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The Reactions of *Pseudomonas* Cytochrome *c*-551 Oxidase with Potassium Cyanide

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The binding of cyanide to both oxidized and ascorbate-reduced forms of *Pseudomonas* cytochrome *c*-551 oxidase was investigated. Spectral studies on the oxidized enzyme and its apoprotein showed that the ligand can bind to both the *c* and d_1 haem components of the molecule, and kinetic observations indicated that both chromophores reacted, under a variety of conditions, with very similar rates. Cyanide combination velocities were dependent on ligand concentration, and increasing the pH also accelerated the reaction; the second-order rate constant was estimated as approx. $0.2\mathrm{M}^{-1}\cdot\mathrm{s}^{-1}$ at pH 7.0 The binding of cyanide to the protein was observed to have a considerable influence on reduction of the enzyme by ascorbate. Spectral and kinetic observations have revealed that the species haem $d_1{}^{3+}$–cyanide and any unbound haem *c* may react relatively rapidly with the reductant, but the behaviour of cyanide-bound haem *c* indicates that it may not be reduced without prior dissociation of the ligand, which occurs relatively slowly. The reaction of reduced *Pseudomonas* cytochrome oxidase with cyanide is radically different from that of the oxidized protein. In this case the ligand only binds to the haem d_1 component and reacts much more rapidly. Stopped-flow kinetic measurements showed the binding to be biphasic in form. Both the rates of these processes were dependent on cyanide concentration, with the fast phase having a second-order rate constant of $9.3\times10^5\mathrm{M}^{-1}\cdot\mathrm{s}^{-1}$ and the slow phase one of $2.3\times10^5\mathrm{M}^{-1}\cdot\mathrm{s}^{-1}$. The relative proportions of the two phases also showed a dependency on cyanide concentration, the slower phase increasing as the cyanide concentration decreased. Computer simulations indicate that a reaction scheme originally proposed for the reaction of the enzyme with CO is capable of providing a reasonable explanation of the experimental results. Static-titration data of the reduced enzyme with cyanide indicated that the binding was non-stoicheiometric, the ligand-binding curve being sigmoidal in shape. A Hill plot of the results yielded a Hill coefficient of 2.6.

Pseudomonas ferrocytochrome *c*-551 oxidoreductase (EC1.9.3.2) is a water-soluble enzyme that may be isolated from cellular extracts of *Pseudomonas aeruginosa* grown anaerobically in the presence of nitrate (Parr *et al.* 1976). A considerable amount of evidence has now been accumulated that supports the view that the enzyme molecule has mol.wt. 120000 and consists of two identical subunits, each containing one haem *c* and one haem d_1 moiety (Kuronen & Ellfolk, 1972; Gudat *et al.*, 1973; Kuronen *et al.*, 1975; Saratse *et al.*, 1977). Enzymically, the protein is capable of accepting electrons from two protein electron donors, *Pseudomonas* ferrocytochrome *c*-551 and reduced azurin, and of accomplishing both the four-electron reduction of O_2 to water and the single-electron reduction of nitrite to NO, although the nitrite reductase activity appears to be more important physiologically (Yamanaka *et al.*, 1963). Rapid-mixing experiments with azurin have revealed haem *c* as the site at which electrons are donated to the enzyme (Wharton *et al.*, 1973; Parr *et al.*, 1977), and spectroscopic work has shown that the haem d_1 component of reduced *Pseudomonas* cytochrome oxidase is capable of binding ligands such as CO and cyanide (Yamanaka & Okunuki, 1963*b*). Both these classical respiratory poisons are potent inhibitors of the oxidase activity, but only cyanide is effective against the nitrite reductase activity (Yamanaka & Okunuki, 1963*a*).

The present study has been undertaken with a view to obtaining results for comparison with previous work (Parr *et al.*, 1975) on the reaction of the enzyme with CO and also with the ligand-binding reactions associated with the enzymic functions of the reduced protein. Furthermore, because cyanide, unlike CO, is able to complex with haemoproteins that are in the ferric state, experiments have been carried out

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with the ligand as a probe of the ligand-binding behaviour of oxidized *Pseudomonas* cytochrome oxidase. No spectra of the oxidized, cyanide-bound enzyme have been published previously, although an ability to bind the ligand has been implied by the magnetic-circular-dichroic studies by Orii *et al.* (1977). It was also of interest to determine whether the haem *c* component of *Pseudomonas* cytochrome oxidase would bind cyanide and thus parallel the behaviour of mammalian ferricytochrome *c* (George & Tsou, 1952).

Materials and Methods

All chemicals were obtained from Fison's Scientific Apparatus, Loughborough, Leics., U.K., and were of Analytical Reagent grade, except for KCN and $AgNO_3$, which was from Hopkin and Williams, Chadwell Heath, Essex, U.K., and ascorbic acid (disodium salt), which was obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. N_2 gas was supplied by British Oxygen Co., London S.W.19, U.K., and was dispensed from the cylinder and stored in a glass vessel over an alkaline solution of anthroquinonesulphonate.

KCN solutions were freshly prepared in phosphate buffer by adjustment to the desired pH with HCl. Cyanide was determined by using a method similar to that described in the AnalaR Handbook (1967). For this 10ml volumes of cyanide solutions were added to mixtures made up from 40ml of water, 5ml of 2M-NH_3 solution and three drops of 0.1M-KI and titrated against a 0.1M-$AgNO_3$ solution. Cyanide determinations were found to be essential in cases where solutions were subjected to the degassing procedure; the repeated evacuations involved were found to decrease cyanide concentration by up to 60%.

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 A_{410}^{ox}/A_{280} and $A_{640}^{ox}/A_{520}^{ox}$ for the oxidized enzyme were 1.18–1.2 and 1.15–1.2 respectively. The concentrations of *Pseudomonas* cytochrome oxidase solutions were obtained by using an absorption coefficient of $A_{410}^{ox} = 288 \times 10^3$ litre·mol^{-1}·cm^{-1} (M. C. Silvestrini, A. Colosimo, M. Brunori & C. Greenwood, unpublished work).

