Biochem. J. (1980) **185**, 139–154
Printed in Great Britain

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 MALMSTRÖM†

**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*

(*Received* 23 *April* 1979)

The reaction of fully reduced soluble bovine heart cytochrome oxidase with O_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_A^{2+}. Quantitative analysis of e.p.r. signals shows that, at the end point of the reaction at 173 K, nearly 100% of Cu_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 into 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_A in the intermediates. Intermediate I contains cytochrome a^{2+} and Cu_A^{+}, intermediate IIA contains low-spin cytochrome a^{3+} and Cu_A^{+}, intermediate IIB contains cytochrome a^{2+} and Cu_A^{2+}, and intermediate III contains low-spin cytochrome a^{3+} and Cu_A^{2+}. The electronic state of the O_2-binding Cu_Ba_3 couple during the reoxidation of cytochrome oxidase is discussed in terms of an integrated structure containing Cu_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.*, 1976*a*), 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_B, forming a spin-coupled dimer of $S=2$ and J (exchange coupling constant) $\leqslant -200\,cm^{-1}$ in the fully oxidized enzyme; in the fully oxidized enzyme neither cytochrome a_3^{3+} nor Cu_B^{2+} 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^{3+} are seen. No e.p.r. signals attributable to Cu_B^{2+} have as yet been observed in any state of

Abbreviations used: m.c.d., magnetic circular dichroism; $S.D._{ln}$, standard deviation of the natural logarithm of an unknown parameter.

0306-3275/80/010139-16 $1.50/1

the enzyme (Aasa *et al.*, 1976*a*; Shaw *et al.*, 1978*a*; 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 O_2 (Clore & Chance, 1978*a*, 1979; Clore, 1979):

$$E + O_2 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} I \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} II \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} III \quad (1)$$

[The notation is that of Clore & Chance (1978*a*, 1979); intermediates I and III are equivalent to compounds A_1 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, 1978*a*, 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, 1978*a*, 1979).

Preliminary studies by Chance *et al.* (1975) indicated that both low-spin ferric haem and Cu_A^{2+} 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 ($< 10\,\mu$M) 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 & Vänngård (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 O_2, 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 $(NH_4)_2SO_4$. The purified enzyme was stored in liquid N_2 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 $A_{603}^{red.}/A_{485}^{red.}$ and $A_{444}^{red.}/A_{424}^{red.}$ 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 $\varepsilon_{red.-ox.}^{605} = 24.0\,mM^{-1}\cdot cm^{-1}$ (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.*, 1976*a*). 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 O_2 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 N_2 (<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 N_2 stream. The N_2 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-internal-diameter quartz e.p.r. tube previously de-aerated with CO and cooled at 250 K. A solution (100 μl) of O_2 in 30% (v/v) ethylene glycol in 50 mM-sodium phosphate buffer, pH 7.4 (containing 2 mM-O_2 at 250 K; Clore & Chance, 1978*a*), 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 CO_2/ethanol bath at 195 K, resulting in the formation of a homogeneous powder sample. This procedure prevents ligand exchange between O_2 and the fully reduced cytochrome oxidase–CO complex (Chance *et al.*, 1975; Clore & Chance, 1978*a*). The e.p.r. tubes were then stored in the dark in liquid N_2 until used.

The fully reduced cytochrome oxidase–CO complex was photolysed at 77 K (a temperature at which neither O_2 nor CO react with the oxidase; Chance *et al.*, 1975; Clore & Chance, 1978*a*,*b*; Denis & Clore, 1979) by using 10–20 flashes from a 10 J xenon-flash lamp (model 610B; 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 O_2 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 N_2 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 N_2 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 N_2 gas was bubbled with liquid N_2 outside. By regulating the N_2 gas flow the temperature could be maintained within $\pm$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 O_2 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_2, and a stream of dry N_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 severalfold (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_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).

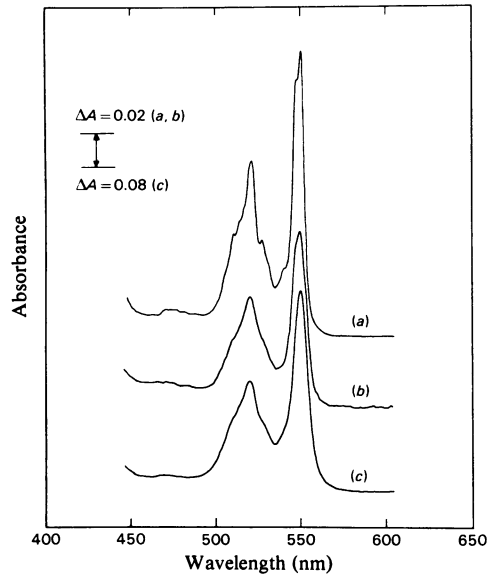


Fig. 1. *Absolute spectra of reduced cytochrome c solution frozen as a glass (a) and as a powder (b) recorded at 77K, together with a spectrum recorded at room temperature (298K) (c)*

Reduction of cytochrome *c* was carried out by the addition of trace amounts of $Na_2S_2O_4$. 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*) 16μM, (*b*) 1μM and (*c*) 16μM, as calculated from $\varepsilon^{550}_{red.-ox.} = 21.1\,mM^{-1}\cdot cm^{-1}$ (Margoliash, 1957). Note the large enhancement in absorption of the bands in sample (*b*) due to scattering (Butler & Norris, 1960).

