A Re-evaluation of some Basic Structural and Functional Properties of *Pseudomonas* Cytochrome Oxidase

By Maria Chiara SILVESTRINI,* Alfredo COLOSIMO,* Maurizio BRUNORI,*

Terence A. WALSH,† Donald BARBER† and Colin GREENWOOD†

*Istituti di Chimica e Biochimica della Facoltà di Medicina, Centro di Biologia Molecolare del C.N.R.,
Università di Roma, 00185 Roma, Italy, and †School of Biological Sciences,
University of East Anglia, Norwich NR4 7TJ, U.K.

(Received 8 May 1979)

Determinations of iron content and dry-weight measurements on samples of *Pseudomonas* cytochrome oxidase were coupled with sodium dodecyl sulphate/polyacrylamide-gel-electrophoresis studies of both the native protein and covalently cross-linked oligomers in order to estimate the enzyme's molecular weight and spectral absorption coefficients. A value of $\epsilon_{410}^{ox} = 282 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ was calculated for a dimeric protein molecule having a total molecular weight of 122000 (based on iron analysis). Steady-state kinetic observations of the enzyme-catalysed oxidation of reduced azurin by nitrite indicated a marked increase in enzyme inactivation as the pH was raised from 5.7 to 7.2. Since NO, a product of the nitrite reductase activity of *Pseudomonas* cytochrome oxidase, is known to bind to the enzyme, a study was undertaken to try to assess the potential of NO as a product inhibitor. Investigations showed that samples of the oxidized protein at pH values 4, 5 and 6 bound NO to both haem *c* and *d*₁ components, but oxidized enzyme samples at pH 7 and above formed their reduced ligand-bound forms when placed under an atmosphere of the gas. Ascorbate-reduced enzyme samples at pH 4, 5, 6 and 7 were also found to bind NO at both haem components, although at pH 7 the rate of haem *c* binding was very slow. At pH 8 and 9 only the ferrohaem *d*₁ bound NO. Titration experiments on the reduced protein over the pH range 5–7, with nitrite as a precursor of NO, showed that the haem *d*₁ had a much higher affinity than the haem *c*: experiments at pH 5.2 and 5.9 with NO-equilibrated solutions revealed the same pattern of behaviour with the oxidized enzyme.

Pseudomonas cytochrome oxidase (ferrocytochrome *c*₅₅₁-O₂ oxidoreductase, EC 1.9.3.2) is a soluble protein that functions as a terminal electron-transfer component in the respiratory system of *Pseudomonas aeruginosa*. The enzyme can accomplish both the four-electron reduction of O₂ to water and the single-electron reduction of nitrite to NO (Yamanaka *et al.*, 1961). The purified protein has been shown to contain two types of prosthetic group, a haem *c* component, which is thought to be the site at which electrons enter the molecule (Wharton *et al.*, 1973; Parr *et al.*, 1977), and a haem *d*₁ component, which in its reduced state will bind CO and CN⁻ (Parr *et al.*, 1975; Barber *et al.*, 1978). Although the protein has been isolated by several groups of workers (Horio *et al.*, 1961; Kuronen & Ellfolk, 1972; Gudat *et al.*, 1973; Parr *et al.*, 1976), there still remain uncertainties as to its minimum molecular weight and molar absorption coefficients. Thus it has been considered essential to redetermine some of the basic analytical parameters of our purified enzyme,

especially since equilibrium and kinetic investigations rely heavily on accurate determinations of the absolute spectral contributions of the two haem components.

At the present stage, little is known about the catalytic activity of *Pseudomonas* cytochrome oxidase in its role as a nitrite reductase, although it is very probable that nitrite reduction represents the most important function of the enzyme within the cell (Yamanaka *et al.*, 1963). This is reflected by the values of the turnover numbers for the oxidation of *Pseudomonas* cytochrome *c*₅₅₁, which are 107 and 139 with O₂ and nitrite respectively as electron acceptors. In fact the enzyme is produced only when the organism is grown in the presence of nitrate.

