lineshape of the Cu\textsubscript{A}\textsuperscript{2+} signal remains constant over the 10–80 K range, in agreement with the findings obtained by Greenaway et al. (1977). (5) The linewidth at half height of the g = 3 'absorption' peak at 10 K remains at
Fig. 3. E.p.r. difference spectra (sample minus fully reduced cytochrome oxidase) at 20s, 200s and 2000s after initiation of the reaction of fully reduced cytochrome oxidase with O₂ at 173 K, and of fully oxidized cytochrome oxidase

The conditions of e.p.r. spectroscopy were: (a) microwave power = 2 mW, microwave frequency = 9.127 GHz, modulation amplitude = 2 mT, temperature = 10 K, scanning time = 500 mT/min, time constant = 0.1 s; (b) as in (a) but scanning rate = 25 mT/min; time constant = 0.3 s and 8-fold higher gain; (c) microwave power = 20 mW, frequency = 9.172 GHz, modulation amplitude = 2 mT, temperature = 77 K, scanning rate = 50 mT/min, time constant = 0.3 s. Experimental conditions were: 106 μM-cytochrome oxidase, 2.67 μM-phenazine methosulphate, 0.6 mM-NADH, 50 mM-sodium phosphate buffer, pH 7.4, 30% (v/v) ethylene glycol, 0.8 mM-CO and 0.67 mM-O₂. An e.p.r. spectrum of fully reduced cytochrome oxidase is also shown; the only signal seen is a small signal around g = 2, which is due to non-reducible extraneous copper; this signal has been subtracted from the other spectra.

Fig. 4. Temperature-dependence of the total intensities (I) of the low-spin cytochrome a³⁺ (a) and Cu₄²⁺ (b) e.p.r. signals over the 5–80 K range at 200s and 2000s after initiation of the fully reduced cytochrome oxidase–O₂ reaction at 173 K, and of fully oxidized cytochrome oxidase

The intensity function \( I_I/T/\sqrt{P_P G_G} \) (where \( I_I \) is the intensity, \( T \) the absolute temperature, \( P_P \) the power and \( G_G \) the gain) is temperature-independent when the paramagnetic species obeys Curie's law. The values of \( I_I/T/\sqrt{P_P G_G} \) have been normalized with respect to the values at 20 K. The conditions of e.p.r. spectroscopy were: frequency 9.12 GHz; microwave power, variable but non-saturating; modulation amplitude 0.2 mT, scanning rate 20 mT/min for low-spin ferric haem and 40 mT/min for Cu₄²⁺, time constant = 0.3 s. The experimental conditions were as given in Fig. 3 legend.

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Fig. 5. Observed and computed kinetics of the reaction of fully reduced cytochrome oxidase with $O_2$ at 173K as measured by optical and e.p.r. spectroscopy

