Some Spectral and Steady-State Kinetic Properties of *Pseudomonas* Cytochrome Oxidase

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(Received 1 March 1976)

Some spectra of *Pseudomonas* cytochrome oxidase are reported, both for comparison with those of other workers and to illustrate the differences between the ascorbate- and dithionite-reduced forms of the enzyme. A spectrum of the reduced enzyme–CO complex, prepared in the absence of added reductants by incubation under CO, is also included. Ultracentrifugation studies yielded a value for the sedimentation coefficient ($s_{20,w}$) of 7.5S, and an isoelectric point of pH 6.9 was determined by isoelectric focusing. Steady-state kinetic constants of the electron donors, quinol, sodium ascorbate, reduced *Pseudomonas* azurin and *Pseudomonas* ferrocycytochrome c_{551} were investigated giving K_m values of 30 mM, 4 mM, 49 μ M and 5.6 μ M respectively. The two protein substrates were observed to be subject to product inhibition and the K_i for oxidized *Pseudomonas* azurin was evaluated at 4.9 μ M. Steady-state kinetics were also used to investigate the effects of the oxidation products of dithionite on the oxidase and nitrite reductase activities of *Pseudomonas* cytochrome oxidase. These experiments showed that whereas the oxidase activity was inhibited, the nitrite reductase activity was slightly enhanced.

Pseudomonas cytochrome *c* oxidase (ferrocycytochrome c_{551} –O₂ oxidoreductase, EC 1.9.3.2) is a dimeric protein, mol.wt. 120000 (Kuronen & Ellfolk, 1972; Gudat *et al.*, 1973), composed of two identical subunits each containing one *c* haem and one d_1 haem (Kuronen & Ellfolk, 1972; Kuronen *et al.*, 1975), the latter being the autoxidizable component. The enzyme functions in terminal electron transfer of cells of *Pseudomonas aeruginosa* grown anaerobically in the presence of nitrate, and is capable of catalysing the four-electron reduction of O₂ to water (Horio *et al.*, 1958) and the one-electron reduction of NO₂[–] to NO (Yamanaka *et al.*, 1961). The latter is considered to be the more important function physiologically. For both oxidase and nitrite reductase activities, either the haem protein *Pseudomonas* cytochrome c_{551} or the copper protein *Pseudomonas* azurin may act as electron donor to the *Pseudomonas* cytochrome oxidase.

In common with other terminal oxidases, *Pseudomonas* cytochrome oxidase binds the classical respiratory inhibitors, the oxidase activity of the enzyme being inhibited by both CN[–] and CO, whereas the nitrite reductase activity, although strongly inhibited in the presence of CN[–], is unaffected by CO (Yamanaka *et al.*, 1961).

In this paper we report some properties of *Pseudomonas* cytochrome oxidase, as isolated in this laboratory (Parr *et al.*, 1976), and compare these properties with the results obtained by other workers. We also present data on the oxidation of *Pseudomonas* ferro-

cytochrome c_{551} and reduced *Pseudomonas* azurin by *Pseudomonas* cytochrome oxidase obtained from steady-state kinetic studies.

Materials and Methods

All chemicals were obtained from Fisons, Loughborough, Leics., U.K., and were of analytical reagent grade, except for sodium dithionite, which was a gift from Hardman and Holden, Miles Platting, Manchester, U.K., ascorbic acid (disodium salt) from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K., and quinol from BDH Chemicals, Poole, Dorset, U.K. Sephadex G-25 (coarse grade) was obtained from Pharmacia (G.B.) Ltd., London W.5, U.K., and Ampholine ampholytes, pH range 6–8, from LKB Instruments, South Croydon, Surrey, U.K. CO and N₂ gases were obtained from the British Oxygen Co., Deer Park Road, London S.W.19, U.K., and were dispensed from cylinders and stored in glass vessels over an alkaline solution of anthraquinonesulphonate.

