The Electron-Transfer Reaction between Azurin and the Cytochrome *c* Oxidase from *Pseudomonas aeruginosa*

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A stopped-flow investigation of the electron-transfer reaction between oxidized azurin and reduced *Pseudomonas aeruginosa* cytochrome *c*-551 oxidase and between reduced azurin and oxidized *Ps. aeruginosa* cytochrome *c*-551 oxidase was performed. Electrons leave and enter the oxidase molecule via its haem *c* component, with the oxidation and reduction of the haem *d*₁ occurring by internal electron transfer. The reaction mechanism in both directions is complex. In the direction of oxidase oxidation, two phases assigned on the basis of difference spectra to haem *c* proceed with rate constants of $3.2 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $2.0 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, whereas the haem *d*₁ oxidation occurs at $0.35 \pm 0.1 \text{ s}^{-1}$. Addition of CO to the reduced enzyme profoundly modifies the rate of haem *c* oxidation, with the faster process tending towards a rate limit of 200 s^{-1} . Reduction of the oxidase was similarly complex, with a fast haem *c* phase tending to a rate limit of 120 s^{-1} , and a slower phase with a second-order rate of $1.5 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$; the internal transfer rate in this direction was $0.25 \pm 0.1 \text{ s}^{-1}$. These results have been applied to a kinetic model originally developed from temperature-jump studies.

Since the initial work of Horio *et al.* (1958), the respiratory-chain system of the denitrifying bacterium *Pseudomonas aeruginosa* has received much attention. Particular interest has centred around the terminal respiratory component, *Pseudomonas* cytochrome *c*-551 oxidase (EC 1.9.3.2), and its electron-donating substrates, all of which are water-soluble and so provide a suitable system for studies *in vitro*.

Pseudomonas cytochrome oxidase is a bifunctional enzyme of two identical subunits each containing one haem *c* and one haem *d*₁ moiety (Kuronen & Ellfolk, 1972) and capable of utilizing either molecular oxygen or inorganic nitrite as the ultimate electron acceptor, although the latter appears to be the more important function physiologically (Yamanaka *et al.*, 1961). The *Pseudomonas* cytochrome oxidase can accomplish both functions, i.e. the four-electron reduction of oxygen to water or the single-electron reduction of nitrite to nitric oxide, by accepting electrons from either of two low-molecular-weight protein substrates, the haem-containing *Pseudomonas* cytochrome *c*-551 or the copper protein azurin (Horio *et al.*, 1961). All three proteins are produced in cells grown anaerobically in the presence of nitrate (Parr *et al.*, 1976), although the exact physiological interrelationship and

pathway of electron transfer *in vivo* between the proteins is not yet clearly defined.

It was shown that electron transfer occurs between *Pseudomonas* cytochrome *c*-551 and azurin, and this reaction has been extensively studied by using both stopped-flow and temperature-jump methods (Brunori *et al.*, 1974; Wilson *et al.*, 1975; Rosen & Pecht, 1976). These investigations have led to the formulation of a kinetic model requiring that the reduced azurin molecule should exist in two forms, only one of which is competent in electron transfer. Similar temperature-jump studies on the azurin-*Pseudomonas* cytochrome oxidase reaction (Brunori *et al.*, 1975) have reinforced this hypothesis and also indicate that electron transfer occurs within a molecular complex of the two proteins. This latter point was also proposed by Wharton *et al.* (1973) from stopped-flow data, although under different experimental conditions.

In the present paper, we report the results of stopped-flow experiments on the reaction between azurin and *Pseudomonas* cytochrome oxidase performed under the same experimental conditions used in our earlier (Brunori *et al.*, 1975) temperature-jump work, in an attempt to quantify some of the kinetic

parameters and extend previous observations on this system.