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_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 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:

$$g_P^{av.} \approx \frac{2}{3}\{(g_x^2+g_y^2+g_z^2)/3\}^{\frac{1}{2}} + \frac{1}{3}(g_x+g_y+g_z)/3 \qquad (2)$$

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

$$c_s = \frac{I_s}{g_P^{av.,s}} \cdot \frac{g_P^{av.,r}}{I_r} \cdot c_r \qquad (3)$$

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 $g_z = 3.03$ peak of the low-spin ferric haem spectrum. The concentration of low-spin ferric haem species, c_s, is then obtained from the relation:

$$c_s = c_r \frac{T_{obs.}}{T} \cdot \frac{g_P^{av.,r}}{I_r} \cdot \frac{\Delta_s}{\Delta_r^2} \qquad (4)$$

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 $I_r = \sum i y_i^r$ with the amplitudes y_i^r in the first derivative spectrum taken at points with equidistant spacing Δ_r, $T_{obs.}$ is the observed area under the isolated g_z peak given by $T_{obs.} = \sum y_i^s$ where y_i^s values are the amplitudes observed at points with equidistant spacing Δ_s, and T is the calculated area under the isolated g_z peak for a spectrum with no hyperfine structure given by the relation:

$$T = \frac{\mu_B}{h\nu} \cdot \frac{g_x^2+g_y^2}{2\{(1-\rho_x)(1-\rho_y)\}^{\frac{1}{2}}} \qquad (5)$$

where $\rho_i = g_i^2/g_z^2$, μ_B is the Bohr magneton, h is Planck's constant and ν 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 $50\,\mu T$ and single integration of the isolated $g = 3.03$ peak of low-spin ferric haem with a step length of $25\,\mu T$.

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 (1978*a*) 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, 1978*a*,*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 (1978*a*,*b*,*c*, 1979), which consist of the following triple requirement: an

S.D. 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, $S.D._{\ln}$) 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:

$$W_i(t) = \sum_l F_l(t)\alpha'_i(l) \qquad (6)$$

where $F_l(t)$ is the concentration of the lth intermediate at time t of a given kinetic scheme obtained by numerical integration of the coupled simultaneous ordinary differential equations representing that scheme, and $\alpha'_i(l)$ is the relative contribution of the lth intermediate to the ith progress curve. The $\alpha'_i(l)$ values are defined relative to two reference species x and z by the relation:

$$\alpha'_i(l) = \frac{\alpha_i(l)-\alpha_i(z)}{\alpha_i(x)-\alpha_i(z)} \qquad (7)$$

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

All the absorbance changes have been digitized and normalized with respect to the difference in absorbance between fully reduced minus fully oxidized cytochrome oxidase [α_i(red.)$-\alpha_i$(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 [α_i(ox.)$-\alpha_i$(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 simultaneous equations of the form:

$$\Delta A_i(t) = \sum_{l=1}^{m}\sum_{i=1}^{n} F_l(t)\,\Delta\varepsilon_i(l-r) \qquad (8)$$

for each wavelength i, where $\Delta A_i(t)$ is the observed difference in absorbance at the ith wavelength between the reaction and reference samples at time t, $F_l(t)$ is the computed concentration of the lth intermediate determined from the optimized values of the rate constants, and $\Delta\varepsilon_i(l-r)$ is the molar difference absorption coefficient at the ith wavelength between the lth 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 sample minus fully reduced cytochrome oxidase) in the visible region following the reaction of fully reduced cytochrome oxidase with O_2 at 173K 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 590nm and a trough around 610nm (20s spectrum), the second by peaks around 595 and 614nm and a small trough around 605nm (60s to 400s spectra), and the third by a small peak around 580nm and a large trough at 604nm (500s to 2000s spectra). It should be noted that at most 10% of the 655nm 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_B^{2+} (Palmer *et al.*, 1976), is seen in the spectra from 500 to 2000s. It should also be noted that the difference spectra at 20s and 2000s are qualitatively 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 614–630nm 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 630nm 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 625–635nm region are very small (<2%) compared with the