Symbols: $\Delta$, 590–630 nm; $\bullet$, 604–630 nm; $\Box$, 614–630 nm; $\bullet$, Cu$_{a}^{2+}$ e.p.r. signal; $\nabla$, low-spin cytochrome $a^{3+}$ e.p.r. signal. Theoretical curves, obtained by using the optimized values of the rate constants and relative contributions of the intermediates for Scheme 3C (Fig. 6) given in Table 3, are shown as continuous lines. The computed kinetics of the individual intermediates are shown in (b) and (d). The initial conditions are: 10$\mu$M fully reduced cytochrome oxidase and 0.67mM $O_2$ for the optical data (a and b); 106$\mu$M fully reduced cytochrome oxidase and 0.67mM $O_2$ for the e.p.r. data (c and d). The overall S.D. of the fit is $2.3\%$; the standard error of the data is $2.5\%$. The value of the mean absolute correlation index, $\overline{C}$, is 0.70, indicating a random distribution of residuals. [For $\overline{C} < 1.0$, the distribution of residuals is random; for $\overline{C} > 1.0$, the deviations between calculated and observed values are systematic; see Clore & Chance (1978a) for the formula of $\overline{C}$.] The absorbance changes are digitized relative to the difference in absorbance between fully reduced minus fully oxidized cytochrome oxidase normalized to 1.0; the e.p.r. intensity changes (computed as described in the Experimental section) are digitized relative to the difference in intensity between fully oxidized minus fully reduced cytochrome oxidase normalized to 1.0. The experimental conditions for the optical and e.p.r. data are given in Figs. 2 and 3 respectively. The conditions for e.p.r. spectroscopy were: modulation amplitude = 2mT; for low-spin cytochrome $a^{3+}$ signal: frequency = 9.127GHz, microwave power = 2mW, temperature = 10K, scanning rate = 25mT/min and time constant = 0.3s; for the Cu$_{a}^{2+}$ signal: frequency = 9.172GHz, microwave power = 20mW, temperature = 77K, scanning rate = 50mT/min and time constant = 0.3s.
5.0 ± 0.2 mT throughout the reaction of fully reduced cytochrome oxidase with O$_2$ at 173 K, but is broadened by approx. 10% in fully oxidized cytochrome oxidase. Both the low-spin cytochrome a$^{3+}$ and Cu$^{2+}_A$ signals are found to obey Curie’s law in samples taken over the entire course of the fully reduced cytochrome oxidase–O$_2$ reaction at 173 K and in fully oxidized cytochrome oxidase over the 5–80 K range (Fig. 4). A further feature is that the new e.p.r. signals at $g = 5$, 1.78 and 1.69 appearing within less than 5 ms on reoxidation of fully reduced cytochrome oxidase with O$_2$ at room temperature.

In all the schemes with the exception of Scheme 1, the relative contribution of a given intermediate to an e.p.r. signal, with respect to the difference in intensity of that signal between fully oxidized and fully reduced cytochrome oxidases, is set equal to 1, if the intermediate contains the paramagnetic species giving rise to the signal, and to 0 if it does not. The contributions of fully reduced cytochrome oxidase to the Cu$^{2+}_A$ and the low-spin ferric haem signals are set to 0, as are the contributions of intermediate I, on account of the lag phase seen in the kinetics of both Cu$^{2+}_A$ and low-spin cytochrome a$^{3+}$. No assumptions on the relative contributions of the intermediates at the monitored wavelength pairs (590–630, 604–630 and 614–630 nm) are made in any of the schemes. Unknown optimized parameters include rate constants and contributions of the different intermediates at the wavelength pairs monitored in the optical studies (see above). In the case of Scheme 1, the set of unknown parameters also includes contributions of intermediates II and III to the Cu$^{2+}_A$ and low-spin ferric haem e.p.r. signals.
temperature (Shaw et al., 1978b) are not seen at any stage of the reaction of fully reduced cytochrome oxidase with O$_2$ at 173 K.

The total intensities (obtained as described in the Experimental section) of the low-spin cytochrome $a^{3+}$ and CuA$_{2+}$ signals (normalized to 1.0 for the difference in intensity between fully oxidized minus fully reduced cytochrome oxidase) are plotted as a function of time in Fig. 5. Thus the only two differences that can be detected in the e.p.r. spectra seen during the fully reduced cytochrome oxidase–O$_2$ reaction at 173 K and that of fully oxidized cytochrome oxidase are (1) a large significant difference in the ratio of the intensities of the low-spin cytochrome $a^{3+}$ to CuA$_{2+}$ signals and (2) a small but significant difference in the linewidth at half-height of the $g = 3$ 'absorption' peak of low-spin cytochrome $a^{3+}$.

**Kinetic analysis of the optical and e.p.r. data**

The kinetics of the absorbance changes at 590–630, 604–630 and 614–630nm and of the intensity changes of the Cu$_{2+}$ and low-spin cytochrome $a^{3+}$ e.p.r. signals are shown in Fig. 5. Both for the optical and e.p.r. data three distinct phases are seen, namely the time intervals 0–40, 40–250 and 250–2000s. At 250s the intensities of the Cu$_{2+}$ and low-spin cytochrome $a^{3+}$ e.p.r. signals are 66 and 40% respectively of their intensities in fully oxidized cytochrome oxidase. At 2000s the corresponding intensities are 90 and 42% respectively.

