Facilitated Intramolecular Electron Transfer in the *Escherichia coli bo*-type Ubiquinol Oxidase Requires Chloride†

Yutaka Orii,*,‡ Tatsushi Mogi,§ Mariko Sato-Watanabe,§ Tomoyasu Hirano,§ and Yasuhiro Anraku§

Department of Public Health, Faculty of Medicine, Kyoto University, Kyoto 606, and Department of Plant Sciences, Graduate School of Science, the University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

Received October 25, 1994; Revised Manuscript Received December 6, 1994⊗

ABSTRACT: Previous flow-flash measurements using the *bo*-type ubiquinol oxidase of *Escherichia coli* have revealed that facilitated heme B−heme O intramolecular electron transfer initiated upon reaction of the fully-reduced enzyme with dioxygen proceeds with a rate constant higher than 5×10^4 s^{-1} at pH 7.4 and 20 °C. Depletion of chloride anions from the enzyme by HPLC performed in the present study considerably decreased the rate constant to ~700 s^{-1}, but the reaction of either dioxygen or carbon monoxide at the binuclear center was not affected at all kinetically. These results strongly suggest that Cl^- is essential in maintaining a subtle molecular structure around the heme B and heme O that enables facilitated intramolecular electron transfer. Furthermore, a series of absorption spectra of the enzyme collected on time scales from microseconds to milliseconds during its single turnover indicate that as heme−heme intramolecular electron transfer is retarded by depletion of Cl^-, an alternative electron transfer pathway is invoked. We discuss a possible role of novel bound Cl^- in electron transfer from bound quinol to the binuclear center to accomplish dioxygen reduction.

Cytochrome *bo*-type ubiquinol oxidase (UQO)[1] of *Escherichia coli* catalyzes reduction of molecular oxygen to water utilizing ubiquinol-8 as an electron donor and, in addition to this scalar protolytic reaction in the cytoplasmic membrane, pumps out protons from the cytoplasm to the periplasm coupled to dioxygen reduction (Puustinen et al., 1989, 1991). This enzyme is similar to mammalian cytochrome *c* oxidase (CCO) with respect to both function and structure and accordingly both belong to the heme−copper respiratory oxidase superfamily (Saraste, 1990; Mogi et al., 1994; Calhoun et al., 1994; Castresana et al., 1994; van der Oost et al., 1994). The *bo*-type quinol oxidase contains a low-spin heme, heme B, a high-spin heme, heme O, and a copper ion (Cu_B) in subunit I. Preliminary Cu-EXAFS analyses of the oxidized quinol oxidase have indicated that both heme O and Cu_B are in a close proximity separated by 2.92 Å (Ingledew & Bacon, 1991), explaining an antiferromagnetic coupling between heme O and Cu_B. Also on the basis of the EXAFS studies of quinol-oxidizing cytochrome aa_3 from *Bacillus subtilis*, which is analogous to *bo*-type UQO, Powers et al. (1994) have proposed that the binuclear center of this enzyme consisting of heme Fe and Cu_B are bridged with a common ligand of either Cl or S. A similar structure has been proposed previously for mammalian CCO in the resting oxidized state (Powers et al., 1981; Chance et al., 1983; Li et al., 1987; George et al., 1993).

The kinetic behavior of UQO during dioxygen reduction, however, contrasts with that of CCO in some respects (Svensson & Nilsson, 1993; Hirota et al., 1994; Orii et al., 1994). UQO reacts with dioxygen appreciably slower than CCO with a maximal rate constant of 5.1×10^4 s^{-1} at pH 7.4 and 20 °C, in contrast to higher than 1.6×10^5 s^{-1} for CCO [Orii et al., 1994; see also Oliveberg et al. (1989) and Verkhovsky et al. (1994)]. On the contrary, a final stage of intramolecular electron transfer (ET) for dioxygen reduction is slow in mammalian CCO with a rate constant of ca. 1×10^3 s^{-1} (Orii, 1984, 1988a). This contrasts with an apparent rate constant of higher than 5×10^4 s^{-1} estimated previously for heme−heme intramolecular ET in *E. coli* cytochrome *bo* during dioxygen reduction. Even higher values are estimated on the basis of photolysis experiments using CO adducts to its partially reduced form (ca. 2×10^5 s^{-1}; Brown et al., 1994; Morgan et al., 1993). Consequently, in the whole dioxygen reduction processes, the initial encounter of UQO with dioxygen is rate-limiting whereas in CCO intramolecular ET becomes rate-limiting. Clarification of the origin of such distinct behavior between UQO and CCO will surely give a clue to understand the mechanism by which dioxygen reduction is coupled to proton pumping.

During investigation of the oxygen reduction kinetics by a flow-flash method, we employed fortuitously chloride-depleted UQO, which was prepared by chromatography on an anion-exchange HPLC column in a Tris-H_2SO_4/Na_2SO_4 buffer system, and found that facilitated intramolecular ET in the bacterial enzyme slowed down appreciably. It is possible that in this preparation the bridging Cl^- is removed and accordingly this brings about an altered structure around the binuclear center resulting in suppressed intramolecular ET. In the present study we report how depletion of Cl^- affects the reaction profiles of UQO during dioxygen reduction. Contrary to the above expectation, depletion of Cl^- did not affect the reaction of UQO with ligand molecules

† This work was supported in part by Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture (Nos. 04266105 and 04225103 to Y.O. and Nos. 04266104 and 04225106 to T.M.). This is a paper XVI in the series "Structure−function studies on the *E. coli* cytochrome *bo* complex".