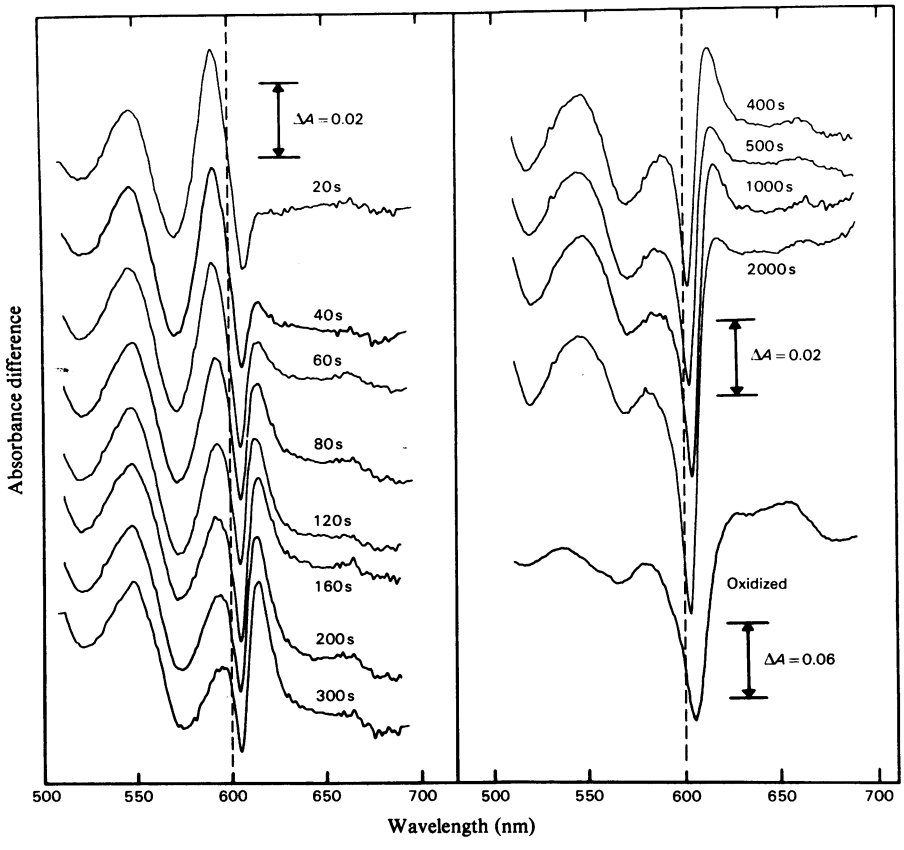


Fig. 2. *Optical difference spectra (reaction sample minus fully reduced cytochrome oxidase) in the visible region obtained at successive times in the reaction of fully reduced cytochrome oxidase with O_2 at 173 K*

Experimental conditions were: 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-CO and 0.67 mM-O_2. A difference spectrum of fully oxidized minus fully reduced cytochrome oxidase is also shown for comparison.

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_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^{3+} ($g = 3.03, 2.21, 1.45$) and Cu_A^{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^{3+} or Cu_A^{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^{3+} to the amplitude of the $g = 2$ Cu_A^{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^{3+} and Cu_A^{2+} are 40 and 90% respectively of those seen in fully oxidized cytochrome oxidase. (4) The lineshape of the Cu_A^{2+} signal remains unchanged throughout the course of the reaction. Further, the lineshape of the Cu_A^{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

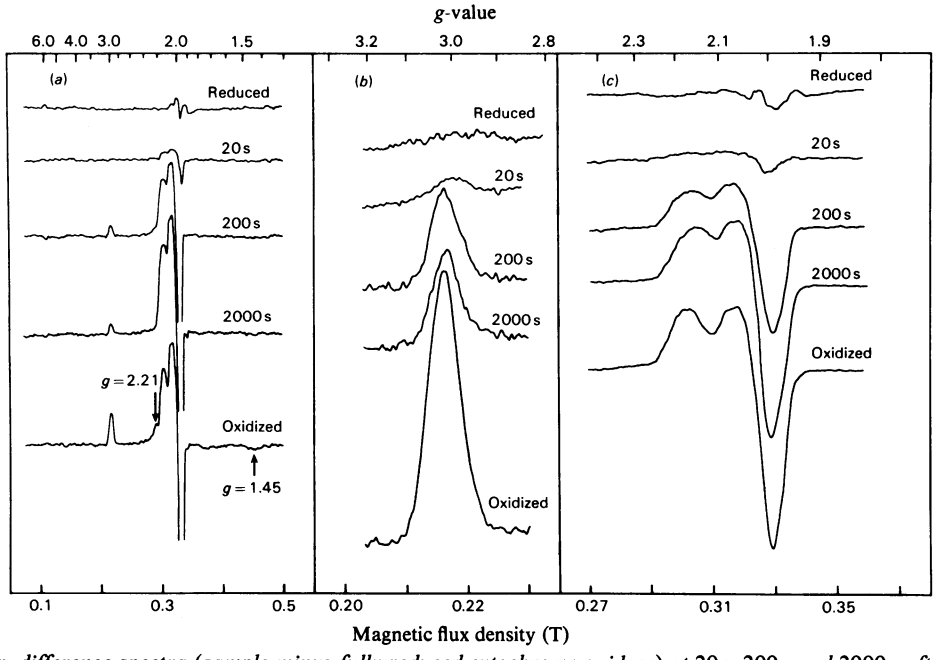


Fig. 3. *E.p.r. difference spectra (sample minus fully reduced cytochrome oxidase) at 20 s, 200 s and 2000 s after initiation of the reaction of fully reduced cytochrome oxidase with O_2 at 173 K, and of fully oxidized cytochrome oxidase*

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-O_2. 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.

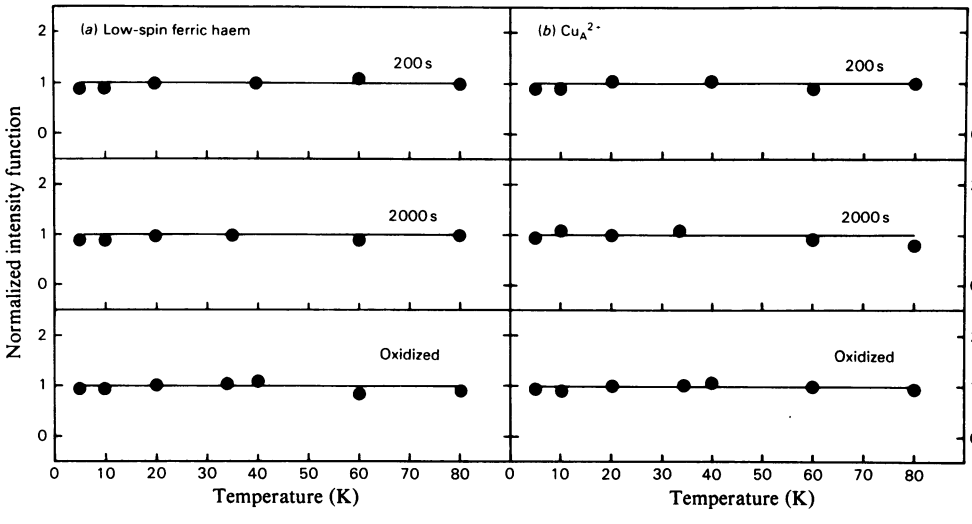


Fig. 4. *Temperature-dependence of the total intensities (I) of the low-spin cytochrome a^{3+} (a) and Cu_A^{2+} (b) e.p.r. signals over the 5–80 K range at 200 s and 2000 s after initiation of the fully reduced cytochrome oxidase–O_2 reaction at 173 K, and of fully oxidized cytochrome oxidase*

