Biochem. J. (1979) **177**, 613–621
Printed in Great Britain

Low-Temperature Kinetics of the Reaction of Fully Reduced Membrane-Bound Cytochrome Oxidase with Oxygen in the Soret, α and Near-Infrared Regions

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(*Received* 14 *June* 1978)

The kinetics of the reaction of fully reduced membrane-bound cytochrome oxidase with O_2 obtained in the Soret, α and near-i.r. regions were analysed, and the contributions of the three intermediates of the reaction [Clore & Chance (1978) *Biochem. J.* **173**, 799–810] to seven wavelength pairs (430–463, 444–463, 590–630, 608–630, 740–940, 790–940 and 830–940 nm) were determined. The nature of the intermediates is discussed on the basis of the data in the present paper together with data in the literature from optical wavelength scanning, e.p.r., i.r. and magnetic-susceptibility studies.

The minimum functioning unit of cytochrome oxidase (EC 1.9.3.1) is thought to consist of two haems, a_3 and a, and two copper atoms (Caughey *et al.*, 1976). One copper atom, termed Cu_A, is e.p.r.-detectable and magnetically isolated; the other copper atom, termed Cu_B, is e.p.r.-undetectable and anti-ferromagnetically coupled to high-spin haem a_3^{3+} when in the cupric state (Palmer *et al.*, 1976; Thomson *et al.*, 1977; Falk *et al.*, 1977).

Recent low-temperature kinetic studies carried out by multi-channel spectroscopy at three wavelength pairs (604–630, 608–630 and 830–940 nm) 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*):

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

[The notation is that of Clore & Chance (1978*a*); intermediates I and III are equivalent to compounds A_1 and B described by Chance *et al.* (1975*a*, 1977).] Optical difference spectra in the α and near-i.r. regions of intermediates I and III minus fully reduced cytochrome oxidase (E) have been obtained (Chance *et al.*, 1975*a*, 1977; Chance & Leigh, 1977). Intermediate I has a 591 nm peak with a shoulder at 600 nm and a trough at 610 nm in the α-region, and no absorption bands in the near i.r. region (700–1000 nm). Intermediate III has a trough at 606 nm and no peak in the α-region, and a 790 nm peak in the near-i.r. region which is blue-shifted by approx. 40 nm with respect to that of fully oxidized cytochrome oxidase at 830 nm.

Abbreviations used: S.D.$_{1n}$ standard deviation of the natural logarithm of an optimized parameter; Hb, haemoglobin; m.c.d., magnetic circular dichroism.

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In the present study we extend our previous work (Clore & Chance, 1978*a*) and correlate the kinetics of the absorbance changes in the Soret (430–463 and 444–463 nm), α (590–630 and 608–630 nm) and near-i.r. (740–940, 790–940 and 830–940 nm) regions to gain further insight into the nature of the intermediates in the reaction of fully reduced membrane-bound cytochrome oxidase with O_2 at low temperatures.

Materials and Methods

The sample preparation, the kinetic recordings, the data digitization and normalization, and the numerical techniques were as described previously (Clore & Chance, 1978*a*).

The experimental conditions were: bovine heart mitochondria (prepared by the method of Low & Vallin, 1963) containing 5 μM-cytochrome oxidase (calculated from $\varepsilon^{605}_{red.-ox.} = 24.0\,mM^{-1}\cdot cm^{-1}$; Van Gelder, 1963); 30% (v/v) ethylene glycol; 0.1 M-mannitol; 50 mM-sodium phosphate buffer, pH 7.2 (stabilized down to temperatures as low as 143 K by the high concentration of protein present in the sample; Clore & Chance, 1978*a*); 5 mM-succinate; 0.6 mM-CO; 750 μM-O_2. The reaction was activated by flash photolysis of the fully reduced cytochrome oxidase–CO complex ($t = 0$ s) by using a 200 J Xenon flash with a pulse width of 1 ms. The flash was approx. 99% saturating, and CO did not recombine to a detectable extent in the presence of the relatively high O_2 concentration used, as shown by control experiments where repeated flashes over the course of the experiment only produced approx. 1% further photolysis of the CO complex, the O_2 intermediates not being susceptible to photolysis at the flash intensity used (Clore, 1978). The kinetics at the seven wavelength pairs were monitored with a Johnson Foundation multi-channel spectrophotometer

(Chance *et al.*, 1975*b*). The wavelengths of light were isolated by filters of appropriate spectral intervals and interlaced, one with another, by synchronized 60Hz rotating discs. The measuring beam was provided by a tungsten iodide lamp; the intensity was not sufficient to perturb the measured kinetics (Clore, 1978).

The normalized experimental data at seven wavelength pairs (430–463, 444–463, 590–630, 608–630, 740–940, 790–940 and 830–940nm) obtained at 176K are shown in Fig. 1 (below). The overall standard error of the data, given by the weighted mean of the standard errors of the individual progress curves, is $2.0\pm0.2\%$.

The total ΔA at 444–463nm was approx. 5 times larger than that at 430–463, 590–630 and 608–630nm, approx. 25 times larger than that at 790–940 and 830–940nm, and approximately 50 times larger than that at 740–940nm. In these experiments where the concentration of cytochrome oxidase was $5\mu M$ and the temperature was 176K, the total ΔA at 444–463nm was of the order of $0.2A$ units. The ratio of the total ΔA values in the near i.r. region for $\Delta A_{790-940}/\Delta A_{830-940}/\Delta A_{740-940}$ was approx. 1:0.90:0.55, a finding that agrees with that of Chance & Leigh (1977).