The extraction of the haem d_1 component of *Pseudomonas* cytochrome was carried out by using the acid acetone method of Yamanaka & Okunuki (1963*c*). This procedure yields a red-coloured protein precipitate, the apoprotein, which contains only the haem *c* chromophore. After the final washing of the precipitate to remove residual acetone, the protein was resuspended in 2–3ml of 0.1M-potassium phosphate buffer, pH 7.0, and redissolved by addition of two or three drops of 2M-KOH. Although previous reports (Horio *et al.*, 1961; Singh, 1973) have noted the insolubility of the apoprotein at neutral pH, the samples of this material prepared during the present studies would not remain fully dissolved at pH values less than 10. For this reason the cyanide-binding experiments on the apoprotein were conducted in 0.1M-potassium phosphate buffer adjusted to pH 10 with KOH. Furthermore, the apoprotein was also observed to become slightly reduced on standing in air, which necessitated the inclusion of a small amount of potassium ferricyanide in the solutions to maintain the haem *c* in its fully oxidized state.

The concentrations of apoprotein solutions were calculated on the assumption that the value of ΔA_{550} of 19.3×10^3 litre·mol^{-1}·cm^{-1} observed on reducing the holoenzyme is not significantly altered by extraction of the haem d_1. This assumption has been based on the work of Barber *et al.* (1977), which shows very little contribution of the haem d_1 to the total difference spectrum at this wavelength.

Spectrophotometry was carried out with a Cary 118c spectrophotometer. The kinetics of the reaction of cyanide with oxidized *Pseudomonas* cytochrome oxidase were followed spectrophotometrically in air at room temperature (22°C). Samples of the oxidized cyano complex of the enzyme resulting from these experiments were then sealed in the cuvette with a vaccine cap, degassed and placed under an atmosphere of N_2. The reduction of the cyanide complex could then be initiated, by injecting a solution of sodium ascorbate into the cell by microsyringe, and the reaction monitored in the spectrophotometer at 22°C. Although the degassing procedure would be expected to decrease the amount of cyanide present, absorption spectra run before and immediately after degassing were found to be identical. It has therefore been assumed that over the time interval between degassing the enzyme and the addition of ascorbate (a few minutes) the decrease in cyanide concentration did not significantly affect the amount of ligand bound to the protein. The very slow rates subsequently observed for the dissociation of cyanide from the enzyme appear to confirm this assumption.

Samples of reduced *Pseudomonas* cytochrome oxidase were all prepared by addition of a large excess of sodium ascorbate to anaerobic enzyme solutions under N_2. Kinetic measurements were, in this case, made with a stopped-flow apparatus identical with that described by Gibson & Milnes (1964), equipped with a 2cm-light-path cell and having a 'dead-time' of 3ms. Static titrations of reduced *Pseudomonas* cytochrome oxidase with KCN were carried out in cuvettes sealed with Suba-Seal vaccine caps (William Freeman, Staincross, Barnsley, Yorkshire, U.K.) and completely filled with anaerobic enzyme solution. Additions of anaerobic KCN solutions were made by microsyringe, displaced volumes of protein solution being allowed

to leave the cuvette by means of a small hypodermic needle inserted through the vaccine cap.

Simulated reaction traces were computed on a Digital Equipment Corporation PDP 8/a mini computer, by using a Runge–Kutta method to solve simultaneous first-order differential equations. The results were displayed on a Tektronix type 564 storage oscilloscope by means of user-defined functions in Lab 4V BASIC subroutines obtained from S. R. Vivian, University of Manitoba, Winnipeg, Canada. If desired the simulated traces were then analysed by means of a two-exponential-fit program based on the general least-squares adjustment program of Wilkins *et al.* (1974). This program computed four parameters, two rate constants and two coefficients corresponding to phase amplitudes, together with estimates of their uncertainties; in all cases the uncertainties were less than 4% of the parameter value.

Results

Reactions of cyanide with oxidized Pseudomonas cytochrome oxidase and its apoprotein

Fig. 1 shows the effect on the spectrum of oxidized *Pseudomonas* cytochrome oxidase produced by the addition of KCN. Clearly the ligand changes the spectrum of the normal oxidized protein throughout the visible region, the principal features being a 2nm red shift of the major Soret band at 410nm (haem *c*) and a 4nm blue shift of the absorption band at 640nm (haem d_1). Cyanide is therefore capable of affecting spectral bands that previous work (Barber *et al.*, 1976) has shown to be associated with both haem components of the enzyme.

The binding of cyanide to the oxidase, as reflected in the difference between the spectra of the oxidized cyanide-bound and oxidized forms of the protein, was found to be dependent on pH (Fig. 2). Although the overall form of the difference spectra at different pH values was similar, increasing the pH from 7 to 8.1 to 9.1 caused a progressive decrease in the amplitude of the extremum at 650nm, but as illustrated in Fig. 2 increases in extrema amplitude were observed at 574 and 420nm.

To help interpret these spectral data, a study was made of the effect of cyanide on the spectrum of the apoprotein of *Pseudomonas* cytochrome oxidase, where removal of the haem d_1 from the molecule allows the haem *c* to be examined in isolation. Fig. 3(*a*) shows the absorption spectra of the cyanide-bound and unbound forms of the apoprotein and Fig. 3(*b*) the corresponding difference spectrum (bound minus unbound) between the two species. These spectra mimic certain features of the corresponding results obtained for the holoenzyme, in particular the red shift of the major Soret band of the haem *c* component. But, whereas the completion of cyanide binding to oxidized *Pseudomonas* cytochrome oxidase required a time period of the order of hours at approximately millimolar ligand concentrations (see below), the reaction of the apoprotein was relatively rapid under the conditions used, being complete within 1 min of addition of the reactant.

Since initial spectral studies had shown that the binding of cyanide to oxidized *Pseudomonas* cytochrome oxidase was a slow process, difference spectroscopy was used to follow the kinetics of the reaction. Fig. 4 gives a typical family of difference spectra, recorded as a function of time, during such

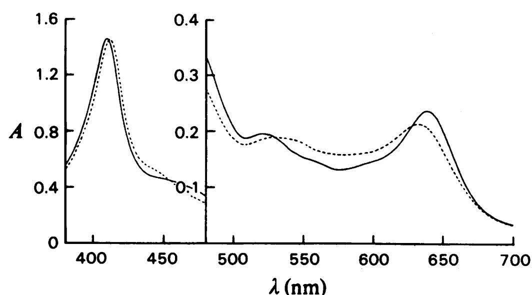


Fig. 1. *Spectrum of oxidized Pseudomonas cytochrome oxidase and its cyanide complex*

The spectra of the oxidized (——) and oxidized cyanide-bound (-----) forms of *Pseudomonas* cytochrome oxidase. The spectra were run in 0.1M-potassium phosphate buffer, pH9.1. The protein concentration was 5.1 μM and the path length 1 cm.