Materials and Methods

All chemicals were obtained from Fisons Scientific Apparatus, Loughborough, Leics., U.K., and were of Analytical-Reagent grade, except for sodium dithionite, which was a gift from Hardman and Holden, Miles Platting, Manchester, U.K., ascorbic acid (disodium salt) from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K., and Sephadex G-25 (coarse grade), which was purchased from Pharmacia (G.B.) Ltd., London W.5, U.K. CO and N₂ gases were obtained from British Oxygen Co., London S.W.19, U.K., and were dispensed from the cylinders and stored in glass vessels over an alkaline solution of sodium anthraquinonesulphonate.

Pseudomonas cytochrome oxidase and azurin were isolated and purified from cells of *Pseudomonas aeruginosa* (N.C.T.C. 6750) as described by Parr *et al.* (1976). The ratios of $A_{410}^{\text{ox}}:A_{280}$ and $A_{640}^{\text{ox}}:A_{520}^{\text{ox}}$ for the oxidized *Pseudomonas* cytochrome oxidase were 1.18–1.2 and 1.15–1.2 respectively, and the azurin used regularly had a ratio of 0.59 for $A_{625}^{\text{ox}}:A_{280}$. The concentration of *Pseudomonas* cytochrome oxidase was obtained by using the value $A_{410} = 149 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for the oxidized protein (Horio *et al.*, 1961). Azurin concentrations were determined by using $A_{625}^{\text{ox}} = 3.5 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Brill *et al.*, 1968).

Reduced *Pseudomonas* azurin was prepared immediately before use by the addition of a few grains

Fig. 1. Reaction between reduced *Pseudomonas* cytochrome oxidase and oxidized azurin

(a) Reaction trace observed on mixing $2.4 \mu\text{M}$ reduced *Pseudomonas* cytochrome oxidase with $220 \mu\text{M}$ oxidized azurin in the stopped-flow apparatus. The vertical scale represents a change of $0.02 A$ unit per division and the horizontal scale corresponds to 100 ms and 1 s per division for the upper and lower traces respectively (the horizontal trace is the base line). The reaction was carried out in 0.1 M-potassium phosphate buffer, pH 7.0 at 25°C , and observed at 422 nm in a 2 cm-path-length cell under an atmosphere of N₂. (b) Reaction trace observed at 460 nm when mixing $6 \mu\text{M}$ *Pseudomonas* cytochrome oxidase with $220 \mu\text{M}$ oxidized azurin in the stopped-flow apparatus. The vertical scale corresponds to a change of $0.02 A$ unit per division and the horizontal scale to 100 ms and 1 s per division for the upper and lower traces respectively (the horizontal trace is the base line). The conditions were as in (a). (c) Semi-logarithmic analysis of Fig. 1(a). The two faster phases ($\circ$, $\bullet$) are plotted by using the time scale on the upper abscissa and the slow phase ($\blacktriangle$) is plotted with reference to the lower abscissa. (d) Semi-logarithmic analysis of the slow phase observed in Fig. 1(b).

of sodium dithionite to the oxidized protein, followed by removal of excess of dithionite and its oxidation products on a Sephadex G-25 column (25cm×1.25cm) (Wilson *et al.*, 1975). Reduced *Pseudomonas* cytochrome oxidase was prepared under an atmosphere of N₂, in a 100 ml glass 1 cm-light-path cuvette that could be sealed with a Suba-Seal vaccine cap. Sodium ascorbate in a stoichiometric excess of 25% was then added and the reduction measured spectrophotometrically; under these conditions the time taken for complete reduction was approx 2h.

For experiments with the reduced *Pseudomonas* cytochrome oxidase-CO complex, the N₂ atmosphere was either replaced by an atmosphere of CO after ascorbate reduction or the *Pseudomonas* oxidase was allowed to autoreduce anaerobically by incubation under an atmosphere of CO (Barber *et al.*, 1976).

Spectrophotometry was carried out with a Cary 118c spectrophotometer. Anaerobic 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 3 ms.