The optimal temperature range for monitoring the fully reduced cytochrome oxidase–O_2 reaction at the time resolution afforded by the multi-channel spectrophotometer is 173–178K (Chance *et al.*, 1975*a*; Clore & Chance, 1978*a*), and the temperature of 176K was chosen so as to allow a direct comparison of the rate constants with those obtained by Clore & Chance (1978*a*).

The kinetics of the haem components of cytochrome oxidase are monitored in the α and Soret regions (Caughey *et al.*, 1976). The choice of the 590–630 and 608–630nm wavelength pairs was based on the difference spectra of intermediates I and III minus fully reduced cytochrome oxidase (Chance *et al.*, 1975*a*). The 444–463nm wavelength pair was chosen on the basis that only free reduced haem a_3^{2+} and haem a^{2+} (i.e. not interacting with external ligands such as O_2 and CO) contribute to the absorbance at 444nm (Lemberg, 1969; Clore & Chance, 1978*b*). The 430–463nm wavelength pair was chosen on the basis that the following species have absorbance maxima in the 428–431nm region: the single intermediate in the fully reduced membrane-bound cytochrome oxidase–O_2 reaction observed at the lowest temperature (233K) at which the regenerative-flow apparatus could be operated (Erecinska & Chance, 1972); the oxygenated oxidase obtained by adding O_2 to fully reduced (Orii & Okunuki, 1963; Gilmour *et al.*, 1969) and mixed-valence-state (Greenwood *et al.*, 1974) soluble cytochrome oxidase at room temperature; and the CO complex of both fully reduced and mixed-valence-state cytochrome oxidase (Caughey *et al.*, 1976).

The kinetics of the copper components of cytochrome oxidase are monitored in the near-i.r. region (Aasa *et al.*, 1976; Wever *et al.*, 1977; Chance & Leigh, 1977; Clore & Chance, 1978*a*,*b*). The choice of the three wavelength pairs (740–940, 790–940 and 830–940nm) was based on the near-i.r. difference spectra of intermediate III and fully oxidized cytochrome oxidase minus fully reduced cytochrome oxidase, and intermediate III_M minus mixed-valence-state cytochrome oxidase (Chance & Leigh, 1977). Intermediate III_M (also known as compound C), the stable end product of the mixed-valence-state cytochrome oxidase–O_2 reaction up to temperatures as high as 265K, has an absorption maximum at 740nm, intermediate III at 790nm and fully oxidized cytochrome oxidase at 830nm.

Although the number of intermediates is known (Clore & Chance, 1978*a*), the data are highly complex in that all the progress curves are multiphasic, more than one intermediate contributes to each progress curve and the contributions of the intermediates to the progress curves (with the exception of the 608–630 and 830–940nm traces) are unknown. To analyse such data, it is essential to have a set of strict quantitative criteria on which to base one's choice of model. Such criteria have been developed by Clore & Chance (1978*a*,*b*) and 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 parameters ($S.D._{ln}$), and a random distribution of residuals. Thus for a given set of data, 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 absorbance change (W_i) in units of concentration is given by:

$$W_i(t) = \sum_i F_l(t)\, \varepsilon'_i(l) \qquad (2)$$

where $F_l(t)$ is the concentration of the lth intermediate at time t given by numerical integration of the set of simultaneous ordinary differential equations derived from eqn. (1), and $\varepsilon'_i(l)$ the relative absorption coefficient of the lth intermediate at the ith wavelength. The $\varepsilon'_i(l)$ are varied relative to a normalized difference absorption coefficient, $\Delta\varepsilon'_i(x-z)$, given by:

$$\begin{aligned}\Delta\varepsilon'_i(x-z) &= \Delta\varepsilon_i(x-z)/\Delta\varepsilon_i(x-z)\\ &= \varepsilon'_i(x)-\varepsilon'_i(z)\\ &= 1.0\end{aligned} \qquad (3)$$

where $\Delta\varepsilon_i(x-z)$ is the molar difference absorption coefficient between species x and z. Thus, the relative

absorption coefficients $\varepsilon_i'(x)$ and $\varepsilon_i'(z)$ of the two reference species x and z are given values of 1.0 and 0 respectively. Therefore, if $\Delta\varepsilon_i^T(x-z)$ at a temperature T is known, $\Delta\varepsilon_i^T(l-z)$ is given by:

$$\Delta\varepsilon_i^T(l-z) = \varepsilon_i'(l)\Delta\varepsilon_i^T(x-z) \quad (4)$$

As the reaction is run to its end point, at which time more than 99% of the total concentration of cytochrome oxidase is in the form of intermediate III at 176K (Clore & Chance, 1978*a*), the crude computed absorbance change for all the progress curves, with the exception of the 590–630nm trace, is normalized by dividing eqn. (2) by the total concentration of cytochrome oxidase in the sample (5μM) and setting the value of the relative absorption coefficient of the intermediate making the largest contribution to the particular trace to 1.0 (i.e. the relative absorption coefficient of intermediate III for the 430–463, 740–940, 790–940 and 830–940nm traces, which are characterized by progressive increases in absorbance, and the relative absorption coefficient of fully reduced cytochrome oxidase for the 444–463 and 608–630nm traces, which are characterized by a progressive decrease in absorbance). The 590–630nm trace is characterized by an initial increase in absorbance corresponding to the formation of intermediate I, followed by a progressive decrease in absorbance corresponding to the formation of intermediates II and III. Since normalization results in the experimental points with the maximum absorbance at 590–630nm being given a value of 1, and since the maximum value of W_{590} is smaller than the total concentration of cytochrome oxidase, an optimized scale factor is required. The value of the scale factor for the 590–630nm trace is 3.96μM (with an S.D.$_{ln}$ of 0.09).

