

Chromous Ion Reduction of Mammalian Cytochrome Oxidase and some of its Derivatives

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The reduction of cytochrome *c* oxidase by Cr^{2+} , followed by means of stopped-flow spectrophotometry, exhibits two phases: the faster Cr^{2+} -concentration-dependent reaction has an initial rate constant of $1.1 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, but reaches a rate limit at high concentration of reductant; the slower phase is concentration-independent with a rate of 0.3 s^{-1} . The activation energies of the fast and the slow processes are 35 and 71 kJ/mol respectively. The reduction kinetics of the mixed-valence CO complex and the cyanide-inhibited enzyme were compared with those of the fully oxidized forms: both the liganded species have a fast phase identical with that found in the oxidized oxidase. A comparison of the kinetic difference spectra obtained for the fast phase of reduction of oxidized oxidase with those obtained on reduction of the liganded species suggests that the rapid phase arises from the reduction of haem *a*, and the slow phase from the reduction of haem *a*₃.

The enzyme cytochrome *c* oxidase contains four redox centres (Muijsers *et al.*, 1972), two haem *a* groups and two non-identical copper atoms (Beinert *et al.*, 1962). The haem groups are identified on the basis of their spectral and ligand-binding properties as belonging to cytochromes *a* and *a*₃ (Keilin & Hartree, 1939). The unusual properties (Yong & Hartree, 1972; Beinert *et al.*, 1971) of undetectable copper $\text{Cu}_{(u)}$ and cytochrome *a*₃ are thought to arise from interactions between these two centres. It has also been proposed that the ligand-binding properties of haem *a*₃ are influenced by the state of haem *a* (Wilson *et al.*, 1972). Site-site interactions have also been proposed to occur in a number of reactions involving cytochrome oxidase (Greenwood *et al.*, 1974; Beinert *et al.*, 1971). The reduction of the oxidized enzyme by ferrocyanide has been extensively investigated (Wilson *et al.*, 1975; Gibson *et al.*, 1965; Beinert & Palmer, 1964), and it has been suggested that the initial reduction of haem *a* is closely accompanied by the reduction of the chromophore responsible for absorption at 830 nm which has been assigned to e.p.r.-detectable copper.

The present paper presents evidence on the reduction of the oxidase molecule by a high-redox-potential metal ion (which was chosen as an alternative to a large protein reductant) as a means of

investigating the possible origins of site-site interactions previously observed in the interaction with the physiological reductant ferrocyanide (Greenwood *et al.*, 1974).

In order to assign the spectral character of the reaction phases to a given haem, the kinetic difference spectrum observed after the reduction of the oxidized protein was compared with those obtained on reduction of the mixed-valence CO complex (Greenwood *et al.*, 1974) and cyanide-inhibited oxidized enzyme, both of which are examples where one of the haems is locked in a particular oxidation state.

Materials and Methods

Cytochrome *c* oxidase (EC 1.9.3.1, ferrocyanide *c*-oxygen oxidoreductase) was prepared essentially by the method of Yonetani (1960), except that EDTA ($10 \mu\text{M}$) was included in the final fractionation steps to ensure removal of adventitious copper. The enzyme concentration was determined by using the extinction coefficients of Yonetani (1961). The mixed-valence CO complex of cytochrome oxidase was prepared by the method of Greenwood *et al.* (1974). The cyanide-inhibited enzyme was prepared by the method of Van Buuren *et al.* (1972) by incubation of the oxidized protein with neutralized 20 mM-KCN for 18 h. CrCl_2 solutions were prepared by the method of Brittain *et al.* (1974). Stopped-flow experiments were performed with an instrument identical with that described by Gibson & Milnes (1964) equipped with 2 mm- and 2 cm-light-path cells with dead times of 1 and 3 ms respectively.

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‡ Abbreviation: e.p.r., electron paramagnetic resonance.

Static spectrophotometric observations were made with a Cary 118C recording spectrophotometer.

