The Mechanism of Reaction of Ferricyanide-Pretreated Mixed-Valence-State Membrane-Bound Cytochrome Oxidase with Oxygen at 173 K

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(Received 19 December 1977)

1. The results of non-linear optimization studies on the mechanism of reaction of ferricyanide-pretreated mixed-valence-state cytochrome oxidase with O₂ at 173 K are presented. The analysis is carried out on data obtained by means of dual-wavelength multi-channel spectroscopy at four wavelengths pairs (444–463 nm, 604–630 nm, 608–630 nm and 830–940 nm) and at two O₂ concentrations (360 µM and 520 µM). The only model that satisfies the triple requirement of a standard deviation within the standard error of the experimental data, a random distribution of residuals and good determination of the optimized parameters, is a three-intermediate sequential mechanism. 2. On the basis of the optimized values of the relative absorption coefficients of the intermediates at each wavelength obtained from the present paper together with data from optical wavelength scanning and e.p.r. spectroscopy obtained by low-temperature trapping studies, the possible valence states of the metal centres in each of the intermediates are discussed.

The minimal functioning unit of cytochrome oxidase (EC 1.9.3.1), the terminal complex of the mitochondrial respiratory chain, is thought to contain two haems, one α, and two copper atoms (Lemberg, 1969; Muijsers et al., 1972; Caughey et al., 1976). One copper atom, termed Cuα, is detectable by e.p.r. spectroscopy (Aasa et al., 1976) and thought to be magnetically isolated (Palmer et al., 1976). The other copper atom, termed Cuβ, is undetectable by e.p.r. (Aasa et al., 1976) and anti-ferromagnetically coupled to high-spin haem α²⁺ when in the cupric state (Palmer et al., 1976; Thomson et al., 1976, 1977; Falk et al., 1977). [The notation used to designate the two copper atoms is that of Clore & Chance (1978) and Palmer et al. (1976), and will be used throughout the present paper.]

In the preceding paper (Clore & Chance, 1978) we analysed the kinetics of the reaction of fully reduced membrane-bound cytochrome oxidase with O₂ at 176 K by means of multi-channel dual-wavelength spectroscopy at three wavelengths pairs, and non-linear stiff numerical integration and optimization techniques to evaluate quantitatively the experimental data. The only model that satisfied the triple requirement of a standard deviation within the standard error of the experimental data, good determination of the optimized parameters and a random distribution of residuals, was a three-species sequential mechanism. This established the presence of another intermediate in the reaction of fully reduced membrane-bound cytochrome oxidase with O₂ at low temperatures that had not been identified by low-temperature trapping and wavelength-scanning optical spectroscopy (Chance et al., 1975b,c).

The present study concerns the elucidation of the kinetics and chemistry of the reaction of ferricyanide-pretreated mixed-valence-state membrane-bound cytochrome oxidase with O₂ at 173 K by means of the techniques developed by Clore & Chance (1978). A combination of spectroscopic, e.p.r. and potentiometric studies (Mackay et al., 1973; Lindsay & Wilson, 1974; Lindsay et al., 1975; Wilson et al., 1975; Chance et al., 1978) have shown that pre-treatment of the fully reduced membrane-bound cytochrome oxidase–CO complex with ferricyanide results in the oxidation of all components of the respiratory chain having midpoint potentials less than 300 mV. Only haem α and Cuβ (midpoint redox potentials at pH 7.2 of 380 ± 10 mV and 340 ± 10 mV respectively) remain reduced, haem α and Cuβ being oxidized together with all the other components of the respiratory chain. This permits one to obtain further insight into the reaction of cytochrome oxidase with O₂ and the interactions of its four metal centres. Two spectroscopically distinct species, A₀ and C, have been identified by using the low-temperature trapping technique (Chance et al., 1975b,c; Denis & Chance, 1977; Chance & Leigh, 1977). Compound A₀ had a 590 nm α-band with a shoulder at 600 nm and a trough at 612 nm, a 546 nm β-band and a negative 444 nm γ-band with a small

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

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423nm peak. Compound C had identical features in the Soret region, a 547nm \( \beta \)-band, and an intense absorption band in the \( \alpha \)-region at 606–609nm. Compound \( A \) was recognized as functional by its conversion into compound C.

The experimental data for the present study were obtained by means of dual-wavelength multi-channel spectroscopy at four wavelength pairs (444–463 nm, 604–630 nm, 608–630 nm and 830–940 nm) and at two \( O_2 \) concentrations (360 \( \mu \)M and 520 \( \mu \)M).

**Experimental**

**Biochemical methods**

The preparation of the CO-inhibited fully reduced mitochondrial suspension is identical with that described (Clore & Chance, 1978) except that \( K_3 \)FeCN \(_4\) (at a final concentration of 5 mm) is added at 253 K and allowed to react for 1 min before the addition of the oxygenated ethylene glycol solution followed by rapid cooling to 195 K. Lanne et al. (1977) have tentatively suggested on the basis of optical and e.p.r. studies that ferricyanide slowly reacts at room temperature (298 K) with purified soluble cytochrome oxidase over a period of approximately 50 min to form a complex whose redox properties differ from those of the free soluble cytochrome oxidase. However, at the low temperatures used in this study any possible complex-formation with ferricyanide over the duration of the experiment will be negligible. Further, there is no evidence for this reaction in the membrane-bound system (B. Chance, unpublished work). The experimental conditions are: 15 nm of bovine heart mitochondria/ml containing 5 \( \mu \)M cytochrome oxidase (calculated from \( \epsilon_{\text{max, 605}} = 24.0 \text{mM}^{-1}\text{cm}^{-1} \); Van Gelder, 1963), 0.2M-mannitol, 0.75M-sucrose, 30mm-sodium phosphate buffer, pH 7.2, 10mm-succinate, 10mm-glutamate, 30% (v/v) ethylene glycol, 5mm-K\(_3\)FeCN \(_4\) and 1.2mm-CO. Two \( O_2 \) concentrations (360 \( \mu \)M and 520 \( \mu \)M) were used and 2mm-path-length cuvettes were used throughout for optical studies. The reaction is activated at 173 K by a laser flash from a 0.1J Rhodamine 6G dye laser that has a wavelength of 585nm and a pulse width of 1 \( \mu \)s.

