Oxygen Kinetics of Frozen Cytochrome Oxidase

THE CAPACITY OF THE OXYGEN POCKET

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Low-temperature kinetics of the reaction between O_2 and cytochrome oxidase suggest the existence of an O_2 pocket of limited capacity in membrane-bound cytochrome oxidase, and one of larger capacity in purified cytochrome oxidase. A model is proposed to explain the difference in capacity of the pockets.

Cytochrome *c* oxidase (EC 1.9.3.1) is the site of O_2 utilization in the inner mitochondrial membrane of all higher organisms. It contains two haems (a and a_3) and two copper atoms. Reduced haem a_3 is able to bind O_2 and catalyse its reduction to water.

Flash photolysis of CO-inhibited cytochrome oxidase in the presence of O_2 at low temperatures (–95 to –125°C) leads to the formation of oxy-cytochrome oxidase in the initial phase of the reaction (Chance *et al.*, 1975c). The equilibrium of O_2 and the enzyme can be demonstrated ($K_D = 320 \mu M$ at –94°C), and a linear increase in the pseudo-first-order rate constant for O_2 binding is observed with increasing O_2 concentration, indicating a bimolecular reaction at the active site of the enzyme, and suggesting the presence of an O_2 pocket for a number of ligand molecules, which retains fluidity well below the macroscopic freezing point of the sample. The O_2 concentration inside the pocket is proportional to that in the bulk of the solution (Chance, 1977).

The kinetics of the reaction of CO with cytochrome oxidase in the frozen state are pseudo-first-order (Sharrock & Yonetani, 1976, 1977). The C–O stretch band at 1963.5 cm^{-1} for carbonmonoxy-cytochrome oxidase has a very narrow bandwidth (Yoshikawa *et al.*, 1977). Both of these observations suggest the existence of a pocket for CO, and that the bound CO is isolated from the polar solvent and is in a hydrophobic environment.

The present study further examines the effect of O_2 on the kinetics of formation of oxycytochrome oxidase in order to estimate the capacity of this pocket in membrane-bound and isolated cytochrome oxidase.

Experimental

Bovine heart mitochondria (Low & Vallin, 1963) were suspended in a medium containing 0.1 M-

mannitol, 0.03 M-sucrose, 15 mM-sodium phosphate buffer (pH 7.4) and 10 mM-succinate, and left for 10 min at 25°C to achieve anaerobiosis. CO (100%) at 0.1 MPa (1 atm) was then bubbled in for 10 min, ethylene glycol was added (final concn. 30%, v/v) and the preparation resaturated with 100% CO. The final concentrations were CO 1.2 mM and bovine heart mitochondria 27 mg/ml (containing 9 μM -cytochrome oxidase). This preparation was injected into a cuvette with a 2 mm optical path, until it was partly filled, and left for 10 min at 25°C to ensure anaerobiosis.

Phospholipid-sufficient purified cytochrome oxidase (Yonetani, 1967) was dissolved in a medium containing 1% cholate, 50 mM-sodium phosphate buffer (pH 7.4) and 30% (v/v) ethylene glycol, giving a clear solution. The sample was placed in a cuvette and phenazine methosulphate, 2,3,5,6-tetramethyl-*p*-phenylenediamine and NADH were added to give concentrations of 10 μM , 10 μM and 2 mM respectively. CO (100%) was then bubbled in for 10 min, and the sample left for 1 h at 20°C to ensure reduction. The final concentration of cytochrome oxidase was 9 μM .

The phospholipid-depleted purified cytochrome oxidase (Yu *et al.*, 1975) (kindly supplied by Dr. T. E. King, Laboratory of Bioenergetics, State University of New York, Albany, NY, U.S.A. was treated exactly the same as the phospholipid-sufficient oxidase (above), except that no cholate was used, resulting in a suspension rather than a clear solution.

In each case, the cuvette containing the CO-bound oxidase was cooled to –20°C, and $K_3Fe(CN)_6$ was added with minimal stirring, to give a concentration of 2 mM, when all components except cytochrome a_3 ·CO were oxidized. At 25 s after the ferricyanide treatment, a precooled (–20°C) O_2 -saturated buffer solution (2 mM- O_2) was added to fill the cuvette, the two layers were mixed carefully, and the sample was frozen rapidly

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at -78°C . The O_2 concentration was varied between 0.25 and 1.5 mM by varying the ratio of oxygenated buffer to CO-saturated preparation. Lower O_2 concentrations were obtained by stirring the sample (Chance *et al.*, 1975c) rather than by using the oxygenated buffer solution. Introduction of O_2 at -20°C causes it to be trapped in the active-site pocket of the enzyme in the close proximity of the carboxyhaem (cytochrome $a_3\cdot\text{CO}$). The sample was then transferred to the Dewar flask of the spectrophotometer, which was maintained at the desired low temperature by a flow of temperature-controlled N_2 .

The reaction with O_2 was initiated at low temperatures by flash photolysis by using a liquid-dye laser (Rhodamine 6G; 100 mJ; 585 nm), which caused about 90% photolysis of cytochrome $a_3\cdot\text{CO}$ (Chance *et al.*, 1975b). Free cytochrome a_3 reacts with O_2 as described by Greenwood (1964). The resulting kinetics were followed by multi-channel dual-wavelength spectroscopy (Chance *et al.*, 1975a,b) at two wavelength pairs (591–630 nm and 445–460 nm). The initial phase of the reaction was analysed by the Guggenheim method (Gutfreund, 1975). Linear plots extending to three half-lives were obtained for the higher O_2 concentrations (0.5–1.5 mM), whereas at lower concentrations linearity was maintained at two half-lives. Both wavelength pairs showed similar kinetics. The absorbance change at 591–630 nm was, in general, more convenient for the analysis of the initial phase, since there is only a small contribution from the second phase of the reaction [giving compound C (Chance *et al.*, 1975c)].

