Short Communications

The Reaction of *Pseudomonas aeruginosa* Cytochrome *c* Oxidase with Sodium Metabisulphite

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Spectrophotometric evidence is presented for the formation of a complex between metabisulphite and reduced *Pseudomonas aeruginosa* cytochrome *c* oxidase. The effects of metabisulphite on the recombination of CO with the reduced enzyme are discussed in terms of alternate binding sites for $S_2O_5^{2-}$ and CO.

Pseudomonas cytochrome *c* oxidase (ferrocytochrome c_{551} - O_2 oxidoreductase, EC 1.9.3.2) is an enzyme that reduces O_2 to water and also can catalyse the reduction of NO_2^- (Yamanaka *et al.*, 1961) to NO. The enzyme was purified and crystallized by Yamanaka *et al.* (1962), who have also studied some of its properties. More recently, Gudat *et al.* (1973) and Kuronen & Ellfolk (1972) have published details of improved preparations and begun investigations of its properties.

The enzyme contains two haem prosthetic groups, one haem *c* and one haem *d*, the latter being the auto-oxidizable component. No metals other than iron have been detected and the molecular weight of the electrophoretically homogeneous enzyme is approx. 120000 (Gudat *et al.*, 1973), corresponding to a dimer, each subunit containing one *c* and one *d* haem (Kuronen & Ellfolk, 1972).

The oxidation of reduced *Pseudomonas* cytochrome *c* oxidase by oxygen is inhibited by the classical respiratory poisons cyanide and CO (Horio, 1958*a,b*), and the CO complex of reduced *Pseudomonas* cytochrome *c* oxidase is photosensitive.

Materials and methods

Pseudomonas aeruginosa N.C.T.C. 6750 was grown in 40-litre stainless-steel vessels at 37°C with stirring, but in essentially anaerobic conditions. Details of the culture conditions are described by Horio *et al.* (1960). After 21 h, when the pH of the culture approached pH 8, the cells were harvested by means of a Sharples centrifuge. The enzyme, along with cytochrome c_{551} and the blue copper protein azurin, were isolated by using a procedure essentially similar to that used by Gudat *et al.* (1973), except that final purification was done by using Amberlite CG 50 column chromatography (Horio *et al.*, 1961).

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The ratio E_{410}/E_{280} for the crystalline homogeneous preparation was found to be 1.2 by Yamanaka *et al.* (1962), and between 1.1 and 1.2 by Kuronen & Ellfolk (1972), and, on this basis, our final preparation is over 90% pure.

The concentrations of *Pseudomonas* cytochrome *c* oxidase were obtained by using the value E_{410} 149 mm⁻¹·cm⁻¹ for the oxidized protein. Spectrophotometry was performed with a Pye-Unicam SP. 700A instrument. Spectrophotometric titrations were made under anaerobic conditions with cuvettes equipped with Suba-Seal vaccine caps. For titrations involving the oxy-anions of sulphur the salts were always dissolved in deoxygenated buffer (0.04 M-potassium phosphate, pH 7.0) just before use. Anaerobic stopped-flow determinations were carried out by using an apparatus identical with that described by Gibson & Milnes (1964), which was equipped with a 2 cm light-path cell and had a dead-time of 3 ms. Flash-photolytic experiments were performed by using an experimental set-up as described by Greenwood & Gibson (1967). CO and N₂ were obtained from the British Oxygen Company, Deer Park Road, London S.W.19, U.K., and were dispensed from the cylinders and stored in glass vessels over an alkaline solution of reduced anthraquinone sulphonate. All other chemicals used were of AnalaR grade and were obtained from BDH Chemicals, Poole, Dorset, U.K., except for sodium dithionite, which was obtained from Hardman and Holdon, Miles Platting, Manchester, U.K., and ascorbic acid (disodium salt) from Sigma (London) Chemical Co., Kingston-upon-Thames, U.K.

