Mixed-Valence Cytochrome Oxidase–Formate Complex

A STEADY-STATE INTERMEDIATE

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At neutral pH, formate binds to the haem a_3 component of cytochrome *c* oxidase to give a complex that reacts differently from the non-liganded enzyme with reducing agents. Addition of sodium dithionite to the formate complex leads directly to the formation of the fully reduced species, whereas reduction with ascorbate/tetramethylenephenylenediamine can lead to the production of a mixed-valence species. The stability of this mixed-valence form was studied, and the species appears to represent a 'steady-state' situation that is stable only in the presence of an excess of O_2 and reducing equivalents. Characterization of the mixed-valence complex by electron paramagnetic resonance and magnetic circular dichroism reveals the presence of reduced low-spin haem *a* together with reduced detectable copper and high-spin ferric haem a_3 .

Cytochrome *c* oxidase contains two haems, *a* and a_3 , originally characterized by their different reactivity towards various ligands, e.g. CO, CN and O_2 (Keilin & Hartree, 1939). There is some evidence that these haems can react co-operatively and consequently exhibit haem–haem interactions (Greenwood *et al.*, 1974; Nicholls & Petersen, 1974). With such a complex enzyme as this, it has been desirable to attempt to simplify the events that can occur, and to this end a number of so-called mixed-valence species have been prepared and characterized (Yonetani, 1960; Greenwood *et al.*, 1974). Until relatively recently the forms normally used were those in which the haem of cytochrome a_3 is 'locked' in the low-spin form, in either the oxidized or the reduced state. It has, however, been demonstrated initially by e.p.r.† and more recently by m.c.d. studies that, in the normal non-liganded form of the enzyme, the haem of cytochrome a_3 is in the high-spin form in both oxidation states (Wilson *et al.*, 1976; Thomson *et al.*, 1976; Babcock *et al.*, 1976). In view of these findings it seemed desirable to investigate the changes occurring in the haem *a* moiety of an enzyme molecule in which the haem of cytochrome a_3 was 'locked' not in the low-spin but in the high-spin form. Nicholls (1975) has introduced the use of formate as an inhibitor of cytochrome oxidase, acting at the level of cytochrome a_3 , and has further shown (Nicholls, 1976) that, in the formate-inhibited enzyme, reduction of the haem of cytochrome *a* can still occur, although the haem of cytochrome a_3 is 'locked' in the high-spin ferric form (Babcock *et al.*, 1976).

† Abbreviations: e.p.r., electron paramagnetic resonance; m.c.d., magnetic circular dichroism.

With this use in mind we have undertaken an investigation of the properties of the mixed-valence formate species, with special reference to situations in which the enzyme might become anaerobic.

Materials and Methods

Cytochrome *c* oxidase was prepared as previously described (Greenwood *et al.*, 1976), and the concentration of protein samples measured by using the absorption coefficients of Yonetani (1960). Spectrophotometric measurements were made by using 1 cm-path-length cells in a Cary 118C recording spectrophotometer.

O_2 utilization was followed by means of a Rank Brothers oxygen-electrode system. Experiments were performed by making additions of microlitre quantities of freshly prepared sodium ascorbate (1 M) and *NNN'*-tetramethylene-*p*-phenylenediamine dihydrochloride (50 mM) solutions to 3 ml of a 10 μ M-cytochrome oxidase–formate solution. O_2 concentrations were obtained by reference to published tables (Kaye & Laby, 1966). All solutions, unless otherwise stated, were prepared in 0.1 M-potassium phosphate buffer, pH 7.4, containing 1% Tween 80.

The formate complex of cytochrome oxidase was prepared as described by Nicholls (1975), by incubation of a sample of the enzyme with a neutralized solution of sodium formate. M.c.d. spectra were recorded by using the equipment and techniques described previously (Thomson *et al.*, 1976) and e.p.r. spectra were recorded at approx. 20 K on a Varian E9 spectrometer operating at 9 GHz.

Sodium ascorbate and *NNN'*-tetramethylene-*p*-phenylenediamine dihydrochloride were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.