* Author to whom correspondence should be addressed.

‡ Kyoto University.

§ The University of Tokyo.

⊗ Abstract published in *Advance ACS Abstracts,* January 1, 1995.

[1] Abbreviations: UQO, ubiquinol oxidase; CCO, cytochrome *c* oxidase; ET, electron transfer; EXAFS, extended X-ray absorption fine structure.

0006-2960/95/0434-1127$09.00/0

like dioxygen and CO for which only the binuclear center is involved, thus existence and essential role of Cl^- other than the bridging ligand being suggested. On the basis of these novel findings, we will discuss the physiological significance of the Cl^- in the *bo*-type ubiquinol oxidase.

MATERIALS AND METHODS

The *E. coli bo*-type quinol oxidase was purified from the cytochrome *bd*-deficient strain GO103 (*cyo*$^+\Delta$*cyd*) harboring a single-copy expression vector pMFO2 which carries the cytochrome *bo* operon (*cyo*$^+$) as described previously (Tsubaki et al., 1993). An essential step in purification is chromatography on an anion-exchange HPLC column in a Tris-HCl/NaCl medium. However, when chloride depletion was intended, the chromatography was carried out in a Tris-H_2SO_4/Na_2SO_4 medium. In the present study we designate the former enzyme preparation chloride-UQO and the latter sulfate-UQO for the sake of brevity. Chloride-UQO was dissolved in 50 mM Tris-HCl buffer, pH 7.4, and sulfate-UQO in 50 mM Tris-H_2SO_4 buffer, pH 7.4, each containing 0.1% (w/v) sucrose monolaurate unless otherwise described. The enzyme concentration was determined spectrophotometrically.

The quinol oxidizing activities of chloride- and sulfate-UQO were determined at 25 °C by following spectrophotometrically the oxidation of different initial concentrations of Q_1H_2 according to the method described previously (Sato-Watanabe et al., 1994).

The reaction of fully-reduced UQO with dioxygen was investigated by using a flow-flash technique on an apparatus as described previously (Orii, 1984, 1993; Orii et al., 1990, 1991) but with three different modes. For measurements of an absorbance change at selected wavelengths, a monitoring monochromatic light was obtained by combination of a 150-W xenon lamp and an appropriate band-path filter, and the light passing through a sample solution was led into a spectrophotometer equipped with a photomultiplier (Hamamatsu Photonics, R2949) (Orii, 1993). The response times of the detector for measurements on time scales of 200−500 μs and 10 ms were 30 ns and 1 μs, respectively. Time-resolved spectral changes were recorded by the double-flash (Orii, 1984, 1993) and rapid-scan techniques (Orii et al., 1990, 1991) depending on the time resolution required. The former records an absorption spectrum of a 200-nm span in 1 μs and the latter in 1 ms. Most of the measurements were carried out by using a 1:1 mixing device, but, in order to achieve a higher oxygen concentration (1.25 mM after mixing), a 1:9 mixing unit was used. In either case 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 at least for 15 min. The reaction medium without the enzyme was placed in the other reservoir and bubbled for 10 min with air oxygen, 100% oxygen, or N_2 gas containing different concentrations of oxygen. The gas mixtures were prepared by using a mass flow controller system (Type SEC-6440, Estec, Co. Ltd., Kyoto).

For CO recombination studies, the enzyme solution placed in one of the reservoirs and that without the enzyme in the other were bubbled with a mixture of N_2 and CO gases of different ratios for 10 min for equilibration. Then the enzyme was reduced with a small amount of sodium dithionite for at least 15 min while the solution surface was being flushed with the gas mixture. A reaction mixture transferred to an observation cell of the flow-flash apparatus was subjected to flash photolysis, and usually 10−50 time traces at 428 nm were averaged. The gas mixtures of different N_2 to CO ratios were prepared by using the mass flow controller system. All of the transient kinetic measurements were carried out at 20 °C.

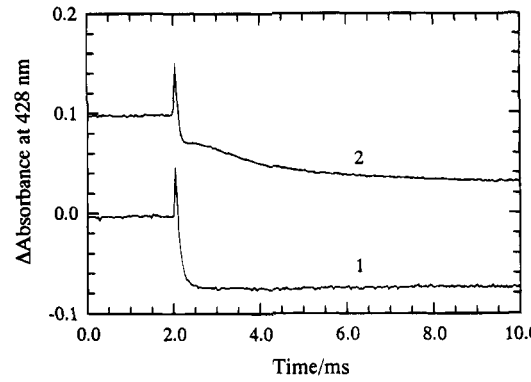

FIGURE 1: Time courses for reaction of fully-reduced chloride- and sulfate-UQO with dioxygen followed at 428 nm. The concentrations of both chloride- (1) and sulfate-UQO (2) dissolved in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% (w/v) sucrose monolaurate were 2.5 μM, respectively, and dioxygen was 145 μM. The absorbance change was followed at 428 nm, and three measurements were accumulated in each case. The traces are positioned with arbitrary offsets for clarity, and the abrupt absorbance increase represents a release of the reduced enzyme upon photolysis of the CO adduct.

