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Facilitated intramolecular electron transfer in cytochrome *bo*-type ubiquinol oxidase initiated upon reaction of the fully reduced enzyme with dioxygen**

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Abstract Flow-flash and double-flash studies of the reaction of fully reduced *bo*-type quinol oxidase with oxygen have revealed that a single turnover of the enzyme proceeds much faster than mammalian cytochrome *c* oxidase. Facilitated intramolecular electron transfer in the *bo*-type oxidase with $k > 5 \times 10^4$ s^{-1} at pH 7.4 and 20°C is responsible for this fast turnover. The kinetics of this reaction indicates that the oxygen reduction does not require electron exchange between quinol oxidase molecules, each having three metal centers. Thus, a bound quinol in the fully reduced enzyme is suggested to be an electron source for complete reduction of dioxygen into water supplementing electrons provided by the metal centers. A single turnover of the quinol oxidase yields a novel spectral species with a Soret maximum at 415 nm corresponding to a 'pulsed' state of mammalian cytochrome *c* oxidase.

Key words: *bo*-Type quinol oxidase; Oxygen reduction; Facilitated electron transfer; Pulsed state; Flow-flash; Double-flash

1. Introduction

Cytochrome *bo*-type ubiquinol oxidase of *Escherichia coli* catalyzes the reduction of molecular oxygen into water, utilizing ubiquinol-8 as an electron donor. Besides this scalar protolytic reaction in the cytoplasmic membrane, it pumps out protons from the cytoplasm to the periplasm [1,2]. The *E. coli* *bo*-type quinol oxidase contains a *hexa*-coordinated low-spin heme, heme B, a *penta*-coordinated high-spin heme, heme O, and a copper ion (Cu_B) in subunit I but lacks another copper ion (Cu_A) present in subunit II of mammalian cytochrome *c* oxidase (see [3] for a review). Both heme O and Cu_B are coupled anti-ferromagnetically and form a heme- copper binuclear center where the four-electron reduction of molecular oxygen takes place. Subunits I, II and III of this quinol oxidase are structurally related to the counterparts of bacterial and mammalian cytochrome *c* oxidase [4]. Based on functional and structural similarities, the *E. coli* *bo*-type quinol oxidase and cytochrome *c* oxidase are classified into the heme–copper respiratory oxidase superfamily [5,6].

The redox metal centers in the fully reduced quinol oxidase can provide three electrons altogether, but one more electron is required for complete reduction of oxygen to water if a single turnover of the enzyme yields the product. A fourth electron may come from another quinol oxidase molecule, or may be supplied from another electron donor within the same oxidase complex. Recently, we have demonstrated that the *E. coli* *bo*-type ubiquinol oxidase contains, in addition to a quinol oxidation site Q_L, a high affinity quinone binding site Q_H to which one molecule of ubiquinone-8 is tightly bound [7]. Therefore, the bound quinol in the fully reduced enzyme is a promising electron donor.

For clarification of this crucial point in the dioxygen reduction mechanism, it is important to establish how a single turnover of *bo*-type quinol oxidase is completed, and to determine whether the fourth electron is supplied inter- or intramolecularly. In the present study, employing the flow-flash, double-flash, and rapid-scan spectro- photometric techniques [8–10], we have investigated the reaction of the dithionite-reduced *bo*-type quinol oxidase with molecular oxygen by changing the concentrations of oxygen as well as the enzyme. We conclude that the binding of molecular oxygen to the fully reduced oxidase is rate-limiting in the oxygen reduction. Consequently, reaction intermediates except for a metastable product, an oxygen 'pulsed' state, do not accumulate to any discernible extent, and it is highly possible that the fourth electron comes from the bound quinol in the fully reduced enzyme. We propose that such facilitated intramolecular electron transfer (ET) originates from a molecular structure of the binuclear center of this oxidase family proposed recently [3].