Results and Discussion

Formate binds to cytochrome oxidase to yield a species with the spectrum shown in Fig. 1. Addition of dithionite to the formate complex instantly produced the fully reduced species as previously reported (Nicholls, 1976). Addition of sodium ascorbate / *NNN'*-tetramethylene-*p*-phenylenediamine dihydrochloride to fully oxidized formate-bound enzyme leads to the production of a mixed-valence species in which the haem of cytochrome *a* is

Fig. 1. Static spectra of some cytochrome oxidase-formate complexes

Incubation of the protein with neutralized sodium formate yields the formate-oxidase complex (—). Addition of dithionite produced the fully reduced species (---), and addition of *NNN'*-tetramethylene-*p*-phenylenediamine dihydrochloride/ascorbate produced the mixed-valence species (-.-). The protein concentration was $10\mu\text{M}$ in all cases and the sodium formate concentration was 10mM. Spectra were recorded in a 1 cm-path-length cell at 25°C .

Fig. 2. Effect of O_2 concentration on the spectral lag phase observed in the reduction of the formate complex of cytochrome oxidase

The length of the lag phase is shown as a function of O_2 concentration. (a) Time course, followed spectrophotometrically, of the reduction in air at 100kPa; (b) time course in air at 50kPa; (c) effect of the addition of *NNN'*-tetramethylene-*p*-phenylenediamine dihydrochloride/ascorbate in the absence of O_2 ; (d) effect of the addition of dithionite in air at 100kPa. The protein concentration was $10\mu\text{M}$ in all cases containing 10mM-sodium formate. The reaction occurred in a 1 cm-path-length cell in a volume of 3 ml. (a), (b) and (c) represent the addition of $60\mu\text{l}$ of 1M-sodium ascorbate and $30\mu\text{l}$ of 50mM-*NNN'*-tetramethylene-*p*-phenylenediamine dihydrochloride, and (d) represents a small excess of dithionite added at zero time.

Fig. 3. Time course of O_2 utilization and reduction of cytochrome oxidase-formate complex by *NNN'*-tetramethylene-*p*-phenylenediamine dihydrochloride/ascorbate

The course of O_2 utilization, followed polarographically (○), and the subsequent reduction, followed spectrophotometrically (●), of the formate complex of cytochrome oxidase is shown. The reaction in both cases was between $60\mu\text{l}$ of 1M-sodium ascorbate / $30\mu\text{l}$ of 50mM-*NNN'*-tetramethylene-*p*-phenylenediamine dihydrochloride and $10\mu\text{M}$ -cytochrome oxidase containing 10mM-sodium formate in a reaction volume of 3 ml at 25°C . The final absorption of the reaction mixture is also shown (t_∞).

reduced but the haem of cytochrome a_3 remains oxidized and bound to formate (Fig. 1). If the filled spectrophotometer cell used in the preparation of the mixed-valence species was stoppered, then after a 'lag' period, when no spectral change occurred, the spectrum changed to that of the fully reduced form. Fig. 2 shows the effect of O_2 concentration on the length of the lag period; traces (a), (b) and (c), in which sodium ascorbate/ NNN' -tetramethylene- p -phenylenediamine dihydrochloride was used as the electron donor, clearly indicate that the length of the lag period is proportional to the O_2 content of the solution. The coincidence of the time course of the dithionite reduction (trace d) with that of trace (c) strongly suggests that the situation represented in traces (a) and (b) arises because of the presence of O_2 in solution and does not reflect the difference in redox potential between dithionite and ascorbate. The immediate reduction of the mixed-valence formate complex by dithionite, added in the presence of O_2 , is undoubtedly due to the ability of dithionite to reduce solution O_2 directly (Nickless, 1968). The rate of reduction of the mixed-valence species, after the lag period, was $6.0 \times 10^{-3} s^{-1}$, irrespective of which of the two reductants was used or of the O_2 concentration initially present. If NNN' -tetramethylene- p -phenylenediamine dihydrochloride/ascorbate was added to the formate complex and the solution allowed to reach the fully reduced state, re-admitting O_2 resulted in the rapid production of the spectrum characteristic of the mixed-valence species (Fig. 1). Thus the enzyme complex may be cycled between the mixed-valence species and the fully reduced form many times, by admitting air to the anaerobic fully reduced material.

