Formation and Decay of the Primary Oxygen Compound of Cytochrome Oxidase at Room Temperature As Observed by Stopped Flow, Laser Flash Photolysis and Rapid Scanning*

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Rapid kinetic and scanning techniques were employed to observe the reaction of dioxygen with fully reduced cytochrome oxidase at room temperature following stopped flow flash photolysis of the CO compound of the enzyme. The initial product of the reaction was an oxygen compound with a spectral profile quite similar to that of the cytochrome oxidase-CO compound. The second order rate constant for formation of the primary oxygen compound was $1.1 \times 10^8 \text{M}^{-1}\text{s}^{-1}$ at pH 7.4 and 25 °C. With an off rate constant of $1.9 \times 10^5 \text{s}^{-1}$, the calculated dissociation constant was $1.8 \times 10^{-8}$ M. The apparent activation energy was 16.7 kJ/mol. The primary oxygen compound underwent further reaction which was independent of oxygen concentration with a rate constant of $1.2 \times 10^{11} \text{s}^{-1}$, and the product was Intermediate III (Orii, Y. (1982) in Oxygenases and Oxygen Metabolism (Nozaki, M., Yamamoto, S., Ishimura, Y., Coon, M. J., Ernst, L., and Estabrook, R., eds) pp. 137–149, Academic Press, New York), an oxidized species presumably identical to the "oxygen pulsed" oxidase. Thus, this reaction was ascribed to intramolecular electron transfer to the oxygen in the compound. The energy of activation was 49.8 kJ/mol below 18°C but above that the rate constant was independent of temperature, and a "tunneling" mechanism was suggested for the intramolecular electron transfer.

"Oxygenated" cytochrome oxidase, which was originally proposed by Okunuki and his co-workers (1–3) as a primary reaction product of fully reduced cytochrome oxidase with oxygen, was later shown to consist of several components (4, 5). Attempts to identify the initial oxygenated intermediate have required either rapid kinetic or low temperature techniques because of its presumed short lifetime at room temperature.

At low temperatures around −100 °C, Chance and his co-workers (6, 7) were able to detect a primary intermediate (Compound A) which, in the visible region, showed a spectral profile similar to that of the CO compound. At room temperatures, limitations in time resolution have hampered unequivocal identification of the primary reaction product even after introduction of the "flow-flash" technique (8–10).

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Materials and Methods

Cytochrome oxidase was purified from bovine heart muscle according to the procedure described previously (13), dissolved in 0.05 M sodium phosphate buffer (pH 7.4), 0.05% Emasol 1130, and stored in liquid nitrogen until used. The molecular activity was the same as reported (14). The concentration (in terms of heme a) was determined spectrophotometrically (13). In flow-flash experiments, cytochrome oxidase in one reservoir of the apparatus was reduced with a small amount of sodium dithionite (about 0.5 mg/ml). A gas mixture of CO:N₂ (1:4, v/v) prepared by a gas mixer (Standard Technology Inc.) model SGD-0.3L was bubbled through the enzyme solution for 1 min at a flow rate of 30 ml/min, and then allowed to flow over the surface of the solution with stirring for 20 min. In the other reservoir, 0.1 M sodium phosphate buffer (pH 7.4) was bubbled with argon gas containing different concentrations of oxygen for at least 15 min. This mixture was prepared by a Toray Oxygen Pump (Toray Co., Ltd., model SEP-104) which utilized a solid state zirconia electrode to regulate the oxygen concentration. Experimental temperature was 25 °C unless otherwise stated.