2. Materials and methods

The *E. coli* *bo*-type quinol oxidase was purified from the cytochrome *bd*-deficient strain GO103 (*cyo*$^+$*Δcyd*) harboring a single copy expression vector pMFO2 which carries the cytochrome *bo* operon (*cyo*$^+$) as described previously [12], and the concentration was determined spectrophotometrically [13]. The purified oxidase was dissolved in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% (v/v) sucrose monolaulate, and the measurements were carried out at 20°C.

The experimental details for the flow-flash measurements are essentially the same as described previously [8,9]. The apparatus for flow-flash and double-flash measurements was also described previously [10]. An enzyme solution was placed in one of the reservoirs of the flow-flash apparatus, and bubbled with a 1:9 mixture of CO and N_2 gases for 10 min. Then, a minimum amount of solid sodium dithionite was added to the enzyme solution, which was allowed to stand for at least 15 min. The reaction medium without the enzyme was placed in the other reservoir, and bubbled for 15 min with N_2 gas containing different concentrations of oxygen prepared by using a Toray Oxygen Pump (Toray, Co.; model SEP-104). Most of the measurements were carried out by using a 1:1 mixing device as described previously [8–10]

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but in order to achieve the highest oxygen concentration of 1.25 mM after mixing, a 1:9 mixing unit was used.

3. Results and discussion

Fig. 1 illustrates the spectral changes during reaction of the fully reduced quinol oxidase with 1.25 mM oxygen at 20°C. For the initial 200 μs the spectral changes are almost homogeneous, contrary to the case observed with mammalian cytochrome *c* oxidase [8,9,14]; a Soret peak of the reduced enzyme at 428 nm changed to a novel spectral species with an absorption maximum at 414.5 nm. This indicates that a large portion of reduced low-spin heme B has been oxidized in this time range, too, via intramolecular ET. From a single exponential decay of absorbance at 428.5 nm as shown in Fig. 2 an apparent rate constant was determined to be 3.4×10^4 s^{-1}. The monophasic decay is clearly different from a biphasic decay which was reported by Svensson and Nilsson [15]. Their use of *bo*-type quinol oxidase purified from the multicopy vector-based overproducing strain RG145 may have yielded the biphasic change. In this strain not only a heme BO-type but also a heme OO-type oxidase are produced to various extents [16,17] due to a gene dosage effect

