The Oxidation of *Pseudomonas* Cytochrome *c*-551 Oxidase by Potassium Ferricyanide

By DONALD BARBER, STEPHEN R. PARR* and COLIN GREENWOOD

School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

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Stopped-flow kinetic studies were made of the reaction between ascorbate-reduced *Pseudomonas* cytochrome oxidase and potassium ferricyanide under both N₂ and CO atmospheres. Under N₂ three kinetic processes were observed, two being dependent on ferricyanide concentration, with second-order rate constants of $9.6 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $1.5 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, whereas the other was concentration-independent, with a first-order rate constant of $0.17 \pm 0.03 \text{ s}^{-1}$. Measurements of their kinetic difference spectra have allowed the fastest and second-fastest phases of the reaction to be assigned to direct bimolecular reactions of ferricyanide with the haem *c* and haem *d*₁ moieties of the enzyme respectively. Under CO, the second-order rate constant for the reaction of the haem *c* was, at $1.3 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, slightly enhanced over the rate in a N₂ atmosphere, but the reaction velocity of the haem *d*₁ component was greatly decreased, being apparently limited to that of the rates of CO dissociation from the molecule (0.15 s^{-1} and 0.03 s^{-1}). The results are compared with those obtained during a previous study of the reaction of reduced *Pseudomonas* cytochrome oxidase with oxidized azurin.

Pseudomonas cytochrome *c*-551 oxidase (EC 1.9.3.2) is a protein found in cellular extracts of cells of *Pseudomonas aeruginosa* grown anaerobically in the presence of nitrate (Yamanaka *et al.*, 1963). Although the first discovered catalytic activity was the ability to reduce oxygen (Horio, 1958), later work has suggested that the true physiological role of the enzyme is the reduction of nitrite (Yamanaka *et al.*, 1961). Studies of the structure of the molecule have revealed that it is composed of two subunits, each containing a haem *c* and a haem *d*₁ prosthetic group, and has a total mol.wt. of approx. 120000 (Kuronen & Ellfolk, 1972; Gudat *et al.*, 1973; Kuronen *et al.*, 1975). Spectroscopic investigations have provided evidence that the haem *d*₁ component is the site of ligand binding (Yamanaka & Okunuki, 1963; Parr *et al.*, 1975), whereas reports on the kinetic behaviour of the enzyme towards azurin have suggested that the haem *c* component is the sole site of electron exchange with this physiological electron donor (Wharton *et al.*, 1973; Parr *et al.*, 1977). More recently, however, kinetic studies of the reduction of the enzyme by Cr²⁺ ions (Barber *et al.*, 1977) have suggested that a direct reaction at the haem *d*₁ may occur with this non-physiological redox reagent. The aim of the work reported here has therefore been to examine the behaviour of reduced *Pseudomonas* cytochrome oxidase towards a non-physiological

oxidant, thus allowing, by comparison, an assessment of the specificity of the protein-protein interaction which occurs when the enzyme reacts with oxidized azurin.

Materials and Methods

All chemicals were from Fisons, Loughborough, Leics., U.K., and were of analytical grade, except for ascorbic acid (disodium salt) from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K., and potassium ferricyanide from Hopkin and Williams, Chadwell Heath, Essex, U.K. CO and N₂ gases were 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 anthroquinonesulphonate.

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 $A_{410}^{\text{ox}}/A_{280}$ and $A_{640}^{\text{ox}}/A_{520}^{\text{ox}}$ for the enzyme were 1.18–1.2 and 1.15–1.2 respectively. The concentrations of oxidase solutions were determined by using an absorption coefficient at 410 nm of $288 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for the oxidized protein (M. C. Silvestrini, A. Colosimo, M. Brunori & C. Greenwood, unpublished work), and an absorption coefficient at 420 nm of $1024 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ has been used for solutions of potassium ferricyanide (Goldberg & Pecht, 1976).