Results and Discussion

The reduction of oxidized cytochrome *c* oxidase by Cr^{2+} in the range 0.5–25 mM was biphasic in nature, showing the presence of two consecutive processes. At 445 and 605 nm both reactions were apparent, and analysis of the concentration-dependence of the rates of the two processes showed the faster process to be concentration-dependent, with a rate constant of $1.1 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ at low Cr^{2+}

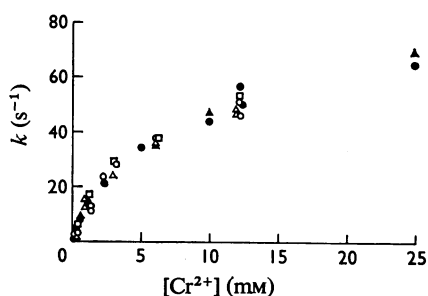


Fig. 1. Concentration-dependence of Cr^{2+} reduction of cytochrome oxidase and its cyanide-inhibited and mixed-valence CO derivatives

The reduction of cytochrome oxidase was followed at 830 nm (○) and 605 nm (△) in a 2 cm-path-length cell, and the reduction of the oxidized enzyme (●), cyanide-inhibited enzyme (□) and mixed-valence CO complex (△) was followed at 445 nm in a 2 mm-path-length cell. The proteins were present at 20 μM before mixing in all cases. The reaction was carried out in 0.1 M-cacodylate/HCl buffer, pH 7.4, containing 1.0% Tween 80 at 20°C.

concentration, and the slower process to be independent, with a rate of 0.3 s^{-1} . The faster phase approached a rate limit, at higher concentrations, of approx. 70 s^{-1} (Fig. 1). When the reduction was observed at 830 nm a single process was apparent, which had the same concentration-dependence as the faster of the two phases observed at 445 or 605 nm. By using reaction data collected over a range of temperatures it was possible to evaluate the activation energies associated with each of the reactions observed as 35 and 71 kJ/mol for the fast and the slow phases respectively.

The kinetic difference spectra obtained for the two kinetic events are presented in Fig. 2. By comparison with previously reported data (Gibson & Greenwood, 1964), it appears that the two phases might be attributable to the reduction of haems *a* and *a*₃ respectively. To investigate this possibility further and also the possible presence of haem-haem interactions, the reduction of two liganded derivatives of the enzyme by Cr^{2+} was investigated. Fig. 1 shows that the rate data obtained for the reduction of mixed-valence CO complex and cyanide-inhibited oxidase corresponds closely to the data obtained for reduction of the oxidized enzyme; in contrast, however, only a single phase was observed at 445 and 605 nm. A comparison of the kinetic difference spectra obtained for the reduction of the two liganded derivatives (Fig. 3) shows clearly that the faster process observed in the reduction of the oxidized oxidase may be attributed to the reduction of haem *a*, since, in liganded derivatives, haem *a*₃ is locked in the reduced low-spin form in the mixed-valence CO complex and in the oxidized low-spin form in the cyanide-inhibited enzyme (Thomson *et al.*, 1976). This is taken as strong evidence that the reduction process observed in these cases can be assigned to the reduction of haem *a*. The differences

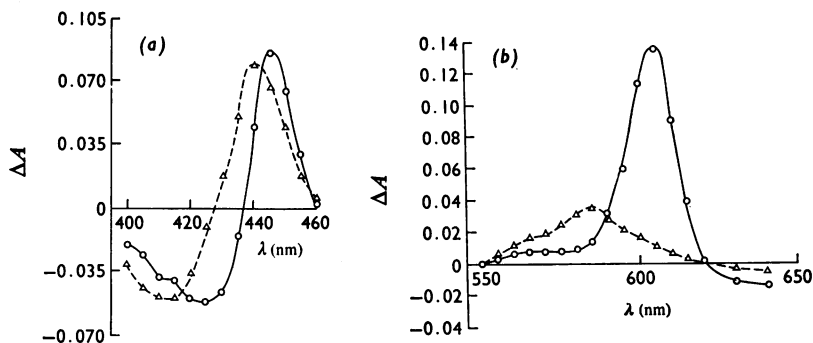


Fig. 2. Kinetic difference spectra for the reduction of cytochrome oxidase by Cr^{2+}