Conceptually, from the purely kinetic stand-point, the simplest scheme required to fit the data in Fig. 5 is the three-intermediate sequential mechanism of Clore & Chance (1978a, 1979) given by Scheme 1 (Fig. 6). The s.d. of the fit is 2.3% (Table 1) and the distribution of residuals is random ($\bar{C} < 1.0$). However, in order to fit the e.p.r. data the concentrations of Cu$_{2+}$ and low-spin cytochrome $a^{3+}$ in intermediates II and III must be smaller than those in fully oxidized cytochrome oxidase (i.e. they must correspond to less than 1mol/mol of cytochrome oxidase). The optimized values, s.D. and 95% confidence limits of the rate constants and relative contributions of the intermediates at each progress curve with respect to the difference in absorbance or intensity between fully reduced and fully oxidized cytochrome oxidase are given in Table 2.

Experimentally we find the following. (a) Both Cu$_{2+}$ and cytochrome $a^{3+}$ are magnetically isolated in partially reduced and fully oxidized cytochrome oxidase, in agreement with magnetic-susceptibility studies over the 1.5–200K range (Tweedle et al., 1978; Moss et al., 1978). (b) Both the CuA$_{2+}$ and

<table>
<thead>
<tr>
<th>Scheme</th>
<th>1</th>
<th>2A</th>
<th>2B</th>
<th>3A</th>
<th>3B</th>
<th>3C</th>
<th>4</th>
</tr>
</thead>
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<tr>
<td>S.D. (%)</td>
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<td>4.0</td>
<td>5.9</td>
<td>4.1</td>
<td>3.3</td>
<td>2.3</td>
<td>3.8</td>
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</table>

**Table 1. Overall standard deviations of the fits for the schemes given in Fig. 6**

The overall standard error of the data is 2.5 ± 0.2% with a 99% confidence interval of 2.0–3.0%. [See Clore & Chance (1978a) for the method of calculation of the S.D.]

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dimensions</th>
<th>Optimized value</th>
<th>s.D. (%)</th>
<th>Confidence limit 5%</th>
<th>Confidence limit 95%</th>
</tr>
</thead>
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<td>$k_{+1}$</td>
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<td>&gt;10</td>
<td>45.3</td>
<td>174</td>
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<tr>
<td>$k_{-1}$</td>
<td>s$^{-1}$</td>
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<td>0.00703</td>
<td>0.0225</td>
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</tr>
<tr>
<td>$k_{+2}$</td>
<td>s$^{-1}$</td>
<td>0.00208</td>
<td>0.00177</td>
<td>0.00244</td>
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</tr>
<tr>
<td>$k_{+3}$</td>
<td>s$^{-1}$</td>
<td>1.64</td>
<td>0.130</td>
<td>1.33</td>
<td>2.04</td>
</tr>
<tr>
<td>$\alpha_{2}^{390-630}$ (I)</td>
<td>s$^{-1}$</td>
<td>0.912</td>
<td>0.0263</td>
<td>1.05</td>
<td>1.14</td>
</tr>
<tr>
<td>$\alpha_{2}^{390-630}$ (II)</td>
<td>s$^{-1}$</td>
<td>0.956</td>
<td>0.0155</td>
<td>0.932</td>
<td>0.981</td>
</tr>
<tr>
<td>$\alpha_{2}^{390-630}$ (III)</td>
<td>s$^{-1}$</td>
<td>0.610</td>
<td>0.0217</td>
<td>0.588</td>
<td>0.632</td>
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<td>$\alpha_{2}^{604-630}$ (I)</td>
<td>s$^{-1}$</td>
<td>0.824</td>
<td>0.0785</td>
<td>0.724</td>
<td>0.938</td>
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<td>$\alpha_{2}^{604-630}$ (II)</td>
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<td>1.82</td>
<td>1.93</td>
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<tr>
<td>$\alpha_{2}^{604-630}$ (III)</td>
<td>s$^{-1}$</td>
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<td>0.0219</td>
<td>1.04</td>
<td>1.12</td>
</tr>
<tr>
<td>$\alpha_{Cu}^{(II)}$ mol of Cu$_{2+}$/mol of $aa_3^*$</td>
<td>s$^{-1}$</td>
<td>0.563</td>
<td>0.0426</td>
<td>0.525</td>
<td>0.604</td>
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<tr>
<td>$\alpha_{Cu}^{(III)}$ mol of Cu$_{2+}$/mol of $aa_3^*$</td>
<td>s$^{-1}$</td>
<td>0.875</td>
<td>0.0169</td>
<td>0.851</td>
<td>0.899</td>
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<tr>
<td>$\alpha_{a}^{(II)}$ mol of low-spin $a^{3+}$/mol of $aa_3^*$</td>
<td>s$^{-1}$</td>
<td>0.408</td>
<td>0.0351</td>
<td>0.386</td>
<td>0.433</td>
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<tr>
<td>$\alpha_{a}^{(III)}$ mol of low-spin $a^{3+}$/mol of $aa_3^*$</td>
<td>s$^{-1}$</td>
<td>0.408</td>
<td>0.0169</td>
<td>0.397</td>
<td>0.420</td>
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</table>