The intensity function $I_T T/\sqrt{P_T} G_T$ (where I_T is the intensity, T the absolute temperature, P_T the power and G_T the gain) is temperature-independent when the paramagnetic species obeys Curie's law. The values of $I_T T/\sqrt{P_T} G_T$ 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 Cu_A^{2+}, time constant = 0.3 s. The experimental conditions were as given in Fig. 3 legend.

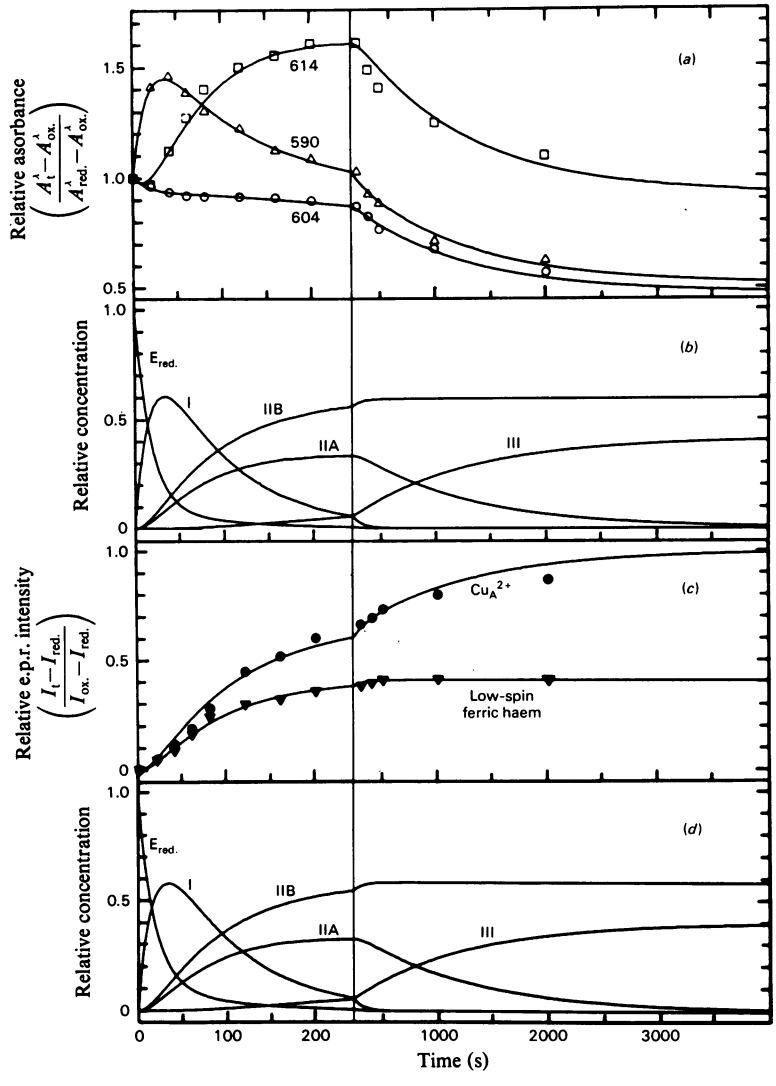
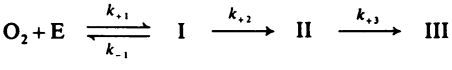


Fig. 5. *Observed and computed kinetics of the reaction of fully reduced cytochrome oxidase with O_2 at 173 K as measured by optical and e.p.r. spectroscopy*

Symbols: △, 590–630nm; ○, 604–630nm; □, 614–630nm; ●, Cu_A^{2+} e.p.r. signal; ▼, 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 μM fully reduced cytochrome oxidase and 0.67 mM-O_2 for the optical data (*a* and *b*); 106 μM fully reduced cytochrome oxidase and 0.67 mM-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 ± 0.2%. The value of the mean absolute correlation index, $\bar{C}$, is 0.70, indicating a random distribution of residuals. [For $\bar{C} < 1.0$, the distribution of residuals is random; for $\bar{C} \gg 1.0$, the deviations between calculated and observed values are systematic; see Clore & Chance (1978*a*) for the formula of $\bar{C}$.] 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 = 2 mT; for low-spin cytochrome a^{3+} signal: frequency = 9.127 GHz, microwave power = 2 mW, temperature = 10 K, scanning rate = 25 mT/min and time constant = 0.3 s; for the Cu_A^{2+} signal: frequency = 9.172 GHz, microwave power = 20 mW, temperature = 77 K, scanning rate = 50 mT/min and time constant = 0.3 s.