An apparent paradox exists in that NO, the product of the nitrite reductase activity, has been shown to be capable of binding to both *c* and *d*₁ haem components in both oxidized and reduced states. There is therefore a possibility that the enzyme may suffer product inhibition. The NO-binding behaviour of the protein has been reported, by Shimada & Orii (1975), to vary with pH, and for this reason we have

Abbreviation used: SDS, sodium dodecyl sulphate.

initiated an examination of both some steady-state kinetic properties of the nitrite reductase activity and some NO-binding characteristics, at different pH values. Because of the inherent ability of the molecule to generate NO from nitrite in the presence of excess reductant, we have been able to carry out titrations of the reduced enzyme with both substrate and product.

Materials and Methods

KNO₂ was obtained from BDH Chemicals, Poole, Dorset, U.K., and sodium ascorbate from Sigma (London) Chemical Co., Poole, Dorset, U.K. O₂-free N₂ was supplied by the British Oxygen Co., London S.W.19, U.K., and was dispensed from the cylinder and stored in glass vessels over an alkaline solution of dithionite-reduced anthraquinonesulphonate before use. NO was acquired from Matheson Gas Products, East Rutherford, NJ, U.S.A., and used straight from the cylinders.

Pseudomonas cytochrome oxidase, and its protein electron donors cytochrome *c*-551 and azurin, were purified from cells of *Pseudomonas aeruginosa* (strain N.C.T.C. 6750) by following the procedure of Parr *et al.* (1976). Samples of oxidized *Pseudomonas* cytochrome oxidase prepared by this method routinely give values for the ratio A_{410}/A_{280} close to 1.2, and on the basis of a similar value determined for the crystallized protein (Kuronen & Ellfolk, 1972) approach a purity of 100%. *Pseudomonas* cytochrome oxidase solutions to be used for iron analysis were extensively dialysed against 0.01 M-sodium phosphate buffer, pH 7.0, containing 1 mM-EDTA to remove extraneous metal ions. After protein digestion, according to the procedure of Cameron (1965), colorimetric analysis of iron was carried out by the method of Bothwell & Mallett (1955). The protein content was determined by dry-weight measurements (at 105°C to constant weight).

Covalent oligomers of *Pseudomonas* cytochrome oxidase were prepared by chemical cross-linking reaction of the protein with dimethylsuberimide by following the procedure of Davies & Stark (1970). SDS/polyacrylamide-gel electrophoresis was carried out according to the procedure of Weber & Osborn (1969) on (i) the untreated protein and (ii) the products of the cross-linking reaction. The molecular-weight standards were ovalbumin and bovine serum albumin monomers and dimers prepared by chemical cross-linking (Davies & Stark, 1970).

On the basis of results described below a molar absorption coefficient of $\epsilon_{410}^{ox} = 282 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ was used for *Pseudomonas* cytochrome oxidase. A molar absorption coefficient of $\epsilon_{225}^{ox} = 3.5 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ was used for *Pseudomonas* azurin.

Spectrophotometric measurements were made with a Cary 14 or a Cary 118C spectrophotometer.

Binding of NO was followed, under anaerobic conditions, by using either Thunberg cuvettes sealed with vaccine caps or standard cuvettes similarly sealed but completely filled with enzyme solution. For reduced *Pseudomonas* cytochrome oxidase a large excess (approx. 1000-fold) of sodium ascorbate was present. Additions of NO or nitrite solutions were made by injection through the vaccine cap with a micro-syringe. Stock solutions of NO were calibrated by titration against human deoxyhaemoglobin.

Results

Iron content

Our estimate of the iron content of purified *Pseudomonas* cytochrome oxidase (0.18%; see Table 1) is in satisfactory agreement with the value reported by Kuronen & Ellfolk (1972), but differs considerably from that given by Gudat *et al.* (1973). The minimum weight per iron atom is calculated to be 30 500 for our preparation of purified oxidase.

Cross-linking experiments

Previous workers (Kuronen & Ellfolk, 1972; Gudat *et al.*, 1973) have noted that the molecular weight of *Pseudomonas* cytochrome oxidase determined by SDS/polyacrylamide-gel electrophoresis is approximately half that which may be obtained from centrifugation studies. This has led to the concept of a dimeric enzyme molecule comprising two subunits of equal molecular weight. Chemical cross-linking offers the possibility of producing a dimeric species of the oxidase which is stable to SDS and allows a determination of molecular size by the electrophoretic technique.

