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

By COLIN GREENWOOD, DONALD BARBER and STEPHEN R. PARR*
School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

and ERALDO ANTONINI, MAURIZIO BRUNORI and ALFREDO COLOSIMO
C.N.R. Centre of Molecular Biology, Institute of Chemistry, Faculty of Medicine, University of Rome, Rome, Italy

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The reaction of ascorbate-reduced *Pseudomonas* cytochrome oxidase with oxygen was studied by using stopped-flow techniques at pH 7.0 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 3.3×10^4 M^{-1}·s^{-1}, was dependent on oxygen concentration. The two slower processes were first-order reactions with rates of 1.0 ± 0.4 s^{-1} and 0.1 ± 0.03 s^{-1}. A kinetic titration experiment revealed that the enzyme had a relatively low affinity constant for oxygen, approx. 10^4 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.

Pseudomonas cytochrome *c* oxidase (ferrocytochrome *c*-551–O_2 oxidoreductase, EC 1.9.3.2) is the enzyme that functions in terminal electron transfer in cells of *Pseudomonas aeruginosa* grown anaerobically in the presence of NO_3^- (Yamanaka *et al.*, 1963). Although the true physiological function of the enzyme appears to be the reduction of nitrite (NO_2^-) to NO (Yamanaka *et al.*, 1961), considerable interest surrounds the initially observed ability (Horio *et al.*, 1958) to reduce oxygen.

Pseudomonas cytochrome oxidase is a water-soluble enzyme composed of two subunits each containing haem *c* and haem d_1 prosthetic groups (Kuronen & Ellfolk, 1972). Ligand-binding studies on the reduced enzyme have shown that both CO and cyanide bind to the haem d_1 component (Parr *et al.*, 1975; Yamanaka & Okunuki, 1963), and on this basis it has been assumed that this particular redox centre is also the site of attack by oxygen.

Two previous attempts at understanding the detailed mechanism of the reaction of *Pseudomonas* cytochrome oxidase with oxygen have been reported. Shimada & Orii (1976) have used rapid-scanning spectrophotometry to observe the reduction of the enzyme by excess ascorbate in the presence of oxygen. They obtained evidence for the formation of a transient 'oxygenated' species of the enzyme during the course of their experiment. A more direct study in which the oxidation of the ascorbate-reduced enzyme by oxygen was monitored during rapid-mixing experiments has been reported by Wharton & Gibson (1976). This latter procedure has also been the experimental basis of the present study. However, as reported below, we have observed significant differences from the previous studies which carry considerable implications with regard to possible mechanisms of oxygen reduction.

* Present address: Department of Biochemistry, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 7AG, Scotland, U.K.

Experimental

All chemicals were obtained from Fisons, Loughborough, Leics., U.K., and were of analytical-reagent grade. O_2-free N_2 was obtained either from British Oxygen Co. (London S.W.19, U.K.) or from the Societa Italiana Ossigeno (Rome, Italy). They were dispensed from the cylinder and stored in glass vessels over an alkaline solution of dithionite-reduced anthraquinonesulphonate before use (Greenwood *et al.*, 1974). Medically pure oxygen was obtained from the same sources, and oxygen-saturated solutions were prepared by bubbling the