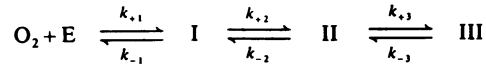
5.0 ± 0.2 mT throughout the reaction of fully reduced cytochrome oxidase with O_2 at 173 K, but is broadened by approx. 10% in fully oxidized cytochrome oxidase. (6) Both the low-spin cytochrome a^{3+} and Cu_A^{2+} signals are found to obey Curie's law in samples taken over the entire course of the fully reduced cytochrome oxidase–O_2 reaction at 173 K and in fully oxidized cytochrome oxidase over the 5–80 K range (Fig. 4). (7) 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

Scheme 1



Unknown parameters = 17

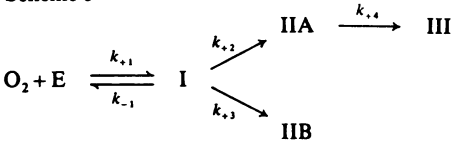
Scheme 2



	Alternatives	Intermediates II	Intermediates III
Paramagnetic species	A	$a^{3+}Cu_A^{2+}$	Cu_A^{2+}
	B	Cu_A^{2+}	$a^{3+}Cu_A^{2+}$

Unknown parameters = 15

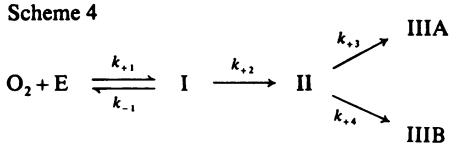
Scheme 3



	Alternatives	Intermediates IIA	Intermediates IIB	Intermediates III
Paramagnetic species	A	$a^{3+}Cu_A^{2+}$	Cu_A^{2+}	$a^{3+}Cu_A^{2+}$
	B	Cu_A^{2+}	$a^{3+}Cu_A^{2+}$	Cu_A^{2+}
	C	a^{3+}	Cu_A^{2+}	$a^{3}Cu_A^{2+}$

Unknown parameters = 14

Scheme 4



	Intermediates II	Intermediates IIIA	Intermediates IIIB
Paramagnetic species	$a^{3+}Cu_A^{2+}$	$a^{3+}Cu_A^{2+}$	Cu_A^{2+}

Unknown parameters = 14

Fig. 6. *Schemes for the reaction of fully reduced cytochrome c oxidase with O_2 at 173 K*

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_A^{2+} 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_A^{2+} 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_A^{2+} and low-spin ferric haem e.p.r. signals.

temperature (Shaw *et al.*, 1978*b*) are not seen at any stage of the reaction of fully reduced cytochrome oxidase with O_2 at 173K.

The total intensities (obtained as described in the Experimental section) of the low-spin cytochrome a^{3+} and Cu_A^{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 173K 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 Cu_A^{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_A^{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_A^{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 (1978*a*, 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 ($\overline{C} < 1.0$). However, in order to fit the e.p.r. data the concentrations of Cu_A^{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, $S.D._{ln}$ and 5–95% 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_A^{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 Cu_A^{2+} and

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 (1978*a*) for the method of calculation of the S.D.]

Scheme ...	1	2A	2B	3A	3B	3C	4
S.D. (%)	2.2	4.0	5.9	4.1	3.3	2.3	3.8

Table 2. *Values of the optimized parameters together with their* $S.D._{ln}$ *values and confidence limits for Scheme* 1

$\alpha_i'(l)$ is defined by eqn. (7) with the reference species x and z being taken as fully reduced and fully oxidized cytochrome oxidase respectively for the optical data and as fully oxidized and fully reduced cytochrome oxidase respectively for the e.p.r. data.

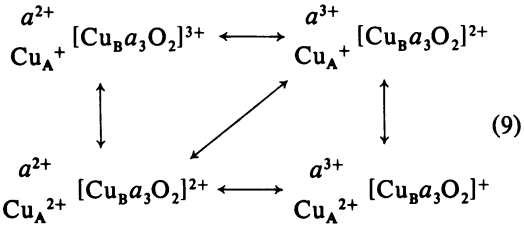
Parameter	Dimensions	Optimized value	$S.D._{ln}$	Confidence limit 5%	Confidence limit 95%
k_{+1}	$M^{-1} \cdot s^{-1}$	88.8	0.408	45.3	174
k_{-1}	s^{-1}	0.00016	>10		
k_{+2}	s^{-1}	0.0126	0.353	0.00703	0.0225
k_{+3}	s^{-1}	0.00208	0.0971	0.00177	0.00244
$\alpha'_{590-630}(I)$		1.64	0.130	1.33	2.04
$\alpha'_{590-630}(II)$		1.09	0.0263	1.05	1.14
$\alpha'_{590-630}(III)$		0.661	0.0285	0.630	0.692
$\alpha'_{604-630}(I)$		0.912	0.0350	0.861	0.966
$\alpha'_{604-630}(II)$		0.956	0.0155	0.932	0.981
$\alpha'_{604-630}(III)$		0.610	0.0217	0.588	0.632
$\alpha'_{614-630}(I)$		0.824	0.0785	0.724	0.938
$\alpha'_{614-630}(II)$		1.87	0.0190	1.82	1.93
$\alpha'_{614-630}(III)$		1.08	0.0219	1.04	1.12
$\alpha'_{Cu_A}(II)$ mol of Cu_A^{2+}/mol of aa_3*		0.563	0.0426	0.525	0.604
$\alpha'_{Cu_A}(III)$ mol of Cu_A^{2+}/mol of aa_3*		0.875	0.0169	0.851	0.899
$\alpha'_a(II)$ mol of low-spin a^{3+}/mol of aa_3*		0.408	0.0351	0.386	0.433
$\alpha'_a(III)$ mol of low-spin a^{3+}/mol of aa_3*		0.408	0.0169	0.397	0.420

* 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^{2+} 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^{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:



(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. $<10^{-5} s^{-1}$), 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^{2+} and low-spin cytochrome a^{3+} at time t are given by:

$$Cu_A^{2+}(t) = [IIB(t) + III(t)] \quad (10)$$

$$a^{3+}(t) = [IIA(t) + III(t)] \quad (11)$$

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 ith wavelength pair (normalized with respect to the difference in absorbance between fully reduced minus fully oxidized cytochrome oxidase) at time t is given by:

$$N_i(t) = [E(t) + \alpha_i'(I)\cdot I(t) + \alpha_i'(IIA)\cdot IIA(t) + \alpha_i'(IIB)\cdot IIB(t) + \alpha_i'(III)\cdot III(t)]/E_o \quad (12)$$

where E is the concentration of fully reduced cytochrome oxidase, E_o the total concentration of cytochrome oxidase and $\alpha_i'(l)$ the relative contribution of the lth intermediate at the ith 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:

$$IIB(t) = [III(t) + IIA(t)]\cdot k_{+3}/k_{+2} \quad (13)$$

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

$$N_i(t) = [E(t) + \alpha_i'(I)\cdot I(t) + \alpha_i'(IIA,IIB)\cdot IIA(t) + \alpha_i'(III,IIB)\cdot III(t)]/E_o \quad (14)$$

where

$$\alpha_i'(IIA,IIB) = \alpha_i'(IIA) + \alpha_i'(IIB)\cdot k_{+3}/k_{+2} \quad (15)$$

$$\alpha_i'(III,IIB) = \alpha_i'(III) + \alpha_i'(IIB)\cdot k_{+3}/k_{+2} \quad (16)$$

As a result, it is impossible to determine $\alpha_i'(IIA)$, $\alpha_i'(IIB)$ and $\alpha_i'(III)$ individually; we can only determine $\alpha_i'(IIA,IIB)$ and $\alpha_i'(III,IIB)$. The optimized values $S.D._{\ln}$ and 5–95% confidence limits of the rate constants, and $\alpha_i'(I)$, $\alpha_i'(IIA,IIB)$ and $\alpha_i'(IIB,III)$ 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).

Table 3. *Values of the optimized parameters together with their* S.D.$_{ln}$ *values and confidence limits for Scheme* 3*C*
α'_i(I), α'_i(IIA,IIB) and α'_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.

Parameter	Dimensions	Optimized value	S.D.$_{ln}$	Confidence limit 5%	Confidence limit 95%
k_{+1}	$M^{-1} \cdot s^{-1}$	80.9	0.122	65.8	99.5
k_{-1}	s^{-1}	0.00400	0.151	0.00312	0.00513
k_{+2}	s^{-1}	0.00561	0.0528	0.00515	0.00612
k_{+3}	s^{-1}	0.00805	0.0585	0.00731	0.00886
k_{+4}	s^{-1}	0.000988	0.0737	0.000875	0.00111
$\alpha'_{590-630}$(I)		1.71	0.0259	1.64	1.79
$\alpha'_{590-630}$(IIA,IIB)		2.59	0.0205	2.50	2.68
$\alpha'_{590-630}$(III,IIB)		1.24	0.0568	1.13	1.36
$\alpha'_{604-630}$(I)		0.913	0.0257	0.887	0.940
$\alpha'_{604-630}$(IIA,IIB)		2.27	0.0140	2.22	2.32
$\alpha'_{604-630}$(III,IIB)		1.14	0.0383	1.06	1.23
$\alpha'_{614-630}$(I)		0.865	0.0176	0.830	0.903
$\alpha'_{614-630}$(IIA,IIB)		4.29	0.0137	4.19	4.39
$\alpha'_{614-630}$(III,IIB)		2.22	0.0443	2.08	2.36