Results and Discussion

The normalized experimental data at seven wavelength pairs (430–463, 444–463, 590–630, 608–630, 740–940, 790–940 and 830–940nm) together with the computed normalized absorbance changes and kinetics of the intermediates are shown in Fig. 1. The overall S.D. of the fit is 1.95% and the distribution of residuals is random. The values and S.D.$_{ln}$ of the rate constants and relative absorption coefficients together with the proposed assignments of the valence states of the four metal centres in the intermediates are given in Table 1. The value of the rate constants and the relative absorption coefficients at 608–630 and 830–940nm are the same as those found by Clore & Chance (1978*a*) within the errors specified.

In discussing the assignment of valence states of the four metal centres to the intermediates, we make the same assumptions as those made by Clore & Chance (1978*a*,*b*). For the purpose of our discussion, we also make the simplifying assumption that electrons are localized on particular metal centres rather than being distributed in some statistical manner between them. Although such a statistical distribution of electrons appears to be established in intermediate reduction states of xanthine oxidase (Olson *et al.*, 1974), we have not enough data at present to specify the exact statistical distribution of electrons among O_2 and the four metal centres in the intermediates of the fully reduced cytochrome oxidase–O_2 reaction. It should be noted that the above simplifying assumption does not affect the general arguments presented. Thus when we assign a valence state of j (where j is an integer) to a particular metal centre or to O_2, we do not exclude that the average charge, $\bar{j}$, located on a particular atom may lie in the range $j-0.5<\bar{j}<j+0.5$.

Contributions of the intermediates to the 430 and 444 nm traces

Table 1 shows that:

$$\varepsilon'_{430}(\mathrm{III}) > \varepsilon'_{430}(\mathrm{II}) > \varepsilon'_{430}(\mathrm{I})$$
$$\varepsilon'_{444}(\mathrm{E}) > \varepsilon'_{444}(\mathrm{I}) > \varepsilon'_{444}(\mathrm{II}) = \varepsilon'_{444}(\mathrm{III}) = 0 \quad (5)$$

This indicates a progressive blue-shift in the Soret band and therefore an increase in the energy of the π–π^* transitions of the haems as one progresses from fully reduced cytochrome oxidase (E) to intermediate III via intermediates I and II. A detailed interpretation of this blue-shift is extremely difficult, because of the presence of two haems with distinct environments, which are capable of extensive interaction (Caughey *et al.*, 1976), and is not possible on the basis of data at present available.

Identity of intermediate I

Clore & Chance (1978*a*) have proposed that the first step involves a one-electron reduction of O_2 to the O_2^- oxidation state and the concomitant oxidation of haem a_3^{2+} to the ferric state. This view was based on the assumption (founded on spectroscopic and kinetic grounds) that the electronic configuration of the haem a_3–O_2 bond is approximately the same in intermediate I and intermediate I_M (the first intermediate in the mixed-valence-state cytochrome oxidase–O_2 reaction) and the finding that intermediate I_M does not contribute to the absorbance at 444, 604 and 608nm (Clore & Chance, 1978*b*). The findings in the present paper are entirely consistent with this view. The absence of any contribution of intermediate I to the 740, 790 and 830nm traces (Table 1) confirms that neither copper atom is oxidized in intermediate I. The decrease of 34% in the contribution of intermediate I with respect to that of fully reduced cytochrome oxidase at 444nm [$\varepsilon'_{444}(\mathrm{E})-\varepsilon'_{444}(\mathrm{I}) = 0.34\pm0.02$; also see Table 1] is entirely consistent with the removal of the contribution of free haem a_3^{2+} (i.e. not interacting with external

