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I. THE POLYPHASIC REDUCTION OF OXYGEN TO WATER BY PURIFIED CYTOCHROME c OXIDASE

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The time course of oxygen consumption by purified cytochrome oxidase has been studied in reactions where the fully reduced enzyme was rapidly mixed with molecular oxygen. Similar to intact mitochondria (Reynafarje & Davies, Am. J. Physiol. 258, 1990), the enzyme reduces oxygen to water in a kinetically and well defined polyphasic event. The initial rates of O_2 consumption depended hyperbolically on O_2 concentration, with a bimolecular rate constant of near 10^7 M-1 s-1. The V_{max} of O_2 uptake was,however, a complex function of the concentrations of both enzyme and cytochrome c. It is concluded that the reduction of oxygen to water takes place in a cyclic process in which the oxidase undergoes redox changes at rates depending on the relative concentration of the enzyme and its 3 substrates: O_2, electrons and protons. No evidence was found for impairments in the intramolecular flow of electrons per se.

INTRODUCTION. Cytochrome c oxidase (ferrocytochrome c: oxygen oxido reductase, E.C. 1.9.3.1) is the terminal link in the chain of respiratory enzymes responsible for the transport of electrons from NADH to oxygen. In mitochondria, the free energy released in the transport of electrons is coupled with the translocation of protons across the inner membrane and the consequent generation of a proton electrochemical potential. Mitchell's chemiosmotic hypothesis suggests that 2 protons are translocated from the matrix to the cytosolic side of the membrane as a pair of electrons traverse (in the opposite direction) each of the loops of the respiratory chain. The hypothesis now suggests that a total of 8 protons (2 per loop, including the "O" loops of cytochrome oxidase) are ejected per pair of electrons transported from NADH to 1/2 O_2 (1). A H+/O stoichiometry of 8 is, however, still debated (2-6). Discrepancies may arise from mere difficulties in assessing the extent of oxygen directly involved in the translocation of protons. In this regard we have shown that respiration, in intact mitochondria, is a cyclic and kinetically polyphasic process (7), and that the pumping

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of protons and the synthesis of ATP (unpublished observations) take place mainly during the first phase of this process. Since in intact mitochondria respiration is extremely complex, it was necessary to know whether the consumption of O_2 by an uncomplicated system, such as that catalyzed by purified cytochrome c oxidase, also takes place in a polyphasic manner.

MATERIALS AND METHODS

Purified bovine- and pig-heart cytochrome c oxidases were gifts of Drs. David Bickar and Bernard Kadenback. The reaction medium consisted of either 250 mM sucrose, 50 mM KCl and 3 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), or 125 mM KCl and 3 mM HEPES. The pH of the medium was 7.0 and the temperature 24°C. The contents of the medium, including the enzyme and its substrates, were stirred by a magnetic bar rotating at a speed of 2,000 rpm in the bottom of an airtight-closed cell of 1.65 ml of volume. A dynamic calibration of the oxygen electrode was performed using glucose oxidase as previously described (7). The concentration of the dithionite-reduced enzyme was determined at the wave length pair of 605-630 nm, using an extinction coefficient of 17.6 mM-1 cm-1 (8).

RESULTS AND DISCUSSION

I. The polyphasic nature of oxygen consumption by purified cytochrome oxidase. Figures 1 & 2 show the time course of O_2 consumption in experiments performed under a variety of conditions. The following four kinetic phases can be distinguished in all the experiments, regardless of type (bovine or porcine) and concentration of the enzyme and its substrates.

1. The first phase is characterized by very fast rates of O_2 uptake. Figure 1 shows that from 12 to 98% of the oxygen added (see cross bar in Y axes) is rapidly consumed in less than 150 ms (the dead time of the apparatus). Traces A, C & D show only the last portion of the first phase, spanning from 150 to 600 ms. Figures 1 D and 2 show that over 90% of the O_2 added is consumed exclusively during the first phase, in a single and apparently monotonic reaction (see inset in Fig. 2).

2. The second phase is characterized by different degrees of impairment in the initial rates of oxygen consumption. In Figs. 1 A and 2 this phase is practically imperceptible. In traces B, C & D of Fig. 1, however, the second phase is marked by an abrupt transition in the initial rates of O_2 uptake. This phenomenon can not be attributed to a sudden impairment in the intramolecular flow of electrons (9), because the enzyme can turn over hundreds of times before the first phase ends (Figs. 1 B & 2). Since the second