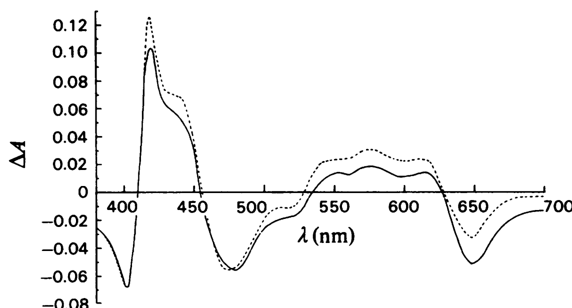


Fig. 2. *Effect of pH on the difference spectra (oxidized cyanide-bound minus oxidized) observed on binding cyanide to Pseudomonas cytochrome oxidase*

The difference spectra observed at pH7.0 (——) and pH9.1 (-----) on addition of 2mM-KCN to 5.3 μM solutions of oxidized *Pseudomonas* cytochrome oxidase in 0.1M-potassium phosphate buffer. The path length was 1 cm.

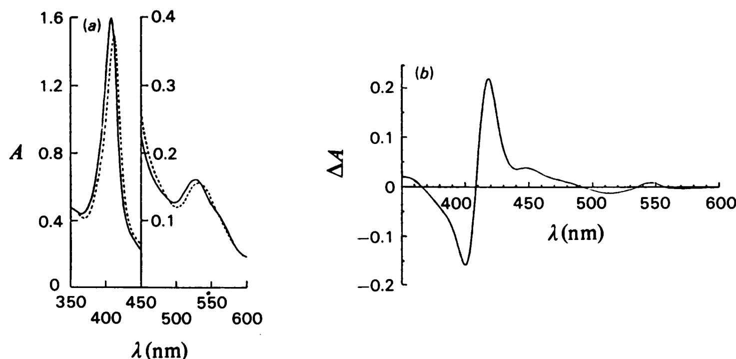


Fig. 3. *Effect of cyanide on the spectral properties of oxidized Pseudomonas cytochrome oxidase apoprotein*
(*a*) shows the absorption spectra of oxidized and oxidized cyanide-bound *Pseudomonas* cytochrome oxidase apoprotein. ——, Absorption spectrum of 4.9μM-*Pseudomonas* cytochrome oxidase apoprotein; -----, absorption spectrum after addition of 1 mM-KCN. The spectra were run in 0.1 M-potassium phosphate buffer, adjusted to pH 10 with KOH, and in the presence of 20μM-potassium ferricyanide. The path length was 1 cm. (*b*) shows the difference spectrum (oxidized cyanide-bound minus oxidized) produced on binding cyanide (1 mM) to *Pseudomonas* cytochrome oxidase apoprotein (4.9μM). The conditions were as for (*a*), with potassium ferricyanide being added in equal amounts to both sample and reference cells.

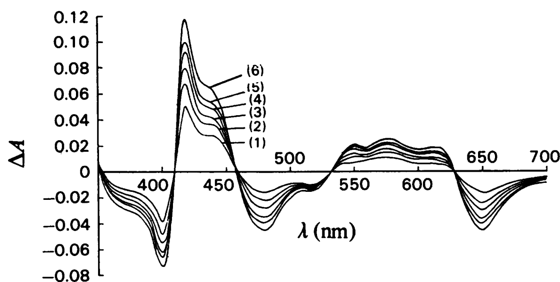


Fig. 4. *Reaction of oxidized Pseudomonas cytochrome oxidase with cyanide*
Difference spectra were run on the Cary 118c spectrophotometer from 700 to 350 nm at times of 7 min (1), 13 min (2), 19 min (3), 30 min (4), 40 min (5) and 220 min (6) after addition of 4 mM-KCN to 5.2μM-*Pseudomonas* cytochrome oxidase in 0.1 M-potassium phosphate buffer, pH 7.0. The scan speed was 2 nm/s and the temperature 22°C. The reference cuvette contained an identical concentration of a solution of the unbound oxidized enzyme.

an experiment, and perhaps the most striking feature is the maintenance of isosbestic points at 350, 410, 454, 535 and 627 nm throughout the reaction, although not all of these points appear to lie on the 'no-difference' line. Similar behaviour was observed in experiments carried out either at different pH values or at different cyanide concentrations. Semi-logarithmic analyses of the results showed that the reaction obeyed simple pseudo-first-order kinetics, the reaction velocity being accelerated by increases either in the cyanide concentration or in the pH of the reaction solution (Table 1). Table 1 also shows that the calculated pseudo-first-order rate constants are, within experimental error, independent of the observation wavelength.

Table 1. *Effect of pH, ligand concentration and observation wavelength on the rate constant of cyanide binding to oxidized Pseudomonas cytochrome oxidase*
The experiments were carried out in 0.1 M-potassium phosphate buffer at 22°C. Approx. 5μM enzyme solutions were used in all experiments.

pH	[KCN] (mM)	$10^3 \times k$ (s^{-1})				
	λ (nm) ...	400	420	440	480	650
7.0	2	0.28	0.32	0.32	0.33	0.32
7.0	4	0.87	0.78	0.67	0.83	0.83
8.1	2	0.78	0.92	0.77	0.97	0.85
9.1	2	2.3	2.1	3.0	2.3	2.2

Ascorbate reduction of the oxidized Pseudomonas cytochrome oxidase–cyanide complex

The oxidized cyanide complex of *Pseudomonas* cytochrome oxidase is reduced by the anaerobic addition of sodium ascorbate (Fig. 5) to yield an end product that is identical with that produced when the ligand cyanide is added to the oxidase after ascorbate reduction (Fig. 7). However, the characteristics of the intermediate spectrum in Fig. 5 run immediately after the addition of ascorbate indicate that, whereas

the haem d_1 reacts rapidly to produce the species haem d_1^{2+}–cyanide, the haem *c*, for the most part, remains in the oxidized cyanide-bound form and is only reduced at a very slow rate. Fig. 6 shows some typical reaction traces produced under a variety of conditions in which the reduction of the cyanide-bound enzyme was monitored with time at a single wavelength, 419nm. This reduction is a complex process, but all the traces have some features in common, i.e. an initial fast phase and a final very slow phase. In addition a lag period may be observed between these two phases in those experiments carried out at a high ascorbate concentration (16mM).

