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The Reaction of *Pseudomonas aeruginosa* Cytochrome *c* Oxidase with Carbon Monoxide

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The binding of CO to ascorbate-reduced *Pseudomonas* cytochrome oxidase was investigated by static-titration, stopped-flow and flash-photolytic techniques. Static-titration data indicated that the binding process was non-stoicheiometric, with a Hill number of 1.44. Stopped-flow kinetics obtained on the binding of CO to reduced *Pseudomonas* cytochrome oxidase were biphasic in form; the faster rate exhibited a linear dependence on CO concentration with a second-order rate constant of $2\times10^4\,M^{-1}\cdot s^{-1}$, whereas the slower reaction rapidly reached a pseudo-first-order rate limit at approx. $1\,s^{-1}$. The relative proportions of the two phases observed in stopped-flow experiments also showed a dependency on CO concentration, the slower phase increasing as the CO concentration decreased. The kinetics of CO recombination after flash-photolytic dissociation of the reduced *Pseudomonas* cytochrome oxidase–CO complex were also biphasic in character, both phases showing a linear pseudo-first-order rate dependence on CO concentration. The second-order rate constants were determined as $3.6\times10^4\,M^{-1}\cdot s^{-1}$ and $1.6\times10^4\,M^{-1}\cdot s^{-1}$ respectively. Again the relative proportions of the two phases varied with CO concentration, the slower phase predominating at low CO concentrations. CO dissociation from the enzyme–CO complex measured in the presence of O_2 and NO indicated the presence of two rates, of the order of $0.03\,s^{-1}$ and $0.15\,s^{-1}$. When sodium dithionite was used as a reducing agent for the *Pseudomonas* cytochrome oxidase, the CO-combination kinetics observed by both stopped flow and flash photolysis were extremely complex and not able to be simply analysed.

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). The oxidase can either reduce O_2 to water (Horio *et al.*, 1958) or catalyse the reduction of NO_2^- to NO (Yamanaka *et al.*, 1961). Evidence suggests that the latter is the more important function physiologically.

Pseudomonas cytochrome oxidase is a water-soluble enzyme that contains two haem prosthetic groups, one haem *c* and one haem *d*, the latter being the autoxidizable component (Horio *et al.*, 1961). No metals other than iron have been detected. The enzyme has been purified to a state of electrophoretic homogeneity (Gudat *et al.*, 1973), corresponding to a dimeric protein of molecular weight 120000, each subunit containing one molecule each of haem *c* and haem *d* (Kuronen & Ellfolk, 1972). The oxidase activity of *Pseudomonas* cytochrome oxidase, in common with other terminal oxidases, is inhibited by the classical respiratory poisons cyanide and CO (Yamanaka & Okunuki, 1963*a*).

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CO provides a useful probe for studying ligand-binding reactions of reduced cytochrome oxidases, since its rate of combination is much slower than that of O_2 and can easily be followed in rapid-mixing experiments. Also the rate of dissociation of CO is low, and hence for certain approximations the reactions can be treated as irreversible. The photosensitivity of the ferrous iron–CO bond allows investigation by the flash-photolytic technique and so provides a useful alternative method for studying the recombination reaction between CO and reduced enzyme.

Experimental

Pseudomonas aeruginosa cytochrome oxidase was isolated and purified from cells of *Pseudomonas aeruginosa* (N.C.T.C. 6750) as described by Parr *et al.* (1974). The ratio $E^{ox.}_{410}/E_{280}$ for the crystalline homogeneous preparation was found to be 1.2 by Yamanaka *et al.* (1962) and between 1.1 and 1.2 by Kuronen & Ellfolk (1972). On these criteria our final preparation is over 90% pure. The concentrations of *Pseudomonas* cytochrome oxidase were obtained by using the value $E_{410} = 149\,mM^{-1}\cdot cm^{-1}$ for the oxidized protein.

Spectrophotometry was performed with a Pye–Unicam SP.700A (Pye Unicam, Cambridge, U.K.) instrument. Spectrophotometric titrations were made under anaerobic conditions with cuvettes fitted with Suba-Seal vaccine caps.

Anaerobic stopped-flow determinations were carried out by using an apparatus identical with that described by Gibson & Milnes (1964), which was equipped with a 2cm-light-path cell and had a dead time of 3ms. The voltage output from the photomultiplier was passed through a log amplifier before display on the oscilloscope screen so that the records obtained were a direct measure of the change in absorbance. This facility also allowed the determination of the final absolute absorbance in the reaction compartment.

Flash-photolytic experiments were performed by using an experimental set-up as described by Greenwood & Gibson (1967).