* $aa_3$ is used to represent cytochrome oxidase (the functional unit of which contains two haem and two copper atoms).
low-spin cytochrome $a^{3+}$ e.p.r. signals, observed over the course of the fully reduced cytochrome oxidase–O$_2$ reaction at 173 K, have the same g-values as in fully oxidized cytochrome oxidase (Fig. 3). (c) Both the Cu$_A$$a^{3+}$ and low-spin cytochrome $a^{3+}$ e.p.r. signals obey Curie’s law over the 5–80 K range in samples taken over the course of the fully reduced cytochrome oxidase–O$_2$ reaction at 173 K (Fig. 4). (d) The linewidth at half height of the $g = 3$ ‘absorption’ peak of low-spin cytochrome $a^{3+}$ seen during the course of the fully reduced cytochrome oxidase–O$_2$ reaction at 173 K is approx. 10% narrower than that of fully oxidized cytochrome oxidase (Fig. 3). (e) The shape of the Cu$_A$$a^{2+}$ e.p.r. signal observed over the course of the fully reduced cytochrome oxidase–O$_2$ reaction at 173 K is identical with that of fully oxidized cytochrome oxidase (Fig. 3).

These five observations exclude all known mechanisms (namely ferromagnetic, anti-ferromagnetic and dipole–dipole coupling) whereby the total integrated intensity of the e.p.r. signal produced by a given concentration of a paramagnetic species may be diminished.

Therefore, if Scheme 1 is to be compatible with the e.p.r. data, species II and III must each be viewed as a mixture of a minimum of three species in very rapid equilibrium. In effect, four species could be in rapid equilibrium of the type:

$$
\begin{align*}
\text{Cu}_A^{a^{2+}} & \leftrightarrow \text{Cu}_A^{a^{3+}} \\
[\text{Cu}_B a_2O_2]^{a^{2+}} & \leftrightarrow [\text{Cu}_B a_2O_2]^{a^{3+}} \\
[\text{Cu}_B a_2O_2]^{a^{2+}} & \leftrightarrow [\text{Cu}_B a_2O_2]^{a^{3+}} \\
\text{Cu}_A^{a^{2+}} & \leftrightarrow \text{Cu}_A^{a^{3+}}
\end{align*}
$$

(9)

The distribution of the four species and therefore the individual equilibrium constants would then be different for species II and III. Such a situation, however, seems highly unlikely on account of observation (c), which would require either (i) that equilibrium was fast and the equilibrium constants for eqn. (9) remained temperature-independent over the 5–80 K range, or (ii) that the activation energies of the rate constants for eqn. (9) were so large that at 173 K the rate constants would be large (i.e. $> 10^3$ s$^{-1}$) and equilibration fast, and below 80 K very small (i.e. $< 10^{-2}$ s$^{-1}$), so that on rapid freezing of the reaction sample in liquid N$_2$ the equilibrium would essentially be ‘frozen’ in its state at 173 K. Both these requirements are so improbable that Scheme 1 can be discounted.