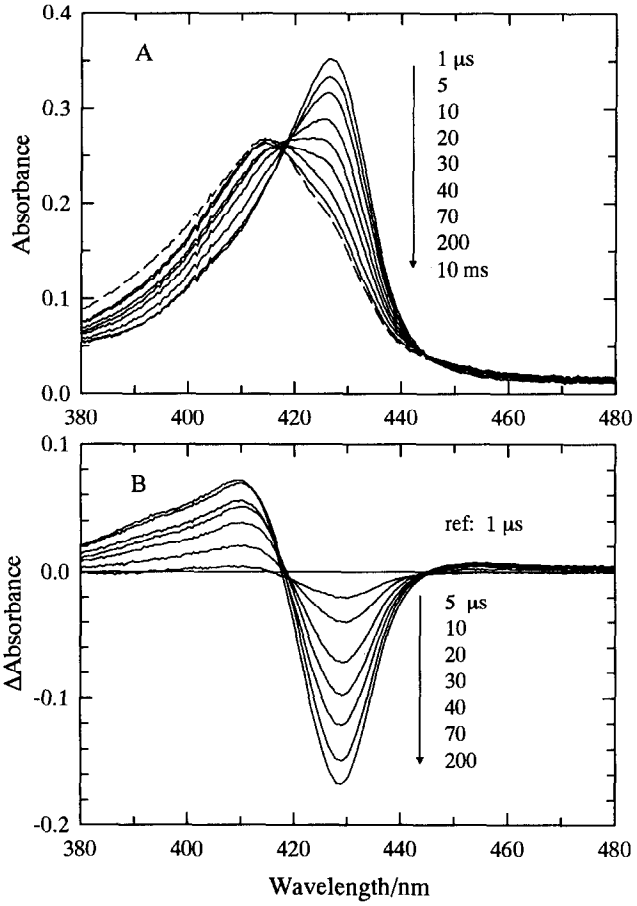

Fig. 1. Absorption spectra of *bo*-type quinol oxidase during reaction with dioxygen. The concentrations of the enzyme and oxygen were 1.4 μM (with respect to heme) and 1.25 mM, respectively. (A) The spectra recorded after initiation of the reaction by the flow-flash, double-flash method at pH 7.4 and 20°C. The reaction times were 1, 5, 10, 20, 30, 40, 70, 200 μs, and 10 ms (dashed line). (B) Time-difference spectra processed with the 1-μs spectrum as reference. The reaction times are as in A but the 10-ms spectrum is omitted.

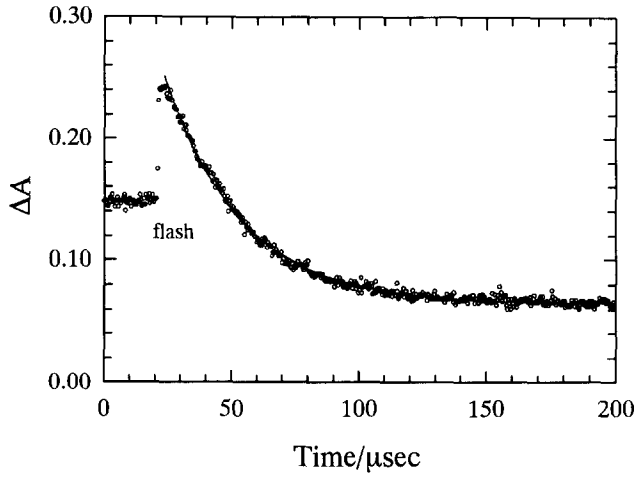

Fig. 2. Time-course for reaction of the fully reduced *bo*-type quinol oxidase with dioxygen followed at 428.5 nm. The experimental conditions are the same for Fig. 1. Three accumulations were made.

of the heme O synthase gene in the cytochrome *bo* operon [11,18–21].

The monophasic change also contrasts with a biphasic change observed with mammalian cytochrome *c* oxidase, which yielded apparent rate constants of 1.63×10^5 s^{-1} and 3.74×10^4 s^{-1}, respectively, in the presence of 1.25 mM oxygen at 20°C (Orii, to be published). The rate constant for the bacterial quinol oxidase, 3.4×10^4 s^{-1}, is apparently much smaller than the largest one of the mammalian enzyme and almost the same as the second largest. This lower rate constant indicates that the reaction of the fully reduced bacterial oxidase with molecular oxygen is fairly suppressed compared with the mammalian oxidase. Consequently, the monophasic change on the 200-μs time scale suggests a reaction scheme in which the initial reaction of the fully reduced enzyme with molecular oxygen is rate-limiting for the reaction observed. This explains why in Fig. 1 there is no spectral indication for accumulation of either the oxy or peroxy form, both of which are supposed to be reaction intermediates as deduced from the reaction mechanism of mammalian cytochrome *c* oxidase [8,9,14,22,23].

Recent time-resolved resonance Raman studies have indicated formation of the oxy and oxoferryl forms as reaction intermediates [24]. Although direct comparison cannot be made because of different experimental conditions, it should be noted that the oxy intermediate, or the primary oxygen compound, appears at 0–20 μs, and the oxoferryl intermediate at 20–40 μs at 5°C [24]. Hence it is considered that at 200 μs and 20°C not only the ferryl form but also subsequent reaction product(s) are generated. Fig. 1A indicates that a difference between the 200-μs and 10-ms spectra is small with a slight decrease and an increase in absorbance around 430 nm and 414.5 nm, respectively. The 414.5-nm species at 10 ms is relatively stable and converted into a resting oxidized state with an apparent rate constant of 0.66 s^{-1}. As this rate constant is much smaller than a maximal turnover number of 10^3 s^{-1} for the *bo*-type quinol oxidase determined by a steady-state measurement, this transition has no relevance to the physiological turnover of this enzyme. Phenomenally this transition corresponds to a conversion of an oxygen 'pulsed' state to the resting state as observed with the mammalian cytochrome *c* oxidase [25,26]. Conse-