As before (Clore & Chance, 1978), the choice of temperature is governed by the time resolution of the multi-channel spectrophotometer, the turbidity of the mitochondrial suspension resulting in a signal-to-noise ratio that is too low at less than 0.1 s to obtain meaningful data.

**Biophysical methods**

All the spectrophotometric data at each \( O_2 \) concentration were recorded simultaneously from the same sample at the same temperature by using a single laser flash by means of three time-sharing Johnson Foundation multi-channel spectrophotometers (Chance et al., 1975a), the first two affording wavelengths appropriate to haem kinetics in the region of the \( \alpha \)- and \( \gamma \)-bands respectively, and the third appropriate to i.r.-absorbance changes attributable to the kinetics of the copper components of cytochrome oxidase. Further details are as described by Clore & Chance (1978).

**Spectroscopic recordings and data digitization**

The kinetics were resolved with an amplifier rise time of approx. 0.1 s and recorded on strip charts over a period of approx. 40 min until equilibrium was reached.

Data were digitized by the method developed in Appendix 1 of Clore & Chance (1978). Fig. 1 shows the percentage absorbance change at four wavelength pairs (444–463, 604–630, 608–630 and 830–940 nm) and at two \( O_2 \) concentrations (360 and 520 \( \mu \)M) plotted as a function of logarithmic time. The overall standard error of the data, given by the weighted mean of the standard errors of the individual spectroscopic curves, is \( \pm 0.26\% \).

**Numerical techniques**

The numerical techniques of Clore & Chance (1978) were used with a computer program FACSIMILE (Curtis, 1976; Curtis & Kirby, 1977) that employs a modified version of Gear's (1971) method of numerical integration and Powell's (1965, 1972) method of non-linear optimization.

We minimize the residual sum squares (RSQ) given by:

\[
\text{RSQ} = \sum_{i=1}^{n} \sum_{j=1}^{m} R_{ij}^2 = \sum_{i=1}^{n} \sum_{j=1}^{m} \left[ \epsilon_{ij} - \left( \frac{a_i}{s_i} \right) \right]^2
\]

where \( j \) identifies the time point and \( i \) the data curve, \( R_{ij} \) are the residuals, \( \epsilon_{ij} \) the observed values, \( a_i \) the corresponding calculated values, \( s_i \) a scale factor and \( \sigma_i \) the standard error for curve \( i \). At the minimum RSQ is equal to chi-squared (\( \chi^2 \)). From the RSQ we calculate the standard deviation that, unlike the RSQ, is independent of the number of experimental points:

\[
\text{s.d.} = \phi \sqrt{\text{RSQ} / (d - p)}
\]

where \( d \) is the total number of experimental points, \( p \) is the number of optimized parameters and \( \phi \) the overall standard error of the data:

\[
\phi = \sum \sigma_i r_i / \sum r_i
\]

(3) (where \( r_i \) is the range of curve \( i \)).

The determination of the optimized parameters is given by the s.d.\(_{1\alpha}\) (standard deviation of the
natural logarithm) of the unknown parameter that is obtained from the non-linear co-variance (Clore & Chance, 1978). A parameter whose s.d. is less than 0.2 is considered to have a well-determined minimum in multidimensional parameter space. For larger values of s.d., up to 1 in magnitude, the parameter value is determined to within a factor of order e ≈ 2.72, and so its order of magnitude is known. Significantly larger values of s.d. show that the observations are inadequate to determine the parameter. From the s.d. of the optimized parameters. A measure of the nature of the distribution of the residuals is given by the correlation index (C_j)

\[ C_j = \left( \frac{\sum_t R_j}{\sum_t} \right) \left( \frac{\sum_t R_j^2}{\sum_t} \right) \]  

(4)

A value of |C_j| significantly greater than 1.0 (the expected root-mean-square value of |C_j| if the residuals were all independent random variables of zero mean and the same variance) indicates that the departures between calculated and observed values are systematic, and statistics such as \( \chi^2 \) and s.d., the variance–covariance matrix and the s.d. of the optimized parameters have to be regarded with suspicion. [For the derivation of eqn. (4) see Appendix 3 of Clore & Chance (1978).]

As before (Clore & Chance, 1978), we wish to emphasize that the choice of model in non-linear optimization problems depends not only on obtaining a s.d. within the standard error of the experimental data, but also on obtaining a fit in which the optimized parameters are well determined and the distribution of residuals is random. By providing a rigorous quantitative framework on which to base one's choice of model, this triple requirement greatly decreases the number of models available. In fact, in stiff non-linear problems, it is usually the case that only a single model will satisfy this triple requirement.

Results

Preliminary attempts at non-linear optimization of the coefficients of the differential equations representing a two-intermediate reaction system was found not to fit the data on the basis of s.d. (≥ 10%) and systematic errors in the distribution of residuals. This indicated the presence of other intermediates in the reaction of ferricyanide-pretreated mixed-valence-state membrane-bound cytochrome oxidase with O_2 that had not been identified by low-temperature trapping and wavelength-scanning optical spectroscopy (Chance et al., 1975b,c; Chance & Leigh, 1977; Denis & Chance, 1977). A number of other models were then tested involving more intermediates and/or branching pathways, and, as in the case of the reaction of fully reduced membrane-bound cytochrome oxidase with O_2 (Clore & Chance, 1978), the only model that satisfies the triple requirement of an s.d. within the standard error of the data (i.e. s.d. <2%), good determination of the optimized parameters and a random distribution of residuals, with no arbitrary constraints, is a three-species sequential mechanism, which is stated as follows:

\[ E_M + O_2 \xrightarrow{k_{44}} I_M \xrightarrow{k_{24}} II_M \xrightarrow{k_{34}} III_M \]  

(5)

where \( E_M \) is the ferricyanide-pretreated mixed-valence-state cytochrome oxidase complex and intermediate III_M is the product of the reaction. [The subscript M is used to differentiate these intermediates from those in the reaction of fully reduced membrane-bound cytochrome oxidase with O_2 (Clore & Chance, 1978).]

The contribution of each intermediate to each wavelength is represented by a relative absorption coefficient. The crude computed absorbance at the i-th wavelength, \( W_i(t) \), in units of concentration, is given by:

\[ W_i(t) = \sum_T F_i(t) \varepsilon_i^*(t) \]  

(6)

where \( F_i(t) \) is the concentration of the i-th intermediate at time t, and \( \varepsilon_i^*(t) \) is the relative absorption coefficient of the i-th intermediate at the i-th wavelength (Clore & Chance, 1978).