RESULTS

Effect of Chloride Depletion on the Kinetics of Dioxygen Reduction. Figure 1 illustrates a typical absorption change at 428 nm following reaction of fully-reduced chloride-UQO with 145 μM oxygen. This absorption assignable to the reduced hemes exhibited a single-exponential decay. This reaction profile as well as the rate constant did not change at all when the reaction solution was prepared in the Tris-H_2SO_4 medium. Sometimes, depending on the sample preparations, the monophasic decay was followed by a slower decrease, the amplitude of which accounted for maximally about 10% of the total change. Contrary to this, sulfate-UQO exhibited a discrete biphasic transient; the initial rapid phase accounting for about two-thirds of the total change was completed within 400 μs and followed by another single-exponential decay extending to longer than 5 ms. The apparent rate constants for these decays are summarized in Table 1. It is to be noted, however, that despite such a distinct difference in the transient kinetics, a conventional steady-state assay gave the same V_{max} value of 460 mol of Q_1H_2 oxidized/mol of enzyme/s for both chloride- and sulfate-UQO. Sodium chloride (0.1 M) added to the reaction mixture did not affect the transient kinetics of chloride-UQO, and incubation of sulfate-UQO with 0.1 M NaCl for 8 h in the Tris-HCl medium also had no effect on the discrete biphasic absorption change. These results indicate that the effect of chloride depletion from UQO on the oxygen kinetics is irreversible. When the initial oxygen concentration was raised to 1.25 mM, the reaction profiles of both chloride- and sulfate-UQO were essentially unchanged except for increased apparent rate constants as summarized in Table 1.

Table 1: Kinetic Parameters for Reaction of Fully-Reduced *bo*-Type Quinol Oxidase with Dioxygen

quinol oxidase	145 μM O_2		1.25 mM O_2	
	$k_{fast}/10^4$ (s^{-1})	k_{slow} (s^{-1})	$k_{fast}/10^4$ (s^{-1})	k_{slow} (s^{-1})
(1) Chloride-UQO	1.17 ± 0.01		3.36 ± 0.03	
(2) 1 + 0.1 M NaCl[a] 30-min incubation	1.23 ± 0.01			
(3) sulfate-UQO[b]	1.40 ± 0.02	700 ± 10	3.34 ± 0.07	550 ± 10
(4) 3 + 0.1 M NaCl[a] 30-min incubation	1.38 ± 0.02	670 ± 10		
(5) 3 + 0.1 M NaCl[c] 8-hr incubation	1.31 ± 0.02	760 ± 10		

[a] Incubation temperature was 20 °C. [b] Sulfate-UQO was dissolved in 50 mM Tris/H_2SO_4, pH 7.4, 0.1% (w/v) sucrose monolaurate. [c] The incubation mixture was allowed to stand in an ice bath.

Spectral Changes of Cytochrome bo during Reaction with Dioxygen. Figure 2A illustrates a series of absolute absorption spectra recorded during reaction of fully-reduced chloride-UQO with 145 μM dioxygen. A 1-μs spectrum after photolysis is typical for the reduced enzyme and shows an absorption maximum at 428 nm. This peak decreased in intensity with time and was replaced by a 415-nm peak within 1 ms. The time difference spectra obtained with the 1-μs spectrum as reference indicate that the spectral changes are almost homogeneous, accompanied by a simultaneous decrease in absorbance at 428 nm and an increase at 410 nm (Figure 2B). Minor spectral irregularities recognized in the initial phase of the reaction up to 20 μs are not well resolved to suggest formation of the primary oxygen compound as found with mammalian cytochrome *c* oxidase (Orii, 1984, 1988b). Indeed, the spectral changes until 990 μs are almost homogeneous suggesting transformation of the reduced form into a product with the 415-nm peak, in accordance with a monophasic absorption change registered at 428 nm (Figure 1). It is to be noted that at 990 μs the Soret peak has a bump around 420−430 nm. This spectrum remained almost unchanged, except for a slight decay of the bump, at least for 10 ms.

Upon reaction with dioxygen the absorption spectrum of sulfate-UQO changed following almost the same time course as that of chloride-UQO until 50 μs (Figure 2C). The absorption decrease at 428 nm slowed down thereafter leaving a fairly strong absorption around 430 nm. At the same time an absorption increased around 405 nm, and consequently a broad and flat band appeared between 405 and 430 nm at 990 μs. The absorption increase around 405 nm is also apparent in the time difference spectra (Figure 2D). Thus, at 990 μs the absorption spectrum of sulfate-UQO is clearly different from that of chloride-UQO. The spectrum of sulfate-UQO further changed continuously in accordance with the absorption decrease at 428 nm (Figure 1). At 10 ms its shape became almost the same as that of chloride-UQO at 990 μs or 10 ms. The different states of chloride- and sulfate-UQO at 990 μs are also apparent spectrally in the visible region (Figure 2, insets). The peak height of chloride-UQO around 560 nm remained almost unchanged between 990 μs and 10 ms whereas sulfate-UQO showed a fairly higher absorption peak at 990 μs. This peak decreased in intensity with time, and accordingly at 10 ms both chloride- and sulfate-UQO became indistinguishable from each other in terms of the absorption spectrum. The 10-ms spectrum further changed into that of the resting oxidized state in either case, an apparent rate constant in the presence of 1.25 mM oxygen being 0.7 s^{-1} for the chloride- and sulfate-oxidases.

