The Reaction of *Pseudomonas aeruginosa* Cytochrome c-551 Oxidase with Oxygen

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The reaction of ascorbate-reduced *Pseudomonas* cytochrome oxidase with oxygen was studied by using stopped-flow techniques at pH7.0 and 25°C. The observed time courses were complex, the reaction consisting of three phases. Of these, only the fastest process, with a second-order rate constant of \(3.3 \times 10^4 \text{M}^{-1}\cdot\text{s}^{-1}\), was dependent on oxygen concentration. The two slower processes were first-order reactions with rates of \(1.0 \pm 0.4\text{s}^{-1}\) and \(0.1 \pm 0.03\text{s}^{-1}\). A kinetic titration experiment revealed that the enzyme had a relatively low affinity constant for oxygen, approx. \(10^4\text{M}^{-1}\). Kinetic difference spectra were determined for all three reaction phases, showing each to have different characteristics. The fast-phase difference spectrum showed that changes occurred at both the haem c and haem d\(_1\) components of the enzyme during this process. These changes were consistent with the haem c becoming oxidized, but with the haem d\(_1\) assuming a form that did not correspond to the normal oxidized state, a situation that was not restored even after the second kinetic phase, which reflected further changes in the haem d\(_1\) component. The results are discussed in terms of a kinetic scheme.

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Pseudomonas cytochrome oxidase was isolated and purified from cells of *Pseudomonas aeruginosa* (N.C.T.C. 6750) as described by Parr et al. (1976). The ratios of $A^{660}_{610}/A^{550}_{520}$ and $A^{550}_{520}/A^{410}_{464}$ were 1.18–1.20 and 1.15–1.20 respectively. The concentrations of *Pseudomonas* cytochrome oxidase solutions were determined by using an absorption coefficient at 410 nm of 149 000 litre·mol$^{-1}$·cm$^{-1}$ for the oxidized protein (Horio et al., 1961). Reduced cytochrome oxidase was prepared under an atmosphere of N$_2$ in a large (70ml) cuvette, sealed with a Suba-Seal (William Freeman and Co., Staincross, Barnsley, Yorks., U.K.) vaccine cap, by anaerobic addition of a slight stoichiometric excess of sodium ascorbate; under these conditions the time taken for complete reduction was of the order of 2h. Spectrophotometry was carried out with either a Cary 14 or a Cary 118c recording spectrophotometer. Stopped-flow experiments were performed by using an apparatus identical with that described by Gibson & Milnes (1964) equipped with a 2 cm-light-path cell and having a dead time of 3 ms. Total kinetic difference spectra have been plotted as the change in absorbance with wavelength occurring between $t = 3$ ms and $t_0$ after mixing in the stopped-flow apparatus. Kinetic difference spectra of reaction phases have been determined from semi-logarithmic analysis of progress curves. Logarithmic plots of slower reactions were used to provide ‘$t_0$’ baselines for faster processes, the spectroscopic amplitude for a particular phase being measured as the difference in absorbance between its extrapolated logarithmic plot, at $t = 3$ ms, and the $t_\infty$ line for the phase (see Gutfreund, 1972).

25°C at an oxygen concentration of 675 μM. The concentration of the enzyme was 9.1 μM before mixing and the reaction was observed in the stopped-flow apparatus by using a 2 cm-path-length cell. (b) A typical oscilloscope trace produced on mixing reduced *Pseudomonas* cytochrome oxidase with oxygen observed at 550 nm. The trace was collected in exactly the same manner and under exactly the same conditions as for (a). (c) Logarithmic plots of the reaction phases seen in Figs. 1(a) and (b). The fast phase of Fig. 1(a) (○) has been plotted with respect to the lower abscissa, the second fastest phase of Fig. 1(a) (●) (increasing absorbance) with respect to the upper abscissa and the fast phase of Fig. 1(b) (△) with respect to the lower abscissa.

![Fig. 1. Reaction of reduced Pseudomonas cytochrome oxidase with oxygen](image)

(a) A typical oscilloscope trace produced on mixing reduced *Pseudomonas* cytochrome oxidase with oxygen (observed at 660 nm). The vertical scale corresponds to Δψ of 0.02 per grid unit, and the horizontal scale represents sweep times of 20 ms, 500 ms and 5 s per grid unit for traces (1), (2) and (3) respectively. Trace (3) was obtained by manually retriggersing the oscilloscope immediately after the completion of trace (2). The reaction was conducted in 0.1 M-potassium phosphate buffer, pH 7.0, at...
Results