On the basis of a qualitative interpretation of the data in Fig. 1, the following assignment of intermediates to each wavelength was made. The free mixed-valence-state cytochrome oxidase (E_M) and intermediates I_M, II_M and III_M were assigned to the 444 nm, 604 nm and 608 nm traces:

\[ \begin{align*}
W_{444} &= [E_M] \varepsilon_{444}(E_M) + [I_M] \varepsilon_{444}(I_M) \\
&\quad + [II_M] \varepsilon_{444}(II_M) + [III_M] \varepsilon_{444}(III_M) \\
W_{604} &= [E_M] \varepsilon_{604}(E_M) + [I_M] \varepsilon_{604}(I_M) \\
&\quad + [II_M] \varepsilon_{604}(II_M) + [III_M] \varepsilon_{604}(III_M) \\
W_{608} &= [E_M] \varepsilon_{608}(E_M) + [I_M] \varepsilon_{608}(I_M) \\
&\quad + [II_M] \varepsilon_{608}(II_M) + [III_M] \varepsilon_{608}(III_M)
\end{align*} \]  

(7)

In the case of the 830 nm traces, the absorbance of E_M (which contains e.p.r.-detectable cupric copper, Cu_{A^2+}) is used as the baseline during digitization so that \( \varepsilon_{830}(E_M) \) is given a priori a value of zero and only intermediates I_M, II_M and III_M are assigned to the 830 nm traces:

\[ \begin{align*}
W_{830} &= [I_M] \varepsilon_{830}(I_M) + [II_M] \varepsilon_{830}(II_M) + [III_M] \varepsilon_{830}(III_M)
\end{align*} \]  

(8)

The crude computed absorbance, in units of concentration (given by eqn. 6), is converted into a
Fig. 1. Observed kinetics of the reaction of ferricyanide-pretreated mixed-valence-state membrane-bound cytochrome oxidase with O₂ at 173 K as measured at four wavelength pairs
Symbols: O, 444–463 nm; ●, 604–630 nm; ▲, 608–630 nm; ▲, 830–940 nm. Theoretical curves are shown as solid lines. The experimental conditions are: bovine heart mitochondria, 15 mg/ml containing 5 μM-cytochrome oxidase, 30% (v/v) ethylene glycol, 0.2 M-mannitol, 0.75 M-sucrose, 50 mM-sodium phosphate buffer, pH 7.2, 10 mM-succinate, 10 mM-glutamate and 1.2 mM-CO in the presence of 360 μM-O₂ (a) and 520 μM-O₂ (b).

percentage absorbance change [N(t)/%] by means of a scale factor and offset:

\[ N(t)\% = \left( \frac{W(t) - S_o}{D_o} \right) \times 100 \]  

where \( S_o \) are the scale factors and \( D_o \) the offsets. [Offsets are only required for the 444, 604 and 608 nm traces. In the former trace, characterized by a progressive decrease in absorbance and in the latter two traces, characterized by an initial decrease in absorbance followed by an absorbance increase, the experimental points with the minimum absorbance were given a value of zero; however, these points do not correspond to a zero value of \( W \) and consequently an offset is required. In the case of the 830 nm traces, which are characterized by a progressive increase in absorbance, the first experimental point (at \( t = 0 \)) was given a value of zero; since \( W_{830} \) at \( t = 0 \) is zero, no offset is required.]

In the initial optimization, all the following parameters were varied simultaneously:

(a) The rate constants \( k_{+1}, k_{-1}, k_{+2}, k_{-2}, k_{+3} \) and \( k_{-3} \).

(b) The relative absorption coefficients of intermediates \( I_M, I_{II_M} \) and \( I_{III_M} \) at 444 nm; these were varied relative to \( \varepsilon_{444}(I_M) \), which was arbitrarily set to 1.0. The relative absorption coefficients of \( E_M \) and intermediates \( I_M, I_{II_M} \) and \( I_{III_M} \) at 504 and 608 nm; these were varied relative to \( \varepsilon_{504}(III_M) \) and \( \varepsilon_{608}(III_M) \) respectively, which were arbitrarily set to 1.0. The relative absorption coefficients of intermediates \( I_M, I_{II_M} \); these were varied relative to \( \varepsilon_{504}(III_M) \), which was arbitrarily set to 1.0.

(c) The scale factors and offsets for each wavelength at each O₂ concentration (S_{444A}, D_{444A}, S_{504A}, D_{504A}, S_{608A}, D_{608A}, S_{630A}, D_{630A}, S_{830A}, D_{830A}; the subscripts A and B refer to the O₂ concentrations of 360 and 520 μM respectively.)

All the parameters were well determined except for the rate constant \( k_{-3} \); the offsets at 444 nm (\( D_{444A} \) and \( D_{444B} \)) and the relative absorption coefficients \( \varepsilon_{444}(I_M), \varepsilon_{444}(I_{II_M}), \varepsilon_{504}(III_M), \varepsilon_{608}(III_M) \) and \( \varepsilon_{630}(III_M) \), whose values were both small and very poorly determined (s.d. > 10), indicating that the value of these parameters was essentially zero. This indicated that the formation of intermediate \( III_M \) is essentially irreversible at this temperature, and that

Table 1. Values of the correlation indices for each curve, the mean absolute correlation index, \( \chi^2 \) and the overall s.d.

The correlation index \( (C) \) is a measure of the distribution of residuals. For \( |C| < 1.0 \), the distribution of residuals is random; for \( |C| \geq 1.0 \), the deviations between the calculated and observed values are systematic. Mean absolute correlation index \( (C) \):

\[ C = \frac{1}{k} \sum_{i=1}^{k} |C_i|, \text{ where } k \text{ is the number of curves} \]

\( \chi^2 \): 213 for 217 degrees of freedom (240 observations and 23 parameters). For values of \( \chi^2 > 100 \), where \( k \) is the number of degrees of freedom, the confidence limits for \( \chi^2 \) are given by \( \sqrt{(2k-1)\chi^2}\)F, where \( \chi^2 \) is the value of the standard normal variable at the \( \alpha/2 \) confidence level and \( \xi \) is the fractional error in the estimation of the overall standard error of the data (in this case 0.13). The 99% confidence interval for \( \chi^2 \) is 1.98. The overall standard error of the data is 2 ± 0.26%, with a 99% confidence interval of 1.33–2.67%.