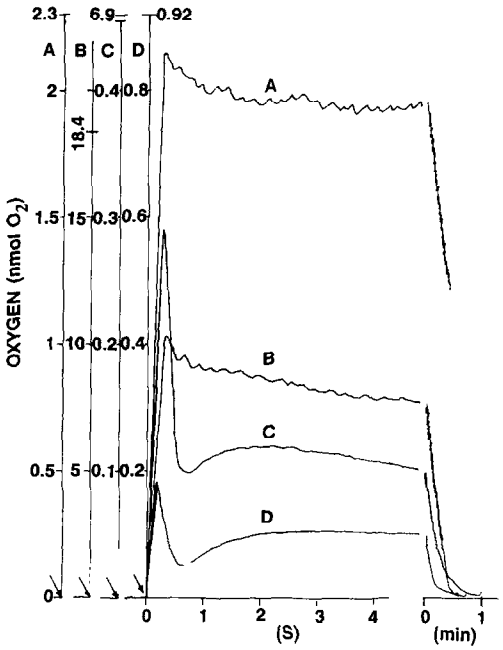

Figure 1. Different phases in the time course of O_2 consumption. The basic medium consisted of 125 mM KCl, 3 mM HEPES and 10 mm Na L(+) ascorbate, pH 7.0 and 25° C. The amount of reactants added to 1.65 ml of medium in traces A, B, C and D were, respectively: 2.3, 18.4, 6.9 and 0.92 nmols O_2 (note cross bars in Y axes); 0.179, 0.164, 2.5, and 2.61 nmol aa_3, and 2, 60, 60 and 2 nmols cytochrome c. Reactions were initiated (see the arrow) by injecting O_2 into the anaerobic reaction medium.

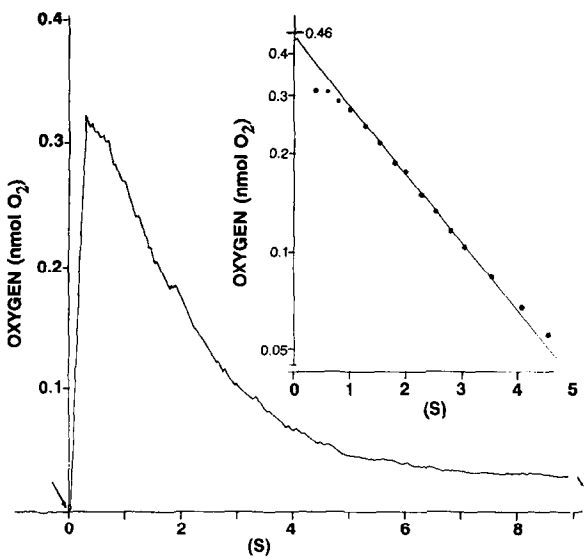

Figure 2. Typical reaction of first order during the initial phase of O_2 uptake. Experimental conditions as in Fig. 1, except that the amount of reactants added were: 0.46 nmol O_2, 0.05 nmol aa_3 and 60 nmol cytochrome c. The first order rate constant ($k = 0.48\ s^{-1}$) was calculated from the plot shown in the inset.

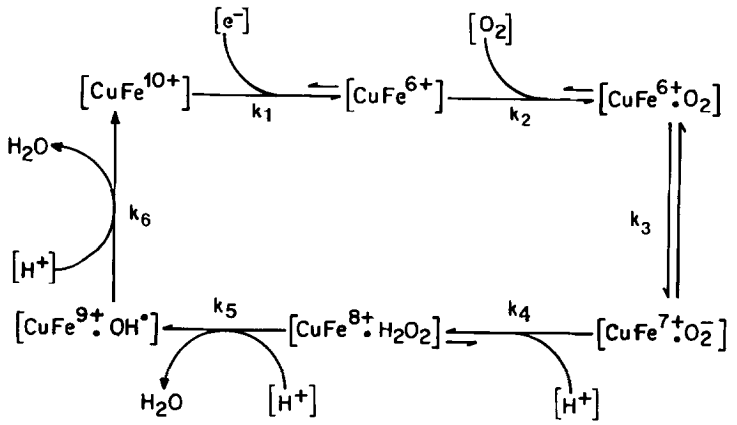

Figure 3. Simplified model of electron flow through cytochrome c oxidase.

phase is most clearly observed in the presence of relatively high concentration of enzyme, O_2, and cytochrome c (Fig. 1, C & D) it is postulated that the impairment is due to limitations in the availability and/or diffusion of protons (steps 2 to 4 of Fig. 3).

3. The third phase is characterized by a spurious steady-state in the rates of the O_2 uptake. Depending on conditions, the concentration of O_2 in the medium can decrease, remain relatively constant and in some instances even increase. In Fig. 1 A & B, for example, O_2 decreases linearly in an apparent reaction of zero order, whereas in Fig. 1, C & D, O_2 is, for more than 1 second, released to the medium. This release is probably attributable to reversal of electron flow, since the enzyme becomes more reduced (7) and protons are simultaneously released to the medium (not shown). Oxygen, however, can not proceed from water (Ref. 10) since there is not enough energy for this reaction to take place. Since the phenomenon is only observed in the presence of rather high concentrations of both oxygen and enzyme, it is assumed that a peroxy intermediate is most likely the source of both oxygen and protons.