The only scheme that accounts for both the optical and e.p.r. data without the need for any unreasonable assumptions, has an s.d. within the standard error of the data (i.e. less than 2.5%), a random distribution of residuals ($\bar{C} < 1.0$) and good determination of the optimized parameters is Scheme 3C (Fig. 6). The only alternative schemes (Schemes 2A, 2B, 3A, 3B and 4), involving a similar number of parameters, are given in Fig. 6, and these fail to fit the data on the basis that their s.d. values are significantly greater than the standard error of the data (see Table 1).

In the case of Scheme 3C, the concentrations of Cu$_A$$a^{2+}$ and low-spin cytochrome $a^{3+}$ at time $t$ are given by:

$$
\begin{align*}
\text{Cu}_A^{a^{2+}}(t) &= [\text{IIB}(t) + \text{III}(t)] \\
\text{Cu}_A^{a^{3+}}(t) &= [\text{IIA}(t) + \text{III}(t)]
\end{align*}
$$

(10)

(11)

where IIA, IIB and III are the concentrations of intermediates IIA, IIB and III at time $t$ respectively. The normalized absorbance change ($N_i$) at the $i$th wavelength pair (normalized with respect to the difference in absorbance between fully reduced and fully oxidized cytochrome oxidase) at time $t$ is given by:

$$
N_i(t) = [E(t) + \alpha'_1(t) - I(t) + \alpha'_3(\text{IIA}, \text{IIB})] - IIA(t) + \alpha'_3(\text{IIA}, \text{IIB}) - \text{III}(t) - E_0
$$

(12)

where $E$ is the concentration of fully reduced cytochrome oxidase, $E_0$ the total concentration of cytochrome oxidase and $\alpha'_i(t)$ the relative contribution of the $i$th intermediate at the $i$th wavelength pair defined by eqn. (7) with the reference species $x$ and $z$ taken as fully reduced and fully oxidized cytochrome oxidase respectively. However, in Scheme 3C the relation:

$$
\text{IIA}(t) = [\text{IIA}(t) + \text{III}(t)] \cdot \kappa_{+1}/k_{+2}
$$

(13)

always holds, so that eqn. (12) is reduced to:

$$
N_i(t) = [E(t) + \alpha'_1(t) - I(t) + \alpha'_3(\text{IIA}, \text{IIB}) - IIA(t)] - [\alpha'_3(\text{IIA}, \text{IIB}) - \text{III}(t)]/E_0
$$

(14)

where

$$
\alpha'_3(\text{IIA}, \text{IIB}) = \alpha'_3(\text{IIA}) + \alpha'_3(\text{IIB}) \cdot k_{+3}/k_{+2}
$$

(15)

$$
\alpha'_3(\text{III}, \text{IIB}) = \alpha'_3(\text{III}) + \alpha'_3(\text{IIB}) \cdot k_{+3}/k_{+2}
$$

(16)

As a result, it is impossible to determine $\alpha'_3(\text{IIA})$, $\alpha'_3(\text{IIB})$ and $\alpha'_3(\text{III})$ individually; we can only determine $\alpha'_3(\text{IIA}, \text{IIB})$ and $\alpha'_3(\text{III}, \text{IIB})$. The optimized values s.d. in and 5–95% confidence limits of the rate constants, and $\alpha'_1(t), \alpha'_3(\text{IIA}, \text{IIB})$ and $\alpha'_3(\text{III}, \text{IIB})$ at 590–630, 604–630 and 614–630 nm, are given in Table 3. It should be noted that the number of parameters describing Scheme 3C is 14 compared with 17 for Scheme 1, and that these are considerably better determined in Scheme 3C (Table 3) than in Scheme 1 (Table 2).
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Table 3. Values of the optimized parameters together with their s.D.$_{\text{im}}$ values and confidence limits for Scheme 3C