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>360 μM-O₂</th>
<th>520 μM-O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>444–463</td>
<td>0.779</td>
<td>-0.162</td>
</tr>
<tr>
<td>604–630</td>
<td>-0.0911</td>
<td>0.00608</td>
</tr>
<tr>
<td>608–630</td>
<td>-0.110</td>
<td>0.174</td>
</tr>
<tr>
<td>830–940</td>
<td>-0.287</td>
<td>0.288</td>
</tr>
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</table>

1978
**Table 2. Optimized values of the parameters together with their S.D. and confidence limits**

<table>
<thead>
<tr>
<th>Parameter number</th>
<th>Parameter</th>
<th>Dimensions</th>
<th>Optimized value</th>
<th>S.D.</th>
<th>Confidence limits</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5%</td>
</tr>
<tr>
<td>1</td>
<td>(k_{a1})</td>
<td>M(^{-1}) s(^{-1})</td>
<td>76.0</td>
<td>0.121</td>
<td>62.2</td>
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<td>2</td>
<td>(k_{b1})</td>
<td>s(^{-1})</td>
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<td>0.0927</td>
<td>0.0193</td>
</tr>
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<td>(k_{a2})</td>
<td>s(^{-1})</td>
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<td>0.0756</td>
<td>0.0141</td>
</tr>
<tr>
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<td>(k_{b2})</td>
<td>s(^{-1})</td>
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<td>0.288</td>
<td>0.000242</td>
</tr>
<tr>
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<td>(k_{a3})</td>
<td>s(^{-1})</td>
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<td>6</td>
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<td>1 \times 10^{-66}</td>
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<td>7</td>
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</tr>
<tr>
<td>8</td>
<td>(\epsilon_{l2}(\lambda_m))^\dagger</td>
<td></td>
<td>0*</td>
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</tr>
<tr>
<td>9</td>
<td>(\epsilon_{l3}(\lambda_m))^\dagger</td>
<td></td>
<td>0*</td>
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<td>(\epsilon_{l6}(\lambda_m))^\dagger</td>
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<td>0.0512</td>
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<td>(\epsilon_{l9}(\lambda_m))^\dagger</td>
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<td>0.0249</td>
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<td>(\epsilon_{l10}(\lambda_m))^\dagger</td>
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<tr>
<td>17</td>
<td>(\epsilon_{l11}(\lambda_m))^\dagger</td>
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</tr>
<tr>
<td>18</td>
<td>(S_{l444})</td>
<td>(\mu M^{-1})</td>
<td>0.200</td>
<td>0.0519</td>
<td>0.184</td>
</tr>
<tr>
<td>19</td>
<td>(D_{l444})</td>
<td>(\mu M^{-1})</td>
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<tr>
<td>20</td>
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<td>(\mu M^{-1})</td>
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<td>21</td>
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<td>(\mu M^{-1})</td>
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<tr>
<td>22</td>
<td>(S_{0044})</td>
<td>(\mu M^{-1})</td>
<td>0.302</td>
<td>0.0433</td>
<td>0.281</td>
</tr>
<tr>
<td>23</td>
<td>(S_{0064})</td>
<td>(\mu M^{-1})</td>
<td>0.504</td>
<td>0.128</td>
<td>0.408</td>
</tr>
<tr>
<td>24</td>
<td>(S_{0066})</td>
<td>(\mu M^{-1})</td>
<td>0.287</td>
<td>0.0385</td>
<td>0.269</td>
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<tr>
<td>25</td>
<td>(S_{0068})</td>
<td>(\mu M^{-1})</td>
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<td>0.128</td>
<td>0.349</td>
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<td>26</td>
<td>(S_{0084})</td>
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<td>0.375</td>
<td>0.0481</td>
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<td>28</td>
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<td>0.0413</td>
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<td>29</td>
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<td>0.497</td>
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<tr>
<td>30</td>
<td>(S_{0204})</td>
<td>(\mu M^{-1})</td>
<td>0.201</td>
<td>0.0139</td>
<td>0.196</td>
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<tr>
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<td>(S_{0208})</td>
<td>(\mu M^{-1})</td>
<td>0.201</td>
<td>0.00633</td>
<td>0.199</td>
</tr>
</tbody>
</table>

* \(k_{a3}\), \(\epsilon_{l4}(\lambda_m)\), \(\epsilon_{l5}(\lambda_m)\), \(\epsilon_{l6}(\lambda_m)\), \(\epsilon_{l7}(\lambda_m)\), \(\epsilon_{l8}(\lambda_m)\), \(\epsilon_{l9}(\lambda_m)\), \(\epsilon_{l10}(\lambda_m)\), \(\epsilon_{l11}(\lambda_m)\), \(S_{l444}\), \(D_{l444}\) and \(S_{l648}\) were constrained at these values on the basis of the initial optimizations in which their values were small and very poorly determined (S.D. > 10).

\dagger The relative absorption coefficients at 444, 604, 606 and 830nm are \(\epsilon_{l4}(\lambda), \epsilon_{l5}(\lambda), \epsilon_{l6}(\lambda), \epsilon_{l7}(\lambda)\) respectively, where \(\lambda\) is the intermediate referred to. The \(\epsilon_{l4}(\lambda)\) were varied relative to \(\epsilon_{l4}(\lambda_{\text{int}})\), which was given a value of 1.0. The \(\epsilon_{l5}(\lambda), \epsilon_{l6}(\lambda)\) and \(\epsilon_{l7}(\lambda)\) were varied relative to \(\epsilon_{l5}(\lambda_{\text{int}}), \epsilon_{l6}(\lambda_{\text{int}})\) and \(\epsilon_{l7}(\lambda_{\text{int}})\) respectively, which were given a value of 1.0.

The 444 nm trace only monitors the kinetics of \(E_{\text{int}}\). In the subsequent optimization these parameters were set to zero except for \(k_{a3}\), which was set to a suitably low value (1 \times 10^{-6} s\(^{-1}\)). In the resulting solution, all the optimized parameters were well determined. The values of the correlation indices for each curve, the mean absolute correlation index, \(\chi^2\) and the overall S.D. of the fit are shown in Table 1; the values of the optimized parameters together with their S.D. and confidence limits are shown in Table 2. The comparison of the experimental and the computed curves is shown in Fig. 1.