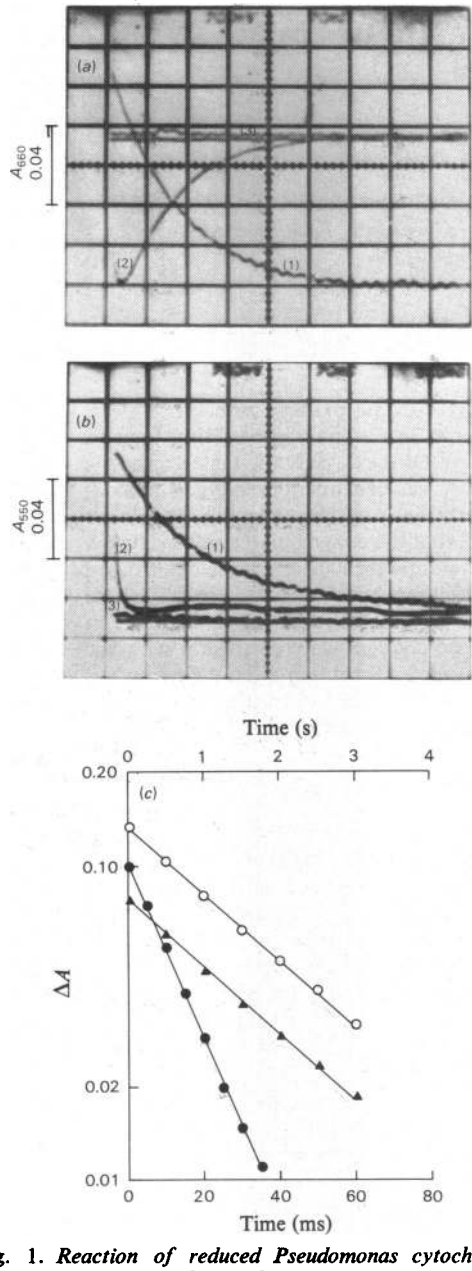

Fig. 1. *Reaction of reduced Pseudomonas cytochrome oxidase with oxygen*

(*a*) A typical oscilloscope trace produced on mixing reduced *Pseudomonas* cytochrome oxidase with oxygen (observed at 660nm). The vertical scale corresponds to ΔA of 0.02 per grid unit, and the horizontal scale represents sweep times of 20ms, 500ms and 5s per grid unit for traces (1), (2) and (3) respectively. Trace (3) was obtained by manually retriggering the oscilloscope immediately after the completion of trace (2). The reaction was conducted in 0.1M-potassium phosphate buffer, pH7.0, at 25°C at an oxygen concentration of 675μM. The concentration of the enzyme was 9.1μM before mixing and the reaction was observed in the stopped-flow apparatus by using a 2cm-path-length cell. (*b*) A typical oscilloscope trace produced on mixing reduced *Pseudomonas* cytochrome oxidase with oxygen observed at 550nm. 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.

gas through the appropriate buffer solution at room temperature (20°C) and atmospheric pressure for 10–15min. The concentrations of oxygen-saturated solutions were found by reference to the appropriate published Tables (Kaye & Laby, 1966). Solutions of oxygen, less than saturated, were prepared by serial dilution of the saturated stock by using buffer equilibrated with O_2-free N_2.

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 $A^{ox.}_{410}/A^{ox.}_{280}$ and $A^{ox.}_{640}/A^{ox.}_{520}$ 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 410nM of 149000 litre$\cdot$mol$^{-1}\cdot$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 stoicheiometric 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 2cm-light-path cell and having a dead time of 3ms. Total kinetic difference spectra have been plotted as the change in absorbance with wavelength occurring between $t = 3$ms and t_∞ 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_∞' 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_∞ line for the phase (see Gutfreund, 1972).