Pseudomonas cytochrome oxidase, *Pseudomonas* cytochrome c_{551} and *Pseudomonas* azurin were isolated as described by Parr *et al.* (1976). The concentrations of these proteins were determined by using the following extinction coefficients: $\epsilon = 3.5 \times 10^3$ litre·mol^{–1}·cm^{–1} at 625 nm for oxidized *Pseudomonas* azurin (Brill *et al.*, 1968), $\epsilon = 149 \times 10^3$ litre·mol^{–1}·cm^{–1} for oxidized *Pseudomonas* cyto-

chrome oxidase at 410 nm (Horio *et al.*, 1961a) and $\epsilon = 28.3 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at 551 nm for reduced *Pseudomonas* cytochrome c_{551} (Horio *et al.*, 1960).

Spectrophotometry was carried out by using either a Cary 118c or a Unicam SP500 series 2 spectrophotometer, the latter being equipped with a logarithmic converter coupled to a Bryans Southern Instruments 28000 chart recorder.

The oxidase activity of *Pseudomonas* cytochrome oxidase was measured either by the rate of O_2 uptake in a Rank Brothers oxygen electrode system linked to an Esterline Angus chart recorder with a 0–1 mV full-scale deflexion, or by the spectral changes associated with the oxidation of the protein substrates. Nitrite reductase activity was always measured by the spectrophotometric method. *Pseudomonas* ferrocycytochrome c_{551} and reduced *Pseudomonas* azurin were prepared immediately before use by the addition of a few grains of sodium dithionite to the oxidized protein followed by removal of excess of

dithionite and its oxidation products by means of a Sephadex G-25 column (25 cm $\times$ 1.25 cm) (Wilson *et al.*, 1975).

Anaerobic spectra were recorded under N_2 or CO (after several cycles of evacuation and N_2 equilibration) of Thunberg cuvettes which were sealed with Suba-Seal vaccine caps.

Ultracentrifugation was carried out in 0.2 M-ammonium phosphate buffer, pH 7.0, in a Beckman model E analytical ultracentrifuge equipped with a mechanical speed control and a rotor temperature-indicating system. A type AnD rotor was used together with a schlieren optical system. The rotor temperature was controlled at 20°C and the operating speed was 42040 rev./min.

Isoelectric-point determinations were made by the method of isoelectric focusing at 600 V and 0.5 mA by using an LKB type 8101 110 ml column maintained at 4°C. pH measurements were performed after a run lasting 72 h by using an E.I.L. model 23A pH-meter

Fig. 1. Absorption spectra of oxidized and ascorbate-reduced *Pseudomonas* cytochrome oxidase

—, Oxidized; ----, ascorbate-reduced *Pseudomonas* cytochrome oxidase in 0.04 M-potassium phosphate buffer, pH 6.9. The enzyme concentration was $8.9 \mu\text{M}$ and the spectra were recorded at room temperature (20°C), under N_2 , in a Thunberg cuvette with a light-path of 1 cm.

equipped with a Russell pH limited CMT micro-electrode.

Results

Spectral properties

Fig. 1 shows the spectra of oxidized and anaerobic ascorbate-reduced *Pseudomonas* cytochrome oxidase. The oxidized spectrum exhibits absorption maxima in the visible region at 411 and 640 nm, with shoulders

at 360, 525 and 561 nm. The absorption maxima for the ascorbate-reduced enzyme are at 418, 460, 522, 549, 553 and 655 nm with a shoulder at 625 nm. A corresponding reduced spectrum produced by anaerobic reduction with sodium dithionite is shown in Fig. 2. The two reduced spectra (Figs. 1 and 2) are similar, except that, in the presence of dithionite, the α band of the haem d_1 centred at 655 nm in the ascorbate-reduced enzyme, is shifted to 625 nm, with a concomitant decrease in the 460 nm band. This

Fig. 2. Absorption spectra of oxidized and dithionite-reduced *Pseudomonas* cytochrome oxidase

—, Oxidized *Pseudomonas* cytochrome oxidase; ----, dithionite-reduced *Pseudomonas* cytochrome oxidase, in 0.04M-potassium phosphate buffer, pH 6.9. The enzyme concentration was $8 \mu\text{M}$ and the spectra were recorded at room temperature (20°C), under N_2 in a Thunberg cuvette with a light-path of 1 cm. The sample volume was 3 ml and the reduction was carried out by allowing 19 mg of sodium dithionite, placed in a side arm of the cuvette before degassing, to dissolve in the solution.

effect of dithionite results from binding of sulphur oxy-anions to the reduced haem d_1 (Parr *et al.*, 1974).