From the above results it appears that the mixed-valence species is stable only in the presence of O_2 , and this conclusion has been further investigated by using polarographic techniques. Fig. 3 shows a plot of O_2 concentration as a function of time for a solution identical with that used in the spectral studies. It is clear that O_2 is being consumed during the spectrophotometric lag phase, and the rate of O_2 consumption is constant with time, suggesting a zero-order process. Increasing the enzyme concentration increased the rate of O_2 consumption, thus implicating enzyme turnover as being responsible for O_2 usage.

The steady-state situation involving the mixed-valence formate complex has been rapidly frozen and its constituents examined by a combination of e.p.r. and m.c.d. spectroscopy. Fig. 4(a) shows that, in the mixed-valence complex, all the normally observed e.p.r. features arising from low-spin ferric haem a and cupric copper are absent. E.p.r. results by themselves cannot, however, eliminate the possibility that O_2 turnover arises from the presence of substantial amounts of fully reduced and non-

Fig. 4. Characterization of the mixed-valence formate complex

(a) (i) and (ii) show the e.p.r. spectrum of the fully oxidized and mixed-valence formate complex respectively. Both spectra, at a protein concentration of $150 \mu M$, were recorded at 300 kHz modulation frequency, at 100 mW microwave power at a microwave frequency of 9.1 GHz at approx. 20 K. The signal amplitude (ordinate) is plotted as a function of increase of which is indicated by the arrow. The spectrum of the mixed-valence formate complex (ii) was recorded at a gain factor of 20 larger than that in (i), and is shifted for clarity. (b) M.c.d. spectrum of the mixed-valence formate species. The protein concentration was $130 \mu M$, containing 30 mM-sodium formate. Spectra recorded at 31 K (---), 19 K (----) and 15 K (—) were measured in a 0.8 mm-path-length cell. B is the magnetic field strength.

Scheme 1. Formation of mixed-valence formate complex in the presence of an excess of reducing equivalents and O_2 . e, Electron from reductant. All species in parentheses are considered to exist in extremely low concentrations.

liganded enzyme. To resolve this problem, m.c.d. spectra of the mixed-valence formate complex were obtained, and these are presented in Fig. 4(b). The m.c.d. spectra are consistent with an interpretation of the mixed-valence species containing high-spin ferric haem a_3 and low-spin ferrous haem a (Thomson *et al.*, 1977). The lack of temperature-dependence in the spectral region centred at 450 nm allows us to conclude that there is virtually no free fully reduced enzyme present, since this material would contain high-spin ferrous haem a_3 , that which previous studies (Thomson *et al.*, 1976) have shown to be m.c.d.-intense in this region. Under the conditions used, it should be noted that even when EDTA is added to the sodium ascorbate, a measurable rate of O_2 reduction is observed for ascorbate/*NNN'*-tetramethyl-*p*-phenylenediamine dihydrochloride, in the absence of the enzyme.

We conclude that the mixed-valence formate complex is stable only in the presence of oxygen. This situation appears to arise from the fact that, in the presence of an excess of reducing equivalents and O_2 , the enzyme cycles between the mixed-valence formate complex and the fully oxidized formate-bound form, via the non-liganded fully reduced and fully oxidized states, the rate of cycling being limited by the formate 'off' rate. This may be represented simplistically by means of Scheme 1.

The non-enzymic *NNN'*-tetramethyl-*p*-phenylenediamine dihydrochloride/ascorbate reaction undoubtedly contributes to the use of O_2 , and it is

possible that a small equilibrium amount of non-liganded enzyme could add to the O_2 turnover. If the rate of cycling is, however, limited by the formate 'off' rate, then most of the enzyme would appear to be present as the mixed-valence species, with only minute quantities present as other forms. In the stopped spectrophotometer cell the O_2 content would eventually be reduced to zero, at which time the cycle is broken at the reduced form. Most of the enzyme is then reduced at the formate 'off' rate. Re-introduction of O_2 to the system would then re-establish the cycle with most of the enzyme held as the mixed-valence formate species until all the O_2 is reduced once more.

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