Results

Reaction of reduced pseudomonas cytochrome oxidase with oxidized azurin

When ascorbate-reduced *Pseudomonas* cytochrome oxidase was mixed anaerobically in the stopped-flow apparatus with oxidized azurin under pseudo-first-order conditions, complex reaction traces were observed, reflecting changes in both the haem *c* and haem *d*₁ components of the oxidase. Fig. 1 shows the time course of oxidation of *Pseudomonas* cytochrome oxidase as followed at two wavelengths and the associated semilogarithmic analysis of the traces. At 422nm the records exhibited a triphasic character, whereas at 460nm the record was essentially monophasic. Figs. 2(a) and 2(b) indicate that the rates of the two faster phases observed at 422nm were dependent on azurin concentration, and, on the basis of Fig. 4, we assign these changes to the haem *c* component of the enzyme. The respective second-order rate constants derived from Figs. 2(a) and 2(b) are $3.2 \pm 0.5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $2.0 \pm 0.5 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, although the small relative amplitude associated with the fast phase leads to some uncertainty of the absolute value. The slowest rate observed at 422nm was found to be independent of the azurin concentration, with a rate constant of $0.35 \pm 0.1 \text{ s}^{-1}$; this process was the major contributor at 460nm, and on the basis of Fig. 4(b) we assigned it to oxidation of the haem *d*₁, with the reservation that, within the 'envelope' of the haem *c* Soret band, errors inherent in the analysis may have resulted in a slight overestimate of the haem *d*₁ contribution (see Barber *et al.*, 1977).

Fig. 2. Dependence on oxidized azurin concentration of the rate of oxidation of reduced *Pseudomonas* cytochrome oxidase

The dependence of the fast phase (a) and intermediate phase (b) are shown. The reactions were carried out anaerobically, in the stopped-flow apparatus, in 0.1 M-potassium phosphate buffer, pH 7.0. The oxidation was followed at 422nm in a 2cm-path-length cell at 25°C. The closed and open circles refer to different experiments. The oxidase concentration used was 6 μM, before mixing.

The effect of any slight excess of ascorbate on the kinetics has been ignored. As may be deduced from the time taken for the ascorbate titration, the reaction between this reducing agent and the enzyme is very slow at the concentrations used. Separate experiments on the ascorbate-reduction kinetics of azurin have shown that the reaction is also very slow (second-order rate constant $0.55 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 7.0, 20°C) and of negligible effect at the concentrations of ascorbate likely to be present.

By repeating experiments of the type described at a number of different wavelengths but at a single azurin concentration it is possible to construct a kinetic difference spectrum; in Fig. 3 this is compared with the static difference spectrum between ascorbate-reduced and oxidized *Pseudomonas* cytochrome oxidase. The small differences between these two spectra are not thought to arise as a result of the azurin con-

Fig. 3. Static and kinetic difference spectra

The total static (—) difference spectrum of ascorbate-reduced minus oxidized *Pseudomonas* cytochrome oxidase (concentration $6\mu\text{M}$) and the total kinetic difference spectrum (●) (t_0-t_∞) determined on anaerobically mixing the protein with $220\mu\text{M}$ -oxidized azurin. The kinetic observations were made in 0.1 M -potassium phosphate buffer, pH 7.0 at 25°C , by using the stopped-flow apparatus equipped with a 2 cm -path-length cell.

tribution, which is negligible in this spectral region. In those spectral regions where the absorption change is sufficient to permit a complete analysis of the progress curves, it is possible to determine the relative contribution of each phase and thereby generate the difference spectrum associated with each process (Figs. 4a and 4b). In experiments in which the azurin concentration was varied over the range $26\text{--}236\mu\text{M}$, no significant change in the relative proportion of the fast and intermediate phases could be detected, the fast phase representing 20–25% of the dependent, haem c, reaction.