Computer simulations were performed on a PDP 10 computer programmed to solve simultaneous first-order differential equations by using a predictor–corrector method with 5ms step-lengths.

CO and N_2 were obtained from British Oxygen Company, Deer Park Road, London S.W. 19, U.K., and were dispensed from the cylinders and stored in glass vessels over an alkaline solution of reduced anthraquinone sulphonate. NO was obtained from Matheson Gas Products, East Rutherford, N.J., U.S.A., and used straight from the cylinders. CO solutions were prepared in a tonometer by several cycles of evacuation and N_2 equilibration before final equilibration under 1 atm. of CO at 20°C. Further solutions of known CO concentration were prepared by serial dilution of the stock solution with a solution of N_2-equilibrated buffer.

Solutions of reduced *Pseudomonas* cytochrome oxidase were usually prepared in sealed test tubes by thorough evacuation of oxidized enzyme solutions followed by N_2 equilibration and reduction by injection of a solution of sodium ascorbate. For flash-photolytic experiments the *Pseudomonas* cytochrome oxidase–CO complex was prepared by dilution of the anaerobic reduced *Pseudomonas* cytochrome oxidase with CO-equilibrated buffer. Serial dilutions of the CO concentration were performed by mixing the initial solution of *Pseudomonas* cytochrome oxidase–CO complex with known volumes of the reduced enzyme. In this way it was possible to maintain a constant enzyme concentration while varying the CO concentration.

All other chemicals were of AnalaR grade and were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., except for sodium dithionite (which was obtained from Hardman and Holdon, Miles Platting, Manchester, U.K.) and ascorbic acid (disodium salt), from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.

Results

Pseudomonas cytochrome oxidase can be reduced by a variety of reductants, and the reduced protein will combine with CO. Fig. 1(*a*) shows the spectra of ascorbate-reduced oxidase in the presence and absence of CO. Clearly those regions of the spectrum associated with the haem *d* are the most affected (Yamanaka & Okunuki, 1963*b*). The Soret peak of the haem *d*, centred at 460nm, is partially bleached and the α band at 655nm is correspondingly attenuated. The rest of the spectrum, mainly associated with the haem *c*, remains largely unaffected. These spectral changes in the reduced enzyme would indicate that CO is binding only to the haem *d*. CO produces analogous changes in the dithionite-reduced enzyme, except that the α band of the haem *d* in the dithionite-reduced protein is centred at 630nm (see Fig. 1*b*). Such differences in the reduced haem *d* spectrum have been associated with the binding of sulphur oxyanions to the protein (Parr *et al.*, 1974)

Fig. 2(*a*) shows the results from a typical titration of ascorbate-reduced *Pseudomonas* cytochrome oxidase with CO-equilibrated buffer. To minimize possible errors arising from baseline shifts the results have been plotted on the ordinate by taking the difference at two wavelengths on either side of an isosbestic point ($E_{470}-E_{520}$). Fig. 2(*a*) shows that the binding of CO by the oxidase is not particularly tight, and thus it was possible to analyse the data by a Hill plot. Fig. 2(*b*) is the Hill plot derived from the data in Fig. 2(*a*), except that the points at 5 and 14μl of added CO which yield fractional saturations ($\bar{Y}$) less than 0.1 have been neglected; see Antonini & Brunori (1971) for a discussion. The slope of the line in Fig. 2(*b*) yields a value of h equal to 1.44 ± 0.09 and a CO concentration at one-half saturation of 16μM.

Stopped-flow kinetics

When ascorbate-reduced enzyme was mixed under strictly anaerobic conditions with CO in the stopped-flow apparatus, complex progress curves were observed at 470nm. An example of one such progress curve is shown in Fig. 3 together with its analysis into two exponential phases. The dependence of the pseudo-first-order rate constants for the two phases of CO concentration is presented in Fig. 4 which shows that the fast phase exhibits second-order behaviour ($k = 2 \times 10^4 \text{M}^{-1} \cdot \text{s}^{-1}$) over the concentration range explored, whereas the slower of the two processes appears to become rate limited at about $1\,\text{s}^{-1}$. The relative proportions of the two phases were also found to depend on the CO concentration, the proportion of the fast phase increasing with increasing CO concentration. Fig. 5 shows a plot of the ratio of the amplitudes of the two phases as a function of CO concentration.