$\alpha_I'(I)$, $\alpha_I''(IIA,IIB)$ and $\alpha_I''(III,IIB)$ are defined by eqns. (7), (15) and (16) respectively with the reference species $x$ and $z$ being taken as fully reduced and fully oxidized cytochrome oxidase respectively.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dimensions</th>
<th>Optimized value</th>
<th>s.D.$_{\text{im}}$</th>
<th>Confidence limit 5%</th>
<th>Confidence limit 95%</th>
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<tr>
<td>$k_{+1}$</td>
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<td>0.151</td>
<td>0.00312</td>
<td>0.00513</td>
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<td>$k_{+4}$</td>
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</tr>
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<td>$\alpha_{590-630}(I)$</td>
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<td>0.0259</td>
<td>1.64</td>
<td>1.79</td>
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<tr>
<td>$\alpha_{590-630}(IIA,IIB)$</td>
<td></td>
<td>2.59</td>
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<tr>
<td>$\alpha_{590-630}(III,IIB)$</td>
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<td>1.24</td>
<td>0.0568</td>
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<td>1.36</td>
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<tr>
<td>$\alpha_{604-630}(I)$</td>
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<td>0.913</td>
<td>0.0257</td>
<td>0.887</td>
<td>0.940</td>
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<tr>
<td>$\alpha_{604-630}(IIA,IIB)$</td>
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<td>2.27</td>
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<tr>
<td>$\alpha_{604-630}(III,IIB)$</td>
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<td>$\alpha_{514-630}(I)$</td>
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<td>$\alpha_{514-630}(IIA,IIB)$</td>
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<td>$\alpha_{514-630}(III,IIB)$</td>
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<td>2.22</td>
<td>0.0443</td>
<td>2.08</td>
<td>2.36</td>
</tr>
</tbody>
</table>

Fig. 7. Computed difference (a) and absolute (b) spectra obtained during the cytochrome c oxidase--$O_2$ reaction at 173 K

The difference spectra of the CO complex of fully reduced cytochrome oxidase, intermediate I and fully oxidized enzyme were obtained as described in the Experimental section, with fully reduced oxidase as the reference. The intensity of the experimentally obtained difference spectrum of intermediate I was corrected with the help of its calculated concentration after 20 s (see Fig. 5) to correspond to the same concentration as that of the enzyme–CO complex and fully oxidized enzyme. The absolute spectra in (b) were obtained by adding the difference spectra in (a) to an absolute spectrum of fully reduced cytochrome oxidase, shown in (b). recorded with a frozen solution of ethylene glycol (30%, v/v) in water as the reference. The experimental conditions were the same as given in Fig. 2 legend.
Computed difference and absolute spectra of intermediate I

Unfortunately, as a consequence of eqn. (13) we cannot use eqn. (8) to calculate the difference spectra of intermediates IIA, IIB and III minus fully reduced cytochrome oxidase (E) individually. However, we can use eqn. (8) to calculate the difference spectrum I minus E with \([I] = [E]\). This is shown in Fig. 7 together with the difference spectra of the fully reduced cytochrome oxidase–CO complex and fully oxidized cytochrome oxidase minus fully reduced cytochrome oxidase. The corresponding absolute spectra are also shown. The optical properties of intermediate I are very similar to those of the enzyme–CO complex with slight but significant differences in the positions of the peaks (547 and 593 nm against 547 and 589 nm for the enzyme–CO complex) and the trough (608 and 612 nm respectively) in the 500–700 nm region of the difference spectrum. The absorption band around 590 nm is also considerably more intense with intermediate I compared with that with the enzyme–CO complex.