Results and Discussion

Fig. 1 shows the pseudo-first-order rate constant (k) for the formation of oxy-cytochrome oxidase (Compound A_2 in Chance *et al.*, 1975c) in ferricyanide-pretreated bovine heart mitochondria, at various O_2 concentrations at four temperatures in the range -98 to -115°C . At O_2 concentrations below about 0.3 mM, k varies linearly with O_2 concentration. At higher concentrations k reaches a constant value, which is O_2 -independent, suggesting a limit to the O_2 capacity of the pocket containing the active site. Arrhenius plots (Fig. 2) for the formation of Compound A_2 , constructed from the data in Fig. 1 at low (0.25 mM) and high (1 mM) O_2 concentrations, yield similar values for the activation energy ($E_a = 41.6 \text{ kJ/mol}$ at 1 mM- O_2 ; $E_a = 45.8 \text{ kJ/mol}$ at 0.25 mM- O_2). The O_2 kinetics of membrane-bound and purified oxidase are compared in Fig. 3. Neither phospholipid-sufficient not delipidated purified oxidase shows an O_2 -limited region, and both show lower rates of O_2 binding compared with membrane-bound oxidase, at the lower O_2 concentrations (cf. Chance *et al.*,

Fig. 1. Effect of O_2 on the pseudo-first-order rate constant (k) for the formation of oxy-cytochrome oxidase in ferricyanide-pretreated bovine heart mitochondria. Kinetic data are obtained at two wavelength pairs: 591–630 nm (Δ , $\triangle$) and 445–460 nm ($\bullet$, $\circ$). The broken line shows the linear relation $k = 0.15 [\text{O}_2]$. Experimental conditions are described in the text.

Fig. 2. Activation energy for formation of oxy-cytochrome oxidase in ferricyanide-pretreated bovine heart mitochondria.

The rate constants are obtained from Fig. 1 at 0.25 mM- O_2 ($\triangle$) and 1 mM- O_2 ($\circ$). The error bars indicate the precision of the temperature measurement ($\pm 1^{\circ}\text{C}$).

Fig. 3. Comparison of the O_2 kinetics of bovine heart mitochondria (---) with phospholipid-sufficient (—), $\circ$ and phospholipid-depleted ($\square$, ---) purified cytochrome oxidase

The curves for mitochondria are computed from the data in Fig. 2. Experimental conditions are described in the text.

1978). The second-order rate constants are, for bovine heart mitochondria, $150 \text{ M}^{-1} \cdot \text{s}^{-1}$ (-103°C), for phospholipid-sufficient purified oxidase, $115 \text{ M}^{-1} \cdot \text{s}^{-1}$ (-101°C) and for delipidated purified oxidase, $140 \text{ M}^{-1} \cdot \text{s}^{-1}$ (-102°C). The data for delipidated oxidase are of poor precision, probably owing to the heterogeneity of the sample. Hence it is noted that the kinetics for delipidated oxidase are very similar to, and certainly not slower than, those for phospholipid-sufficient purified oxidase, which is consistent with previous observations (Yu *et al.*, 1975) on the reaction of cytochrome a_3^{2+} with O_2 at about 25°C .

We attempt to rationalize the data by using a model (Fig. 4) that is able to explain the O_2 -limited region in the membrane-bound oxidase and the lower O_2 -binding rate (at low O_2 concentrations) in the absence of the membrane.

We propose that the haem environment in the membrane-bound state contains two pockets (P_1 and P_2), which are in equilibrium with each other at the temperature of O_2 addition and trapping (-20°C), but are isolated from each other at the flash-photolysis temperatures (-98 to -115°C). The O_2 concentration in the outer pocket (P_2) is proportional to that in the bulk of the solution, whereas the inner pocket (P_1), which contains all the O_2 molecules capable of binding haem a_3 , saturates when the bulk O_2 concentration is about 0.4 mM . We propose that

Fig. 4. Model for the environment of the active site of membrane-bound cytochrome oxidase

The broken line suggests the change caused by purification of the oxidase. Abbreviations: Fe, haem a_3 ; P_1 and P_2 , pockets; B_1 and B_2 , activation barriers; E_1 and E_2 , activation energies.

purification removes the inter-pocket activation barrier (B_2), resulting in a pocket with a larger O_2 capacity, and in a higher activation energy for O_2 binding.

Similar models, with several regions among which the ligand molecules can move, have been used to rationalize data on CO recombination with cytochrome oxidase (Sharrock & Yonetani, 1976, 1977) and myoglobin (Austin *et al.*, 1975). Striking differences can be observed between the reactions of O_2 and CO. The rate of CO recombination with cytochrome oxidase is independent of CO concentration at the low temperatures used in the present study, and have been rationalized on the basis of an inner pocket with a capacity for only one CO molecule (Sharrock & Yonetani, 1976, 1977). Such a pocket is unable to explain the dependence on O_2 concentration observed in the present work at low concentrations.

It is difficult to give a physical interpretation of the various features shown in Fig. 4 on the basis of current knowledge on the structural features of cytochrome oxidase. It is likely that the pockets are lipid in character, and, as such, their O_2 concentrations are much higher than that in the bulk of the solution, since O_2 is about 5 times more soluble in lipid than in water (*International Critical Tables of Numerical Data for Physics, Chemistry and Technology*, 1928).

In conclusion, the decrease in activity that accompanies the purification of cytochrome oxidase may be partly due to changes in the nature of the O_2 pocket caused by removal of the membrane phospholipid.

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