The experimental conditions used were as close as possible to those used in a previous study of the

* Present address: Department of Biochemistry, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, Scotland, U.K.

reaction of the enzyme with azurin (Parr *et al.*, 1977) and all kinetic work has been carried out in 0.1M-potassium phosphate buffer, pH 7.0, at 25°C. Reduced *Pseudomonas* cytochrome oxidase solutions were prepared under an atmosphere of either N₂ or CO, in a large (approx. 70ml) cuvette sealed with a Suba-Seal vaccine cap, by anaerobic addition of a 50% stoichiometric excess of sodium ascorbate. Under these conditions the time taken for complete reduction was about 2h, the reaction being monitored spectrophotometrically in a Cary 118C recording spectrophotometer; spectra run before the addition of ascorbate and after completion of the reduction reaction have been used to construct the total static reduced-minus-oxidized difference spectra. An excess of sodium ascorbate was used to ensure the removal of any residual O₂ remaining in the oxidase solution after the degassing procedure (it is reduced by the enzyme). This allows the protein solution to become fully reduced, but as may be deduced from the long time required for the reduction any small amounts of ascorbate that are left would not be expected to affect the relatively much faster reactions of the enzyme with ferricyanide.

Stopped-flow kinetic studies were carried out anaerobically, in an apparatus identical with that described by Gibson & Milnes (1964). A 2cm-light-path cell with a dead time of 3ms was used, and reaction traces were displayed on a Tektronix type 7514 dual-time-base storage oscilloscope. Total kinetic difference spectra have been plotted as the change in absorbance, with wavelength, occurring between $t=3\text{ms}$ and t_{∞} after mixing in the stopped-flow apparatus. Kinetic difference spectra of reaction phases have been determined from semilogarithmic analysis of progress curves. Log plots of slower reactions were used to provide ' t_{∞} ' baselines for faster processes, the spectroscopic amplitude for a particular phase being measured as the difference in absorbance between its extrapolated log plot, at $t=3\text{ms}$, and the t_{∞} line for the phase. The concentrations of oxidase solutions used in the determination of the kinetic difference spectra were varied according to the size of the absorption changes expected over a particular wavelength region. However, the results have been presented in terms of the highest concentration used, $6.8\mu\text{M}$. Estimates of errors in second-order rate constants have been made from the plots used for their determination. Errors of first-order rate constants are calculated standard errors.

Results

Kinetic behaviour of reduced Pseudomonas cytochrome oxidase towards oxidation by potassium ferricyanide

Initial studies showed that when ascorbate-reduced *Pseudomonas* cytochrome oxidase was mixed with ferricyanide, under a N₂ atmosphere, complex

reaction traces were produced, with three observable kinetic phases arising from redox changes at both the haem *c* and haem *d*₁ components. Previous studies (Barber *et al.*, 1977) have shown that the major contributor to the difference spectrum at 550nm is the haem *c* component, and hence this wavelength was chosen to define the reaction rate of the haem *c*. It was found that a particularly favourable set of circumstances existed at 520nm in which all three kinetic processes could be clearly observed and easily separated on analysis, and so this wavelength was also used in experiments to determine the dependence of reaction rates on varying ferricyanide concentration.

Figs. 1(a) and 1(b) show two typical progress curves observed at 550 and 520nm respectively when reduced *Pseudomonas* cytochrome oxidase reacted with ferricyanide, under N₂. Figs. 2(a) and 2(b) show the corresponding semilogarithmic plots for these traces; of the three reaction phases monitored at 520nm, only the fastest phase is significant at 550nm. Of the three processes, the rate of the slowest was independent of ferricyanide concentration, with a first-order rate constant of $0.17 \pm 0.03\text{s}^{-1}$. However, the other two reaction phases were linearly dependent on oxidant concentration (Figs. 3a and 3b), with second-order rate constants for the second-fastest and fastest phases of $1.5 \times 10^4 \pm 0.1 \times 10^4\text{M}^{-1}\cdot\text{s}^{-1}$ and $9.6 \times 10^4 \pm 0.15 \times 10^4\text{M}^{-1}\cdot\text{s}^{-1}$ respectively.