In common with mammalian cytochrome oxidase (Greenwood *et al.*, 1974), incubation of oxidized anaerobic *Pseudomonas* cytochrome oxidase under an atmosphere of CO, produced, over a period of approximately 2 h, the reduced enzyme-CO complex, despite the absence of added reducing agents. Under these conditions, however, and in contrast with the mammalian oxidase, both haem components of the *Pseudomonas* cytochrome oxidase become reduced. Fig. 3 shows a spectrum of the reduced enzyme-CO complex generated as described and is identical with that of the ascorbate reduced enzyme-CO complex in the visible region (Parr *et al.*, 1975). Additionally it is possible, by this method, to obtain a reduced spectrum in the u.v. region, the major feature of which is the shift of the δ band of the haem c from 360 nm in the oxidized enzyme to 317 nm in the reduced protein. The u.v. spectrum of the reduced protein is not

normally observable because of the high absorption exhibited by standard reducing agents in this region.

General properties

Velocity sedimentation of *Pseudomonas* cytochrome oxidase (6.2 mg/ml) in the analytical ultracentrifuge gave a single peak of material with a sedimentation coefficient ($s_{20,w}$) of 7.5S. This value is in close agreement with that obtained by Kuronen & Ellfolk (1972). The isoelectric point of the protein at 4°C as measured after 72 h of isoelectric focusing, was 6.9. This value is more consistent with the behaviour of *Pseudomonas* cytochrome oxidase in ion-exchange chromatography than the value of 5.8 measured by Horio *et al.* (1961a,b).

Kinetic properties

As well as the physiological protein substrates *Pseudomonas* ferrocyclochrome c_{551} and *Pseudomonas* azurin, *Pseudomonas* cytochrome oxidase can utilize

Fig. 3. Absorption spectra of oxidized and CO-reduced *Pseudomonas* cytochrome oxidase

—, Oxidized *Pseudomonas* cytochrome oxidase under N_2 ; ----, reduced *Pseudomonas* cytochrome oxidase-CO complex formed after 2 h incubation under 1 atm of CO, in 0.04 M-potassium phosphate buffer, pH 6.9. The enzyme concentration was $9.5 \mu M$ and the spectra were recorded at room temperature (20°C) in a Thunberg cuvette of path-length 1 cm. The sample volume was 3 ml.

two chemical substrates, sodium ascorbate and hydroquinone, as electron donors in the reduction of both oxygen and nitrite. Fig. 4 shows the Lineweaver-Burk plots for sodium ascorbate and quinol in the enzyme-catalysed reduction of O_2 from which the K_m values at pH 6.4 and 30°C of 4 and 30 mM respectively were derived.

Fig. 5(a) shows a typical reaction trace for the aerobic oxidation of *Pseudomonas* ferrocyclochrome c_{551} in the presence of *Pseudomonas* cytochrome oxidase. It can be seen that the rate of reaction

decreases rapidly with time. Such behaviour can occur as a result of product inhibition in which the affinity of the enzyme for the product is greater than that for the substrate (Philo & Selwyn, 1973). This was confirmed by comparing the initial reaction velocities at identical concentrations of ferrocyclochrome c_{551} in the absence and presence of various concentrations of ferrocyclochrome c_{551} . Addition of oxidized substrate produced an inhibitory effect resulting in a decrease in reaction rate. The aerobic oxidation of reduced *Pseudomonas* azurin by *Pseudomonas* cyto-

Fig. 4. Lineweaver-Burk plots of initial reaction rates observed with sodium ascorbate and quinol as electron donors

Experiments were performed at 30°C in 0.04M-potassium phosphate buffer, pH 6.4, (a) with sodium ascorbate as substrate and (b) with quinol as substrate, with a concentration of 0.1 μ M-*Pseudomonas* cytochrome oxidase. The reactions were followed in an oxygen electrode containing 3 ml volumes of buffer/substrate mixtures.