A brief description of the current flow-flash apparatus is as follows. The reservoirs for reactants were glass cylinders of 5-ml capacity equipped with the inlet and outlet for gases at the top. The solutions were stirred with magnetic stirrers installed in the bottom. The reactant solutions were transferred to horizontal drive syringes by pulling a rod block to which were attached the plungers, and then forced to pass through a jet mixer into an observation tube by a stroke of a pneumatic piston (4.0 kg/cm²). Both reservoirs and drive syringes were mounted in a thermostated aluminum block. The volume of reactant required for one shot was 260 μl for each reservoir. The observation cell was fabricated from a block of glass ceramics. A hole 2 mm in diameter was drilled and the two ends were sealed with quartz windows (10 mm apart). The top half of the block was removed by cutting horizontally through the middle of the hole leaving a rectangular opening (2 × 8.5 mm), which was covered with a quartz window to receive the photolyzing beam.

Photolysis was achieved by pulsing rhodamine 590 in a Phase-R flash lamp (DL-10). The dye pumped by a coaxial xenon flash (50 J) gave a laser pulse with rated duration of 300 ns. The laser beam was reflected 90° with a prism, and converged by a cylindrical lens but defocused to give a lanceolate spot on the top window of the observation cell. A cutoff filter (Toshiba, 0-56) was placed over the top window and a color filter (Toshiba, V-40) in front of the photomultiplier to minimize overloading of the photomultiplier with scattered excitation light.
Spectrophotometric changes were followed either as monochromatically optical transmittance or as time-resolved spectra. In the former case, monochromatic light passed through the observation tube to the detector, a photomultiplier (Hamamatsu, R647-04) with an integrator (response time = 200 ns). The light source was a 100-Watt tungsten iodine lamp. The output of the amplifier was fed to a transient recorder with 128 channels (Kawasaki Electronics Co. Ltd., model MR-903E), and displayed on an oscilloscope (Kikusui Electronics Corp., model C55041). In the latter case, a xenon flash lamp (1.6 J, pulse duration = 3 μs) was fired at a preset time delay after the main laser flash to record a transient spectrum on a 256-channel diode array. This was a component of a Union Giken stopped flow rapid scan spectrophotometer.

A Sharp MZ20000 desk-top computer with appropriate interfaces and programs written in BASIC served as both controller and data analyzer. As the controller, it activated the pneumatic piston to drive the plungers, sensed the stroke end, and created a time delay of 5 ms to fire the laser flash and trigger the transient recorder. When spectra were being recorded, it also fired the smaller xenon flash lamp at 5-μs intervals after the main laser flash. In this case, the diode array was triggered as the plungers hit the stop.

Data were stored on floppy discs, and retrieved for data analysis on the computer or recording on an X-Y plotter. In analyzing the time course data, the transmittance change was assumed to be proportional to the absorbance change; this relation held as long as the concentration of cytochrome oxidase after mixing did not exceed 0.85 μM, the absorbance change; this relation held as long as the concentration of cytochrome oxidase after mixing did not exceed 0.85 μM, the absorbance change. These parameters were determined by a "peeling" procedure and validity of the values calculated in each case was confirmed by comparing the theoretical curve with the experimental trace. The fit between them was always excellent (see Fig. 1A).

Results and Discussion

Fig. 1A illustrates the biphasic transmittance change at 445 nm following the reaction of fully reduced cytochrome oxidase with dioxygen at pH 7.4 and 25°C, which was completed in a few milliseconds. The lifetimes (τ = 1/k) of each step were 65 and 900 μs, respectively, when the reaction mixture contained 140 μM oxygen, 100 μM carbon monoxide, and 0.85 μM cytochrome oxidase. The biphasic profile, however, was affected by the oxygen concentration; below 30 μM oxygen, the separation of the two phases became less distinct, and above 300 μM, a plateau appeared in between. The contribution of each step to the total change was dependent on the observation wavelength; for example, at 415 nm, the slow change dominated whereas only the rapid change was observed at 430 nm (Fig. 1C). This latter wavelength, therefore, may be an isosbestic point between the spectrum of the intermediate undergoing the rapid formation and the spectrum of the product after the slow change. These results suggest that the fast and slow reactions are of quite different nature.