The reaction of dimethylsuberimide with native *Pseudomonas* cytochrome oxidase yields a heterogeneous mixture of altered protein, covalent dimers and other oligomers of higher size, with the dimers representing more than 60% of the total, as judged by SDS/polyacrylamide-gel electrophoresis. However, when examined by isoelectric focusing the reaction mixture displayed only a single broad band with an isoelectric point higher than that of the native enzyme (pI 6.9; Barber *et al.*, 1976). The similarity in their isoelectric-focusing behaviour and the identical molecular weights of the non-covalent dimers have until now prevented the isolation of the latter from the reaction mixture and therefore its more complete characterization.

Table 1 summarizes our estimates of the molecular weights of the monomer and the covalent dimer, as well as the minimum molecular weight. Our results are compared with those given previously by Kuronen & Ellfolk (1972) and Gudat *et al.* (1973). The molecular weight of 90 000 originally reported by Horio *et al.* (1961) has not been confirmed.

Table 1. Some properties of *Pseudomonas cytochrome oxidase*

	Kuronen & Ellfolk (1972)	Gudat <i>et al.</i> (1973)	This work
Iron content (w/w)	0.166%	0.108%	$0.18 \pm 0.002\%$
Minimum mol.wt. (per iron)	33 500	52 000	$30\,500 \pm 350$
Mol.wt. of subunit	63 000	58 000	60 000–65 000
	(SDS/polyacrylamide-gel electrophoresis)	(SDS/polyacrylamide-gel electrophoresis)	(SDS/polyacrylamide-gel electrophoresis)
Mol.wt. in solution	119 000	121 200	130 000
	(Sedimentation equilibrium and diffusion)	(Sedimentation equilibrium)	(SDS/polyacrylamide-gel electrophoresis of cross-linked oxidase)

Table 2. Absorption coefficients of *Pseudomonas cytochrome oxidase*

The values are expressed as millimolar absorption coefficients in 1 cm light-path, and the molecular weight is that of a unit containing four iron atoms, i.e. $30\,500 \times 4 = 122\,000$. They are the means of two determinations on two different samples electrophoretically homogeneous, with ratios $A_{410}^{ox}/A_{280}^{ox} = 1.21$ and $A_{640}^{ox}/A_{520}^{ox} = 1.12$.

Derivative	Wavelength (nm)	$10^{-3} \times \epsilon$ (litre · mol ⁻¹ · cm ⁻¹)
Oxidized	640	41 ± 3
	410	282 ± 15
Reduced by sodium ascorbate	650	37 ± 2
	548	52 ± 3
	417	355 ± 17

Absorption coefficients

From the results given above and the absorption spectra measured in parallel on the same solution, we have calculated the millimolar absorption coefficients of *Pseudomonas cytochrome oxidase* in the visible and Soret regions. The data, expressed on the basis of an enzyme molecule containing four iron atoms (two haems *c* and two haems *d₁*) and having a molecular weight of 122 000 (i.e. $30\,500 \times 4$), are given in Table 2.

Reactions with NO and nitrite

Fig. 1 shows some steady-state-kinetic reaction traces that follow the enzyme-catalysed oxidation of azurin by nitrite under anaerobic conditions and at different pH values. The results clearly illustrate that, particularly at pH values near neutrality, the reaction is rapidly terminated, in spite of the presence of oxidant and reductant in the cell. We have also observed similar results with *Pseudomonas ferrocytochrome c-551* as electron donor. Experiments conducted at pH 7.0 (results not shown) have demonstrated that the extent of the fast initial burst of electron-donor oxidation may be amplified by increasing the concentration of nitrite added to the cell. These results are consistent with those obtained by Yamanaka *et al.* (1961), and are strongly suggest-

Fig. 1. Effect of pH on the nitrite reductase activity of *Pseudomonas cytochrome oxidase*

The experiments were conducted with in all cases a reduced azurin concentration of $46\,\mu\text{M}$, a nitrite concentration of $600\,\mu\text{M}$ and a concentration of *Pseudomonas cytochrome oxidase* of $0.1\,\mu\text{M}$. The temperature was 30°C and the assay volume 2.5 ml , and the reaction was initiated by addition of nitrite at X, the enzyme having been added previously. The measurements were carried out in a Thunberg cuvette of path length 1 cm, under an atmosphere of N_2 . The buffer used was 0.04 M -potassium phosphate, with pH values of (1) 7.2, (2) 6.9, (3) 6.6, (4) 6.2, (5) 5.95 and (6) 5.7. The broken line shows the absorbance of the system when fully oxidized.

ive of a product inhibition that is counteracted by low pH and increased substrate concentration.