Discussion

The experimental results and kinetic analysis presented in the Results section demonstrate that with isolated cytochrome oxidase the reaction of the fully reduced enzyme with \(O_2\) displays three distinct kinetic phases, as shown previously with membrane-bound cytochrome oxidase (Clore & Chance, 1978a, 1979). The e.p.r. results allow the unambiguous assignment of the valence states of cytochrome \(a\) and Cu in the intermediates and require a modification of the previously suggested reaction scheme, which corresponds to Scheme I in Fig. 6. In particular, the e.p.r. data show that in the second reaction phase cytochrome \(a\) as well as Cu transfers electrons to other sites, but the transfers are not complete as neither component becomes fully re-oxidized. This suggests that there are two parallel reactions in the second phase and that intermediate II is really a mixture of two intermediates, IIA and IIB. These considerations are incorporated into reaction Scheme 3C (Fig. 6), and it has already been shown that this is the only one of the schemes tested that can satisfactorily account for both the optical and the e.p.r. data.

Unfortunately neither the optical nor the e.p.r. spectra provide any direct information on the valence states of cytochrome \(a\) and Cu in the intermediates. As cytochrome \(a\) and Cu constitute a coupled binuclear metal centre (Falk et al., 1977; Tweedle et al., 1978), and as \(O_2\) is known to react with reduced cytochrome \(a\), it may be convenient to consider cytochrome \(a\), Cu and \(O_2\) as a single unit, \([Cu_{a3}O_2]\). Although at present it appears impossible to specify the exact electron distribution among the components of this unit, we can assign its overall charge for each intermediate, as the total charge on [cytochrome oxidase metal centres plus \(O_2\)] must always be equal to +6. Bearing this in mind, we have summarized the mechanism of the reaction of fully reduced cytochrome oxidase with \(O_2\) at 173 K in Fig. 8. It should be noted that the formulae given are intended to show electron distributions only and do not imply steric structures. Thus we are not excluding that \(O_2\) itself may form a bridging ligand between Cu and cytochrome \(a\). On the other hand, investigations with simpler inorganic complexes suggest that electron redistributions within binuclear centres are rapid, and that two-electron donation to \(O_2\) from such a centre may occur even if the \(O_2\) molecule is co-ordinated end-on to one of the metals alone (J. Halpern, personal communication). If \(O_2\) forms a bridging ligand, this would exclude the proposal by Palmer and his colleagues (Palmer et al., 1976; Babcock et al., 1976, 1978; Tweedle et al., 1978) that imidazole serves this function. This hypothesis, however, is made unlikely, as model compounds (Kolks & Lippard, 1977) suggest that bridging imidazole cannot result in the strong exchange coupling observed with cytochrome oxidase \((J \leq -200 \text{ cm}^{-1})\).

![Fig. 8. Scheme for the reaction of fully reduced cytochrome oxidase with \(O_2\) at 173 K derived from Scheme 3C (Fig. 6)](image-url)
The first step in the reaction between fully reduced cytochrome oxidase and $O_2$ involves the binding of $O_2$ to the cytochrome $a_1$ moiety of the $[a_1C_u]_n$ couple. E.p.r. shows that no transfer of electrons from either cytochrome $a$ or $C_u$ to the $[Cu_u a_1]$ unit occurs, so that the charge on $[Cu_u a_1]_2+O_2$ remains at $+3$. The nature of the cytochrome $a_1-O_2$ bond in intermediate I has been discussed at length by Clore & Chance (1979). On the basis of a comparison of its absorption spectrum with those of model haem compounds, it is suggested to be best represented by the configuration $a_1^{2+}O_2^{-6}$, in which the charge localized on the iron of cytochrome $a_1$ is greater than $+2.5$ ($\delta > 0.5$). In view of the considerations just given, a more correct formulation should probably involve $Cu_u$ as well, in which case the configuration can be given as $Cu_u^{1+}a_1^{2+}O_2^{-6}$, with ($\delta_1^+ + \delta_2^\circ\approx 1$). Such a delocalization of electrons from the metal centres to $O_2$ would be consistent with the absorption spectrum of intermediate I (Fig. 7), which has similar features to that of oxymyoglobin (Antonini & Brunori, 1971).