The spectral changes during the reaction were recorded by rapid scanning spectrophotometry. As a control, Fig. 2A shows a series of spectra recorded during simple recombination of CO to cytochrome oxidase in the absence of oxygen. The top spectrum obtained 5 μs after photolysis of the CO compound of cytochrome oxidase indicates that the CO was released almost completely from the fully reduced enzyme. Even after 1 ms, the formation of the CO compound was negligible, and it took about 30 ms for completion (τ = 12.7 ms determined in separate runs). The spectral changes during the reaction of the fully reduced oxidase with dioxygen proceeded much more rapidly (Fig. 2B). The 5-μs spectrum already lost some intensity at the peak and showed an increased absorbance around 400-410 nm. At 100 μs, a broad peak, possibly a composite of at least three components, appeared, and at 200 μs, a peak was most prominent at 431 nm. At 1 ms, the peak shifted to 425 nm in agreement with previous observations (4, 5) and the 5-ms spectrum represents the final product in a time range of tens of milliseconds. This corresponds to a conformer variant of oxidized cytochrome oxidase, which was designated as Compound III previously (4) and later renamed Intermediate III (15).

![Fig. 1. Reaction of cytochrome oxidase with dioxygen. A, time course for reaction of fully reduced cytochrome oxidase with dioxygen followed at 445 nm. The concentrations of cytochrome oxidase and oxygen after mixing were 0.85 and 122.5 μM, respectively. The dots are digitalized data which were collected every 1 μs over the period of 1 ms but only every other point, 500 points total, were plotted. The solid line is the theoretical curve derived from Equation 1 with T1 = 3.53%, T2 = 2.1% in transmittance, b1 = 1.53 × 10^4 s^-1, and k = 1.11 × 10^4 s^-1. B, simulated time courses for fractional changes of reduced cytochrome oxidase (1), its oxygen compound (2), and Intermediate III (3). Calculations were based on the reaction model expressed by Equation 2 and parameters cited therein. C, time courses for reaction of reduced cytochrome oxidase with oxygen followed at 445 (1), 415 (2), and 430 nm (3). The concentrations of the enzyme and oxygen after mixing were 0.85 and 143 μM, respectively.](image_url)
Primary Oxygen Compound of Cytochrome Oxidase

The spectral species involved were further characterized by time difference spectra (Fig. 2A2, k through n). Spectrum k had a deep trough at 445 nm compared with a rather low peak at 425 nm and resembled the spectra presented (but in the reversed mode) by Gibson and his co-workers for the rapid phase of Paracoccus (11) and mammalian cytochrome oxidase (12). If the primary reaction intermediate decayed rapidly, its spectral characteristics would be elicited more precisely by taking the spectrum in earlier stages. Accordingly, a time difference between 50 and 5 μs was recorded as shown in Fig. 2B2. The peak and trough were at 427 and 445 nm, respectively. This was different from the computed difference spectrum (reduced—oxidized) of heme a3 (16), but was similar to the time difference spectrum for the rebinding of photodissociated CO (Fig. 2B1) with the peak and trough at 428 and 445 nm, respectively. In both cases, the trough and peak had nearly equal intensities. Thus, it can be concluded that the primary reaction intermediate formed between the fully re-
duced cytochrome oxidase and dioxigen in the fast reaction at room temperature is an oxygen compound having the same electronic properties as the CO compound. Based on the observation of spectral changes in the visible region but made around −100 °C, Chance et al. (6) have derived the same conclusion as to the nature of Compound A.

Since the fast phase corresponds to the formation of the oxygen compound, the succeeding process resulting in the formation of Intermediate III would be the intramolecular electron transfer from the redox centers in the compound to the bound oxygen. This process brought about the appearance of the 415-nm peak in the time difference spectrum m and n.