On mixing reduced *Pseudomonas* cytochrome oxidase with oxygen in the stopped-flow apparatus at pH 7.0, complex reaction profiles were observed. Figs. 1(c) and 1(d) show the progress curves at 660 and 550 nm respectively and their associated semilogarithmic analyses are given in Figs. 1(c) and 1(d). The overall reaction thus comprises three processes: a fast phase clearly seen at both wavelengths, followed by an intermediate phase most easily distinguished at 660 nm, and finally a much slower process of small amplitude at these wavelengths, but clearly visible in Fig. 2, where measurements were made at 460 and 395 nm. Of these three phases, only the rate of initial process was dependent on oxygen concentration over the range used in Fig. 3, which would indicate that this is a simple bimolecular process with a second-order rate constant of $3.3 \times 10^4 \text{M}^{-1}\cdot\text{s}^{-1}$. Fig. 3, which incorporates data collected at a number of wavelengths, also illustrates another important

\[ k (s^{-1}) \]
\[ [O_2] (\mu M) \]

**Fig. 3. Oxygen-dependence of the rate of the fast phase of the oxidation of reduced *Pseudomonas* cytochrome oxidase**

A plot of the observed pseudo-first-order rate constant ($k$) for the fast phase of the reaction of *Pseudomonas* cytochrome oxidase against oxygen concentration. The observations were made at: •, 660 nm; ○, 550 nm; ▲, 395 nm; ■, 460 nm.

pH 7.0, at 25°C at an oxygen concentration of 354 μM. The concentration of the enzyme was 4.5 μM before mixing and the reaction was observed in the stopped-flow apparatus in a 2cm-path-length cell. (b) A typical oscilloscope trace produced on mixing *Pseudomonas* cytochrome oxidase with oxygen at 395 nm. The trace was collected in exactly the same manner and under exactly the same conditions as for (a). (c) Logarithmic plots of the reaction phases seen in Figs. 2(a) and 2(b). The fast phases of Fig. 2(a) (○) and 2(b) (●) (increasing absorbance) have been plotted by using the lower abscissa. The second fastest (▲) and slowest (▲) phases of Fig. 2(b) (both increasing absorbance) have been plotted with respect to the upper abscissa.
feature of this reaction, namely that the rate of the fast phase was wavelength-independent. The inter-
mediate and slow phases were independent of oxygen concentration, with first-order rate constants of

\[ \text{Rate} = k \cdot [\text{O}_2] \]

where \( k \) is the rate constant and \([\text{O}_2]\) is the oxygen concentration. The difference spectrum of this reaction is shown in Fig. 4.

**Fig. 4. Total static difference spectrum and total kinetic difference spectrum for the reaction of oxygen with reduced Pseudomonas cytochrome oxidase**

The total static difference spectrum (-----) obtained on reducing 9.1 \( \mu \)M Pseudomonas cytochrome oxidase with a minimum excess of ascorbate, and the total kinetic difference spectrum determined after the protein had reacted with oxygen (○). The reactions were conducted in 0.1 M-potassium phosphate buffer, pH7.0 at 25°C.

![Difference spectrum](image)

**Fig. 5. Difference spectra of the fast and middle phases of the reaction of reduced Pseudomonas cytochrome oxidase with oxygen**

○, Kinetic difference of the fast phase (reduced Pseudomonas cytochrome oxidase minus species A in eqn. 1), and ○, the kinetic difference spectrum of the intermediate phase (species A minus species B of eqn. 1). The spectra were resolved as described in the Experimental section; the fast phase was effectively complete by \( t = 200 \text{ ms} \) and the intermediate phase by \( t = 6 \text{s} \).

![Difference spectra](image)

**Fig. 6. Kinetic difference spectrum of the slowest phase of the reaction of reduced Pseudomonas cytochrome oxidase with oxygen**

The difference spectrum of the slow phase (species B minus species C of eqn. 1) was determined from semi-logarithmic plots as described in the Experimental section.

![Difference spectrum](image)
Fig. 7. Absorption spectra of oxidized and reduced Pseudomonas cytochrome oxidase, and species A and B of eqn. (1) seen during the reaction with oxygen.

The absorption spectra of oxidized (-----) and reduced (-----) Pseudomonas cytochrome oxidase and the spectra of species A (○), and species B (●) seen during the reaction with oxygen. The spectrum of species A was generated by subtracting the amplitude of the fast phase of the oxygen reaction, wavelength by wavelength, from the spectrum of the reduced enzyme. The spectrum of species B was then produced by adding the amplitude of the intermediate phase to the spectrum of species A. All the spectra are for an enzyme concentration of 9.1 μM, in 0.1 M-potassium phosphate buffer, pH 7.0, and a path length of 1 cm.

Fig. 8. Variation of the amplitude of the fast phase during a 'kinetic' titration with oxygen.