Fig. 2 illustrates the kinetics of the individual intermediates and their relationship to the absorbance changes at 444-463, 604-630, 608-630 and 830-940 nm. Starting 1s after photolysis of the CO-inhibited compound the traces begin with the free ferricyanide-pretreated mixed-valence-state cytochrome oxidase at a concentration greater than 95% of its initial value (i.e. 5 \(\mu M\)). Intermediate \(E_{\text{int}}\) rises first to a plateau of 35 and 42% of the total enzyme concentration at 360 \(\mu M\)- and 520 \(\mu M\)-O\(_2\) respectively. Intermediate \(I_{\text{int}}\) is rapidly converted into intermediate \(I_{\text{int}}\), which rises to a later and larger maximum (62 and 65% at 360 \(\mu M\)- and 520 \(\mu M\)-O\(_2\) respectively). The reason for the low maxima of intermediates \(I_{\text{int}}\) and \(I_{\text{int}}\) is because of their rapid conversion into intermediate \(I_{\text{int}}\), which is the final product of the reaction. Table 3 shows the calculated values of the half-times of formation and disappearance of the intermediates at the two O\(_2\) concentrations.
Fig. 2. Computed reaction kinetics of the individual intermediates and their relationship to the theoretical absorbance changes (in units of concentration) at 444–463, 604–630, 608–630 and 830–940 nm in the reaction of ferricyanide-pretreated mixed-valence-state membrane-bound cytochrome oxidase with O$_2$ at 173 K, as obtained by numerical integration of the differential equations representing a three-intermediate sequential mechanism, by using the values of the rate constants and relative absorption coefficients obtained by optimization.

An estimate of the stiffness of the system is given by the ratio of the longest to the shortest time constant: the shortest time constant is $1/(76.0(O_2)) = 25.3$ s, and the longest is $10^8$ s, giving a ratio of $3.95 \times 10^4$. Initial conditions: 5$\mu$m-free ferricyanide-pretreated mixed-valence-state cytochrome oxidase (E$_m$) in the presence of 360$\mu$m-O$_2$ (a) and 520$\mu$m-O$_2$ (b).

| Table 3. Calculated half-times of formation ($t_{1/2}^+$) and disappearance ($t_{1/2}^-$) of the intermediates at 360$\mu$m- and 520$\mu$m-O$_2$ at 173 K |
|-----------------|-----------------|-----------------|-----------------|
|                 | Intermediate    | Intermediate    | Intermediate    |
|                 | E$_m$           | I$_m$           | II$_m$          | III$_m$         |
| $t_{1/2}^+$ (s) at: |                 |                 |                 |                 |
| 360$\mu$m-O$_2$  | 8.41            | 70.8            | 596             |                 |
| 520$\mu$m-O$_2$  | 7.08            | 63.1            | 562             |                 |
| $t_{1/2}^-$ (s) at: |                 |                 |                 |                 |
| 360$\mu$m-O$_2$  | 37.6            | 168             | 841             |                 |
| 520$\mu$m-O$_2$  | 23.7            | 133             | 794             |                 |

**Discussion**

General assumptions in the assignment of valence states of the four metal centres to the intermediates

In the following sections we attempt to assign valence states to the four metal centres of cytochrome oxidase in each of the intermediates. In doing so we recognize that it may be an oversimplification to assume that electrons are localized on particular metal centres rather than being distributed in some statistical manner among them.

Only two species A$_2$ and C have been trapped at low temperatures in the reaction of ferricyanide-pretreated mixed-valence-state membrane-bound cytochrome oxidase with O$_2$ and characterized by optical wavelength scanning and e.p.r. spectroscopy (Chance et al., 1975b,c; Chance & Leigh, 1977; Denis & Chance, 1977). In the discussion that follows, we make the following general assumptions about the relationship of these two species with the three kinetically identified intermediates of this study.

(1) Compound A$_2$ is equivalent to intermediate II$_m$. This seems reasonable, since compound A$_2$ was trapped at 177 K 10s after flash photolysis (Chance et al., 1975c), and Fig. 3 shows that at 173 K the concentration of intermediates II$_m$ and III$_m$ is negligible relative to that of intermediate I$_m$ up to about 30s after flash photolysis.

(2) Compound C is equivalent to intermediate III$_m$. This seems reasonable, since compound C is the stable end product of the reaction in the range 163–265 K (Chance & Leigh, 1977).

Therefore in the following discussion the first and third intermediates in the reaction will be referred to as intermediates I$_m$ and III$_m$ rather than compounds A$_2$ and C.

In addition, we make the following assumptions about the nature of the contributions to the 444, 604, 608 and 830 nm traces.

(1) Only free haem a$_3$$^{2+}$ and free haem a$^{2+}$ (i.e. not interacting with O$_2$) contribute to the absorbance at 444 nm (Lemberg, 1969). Thus haem a$_3$$^{2+}$ and haem a$^{2+}$ directly interacting with O$_2$, haem a$_3$$^{3+}$ and haem a$^{3+}$, and higher valence states of haem iron [Fe(IV) and greater], do not contribute to the 444 nm traces. The latter assumption is by analogy with compound I of horseradish peroxidase in which haem iron is in the Fe(IV) state (Dolphin et al., 1971).

(2) Haem a$_3$$^{3+}$ and haem a$^{3+}$ do not contribute to the absorbance of the 600–610 nm band in the $\alpha$-region of the cytochrome oxidase spectrum (Wikström et al., 1976). Thus in the presence of both haem a$_3$$^{3+}$ and haem a$^{3+}$ the relative absorption coefficients at both 604 and 608 nm are zero.

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(3) The relative contribution of haem $a_{2}^{2+}$ and haem $a_{2}^{2+}$ at 604 nm and 608 nm may be affected by haem–haem (Wikström et al., 1976) and haem–copper (Palmer et al., 1976) interactions (by which is meant all mechanisms by which any modification in one of the metal components of cytochrome oxidase may affect the properties of the others), and by interactions with ligands (Wilson & Leigh, 1974).

(4) Copper is the major, if not only, contributor to the 830 nm traces (Aasa et al., 1976; Wever et al., 1977).