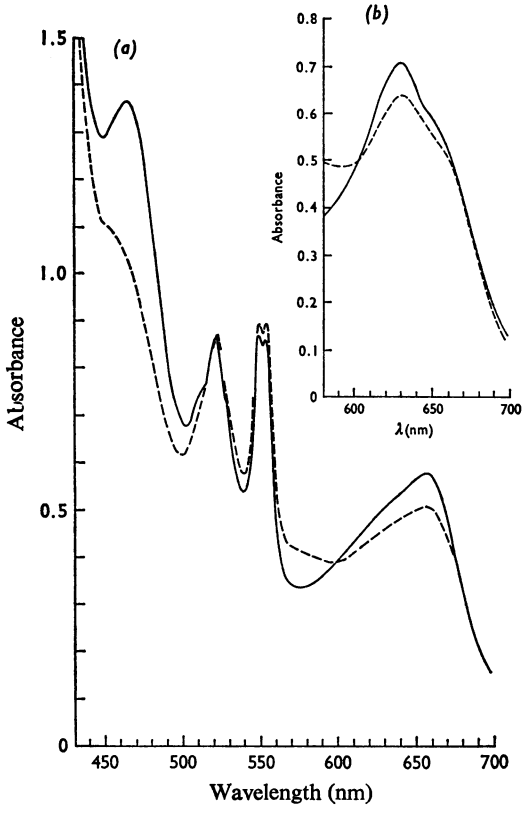


Fig. 1. *Absorption spectra of reduced Pseudomonas cytochrome oxidase in the absence and presence of CO*

(*a*) ——, Ascorbate-reduced *Pseudomonas* cytochrome oxidase, ----, ascorbate-reduced *Pseudomonas* cytochrome oxidase–CO complex, in 0.04M-potassium phosphate buffer, pH7.0. The enzyme concentration was 22μM; CO concentration was 1mM. Spectra were recorded at room temperature (20°C) and the light-path was 1cm. (*b*) ——, Dithionite-reduced *Pseudomonas* cytochrome oxidase in the red region; ----, dithionite-reduced *Pseudomonas* cytochrome oxidase–CO complex. Enzyme concentration was 26μM, CO concentration was 1mM. All other conditions were as above.

The dissociation velocity constant of CO from the CO–enzyme complex has been determined by mixing the latter with O_2 or NO-containing buffer in the stopped-flow apparatus. On mixing the *Pseudomonas* cytochrome oxidase–CO complex with O_2, biphasic kinetics were observed and the rates of both phases were found to be independent of O_2 concentration (see Table 1). Because a change of valence of the haem *d* iron is involved in this procedure, it was obviously important to confirm these findings by using a purely competitive ligand, and Table 1 also shows the results obtained with NO. The rates k_1 and k_2, which are the same for both situations, are considered to be the 'CO off' rates. However, a third, very slow, rate was observed with NO, which may represent the attack of NO on the reduced haem *c*. The spectrum of the material in the stopped-flow reaction compartment on completion of the reaction showed that the α and β peaks of the haem *c* were abolished; this is consistent with the findings of Yamanaka & Okunuki (1963*b*).

Flash-photolysis kinetics

As with many haemoproteins, the CO complex of *Pseudomonas* cytochrome oxidase is photosensitive and so offers the possibility of investigating CO recombination after a brief but intense photolytic flash. Fig. 6 shows a progress curve for CO recombination to ascorbate-reduced enzyme. As was found with flow kinetics, complex progress curves were again observed which could be analysed into two simple rate processes (see Fig. 6). In contrast with the flow data, the rates of both phases were second order, the slopes in Fig. 7 yielding second-order rate constants of 3.6×10^4M$^{-1}\cdot$s^{-1} and 1.6×10^4M$^{-1}\cdot$s^{-1}. Because of the close similarity between these two values and the change in proportions of the two phases as a function of CO concentration (see Fig. 8), the analysis of progress curves under certain conditions has proved difficult.

By using the photolytic procedure and progressively varying the wavelength of observation it was possible to generate a difference spectrum between the reduced enzyme and the reduced enzyme–CO complex at the end of the reaction. This form of difference spectrum we refer to as a kinetic difference spectrum, and in Fig. 9 the kinetic difference spectrum is compared with the static difference spectrum for the ascorbate-reduced enzyme. Also shown in Fig. 9 is the kinetic difference spectrum for enzyme reduced by dithionite, which shows similarities to the kinetic difference spectrum of the ascorbate-reduced enzyme in the presence of sodium metabisulphite (Parr *et al.*, 1974). Accompanying the change in the difference spectrum induced by dithionite is a vastly more complicated pattern of CO-recombination kinetics. In the presence of dithionite it was impossible to resolve the progress curves into a small number of rate processes in any reproducible manner.