The experiments were conducted in the stopped-flow apparatus with a concentration of 79 μM reduced Pseudomonas cytochrome oxidase before mixing. The reactions were carried out in 0.1 M-potassium phosphate buffer, pH 7.0, at 25°C, and were monitored at 665 nm. The amplitudes were calculated as the ΔA from the start of the reaction to that point in the reaction time course corresponding to the maximum difference in absorbance from the initial value.

Fig. 9. Dependence on oxygen concentration of the time course of oxidation of reduced Pseudomonas cytochrome oxidase.

Two typical reaction time courses produced during the titration shown in Fig. 8. Traces (1) and (2) were obtained at oxygen concentrations, after mixing, of 675 and 135 μM respectively. The reactions were followed in the stopped-flow apparatus at 665 nm, at an enzyme concentration of 79 μM before mixing, in 0.1 M-potassium phosphate buffer, pH 7.0, and at 25°C. The points in the Figure are the experimentally observed values, and the solid lines have been calculated as described in the text, by using values of $3 \times 10^4$ M$^{-1}$ s$^{-1}$, $1.5$ s$^{-1}$, and $2.6 s^{-1}$ for $k_{+1}$, $k_{-1}$ and $k_{+2}$, and $15 \times 10^3$, $5 \times 10^3$ and $12.5 \times 10^3$ litre mol$^{-1}$ cm$^{-1}$ for the absorption coefficients of the reduced enzyme and species A and B of eqn. (1).
9830A mini-computer and the changes in concentrations with time used to calculate changes in absorption by the application of absorption coefficients for reduced *Pseudomonas* cytochrome oxidase and species A and B of eqn. (1). These absorption coefficients may be estimated from the spectra given in Fig. 7, and values of $15 \times 10^3$, $5 \times 10^3$ and $12.5 \times 10^3$ litre mol$^{-1}$ cm$^{-1}$ have been used for reduced *Pseudomonas* cytochrome oxidase, species A and species B respectively. The values of $k_{+1}$, $k_{-1}$ and $k_{+2}$ in eqn. (1) were then derived empirically such that it was possible to reproduce closely the events observed during the course of the titration experiment in Fig. 8. In fact, some computations were carried out in which the absorption coefficient of species A was also varied, but the final value (quoted above) is very close to that which may be obtained from Fig. 7. By this method $k_{+1}$, $k_{-1}$ and $k_{+2}$ have been evaluated as $3 \times 10^4$ M$^{-1}$ s$^{-1}$, 1.5 s$^{-1}$ and 2.6 s$^{-1}$ respectively, and are thus in reasonable agreement with those rate constants derived from standard semi-logarithmic analysis.

The values of $k_{+1}$ and $k_{-1}$ lead to a value of approx. $10^4$ M$^{-1}$ for the affinity constant for oxygen binding to reduced *Pseudomonas* cytochrome oxidase and thus offer a rationalization of the non-stoichiometric binding behaviour seen in Fig. 8.

Discussion

Previous observations on the reaction of oxygen with reduced *Pseudomonas* cytochrome oxidase (Wharton & Gibson, 1976) led to the postulation of a sequential transfer of electrons through the enzyme to O$_2$. The basis of this hypothesis was these authors’ interpretation of the kinetic events in terms of a slower oxidation of the haem c than of haem $d_1$. The results obtained in the present investigation are in marked contrast with this, the reaction of both haem components occurring at the same rate. We have observed rates of haem c oxidation considerably in excess of the apparent rate limit of 8 s$^{-1}$ suggested by Wharton & Gibson (1976) to be the monomolecular internal electron-transfer rate, with no evidence of any limit to this rate over the range of oxygen concentrations that we have used.

Barber et al. (1977) have been able to resolve kinetically the difference spectra for the haem c and haem $d_1$ components of the enzyme, and a comparison of their results with those of the fast phase in Fig. 5 clearly shows that the haem c is oxidized during the fast initial process. The events occurring during the fast phase of oxidation appear to represent changes at both haems c and $d_1$. However, the absolute spectrum of species A, the first-formed intermediate in eqn. (1), shown in Fig. 7, is not consistent with haem $d_1$ having assumed its normal oxidized state. The same is also true for the absolute spectrum, in Fig. 7, which corresponds to species B in eqn. (1), the intermediate formed as a result of the middle phase of the overall reaction.