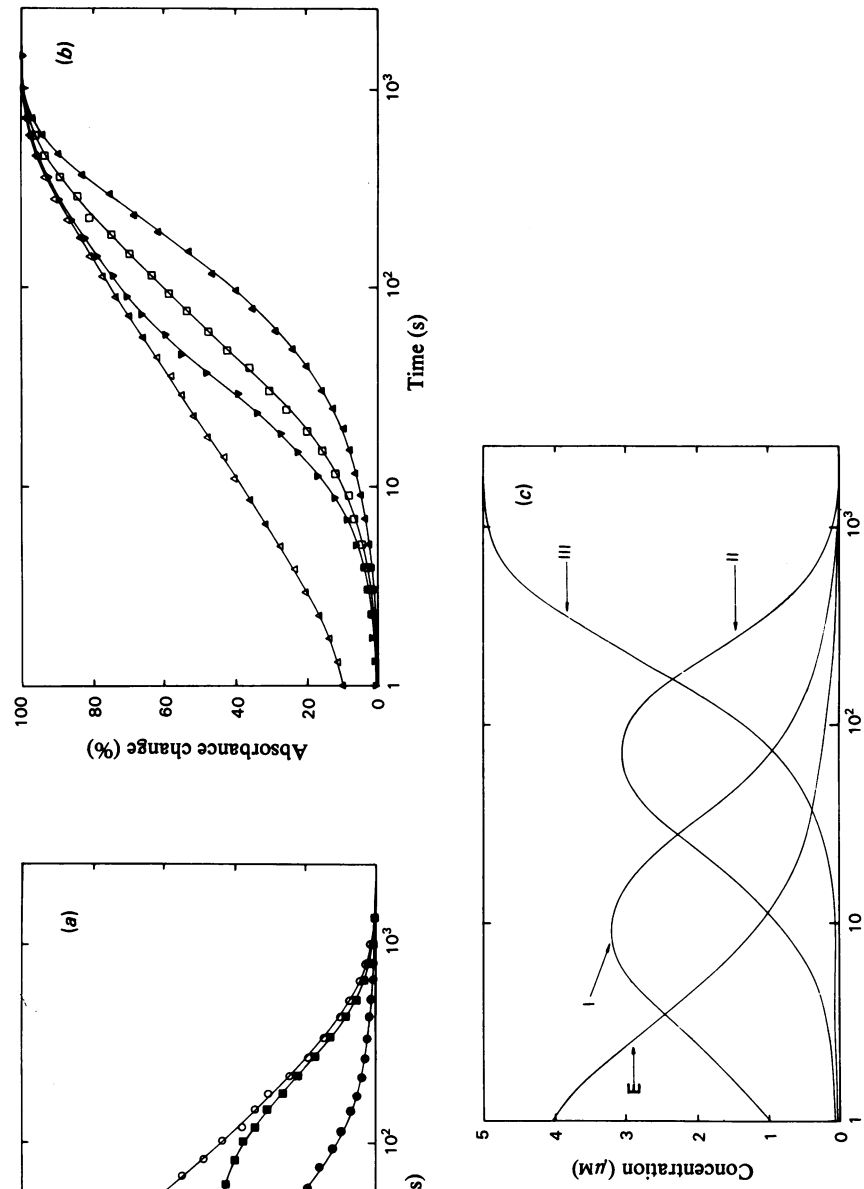

Fig. 1. *Normalized observed and computed kinetics of the reaction of fully reduced membrane-bound cytochrome oxidase with O_2 at 176 K as measured at seven wavelength pairs*

Symbols: △, 430–463 nm; ○, 590–630 nm; ■, 608–630 nm; ▲, 740–940 nm; □, 790–940 nm; ▼, 830–940 nm. The normalized computed absorbance changes are shown as solid lines. The computed kinetics of the individual intermediates are shown in (*c*). The overall S.D. of the fit is 1.95%; the standard error of the data is 2±0.2%. A measure of the distribution of residuals is provided by the mean absolute correlation index ($\bar{C}$) which has a value of 0.8.

$$\bar{C} = \frac{1}{k}\sum_{i=1}^{k}\left|\left(\sum_{j=1}^{m} R_{ij}\right)\Big/\left(\sum_{j=1}^{m} R_{ij}^2\right)^{\frac{1}{2}}\right|$$

where R_{ij} is the residual at the *j*th data point of the *i*th curve, *m* the number of data points for the *i*th curve and *k* the number of curves. 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 (Clore & Chance, 1978*a*). Initial conditions: 5 μM fully reduced cytochrome oxidase (E) in the presence of 750 μM-O_2.

Table 1. *Optimized values of the rate constants and relative absorption coefficients of the intermediates of the reaction of fully reduced membrane-bound cytochrome oxidase with O_2 at 176K, together with the proposed chemical identity of the intermediates*

The S.D.$_{ln}$ of the optimized parameters are shown in parentheses. Because of the linearity of logarithms less than 0.2, a parameter whose S.D.$_{ln}$ lies below this value has a relative standard deviation (i.e. $\Delta x/x$) equal to the S.D.$_{ln}$. For larger values of S.D.$_{ln}$, up to 1.0 in magnitude, the parameter value is determined to within a factor of the order $e \simeq 2.72$, and so its order of magnitude is known. Significantly larger values of S.D.$_{ln}$ show that the observations are inadequate to determine the parameter.