The kinetic difference spectra of the fast (0–100 ms) (○) and slow (100 ms– t_{∞}) (△) phases observed on the reduction of cytochrome oxidase by 12.5 mM- Cr^{2+} are shown. A 2 mm-path-length cell was used in the 400–460 nm region (a) and a 2 cm-path-length cell in the 550–650 nm region (b). The protein concentrations used were 20 μM before mixing.

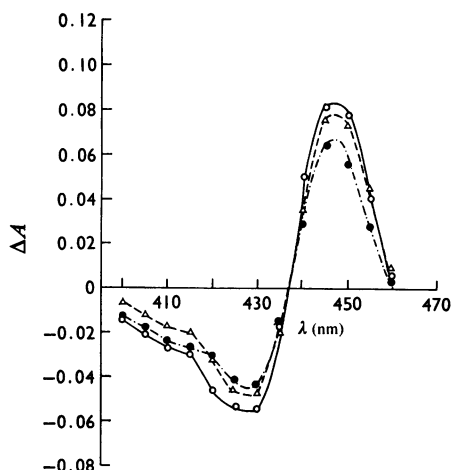


Fig. 3. Comparison of the kinetic difference spectra for the reduction of cytochrome oxidase and its cyanide-inhibited and mixed-valence CO derivatives

The kinetic difference spectrum of the fast phase seen on the reduction of cytochrome oxidase (○) is compared with those observed for the reduction of the cyanide-inhibited (●) and mixed-valence CO derivatives (Δ). The protein concentrations used were all $20\mu\text{M}$ before mixing. Reductions were carried out by mixing 12.5mM-Cr^{2+} with the proteins in a 2mm-path-length cell.

in the associated absorbance changes and slight shift in wavelength of maxima seen in Fig. 3 arises from the fact that reduction in each case leads to a different final product (Nicholls *et al.*, 1976; Greenwood *et al.*, 1974). These data, together with the concentration-independence of the slower phase and comparison with previously reported spectra (Gibson & Greenwood, 1964), lead to the conclusion that the slower phase observed in the reduction of the oxidized oxidase by Cr^{2+} is due to the reduction of haem a_3 after intramolecular electron transfer from some other reduced site in the enzyme. Although the rate of reduction of haem a_3 is slow, it is not dramatically different from the value of 0.6s^{-1} found by Gibson *et al.* (1965) for this process.

The similarity of the concentration-dependence of the rates depicted in Fig. 1 shows that the reaction of Cr^{2+} with haem a is not apparently affected by the redox state of haem a_3 . Thus during Cr^{2+} reduction, in contrast with the reduction by the physiological reductant ferrocyanide c (Greenwood *et al.*, 1974), no haem-haem interaction is observed. This may be due to the fact that Cr^{2+} requires only to enter the protein to obtain close proximity to the haem a site in order to reduce the iron, as has been proposed for cytochrome c reduction (Sutin &

Yandell, 1972). If this is the situation, then the rate limit approached at high reductant concentration (Fig. 1) might reflect some conformational change that is necessary to allow access for the Cr^{2+} into the protein. Alternatively it is possible that this rate limit may reflect the formation of a complex between the enzyme and the reductant. The temporal relationship between the reduction of haem a and that of the chromophore responsible for the 830nm band could arise from either simultaneous reduction or a very rapid exchange between the two sites, as suggested by Wilson *et al.* (1975). The exact correlation between the concentration-dependence of the reduction of these two sites, taken with previous results (Greenwood *et al.*, 1976; Wilson *et al.*, 1975), leads up to support the latter of these two propositions.

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