In the discussion that follows, we attempt to assign particular valence states to the haem and copper moieties in each intermediate on the basis of the optimized values of the relative absorption coefficients of the intermediates at each wavelength obtained from the present paper, and the optical wavelength scanning and e.p.r. data on intermediates I$_{m}$ and II$_{m}$ (Chance et al., 1975b,c; Chance & Leigh, 1977; Denis & Chance, 1977). The reaction sequence, together with the optimized values of the relative absorption coefficients, rate constants and equilibrium constants at 173 K, and the proposed valence states of the four metal centres in each intermediate, is summarized in Table 4.

Assignment of valence states of the four metal centres to the intermediates

The 830 nm traces are best fitted by nearly equal contributions from intermediates II$_{m}$ and III$_{m}$, and no contribution from E$_{m}$ and intermediate I$_{m}$ (see Tables 2 and 4). Since the absorbance of E$_{m}$ (in which Cu$_{A}$ is in the cupric state) is used as the baseline during digitization so that $e_{830}^{E}(E_{m})$ is given a priori a value of zero, and since the intensity of the e.p.r. signal due to cupric copper at $g = 2$ is approximately the same in E$_{m}$ and intermediates I$_{m}$ and III$_{m}$ (Chance & Leigh, 1977), we deduce, on the basis of our initial assumptions, that Cu$_{A}$ remains in the cupric state in intermediates I$_{m}$ and III$_{m}$, and that Cu$_{B}$ is in the cuprous state in intermediate I$_{m}$ and in the cupric state in intermediate III$_{m}$. The finding that $e_{830}^{E}(I_{m})$ is nearly equal to $e_{830}^{E}(III_{m})$ strongly suggests that the configuration of the copper atoms in intermediate III$_{m}$ is the same as that in intermediate III$_{m}$, namely Cu$_{A}^{2+}$Cu$_{B}^{2+}$. It should be noted, however, that the difference between $e_{830}^{E}(II_{m})$ and $e_{830}^{E}(II_{m})$, though small, is significant $e_{830}^{E}(II_{m}) - e_{830}^{E}(II_{m}) = 0.083 \pm 0.019$; also see Table 2), and we attribute this to copper–copper and/or copper–haem interactions.

The observation that the intensity of the e.p.r. signal due to low-spin ferric haem at $g = 3.05$ is approximately the same in E$_{m}$ and intermediates I$_{m}$ and III$_{m}$ strongly suggests that haem $a$ is in the ferric state in intermediates I$_{m}$ and III$_{m}$. Further evidence that haem $a$ remains in the ferric state in intermediate III$_{m}$ is afforded by the formation of intermediate III$_{m}$ at temperatures as high as 265 K (Chance & Leigh, 1977), where ferricyanide is the active oxidant of haem $a$ and its reduction to any significant extent would seem unlikely.

Tables 2 and 4 show that, within the errors specified, for the 444 nm traces:

$$
\begin{align*}
\epsilon_{444}^{E}(E_{m}) &= 1.0 \\
\epsilon_{444}^{E}(I_{m}) &= \epsilon_{444}^{E}(II_{m}) = \epsilon_{444}^{E}(III_{m}) = 0
\end{align*}
$$

Table 4. Proposed chemical identity of the intermediates and the reaction sequence at 173 K, with the optimized values of the rate constants and the reaction constants, and the calculated value of the equilibrium constants together with their s.d. and confidence limits

The s.d.$_{m}$ and 5–95% confidence limits of the equilibrium constants are calculated from the s.d.$_{m}$ of the individual rate constants (given in Table 2) and the correlation coefficient ($r$) relating the forward and backward rate constants by using the formula $(\psi_{r}^{2} - 2\psi_{r}\psi_{j} + \psi_{j})^{0.5}$, where $\psi_{r}$ and $\psi_{j}$ are the s.d.$_{m}$ of the forward and backward rate constants.

<table>
<thead>
<tr>
<th>Reaction sequence and rate constants at 173 K</th>
<th>$O_{2} + E_{m}$</th>
<th>$I_{m}$</th>
<th>$II_{m}$</th>
<th>$III_{m}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{-1} / k_{+1}$</td>
<td>76.0 M$^{-1}$ s$^{-1}$</td>
<td>0.0225 s$^{-1}$</td>
<td>0.0159 s$^{-1}$</td>
<td>0.01168 s$^{-1}$</td>
</tr>
<tr>
<td>$k_{+1} / k_{-1}$</td>
<td>0.00068 s$^{-1}$</td>
<td>0.0159 s$^{-1}$</td>
<td>0.00068 s$^{-1}$</td>
<td>0.01168 s$^{-1}$</td>
</tr>
<tr>
<td>Equilibrium constants ($k_{+1}/k_{-1}$)</td>
<td>3380 M$^{-1}$</td>
<td>23.4</td>
<td>1680</td>
<td></td>
</tr>
<tr>
<td>Correlation coefficient ($r$)</td>
<td>0.624</td>
<td>0.106</td>
<td>0.0961</td>
<td></td>
</tr>
<tr>
<td>s.d.$_{m}$</td>
<td>0.0961</td>
<td>0.290</td>
<td>0.0961</td>
<td></td>
</tr>
<tr>
<td>5–95% confidence limits</td>
<td>2890–3960 M$^{-1}$</td>
<td>14.5–37.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Relative absorption coefficients at:

<table>
<thead>
<tr>
<th>Absorption Coefficient at:</th>
<th>444–463 nm</th>
<th>604–630 nm</th>
<th>608–630 nm</th>
<th>830–940 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\epsilon_{444}(l)$</td>
<td>1.0 (100%)</td>
<td>0.0 (0%)</td>
<td>0.0 (0%)</td>
<td>0.0 (0%)</td>
</tr>
<tr>
<td>$\epsilon_{604}(l)$</td>
<td>0.422 (17.4%)</td>
<td>0.0 (0%)</td>
<td>1.00 (41.3%)</td>
<td>1.0 (41.3%)</td>
</tr>
<tr>
<td>$\epsilon_{608}(l)$</td>
<td>0.636 (27.2%)</td>
<td>0.0 (0%)</td>
<td>0.703 (30.0%)</td>
<td>1.0 (42.8%)</td>
</tr>
<tr>
<td>$\epsilon_{830}(l)$</td>
<td>0.0 (0%)</td>
<td>0.971 (47.8%)</td>
<td>1.0 (52.2%)</td>
<td></td>
</tr>
</tbody>
</table>

Proposed chemical identity of the intermediates:

$\frac{a_{2}^{2+}Cu_{A}^{2+}}{a_{2}^{2+}Cu_{A}^{2+}} \quad \frac{a_{2}^{2+}Cu_{A}^{2+}O_{2}^{-}}{a_{2}^{2+}Cu_{A}^{2+}O_{2}^{-}} \quad \frac{a_{2}^{2+}Cu_{A}^{2+}}{a_{2}^{2+}Cu_{A}^{2+}O_{2}^{-}} \quad \frac{a_{2}^{2+}Cu_{A}^{2+}O_{2}^{-}}{a_{2}^{2+}Cu_{A}^{2+}O_{2}^{-}}$

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for the 604 nm traces:
\[ e_{604}(III_M) = e_{604}(II_M) \]
\[ e_{604}(II_M) > e_{604}(I_M) \]
\[ e_{604}(I_M) = 0 \]  
(11)
and for the 608 nm traces:
\[ e_{608}(III_M) > e_{608}(II_M) > e_{608}(I_M) \]
\[ e_{608}(I_M) = 0 \]  
(12)

Since the relative absorption coefficients of intermediate \( I_M \) at both 604 and 608 nm are zero, we deduce, on the basis of our initial assumptions, that both haem \( a \) and haem \( a \) are in the ferric state in intermediate \( I_M \).

On the basis of the evidence presented so far, a basic scheme can be constructed (see Table 4). The valence states of haem \( a \) and haem \( a \) in intermediate \( II_M \), the valence state of haem \( a \) in intermediate \( III_M \) and the number of electrons donated to \( O_2 \) in intermediates \( II_M \) and \( III_M \) are not specified. The first step involves a one-electron reduction of \( O_2 \) resulting in the production of a superoxide ion \( (O_2^-) \) and the oxidation of haem \( a \) to the ferric state \( (a^{3+}O_2^-) \), rather than simple ligand binding without electron transfer to form an oxy compound of the ferrous haem \( (a^{1+}O_2) \) as suggested by Chance et al. (1975b,c). These authors have argued against the presence of \( O_2^- \) in intermediate \( I_M \) on the basis that they did not observe a high-spin haem e.p.r. signal at \( g = 6 \) (Chance et al., 1978) attributable to high-spin haem \( a^{3+} \) in the absence of anti-ferromagnetic coupling of haem \( a^{3+} \) to \( Cu^{2+} \) (Palmer et al., 1976) and broadening of the e.p.r. signals at \( g = 2.05 \) and \( g = 2 \) attributable to the presence of the paramagnetic \( O_2^- \) (Chance et al., 1975b,c). However, just as one of the unpaired electrons on high-spin haem \( a^{3+} \) is spin-coupled with the unpaired electron on \( Cu^{2+} \) resulting in the formation of an anti-ferromagnetically spin-coupled haem-copper dimer that is not visible by e.p.r. (Palmer et al., 1976; Thomson et al., 1976, 1977; Falk et al., 1977), it seems likely that one of the unpaired electrons on high-spin haem \( a^{3+} \) could also undergo spin-coupling with the unpaired electron \( O_2^- \) resulting in the formation of an exchange-coupled complex of even spin \( (S = 2 \) or 3\), which would be difficult or impossible to observe by e.p.r.

Any assignment of valence states of haem \( a \) and haem \( a \) in intermediate \( II_M \), and haem \( a \) in intermediate \( III_M \) must account for the following observations.

(a) The absence of any contribution from intermediates \( II_M \) and \( III_M \) to the 444 nm traces.

(b) The intense absorption band at 606-609 nm associated with intermediate \( III_M \) (Chance et al., 1975b,c; Chance & Leigh, 1977; Denis & Chance, 1977).

(c) The finding that there is a significant increase in the relative absorption coefficient of intermediate \( III_M \) with respect to that of intermediate \( II_M \) at 608 nm (\( e_{608}(III_M) - e_{608}(II_M) = 0.297 \pm 0.017 \); also see Table 2), but that the relative absorption coefficients of intermediates \( II_M \) and \( III_M \) at 604 nm are equal (i.e. this suggests the presence of a 606-609 nm band in intermediate \( II_M \), which is blue-shifted with respect to that of intermediate \( III_M \)).

(d) The 655 nm band, which, on the basis of e.p.r. (Beinert et al., 1976) and m.c.d. (Palmer et al., 1976) studies, is thought to arise as a consequence of anti-ferromagnetic coupling between high-spin haem \( a^{3+} \) and \( Cu^{2+} \), is not formed during the course of the reaction (Chance & Leigh, 1977; Denis & Chance, 1977).

(e) The stability of intermediate \( III_M \) at temperatures up to as high as 265 K (Chance & Leigh, 1977).

If we exclude all states where haem \( a^{3+} \) and haem \( a^{2+} \) coexist on the basis that the relative absorption coefficients of intermediates \( II_M \) and \( III_M \) are not zero at 604 and 608 nm, and assume that the maximum number of electrons that can be donated to \( O_2 \) is four, and that reduced \( O_2 \) species are not reoxidized, we are left with three alternative schemes to account for the above observations (see Fig. 3).

In Fig. 3 (Scheme 1), the formation of intermediate \( II_M \) involves an internal oxidation-reduction resulting in the oxidation of \( Cu^{2+} \) to the cupric state and the reduction of haem \( a \) to the ferrous state. This is followed by a further internal oxidation-reduction resulting in the re-oxidation of haem \( a \) to the ferric state and the reduction of haem \( a \) to the ferrous state in intermediate \( III_M \). Although the intense absorption at 606-609 nm could be accounted for by interaction between haem \( a^{2+} \), \( Cu^{2+} \) and \( O_2^- \) in intermediate \( II_M \), and between haem \( a^{3+} \), \( Cu^{2+} \) and \( O_2^- \) in intermediate \( III_M \) resulting in the generation of a charge transfer band, this scheme would have difficulty in accounting for both the absence of a 655 nm band and the absence of any contribution from intermediate \( II_M \) to the 444 nm traces. The unpaired electron on \( O_2^- \) could undergo spin-coupling with one of the unpaired electrons on high-spin haem \( a^{3+} \) and thereby prevent anti-ferromagnetic coupling between high-spin haem \( a^{3+} \) and \( Cu^{2+} \) (which would account for the absence of the 655 nm band), but it seems unlikely that \( O_2^- \) could interact at the same time with haem \( a^{2+} \) to account for the absence of any contribution of intermediate \( III_M \) to the 444 nm traces.