Figure 3 shows the dependence on the oxygen concentration of apparent rate constants determined for the reaction of fully-reduced sulfate-UQO with dioxygen. A hyperbolic relationship for the fast phase is quite similar to that obtained with chloride-UQO (Orii et al., 1994), suggesting a two-step dioxygen binding model (Orii, 1988a) for both ubiquinol oxidases. Table 2 compares the kinetic parameters between chloride- and sulfate-UQO. The apparent rate constants for the slow phase are independent of the dioxygen concentration giving 686 s^{-1} as an average. This feature is the same as originally observed with mammalian CCO and assigned to intramolecular ET (Orii, 1984, 1988a).

Reaction of the Fully-reduced Enzyme with Carbon Monoxide. The recombination of CO to fully reduced UQO following photolysis obeyed first-order kinetics in the concentration range between 0.1 and 1.0 mM CO. The apparent rate constant for the CO binding increased linearly with the CO concentration [Figure 4; but see Lemon et al. (1993)], in contrast to the hyperbolic relationship in the dioxygen reaction. It is to be noticed that both chloride- and sulfate-UQO gave nearly identical plots. In either case, the binding rate constant at 1.0 mM CO is ~70 s^{-1} [see also Brown et al. (1994) and Mitchell and Rich (1994)]. This is much smaller than the binding rate constant of ~3 × 10^4 s^{-1} determined for UQO in the presence of 1 mM O_2. Table 2 summarizes the kinetic parameters for the CO reaction; the on- and off- rate constants as well as the dissociation constants derived therefrom are the same between the two enzyme preparations. Interestingly, the ligand binding reactions confined to the binuclear center do not differentiate sulfate-UQO from chloride-UQO.

DISCUSSION

This communication reports for the first time that during dioxygen reduction the rate of intramolecular ET in the *E. coli bo*-type ubiquinol oxidase prepared in the chloride-containing medium decreases more than 70 times by depletion of Cl^-.

Previously we have shown that this enzyme, or chloride-UQO as designated in the present study, undergoes a fast single turnover when its fully-reduced form reacts with dioxygen (Orii et al., 1994). The rate-determining step is in the initial binding with dioxygen (an apparent maximal rate constant is 5 × 10^4 s^{-1} at pH 7.4 and 20 °C) and intramolecular ET from heme B (and bound quinol) to heme O proceeds with a rate constant larger than this. This behavior contrasts with that of mammalian CCO in which final heme-to-heme intramolecular ET proceeding with a rate constant of ~10^3 s^{-1} becomes rate-limiting (Orii, 1984, 1988a). It is to be noted, however, that during a rapid decay of the primary oxygen compound in mammalian CCO there occurs initial intramolecular ET from heme A to the binuclear center with an apparent rate constant of 3 × 10^4 s^{-1} (Han et al., 1990). This, of course, does not constitute a rate-determining step. Reflecting a difference in the rate-determining step, the absorption decrease of the reduced hemes at 428 nm for UQO is monophasic whereas that at 445 nm for CCO is biphasic in the time range of a few milliseconds.

In the present study we have observed fortuitously that sulfate-UQO shows a biphasic absorption change at 428 nm