4. During the fourth phase the rates of O_2 uptake decrease until O_2 is totally exhausted. This phase (of apparent first order) and the linear portion in the third phase (of apparent zero order) have been used in the expression of the Michaelis-Menten equation, the rate equation for a one-substrate enzyme-catalyzed reaction. During the third and fourth phases, however, there is a change in concentration of at least two substrates. Thus, while O_2 decreases, both cytochrome c and enzyme (7) become more reduced. Consequently, methods have been developed to calculate the Km of the oxidase for

Table I. Effect of the concentration of enzyme, oxygen and cytochrome c on the kinetics of oxygen uptake

Cyt. c (μM)	Oxygen (μM)	Enzyme (μM aa_3)	k (s^{-1})	$Kx10^{-7}$ (M^{-1} s^{-1})
12	0.084	0.22	1.86	0.22
12	0.112	0.22	1.98	0.22
12	0.139	0.22	1.89	0.22
12	0.279	0.22	1.95	0.22
12	0.055	0.22	1.88	0.22
24	0.055	0.18	3.73	0.52
36	0.055	0.18	3.94	0.55
60	0.055	0.14	3.24	0.58
60	0.055	1.04	14.95	0.36

Experimental conditions as described in Fig. 1.

O_2 using neither the last phase of the reaction nor the oxygen-pulse technique (11,12). That the phases here described are not due to defects of the system or artifacts of the electrode, are indicated by the following observations. a) The O_2 consumption by either glucose oxidase (7), or dithionite (not shown) takes place in a single phase, following the kinetics of a reaction of first order. b) Conventional electrodes of the Clark type can also detect these phases, but because of response lags of 1 to 2 seconds, only the last two phases can be recorded, including the portion where a small, but measurable, amount of O_2 is released into the medium.

II. The extent and rates of oxygen consumption during the first phase. For the first time, extremely high initial rates of O_2 uptake were directly measured and characterized with a fast responding electrode. As expected, this phase coincides with a period of net oxidation of both enzyme and cytochrome c (7,13,14). The first phase, however, should not be considered a pre-steady state because, under certain conditions, it can account for the entire reaction (Fig. 2).

Table I shows the effect of the concentration of enzyme, O_2 and cytochrome c on the kinetics of O_2 consumption. At a constant concentration of both enzyme (0.22 μM) and cytochrome c (12 μM), the rates of O_2 consumption depend on O_2 concentration ($v = K[O_2]$). Table 1 also shows that, at constant concentrations of O_2 (55 nM) and enzyme (~17 μM), the rates are proportional to cytochrome c concentration. The bimolecular rate constant (K), however, decreases significantly when the concentration of enzyme increases from 0.14 to 1.04 μM, a phenomenon that is discussed elsewhere.

III. The physiological relevance of the polyphasic nature of oxygen consumption. The basic role of the oxidase in respiration is to transport electrons from cytochrome c to O_2. In order to better

accomplish this transport, the enzyme must change from a maximal state of reduction (filled with electrons) to a maximal state of oxidation (empty of electrons). Optimally, the enzyme binds ferrocytochrome when fully oxidized and binds O_2 when fully reduced. Therefore, the rates of oxygen reduction to water (respiration) depend not only on the concentration of O_2 and cytochrome c but also on the redox state and concentration of cytochrome oxidase. Thus, the enzyme itself may have an elementary mechanism to control respiration based on the relative concentration of four elements: enzyme, O_2, cytochrome c and protons. The results of this study provide evidence that the first phase can not proceed without control for unlimited periods of time, because higher concentrations of enzyme and oxygen result in faster initial rates of O_2 uptake and stronger impairment in the complete reduction of O_2 to water. The impairment is, in all probability, due to the accumulation of partially reduced forms of oxygen (steps 2 to 5 in Fig. 3). These enzyme-substrate complexes will not be totally reduced to water unless protons are available at the active site of the enzyme. Conformational changes induced by ATP, for example (15), could facilitate the diffusion of protons thus releasing the respiratory impairment and allowing the reaction to take place as long as ATP is synthesized.

Fig. 3 shows a simplified model of electron flow through the oxidase, in which the rates of O_2 uptake depend on the effective collision of the enzyme with its substrates, including protons. In fact, experimental evidence (not shown) indicates that the k_{cat}/K_m ratio of the enzyme is at least 10^8 M^{-1} s^{-1}, i.e. the enzyme has attained kinetic perfection, with rates only limited by the diffusion-controlled encounter of the enzyme and its substrates.

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