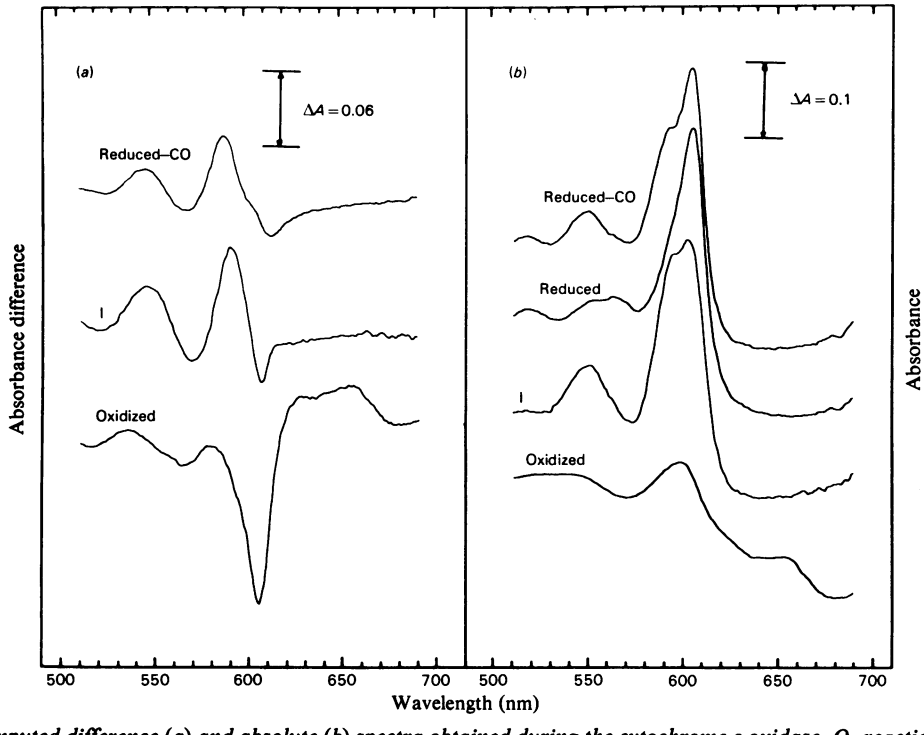


Fig. 7. *Computed difference* (*a*) *and absolute* (*b*) *spectra obtained during the cytochrome c oxidase–O_2 reaction at* 173 *K*
The difference spectra of the CO complex of fully reduced cytochrome oxidase, intermediate I and fully oxidized enzyme were obtained as described in the Experimental section, 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 593nm against 547 and 589nm for the enzyme–CO complex) and the trough (608 and 612nm respectively) in the 500–700nm region of the difference spectrum. The absorption band around 590nm 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, 1978*a*, 1979). The e.p.r. results allow the unambiguous assignment of the valence states of cytochrome *a* and Cu_A in the intermediates and require a modification of the previously suggested reaction scheme, which corresponds to Scheme 1 in Fig. 6. In particular, the e.p.r. data show that in the second reaction phase cytochrome *a* as well as Cu_A 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_3 and Cu_B in the intermediates. As cytochrome a_3 and Cu_B 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_3, it may be convenient to consider cytochrome a_3, Cu_B and O_2 as a single unit, $[Cu_Ba_3{\cdot}O_2]$. 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 173K 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_B and cytochrome a_3. 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 ($J \leqslant -200\,cm^{-1}$).

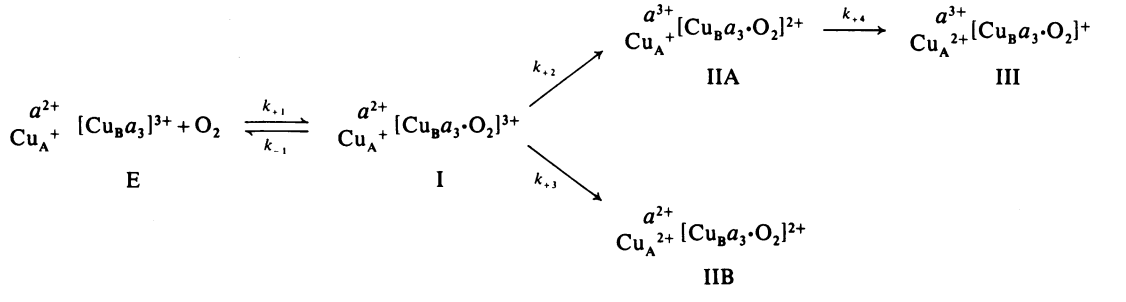


Fig. 8. *Scheme for the reaction of fully reduced cytochrome oxidase with O_2 at 173K derived from Scheme 3C (Fig. 6)*

The first step in the reaction between fully reduced cytochrome oxidase and O_2 involves the binding of O_2 to the cytochrome a_3 moiety of the $[a_3Cu_B]$ couple. E.p.r. shows that no transfer of electrons from either cytochrome a or Cu_A to the $[Cu_Ba_3]$ unit occurs, so that the charge on $[Cu_Ba_3{\cdot}O_2]$ remains at +3. The nature of the cytochrome a_3–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 $a_3^{2+\delta}$–$O_2^{-\delta}$, in which the charge localized on the iron of cytochrome a_3 is greater than +2.5 ($\delta > 0.5$). In view of the considerations just given, a more correct formulation should probably involve Cu_B as well, in which case the configuration can be given as $Cu_B^{1+\delta_1}a_3^{2+\delta_2}$–$O_2^{-(\delta_1+\delta_2)}$, with $(\delta_1+\delta_2)\approx 1$. 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 $[Cu_Ba_3{\cdot}O_2]^{3+}$ 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_3^{3+}–O_2^-).

Intermediate I is converted into either intermediate IIA or intermediate IIB by a one-electron reduction of the $[Cu_Ba_3{\cdot}O_2]^{3+}$ unit to the $[Cu_Ba_3{\cdot}O_2]^{2+}$ unit, coupled to the oxidation of cytochrome a^{2+} to cytochrome a^{3+} in the case of intermediate IIA and to oxidation of Cu_A^+ to Cu_A^{2+} in the case of intermediate IIB. It should be noted that electron transfer between cytochrome a and Cu_A 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 ($<10^{-6}\,s^{-1}$) and very poorly determined ($S.D._{\cdot\ln} \gg 10$).

No matter what exact electron configuration we assign to the $[Cu_Ba_3{\cdot}O_2]^{2+}$ 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.*, 1976*b*,*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 $[Cu_Ba_3{\cdot}O_2]^{2+}$ 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 $[Cu_Ba_3{\cdot}O_2]^{2+}$ to $[Cu_Ba_3{\cdot}O_2]^+$ coupled to the oxidation of Cu_A^+ to Cu_A^{2+}. Formally the $[Cu_Ba_3{\cdot}O_2]^+$ 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 reactivities of intermediates IIA and IIB would be that electrons from Cu_A and cytochrome a enter the $[Cu_Ba_3{\cdot}O_2]^{3+}$ unit at different sites and that their site of entry governs the resulting configuration of the $[Cu_Ba_3{\cdot}O_2]^{2+}$ 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+} to the $[Cu_Ba_3{\cdot}O_2]^{2+}$ unit occurs only when Cu_A 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 $[Cu_Ba_3]^{3+}$ unit to involve molecules having cytochrome a and Cu_A 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_A are initially oxidized.