Fig. 5. Aerobic oxidation of *Pseudomonas* ferrocyclochrome c_{551} by *Pseudomonas* cytochrome oxidase

(a) Typical reaction trace for the oxidation of *Pseudomonas* ferrocyclochrome c_{551} by *Pseudomonas* cytochrome oxidase observed at 551 nm. The concentration of substrate was 4.7 μ M, that of the enzyme 0.02 μ M. The reaction was carried out in 2.5 ml of 0.04M-potassium phosphate buffer, pH 7.0, and 1 mM-EDTA, at a temperature of 30°C in a 1 cm-path-length cell. ----, Absorbance of the system when fully oxidized. Pseudomonas cytochrome oxidase was added at X. (b) Lineweaver-Burk plot of the initial reaction rates observed with *Pseudomonas* ferrocyclochrome c_{551} as an electron donor. The conditions used were as described in (a).

Fig. 6. Oxidation of reduced *Pseudomonas* azurin by *Pseudomonas* cytochrome oxidase

(a) Lineweaver-Burk plot of the initial reaction rates observed at 625 nm with reduced *Pseudomonas* azurin as an electron donor. The reactions were carried out in 2.5 ml of 0.04 M-potassium phosphate buffer, pH 7.0, and 1 mM-EDTA, at a temperature of 30°C, in a 1 cm-path-length cell. The concentration of *Pseudomonas* cytochrome oxidase used was 0.1 μM . (b) Dixon plot to determine the K_i for oxidized *Pseudomonas* azurin acting as a competitive inhibitor for reduced *Pseudomonas* azurin. The conditions used were as described in (a), the concentrations of reduced *Pseudomonas* azurin being: $\bullet$, 22.7 μM ; Δ , 27.8 μM ; $\blacksquare$, 31.05 μM ; $\blacktriangle$, 58.8 μM . ----, V_{max} value obtained from (a).

chrome oxidase exhibited a similar reaction profile due to inhibition resulting from the production of oxidized azurin. Both *Pseudomonas* ferrocyclochrome c_{551} and oxidized azurin were also inhibitory when nitrite was used as an electron acceptor under anaerobic conditions.

A Lineweaver-Burk plot for the aerobic oxidation of *Pseudomonas* ferrocyclochrome c_{551} (Fig. 5b) yielded a K_m of 5.6 μM and a V_{max} of 168 mol of ferrocyclochrome c_{551} oxidized/min per mol of *Pseudomonas* cytochrome oxidase at pH 7.0 and 30°C. The corresponding plot for reduced *Pseudomonas* azurin is shown in Fig. 6(a) and gives a K_m of 49 μM and a V_{max} of 192 mol of *Pseudomonas* azurin oxidized/min per mol of *Pseudomonas* cytochrome oxidase, under the same conditions. For competitive inhibition, a Dixon plot can be used to determine the affinity of the inhibitor (K_i). A Dixon plot for the inhibition of

the oxidation of reduced *Pseudomonas* azurin by oxidized *Pseudomonas* azurin is shown in Fig. 6(b), from which a value for the K_i of 4.9 μM was obtained. The K_i of *Pseudomonas* ferrocyclochrome c_{551} was estimated to be 1–2 μM , but it was difficult to determine an accurate value because the high affinity led to inaccuracies in the measurements of initial reaction rates.

In all the steady-state kinetic experiments cited above the reduced protein substrates were prepared as described in the Materials and Methods section. However, Gudat *et al.* (1973) have used an assay system in which *Pseudomonas* cytochrome c_{551} is reduced by the addition of a few grains of solid sodium dithionite, excess of reducing agent being removed by gently shaking the solution for a few minutes in air. In view of previous observations (Parr *et al.*, 1974) on the effects of certain sulphur oxy-anions on the spectrum of ascorbate-reduced *Pseudomonas* cytochrome oxidase, the oxidation of *Pseudomonas* ferrocyclochrome c_{551} was followed by the method described by Gudat *et al.* (1973) and also by the method described above in which the oxidation products of dithionite are removed by filtration chromatography. Fig. 7(a) shows that the presence of dithionite oxidation products inhibits the rate of aerobic oxidation of *Pseudomonas* ferrocyclochrome c_{551} by *Pseudomonas* cytochrome oxidase. Conversely, the nitrite reductase activity of *Pseudomonas* cytochrome oxidase exhibits a slight enhancement when dithionite is present (Fig. 7b). The oxidase and nitrite reductase activities of the enzyme are similarly affected by sodium metabisulphite.