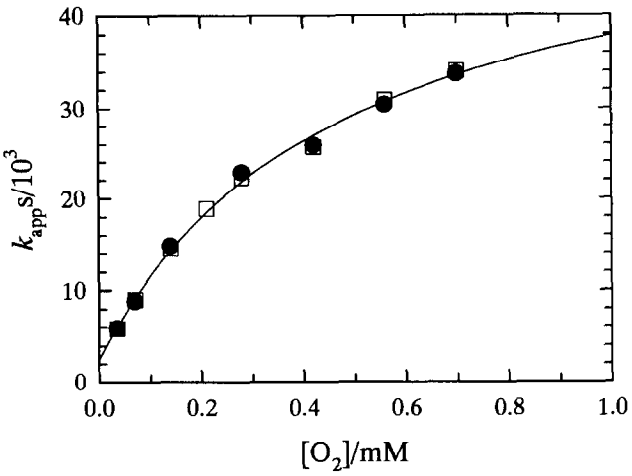

Fig. 3. Dependence of apparent rate constants on the oxygen concentration. The concentrations of the enzyme were 1.1 (□) and 0.56 μM (●), respectively. The oxygen concentration was 145 μM. The solid line is a theoretical curve drawn with $K = 0.44$ mM, $k_{+2} = 5.1 \times 10^4$ s^{-1}, and $k_{-2} = 2.2 \times 10^3$ s^{-1} and Eq. 2.

quently, the 414.5-nm species which is predominant at 200 μs probably consists of the 'pulsed' state in addition to the ferryl form. In other words, even at 200 μs an appreciable portion of the enzyme is in the 'pulsed' state as a consequence of having supplied four electrons to the bound dioxygen. This reaction profile makes it less likely that the fourth electron comes from another oxidase molecule through molecular collision. If we assume a second order rate constant of diffusion-controlled 10^9 $M^{-1} \cdot s^{-1}$ for the collision between micromolar concentrations of the oxidase for a presumed electron exchange, an apparent rate constant of 10^3 s^{-1} is obtained. It should be noted that this is much smaller than 3.4×10^4 s^{-1} determined experimentally. Also it is speculated that the rate- limiting step in the quinol oxidase reaction resides not in the intramolecular process but in the encounter of the substrate, ubiquinol, with the enzyme.

Fig. 3 illustrates the dependence on the oxygen concentration of apparent rate constants for the reaction of the fully reduced quinol oxidase with dioxygen. It is remarkable that measurements using two different concentrations of the enzyme give almost the identical plot. The hyperbolic relationship presented here is the same as that initially reported for mammalian cytochrome *c* oxidase [14], and accordingly each plot was analyzed based on the following two-step binding model.

$$bo + O_2 \overset{K}{\longleftrightarrow} \langle bo\ O_2 \rangle \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} bo \cdot O_2 \qquad (1)$$

In this scheme $\langle bo\ O_2 \rangle$ denotes an intermediary state of the enzyme leading to formation of the primary oxygen compound, $bo \cdot O_2$. Oxygen may have been trapped in a heme pocket or bound to Cu_B. We assume that the equilibration of the initial step is established much faster than for the second, and that the observed signal reflects the decay of the reduced enzyme.