Results and discussion

Sodium dithionite is a widely used and very versatile reducing agent, and along with most workers in the haemoprotein field we have used it as a routine in our studies of a number of cytochromes and

Fig. 1. Anaerobic titration of *Pseudomonas* cytochrome *c* oxidase with sodium metabisulphite

Fig. 1(a) shows the spectra obtained on titrating ascorbate-reduced *Pseudomonas* cytochrome *c* oxidase anaerobically with $\text{Na}_2\text{S}_2\text{O}_5$. Samples of 0.2M- $\text{Na}_2\text{S}_2\text{O}_5$ (of varying size) were injected through a vaccine cap into a 4cm-path-length cuvette containing 4.4ml of 8.3 μM -ascorbate-reduced *Pseudomonas* cytochrome *c* oxidase in 0.04M-potassium phosphate buffer, pH7.0. The spectra (1) to (7) inclusive correspond to the following concentrations of $\text{S}_2\text{O}_5^{2-}$: 0, 0.68, 1.57, 3.34, 5.51, 7.63 and 10.72mM respectively. Fig. 1(b) is a Hill plot of the titration data. $\bar{Y}$ is the proportion of the enzyme saturated with metabisulphite after each addition.

other redox proteins. However, during the course of investigating the properties of *Pseudomonas* cytochrome *c* oxidase we have consistently observed significant differences in the rate and form of the CO-binding kinetics that depended on whether the enzyme had been reduced by sodium dithionite or by sodium ascorbate. The position of the α peak of the ferrous *d* haem was also found to depend on the nature of the reductant, this peak being located at 635nm when the enzyme was reduced by sodium dithionite, and at 655nm when sodium ascorbate was used. Anaerobic addition of sodium dithionite to an ascorbate-reduced preparation resulted in a shift of the band from 655 to 635nm, which suggested the possibility of complex-formation. Therefore we have examined the effect of adding a number of oxy-anions of sulphur to *Pseudomonas* cytochrome *c* oxidase reduced with ascorbate. Out of the series SO_3^{2-} , SO_4^{2-} , $\text{S}_2\text{O}_3^{2-}$ and $\text{S}_2\text{O}_5^{2-}$, SO_3^{2-} and $\text{S}_2\text{O}_5^{2-}$ were both observed to produce a spectral shift similar to that seen with sodium dithionite. SO_4^{2-} and $\text{S}_2\text{O}_3^{2-}$, however, proved to have no effect on the spectrum of reduced oxidase. Fig. 1(a) shows the results of an anaerobic titration of ascorbate-reduced *Pseudomonas* cytochrome *c* oxi-

dase with $\text{Na}_2\text{S}_2\text{O}_5$ over the wavelength range 580–680nm. This titration data appear to reflect a simple binding process such as:

where X may denote either the $\text{S}_2\text{O}_5^{2-}$ ion itself or some product formed by metabisulphite in aqueous solution. K is the affinity constant, k_{+1} is the second-order combination constant and k_{-1} is the first-order dissociation constant. Fig. 1(b) shows a Hill plot of the data in Fig. 1(a), which yields an h value close to unity (1.2) and an affinity constant, K , of $3.2 \times 10^2 \text{M}^{-1}$.

In stopped-flow experiments where ascorbate-reduced *Pseudomonas* cytochrome *c* oxidase was mixed anaerobically with a large excess of $\text{Na}_2\text{S}_2\text{O}_5$ the progress curves, monitored at 655nm, were found to be simple exponentials. A plot of the pseudo-first-order rate constant against metabisulphite concentration was linear over the concentration range explored (up to 50mM-metabisulphite) and yielded a combination constant, k_{+1} , of $2.8 \text{M}^{-1} \cdot \text{s}^{-1}$ and a dissociation constant, k_{-1} , of $5 \times 10^{-3} \text{s}^{-1}$. These two values taken together give a value of $5.6 \times$

Fig. 2. Effect of metabisulphite on the kinetic difference spectrum reduced *Pseudomonas* cytochrome *c* oxidase minus reduced *Pseudomonas* cytochrome *c* oxidase-CO complex