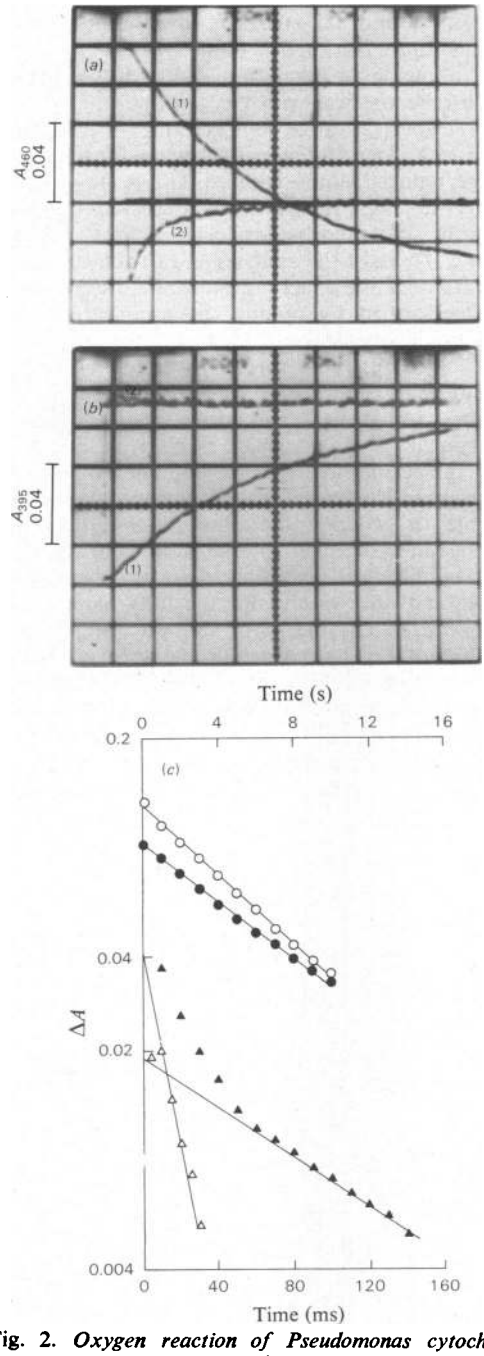

Fig. 2. *Oxygen reaction of Pseudomonas cytochrome oxidase*

(*a*) A typical oscilloscope trace produced on mixing *Pseudomonas* cytochrome oxidase with oxygen, observed at 460nm. The vertical scale corresponds to ΔA of 0.02 per grid unit, and the horizontal scale represents sweep times of 20ms and 5s per grid unit for traces (1) and (2) respectively. The reaction was conducted in 0.1M-potassium phosphate buffer, pH7.0, at 25°C at an oxygen concentration of 354μM. The concentration of the enzyme was 4.5μM 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 395nm. 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.

Results

On mixing reduced *Pseudomonas* cytochrome oxidase with oxygen in the stopped-flow apparatus at pH7.0, complex reaction profiles were observed. Figs. 1(*a*) and 1(*b*) show the progress curves at 660 and 550nm respectively and their associated semi-logarithmic 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 660nm, 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 395nm. 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 $3.3\times10^4\,M^{-1}\cdot s^{-1}$. Fig. 3, which incorporates data collected at a number of wavelengths, also illustrates another important

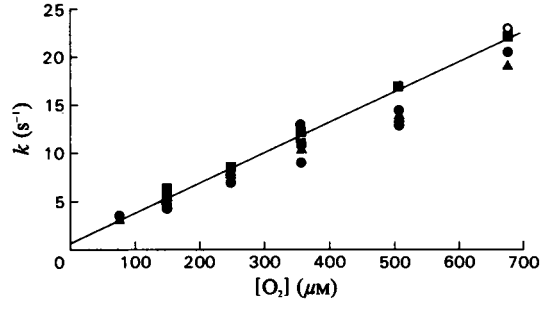

Fig. 3. *Oxygen-dependence of the rate of the fast phase of the oxidation of reduced Pseudomonas cytochrome oxidase*

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: ●, 660nm; ○, 550nm; ▲, 395nm; ■, 460nm.

feature of this reaction, namely that the rate of the fast phase was wavelength-independent. The intermediate and slow phases were independent of oxygen concentration, with first-order rate constants of $1.0\pm0.4\,s^{-1}$ and $0.1\pm0.03\,s^{-1}$, over the range of oxidant concentration 675–75 μM.