Discussion

Since the initial isolation and purification of *Pseudomonas* cytochrome oxidase (Horio *et al.*, 1961a), several different procedures for purifying the enzyme have been devised (Newton, 1969; Kuronen & Ellfolk, 1972; Gudat *et al.*, 1973), mainly because of difficulties in reproducing the original work of Horio *et al.* (1961a). Indeed, it has since become apparent that there are certain discrepancies between the properties of *Pseudomonas* cytochrome oxidase as originally purified and those of the enzyme prepared by the more recent isolation procedures. Our values of sedimentation coefficient and isoelectric point tend to confirm the results of the later workers (Kuronen & Ellfolk, 1972; Gudat *et al.*, 1973), which indicate that *Pseudomonas* cytochrome oxidase exists as a dimeric protein containing four haem groups per 120000 mol.wt.

The spectral properties of *Pseudomonas* cytochrome oxidase reported here are similar to those described by previous workers (Yamanaka & Okunuki, 1963; Gudat *et al.*, 1973) and reflect the absorption charac-

Fig. 7. Effects of sodium dithionite oxidation products on the oxidase and nitrite reductase activities of *Pseudomonas* cytochrome oxidase

(a) Reaction traces showing the oxidation of *Pseudomonas* ferrocytochrome c_{551} in the presence (----) and absence (—) of dithionite oxidation products. The reactions were observed at 551 nm at a temperature of 20°C with 14 μM -*Pseudomonas* ferrocytochrome c_{551} in air-equilibrated 0.04 M-potassium phosphate buffer, pH 7.0. *Pseudomonas* cytochrome oxidase was added to a concentration of 0.06 μM at X and potassium nitrite to a concentration of 300 μM at Y. ---, Absorbance of the system when fully oxidized. The sample volume was 2.5 ml and the path-length 1 cm. (b) Reaction traces showing the oxidation of *Pseudomonas* ferrocytochrome c_{551} in the presence of 300 μM -potassium nitrite, with (—) and without (----) the removal of dithionite oxidation products. The conditions used were as for (a), with addition of *Pseudomonas* cytochrome oxidase at Z.

teristics typical of a protein containing haem *c* in association with another component with absorption peaks at 640 nm in the oxidized protein and 460 and 655 nm in the reduced protein. This component has been classified as haem d_1 (Lemberg & Barrett, 1972) and is the chromophore affected when ligands, such as CN^- and CO, bind to the enzyme.

It has been shown that both oxidase and nitrite reductase activities of *Pseudomonas* cytochrome oxidase are severely inhibited by *Pseudomonas* ferricytochrome c_{551} and oxidized *Pseudomonas* azurin when the reduced forms of those proteins are used as substrates. Physiologically it seems therefore, that for the *Pseudomonas* cytochrome oxidase to function efficiently the natural protein substrates, within the cell, must be maintained at a high degree of reduction. It is noteworthy that a similar situation occurs in mammalian systems where ferricytochrome *c* inhibits the cytochrome oxidase (Yonetani & Ray, 1965), and it is open to speculation as to whether this product inhibition provides a means of respiratory control at the cellular level.

Product inhibition, however, poses a number of problems in steady-state kinetic experiments. The rapid decrease in rate as a function of time caused by the accumulation of product leads to inaccuracies in measuring rates. These errors are compounded because, even immediately after reduction, there is always a small percentage (approx. 2%) of the protein substrate in the oxidized form. This becomes significant in view of the very low values of K_1 for *Pseudomonas* ferricytochrome c_{551} and oxidized *Pseudomonas* azurin and will necessarily affect the initial rate observed.

In spite of these problems, it is quite clear that the natural substrates have far lower K_m values than the two chemical substrates investigated, although the latter have much more negative reducing potentials.