$$k_{app} = k_{-2} + \frac{k_{+2}}{1 + K/[O_2]} \qquad (2)$$

Eq. 2 derived from the reaction scheme is fitted to the experimental data to give kinetic parameters as summarized in Table 1, which confirms the independence of the parameters on the enzyme concentration. This result again argues against a mechanism that a single turnover of the bacterial enzyme to reduce dioxygen into water requires molecular collision to gain the fourth electron. As the fully reduced quinol oxidase invariably contains a bound quinol molecule at the Q_H site [7], it is highly possible that this is a source for essential electrons. If this is the case, the intramolecular ET from the bound quinol to the binuclear center must proceed faster than the initial oxygen binding step, and an apparent rate constant 5×10^4 s^{-1} (Table 1) is the lowest value assignable to the facilitated intramolecular ET, in which not only heme B but also quinol participates. This value is much larger than ~10^3 s^{-1} which describes the heme-to-heme intramolecular ET in mammalian cytochrome *c* oxidase [8,14], and hence explains a much faster single turnover of the *bo*-type quinol oxidase compared with the mammalian oxidase. Here, it should be made clear that in the present definition the facilitated intramolecular ET is concerned with the heme (quinol)-to-heme ET initiated upon reaction of the enzyme with dioxygen, and is compared with mammalian cytochrome *c* oxidase. In another type of experiment a reaction can be initiated by photolyzing a CO adduct of the partially reduced quinol oxidase, and apparent rate constants of 2.3–2.7 × 10^5 s^{-1} are reported for intramolecular ET from the high-spin heme O to the low-spin heme B [17,27], a reverse course to the present case. At present, there is no a priori reasoning to equate the forward rate constant with the backward one for this specific reaction. Recently, Mogi et al. have proposed a tertiary structure model of the heme–copper metal center [3]. The invariant histidines in helix X, His^{421} and His^{419}, ligate heme B and heme O, respectively [28–30]. The two hemes are 16.4 Å apart but are directly connected by side chains of the ligand histidines and the peptide bonds His^{421}-Phe^{420}-His^{419} [3]. A local molecular structure around the ligand histidines in the *bo*-type quinol oxidase may help to facilitate the heme-to-heme intramolecular ET, in contrast to mammalian cytochrome *c* oxidase.

Eq. 2 also gives a value of 3.7×10^4 s^{-1} at 1.25 mM oxygen, in agreement with the experimental value. On the other hand, an equation $k_{obs} = kk'[O_2]/(k + k'[O_2])$, which was employed by Svensson and Nilsson [15] to analyze the similar hyperbola and predicts $k_{obs} = 0$ at the zero oxygen concentration, could never be fitted reasonably to the present experimental data. Based on the average values of the kinetic parameters in Table 1, an apparent dissociation constant of a putative $bo \cdot O_2$ compound was calculated to be 1.8×10^{-5} M according to $K_{diss} = K/(1 + k_{+2}/k_{-2})$. Interestingly, this is numerically the same to $K_{diss} = 1.6 \times 10^{-5}$ M determined by Poole et al. for the membrane-bound *bo*-type ubiquinol oxidase at −95°C [31].

In conclusion, the initial step of the reaction of the fully reduced quinol oxidase with molecular oxygen is much slower

Table 1
Kinetic parameters for the reaction of fully reduced cytochrome *bo* with dioxygen

		Quinol oxidase (μM)		Average
		1.1	0.56	
K	(mM)	0.47	0.41	0.44
k_{+2}	(s^{-1})	5.23×10^4	4.96×10^4	5.1×10^4
k_{-2}	(s^{-1})	2.37×10^3	1.97×10^3	2.2×10^3

The experimental conditions are described in the legend to Fig. 3. Each parameter is for Eq. 2.

than that of mammalian cytochrome *c* oxidase. This step is rate-limiting for a single turnover of the quinol oxidase, in which 'oxy', 'peroxy', and 'ferryl' forms are supposed to be reaction intermediates though unidentifiable spectrophotometrically. A single turnover of the fully reduced enzyme, which utilizes the bound quinol as an electron source to completely reduce molecular oxygen into water, yields the oxygen 'pulsed' state. This is distinct from the resting oxidized state and will participate in the cyclic turnover.

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