In control experiments at pH7.0, 8.1 and 9.1 in which ascorbate was added to unbound oxidized

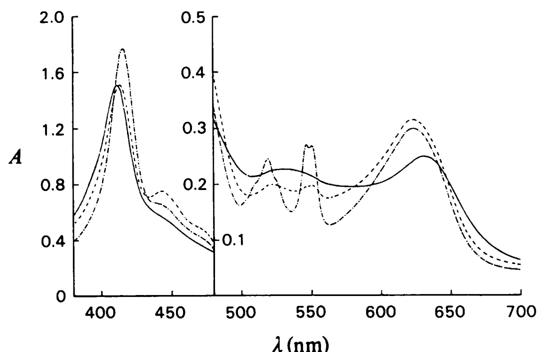


Fig. 5. *Absorption spectra of fully oxidized Pseudomonas cytochrome oxidase–cyanide and the species produced during its reduction by ascorbate*

——, Spectrum of the fully reduced *Pseudomonas* cytochrome oxidase–cyanide complex; -----, spectrum of the species produced immediately after the end of the fast phase of the reduction by ascorbate (5min after addition of reductant); –·–·, spectrum of the fully reduced cyanide-bound form of *Pseudomonas* cytochrome oxidase (150min after addition of reductant). The spectra were obtained at an enzyme concentration of 5.1 μM in 0.1 M-potassium phosphate buffer, pH9.1. The spectrum of oxidized *Pseudomonas* cytochrome oxidase–cyanide was recorded in air, in the presence of 2mM-KCN, and the spectra of the reduced forms were recorded under N_2 after the addition of 16mM-sodium ascorbate. The path length was 1cm.

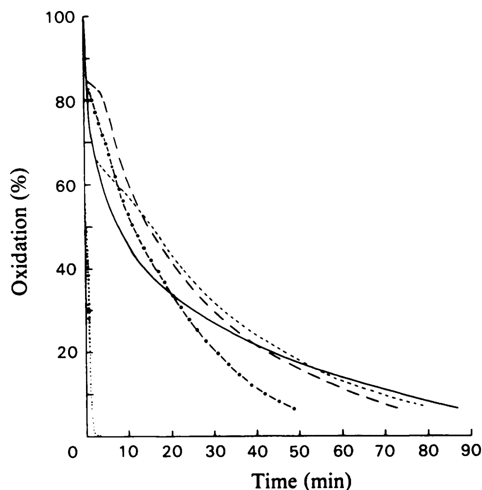


Fig. 6. *Ascorbate reduction of oxidized Pseudomonas cytochrome oxidase–cyanide*

Some typical time courses obtained at 419nm for the ascorbate reduction of *Pseudomonas* cytochrome oxidase–cyanide. The conditions were as follows: ——, pH7.0, 2mM-KCN, 0.8mM-sodium ascorbate; ----, pH7.0, 4mM-KCN, 16mM-sodium ascorbate, ——, pH8.1, 2mM-KCN, 16mM-sodium ascorbate; –·–·, pH9.1, 2mM-KCN, 16mM-sodium ascorbate. The experiments were carried out in 0.1 M-potassium phosphate buffer, at 22°C, under an atmosphere of N_2. The path length was 1cm. The concentrations of cyanide given are those before degassing the samples. ----- shows the result of a control experiment carried out on the unbound enzyme at pH8.1 at an ascorbate concentration of 16mM.

Table 2. *Variation of the amplitude of the fast phase and the rate of the slow phase observed during ascorbate reductions of the oxidized Pseudomonas cytochrome oxidase–cyanide complex*

The experiments were carried out under a N_2 atmosphere, in 0.1 M-potassium phosphate buffer and at 22°C. Ascorbate solutions were added by means of microsyringe

Expt. no.	[Ascorbate] (mM)	pH	[KCN] (mM)	ΔA_{fast}†	$10^4 \times k_{slow}(s^{-1})$
1	16	7.0	2	0.208	5.0
2	0.8	7.0	2	0.233	4.0
3	16	7.0	4	0.166	4.7
4	16	8.1	2	0.072	5.7
5	16*	8.1	2	0.276	4.2
6	16	9.1	2	0.074	8.7

* Ascorbate added before completion of the cyanide-binding reaction.

† Observed absorbance changes have been normalized to an enzyme concentration of 5 μM.

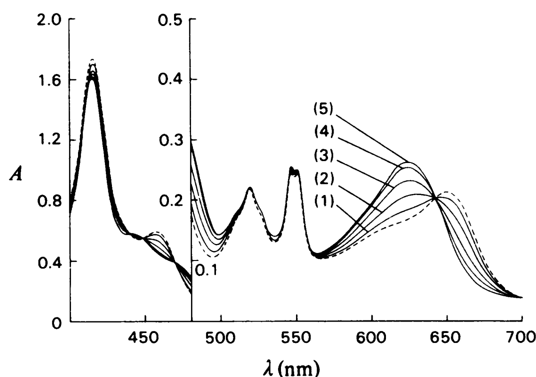


Fig. 7. *Titration of reduced Pseudomonas cytochrome oxidase with KCN*

----, Spectrum of 4.8 μM-reduced *Pseudomonas* cytochrome oxidase in 0.1 M-potassium phosphate buffer, pH 7.0, under an atmosphere of N_2. Lines (1), (2), (3) and (4) show spectra run after the addition of KCN to concentrations of 14.1 μM, 24.6 μM, 38.7 μM and 59.8 μM respectively, and line (5) shows the spectrum of the fully bound enzyme. Some of the spectra obtained during the titration have been omitted for clarity. Spectral measurements were made 5 min after each addition of KCN solution.