Examination of Fig. 4 reveals that, although there is a good correlation between the overall kinetic and static difference spectra (reduced-minus-oxidized), small discrepancies are particularly evident in the red (600–700nm) region of the spectra. This region is largely assigned to the $d_1$ haem (Barber et al., 1977), and possible explanations for the spectral differences between the final product, species C, and the normal oxidized enzyme must presumably be sought largely in terms of this component. Haem $d_1$ has been implicated by Shimada & Orii (1976) in the formation of an ‘oxygenated’ species of *Pseudomonas* cytochrome oxidase, which has spectral characteristics very similar to those of species B and C (the absorption changes associated with the slow phase are relatively small). However, it is not clear whether the ‘oxygenated’ species found by Shimada & Orii (1976) has a ‘real’ existence, or is, for example, a steady-state mixture of species A, B, C and the reduced and oxidized forms of *Pseudomonas* cytochrome oxidase. If this latter hypothesis is true, then, since the lifetime of species A is much less than that of either species B or C, a steady-state experiment of the type performed by Shimada & Orii (1976) would be expected for the most part to reflect these longer-lived intermediates.

Although the ‘oxygenated’ form was found to exist only transiently in the presence of a large excess of reductant, it does not follow that this is the case when the reagent in excess is oxygen. Nevertheless, although it is possible that species C may correspond to the ‘oxygenated’ enzyme and may decay only very slowly to the normal oxidized form of *Pseudomonas* cytochrome oxidase, it is also conceivable that the spectral anomalies seen in Fig. 4 arise from a slight denaturation of the protein over the course of the experiment; however, the good agreement between the total static and kinetic difference spectra (Fig. 4) over most of the wavelength range studied would argue against this latter explanation. The difference spectrum associated with the slow conversion of species B into C is very reminiscent of that which we have observed in the slowest phase of the reaction of reduced *Pseudomonas* cytochrome oxidase (Barber et al., 1978) with ferricyanide. Data from these experiments suggested that the haem $d_1$ had in fact assumed its oxidized state before this slow change, which, on this basis, might therefore be assigned to a conformational event. By analogy with the ferricyanide experiment, we would therefore conclude that, in the reaction of reduced *Pseudomonas* cytochrome oxidase with oxygen, all the expected redox changes in the enzyme occur before the conversion of species B into C.

The present kinetic results do not allow us to say which haem, c or $d_1$, is the binding site for oxygen, but ligand-binding evidence (Parr et al., 1975;
Yamanaka & Okunuki, 1963) suggests that the haem $d_1$ is the most likely site of attack for oxygen. A number of mechanisms may be written to describe possible electronic structures of the enzyme in species A and B of eqn. (1), although detailed discussion of them would not appear to be justified on the basis of the results shown in the present paper. Nevertheless, our data demand that in all such mechanisms the haem c component must be in the oxidized state in species A, and therefore differ fundamentally from the work of Wharton & Gibson (1976); the reasons for the discrepancies between that previous work and our results are not clear, but may lie in that the conditions used in the two cases were slightly different (0.05 M-potassium phosphate buffer, pH 6.6, 20°C, as opposed to 0.1 M-potassium phosphate buffer, pH 7.0, 25°C).

It is interesting to note an apparent contrast in the kinetic behaviour of the reduced enzyme towards the classical respiratory inhibitors CO and CN$^-$ and the reaction with oxygen. Experiments conducted by Parr et al. (1975) have shown that the CO-combination reaction is biphasic, and we have shown (D. Barber, S. R. Parr & C. Greenwood, unpublished work) that a similar situation is found with CN$^-$; with both these ligands the enzyme would be expected to offer two binding sites per molecule. The initial fast phase in the reaction with oxygen would, however, appear to be a monophasic process, although it apparently corresponds to a concerted ligand-binding and electron-transfer reaction. Taken together these facts may indicate that the binding of oxygen to the enzyme is not comparable with the binding of the respiratory poisons, i.e. the enzyme may bind only a single oxygen molecule.

If we are correct in our assumption that oxygen binding occurs only at the haem $d_1$, then the fast oxygen-dependent reaction of the haem c calls for internal electron-transfer rates of at least 100 s$^{-1}$. Rates of this order are greatly in excess of those that have been observed for the electron transfer from haem c to haem $d_1$ in the anaerobic reduction of oxidized *Pseudomonas* cytochrome oxidase by azurin, i.e. 0.25 s$^{-1}$ (Parr et al., 1977).

This behaviour would therefore appear to parallel that of mammalian cytochrome c oxidase (Gibson et al., 1965; Greenwood & Gibson, 1967), in which the presence of the substrate, oxygen, greatly increased the electron-transfer rates within the protein, although it is clear that the rate of oxygen reduction by the bacterial enzyme is very much lower than that of the mitochondrial oxidase. It is noteworthy that Parr et al. (1977) have observed that changes in the electron-transfer behaviour of the haem c may also be brought about by binding CO to reduced *Pseudomonas* cytochrome oxidase.

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References


Shimada, H. & Orii, Y. (1976) *J. Biochem. (Tokyo)* 80, 135–140