Effect of CO on the reaction of reduced Pseudomonas cytochrome oxidase with potassium ferricyanide

Figs. 4(a) and 4(b) are typical reaction time courses observed at 550 and 490nm respectively on mixing the reduced carbonmonooxy complex of *Pseudomonas* cytochrome oxidase with potassium ferricyanide in stopped-flow apparatus under an atmosphere of CO. The semilogarithmic analyses of these two traces in Figs. 5(a) and 5(b) show that the reaction at 550nm is very similar in form to that seen in the experiments under N₂ at this wavelength, but the second-order rate constant of $1.3 \times 10^5 \pm 0.15 \times 10^5\text{M}^{-1}\cdot\text{s}^{-1}$ is slightly enhanced with respect to that of the fast phase observed in the absence of CO (Fig. 3b). Fig. 5(b) shows that there is also a small amount of fast phase at 490nm, but here the predominant part of the absorption change is due to very much slower processes, with rates of $0.15 \pm 0.03\text{s}^{-1}$ and $0.03 \pm 0.01\text{s}^{-1}$. These slower rates were independent of ferricyanide concentration, provided that this was large ($> 500\mu\text{M}$), and compare well with the values reported by Parr *et al.* (1975) for the rates of dissociation of CO from the haem *d*₁ of the reduced *Pseudomonas* cytochrome oxidase-CO complex.

As may be deduced from the spectra of the oxidized, reduced and reduced CO-bound forms of *Pseudomonas* cytochrome (see Barber *et al.*, 1976)

Fig. 1. Reaction progress curves for the oxidation of reduced *Pseudomonas* cytochrome oxidase by potassium ferricyanide (a) An oscilloscope trace produced by mixing $4.1 \mu\text{M}$ -*Pseudomonas* cytochrome oxidase with $1000 \mu\text{M}$ -potassium ferricyanide in 0.1M -potassium phosphate buffer, pH 7.0. The reaction was observed at 550 nm in a 2 cm path-length cell at a temperature of 25°C under an atmosphere of N_2 . The vertical scale represents a change of $0.01 A$ unit per division and the horizontal scale 10 ms and 50 ms per division for upper and lower traces respectively. (b) An oscilloscope trace produced on mixing $6.8 \mu\text{M}$ -*Pseudomonas* cytochrome oxidase with $125 \mu\text{M}$ -potassium ferricyanide. The conditions were as described in (a) except that the reaction was observed at 520 nm . The vertical scale corresponds to a change of $0.005 A$ unit per division, and the horizontal scale represents sweep times of 200 ms and 2 s per division for traces (1) and (2) respectively.

the reaction trace at 550 nm would also be expected to exhibit substantial amounts of these slow absorption changes. Experiments showed that such behaviour did indeed occur. However, reactions monitored under CO were generally followed as shown in Fig. 5(a), with the fast reaction being observed by using one time base of the oscilloscope and the baseline for this reaction being collected by using the second time base at a slower scan speed. Such a method was feasible, because the subsequent processes associated with haem d_1 oxidation were comparatively so slow that their effect on the baseline could be neglected.

Fig. 2. Analysis of the reaction of potassium ferricyanide with reduced *Pseudomonas* cytochrome oxidase (a) Log plot of Fig. 1(a); (b) log plots of the reaction phases of Fig. 1(b). The fastest phase ($\circ$) and the second-fastest phase ($\bullet$) have been plotted using the upper abscissa and the inner and outer ordinates respectively. The slowest phase ($\blacktriangle$) has been plotted with respect to the lower abscissa and the innermost ordinate.

The use of slower scans to obtain fast-phase baselines was particularly important at low ferricyanide concentrations, where the amplitudes of the two slowest processes were found to decrease with the amount of oxidant present until eventually 'overshoots' were seen, when the slow oxidations were over-ridden by another process which appeared to

Fig. 3. Ferricyanide-dependence of the fastest and second-fastest phases of the oxidation of reduced *Pseudomonas* cytochrome oxidase

(a) The dependence of the second-fastest phase observed at 520 nm under N₂. (b) The dependence of the fast phase observed at 550 (●) and 520 (○) nm under N₂ and (▲) at 550 nm under CO. The experiments were performed in 0.1 M-potassium phosphate buffer, pH 7.0, in the stopped-flow apparatus, in a 2 cm cell. The temperature was 25 °C.

correspond to reduction of the protein. Such an occurrence was not totally unexpected, in view of the previous finding (Barber *et al.*, 1976) that incubation of oxidized *Pseudomonas* cytochrome oxidase under an atmosphere of CO could produce the fully reduced carbonmonoxy complex in the absence of any added reducing agents. The nature of the reductant operating under these conditions is unknown, although it must be present in quite large amounts, since substantial concentrations of ferricyanide are also reduced. Nevertheless, despite the fact that the 'auto-reduction' process is able to compete kinetically with the slow oxidation of the haem d_1 under these conditions, the rate would not appear to be such that any significant influence on the fast oxidation process would be expected.