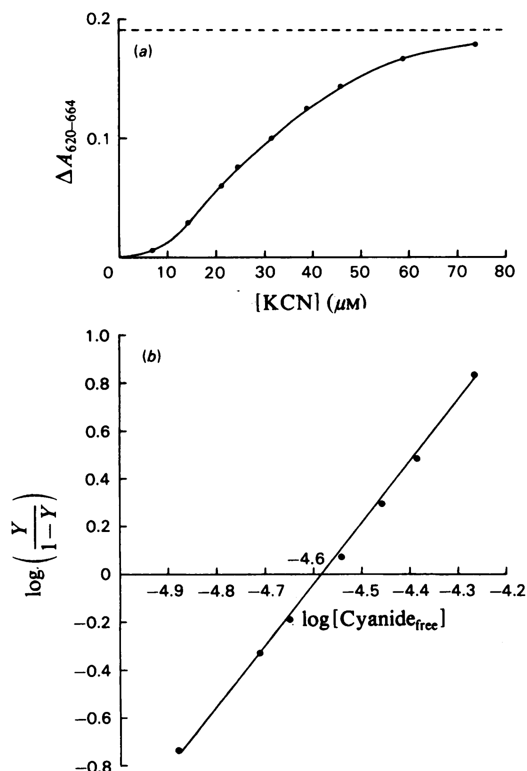


Fig. 8. *Analysis of the data obtained during a titration of reduced Pseudomonas cytochrome oxidase with KCN*

(*a*) The results in Fig. 7 are plotted as the difference in absorbance at 620 nm and 664 mn against total KCN concentration. The broken line represents the end point of the titration. The path length was 1 cm. (*b*) A Hill plot of the data in (*a*), where *Y* is the fractional saturation of the enzyme with ligand and [cyanide$_{free}$] represents the concentration of unbound ligand present in the cuvette.

enzyme, the reduction was in all cases complete in 3 min. Table 2 summarizes the data obtained from a number of experiments of this type. The rate constants given are those of the final, slowest phase. That these rate constants are effectively invariant over a 20-fold change in ascorbate concentration suggests that this reduction can only occur after a preceding monomolecular step, namely the dissociation of cyanide from the oxidized haem *c* component.

Table 2 shows that both pH and the concentration of cyanide used during the ligand-binding reaction can affect the amplitude of the fast phase in the subsequent reduction of the oxidase. Furthermore, if the ascorbate reduction is carried out after the cyanide reaction has only been allowed to progress for approximately one half-time (Table 2, Expt. 5), a large increase in the absorbance change of the fast phase is observed compared with the control (Table 2, Expt. 4) in which the cyanide binding was allowed to proceed to completion.

Reaction of cyanide with reduced Pseudomonas cytochrome oxidase

In contrast with the oxidized protein, the reaction of cyanide with reduced *Pseudomonas* cytochrome oxidase was much more rapid. For this reason it has been practical to study the equilibrium binding behaviour of the reduced protein, and Fig. 7 shows a family of spectra recorded during a titration of the reduced enzyme with cyanide. The ligand has a marked effect on those absorption bands associated with the haem d_1, whereas any changes to those absorption bands associated with haem *c* are merely of band height rather than position or shape. These observations are consistent with previous work (Yamanaka & Okunuki, 1963*b*) and may be interpreted as indicating that cyanide only binds to the haem d_1 component of reduced *Pseudomonas* cytochrome oxidase. The experimental results provide clear evidence, in the form of isosbestic points maintained throughout the titration, that only two species contribute significantly to the observed spectra, $c^{2+}\cdot d_1^{2+}$ and $c^{2+}\cdot d_1^{2+}$–cyanide.

The ligand-binding curve (Fig. 8*a*), plotted as the difference in absorption at two wavelengths on either side of an isosbestic point to minimize non-specific