In the oxygen concentration range in which the transmittance at 445 nm changed biphasically, the pseudo-first order rate constants for the two phases were determined, and plotted against the oxygen concentration (Fig. 3). The plot for the fast phase was linear with an intercept, a good indication of formation of a reversible oxygen compound. The slope gave a second order rate constant of $1.05 \times 10^6$ M$^{-1}$ s$^{-1}$ and the intercept or the off-rate constant was $1.9 \times 10^3$ s$^{-1}$. Accordinglly, the dissociation constant for the oxygen compound was calculated to be $1.8 \times 10^{-3}$ M, which is 1 order of magnitude smaller than that reported for Compound A, $4.8 \times 10^{-4}$ M at $-91$ °C (6). The pseudo-first order rate constant of the slow reaction was independent of the oxygen concentration and was $1.2 \times 10^3$ s$^{-1}$. The simplest reaction scheme to explain the present kinetics is

$$k_1 = 1.05 \times 10^6$ M$^{-1}$ s$^{-1}$
$$k_2 = 1.9 \times 10^3$ s$^{-1}$
$$k_4 = 2.5 \times 10^3$ s$^{-1}$

Based on this scheme and using the above parameters as well as the oxygen concentration of 140 μM, the fractional changes of the reduced form, the oxygen compound and Intermediate III were calculated as illustrated in Fig. 1B. Furthermore, in order to simulate the transmittance change as shown in Fig. 1A, the extents of contribution of the initial rapid change and of the slow change to the total at 445 nm were determined by numerical calculation to be 0.75 and 0.25, respectively. The simulated transmittance (not shown) showed a good fit of the experimental data, thus supporting the adequacy of the parameters determined. It is to be noted in Fig. 1B that 1 ms after the initiation of the reaction there still remained 30% of the oxygen compound in addition to Intermediate III (65%) and the reduced form (5%). These calculated time courses enabled the assignment of a small shoulder around 430 nm in spectrum i (1 ms) and the peak at 431 nm in spectrum h (200 μs) as due to the oxygen compound (Fig. 2A).

Fig. 4 shows the temperature dependence of the rate constants for the fast and slow changes. An apparent activation energy for the oxygen binding was 16.7 kJ/mol, in agreement with that reported for the reaction of cytochrome oxidase in intact pigeon heart mitochondria with oxygen (17). An Arrhenius plot for the slow change had a break around 18 °C. Below this temperature, the activation energy was 49.8 kJ/mol whereas above that the rate constant remained essentially unchanged. A similar temperature independent process has been observed for the intramolecular electron transfer in cytochrome oxidase initiated by aerobic addition of sodium dithionite to the oxidized enzyme (18). Therefore, it is concluded that irrespective of the means to initiate the reaction, there is a temperature range in which the activation energy for intramolecular electron transfer becomes negligibly small, and this result could be explained by an electron tunneling mechanism (19-22). This relationship between the Arrhenius break and the proposed electron tunneling mechanism is supported by the following considerations. The transition temperature around 20 °C has been observed in some kinetic studies (13, 18, 23) and correlated with the structural transition of cytochrome oxidase between "hot" and "cold" conformers (13), which may accompany rearrangement of the constituent subunits. The intrinsic conformational change has been confirmed in nanosecond fluorescence depolarization studies as well (24). On the other hand, the tunneling would require proper orientation of the redox centers involved and alignment of amino acid residues of the polypeptide skeleton to provide the pathway for electron transfer. A recent model for the structure of the oxygen-binding site in subunit I of cytochrome oxidase (25) is consistent with this idea. The model assumes that clusters of aromatic residues are arranged to line the binding site and in one of the transmembrane segments enabling a π-type interaction for electron transfer. Therefore, oxygenation of the hot conformer might induce alignment of the aromatic residues to "pave" the pathway for the intramolecular electron transfer. This idea also explains the more efficient electron transfer in Intermediate III or the "oxygen pulsed" (26) oxidase compared with the "resting" oxidase (12). Verification of the present proposal needs more extensive studies.

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