By following the reaction of reduced *Pseudomonas* cytochrome oxidase with oxygen at 5nm intervals over the spectral range 400–700nm, it is possible to construct a kinetic difference spectrum for the overall change, reduced minus oxidized. This is shown in Fig. 4, together with the statically observed difference spectrum of ascorbate-reduced minus oxidized enzyme. To assist the analysis and to achieve a clear separation of the kinetic events, these experiments were performed by mixing the ascorbate-reduced enzyme with oxygen-saturated buffer. Fig. 5 shows the resolved difference spectra for the fast and intermediate phases seen during the reaction of the reduced enzyme with oxygen. The corresponding difference spectrum for the slow phase is given in Fig. 6. By using the results in Fig. 5 in conjunction with the spectrum of the fully reduced enzyme, it has been possible to generate the absolute spectra of the intermediates (species A and B in eqn. 1 below) observed during the reaction; these are given in Fig. 7 along with the spectra for the fully oxidized and fully reduced enzyme, over the wavelength range 580–700nm. Fig. 8 gives details of a 'kinetic' titration experiment in which the amplitude of the fast phase has been used to measure the affinity of the enzyme for oxygen. To perform this type of experiment it is necessary to use a high concentration of enzyme, with the result that, at the lower oxygen concentra-

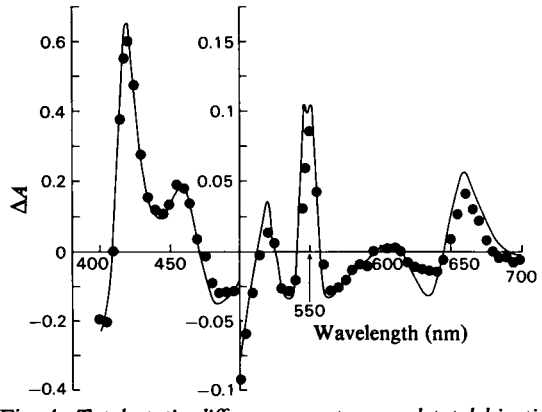

Fig. 4. *Total static difference spectrum and total kinetic difference spectrum for the reaction of oxygen with reduced Pseudomonas cytochrome oxidase*

The total static difference spectrum (——) obtained on reducing 9.1 μM *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, pH 7.0 at 25°C.

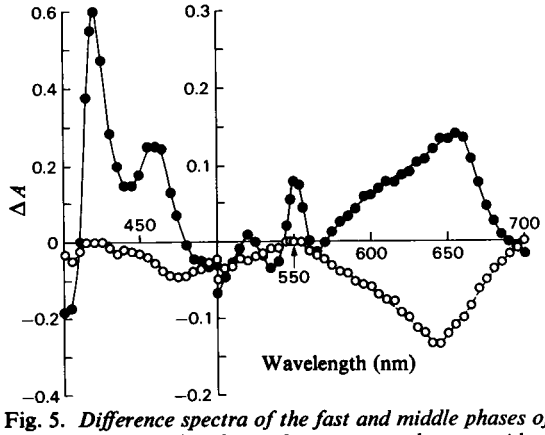

Fig. 5. *Difference spectra of the fast and middle phases of the reaction of reduced Pseudomonas cytochrome oxidase with oxygen*

●, 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 $t = 200$ms and the intermediate phase by $t = 6$s.

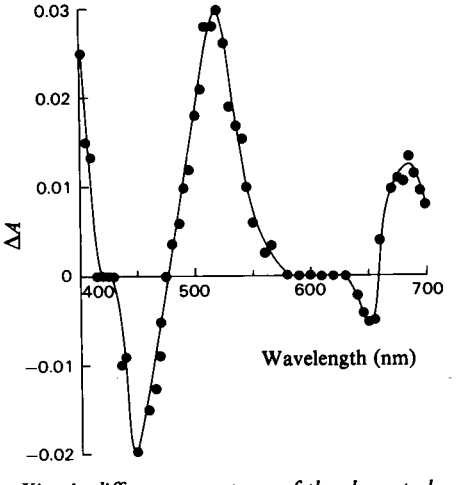

Fig. 6. *Kinetic difference spectrum of the slowest phase of the reaction of reduced Pseudomonas cytochrome oxidase with oxygen*

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.