Reaction sequence and rate constants at 176K	$O_2+E \underset{k_{-1}}{\overset{k_{+1}}{\underset{0.0428\ s^{-1}\ (0.15)}{\overset{360M^{-1}\cdot s^{-1}\ (0.10)}{\rightleftharpoons}}}} I \underset{k_{-2}}{\overset{k_{+2}}{\underset{0.00442\,s^{-1}\ (0.10)}{\overset{0.0341s^{-1}\ (0.09)}{\rightleftharpoons}}}} II \underset{k_{-3}}{\overset{k_{+3}}{\underset{1\times10^{-6}\ s^{-1}\dagger}{\overset{0.00551s^{-1}\ (0.10)}{\rightleftharpoons}}}} III$			
Relative absorption coefficient at:				
430–463nm [$\varepsilon'_{430}(l)$]	0*	0.404 (0.04)	0.743 (0.05)	1.0*
444–463nm [$\varepsilon'_{444}(l)$]	1.0*	0.662 (0.03)	0†	0*
590–630nm [$\varepsilon'_{590}(l)$]	0.461 (0.03)	1.0*	0.357 (0.24)	0*
608–630nm [$\varepsilon'_{608}(l)$]	1.0*	0.283 (0.15)	0.515 (0.10)	0*
740–940nm [$\varepsilon'_{740}(l)$]	0*	0†	0.246 (0.04)	1.0*
790–940nm [$\varepsilon'_{790}(l)$]	0*	0†	0.572 (0.03)	1.0*
830–940nm [$\varepsilon'_{830}(l)$]	0*	0†	0.723 (0.04)	1.0*
Proposed chemical identity of the intermediates	$a_3^{2+}Cu_B^{+}$ $a^{2+}Cu_A^{+}$	$a_3^{3+}Cu_B^{+}\cdot O_2^{-}$ $a^{2+}Cu_A^{+}$	$a_3^{2+}Cu_B^{2+}\cdot O_2^{2-}$ $a^{3+}Cu_A^{+}$	$a_3^{2+}Cu_B^{2+}\cdot O_2^{3-}$ $a^{3+}Cu_A^{2+}$

* The relative absorption coefficients are varied relative to a normalized difference absorption coefficient $\Delta\varepsilon'_i(x-z)$ defined by eqn. (3) so that $\varepsilon'_i(x)$ and $\varepsilon'_i(z)$ are given values of 1.0 and 0 respectively. The $\varepsilon'_{430}(l)$, $\varepsilon'_{444}(l)$, $\varepsilon'_{590}(l)$, $\varepsilon'_{608}(l)$, $\varepsilon'_{740}(l)$, $\varepsilon'_{790}(l)$ and $\varepsilon'_{830}(l)$ are varied relative to $\Delta\varepsilon'_{430}$(III-E), $\Delta\varepsilon'_{444}$(E-III), $\Delta\varepsilon'_{590}$(I-III), $\Delta\varepsilon'_{608}$(E-III), $\Delta\varepsilon'_{740}$(III-E), $\Delta\varepsilon'_{790}$(III-E) and $\Delta\varepsilon'_{830}$(III-E) respectively.

† These parameters were set to zero, except for k_{-3}, which was set to a suitably low value ($1\times10^{-6}\cdot s^{-1}$) on the basis of initial optimizations in which their values were found to be small and very poorly determined (S.D.$_{ln} \gg 10$).

ligands) to the absorbance at 444nm as a result of direct interaction of haem a_3 with O_2. The confirmation (Table 1) that:

$$\varepsilon'_{608}(E) > \varepsilon'_{608}(II) > \varepsilon'_{608}(I) \qquad (6)$$

also supports the formal assignment of the iron of haem a_3 to the Fe(III) state in intermediate I, since, if the iron of haem a_3 were formally in the Fe(II) state, we would expect any subsequent transfer of electrons from cytochrome oxidase to O_2 involving the oxidation of haem a_3^{2+} to result in the formation of an intermediate which made a smaller contribution than intermediate I to the absorbance at 608nm, rather than a larger one.

Any discussion of the nature of the haem a_3–O_2 bond in intermediate I must take into account the nature of the haem a_3–CO, Hb–CO and Hb–O_2 bonds. I.r. studies on the cytochrome oxidase–CO complex and HbCO have demonstrated the existence of a single C—O stretching frequency at 1963.5 and 1951 cm^{-1} respectively, which is entirely consistent with a synergic bonding mechanism for the Fe–CO bond in these compounds (Yoshikawa *et al.*, 1977) involving σ overlap between an sp hybrid in the carbon of CO and a d^2sp_3 hybrid of iron, supported by overlap of two dπ orbitals of iron with two empty pπ^* orbitals of CO (The t_{2g} and e_g terminology is not used, since the symmetry is lower than tetragonal). HbO_2 has an i.r. O—O stretching frequency at 1107cm^{-1} consistent with the presence of a superoxide anion (Yoshikawa *et al.*, 1977) (e.g. compared to the i.r. O—O stretching frequencies of $Na^+O_2^-$ and $Li^+O_2^-$ at 1080 and 1097 cm^{-1} respectively; Andrews, 1969) and a haem resonance Raman band at 1375cm^{-1} consistent with the presence of Fe(III) (Yamamoto *et al.*, 1973; Spiro & Strekas, 1974). However, HbCO also has a haem resonance Raman band around 1375cm^{-1} (Spiro & Strekas, 1974). It therefore seems likely that π acid ligands such as O_2 and CO selectively deplete the iron dπ orbitals, and their effect on back donation to porphyrin appears to be approximately the same as actual removal of one electron from Fe(II) (Spiro & Strekas, 1974; Spiro & Loehr, 1975).