Discussion

In attempting to explain the present data we make the initial assumption that each haem *d* molecule binds only one CO molecule, and clearly, if this were a simple haemoprotein, the kinetic consequences would be that we should observe monophasic

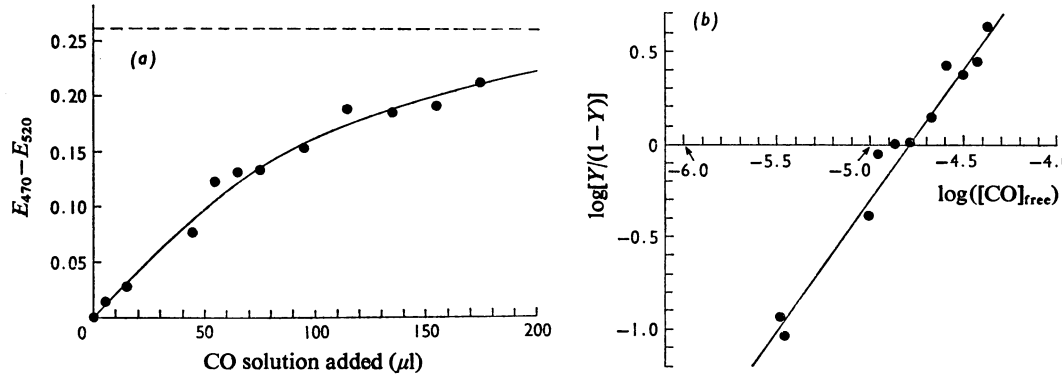


Fig. 2. *Anaerobic titration of reduced Pseudomonas cytochrome oxidase with CO*

Ascorbate-reduced *Pseudomonas* cytochrome oxidase (13 μM) in 0.04 M-potassium phosphate buffer, pH 7.0, was titrated anaerobically with portions of a 1 mM-CO solution at 20°C. The light-path was 1 cm. (*a*) Titration in which the difference $E_{470}-E_{520}$ is plotted on the ordinate against μl of CO solution on the abscissa. The initial extinction difference at zero CO has been assigned the value of zero. (*b*) Hill plot of the data in Fig. 2(*a*) where Y represents the proportion of the enzyme saturated with CO after each addition.

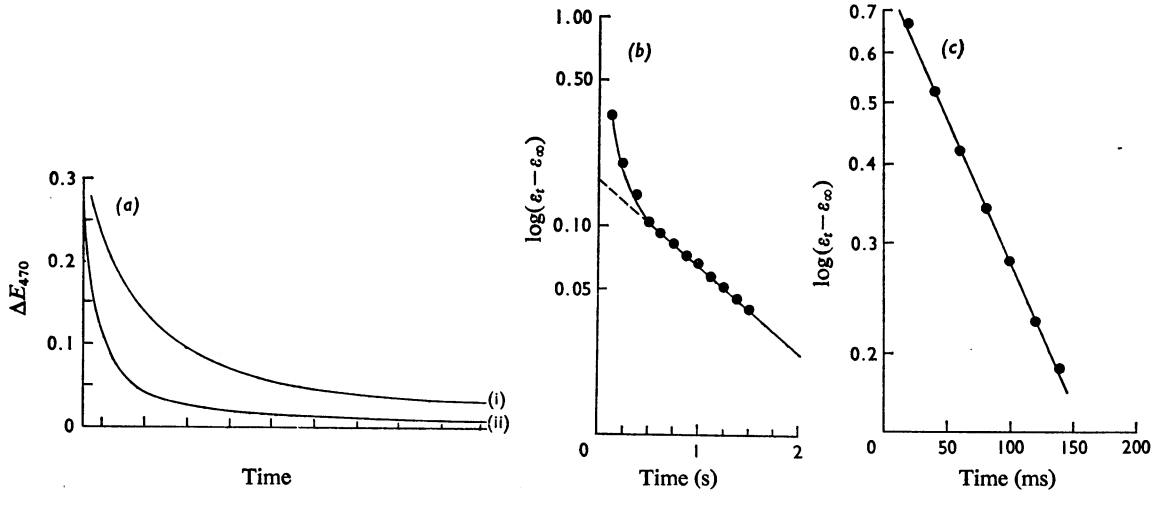


Fig. 3. *Reaction of ascorbate-reduced Pseudomonas cytochrome oxidase with CO as observed by rapid-mixing experiments*

(*a*) Form of the progress curves obtained in the stopped-flow apparatus. The reaction was observed at 470 nm on mixing the ascorbate-reduced enzyme (10 μM before mixing) in 0.04 M-potassium phosphate buffer, pH 7.0, with the same buffer solution containing 1 mM-CO (before mixing). The stopped-flow apparatus was fitted with a 2 cm-light-path-cell and the light had a nominal band-width of 1.8 nm. The temperature was 20°C. The sweep times were 50 ms (curve i) and 200 ms (curve ii) per grid unit respectively and each vertical grid unit represented an extinction change of 0.05. (*b*) Pseudo-first-order plot of the whole reaction shown above (CO = 500 μM, after mixing), where ε_t and ε_∞ are the extinctions at any time t and at the end of the reaction respectively. The rate of the slow process was calculated from the slope of the linear section of the plot at long times. (*c*) Pseudo-first-order plot of the fast reaction obtained by extrapolating the slow reaction to zero time and subtracting this line from the whole reaction curve (*b*).