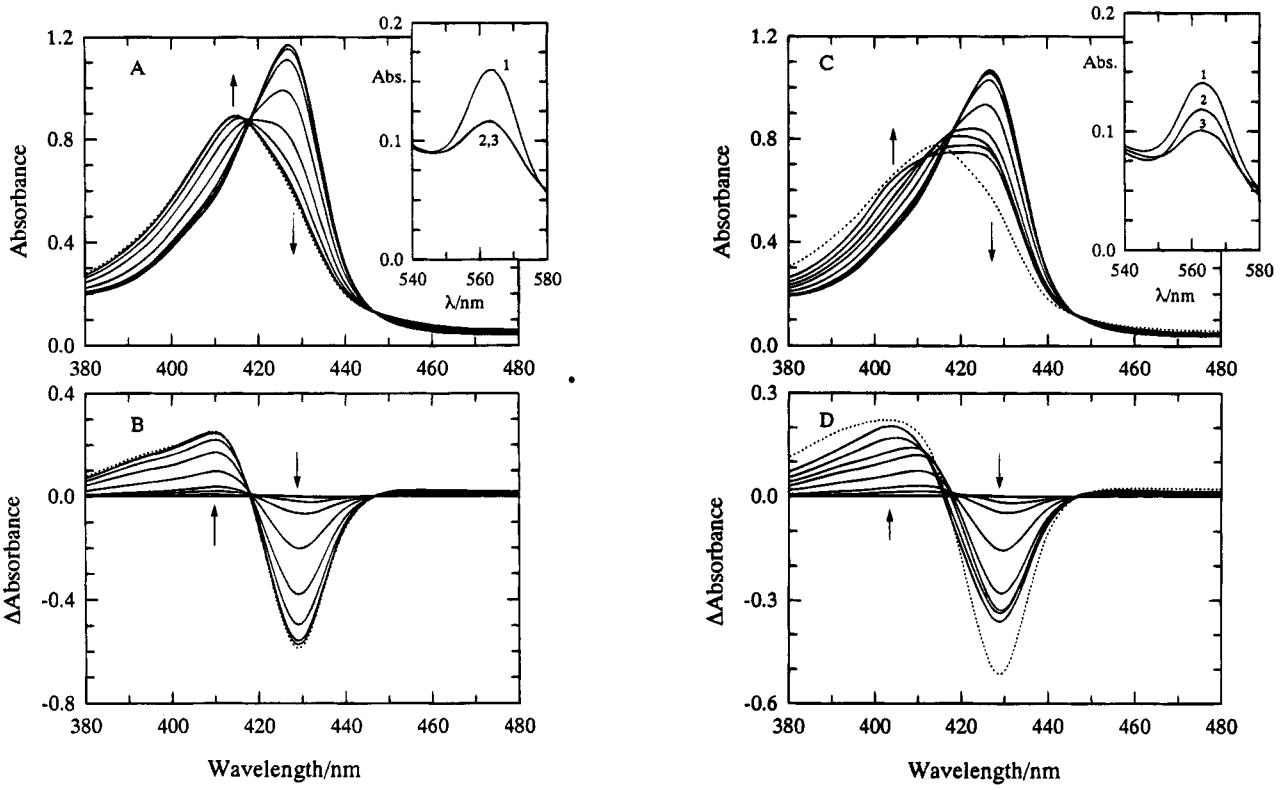

FIGURE 2: Spectral changes during reaction of fully-reduced chloride- and sulfate-UQO with dioxygen. The concentrations of chloride-UQO, sulfate-UQO, and dioxygen dissolved in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% (w/v) sucrose monolaurate were 4.7, 4.9, and 145 μM, respectively. (A, C) The absorption spectra were recorded after initiation of the reaction by the flow-flash, double-flash method. The reaction times were 1, 5, 10, 20, 50, 100, 200, 500, 990 μs, and 10 ms (-). (B, D) Time-difference spectra processed with the 1-μs spectrum as reference. The reaction times are as in panels A and C. The absorption spectra in the insets are for 1 μs (1), 990 μs (2), and 10 ms (3), respectively.

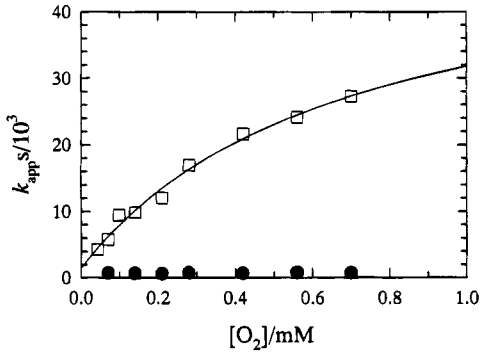

FIGURE 3: Effect of dioxygen concentration on the apparent rate constants for reaction of fully reduced sulfate-UQO with dioxygen. The concentration of sulfate-UQO dissolved in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% (w/v) sucrose monolaurate was 1.8 μM. The equation $k_{app} = k_{-2} + k_{+2}/(1 + K[O_2])$ derived from a two-step model (Orii, 1988a) was fitted to the rate constants for the fast phase (□) yielding K = 0.68 mM, $k_{+2} = 5.1 \times 10^4$ s^{-1}, and $k_{-2} = 1.5 \times 10^3$ s^{-1}. The solid line is a theoretical curve. The apparent rate constants for the slow phase (●) gave an average value of 686 s^{-1}.

like mammalian CCO. Retardation of heme B−heme O intramolecular ET is responsible to this phenomenon. Figure 2C shows that at 990 μs the absorptions at both 428 and 560 nm of sulfate-UQO are appreciably higher than those of chloride-UQO, respectively, representing persistence of the reduced heme(s) in the former. It is noteworthy, however, that the initial reaction step is indistinguishable between chloride- and sulfate-UQO; the limiting rate constants for the reaction with dioxygen (Table 1) and the spectral changes up to 50 μs (Figure 2A,B) are the same. In

Table 2: Reaction of Fully-Reduced *bo*-Type Quinol Oxidase with Dioxygen and Carbon Monoxide

	chloride-UQO	sulfate-UQO
O$_2$[a]		
K (mM)	0.44[b]	0.68
$k_{+2}/10^4$ (s^{-1})	5.1[b]	5.1
$k_{-2}/10^3$ (s^{-1})	2.2[b]	1.5
CO		
$k_{on}/10^4$ (M^{-1} s^{-1})	6.46 ± 0.20	6.42 ± 0.25
k_{off} (s^{-1})	6.7 ± 1.2	6.1 ± 1.0
K_d (mM)	0.104	0.095

[a] Kinetic parameters are for the equation as appeared in the legend to Figure 3. [b] The data were taken from Orii et al. (1994) for comparison.