To try to elucidate these phenomena we have

attempted to characterize some properties of the NO complexes of *Pseudomonas* cytochrome oxidase. Fig. 2 shows the absolute spectra of the oxidized enzyme and the oxidized enzyme-NO complex at pH 6.0, and Fig. 3 shows the NO-bound and unbound forms of the reduced protein at pH 5.0. Addition of 0.1 MPa (1 atm) of NO gas to samples of oxidized *Pseudomonas* cytochrome oxidase at pH 7.0 and above gave spectra that were consistent with those of the reduced protein with the haem d_1 component NO-bound. Although unaffected by NO at pH 8.0 and 9.0, it was observed that the ferrohaem c underwent a slow reaction over a period of hours, that resulted in the formation of the reduced enzyme-NO complex at pH 7.0. Spectra run after addition of NO gas to oxidized enzyme samples at pH 4, 5 and 6 did not show these autoreductive phenomena. The spectrum in Fig. 2 shows that NO affects the absorption bands of both the haem c and haem d_1 components of the protein. The bands at 416, 528 and 562 nm are those expected of a complex between NO and a ferricytochrome c (Ehrenberg & Szczepkowski, 1960). These haem c bands were of similar intensity at pH 5.0 and 6.0, but appeared to be slightly enhanced at pH 4.0. Slight differences were also observed in the α -band of the haem d_1 moiety at 634 nm over the pH range 6-4.

The spectra given in Fig. 3 show that addition of NO gas to ascorbate-reduced *Pseudomonas* cytochrome oxidase leads to ligand binding to both haem components at pH 5.0. We have also observed similar behaviour at pH 4.0 and 6.0, although, as mentioned above, at alkaline pH values the reduced haem c does not bind NO. In general, over the pH range where the haem c does bind NO the spectrum of the complex is that expected from a ferrocycytochrome c (Ehrenberg & Szczepkowski, 1960), with absorption maxima at 412, 534 and 568 nm. Unlike the haem c , the reduced haem d_1 component appeared to bind NO over all the pH range from 4 to 9. This binding abolished the reduced haem d_1 peak at 460 nm and also produced changes in the α -band region of this component around 650 nm. It was apparent that minor variations in the shape of the reduced haem d_1 -NO complex α -band did take place between pH 4 and pH 9.

To try to gauge the possible importance of NO binding to the catalytic functioning of the enzyme in its nitrite reductase activity, we have examined the effect of ligand concentration on the degree and nature of binding. Fig. 4(a) shows a family of spectra produced during a titration of the ascorbate-reduced enzyme with nitrite at pH 6.1. It is evident from a comparison of the final spectrum achieved in Fig. 4(a) with the NO-bound spectrum in Fig. 2 (i) that the use

Fig. 2. Spectra of oxidized *Pseudomonas* cytochrome oxidase and its NO complex

The spectra of oxidized (—) and oxidized NO-bound (----) forms of *Pseudomonas* cytochrome oxidase are shown. The spectra were run in 0.1 M-potassium phosphate buffer, pH 6.0, in a Thunberg cuvette of path length 1 cm. The NO-bound species was made by replacing the atmosphere of N_2 used for the unbound enzyme with one of NO.

Fig. 3. Spectra of ascorbate-reduced *Pseudomonas* cytochrome oxidase and its NO complex

The spectra of reduced (—) and reduced NO-bound forms (----) of *Pseudomonas* cytochrome oxidase are shown. The spectra were run in 0.1 M-potassium phosphate buffer, pH 5.0, in a Thunberg cuvette of path length 1 cm. The NO-bound species was made by replacing the atmosphere of N_2 used for the unbound enzyme with one of NO.