It may be noted that the $[Cu_u a_1]_2+O_2$ unit in intermediate I contains an even number of electrons, which can readily account for the absence of an e.p.r. signal in this state. The lack of e.p.r. absorption is expected even if there are delocalizations of the type suggested, as there can always be couplings between potentially paramagnetic centres (e.g. $a_1^{3+}O_2^-$.).

Intermediate I is converted into either intermediate IIA or intermediate IIB by a one-electron reduction of the $[Cu_u a_1]_2+O_2$ unit to the $[Cu_u a_1]_2+O_2$ unit, coupled to the oxidation of cytochrome $a_1^{2+}$ to cytochrome $a_1^{3+}$ in the case of intermediate IIA and to oxidation of $Cu_u^{1+}$ to $Cu_u^{2+}$ in the case of intermediate IIB. It should be noted that electron transfer between cytochrome $a$ and $Cu_u$ in intermediates IIA and IIB is insignificant at 173 K: if a rate constant for the conversion of intermediate IIA into IIB (or vice versa) is introduced into Scheme 3C, its optimized value is very small ($<10^{-4}$ s$^{-1}$) and very poorly determined ($s.D. e^{10} > 10$).

No matter what exact electron configuration we assign to the $[Cu_u a_1]_2+O_2$ unit in intermediates IIA and IIB, this unit must contain at least one unpaired electron. Formally $O_2$ is in the same oxidation state as in the paramagnetic oxygen intermediates described for laccase (Aasa et al., 1976b,c). In contrast with the cytochrome oxidase intermediates IIA and IIB, the laccase intermediate gives a specific e.p.r. signal associated with an oxygen radical. It is quite difficult to detect however, and couplings within the $[Cu_u a_1]_2+O_2$ unit may very well produce a spectrum so broad that it easily accounts for our failure to detect any new e.p.r. signals.

Intermediate IIB is a stable end product at 173 K. Intermediate IIA, on the other hand, is converted into intermediate III by a one-electron reduction of $[Cu_u a_1]_2+O_2$ to $[Cu_u a_1]_2+O_2$ coupled to the oxidation of $Cu_u^{1+}$ to $Cu_u^{2+}$. Formally the $[Cu_u a_1]_2+O_2$ unit has the same valence as fully oxidized oxidase and two molecules of water. The absence of a 655 nm band (Fig. 2) shows, however, that intermediate III is not identical with the oxidized enzyme and thus represents a true intermediate in the reaction.

One possible explanation of the different reactionalities of intermediates IIA and IIB would be that electrons from $Cu_u$ and cytochrome $a$ enter the $[Cu_u a_1]_2+O_2$ unit at different sites and that their site of entry governs the resulting configuration of the $[Cu_u a_1]_2+O_2$ unit. It has, however, already been pointed out that electron redistributions within the unit might be expected to be rapid. Thus another possibility would be that rapid electron donation from cytochrome $a_2^{2+}$ to the $[Cu_u a_1]_2+O_2$ unit occurs only when $Cu_u$ is reduced. It should be noted that starting with the fully reduced enzyme may represent an artificial condition compared with the redox states found during turnover. In the catalytic reaction, with excess ferrocytochrome $c$ and $O_2$ present, one might expect the initial reaction of $O_2$ with the $[Cu_u a_1]_2$ unit to involve molecules having cytochrome $a$ and $Cu_u$ oxidized. Further considerations of the relevance of the observations described here to our understanding of the catalytic mechanism of oxygen reduction in cytochrome oxidase are, however, deferred to the accompanying paper (Clore et al., 1980). This gives a characterization of the intermediates in the reaction of $O_2$ with the enzyme in the so-called mixed-valence state, in which cytochrome $a$ and $Cu_u$ are initially oxidized.

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