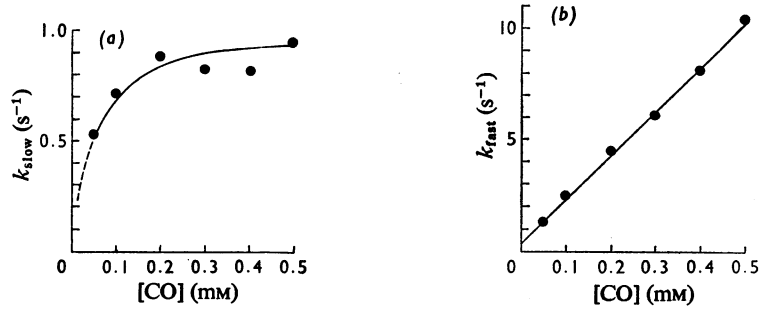


Fig. 4. *Dependence on CO concentration of the rate of formation of the ascorbate-reduced Pseudomonas oxidase–CO complex*

(*a*) Dependence of the pseudo-first-order rate constant (k_{slow}) of the slow phase and (*b*) fast phase (k_{fast}) on the CO concentration. Conditions were as in Fig. 3.

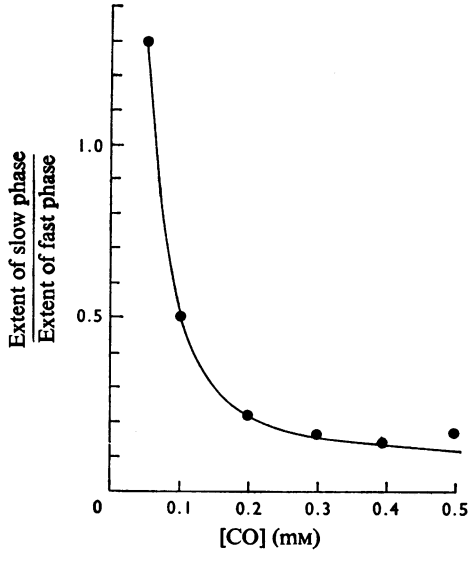


Fig. 5. *Dependence on CO concentration of the ratio of the amplitudes of the two phases observed by stopped-flow experiments*

Conditions were as in Fig. 3. The extent of the slow phase was determined by extrapolation of the linear section of the whole reaction plots at long times to zero time. The extent of the fast phase was determined from the total absorption change minus that absorption change due to the slow phase.

exponential combination kinetics by both stopped-flow and flash photolysis. This is indeed the case for simple single-haem proteins such as myoglobin (Gibson, 1956), or even for more complex proteins such as mammalian cytochrome oxidase (Gibson & Greenwood, 1963), which nevertheless contains only

Table 1. *Dissociation of CO from the Pseudomonas cytochrome oxidase–CO complex*

The first-order rate constants for CO dissociation from the *Pseudomonas* cytochrome oxidase–CO complex (4.4 μM) when mixed with either O_2 or NO in the stopped-flow apparatus at 20°C are shown. [CO] 125 μM.

Ligand	Concentration	k_{+1} (s^{-1})	k_{+2} (s^{-1})	k_{+3} (s^{-1})
[O_2]	670 μM	0.18	0.043	—
[O_2]	135 μM	0.14	0.021	—
[O_2]	67 μM	0.17	0.034	—
[NO]	1.02 mM	0.14	0.017	0.002

one CO-binding site; such a mechanism can be represented as shown in eqn. (1):

$$\text{Oxidase} + \text{CO} \rightleftharpoons \text{oxidase–CO} \qquad (1)$$

As indicated by the data in Fig 3(*b*) we have observed biphasic combination kinetics that clearly eliminate the mechanism in eqn. (1) above but which might be explainable on the basis that two forms of oxidase exist in equilibrium, only one of which can react directly with CO. Eqn. (2) shows one such system:

$$\text{Oxidase}^* \rightleftharpoons \text{oxidase} + \text{CO} \rightarrow \text{oxidase–CO} \qquad (2)$$

Although this scheme can generate biphasic kinetics, the relative amplitudes of the phases will be fixed by the position of the equilibrium, and not, as we have observed, be sensitive to CO concentration (see Fig. 5).