fact, the apparent rate constants for the fast phase of dioxygen reduction by sulfate-UQO showed the same dependence on the dioxygen concentration, yielding kinetic parameters comparable to those determined with chloride-UQO (Table 2). These results suggest that the initial molecular event occurring at and confined to the binuclear center is not affected by depletion of Cl$^-$ from the enzyme. This conclusion is supported further by the identical reaction profiles of both chloride- and sulfate-UQO with CO. Flash photolysis experiments using the CO adducts indicate that not only the association but also the dissociation rate constants of CO, and accordingly the dissociation constants of the CO adducts calculated from these parameters, are the same (Table 2). Finally, as an *ex post facto* step, both chloride- and sulfate-UQO took the same time course in the decay of the "pulsed" form to the "resting" with a rate constant of 0.7 s^{-1} following a single turnover for reduction

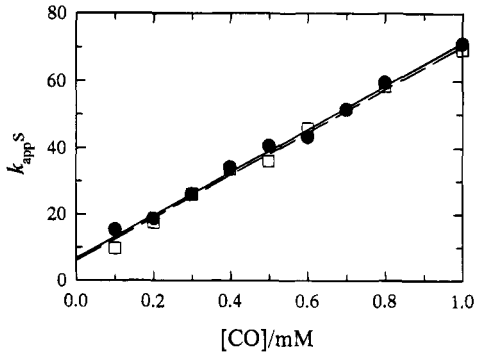

FIGURE 4: Effect of CO concentration on the reaction of fully-reduced chloride- and sulfate-UQO with CO. The concentrations of chloride- (□) and sulfate-UQO (●) were 1.1 μM, respectively. The absorbance change was followed at 428.5 nm upon flash photolysis of the CO adduct, and 10 traces were accumulated to give a single-exponential decay in each case.

of dioxygen to water. Thus, it is concluded that the Cl^- depletion does not affect the kinetics of ligand binding in which only the binuclear center of the fully-reduced enzyme participates but retards facilitated intramolecular ET from heme B to heme O by a factor larger than 70 (5×10^4 s^{-1}/700 s^{-1} for chloride- vs sulfate-UQO).

As heme B-to-heme O intramolecular ET is retarded, a peroxy intermediate (or its analogue) is expected to accumulate transiently if we assume that only two electrons from reduced heme O and Cu_B are supplied preferentially to the bound oxygen at the binuclear center. Comparison of the 990-μs spectra of both chloride- and sulfate-UQO shows that the result is contrary to the expectation. We have reasoned previously that the 415-nm species of chloride-UQO consists of the "pulsed" state in addition to the ferryl form (Orii et al., 1994). This spectral species is not apparent with sulfate-UQO, and at 990 μs it shows a broad and flat peak extending from ~405 to ~428 nm. Persistence of the absorption around 428 nm is ascribed to the reduced heme B because its oxidation is suppressed. On the other hand, the strong absorption around 405 nm shown in Figure 2C does not have a counterpart in the spectra of chloride-UQO and is distinct from either the 415-nm peak of the "pulsed" plus ferryl (Orii et al., 1994) or a 411-nm peak ascribed to an "oxoferryl" form (Watmough et al., 1994). Accordingly, on the basis of a spectral similarity to the resting oxidized state, we assume that heme O showing the 405-nm absorption is in an oxidized state though its exact nature remains to be determined. Anyway, this result shows that heme O has gone beyond a peroxy intermediate within 1 ms, and it is possible that sulfate-UQO undergoes a single turnover in this time period, too, notwithstanding suppressed intramolecular ET from reduced heme B to the binuclear center. The same V_{max} value of 460 mol of Q_1H_2 oxidized/mol of enzyme/s for both chloride- and sulfate-UQO does not contradict this possibility.

We have proposed previously that not only reduced heme B but also bound quinol at the Q_H (i.e., a high-affinity quinone-binding site) site would provide electron(s) needed to fully reduce dioxygen to water (Orii et al., 1994). Although we are not successful in determining directly the rate of oxidation of the bound quinol, completion of a rapid single turnover of chloride-UQO within 1 ms suggests that a fourth electron originating from the quinol travels an ET pathway as rapidly as electron going from heme B to heme O. Then it is possible that even when facilitated heme B-to-heme O ET is suppressed by the chloride depletion, dioxygen reduction proceeds in sulfate-UQO by utilizing two electrons coming from the bound quinol to the binuclear center through an alternative pathway bypassing the heme B-heme O path. The delayed oxidation of reduced heme B in sulfate-UQO with a rate constant around 700 s^{-1} may represent a supply of an electron to the oxidized binuclear center, or the electron may be used to reduce the oxidized quinone at the Q_H site. The latter possibility implicates that heme B-heme O ET is suppressed prohibitively by the chloride depletion (but see discussions below). Such a mechanism of switching intramolecular ET to accomplish dioxygen reduction may be regarded as one of "fail-safe" mechanisms incorporated into a molecular structure of this enzyme. This concept explains adequately that the steady-state activity assay does not differentiate sulfate-UQO from chloride-UQO. Thus, the kinetic parameters estimated experimentally in the present study favor that sulfate-UQO undergoes a single turnover as rapidly as chloride-UQO. At present, however, we cannot eliminate completely the possibility that heme B-to-heme O ET is still obligatory for dioxygen reduction in sulfate-UQO. Disposal of this possibility depends on how we evaluate the difference between the rate constant of 700 s^{-1} for intramolecular ET and V_{max} of 460 mol of Q_1H_2 oxidized/mol of enzyme/s which gives 920 s^{-1} in terms of electron equivalents. Further studies will decisively settle the present issue.