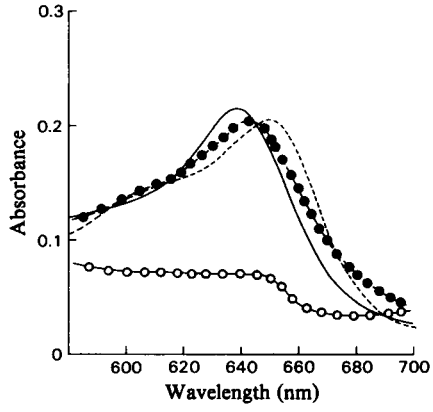

Fig. 7. *Absorption spectra of oxidized and reduced Pseudomonas cytochrome oxidase, and species A and B of eqn.* (1) *seen during the reaction with oxygen*

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.

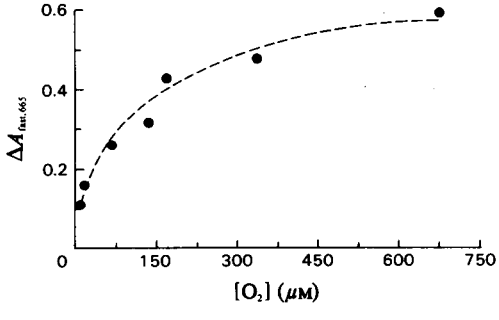

Fig. 8. *Variation of the amplitude of the fast phase during a 'kinetic' titration with oxygen*

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.

tions used, the amount of terminal electron acceptor was sub-stoicheiometric. These conditions have led to a situation where the fast and intermediate phases approach each other in rate and have necessitated a much more rigorous kinetic analysis of the type presented in Fig. 9. The solid lines in Fig. 9 represent the solution of the differential equations arising from eqn. (1) below, which represents the minimum explanation of the kinetic data:

$$O_2 + \text{fully reduced enzyme} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} A \xrightarrow{k_{+2}} B \rightarrow C \qquad (1)$$

For the purposes of the present calculations a simplified model of eqn. (1) has been used: the final very slow phase leading to the formation of species C has been neglected and the conversion of the reduced enzyme into species A treated as a first-order process. A bimolecular character was imparted to the reaction forming species A by allowing the corresponding first-order rate constant to vary, in a manner consistent with a linear dependence on oxygen concentration. The simultaneous differential equations arising from the model were solved by a Runge–Kutta method on a Hewlett Packard

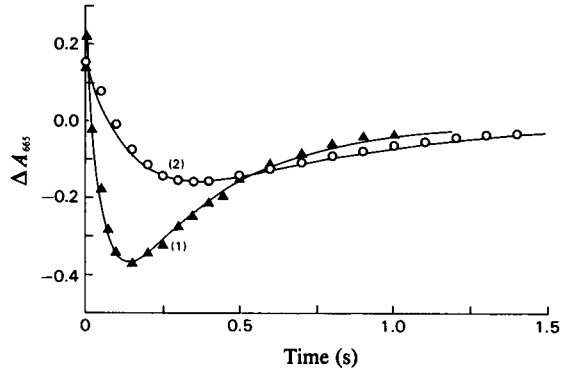

Fig. 9. *Dependence on oxygen concentration of the time course of oxidation of reduced Pseudomonas cytochrome oxidase*

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 3×10^4 M$^{-1}\cdot$s^{-1}, 1.5 s^{-1} and 2.6 s^{-1} for k_{+1}, k_{-1} and k_{+2}, and 15×10^3, 5×10^3 and 12.5×10^3 litre$\cdot$mol$^{-1}\cdot$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 15×10^3, 5×10^3 and 12.5×10^3 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 3×10^4 M^{-1}·s^{-1}, 1.5s^{-1} and 2.6s^{-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-stoicheiometric 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 8s^{-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 (0.05M-potassium phosphate buffer, pH6.6, 20°C, as opposed to 0.1M-potassium phosphate buffer, pH7.0, 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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