Difference spectra

By repeating experiments of the type described above over a series of wavelengths, it is possible to construct kinetic difference spectra for both complete reactions and reaction phases. Fig. 6 shows the

Fig. 4. Reaction progress curves for the oxidation of the reduced *Pseudomonas* cytochrome oxidase-CO complex by potassium ferricyanide

(a) An oscilloscope trace produced on mixing 3.5 μ M-*Pseudomonas* cytochrome oxidase-CO with 1000 μ M-potassium ferricyanide in 0.1 M-potassium phosphate buffer, pH 7.0. The reaction was observed at 550 nm in a 2 cm-path-length cell, at 25 °C and under an atmosphere of CO. The vertical scale represents a change of 0.01 A unit per division and the horizontal scale 10 ms and 50 ms for the upper and lower traces respectively. (b) An oscilloscope trace produced on mixing 4.1 μ M-*Pseudomonas* cytochrome oxidase-CO with 1000 μ M-potassium ferricyanide. The conditions were as described in (a) except that the reaction was observed at 490 nm. The vertical scale corresponds to a change of 0.01 A unit per division, and the horizontal scale represents sweep times of 20 ms and 15 s per division for the lower and upper traces respectively.

total static and kinetic difference spectra for the reaction of reduced *Pseudomonas* cytochrome oxidase with ferricyanide under an atmosphere of N₂. Most of the difference spectrum (440–700 nm) was determined at using a ferricyanide concentration of 250 μ M after mixing. However, ferricyanide itself has a substantial absorption band over the range 400–440 nm, and to lower this 'background' absorption, experiments over these wavelengths were carried out at an oxidant concentration of 100 μ M after mixing. The presence of the ferricyanide absorption band

Fig. 5. Analysis of the reaction of potassium ferricyanide with the reduced *Pseudomonas* cytochrome oxidase-CO complex

(a) Log plot of Fig. 4(a); (b) log plot of Fig. 4(b). The two slow phases (○, ●) are plotted by using the time scale on the upper abscissa, and the fast phase (▲) has been plotted with reference to the lower abscissa.

would be expected to have a significant effect on the absorption changes over this region. To lessen these errors, corrections have been made to the observed reaction traces on the basis of measured spectra of potassium ferri- and ferro-cyanide and on the assumption that each molecule of oxidase possesses two c and two d_1 haems. Despite such precautions there still remains the possibility that any residual ascorbate present in the oxidase solution may affect the observed absorption changes by reaction with ferricyanide. An exact correction for this has not been possible, since the amount of ascorbate present will be dependent on the unknown amount of O_2 that remains in the protein solution after the degassing procedure. Nevertheless, from the information given in the Materials and Methods section, the maximum error, owing to excess ascorbate, in the results presented here would not be expected to exceed 0.01 absorbance unit. Thus the reasonable correlation between the static and kinetic difference spectra in Fig. 6 may be seen to indicate that no reaction processes are lost in the 'dead time' of the stopped-flow apparatus.

Fig. 7 illustrates the total static difference spectrum between reduced carbonmonoxy *Pseudomonas* cytochrome oxidase and the oxidized form of the enzyme, together with the kinetic difference spectrum of the fast phase observed under these conditions. Clearly, there are substantial differences between the two spectra in this case, with the kinetically derived spectrum showing only the characteristic α -, β - and γ -bands of a haem c chromophore (see Barber *et al.*, 1977). Thus the discrepancies between the two spectra in Fig. 7 can be ascribed to differences between the spectrum of the reduced haem d_1 -CO complex and the oxidized spectrum of this component.