The kinetic results presented in this paper suggest that we have two classes of CO-binding site, characterized by different rates of combination and dissociation. Further, the data in Figs. 3 and 8 indicate that these two classes of site are in some way interconvertible. Since it has been shown that the active

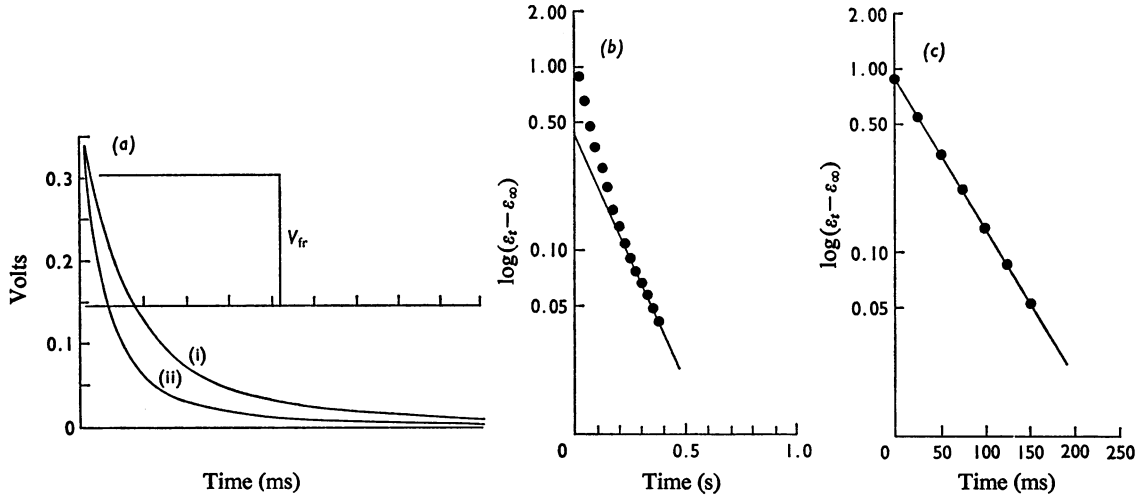


Fig. 6. *Recombination of CO with ascorbate-reduced Pseudomonas cytochrome oxidase after flash photolysis*

(*a*) Form of the progress curves obtained. The reaction was observed at 460nm after a photolytic flash of 250J was used to give 100% breakdown of the *Pseudomonas* cytochrome oxidase–CO complex (3.5μM). The reaction was performed at room temperature in 0.04M-potassium phosphate buffer, pH 6.8, in a 4cm-light-path cell and in the presence of 600μM-CO. The band width of the observing light was 0.8nm. The sweep times were 50ms (curve i) and 100ms (curve ii) per grid unit respectively and the traces were recorded at 50mV per vertical grid unit. The V_{fr} (difference between no light and the fully reacted state) was recorded at 500mV per vertical grid unit. (*b*) and (*c*) show the pseudo-first-order analysis of the reaction trace above. The whole reaction trace in (*b*) can be resolved into two rate processes as described in Fig. 3. (*c*) shows the plot obtained for the faster phase. ε_t and ε_∞ are the extinctions at any time t and at the end of the reaction respectively.

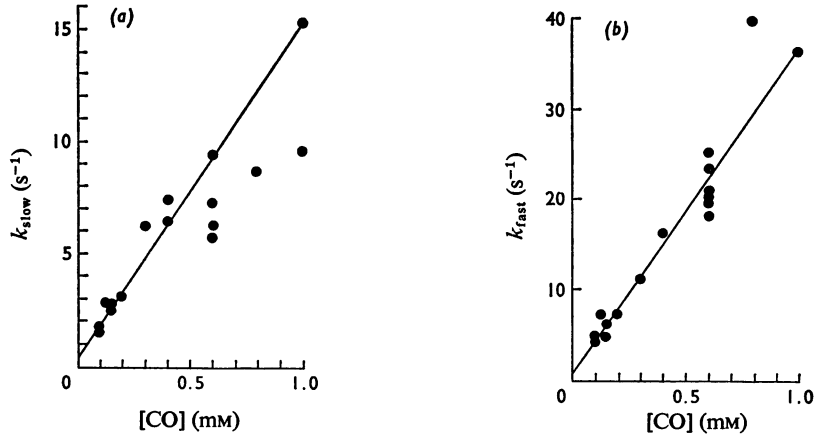


Fig. 7. *Dependence on CO concentration of the rates of recombination of CO with ascorbate-reduced Pseudomonas cytochrome oxidase*

(*a*) Dependence of the slower phase (k_{slow}); (*b*) dependence of the faster phase (k_{fast}). Conditions were as in Fig. 6.

enzyme is a dimer containing two haem *d* prosthetic groups (Kuronen & Ellfolk, 1972), the reasons for the observed complexity may reside in some form of haem–haem interaction. The titration data presented in Fig. 2, which yield a Hill coefficient (*h*) of 1.44± 0.09, strongly suggest that postive co-operativity is manifest in this system.