The formation of HbCO results in the splitting of the single band of deoxyHb in the visible region at 555nm into an α-band at 569nm and a β-band at 540nm; no shoulder is seen in the α-band of HbCO (Antonini & Brunori, 1971). Fully reduced cytochrome oxidase has absorption maxima at 603 and 560nm in the α- and β-regions respectively (Caughey *et al.*, 1976). The formation of the fully reduced cytochrome oxidase–CO complex is associated with

the formation of a shoulder at 590nm in the α-band, the α-peak remaining at 603nm, and a blue-shift in the β-band to 551nm (Caughey *et al.*, 1976). This shoulder can be attributed to a charge-transfer transition ($d\pi_{Fe} \rightarrow p\pi^*_{CO}$) from the iron of haem a_3 to CO. The 590nm shoulder is seen as a peak at 588.5nm in the difference spectrum for the fully reduced cytochrome oxidase–CO complex minus fully reduced cytochrome oxidase at 77K (Chance *et al.*, 1975*a*). Intermediate I also exhibits a shoulder around 590nm in the α-band that is reflected by the large increase in the contribution of intermediate I relative to both fully reduced cytochrome oxidase and intermediate II to the 590nm trace (see Table 1) and a peak at 591nm in the difference spectrum for intermediate I (compound A_1) minus fully reduced cytochrome oxidase at 77K (Chance *et al.*, 1975*a*). The red-shift of 2.5nm in the α-band shoulder of intermediate I relative to the fully reduced cytochrome oxidase–CO complex (corresponding to a decrease in the energy of the $d\pi_{Fe} \rightarrow p\pi^*_{ligand}$ transition of $\sim 0.9\,kJ \cdot mol^{-1}$) is to be expected, since the energy of charge transfer to a ligand is decreased as the acceptor power of the $p\pi^*$ orbital of the ligand is increased (Williams, 1955) (i.e. as the oxidizing power of the ligand increases, so the energy of the $d\pi_{Fe} \rightarrow p\pi^*_{ligand}$ transition decreases, and the absorption maximum of the charge-transfer band is shifted to the red).

On the basis of the above evidence, we suggest that the charge localized on the iron of haem a_3 in intermediate I is greater than +2.5. We therefore assign the iron of haem a_3 formally to the Fe(III) state and represent intermediate I by the configuration

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

Identity of intermediates II and III

Clore & Chance (1978*a*) proposed that O_2 is reduced to the O_2^{2-} oxidation state in the second step and that the configuration of intermediate II is

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

The difference in the relative absorption coefficients of intermediates I and II at 608nm was attributed to haem–haem, haem–ligand and/or haem–copper interactions. Three possible configurations were proposed for intermediate III (Clore & Chance, 1978*a*) to account for the decrease in the relative absorption coefficient of intermediate III with respect to that of intermediate II, and the observation that, by the time equilibrium is reached in the 173–195K range, only 40% of the total oxidized–reduced absorbance change at 605nm (at the corresponding temperature) has taken place (Chance *et al.*, 1977):

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

(x) (y) (z)

None of the configurations proposed by Clore & Chance (1978*a*) for intermediates II and III allows for anti-ferromagnetic coupling between high-spin haem a_3^{3+} and Cu_B^{2+}, thereby accounting for the observation that no 655nm band (which is thought to arise from such coupling, Palmer *et al.*, 1976) is formed during the course of the reaction of fully reduced cytochrome oxidase with O_2 below 195K (Chance *et al.*, 1977; Denis, 1977).

The absence of any contribution from intermediates II and III to the 444nm trace (see Table 1) is entirely consistent with the above configurations, and implies, on the basis of the assumptions of Clore & Chance (1978*b*), that haem a_3^{2+} in intermediates II and III interacts directly with the reduced O_2 species.

Chance & Leigh (1977) have shown that, although the near-i.r. peak of fully oxidized cytochrome oxidase at 820–830nm is blue-shifted to 790–800nm in intermediate III, the intensities of the near-i.r. peaks of intermediate III and fully oxidized cytochrome oxidase are approximately equal. Table 1 shows that

$$\varepsilon'_{830}(II) > \varepsilon'_{790}(II) > \varepsilon'_{740}(II) \qquad (7)$$

and that the increase in the relative absorption coefficients of intermediate III with respect to those of intermediate II at 740, 790 and 830nm are highly significant. This strongly suggests that intermediate II contains one atom of cupric copper, Cu_B^{2+}, and intermediate III two atoms of cupric copper, Cu_A^{2+} and Cu_B^{2+}, and therefore that the configuration of intermediate III is configuration (y).

The ratios $\Delta A^{III}_{830-940}/\Delta A^{III}_{790-940}$ and $\Delta A^{III}_{740-940}/\Delta A^{III}_{790-940}$ are approximately 0.90 and 0.55 respectively (derived from Fig. 2 of Chance & Leigh, 1977). These ratios allow one to normalize the contributions of intermediates II and III at 740 and 830nm with respect to those at 790nm (Table 2). On so doing we find that

$$\Delta A^{II}_{830-940}/\Delta A^{III}_{790-940} > \Delta A^{II}_{790-940}/\Delta A^{III}_{790-940} > \Delta A^{II}_{740-940}/\Delta A^{III}_{790-940} \qquad (8)$$

indicating that the near-i.r. peak of intermediate II is red-shifted with respect to that of intermediate III. This is clearly seen in Fig. 2, where the near-i.r. difference spectra of intermediates II and III minus fully reduced cytochrome oxidase have been constructed from the data in Table 2. Because of the relatively low symmetry (i.e. less than cubic) of the environments in which cupric copper is characteristically found (Cotton & Wilkinson, 1972), a detailed

Table 2. *Contributions of intermediates II and III at* 740–940, 790–940 *and* 830–940 *nm normalized relative to the contribution of intermediate III at* 790–940 *nm*

Normalization is carried out by using the ratios $\Delta A^{III}_{830-940}/\Delta A^{III}_{790-940}$ and $\Delta A^{III}_{740-940}/\Delta A^{III}_{790-940}$, which have values of approx. 0.90 and 0.55 respectively (derived from Fig. 2 of Chance & Leigh, 1977), and the optimized values of the relative absorption coefficients of intermediate II at 740–940, 790–940 and 830–940 nm given in Table 1.