Fig. 8 gives details of the difference spectra, over the range 440–700nm, of the fast and second-fastest phases of the reaction data in Fig. 6. The difference spectrum of the fast process shows clearly the presence of the typical cytochrome c α - and β -bands around 550 and 520nm respectively, confirming that it is the haem c component of *Pseudomonas* cytochrome oxidase that gives rise to the fast kinetic phases observed under both N_2 and CO atmospheres. In contrast, the difference spectrum of the second-fastest phase of the reaction under N_2 reveals that the largest spectral contributions of this process are found at wavelengths which, from previous studies (Barber *et al.*, 1977), may be associated with the haem d_1 moiety, i.e. the extrema at 460, 500 and 660nm. The second-fastest phase therefore appears to represent a direct bimolecular reaction of the haem d_1 component with ferricyanide.

Difficulties were encountered in analysing reaction traces, obtained under a N_2 atmosphere, over the range 400–440nm. This situation arose because over much of this region the absorption changes were so

Fig. 6. Static and total kinetic difference spectra for the oxidation of reduced *Pseudomonas* cytochrome oxidase by potassium ferricyanide

The static difference spectrum (—; reduced minus oxidized) of $6.8 \mu\text{M}$ -*Pseudomonas* cytochrome oxidase and the total kinetic difference spectrum (●, ○) obtained by treating the protein with either $500 \mu\text{M}$ - (●) or $200 \mu\text{M}$ - (○) potassium ferricyanide in the stopped-flow apparatus. The reactions were carried out in 0.1 M -potassium phosphate buffer, pH 7.0, at 25°C in a 2 cm path-length cell and under an atmosphere of N_2 . The kinetic difference spectrum has been corrected for the contribution of ferricyanide.

dominated by one or other of the haem components that an accurate assessment of the proportions of the minor phases was not possible. To estimate the contribution of the haem d_1 to the difference spectrum over these wavelengths, the difference spectrum of the fast phase observed under CO has been subtracted from the total difference spectrum obtained under N_2 . Since the haem d_1 is effectively 'locked' in the reduced state when complexed with CO, the uncertainty in determining the difference spectrum of the haem c is considerably lessened in this case with respect to the conditions prevailing under a N_2 atmosphere. However, this method assumes that not only does the binding of CO to the enzyme have no effect on the difference spectrum of the haem c over the wavelength range in question, but also that the haem c makes no contribution to the slow ferricyanide-independent process observed under N_2 . These assumptions will be discussed below, but the form of the difference spectrum of the haem d_1 obtained in this way (see Fig. 9) compares reasonably well with that determined for this chromophore in chromium-reduction experiments (Barber *et al.*, 1977).

The difference spectrum of the slowest phase of the reaction observed under N_2 over the wavelength range $445\text{--}700 \text{ nm}$ (Fig. 9) represents the change in the spectral properties of the enzyme which occurs as the intermediate formed after the two ferricyanide-

dependent processes decays into the normal oxidized form of the protein. Examination of Figs. 7, 8 and 9 indicates that the slow phase has spectral characteristics which differ from those of both the fast and intermediate phases of the reaction, and thus the origin of the ferricyanide-independent process is in doubt.

Discussion

The mechanisms in Schemes 1 and 2 may be put forward as explanations of the kinetic behaviour of reduced *Pseudomonas* cytochrome oxidase towards ferricyanide oxidation, under N_2 and CO atmospheres.

In Scheme 1 it is proposed that the slow ferricyanide-independent process represents a conformational change in the protein which occurs after the electron-transfer reactions have taken place. It would seem unlikely that the slow phase involves a redox reaction of the enzyme, since the two ferricyanide-dependent processes appear to represent the direct transfer of electrons from the haem c and d_1 components to ferricyanide.

An important question is whether the slow phase arises from absorbance changes associated with a particular haem component or from both haems. The fact that the slow phase may be observed at wavelengths in the red region of the spectrum would

Fig. 7. Static and fast-phase kinetic difference spectra for the oxidation of the reduced *Pseudomonas* cytochrome oxidase-CO complex by potassium ferricyanide

The static difference spectrum (—, reduced, CO-bound minus oxidized) of $6.8 \mu\text{M}$ -*Pseudomonas* cytochrome oxidase-CO and the kinetic difference spectrum (●) of the fast phase observed on reaction of the protein with $200 \mu\text{M}$ -potassium ferricyanide in the stopped-flow apparatus. The reactions were carried out in 0.1 M -potassium phosphate buffer, pH 7.0, at 25°C , in a 2 cm path-length cell and under an atmosphere of CO. The kinetic difference spectrum has been corrected for the contribution of ferricyanide.