Effect of CO on the reaction of reduced *Pseudomonas* cytochrome oxidase with azurin

Use of enzyme that had been either allowed to autoreduce in the presence of CO or prepared by the addition of CO to ascorbate-reduced *Pseudomonas* cytochrome oxidase before anaerobic mixing in the stopped-flow apparatus with oxidized azurin resulted in a dramatic change in the reaction profile,

Fig. 4. Difference spectra of kinetic phases observed during oxidation of reduced *Pseudomonas* cytochrome oxidase by oxidized azurin

(a) The difference spectra of the fast (○) ($t_0-t_{150\text{ ms}}$) and intermediate (●) (completed by $t = 2\text{ s}$) phases seen on mixing $6\mu\text{M}$ -reduced *Pseudomonas* cytochrome oxidase with $220\mu\text{M}$ -oxidized azurin in the stopped-flow apparatus. The reactions were carried out anaerobically in 0.1 M -potassium phosphate buffer, pH 7.0 at 25°C , in a 2 cm -path-length cell. The amplitudes of the two phases were determined by extrapolation of semilogarithmic plots to zero time. (b) The difference spectra of the sum of the fast and intermediate phases (○) and the slow phase (▲) produced on analysis of traces recorded under the conditions in (a).

Fig. 5. Reaction of oxidized azurin with the reduced CO complex of *Pseudomonas* cytochrome oxidase

(a) Oscilloscope trace produced on mixing $2.4\mu\text{M}$ *Pseudomonas* cytochrome oxidase-CO complex with $120\mu\text{M}$ oxidized azurin in the stopped-flow apparatus under an atmosphere of CO. The vertical scale corresponds to a change of $0.02A$ unit per division, and the horizontal scale represents time-scales of 10ms and 100ms per division for the upper and lower traces respectively (the horizontal trace is the base line). The reaction was carried out in 0.1 M-potassium phosphate buffer, pH7.0 at 25°C , in a 2cm-path-length cell and monitored at 422nm. (b) Semilogarithmic analysis of (a). The slow phase (●) was plotted by using the time scale on the lower abscissa, and the fast phase (○) is related to that of the upper abscissa.

Fig. 6. Dependence on oxidized azurin concentration of the rate of oxidation of the reduced *Pseudomonas* cytochrome oxidase-CO complex

(a) Dependence of the fast phase (●, plotted with reference to the right-hand ordinate) and the slow phase (○, plotted with reference to the left-hand ordinate) upon the concentration of oxidized azurin. The reactions were carried out in 0.1 M-potassium phosphate buffer, pH7.0 at 25°C under an atmosphere of CO. The time courses were followed at 422nm in the stopped-flow apparatus by using a 2cm-path-length cell and an oxidase concentration of $6\mu\text{M}$ before mixing. (b) A double-reciprocal plot of the fast-phase data in (a).

although the extent of the observed change is comparable with that observed for the two initial phases seen in the absence of CO. Fig. 5(a) shows that the oxidation process at 422nm is now biphasic, with the fast phase comprising the major portion (80%, after allowing for dead-time loss) of the change. Both phases were found to exhibit a concentration dependence with azurin (Fig. 6), with the fast phase tending towards a rate limit of 200s^{-1} (Fig. 6b). The slower rate in Fig. 6(a) showed a linear dependence, yielding a second-order rate constant of $2.4 \times 10^4 \text{M}^{-1} \cdot \text{s}^{-1}$, which is comparable with the intermediate phase seen in the absence of CO. The kinetic difference spectra of the two phases in Fig. 7 seem to indicate that both are signalling changes in the haem *c* and there is no detectable change in the haem *d*₁: this is consistent with previous observations by Wharton *et al.* (1973) and Brunori *et al.* (1975).