The finding, seen in Fig. 4, that the slow process becomes rate limited imposes considerable restrictions on any model for the ligand-binding processes.

One scheme which can in principle fit the stopped-flow data is shown in Scheme 1 below, in which the molecule is depicted as a dimer of two *c* and two *d* haem molecules.

Open symbols refer to the haem *d* and closed symbols to the haem *c*. A is the reduced dimeric enzyme and B and C are the mono- and di-liganded forms respectively. Species D represents a form of the enzyme incapable of binding the second CO molecule and is in equilibrium with species B. It may be noted that species D is only formed after binding one CO molecule, and thus this represents site–site interaction. X is the ligand and k_1, k_2, k_3 and k_{-3} are the rate constants in the directions shown.

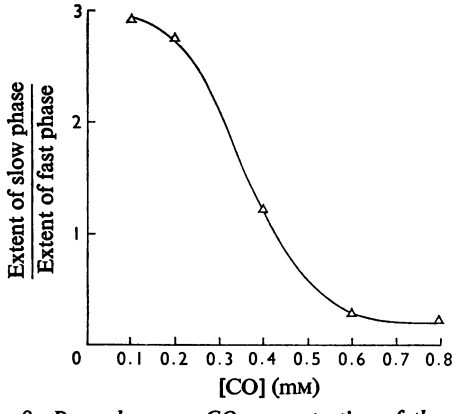


Fig. 8. *Dependence on CO concentration of the relative amplitudes of the two phases observed by flash photolysis*

Conditions were as in Fig. 6 and the same method of calculation was used as in Fig. 5.

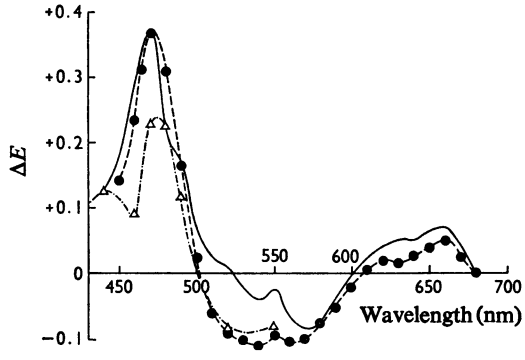


Fig. 9. *Difference spectra of reduced Pseudomonas cytochrome oxidase minus reduced Pseudomonas cytochrome oxidase–CO complex*

——, Static difference spectrum obtained with ascorbate-reduced enzyme. ●, Kinetic difference spectrum obtained from flash photolysis with ascorbate-reduced enzyme–CO complex. △, Kinetic difference spectrum obtained from flash photolysis with dithionite-reduced enzyme–CO complex. All spectra were normalized to 22 μM-cytochrome oxidase in 1 cm light-path. Measurements were made in all cases in 0.04 M-potassium phosphate buffer, pH 7.0, at room temperature.

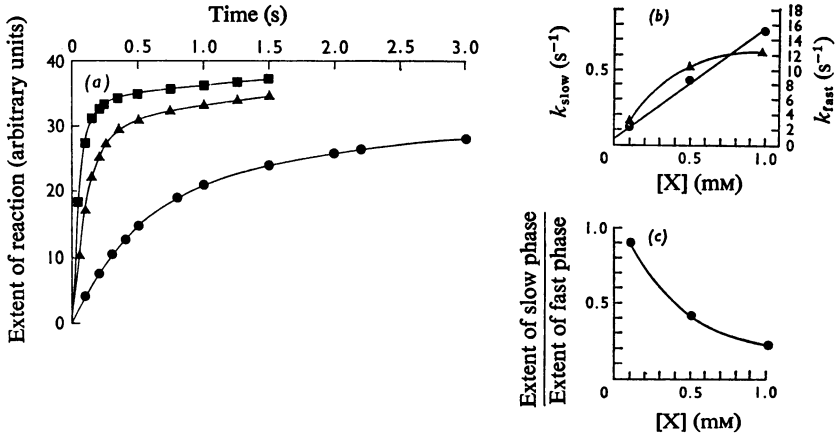
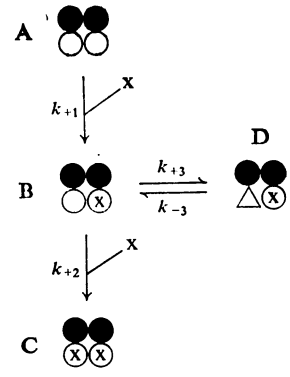