implicate at least the haem d_1 as a progenitor. Furthermore, it is noteworthy that although there is a considerable similarity between the results obtained for the difference spectrum of the haem d_1 by Barber *et al.* (1977) and the difference spectrum of the second-fastest phase in Fig. 8, the agreement is improved if, as in Fig. 9, the sum of the second-fastest and slow-phase difference spectra of the reaction are compared with the earlier result. Such a comparison requires an allowance to be made for the different absorption coefficients used to assess the concentration of *Pseudomonas* cytochrome oxidase (see Horio *et al.*, 1961; M. C. Silvestrini, A. Colosimo, M. Brunori & C. Greenwood, unpublished work), but suggests that a conformational change associated with the haem d_1 alone may cause the ferricyanide-independent process.

The difference spectra of the haem c component in Figs. 7 and 8 and that obtained during the chromium-reduction studies also show the same general form, although there are some differences in detail between the results. These could have arisen for a number of

reasons. It is possible that the haem c may make a spectral contribution to the slow phase of the ferricyanide oxidation under N_2 , and furthermore, in view of the fact that CO binding to the haem d_1 component affected the rate of the haem c reaction, it is conceivable that such ligand binding may also produce changes in its difference spectrum. However, if such effects do occur they would seem to have only a minor influence on the spectral properties of the haem c , particularly over the region $400\text{--}440 \text{ nm}$ (see Fig. 7 and Barber *et al.*, 1977) and hence the error from these sources in Fig. 9, the difference spectrum of the haem d_1 , would not be expected to be large. The most likely cause of the discrepancies in the difference spectra are the approximations inherent in the analysis procedure. It should be noted that the larger the separation in the reaction rates of the haem c and d_1 chromophores, the less the uncertainty in the determination of their difference spectra. On this basis the results obtained for the fastest and second-fastest phases of the ferricyanide oxidation under N_2 are subject to the largest error, the rates

Fig. 8. Difference spectra of the fastest and second-fastest phases determined from the oxidation of reduced *Pseudomonas* cytochrome oxidase by potassium ferricyanide

The reduced minus oxidized difference spectra produced on analysis of the total kinetic difference spectrum in Fig. 6 into fast (●) and middle (○) phases.

Fig. 9. Difference spectra of the slow and slow plus second-fastest phases of the ferricyanide oxidation of *Pseudomonas* cytochrome oxidase

○, Difference spectrum obtained by summing the amplitudes of the middle and slow phases observed, over the region 445–700 nm, for the reaction of reduced *Pseudomonas* cytochrome oxidase with ferricyanide under N_2 . ■, Difference spectrum determined for the wavelength range 400–440 nm on subtraction of the amplitude of the fast phase observed under CO from the total kinetic difference spectrum measured under N_2 . Both these spectra have been plotted with reference to the left-hand ordinate. ●, Difference spectrum of the slow phase alone and has been plotted with reference to the right-hand ordinate. All the spectra are for a protein concentration of $6.8 \mu M$ and a path-length of 1 cm.

of these two reactions being within one order of magnitude. Conversely, the fast-phase difference spectrum determined under CO should be the most accurate.

In comparing the results of the present study with those obtained by Parr *et al.* (1977) for the reaction of reduced *Pseudomonas* cytochrome oxidase with oxidized azurin, it may be seen that the slight enhance-

Scheme 1. Ferricyanide oxidation of reduced *Pseudomonas* cytochrome oxidase under N_2

The species $[c^{3+}-d_1^{3+}]$ represents a state of the enzyme in which both haems are formally oxidized, but which differs from that of the normal 'resting' oxidized enzyme.