A direct comparison of steady-state kinetic constants with those published by other workers is hampered by the variety of conditions used. Nevertheless, the K_m for quinol is of the same order as that found by Horio *et al.* (1961a), whereas the Michaelis constants for *Pseudomonas* ferrocytochrome c_{551} and

reduced *Pseudomonas* azurin are similar to those found by Gudat *et al.* (1973), if one allows for the discrepancies in the values of the extinction coefficient of azurin at 625 nm (Brill *et al.*, 1968; Horio *et al.*, 1961b).

That certain oxy-anions of sulphur, including the oxidation products of dithionite, affect the steady-state oxidation kinetics of *Pseudomonas* cytochrome oxidase is, perhaps, not surprising in view of previous observations on the spectral changes which those reagents exert on the ascorbate-reduced enzyme (Parr *et al.*, 1974). The differential behaviour of the sulphur oxy-anions towards the oxidase and nitrite reductase activities seem to reinforce the hypothesis that oxygen and nitrite react with different enzymic sites. However, the slight enhancement in nitrite reductase activity appears not to be consistent with SO_2^- and NO_2^- ions occupying the same site.

Sodium metabisulphite, in concentrations that from known binding constants would not compete directly with CO, causes a significant decrease in the rate of CO recombination as observed after flash photolysis (Parr *et al.*, 1974) and presumably this effect on the reduced enzyme is also manifested in the inhibition of oxidase activity. Clearly, at present, it is difficult to reconcile the variable affects of the inhibitors (CO , CN^- and SO_2^-) towards the oxidase and nitrite reductase activities of *Pseudomonas* cytochrome oxidase and further work is necessary to resolve this problem.

D. B. thanks the S.R.C. for a graduate studentship. S. R. P. is the recipient of an S.R.C. fellowship. C. G. gratefully acknowledges a grant from the Royal Society for the purchase of a Cary 118C spectrophotometer. This work was supported by a grant, B/RG/1537, from the Science Research Council.

References

- Brill, A. S., Bryce, G. F. & Maria, H. (1968) *Biochim. Biophys. Acta* **154**, 342–351
- Greenwood, C., Wilson, M. T. & Brunori, M. (1974) *Biochem. J.* **137**, 205–215
- Gudat, J. C., Singh, J. & Wharton, D. C. (1973) *Biochim. Biophys. Acta* **292**, 376–390
- Horio, T., Matsubara, H., Kusai, K., Nakai, M. & Okunuki, K. (1958) *Biochim. Biophys. Acta* **29**, 297–302
- Horio, T., Higashi, T., Sasagawa, M., Kusai, K., Naki, M. & Okunuki, K. (1960) *Biochem. J.* **77**, 197–201
- Horio, T., Higashi, T., Yamanaka, T., Matsubara, H. & Okunuki, K. (1961a) *J. Biol. Chem.* **236**, 944–951
- Horio, T., Sekuzu, I., Higashi, T. & Okunuki, K. (1961b) in *Hematin Enzymes* (Falk, J. E., Lemberg, R. & Morton, R. K., eds.), pp. 302–311, Pergamon Press, London
- Kuronen, T. & Ellfolk, N. (1972) *Biochim. Biophys. Acta* **275**, 308–318
- Kuronen, T., Saratse, M. & Ellfolk, N. (1975) *Biochim. Biophys. Acta* **393**, 48–54
- Lemberg, R. & Barret, J. (1972) *The Cytochromes*, p. 243, Academic Press, New York
- Newton, N. (1969) *Biochim. Biophys. Acta* **185**, 316–331
- Parr, S. R., Wilson, M. T. & Greenwood, C. (1974) *Biochem. J.* **139**, 273–276
- 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
- Philo, R. & Selwyn, M. J. (1973) *Biochem. J.* **135**, 525–530
- Wilson, M. T., Greenwood, C., Brunoir, M. & Antonini, E. (1975) *Biochem. J.* **145**, 449–457
- Yamanaka, T. & Okunuki, K. (1963) *Biochim. Biophys. Acta* **67**, 394–406
- Yamanaka, T., Ota, A. & Okunuki, K. (1961) *Biochim. Biophys. Acta* **53**, 294–308
- Yonetani, T. & Ray, G. S. (1965) *J. Biol. Chem.* **240**, 3392–3398