CO, under 0.25 atm (25 kPa) pressure (= 250 μ M) was introduced into a Thunberg cuvette (1 cm path-length) containing 4 ml of 5.5 μ M-ascorbate-reduced *Pseudomonas* cytochrome *c* oxidase in 0.04 M-potassium phosphate buffer, pH 7.0. The side arm contained 13 mg of $\text{Na}_2\text{S}_2\text{O}_5$ to give a final concentration of $\text{S}_2\text{O}_5^{2-}$, after tipping, of 17 mM. Reduced oxidase minus reduced oxidase-CO difference spectra: (a) $\blacktriangle$, in the absence of $\text{Na}_2\text{S}_2\text{O}_5$; (b) $\bullet$, in the presence of $\text{Na}_2\text{S}_2\text{O}_5$. The band width of the observing light was 0.8 nm and a photolytic flash of 250 J was used to give 100% breakdown of the enzyme-CO complex.

10^2 M^{-1} for the affinity constant, in fair agreement with that obtained from static measurements.

To investigate the effect of metabisulphite on the reaction between CO and the ascorbate-reduced enzyme, we prepared the enzyme-CO complex in a sealed Thunberg cuvette, which contained a few milligrams of $\text{Na}_2\text{S}_2\text{O}_5$ in the side arm. The rate of recombination of CO with the reduced enzyme was monitored after displacement of the CO from the haem *d* by an intense photolytic flash. At 0.25 mM-CO the recombination was simple and corresponded to a second-order rate constant of $3 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 20°C. After mixing with the metabisulphite from the side arm the recombination rate was found to have decreased to $5.4 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$.

Fig. 2 shows that the addition of metabisulphite also had a profound effect on the kinetic difference spectrum reduced *Pseudomonas* cytochrome oxidase minus reduced *Pseudomonas* cytochrome oxidase-CO complex. The kinetic difference spectrum in the presence of metabisulphite corresponds closely to that observed kinetically for the dithionite-reduced enzyme and is not influenced over a fourfold range of CO (0.25–1 mM) concentration. Although there is a close similarity between the effects of metabisulphite

and dithionite we have observed (S. R. Parr, M. T. Wilson & C. Greenwood, unpublished work) that dithionite produces, in addition, complex CO-recombination kinetics.

The metabisulphite titration and Hill plot, taken in conjunction with the observed second-order character of the reaction between metabisulphite and ascorbate-reduced enzyme, seems to indicate that the binding site involves or is closely associated with the *d* haem. This conclusion is further supported by the effect of $\text{S}_2\text{O}_5^{2-}$ on the kinetic difference spectrum seen in Fig. 2 and on the rate of CO recombination.

Direct combination between metabisulphite and the haem *d* iron would, however, seem to be excluded in view of the 10000-fold greater rate of combination of CO with the enzyme as compared with metabisulphite, which would favour CO at all times in a competitive situation. This, taken together with the observed effect of metabisulphite on the CO-recombination rate, and the fact that the kinetic difference spectrum in the presence of metabisulphite is unchanged when the CO concentration is increased, argues strongly for alternative binding sites. Preliminary stopped-flow experiments (S. R. Parr, M. T. Wilson & C. Greenwood, unpublished work), recently carried out in this laboratory, which show that NaNO_2 oxidizes the CO complex of *Pseudomonas* cytochrome *c* oxidase at rates greater than the CO dissociation constant, support this idea.

It has been known for many years (Yamanaka *et al.*, 1961) that, although CO inhibits the oxygen reaction of this enzyme, it has no effect on the reduction of NO_2^- , which is suggestive of alternate binding sites for NO_2^- and O_2 . Whether the binding sites of NO_2^- and the sulphur oxy-anions are identical remains to be proved; however, it may be worth noting that only those sulphur oxy-anions that can form SO_3^{2-} ions (sulphite and metabisulphite) or SO_2^- (dithionite; see Lambeth & Palmer, 1973) in solution produce the spectral shift in the α band of the ferrous *d* haem. It is perhaps reasonable to suppose that SO_3^{2-} and SO_2^- ions may occupy the same site as the NO_2^- ion.

The present results nevertheless clearly preclude the use of dithionite as a reductant for the enzyme.

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