Intermediate	$\Delta A^{I}_{740-940}/\Delta A^{III}_{790-940}$	$\Delta A^{I}_{790-940}/\Delta A^{III}_{790-940}$	$\Delta A^{I}_{830-940}/\Delta A^{III}_{790-940}$
II	0.14	0.57	0.65
III	0.55	1.0	0.90

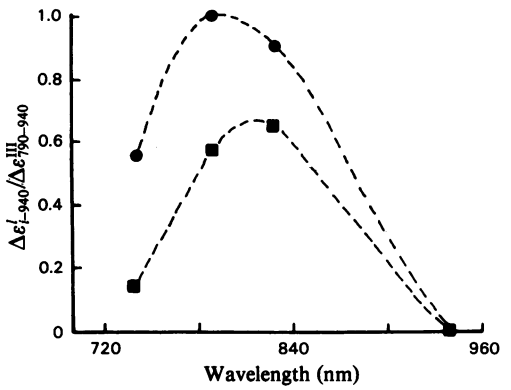

Fig. 2. *Near-i.r. difference spectra of intermediates II* (■) *and III* (●) *minus fully reduced cytochrome oxidase normalized relative to the absorbance of intermediate III minus fully reduced cytochrome oxidase at* 790–940 *nm* (*constructed from the data in Table* 2)

interpretation of the different positions of the near-i.r. absorption maxima of fully oxidized cytochrome oxidase, intermediate II and intermediate III is extremely complicated and is not possible on data available at present. We note, however, that the magnitude of the shift between the near-i.r. peaks of fully oxidized cytochrome oxidase and intermediate III is relatively small (approx. 500 cm^{-1}) for a broad absorption band with a $\Delta\nu_{1/2} \sim 2100\,cm^{-1}$ (Chance & Leigh, 1977), and corresponds to a difference in the energy of the d–d* transitions in the d^9 system of cupric copper of $\sim 6\,kJ \cdot mol^{-1}$; this could easily be accounted for either by direct interaction or by indirect interaction via the haems of one or both copper atoms with O_2.

Scheme for the elementary steps in the reduction of O_2 by fully reduced membrane-bound cytochrome oxidase

On the basis of the present evidence and that in our previous paper (Clore & Chance, 1978*a*), we propose the following scheme for the mechanism of the reaction of fully reduced membrane-bound cytochrome oxidase with O_2 at low temperatures (see Table 1).

The first step involves the binding and simultaneous reduction of O_2 to the O_2^- oxidation state and the concomitant oxidation of haem a_3^{2+} to the ferric state. Thus, the first step can be viewed as an oxidative addition reaction. We do not, however, wish to imply that intermediate I is necessarily an ionic complex, but simply that the average number of electrons transferred to O_2 is greater than 0.5. The spin state of haem a^{2+} is presumed to remain low spin, as in fully reduced cytochrome oxidase (Babcock *et al.*, 1976, 1978*a*; Palmer *et al.*, 1976; Thomson *et al.*, 1977; Falk *et al.*, 1977). Haem a_3^{3+} could be either low ($S = 1/2$) or high ($S = 5/2$) spin, and an unpaired electron on haem a_3^{3+} must be anti-ferromagnetically or ferromagnetically coupled to the unpaired electron on O_2^-, forming an exchange-coupled complex of whole spin ($S = 0$ or 2 respectively if haem a_3^{3+} is low spin; $S = 2$ or 3 respectively if haem a_3^{3+} is high spin), which would be difficult or impossible to observe by e.p.r., thereby accounting for the absence of low- and high-spin ferric haem e.p.r. signals in intermediate I (Chance *et al.*, 1975*a*, 1977; Chance & Leigh, 1977). Of these possibilities, low-spin haem a_3^{3+} anti-ferromagnetically coupled to O_2^- is favoured by analogy with HbO_2 where magnetic-susceptibility studies conducted over a wide temperature range have shown that the haem iron is low-spin ($S = 1/2$) and anti-ferromagnetically coupled to a $S = 1/2$ O_2 species (formally O_2^-) (Cardonio *et al.*, 1977). However, any distinction between these various possibilities will require careful magnetic-susceptibility studies.

The second step is a one-electron reduction of O_2^- to the O_2^{2-} oxidation state coupled with the oxidation of Cu_B^+ and haem a^{2+} to the cupric and ferric states respectively, and the reduction of haem a_3^{3+} to the ferrous state. Since the only ferric haem e.p.r. signal observed during the course of the reaction is the low-spin g = 3.05 signal (G. M. Clore & J. S. Leigh, unpublished work; Chance *et al.*, 1978*a*), haem a^{3+} must be low-spin. To account for the absence of any contribution from intermediate II to the 444 nm trace, haem a_3^{2+} is required to interact directly with O_2^{2-}. In the presence of a strong nucleophile such as O_2^{2-} (Jenks, 1969) haem a_3^{2+} would be expected to be intermediate or high-spin. It is known that the reduction of O_2 induced