Fig. 4. Titration of the reduced haem d_1 component of *Pseudomonas* cytochrome oxidase with nitrite

(a) ----, Spectra of $4.86 \mu\text{M}$ -reduced *Pseudomonas* cytochrome oxidase in 0.1 M-potassium phosphate buffer, pH 6.1. The enzyme was prepared anaerobically under N_2 so as to completely fill a sealed 1 cm-path-length cuvette (volume 3.7 ml) and reduced with $25 \mu\text{l}$ of 1 M-sodium ascorbate. Curves (1), (2), (3) and (4) show spectra after additions of KNO_2 to concentrations of 1.6, 3.8, 5.91 and $8.04 \mu\text{M}$ respectively. Curve (5), used as the end point, corresponds to a nitrite concentration of $27.3 \mu\text{M}$. Some of the spectra obtained during the titration have been omitted for clarity. The temperature was 20°C . (b) The results of three experiments carried out under similar conditions to (a), at pH values of 5.1 ($\bullet$), 6.1 ($\blacktriangle$) and 7.1 ($\square$). The changes in absorbance at 456 nm were corrected for dilution and are plotted as percentages of the total against the ratio of the total concentration of nitrite added to the concentration of enzyme.

of nitrite in concentrations close to stoichiometric results in binding solely at the haem d_1 and (ii) that the ligand-bound species formed on adding nitrite is indeed the reduced haem d_1 -NO complex. Fig. 4(b) gives results obtained from three experiments of this type at pH values of 5.1, 6.1 and 7.1. It is apparent that there is no effect of pH on the binding characteristics over this range and that the affinity of the reduced haem d_1 component for NO is very high. Examination of a number of enzyme preparations has shown a close correspondence in the binding behaviour observed with both nitrite and NO solutions. However, although the data in Fig. 4(b) are consistent with there being two binding sites per protein molecule, some enzyme samples have given stoichiometries between one and two.

The restriction of our titration data on NO binding to the reduced haem d_1 to pH values below 7 has been imposed because the enzyme apparently exhibits an NO reductase activity. This was apparent when, after completion of experiments of the type in Fig. 4(a), spectra run after the sample had been left overnight showed that the enzyme had reverted to an unbound state. At pH 8.0 and above the rate of this

process was such as to interfere seriously with our haem d_1 titration experiments, which involved close to stoichiometric amounts of NO_2^- , but at lower pH values these effects were not significant over the time-scales involved.

By continuation of the titrations beyond the final state illustrated in Fig. 4(a), where the haem d_1 is effectively saturated, it has been possible, by use of higher nitrite solution concentrations, to examine the binding characteristics of the haem c . In fact nitrite was the reagent of choice for these experiments because of the limitations that NO solubility places on preparing a concentrated enough solution. Fig. 5(a) shows a family of spectra measured during such a titration at pH 6.0, and Fig. 5(b) illustrates the corresponding binding curve plotted as the difference in absorbance at two wavelengths either side of an isosbestic point at 556 nm. Similar results were also obtained from a titration at pH 5.2. Difficulties were encountered in establishing the limits of the titration of the reduced haem c with NO, especially the starting point. This was due to the fact that NO binding to the haem d_1 produces changes in the 'background' absorbance underlying the haem c absorption peaks.

Fig. 5. Titration of the reduced haem c component of *Pseudomonas* cytochrome oxidase with nitrite (a) ----, Spectrum of 6.55 μM-reduced *Pseudomonas* cytochrome oxidase after titration of the haem d_1 component by addition of nitrite to a concentration of 13 μM. Curves (1), (2), (3) and (4) were obtained on increasing the nitrite concentration by 52.8, 146.5, 198.7 and 588 μM respectively. Some of the spectra obtained have been omitted for clarity. The conditions used were similar to those in Fig. 4(a), the pH being 6.0. (b) The results of the experiment in (a) were corrected for dilution and are plotted as the difference in absorption at 548 and 570 nm against increases in nitrite concentration after titration of haem d_1 . The broken line corresponds to a total nitrite concentration of 601 μM.