Scheme 2. Ferricyanide oxidation of the carbonmonoxy complex of reduced *Pseudomonas* cytochrome oxidase under an atmosphere of CO

The oxidation of the d_1^{2+} haem is prevented from occurring in a direct reaction and must await the dissociation of CO from the d_1^{2+} site.

ment in the reaction rate of the haem c caused by the presence of CO parallels a similar phenomenon observed with the protein oxidant. However, aside from this one feature the behaviour of the enzyme towards ferricyanide is very different from that found with azurin. Parr *et al.* (1977) have noted that, under both N_2 and CO atmospheres, the haem c component of *Pseudomonas* cytochrome oxidase undergoes a biphasic reaction with the copper protein; this contrasts markedly with the apparently simple oxidation of the haem c by ferricyanide. A large difference is also apparent in the reaction of the haem d_1 moiety. Parr *et al.* (1977) and Wharton *et al.* (1973) have both concluded that direct electron transfer between the haem d_1 and azurin cannot take place, with changes in the redox state of this haem component occurring solely by means of an intramolecular electron exchange with the haem c . Put in this context, the bimolecular reaction of the haem d_1 with ferricyanide follows the simpler behaviour seen with the haem c and indicates a considerable decrease in the specificity of the interaction between oxidant and oxidase compared with azurin. Indeed, the rate of oxidation of the haem d_1 by ferricyanide is such that, under the conditions used here, the slow internal electron-transfer processes between the haem components of *Pseudomonas* cytochrome oxidase would be expected to have a negligible influence on the overall reaction. Nevertheless, it should be stressed that, despite having a similar first-order rate constant, the slow phase observed in the ferricyanide oxidation experiments under N_2 has an entirely different spectral character from the slow internal electron transfer

seen by Parr *et al.* (1977) in the reaction between reduced *Pseudomonas* cytochrome oxidase and oxidized azurin. There is a possibility that the slow phase arises as a result of a small percentage of the enzyme being deficient in haem d_1 and reacting differently. However, it would be necessary for such molecules not only to react differently, but also to possess different spectral characteristics to the normal enzyme. For this reason we prefer the simpler explanation of the results proposed in Scheme 1. It is interesting that a similar, though not identical, slow phase has been observed in the reaction between the reduced *Pseudomonas* cytochrome oxidase and O_2 (Greenwood *et al.*, 1978) which also involves a rapid bimolecular reaction of the haem d_1 moiety.

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References

- Barber, D., Parr, S. R. & Greenwood, C. (1976) *Biochem. J.* **157**, 431–438
- Barber, D., Parr, S. R. & Greenwood, C. (1977) *Biochem. J.* **163**, 629–632
- Gibson, Q. H. & Milnes, L. (1964) *Biochem. J.* **91**, 161–171
- Goldberg, M. & Pecht, I. (1976) *Biochemistry* **15**, 4197–4208
- Greenwood, C., Barber, D., Parr, S. R., Antonini, E., Brunori, M. & Colosimo, A. (1978) *Biochem. J.* **173**, 11–17

- Gudat, J. C., Singh, J. & Wharton, D. C. (1973) *Biochim. Biophys. Acta* **292**, 376–390
- Horio, T. (1958) *J. Biochem. (Tokyo)* **45**, 267–279
- Horio, T., Higashi, T., Yamanaka, T., Matsubara, H. & Okunuki, K. (1961) *J. Biol. Chem.* **236**, 944–951
- Kuronen, T. & Ellfolk, N. (1972) *Biochim. Biophys. Acta* **275**, 308–318
- Kuronen, T., Saratse, M. & Ellfolk, N. (1975) *Biochim. Biophys. Acta* **393**, 48–54
- Parr, S. R., Wilson, M. T. & Greenwood, C. (1975) *Biochem. J.* **151**, 51–59
- Parr, S. R., Barber, D., Greenwood, C., Phillips, B. W. & Melling, J. (1976) *Biochem. J.* **157**, 423–430
- Parr, S. R., Barber, D., Greenwood, C. & Brunori, M. (1977) *Biochem. J.* **167**, 447–455
- Wharton, D. C., Gudat, J. C. & Gibson, Q. H. (1973) *Biochim. Biophys. Acta* **292**, 611–620
- Yamanaka, T. & Okunuki, K. (1963) *Biochim. Biophys. Acta* **67**, 394–406
- Yamanaka, T., Ota, A. & Okunuki, K. (1961) *Biochim. Biophys. Acta* **53**, 294–308
- Yamanaka, T., Kijimoto, S. & Okunuki, K. (1963) *J. Biochem. (Tokyo)* **53**, 416–421