Fig. 10. *Digital computer simulation of stopped-flow experiments calculated on the basis of the model for CO binding to ascorbate-reduced Pseudomonas cytochrome oxidase shown in Scheme* 1

(*a*) Progress curves generated at three different CO concentrations: ●, 0.1 mM; ▲, 0.5 mM; ■, 1 mM. The enzyme concentration was fixed at 2 μM. See the text for detail of the proposed model and assignments of rate constants. (*b*) Dependence of the two pseudo-first-order rate constants, obtained from each trace, on the CO concentration. ▲, slower phase (k_{slow}); ●, faster phase (k_{fast}). (*c*) Dependence on CO concentration of the ratio of the amplitudes of the two phases. In performing these calculations it has been assumed that the binding of each ligand molecule produces the same change in extinction, e.g. $E_{(B-A)} = E_{(C-B)}$, where A, B and C are defined in Scheme 1.



Scheme 1. *Model for the ligand-binding process*

○, △, Haem *d*; ●, haem *c*. A, Reduced dimeric enzyme; B and C, mono- and di-liganded forms respectively. D, Enzyme form incapable of binding the second CO molecule. For further details see the text.

Extensive data-fitting computation has not yet been attempted, but Fig. 10 shows that Scheme 1 can reproduce many of the features of the stopped-flow experiments. For the computed progress curves shown in Fig. 10 k_{+1} and k_{+2} were assigned the value $2\times10^4 M^{-1}\cdot s^{-1}$, i.e. equal to that for the fast phase found experimentally. As the return from species D to B represents the monomolecular rate limit, we have assigned to it the observed value of $1 s^{-1}$. Finally k_{+3} was arbitrarily assigned the value of $10 s^{-1}$ so that it could effectively compete with k_{+2}; the extent of this competition should clearly be dependent on CO concentration.

The model in Scheme 1 predicts biphasic progress curves for the ligand-binding reaction, the exact form of which are dependent on ligand concentration (Fig. 10*a*). Fig. 10(*b*) shows the dependences of the two observed rates on ligand concentration. The rate of the faster process varies linearly with the ligand concentration, whereas that of the slower process reaches a rate limit (cf. the experimental results in Fig. 4). The model also predicts that the ratio of the amplitudes of these two processes is concentration dependent on ligand (Fig. 10*c*; cf. experimental results in Fig. 5). This computation is qualitatively in agreement with our experimental findings, although the actual values of the constants derived from Fig. 10 differ from those found experimentally.

Although Scheme 1 is certainly not unique in accounting qualitatively for the experimental data we believe that it may contain the feature essential to any scheme compatible with the stopped-flow experiments, namely a non-binding form of the protein such as species D of Scheme 1. We were led to this conclusion by the fact that we were unable to obtain even a qualitative fit to the experimental data when computations were performed on the basis of a variety of models that did not include such a non-binding form of the enzyme.

Although we feel that Scheme 1 is the simplest one that is consistent with stopped-flow observations, it is unable to accommodate the static-titration data and photolytic findings. For example, it is difficult to envisage how haem–haem interactions leading to a non-binding species D could produce an '*h*' value indicative of positive co-operativity. Also the scheme does not provide for the faster binding process ($k = 3.6\times10^4 M^{-1}\cdot s^{-1}$) seen in flash-photolytic studies. It is possible to write schemes that can reconcile our static-titration, flow and photolytic data but the results presented in this paper do not appear to justify such a detailed treatment.

In conclusion we feel that the most revealing observation to emerge from this investigation is the existence of positive co-operativity between CO-binding sites. This feature may have real significance when considering the reaction of the reduced enzyme with other ligands and in particular electron-accepting substrate molecules. An oxidase molecule possessing complex interactions of the type that are discussed above is perhaps not unreasonable in view of the known diverse functions of this enzyme. On the one hand it can accomplish the one-electron reduction of NO_2^- to NO, the two-electron reduction of NH_2OH to NH_3 (Singh, 1973) and also act like other terminal oxidases by reducing molecular O_2 to water, i.e. a four-electron process. For this latter reaction the existence of an active dimer of four redox centres able to interact is attractive.

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