Fig. 6. Reaction of the oxidized haem d_1 component of *Pseudomonas* cytochrome oxidase with NO. ----, Spectrum of $5.8\mu\text{M}$ -oxidized *Pseudomonas* cytochrome oxidase in 0.1 M-potassium phosphate buffer, pH 5.2. The enzyme was prepared anaerobically under N_2 so as to fill completely a sealed 1 cm-path-length cuvette (volume 3.7 ml). Curves (1) and (2) are spectra run 5 and 55 min after the addition of NO to a concentration of $40.2\mu\text{M}$. The temperature was 20°C .

It should therefore be noted that the 'zero' binding point in Fig. 5(b) was subject to some uncertainty. Because of these problems we restrict our analysis to an estimate of the half-saturation value as approx. $150\mu\text{M}$.

Titration of the reduced haem c moiety at pH 7.0 proved to be impracticable, owing to the low rate of NO binding. However, we do have some data suggesting that the affinity at this pH is comparable with that found at the lower pH values. In experiments carried out under conditions similar to those described for Fig. 5(a), but with an enzyme concentration of $5.4\mu\text{M}$, the addition of nitrite to a concentration of $588\mu\text{M}$ led to virtually complete binding of the ferrohaem c component at both pH 6.1 and pH 7. Nevertheless, whereas the binding of NO at pH 6.1 took approx. 5 min to attain 90% saturation, the experiment at pH 7.0 required approx. 45 min.

In view of problems associated with autoreduction of the oxidized enzyme by NO (at pH values above neutrality), our examinations of the binding characteristics have been made within the pH range 5–6. Fig. 6 illustrates the extremely slow reaction that occurs when NO is added to oxidized *Pseudomonas* cytochrome oxidase at concentrations close to

stoichiometric. Comparison of Fig. 6 with Fig. 2 shows clearly that again the haem d_1 component exhibits a much higher affinity for NO than does the haem c . The slowness of the binding reaction has precluded a formal titration experiment. However, the fact that experiments carried out under conditions comparable with those in Fig. 6, but with $10.2\mu\text{M}$ - and $40.2\mu\text{M}$ -NO concentrations, proceeded to similar extents implies a high affinity of the oxidized haem d_1 for the ligand. Results obtained at pH 5.2 and pH 5.9 were in good agreement, although, as may be seen by comparing the haem d_1 α -band spectra in Figs. 2 and 6, changes in pH did produce effects on the shape of the absorption-band envelope.

The technical difficulties associated with the achievement of high NO concentrations in solution have so far prevented us from obtaining consistent binding results for the oxidized haem c .

Discussion

The results given in Table 1 show that the soluble cytochrome oxidase, purified from *Pseudomonas aeruginosa* by the method of Parr *et al.* (1976), is a

molecule with molecular properties (iron content, molecular weight in solution) very similar to those characterizing the enzyme purified by Kuronen & Ellfolk (1972). Covalent cross-linking of the native molecule with dimethylsuberimide under mild conditions yields polymers (mostly dimers) whose molecular weight, estimated by SDS/polyacrylamide-gel electrophoresis, confirms the value independently assigned to the native molecule (120 000–130 000). Although purification of the covalent dimer has not been achieved, it is possible that such a derivative may prove useful in investigating the stoichiometry of O₂ binding, not yet elucidated (Greenwood *et al.*, 1978).

The spectral properties of the NO complexes of *Pseudomonas* cytochrome oxidase reported above follow the general features reported by Shimada & Orii (1975), including their observation of the autoreduction of the enzyme in alkaline media. However, Shimada & Orii (1975) have attempted to use the variation in the spectra of the enzyme with pH to assess the affinities of the oxidized haems *c* and *d*₁ for NO. They have suggested a value of 1 mM for the dissociation constant for both haem components. Our results show that the dissociation constant for the haem *d*₁ must be much lower than this value, since in experiments of the type reported in Fig. 6 the reaction with NO appeared to be proceeding to completion at concentrations less than 40 μ M. We therefore believe that the modifications to the spectral properties of the oxidized haem *d*₁–NO complex associated with changes in pH are due to alterations in the spectra of the fully bound species. Whether such behaviour is also exhibited by the oxidized haem *c*, or whether it is indeed subject to variations in ligand affinity with pH, is open to speculation.