In Fig. 3 (Scheme 2), the formation of intermediate \( II_M \) involves an internal oxidation-reduction resulting in the oxidation of \( Cu^{2+} \) to the cupric state and the reduction of haem \( a \) to the ferrous state. The formation of intermediate \( III_M \) is then associated with a one-electron reduction of \( O_2^- \) to the \( O_2^{2-} \) state and the production of a free cation radical \( X^+ \).
Scheme 1
\[ \text{aq}^{+2}\text{Cu}^{2+}_b + \text{O}_2 \rightleftharpoons \text{aq}^{+2}\text{Cu}^{2+}_a \cdot \text{O}_2^- \]
\[ \text{aq}^{+2}\text{Cu}^{2+}_a \rightleftharpoons \text{aq}^{+2}\text{Cu}^{2+}_a \cdot \text{O}_2^- \]
\[ \text{aq}^{+2}\text{Cu}^{2+}_a \rightleftharpoons \text{aq}^{+2}\text{Cu}^{2+}_a \cdot \text{O}_2^- \]

Scheme 2
\[ \text{aq}^{+2}\text{Cu}^{2+}_b + \text{O}_2 \rightleftharpoons \text{aq}^{+2}\text{Cu}^{2+}_a \cdot \text{O}_2^- \]
\[ \text{aq}^{+2}\text{Cu}^{2+}_a \rightleftharpoons \text{aq}^{+2}\text{Cu}^{2+}_a \cdot \text{O}_2^- \]
\[ \text{aq}^{+2}\text{Cu}^{2+}_a \rightleftharpoons \text{aq}^{+2}\text{Cu}^{2+}_a \cdot \text{O}_2^- \]

Scheme 3
\[ \text{aq}^{+2}\text{Cu}^{2+}_b + \text{O}_2 \rightleftharpoons \text{aq}^{+2}\text{Cu}^{2+}_a \cdot \text{O}_2^- \]
\[ \text{aq}^{+2}\text{Cu}^{2+}_a \rightleftharpoons \text{aq}^{+2}\text{Cu}^{2+}_a \cdot \text{O}_2^- \]
\[ \text{aq}^{+2}\text{Cu}^{2+}_a \rightleftharpoons \text{aq}^{+2}\text{Cu}^{2+}_a \cdot \text{O}_2^- \]

Fig. 3. Three schemes for the reaction of ferricyanide-pretreated mixed-valence-state membrane-bound cytochrome oxidase with \( \text{O}_2 \).

Since it is not known at present at what stage cleavage of the O–O bond occurs, whether cleavage is homolytic or heterolytic, and whether protonation takes place at this low temperature, the reduced \( \text{O}_2 \) species are represented as \( \text{O}_2^- \), where \( x \) indicates the number of electrons donated to \( \text{O}_2 \).

In Fig. 3 (Scheme 3), the formation of intermediate \( \text{II}_M \) involves a two-electron reduction of \( \text{O}_2^- \) to the \( \text{O}_2^2^- \) state resulting in the oxidation of haem \( a \) and Cu\( b \) to the quadrivalent [Fe(IV)] and cupric states respectively. The formation of intermediate \( \text{III}_M \) is then associated with a one-electron reduction of \( \text{O}_2^2^- \) to the \( \text{O}_2^4^- \) state and the production of a free cation radical \( X^* \).

In Schemes 2 and 3 of Fig. 3, the free cation radical \( X^* \) in intermediate \( \text{III}_M \) is envisaged as a porphyrin \( \pi \)-cation radical as in compound I of horseradish peroxidase (Dolphin et al., 1971) rather than a protein–cation radical as in compound ES of cytochrome \( c \) peroxidase (Yonetani, 1976) on the basis that the free-radical e.p.r. signal at \( g = 2 \) in compound ES has not been observed in intermediate \( \text{III}_M \) (Chance et al., 1975b). In the case of a porphyrin \( \pi \)-cation radical, an electron localized on the porphyrin will couple through exchange interactions with spin-localized on the haem iron (Dolphin et al., 1971), resulting in broadening of the e.p.r. signal beyond the limit of detection. The known stability of a number of porphyrin \( \pi \)-cation radicals (Dolphin et al., 1971) would then explain the observed stability of intermediate \( \text{III}_M \) at temperatures as high as 265 K.

Both Schemes 2 and 3 of Fig. 3 account for the absence of a 655 nm band since neither scheme allows for anti-ferromagnetic coupling between high-spin haem \( a \) and Cu\( b \). The absence of any contribution of intermediates \( \text{II}_M \) and \( \text{III}_M \) to the 444 nm traces is also accounted for since in Scheme 2 haem \( a \) can interact directly with \( \text{O}_2^- \) and \( \text{O}_2^2^- \) in intermediates \( \text{II}_M \) and \( \text{III}_M \) respectively, and in Scheme 3 haem \( a \) and haem \( b \) are in the quadrivalent and ferric states respectively in intermediates \( \text{II}_M \) and \( \text{III}_M \). In the case of Scheme 2, the intense absorption band at 606–609 nm is attributed to a charge-transfer band arising from interaction between haem \( a \), Cu\( b \) and \( \text{O}_2^- \) in intermediate \( \text{II}_M \), and between haem \( a \), Cu\( b \), \( X^* \) and \( \text{O}_2^2^- \) in intermediate \( \text{III}_M \). In the case of Scheme 3, the 606–609 nm band is attributed to the presence of quadrivalent iron in intermediates \( \text{II}_M \) and \( \text{III}_M \) by analogy with the absorption bands in the 600–700 nm region attributed to quadravalent iron in compounds I and II of horseradish peroxidase (Dolphin et al., 1971) and compound ES of cytochrome \( c \) peroxidase (Yonetani, 1976).

On the basis of the evidence and arguments presented, Scheme 1 of Fig. 3 seems highly unlikely. Schemes 2 and 3 of Fig. 3, however, can both account equally well for the present data, and will require Mössbauer spectroscopy and low-temperature magnetic-susceptibility measurements to discriminate between them.

We thank Dr. B. Chance and Mr. A. R. Curtis for stimulating discussions, criticism and continual encouragement. We thank the Johnson Research Foundation for experimental facilities and the University College London Computer Centre for computing facilities. G. M. G. acknowledges a Carlo Camplin Scholarship.

References