Fig. 7. Kinetic difference spectra of the phases observed during oxidation of the reduced *Pseudomonas* cytochrome oxidase-CO complex by oxidized azurin

The difference spectra of fast ($\circ$) ($t_0 - t_{200\text{ ms}}$) and slow ($\bullet$) (completed by t_∞) phases determined by analysis of reaction traces produced on mixing $6\text{ }\mu\text{M}$ -*Pseudomonas* cytochrome oxidase-CO complex with $120\text{ }\mu\text{M}$ oxidized azurin in the stopped-flow apparatus. The observations were made in 0.1 M -potassium phosphate buffer, pH 7.0 at 25°C under an atmosphere of CO, in a 2 cm -path-length cell. (See Fig. 4 for details of analysis procedure.)

Reaction of oxidized *Pseudomonas* cytochrome oxidase with reduced azurin

Fig. 8 shows that, when oxidized *Pseudomonas* cytochrome oxidase was mixed in anaerobic conditions with reduced azurin, a triphasic process was observed at 422 nm . As with the reaction in the opposite direction, the two faster phases were found to depend on azurin concentration and we attribute them to the reduction of haem *c* in the enzyme. The slowest phase was found to be independent of azurin concentration, with a rate constant of $0.25 \pm 0.10\text{ s}^{-1}$ and this we ascribe to reduction of haem d_1 caused by the internal electron transfer to this component from haem *c*. Fig. 9(a) shows the variation in the pseudo-first-order rate of the fast and intermediate phases. The fast phase approaches a rate limit of 120 s^{-1} (Fig. 9b), whereas the intermediate phase is linearly dependent on azurin concentration with a second-order rate constant of $1.46 \times 10^4\text{ M}^{-1}\cdot\text{s}^{-1}$. The relative proportions of the two haem *c* reduction phases observed were independent of the azurin concentration over the range $20\text{--}353\text{ }\mu\text{M}$, the faster process contributing 30–40% of the dependent reaction. In general the experiments conducted in this direction are technically difficult because of the problem of maintaining strict anaerobic conditions, which affected the analysis of the slow phase. For this reason the kinetic difference spectra that we have determined have shown a wide variability.

Fig. 8. Reaction of oxidized *Pseudomonas* cytochrome oxidase with reduced azurin

(a) Oscilloscope trace produced on mixing $6\text{ }\mu\text{M}$ -*Pseudomonas* cytochrome oxidase with $706\text{ }\mu\text{M}$ -reduced azurin, anaerobically, in the stopped-flow apparatus. The reaction was carried out in 0.1 M -potassium phosphate buffer, pH 7.0 at 25°C , and monitored at 422 nm in a 2 cm -path-length cell. The vertical scale corresponds to a change of $0.02A$ per division and the horizontal scale represents time scales of 20 ms and 1 s per division for the lower and upper

Fig. 9. Dependence of the rate of reduction of oxidized *Pseudomonas* cytochrome oxidase on reduced azurin concentrations

(a) Dependence of the fast phase (●, plotted with reference to the right-hand ordinate) and the intermediate phase (○, plotted with reference to the left-hand ordinate) upon the concentration of reduced azurin. The experiments were conducted anaerobically, in the stopped-flow apparatus, in 0.1M-potassium phosphate, pH7.0 at 25°C, by using an oxidase concentration of 6 μM before mixing. The observations were made at 422nm. (b) A double-reciprocal plot of the fast-phase data in (a).

Discussion

Brunori *et al.* (1975) proposed that the electron-transfer reactions between *Pseudomonas* cytochrome oxidase and azurin could be described by the following scheme:

in which X_1 and X_2 represent forms of a molecular complex between azurin (A) and cytochrome oxidase (C) and A^* is the catalytically incompetent form of azurin. In this scheme the haem d_1 of the oxidase has been omitted, since it was 'locked' in the ferrous state by combination with CO; all temperature-jump experiments were performed under 101 kPa (1 atm) of this gas. The present experiments were performed under as near as possible identical conditions of pH, ionic strength and temperature in an attempt to probe further the scheme in eqn. (1) and to evaluate the kinetic constants involved. Throughout these studies we have regularly used the value for the extinction coefficient of azurin quoted in the Materials and Methods section, although there is some controversy in the literature about this quantity (Horio *et al.*, 1961; Wharton *et al.*, 1973; Rosen & Pecht, 1976). The present results strongly indicate that the initial transfer of electrons is between azurin and the haem c component of the oxidase, in confirmation of the work of Wharton *et al.* (1973). However, contrary to the findings of these workers, it is evident that the process is not a simple single reaction, but, as shown by the kinetic difference spectra in Figs. 4 and 7, both the azurin-dependent phases correspond to redox changes involving the haem c component. We have also observed a similar biphasic reduction of the haem c when *Pseudomonas* cytochrome oxidase was mixed with Cr^{2+} cations at pH7.0 and 25°C (Barber *et al.*, 1977).

Since the enzyme has been shown to be a dimer consisting of two c and two d haems (Kuronen & Ellfolk, 1972; Kuronen *et al.*, 1975) a biphasic reaction at the level of the haem c component would not be inconsistent if the two sites were not equivalent. However, this should result in both the concentration-dependent phases contributing equally to the observed absorption change in the haem c , assuming of course that the changes in absorption coefficients are identical. Our results are not compatible with such a simplistic scheme, since the ratio of the fast phase to the intermediate phase is 1:3 for oxidation of haem c and 1:2 for reduction of haem c . Moreover the proportion of the two phases in the reduced enzyme are greatly affected by the presence of CO, which changes the ratio of the fast phase to the intermediate phase from 1:3 to 12:3. These observations suggest that there may be a heterogeneous population of oxidase molecules manifesting, at the haem c , two different reactivities towards azurin. Furthermore the dramatic effect of CO demonstrates that the two forms indicated above are in equilibrium and

traces respectively (horizontal trace is the base line). (b) Semi-logarithmic analysis of (a). The fast (○) and intermediate (●) phases are plotted with reference to the time scale on the upper abscissa, and the slow process (▲) is plotted with reference to the lower abscissa.

readily interconvertible. This interconversion is of necessity slow, as the distribution of phases remains unaltered as a function of azurin concentration. Since CO combines with the haem d_1 component (Parr *et al.*, 1975) its influence on the reactivity of haem c implies some form of haem-haem interaction within the molecule. Over the range of azurin concentrations used, we have observed that the rate of the fast process under CO was enhanced with respect to the corresponding phase seen under N_2 .

In addition to the changes that we have attributed to the haem c component in this enzyme, we also observed in the absence of CO much slower monomolecular processes associated with redox changes in the haem d_1 component subsequent to internal electron transfer from or to haem c . The rate of $0.35 \pm 0.10 \text{ s}^{-1}$ that we have measured for the oxidation of reduced haem d_1 contrasts markedly with the rate of 10 s^{-1} reported by Wharton *et al.* (1973), allegedly for the same process, although our rate of $0.25 \pm 0.10 \text{ s}^{-1}$ compares reasonably with their value ($0.2\text{--}2.0 \text{ s}^{-1}$) for the reverse process. From these data on the relative rates of internal electron transfer between the haem c and haem d_1 components of the enzyme, it is possible to arrive at a value for the difference in redox potential between the two components of $+9$ to -28 mV , which is consistent with the published value of Shimada & Oriei (1976) of -20 mV .