Figs. 4(a) and 5(a) illustrate that NO is formed by the enzyme during its reaction with nitrite. The form of the binding curve in Fig. 4(b) is consistent with the experiments having been carried out under conditions where the protein concentration was greater than the ligand dissociation constant of the reduced haem *d*₁ component. Such conditions are required for determinations of stoichiometry, but are opposed to those best suited for accurate examination of binding characteristics and affinity constants (Brewer *et al.*, 1974), and we have therefore not attempted to fit the data to a binding equation. Nevertheless it is clear that the reduced haem *d*₁ has a high affinity for NO, which would appear to possess the potential to function as an efficient product inhibitor under anaerobic conditions. In contrast, the relatively low affinity of the reduced haem *c* for NO might be thought to cast doubt on the relevance of this reaction to the catalytic activity. However, during our titrations of the reduced haem *c* with nitrite it was observed that the enzyme approached each point of equilibrium via states that corresponded to greater

NO binding. This result is in agreement with the report by Shimada & Orii (1975) that the reduced haem *c*–NO complex is significantly involved during enzyme turnover. It is also notable that the ability of the ferrohaem *c* moiety of *Pseudomonas* cytochrome oxidase to form an NO complex directly at pH values at or below neutrality is in marked contrast with the behaviour of mammalian ferrocytochrome *c*, which has first to be taken to very alkaline pH in order to bind the ligand (Ehrenberg & Szczepkowski, 1960).

Consideration of the steady-state kinetic traces in Fig. 1 indicates that a fundamental change in the behaviour of the experimental system occurs between pH 5 and 7. Although we have no results for the binding of NO to the oxidized haem *d*₁ at pH 7.0, experiments at pH 5.2 and 5.9 have not revealed any differences in affinity. Furthermore, neither does the reduced haem *d*₁ display any evidence of changes in its affinity for NO over the pH range 5–7. These results therefore do not offer a ready explanation of the enhanced inhibition, shown in Fig. 1, at pH values close to neutrality. However, we have observed some changes in the NO-binding properties of the enzyme over the pH range of interest, notably the autoreductive phenomena and the marked reluctance of NO to bind to the ferrohaem *c* component as the pH approaches 7. The possible significance of such behaviour must be viewed against a background of possible pH variations in the affinity of *Pseudomonas* cytochrome oxidase for nitrite, or changes in the reactive form of the latter with pH (Shimada & Orii, 1975). It is therefore clear that further work is required in order to assess the relative importance of these factors in the nitrite reductase activity of the enzyme.

This work was supported by N.A.T.O. and by the Science Research Council of G. B.

References

- Barber, D., Parr, S. R. & Greenwood, C. (1976) *Biochem. J.* **157**, 431–438
- Barber, D., Parr, S. R. & Greenwood, C. (1978) *Biochem. J.* **175**, 239–249
- Bothwell, T. H. & Mallett, B. (1955) *Biochem. J.* **59**, 599–602
- Brewer, J. M., Pesce, A. J. & Ashworth, R. R. (1974) *Experimental Techniques in Biochemistry*, pp. 248–250, Prentice-Hall, Englewood Cliffs
- Cameron, B. F. (1965) *Anal. Biochem.* **11**, 164–169
- Davies, C. E. & Stark, G. R. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **66**, 651–656
- Ehrenberg, A. & Szczepkowski, T. W. (1960) *Acta Chem. Scand.* **14**, 1684–1692
- 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., Higashi, T., Yamanaka, T., Matsubara, H. & Okunuki, K. (1961) *J. Biol. Chem.* **236**, 944–961
- Kuronen, T. & Ellfolk, N. (1972) *Biochim. Biophys. Acta* **275**, 308–318
- 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
- Shimada, H. & Orii, Y. (1975) *FEBS Lett.* **54**, 231–240
- Weber, R. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Wharton, D. C., Gudat, J. C. & Gibson, Q. H. (1973) *Biochim. Biophys. Acta* **292**, 611–620
- Yamanaka, T., Ota, A. & Okunuki, K. (1961) *Biochim. Biophys. Acta* **53**, 294–308
- Yamanaka, T., Kijimoto, S. & Okunuki, K. (1963) *J. Biochem. (Tokyo)* **53**, 416–423