Kinetic Studies on the Binding of Cyanide to Oxygenated Cytochrome c Oxidase

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(Received 4 February 1976)

The reaction of cyanide with oxygenated cytochrome c oxidase was followed by means of flow-flash techniques. The oxygenated form, produced after photolysis of the partially reduced CO complex in the presence of cyanide and O₂, shows cyanide-binding properties distinct from those of both the oxidized and the reduced forms of the protein. The binding is a simple process (k = 22m⁻¹s⁻¹) linearly dependent on cyanide concentration to a high as 75 mm. It is suggested that the oxygenated form is a conformational variant of the oxidized protein.

Cytochrome c oxidase (EC 1.9.3.1) is capable of forming an oxygenated species that contains four oxidized sites (Tiesjema et al., 1972). The production of this species was first thought to be due to the formation of a peroxidic compound (Takemori et al., 1958); however, more recently the oxygenated species has been prepared under conditions that preclude the formation of peroxidic compounds (Lemberg & Stanbury, 1967). This has led a number of workers to think that the oxygenated species is a transitory conformational form of the oxidized protein (Tiesjema et al., 1972). A major problem in studying the reactions of the oxygenated material is that it decays spontaneously to the oxidized form (Lemberg & Mansley, 1966), and to avoid this we have taken advantage of the observation that the mixed-valence CO complex, when subjected to flow-flash in the presence of O₂, forms the oxygenated material (Greenwood et al., 1974). By using this technique it was possible to form the oxygenated species quantitatively in situ, in the presence of cyanide. The subsequent reactions of the oxygenated species with cyanide were followed and compared with the extensively studied reactions of the fully reduced and the fully oxidized forms of the protein with this ligand. These experiments were performed to investigate the hypothesis that the oxygenated species is a conformational variant of the oxidized form of the oxidase.

Materials and Methods

The cytochrome c oxidase used in these experiments was prepared by the method of Yonetani (1960), and the concentrations of oxidase samples were established by using the extinction coefficients given by Yonetani (1961). The mixed-valence species was prepared by incubation of the oxidized protein under 1 atm (0.1 MPa) of CO (Greenwood et al., 1974). CO and O₂-free N₂ used in these experiments were dispensed from cylinders and stored, before use, in glass vessels over an alkaline solution of dithionite-reduced anthraquinonesulphonate. All chemicals were of AnalaR grade and were obtained from BDH Chemicals (Poole, Dorset, U.K.), except for Tween 80, which was obtained from Sigma (London) Chemical Co. (Kingston-upon-Thames, Surrey, U.K.).

For static spectrophotometry, the oxygenated species was formed by exposing a sample of the mixed-valence material to O₂ and recording the spectrum (within about 1 min); this and other spectra were obtained by using a Cary 118C instrument. Flash-photolytic experiments were conducted by using the instrumentation and methods described by Greenwood & Gibson (1967), a Bausch and Lomb monochromator (500 mm grating; 1200 grooves/mm; f = 4.4) being used in conjunction with the flash-photolysis apparatus.

Results

Oxygenated cytochrome c oxidase, prepared after photolysis, as described, showed a monophasic binding phase at 428 nm over a wide range of cyanide concentration; the pseudo-first-order rate of this process was found to be linearly dependent on cyanide concentration up to 75 mm, and the second-order rate constant was 22m⁻¹s⁻¹ (Fig. 1). Another, much slower, process accounting for approx. 10% of the overall reaction was observed at all concentrations and occurred in the second (s) time-range. However, this slower phase was found to be independent of cyanide concentration in both rate and extent. The rate of this process (k = 0.1s⁻¹) was identical with that of the CO 'off' rate of the mixed-valence species under these conditions; under the experimental conditions employed some re-formation of the
cytochrome $a_2$–CO complex may occur after photolysis. This finding, together with the lack of concentration-dependence, leads us to suggest that this small amount of slow phase is due to cyanide binding to the small amount of re-formed material rate-limited by the CO 'off' rate.

A comparison of the spectrum of the product formed after the reaction between cyanide and the oxygenated species with that of the normal oxidized cyanide complex showed them to be identical.

The kinetic difference spectrum produced between 580 and 630 nm (Fig. 2) compares the overall kinetic difference spectrum with the difference spectrum obtained statically between the oxygenated cytochrome $c$ oxidase and the oxidized cyanide complex.

**Discussion**

Comparison of the static and kinetic difference spectra shown in Fig. 2 confirms that the first product of $O_2$ reaction with the mixed-valence species is the oxygenated protein (Greenwood et al., 1974), unlike the reaction with the fully reduced CO complex, which apparently yields the oxidized protein as the first product (Greenwood & Gibson, 1967). The comparison also indicates that the reaction being followed after the rapid $O_2$ reaction is in fact the binding of cyanide to the oxygenated species, which leads to the production of the fully oxidized cyanide complex.

To gain an insight into the nature of the oxygenated species a comparison of its cyanide-binding kinetics with those of the fully reduced and the fully oxidized protein is informative. Van Buuren et al. (1972) have made an extensive study of the cyanide-binding properties of the cytochrome oxidase molecule in both oxidation states, and find that the reduced protein binds cyanide in a simple process with a rate constant of $150 M^{-1} s^{-1}$ whereas the oxidized protein binds cyanide with a rate constant of $1.8 M^{-1} s^{-1}$, which is in fact limited at $0.018 s^{-1}$. Thus, even though the oxidized material has all four sites oxidized (Tiesjema et al., 1972) and is electronically at least fully oxidized, it can be kinetically distinguished from the fully oxidized protein by means of its reaction with cyanide. When oxidized oxidase reacts with cyanide in the presence of azide the rate constant is $25 M^{-1} s^{-1}$ (Van Buuren et al., 1972), i.e. very similar to that of the oxygenated species, although the process is still limited at $0.018 s^{-1}$. Van Buuren et al. (1972) have suggested that a conformational change accompanies azide binding that facilitates cyanide binding. It may thus be that the oxygenated protein represents a similar conformational variant of the oxidized protein. However, with the oxygenated species no rate limit is observed, as cyanide does not displace bound azide before binding occurs.

We thus suggest that the oxygenated protein is in fact a conformational variant of the oxidized protein in which the protein is in an open structural form, more closely related to the structure of the reduced protein, which may be stabilized by the presence of $O_2$ (Lemberg & Gilmour, 1967; Davison & Wainio, 1968).
T. B. thanks the Science Research Council for a Senior Research Associateship. C. G. thanks The Royal Society for a grant for the purchase of the oscilloscope type 7514 and Cary 118C spectrophotometer. We thank Mr. Adrian Thompson for his skilled technical assistance in the preparation of the enzyme used in these experiments. This work was supported by S.R.C. Grant B/GR/8048.9.

References