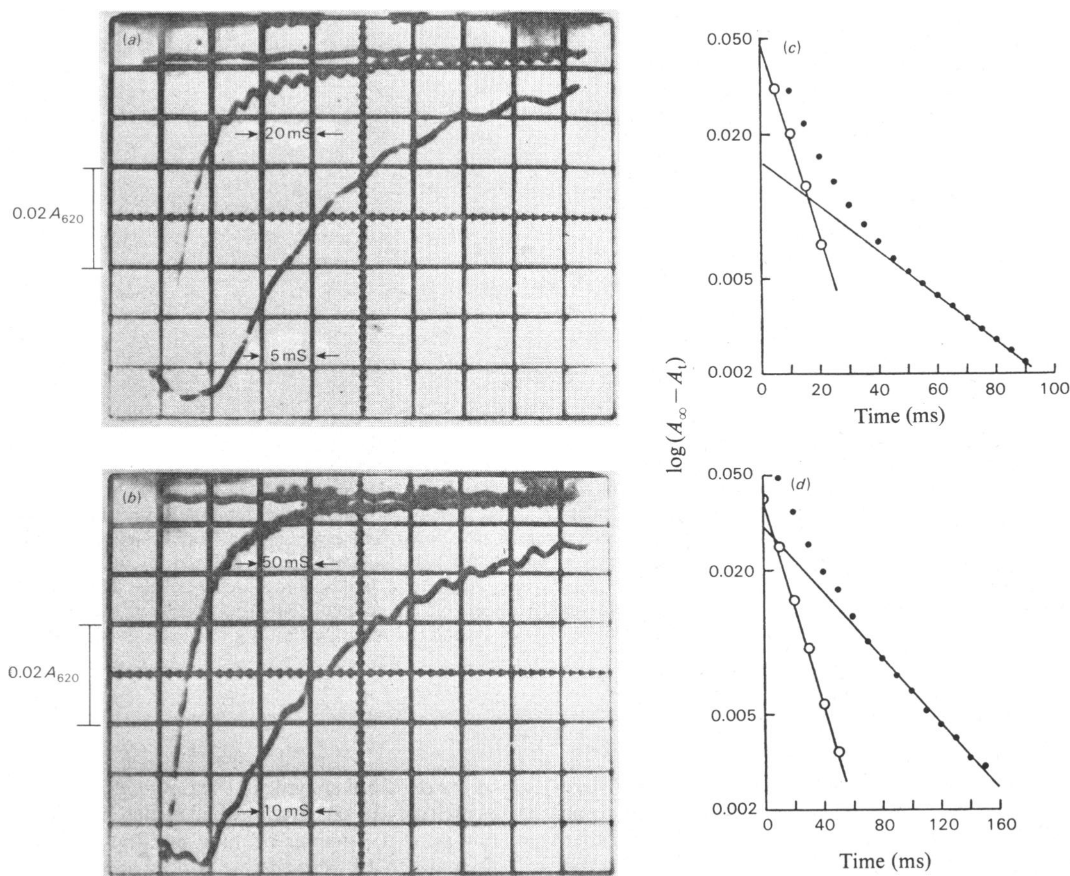


Fig. 9. *Reaction of reduced Pseudomonas cytochrome oxidase with cyanide*

(*a*) The reaction of ascorbate-reduced *Pseudomonas* cytochrome oxidase (4 μM) with 195 μM-KCN potassium cyanide on mixing the two solutions in the stopped-flow apparatus. The experiment was carried out in 0.1 M-potassium phosphate buffer, pH 7.0, under N_2 at 20°C, and in a 2 cm-light-path cell. The vertical scale corresponds to a value of 0.01 *A* unit per division, and the horizontal scale represents changes of 5 ms and 20 ms per division for the lower and upper traces respectively. (*b*) The reaction of ascorbate-reduced *Pseudomonas* cytochrome oxidase (4 μM) with 82 μM-KCN on mixing the two solutions in the stopped-flow apparatus. The conditions were as for (*a*) except that horizontal scale represents changes of 10 ms and 50 ms per division for the lower and upper traces respectively. (*c*) Semi-logarithmic analysis of the fast (○) and slow (●) phases in (*a*). (*d*) Semi-logarithmic analysis of the fast (○) and slow (●) phases in (*b*).

variations, shows that the binding of cyanide to the reduced enzyme is not stoicheiometric, with the titration curve being sigmoidal in shape. Fig. 8(*b*) shows a Hill plot of those data points in Fig. 8(*a*) with fractional saturations between 0.1 and 0.9 (see Antonini & Brunori, 1971) and yields a value for the Hill coefficient (*h*) of 2.6 together with an apparent affinity constant of 5×10^{11} M^{-1}.

Kinetic studies on the reaction of the reduced enzyme with cyanide were carried out by using rapid-mixing techniques. Fig. 9 shows two reaction traces obtained at different ligand concentrations together with their respective semi-logarithmic analyses. The binding process is kinetically complex, consisting of two reaction phases. The pseudo-first-order rate constants of both reaction phases exhibit a linear dependence on cyanide concentration over the experimental range explored (Figs. 10*a* and 10*b*), yielding apparent second-order rate constants of 9.3×10^5 M$^{-1} \cdot$ s^{-1} and 2.3×10^5 M$^{-1} \cdot$ s^{-1}. Another important feature of the reaction is that the relative proportions of the two component phases were also found to vary with cyanide concentration (Fig. 10*c*), so that the slow process was a minor contributor to the overall reaction at high ligand concentrations, but the major contributor at low concentrations.

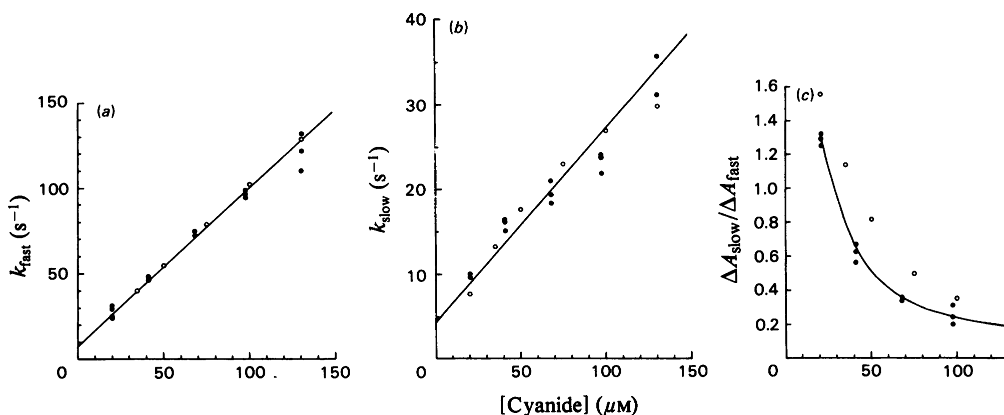


Fig. 10. *Dependence on cyanide concentration of the rates and relative proportions of the fast and slow phases observed in the reaction with reduced Pseudomonas cytochrome oxidase*

(*a*) The dependence of the fast phase, (*b*) the dependence of the slow phase and (*c*) the dependence of the ratio of the amplitudes of the slow to fast phases on cyanide concentration are shown. The experimental results (●) were observed under the same conditions as Fig. 9. The computer-simulated results (○) were calculated by using values of 1.3×10^6M$^{-1}\cdot$s^{-1}, 1.1×10^6M$^{-1}\cdot$s^{-1},50s^{-1} and 45s^{-1} for k_{+1}, k_{+2}, k_{+3} and k_{-3} of Scheme 2 respectively. The lines drawn through the experimental points have no theoretical significance. The enzyme concentration used for the calculations was 2μM and it has been assumed that the absorbance change associated with the binding of both ligand molecules is the same.

Although the kinetic results presented here were obtained by following the reaction at 620nm, some time courses were also followed at a number of other wavelengths (400, 460, 490 and 660nm), but the reaction was observed to have similar characteristics wherever monitored.

Discussion

Contrary to the report of Yamanaka & Okunuki (1963*b*) that no spectral change was observed on addition of cyanide to oxidized *Pseudomonas* cytochrome oxidase, the results presented here seem more consistent with the findings of Gudat *et al.* (1973), who found that this ligand caused a loss of e.p.r. signals associated with the haem d_1 component. Spectral evidence gained during the present study indicates that cyanide not only binds to the haem d_1 of the oxidized protein, but is also able to bind to the haem *c* component. The interpretation of the spectral changes that occur when cyanide binds to oxidized *Pseudomonas* cytochrome oxidase is assisted by the examination of the spectra produced on reaction of cyanide with the apoprotein from which the haem d_1 has been removed, although some caution must be exercised before definite conclusions can be drawn, since removal of the haem d_1 obviously has drastic effects on the physical properties of the enzyme. Nevertheless, comparison of Figs. 1 and 2 with Fig. 3 indicates that the influence of cyanide on the spectrum of the oxidized enzyme in the range 350–425nm is due principally to changes in the spectrum of the haem *c* component, whereas cyanide binding to the haem d_1 appears to be responsible for most of the changes observed throughout the rest of the spectrum.