Powers et al. (1994) have proposed, on the basis of EXAFS studies, that quinol-oxidizing cytochrome aa_3 from *B. subtilis* which is structurally related to the *E. coli bo*-type UQO contains the binuclear center consisting of heme Fe and Cu_B with a bridge ligand of either Cl or S in between. In fact, previous spectroscopic studies on cytochrome *bo* of *E. coli* support the presence of the bridging ligand mediating spin–spin coupling at the binuclear center (Tsubaki et al., 1993, 1994; Uno et al., 1994). Since there is no conserved cysteine or methionine at the binuclear center (Saraste, 1990; Mogi et al., 1994), it is considered that the binuclear center of UQO also has the bridging chloride ion and that the presence and absence of this ligand may modulate intramolecular ET from reduced heme B to heme O. It is to be noted, however, that the initial rapid reaction of the enzyme with dioxygen at least up to 50 μs are almost the same irrespective of whether the enzyme preparation has been subjected to chloride depletion or not (Figure 2 and Tables 1 and 2), and the reaction profiles with CO are almost identical between chloride- and sulfate-UQO (Table 2). These results can be explained if we assume that heme O loses the bridging chloride bond prior to the binding of either dioxygen or CO as speculated for mammalian CCO (Chance et al., 1983). On the contrary, it seems less likely that depletion or replacement of the essential chloride ion at the binuclear center does not affect the direct oxygen or CO binding but exerts the effect on intramolecular heme B–heme O ET. In line with this, resonance Raman spectra of chloride- and sulfate-UQO in the air-oxidized, dithionite-reduced, and CO-bound states were indistinguishable from each other, suggesting that the bound chloride(s) of present concern is distal to the metal centers (Hirota et al., unpublished results). Therefore, we speculate that the chloride ion supporting facilitated intramolecular ET resides in another locus different from the binuclear center. Our preliminary chloride binding experiments using sulfate-UQO

suggest the presence of a novel chloride binding site with a K_d value of 3.6 mM (Hirano et al., unpublished results).

We have pointed out that facilitated intramolecular ET is associated with a specific local structure around heme B and heme O in UQO (Orii et al., 1994), which are separated by 16.4 Å but connected directly by side chains of the ligand histidines and the peptide bonds His421−Phe420−His419 (Woodruff, 1993; Mogi et al., 1994). Interestingly, the rate constant of 5×10^4 s^{-1} estimated as the lowest for heme B−heme O ET is numerically comparable to $\sim 6 \times 10^4$ s^{-1} determined for ET from Zn porphyrin to Ru attached to His48, which are 11.8−16.6 Å apart, in ruthenated sperm whale myoglobin (Axup et al., 1988). On the other hand, using the equations given by Moser et al. (1992), Woodruff (1993) estimated a rate constant for ET through 16 covalent bonds between heme A moieties in mammalian CCO to be 1.9×10^5 s^{-1}, in good agreement with the measured value of 2.5×10^5 s^{-1} (Oliveberg & Malmström, 1992). A structural similarity between mammalian CCO and bacterial UQO suggests that this value represents the upper limiting value for intramolecular ET between heme B and heme O. In fact, the rate constant of $\sim 2 \times 10^5$ s^{-1} has been estimated experimentally for heme O-to-heme B ET (Morgan et al., 1993; Brown et al., 1994).

According to the electron tunneling mechanism to which through-bond and through-space contributions are of primary significance, it is speculated that the rate difference of >20 is easily brought about by subtle orientation differences (Beratan & Onuchic, 1989). Thus, Cl^- may maintain the integrity of this local structure by lessening electrostatic repulsion between localized positive charges on both histidines with intervention in between, or an electrostatic stabilizing effect of Cl^- may be exerted from a distant binding site. Removal of such effects possibly brings about subtle orientation changes among these residues and retards facilitated ET. Concerning the location of this specific ion, a recent report by Wang et al. (1993) may be pertinent. Spectral tuning of color vision pigments is known to be partly due to a bound chloride ion. Site-directed mutagenesis studies have identified His197 and Lys200 in loop IV/V as chloride-binding sites of human red and green pigments (Wang et al., 1993). It can alter the electronic state of a Schiff base-linked retinal molecule surrounded by transmembrane helices to induce a red-shift of its absorption maximum to 530 nm from 500 nm. If we take an analogy from this, the metal centers of UQO are expected to be near the periplasmic surface, and basic residues in loop I/II such as an essential Arg80 might provide a chloride binding site and regulate the ligation states of the low-spin heme B (Mogi et al., 1994). Thus, determination of the chloride binding site and the way it modulates intramolecular ET will surely help understand the physiological significance of Cl^- not only in the ET mechanism but also in the osmochemical behavior of the organism.