by aqueous Fe(II) (George, 1954), Cu(I) (De Marco *et al.*, 1971), Cr(II) (Joyen & Wilmarth, 1961), Co(II) (Mori & Weil, 1967) and isolated ferrous haem (Cohen & Caughey, 1966) involves the formation of an intermediate compound with a peroxo bridge between two metals. Further, examples of mechanisms in which a terminal non-bridging O_2 is reduced to the oxidation level of water are not known (Caughey *et al.*, 1975). We therefore suggest that O_2^{2-} in intermediate II serves as a bridging ligand between haem a_3^{2+} and Cu_B^{2+}. The formation of such a bridge structure requires that the iron of haem a_3 and Cu_B are within a distance of less than 0.4nm from each other. Magnetic-susceptibility measurements (Tsudzuki & Okonuki, 1975) have shown that the magnetic susceptibility of both aqueous and fluoro fully oxidized cytochrome oxidase is linear over the 77–250K range indicating that anti-ferromagnetic coupling between high-spin haem a_3^{2+} and Cu_B^{2+} is strong with J (exchange coupling constant) $\leqslant -200\,cm^{-1}$. Such strong coupling requires electronic overlap between the iron of haem a_3 and Cu_B so that they must certainly be within a distance of 0.4nm from each other. Although Caughey *et al.* (1975, 1976) have suggested that a peroxo-bridge structure could be formed between haems *a* and a_3, we consider this highly unlikely for the following reasons. Firstly, the distance separating the two haem planes appears to be of the order of 1.5–2.0nm (Chance *et al.*, 1977), a distance far too great to allow the formation of a peroxo bridge. Secondly, the inability of haem *a* to react with external ligands (e.g. CO and CN^-), together with the low γ/α ratio caused by a strong α-band, indicates that haem *a*, like the haem of cytochrome *c*, is co-ordinated in both the fifth and sixth positions to amino acid residues of the protein (Lemberg, 1969). The view that haem *a* is co-ordinated in both the fifth and sixth positions is further supported by the following evidence. M.c.d. studies on isolated cytochrome oxidase (Babcock *et al.*, 1976, 1978*a*; Palmer *et al.*, 1976; Thomson *et al.*, 1977) have shown that haem *a* is low-spin in both the fully reduced and fully oxidized enzyme. If haem *a* is to be low-spin, it must be co-ordinated in both the fifth and sixth positions to ligands with a strong ligand field (e.g. the imidazole of a histidine residue). Further, the characteristics of the m.c.d. and laser Raman spectra of the low-spin bisimidazole and bis(*N*-methylimidazole) derivatives of isolated haem A are almost identical with those of haem *a* in intact cytochrome oxidase (Babcock *et al.*, 1978*b*).

The third step involves a one-electron reduction of O_2^{2-} to the O_2^{3-} oxidation state and the concomitant oxidation of Cu_A^+ to the cupric state. The spin states of the haems are the same as in intermediate II, namely low-spin haem a^{3+}, accounting for the low-spin ferric haem e.p.r. signal at $g = 3.05$, and high-spin haem a_3^{2+} anti-ferromagnetically coupled to Cu_B^{2+}, accounting for the absence of any e.p.r. signals around $g = 2$ attributable to Cu_B^{2+}. The reduction of O_2^{2-} to the O_2^{3-} oxidation state may involve heterolytic cleavage of the O—O bond, resulting in the production of the $OH^\bullet$ and OH^- species (i.e. protonated $O^{-\bullet}$ and O^{2-} respectively). However, this seems highly unlikely in view of the very great reactivity of the hydroxyl radical $OH^\bullet$ (Cotton & Wilkinson, 1972). We therefore suggest that the additional electron is accommodated in the π orbitals of the haem-a_3–O_2–Cu_B bridge by resonance stabilization, which can be represented by the structures

$$(a_3^{2+}\text{—}O^-\text{—}O\text{—}Cu_B^{2+} \leftrightarrow a_3^{2+}\text{—}O\text{—}O^-\text{—}Cu_B^{2+}) \equiv (a_3^{2+}{=}O{\overset{\ominus}{=}}O{=}Cu_B^{2+})$$

The absence of any contribution from intermediate III to the 444nm trace is then easily accounted for by the interaction of O_2^{3-} with haem a_3^{2+}, and the blue-shift in the near i.r. band from 830nm in fully oxidized cytochrome oxidase to 790nm in intermediate III by the interaction of O_2^{3-} with Cu_B^{2+}.

Below 195 K, intermediate III is the stable end-product of the fully reduced membrane-bound cytochrome oxidase–O_2 reaction (Chance *et al.*, 1977). As n.m.r. studies (Chance *et al.*, 1978*b*) have demonstrated that no detectable fluid phase is present in mitochondrial suspensions in 20% (v/v) ethylene glycol below 195K, this may be due to extensive restriction of lateral diffusion of cytochrome *c* in the inner mitochondrial membrane so that the oxidation of cytochrome *c* by cytochrome oxidase, which would provide the driving force for the reduction of O_2^{3-} to the O_2^{4-} oxidation state, cannot occur.

We thank Dr. M. R. Hollaway and Dr. B. Chance for several stimulating discussions. We also thank the Johnson Research Foundation for experimental facilities and the University College London Computer Centre for computing facilities. G. M. C. acknowledges the tenure of a Boehringer–Mannheim Travelling Fellowship during part of this study.

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