We thank Miss A.-C. Carlsson and Mrs. B. Bejke for participation in the enzyme purification. The investigation was supported by a grant from Statens Naturvetenskapliga Forskningsråd. We are grateful to the University College London Computer Centre for computer facilities. G. M. C. acknowledges a short-term E.M.B.O. fellowship.

References

Aasa, R. & Vänngård, T. (1975) *J. Magn. Reson.* **19**, 308–315

Aasa, R., Albracht, S. P. J., Falk, K.-E., Lanne, B. & Vänngård, T. (1976*a*) *Biochim. Biophys. Acta* **422**, 260–272

Aasa, R. Brändén, R., Deinum, J., Malmström, B. G., Reinhammar, B. & Vänngård, T. (1976*b*) *FEBS Lett.* **61**, 115–119

Aasa, R., Brändén, R., Deinum, J., Malmström, B. G., Reinhammar, B. & Vänngård, T. (1976*c*) *Biochem. Biophys. Res. Commun.* **70**, 1204–1209

Adar, F. & Yonetani, T. (1978) *Biochim. Biophys. Acta* **502**, 30–36

Antonini, E. & Brunori, M. (1971) *Hemoglobin and Myoglobin in their Reactions with Ligands*, North-Holland, Amsterdam
Babcock, G. T., Vickery, L. E. & Palmer, G. (1976) *J. Biol. Chem.* **251**, 7907–7919
Babcock, G. T., Vickery, L. E. & Palmer, G. (1978) *J. Biol. Chem.* **253**, 2400–2411
Beinert, H., Hansen, R. E. & Hartzell, C. R. (1976) *Biochim. Biophys. Acta* **423**, 339–355
Butler, W. L. & Norris, K. H. (1960) *Arch. Biochem. Biophys.* **87**, 31–40
Chance, B. & Graham, N. (1971) *Rev. Sci. Instrum.* **42**, 941–945
Chance, B. & Leigh, J. S. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4777–4780
Chance, B., Saronio, C. & Leigh, J. S. (1975) *J. Biol. Chem.* **250**, 9226–9237
Chance, B., Saronio, C., Leigh, J. S., Ingledew, W. J. & King, T. E. (1978) *Biochem. J.* **171**, 787–798
Clore, G. M. (1979) in *Cytochrome Oxidase* (King, T. E., Orii, Y., Chance, B. & Okunuki, K., eds.), pp. 341–352, Elsevier/North-Holland, Amsterdam
Clore, G. M. & Chance, E. M. (1978*a*) *Biochem. J.* **173**, 799–810
Clore, G. M. & Chance, E. M. (1978*b*) *Biochem. J.* **173**, 811–820
Clore, G. M. & Chance, E. M. (1978*c*) *Biochem. J.* **175**, 709–725
Clore, G. M. & Chance, E. M. (1979) *Biochem. J.* **177**, 613–621
Clore, G. M., Andréasson, L.-E., Karlsson, B., Aasa, R. & Malmström, B. G. (1980) *Biochem. J.* **185**, 155–167
Denis, M. & Clore, G. M. (1979) *Biochim. Biophys. Acta* **545**, 483–495
Falk, K.-E., Vänngård, T. & Ångström, J. (1977) *FEBS Lett.* **75**, 23–27
Greenaway, F. T., Chan, S. H. P. & Vincow, G. (1977) *Biochim. Biophys. Acta* **490**, 62–78
Greenwood, C., Wilson, M. T. & Brunori, M. (1974) *Biochem. J.* **137**, 205–215
Hartzell, C. R. & Beinert, H. (1974) *Biochim. Biophys. Acta* **368**, 318–338
Kolks, G. & Lippard, S. J. (1977) *J. Am. Chem. Soc.* **99**, 5804–5806
Malmström, B. G. (1973) *Q. Rev. Biophys.* **6**, 389–431
Margoliash, E. (1957) *Biochem. Prep.* **5**, 23–29
Meites, L. & Meites, T. (1948) *Anal. Chem.* **20**, 984–985
Moss, T. H., Shapiro, E., King, T. E., Beinert, H. & Hartzell, C. R. (1978) *J. Biol. Chem.* **253**, 8072–8073
Palmer, G., Babcock, G. T. & Vickery, L. E. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2206–2210
Shaw, R. W., Hansen, R. E. & Beinert, H. (1978*a*) *Biochim. Biophys. Acta* **504**, 187–199
Shaw, R. W., Hansen, R. E. & Beinert, H. (1978*b*) *J. Biol. Chem.* **253**, 6637–6640
Thomson, A. J., Brittain, T., Greenwood, C. & Springall, J. P. (1976) *FEBS Lett.* **67**, 94–98
Thomson, A. J., Brittain, T., Greenwood, C. & Springall, J. P. (1977) *Biochem. J.* **165**, 327–336
Tweedle, M. F., Wilson, L. J., García-Iñiguez, L., Babcock, G. T. & Palmer, G. (1978) *J. Biol. Chem.* **253**, 8065–8071
Van Buuren, K. J. H. (1972) Ph.D. Thesis, University of Amsterdam (*Binding of Cyanide to Cytochrome aa_3*, Gerja, Waarland)
Van Gelder, B. F. (1963) *Biochim. Biophys. Acta* **73**, 663–665
Vanneste, V. W., Ysebaert-Vanneste, M. & Mason, H. S. (1974) *J. Biol. Chem.* **249**, 7390–7401
Williams-Smith, D. L., Bray, R. C., Barter, M. J., Tsoponakis, A. D. & Vincent, S. P. (1977) *Biochem. J.* **167**, 593–600