Fig. 2 shows that changes in pH are capable of altering the difference spectrum between the cyanide-bound and unbound forms of the oxidized protein. At first sight it might appear from the decrease in amplitude of the difference extremum at 650nm that raising the pH shifts the haem d_1/d_1–cyanide equilibrium towards the unbound form. However, exactly the reverse occurs at other extrema, which, for reasons given above, would also be associated with the haem d_1 moiety. These results therefore suggest that pH may have a direct effect on the spectrum of the species haem $d_1{}^{3+}$–cyanide. Any effects of pH on the species haem c^{3+}–cyanide are more difficult to assess since, as discussed below, the difference spectrum of this component does appear to be influenced by the exact position of the ligand-binding equilibrium under a particular set of circumstances.

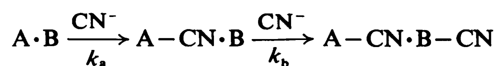
A further insight into the nature of the oxidized enzyme–cyanide complex was obtained by following the progress of reduction of this species after addition of sodium ascorbate. Despite the complexity of the overall reaction, it is reasonable to conclude, on the basis of Figs. 5 and 6 and the data in Table 2, that the initial fast phase represents, not only the reduction of all the haem d_1, but also the reduction of any uncom-

plexed haem *c*, the latter being a function of both ligand concentration and pH. The data in Fig. 6 and Table 2 indicate that between pH 8.1 and 9.1 the proportion of the fast phase is the same, implying that in these two cases the haem *c* has become fully bound and that the fast phase represents only the effect of haem d_1 reduction. The subsequent slow phase appears to monitor only the reduction of cyanide-bound haem *c*, which is limited by the rate of cyanide dissociation. The spectral evidence collected during the present study suggests that cyanide does not bind to the ferrous haem *c* under the experimental conditions used. With an average value of $4.6 \times 10^{-4}\,s^{-1}$ for the dissociation rate constant of cyanide from the haem *c* component at pH 7.0 (Table 2), in conjunction with a second-order rate constant for cyanide combination of $0.2\,M^{-1} \cdot s^{-1}$, which may be derived from Table 1, the haem *c*–cyanide dissociation equilibrium constant, at pH 7.0, may be calculated as approximately $2.3 \times 10^{-3}\,M$. However, the haem *c*–cyanide dissociation rate used is that for the species c^{3+}–cyanide$\cdot d_1{}^{2+}$–cyanide and not c^{3+}–cyanide$\cdot d_1{}^{3+}$–cyanide, and this difference in redox states could produce analogous effects to those observed in mammalian cytochrome oxidase (Van Buuren *et al.* 1972*a*), where changing the redox state of haem *a* from ferric to ferrous enhances the cyanide dissociation rate from ferric haem a_3. It is possible that the lag phase observed in some cases after the fast reduction phase is, in fact, signalling a conformational change within the mixed-valence species, haem c^{3+}–cyanide$\cdot d_1{}^{2+}$–cyanide, from one state to another with an accelerated haem *c*–cyanide dissociation rate.

The behaviour of the haem d_1 towards reduction by ascorbate does not allow an unequivocal determination as to whether any fraction of this component is uncomplexed after the cyanide reaction has reached equilibrium. As part of this study we have examined the kinetic behaviour of oxidized *Pseudomonas* cytochrome oxidase towards reduction by ascorbate under anaerobic conditions. These experiments (results not illustrated) showed that, over the ascorbate concentration range used for the reduction of the cyanide complex (<16 mM), the rates of reduction of the haems *c* and d_1, as measured at 419 and 660 nm respectively, were the same, the second-order rate constant being $1.7\,M^{-1} \cdot s^{-1}$ at pH 7.0 and 25°C. This behaviour is consistent with the results of Parr *et al.* (1977), since electrons would be transferred between the haem components at rates faster than ascorbate reacts with the enzyme. Unfortunately this situation does not allow us to decide whether a particular haem or both haem groups may be attacked directly by ascorbate. However, there is strong indirect evidence to suggest that the species haem $d_1{}^{3+}$–cyanide can undergo direct reduction by ascorbate without prior dissociation of the ligand. The fast phase in the reduction of oxidized cyano-*Pseudomonas* cytochrome oxidase, during which the haem d_1 is reduced, has a half-time of less than 60 s at an ascorbate concentration of 16 mM, which means that, if cyanide dissociation is to precede the reduction, it must occur at a rate of at least $0.01\,s^{-1}$. This value is considerably in excess of any of the cyanide-combination rates (Table 1), and it would seem unlikely from the results (Fig. 4) that the equilibrium between bound and unbound haem d_1 lies so far towards the latter. Nevertheless, any haem d_1 that did remain unbound after completion of the cyanide-binding reaction might be reduced quickly on addition of ascorbate, as indicated above, and then rapidly bind the ligand with a high affinity (Figs. 9 and 10).

Perhaps the most interesting aspect of the reaction of oxidized *Pseudomonas* cytochrome oxidase with cyanide is the coincidence, under a variety of conditions, of the combination rate constants for the haem *c* and d_1 components. This identity in rates appears to be responsible for the maintenance of isosbestic points during the reaction, although the reason why not all of them lie on the 'no-difference' line (the line of zero ΔA in Fig. 4, corresponding to both reference and sample cells containing only oxidized enzyme) is not clear, but may be due to a very small proportion of the protein reacting more rapidly than the rest. It is possible that the two haem chromophores react individually at very similar rates under the conditions used, but it seems equally possible that the experimental results indicate a haem–haem co-operativity between the two centres. Scheme 1 seeks to illustrate this latter effect where A and B represent two cyanide-binding components. Provided that k_b is much greater than k_a then, although cyanide binding to B can only occur after the ligand has bound to A, the two reactions will appear to proceed simultaneously with rate k_a. Such a mechanism, however, does not allow any conclusions to be drawn as to which haem is the first to bind cyanide, or indeed if there is a preferred first site.