Eqn. (1) was derived for an enzyme species in which the haem d_1 has been effectively removed from the reaction by combination with CO, and only those stopped-flow experiments in which the haem c oxidation proceeds in the presence of haem d_1^{2+} -CO can be considered strictly applicable to eqn. (1). In such conditions the reaction essentially consists of the fast concentration-dependent process, and we postulate that the reaction of this species is the one monitored in the temperature-jump experiments (Brunori *et al.*, 1975). The form of the dependence of the rate of oxidation on azurin concentration shows a hyperbolic character (Figs. 6a and 6b) and this is consistent with the scheme in eqn. (1), which itself assumes a very rapid equilibrium that is spectrally silent, leading to complex-formation, wherein electron exchange occurs. Evidence for the validity of this assumption is provided by the temperature-jump experiments as well as by the absence of a lag phase in the stopped-flow experiments (see Strickland *et al.*, 1975); a similar result was also obtained by Wharton *et al.* (1973). From Fig. 6(b) it is possible to assign values of 200 s^{-1} to k_{-2} (the rate-limiting transfer of electrons within the complex) and $1.1 \times 10^{-4} \text{ M}$ to

$k_3 \left(\frac{k_{+3}}{k_{-3}} \right)$. Assuming that complex-formation occurs at a diffusion rate limit of approx. 10^9 s^{-1} , this would confer a value of approx. 10^5 s^{-1} on k_{+3} .

Experiments conducted in the reverse direction,

i.e. the reduction of the oxidized enzyme by reduced azurin, are not strictly comparable with the scheme in eqn. (1), since it has not been possible to prepare the half-reduced enzyme species containing $c^{3+}d_1^{2+}$ -CO. However, in treating the data we have assumed that the fastest azurin-dependent phase is that operating in the temperature-jump scheme. The scheme predicts that the rate of this reaction should also vary in a hyperbolic fashion as a function of azurin concentration and, as Figs. 9(a) and 9(b) show, this is the case. Applying the same criterion of spectrally silent diffusion-limited complex-formation, followed by electron exchange, it is possible to derive values for

k_{+2} of 120 s^{-1} and $\frac{1}{K_1} \left(\frac{k_{-1}}{k_{+1}} \right)$ of $7.35 \times 10^{-5} \text{ M}$ from

Fig. 9(b), the latter yielding a value for k_{-1} of approx. 10^5 s^{-1} . The abscissa in Fig. 9(b) represents the reciprocal of half the total reduced azurin concentration, to allow for the observation (Wilson *et al.*, 1975) that only half the azurin is present in a kinetically competent form. From this result $k_{+2} + k_{-2}$ can be evaluated as 320 s^{-1} , and this compares favourably with the value of 280 s^{-1} (Brunori *et al.*, 1975) obtained in temperature-jump studies.

The present stopped-flow data support and have enabled us to evaluate some of the kinetic parameters in the scheme in eqn. (1) which proposes that electron transfer occurs within a complex formed rapidly between the *Pseudomonas* cytochrome oxidase and azurin. Nevertheless, it is clear that eqn. (1) does not fully describe the events that take place between azurin and the haem c component of the oxidase. The mechanistic significance and nature of the slower azurin-dependent phases seen during both oxidation and reduction of the oxidase, particularly in the absence of CO, when they contribute substantially to the observed reaction, remain open to speculation. The reason for the observed, non-zero intercepts, for the intermediate phases seen in Figs. 2(b), 6(a) and 9(a) is unclear, but may be due to the appropriate reverse reactions or else to the influence of the equilibrium between the two reactive forms of the haem c . Presumably this matter could be resolved by extensive computer simulation of the complete data. However, since we have only been concerned with the applicability of the fast phase to eqn. (1), this does not seem appropriate at present. Whatever the nature of the slower azurin-dependent phases, their substantial contribution to the reaction of the haem c component under N_2 may lead to a merging of the resultant temperature-jump relaxation process into that of the azurin-re-equilibration step (K_4), which might account for the very small absorbance changes that we have observed in this type of experiment (M. Brunori, C. Greenwood, S. R. Parr & D. Barber, unpublished work).

A puzzling feature of our results is that they differ

considerably from those obtained by Wharton *et al.* (1973), even though the experimental conditions were not dramatically different. It is clear that further work is called for before a complete understanding of this system emerges.

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