REFERENCES

Axup, A. W., Albin, M., Mayo, S. L., Crutchley, R. J., & Gray, H. B. (1988) *J. Am. Chem. Soc. 110*, 435−439.

Beratan, D. N., & Onuchic, J. N. (1989) *Photosynth. Res. 22*, 173−186.

Brown, S., Rumbley, J. N., Moody, A. J., Thomas, J. W., Gennis, R. B., & Rich, P. R. (1994) *Biochim. Biophys. Acta 1183*, 521−532.

Calhoun, M. W., Thomas, J. W., & Gennis, R. B. (1994) *Trends Biochem. Sci. 19*, 325−330.

Castresana, J., Lübben, M., Saraste, M., & Higgins, D. G. (1994) *EMBO J. 13*, 2516−2525.

Chance, B., Kumar, C., Powers, L., & Ching, Y.-C. (1983) *Biophys. J. 44*, 353−363.

George, G. N., Cramer, S. P., Frey, T. G., & Prince, R. C. (1993) *Biochim. Biophys. Acta 1142*, 240−252.

Han, S., Ching, Y.-C., & Rousseau, D. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8408−8412.

Hirota, S., Mogi, T., Ogura, T., Hirano, T., Anraku, Y., & Kitagawa, T. (1994) *FEBS Lett. 352*, 67−70.

Ingledew, W. J., & Bacon, M. (1991) *Biochem. Soc. Trans. 19*, 613−616.

Lemon, D. D., Calhoun, M. W., Gennis, R. B., & Woodruff, W. H. (1993) *Biochemistry 32*, 11953−11956.

Li, P. M., Gelles, J., Chan, S. I., Sullivan, R. J., & Scott, R. A. (1987) *Biochemistry 26*, 2091−2095.

Mogi, T., Nakamura, H., & Anraku, Y. (1994) *J. Biochem. (Tokyo) 116*, 471−477.

Morgan, J. E., Verkhovsky, M. I., Puustinen, A., & Wikström, M. (1993) *Biochemistry 32*, 11413−11418.

Moser, C. C., Keske, J. M., Warncke, K., Farid, R. S., & Dutton, P. L. (1992) *Nature 355*, 796−802.

Oliveberg, M., & Malmström, B. G. (1991) *Biochemistry 30*, 7053−7057.

Oliveberg, M., Brzezinski, P., & Malmström, B. G. (1989) *Biochim. Biophys. Acta 977*, 322−328.

Orii, Y. (1984) *J. Biol. Chem. 259*, 7187−7190.

Orii, Y. (1988a) *Chem. Scr. 28A*, 63−69.

Orii, Y. (1988b) *Ann. N.Y. Acad. Sci. 550*, 105−117.

Orii, Y. (1993) *Biochemistry 32*, 11910−11914.

Orii, Y., & Anne, H. (1990) *FEBS Lett. 267*, 117−120.

Orii, Y., Yumoto, I., Fukumori, Y., & Yamanaka, T. (1991) *J. Biol. Chem. 266*, 14310−14316.

Orii, Y., Mogi, T., Kawasaki, M., & Anraku, Y. (1994) *FEBS Lett. 352*, 151−154.

Powers, L., Chance, B., Ching, Y., & Angiolillo, P. (1981) *Biophys. J. 34*, 465−498.

Powers, L., Lauraeus, M., Reddy, K. S., Chance, B., & Wikström, M. (1994) *Biochim. Biophys. Acta 1183*, 504−512.

Puustinen, A., & Wikström, M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6122−6126.

Puustinen, A., Finel, M., Virkki, M., & Wikström, M. (1989) *FEBS Lett. 249*, 163−167.

Saraste, M. (1990) *Q. Rev. Biophys. 23*, 331−366.

Sato-Watanabe, M., Mogi, T., Ogura, T., Kitagawa, T., Miyoshi, H., Iwamura, H., & Anraku, Y. (1994) *J. Biol. Chem. 269*, 28908−28912.

Svensson, M., & Nilsson, T. (1993) *Biochemistry 32*, 5442−5447.

Tsubaki, M., Mogi, T., Anraku, Y., & Hori, H. (1993) *Biochemistry 32*, 6065−6072.

Tsubaki, M., Mogi, T., Hori, H., Ogura, T., Hirota, S., Kitagawa, T., & Anraku, Y. (1994) *J. Biol. Chem.* (in press).

Uno, T., Mogi, T., Tsubaki, M., Nishimura, Y., & Anraku, Y. (1994) *J. Biol. Chem. 269*, 11912−11920.

Van der Oost, J., de Boer, A. P. N., de Gier, J.-W. L., Zumft, W. G., Stouthamer, A. H., & Van Spanning, R. J. M. (1994) *FEMS Microbiol. Lett. 121*, 1−10.

Verkhovsky, M. I., Morgan, J. E., & Wikström, M. (1994) *Biochemistry 33*, 3079−3086.

Wang, Z., Asenjo, A. B., & Oprian, D. D. (1993) *Biochemistry 32*, 2125−2130.

Watmough, N. J., Cheesman, M. R., Greenwood, C., & Thomson, A. J. (1994) *EBEC Short Rep. 8*, 53.

Woodruff, W. H. (1993) *J. Bioenerg. Biomembr. 25*, 177−188.

BI942494G