Whatever the exact mechanism for the reaction of the oxidized protein, the reaction of the reduced enzyme is very different. In a sense the observed behaviour of the two redox states appears to parallel that of mammalian cytochrome oxidase, where the reduced enzyme has also been found to



$$A \cdot B \xrightarrow[k_a]{CN^-} A-CN \cdot B \xrightarrow[k_b]{CN^-} A-CN \cdot B-CN$$

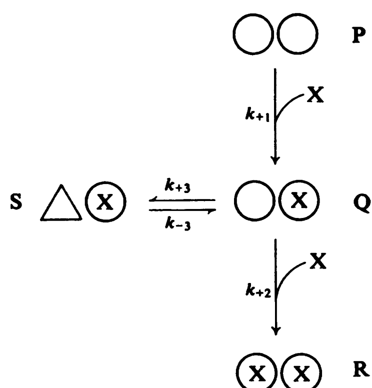
Scheme 1. *A possible kinetic scheme for the binding of cyanide to oxidized Pseudomonas cytochrome oxidase*
A and B represent two cyanide–binding components of a single molecular species.

react much more rapidly than the oxidized (Van Buuren *et al.*, 1972*b*).

The kinetic data for the reaction of reduced *Pseudomonas* cytochrome oxidase with cyanide shows a striking similarity to results obtained previously for the reaction of the enzyme with CO (Parr *et al.*, 1975). This earlier work lead to the postulation of Scheme 2, in which species S cannot bind a second ligand molecule.

The open circles in Fig. 10 show the results of analyses of computer-simulated reaction traces for Scheme 2. Although the rate constants used in the example given were determined empirically and need not necessarily represent a 'best fit' of the scheme to the experimental data, the scheme appears to be capable of reproducing most features of the observed results. The most important difference between the observed and simulated data (Fig. 10*b*) lies in the latter predicting that the slow phase is not linearly dependent on ligand concentration, but tends to a rate limit at high ligand concentrations ($>130\,\mu$M). However, this may be very difficult to observe experimentally, since, under such conditions, the relative proportion of the slow phase becomes very small and hence subject to large errors in analysis. These errors are compounded by the fact that the rates of the fast and slow processes are relatively close to each other and thus their analytical separation is also subject to some uncertainty. We therefore consider that in spite of its shortcomings Scheme 2 does represent a reasonable explanation of the experimental results.

The equilibrium binding studies of the reduced enzyme with cyanide also, to some degree, follow the earlier work with CO in that they appear to support the concept of co-operativity between haem d_1 groups, the observed Hill coefficient (h) being greater than unity. It is, however, difficult to reconcile an h value of 2.6 with structural evidence for *Pseudomonas* cytochrome oxidase being a dimer (Kuronen & Ellfolk, 1972), since the value of h should never exceed the number of binding sites per molecule (Antonini & Brunori, 1971). It is interesting that anomalous behaviour towards cyanide binding has also been shown by another haem protein, ferric horse myoglobin. This single-site protein has been bound to give h values of up to 2.5 (see Antonini & Brunori, 1971). But the fact that the binding appears to be anomalous for *Pseudomonas* cytochrome oxidase also tends to lessen the weight of evidence for there being a co-operative interaction between the haem d_1 groups of this enzyme molecule.



Scheme 2. *Proposed kinetic scheme for the binding of cyanide to reduced Pseudomonas cytochrome oxidase*
The circles represent the haem d_1 components of the *Pseudomonas* oxidase dimer that can bind or are bound with the ligand X. The triangle in species S represents a situation where one of the haems d_1 has assumed a state in which it cannot bind the ligand.

The results in the present paper show that cyanide may react, in a complex manner, with both redox states of *Pseudomonas* cytochrome oxidase. In terms of the reaction of cyanide with the oxidized enzyme the possibility of co-operativity between the c and d_1 haems may have physiological consequences, in that Shimada & Orii (1975) have found that NO, the product of nitrite reductase activity, is also capable of binding to both the oxidized haem components. Furthermore, the fact that cyanide, once bound to the haem c, may inhibit its reduction by ascorbate also raises the possibility that part of the inhibitory effect of the ligand on the enzymic functions of the protein may be due to an interruption of the electron-transfer reactions with its two physiological electron donors *Pseudomonas* ferrocytochrome c-551 and reduced azurin.

As regards the reaction with reduced *Pseudomonas* cytochrome oxidase, the rate of cyanide binding is much faster than those rates reported for the reactions with both O_2 (Greenwood *et al.*, 1978) and CO (Parr *et al.*, 1975), although the overall character of the kinetic behaviour resembles that found for CO much more closely than that observed with the electron acceptor. An explanation for the differential effects of CO and cyanide on the oxidase and nitrite reductase activities as seen in steady-state kinetic experiments should, however, be attempted in terms of affinity rather than kinetic constants. Greenwood *et al.* (1978) have reported a value of approx. $10^4\,$M^{-1} as the affinity constant for O_2 of reduced *Pseudomonas* cytochrome oxidase, based on results of rapid-mixing experiments. Kijimoto (1968) has determined a similar value of $1.67\times10^4\,$M^{-1} for O_2, but a much higher value of $1.25\times10^6\,$M^{-1} for the affinity of nitrite for the haem d_1 on the basis of steady-state kinetic experiments and has pointed out that an inhibitor may be able to compete effectively with one electron acceptor (O_2), but not the other.

Viewed in this way, the Hill-plot data of Parr *et al.*

(1975), which give an apparent affinity constant of CO for reduced *Pseudomonas* cytochrome oxidase of $5\times10^{6}\,\text{M}^{-1}$, may be compared with the value of $5\times10^{11}\,\text{M}^{-1}$ reported above for cyanide, and appear to be consistent with the steady-state behaviour. However, some caution is necessary, since the precise meaning of the affinity constants derived from Hill plots is not clear. For example, despite its lower affinity constant the concentration of CO required for half saturation of the enzyme is less than the concentration of cyanide required for half saturation, although the enzyme concentrations used in the two titrations were approximately the same. This effect is a result of the highly sigmoidal cyanide-binding curve. It is therefore still uncertain whether the different effects of CO and cyanide on the two enzymic activities of *Pseudomonas* sytochrome oxidase can be rationalized purely in terms of relative affinities of substrates and inhibitors for the reduced haem d_1.

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