Cu_B promotes both binding and reduction of dioxygen at the heme-copper binuclear center in the *Escherichia coli bo*-type ubiquinol oxidase**

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Received 16 June 1995; revised version received 17 July 1995

Abstract **A Cu_B-deficient mutant of the *Escherichia coli bo*-type ubiquinol oxidase exhibits a very low oxidase activity that is consistent with a decreased dioxygen binding rate. During the turnover, a photolabile reaction intermediate persists for a few hundred milliseconds, due to much slower heme *o*-to-ligand electron transfer. Thus, the lack of Cu_B seems to have endowed the mutant enzyme with myoglobin-like properties, thereby stabilizing the CO-bound form, too. Accordingly we conclude that Cu_B plays a pivotal role in preferential trapping and efficient reduction of dioxygen at the heme-copper binuclear center.**

Key words: *E. coli bo*-type ubiquinol oxidase; Heme-copper binuclear center; Cu_B-deficient mutant; Intramolecular electron transfer; Dioxygen reduction chemistry

1. Introduction

Cytochrome *bo*-type ubiquinol oxidase (UQO) in *Escherichia coli*, which was first identified as cytochrome *o* by Castor and Chance in 1959 [1], belongs to the heme-copper respiratory oxidase superfamily [2,3]. It catalyzes the two-electron oxidation of ubiquinol-8 and the four-electron reduction of dioxygen [4–6] and functions as a redox-coupled proton pump [7]. Recent time-resolved resonance Raman studies with the wild-type UQO revealed that the oxy intermediate appears in the time range of 0–20 μs and the oxoferryl intermediate develops in the range of 20–40 μs at 4°C [8]. At ambient temperature, these intermediates seem not to accumulate in the wild-type UQO [9,10], although low-temperature spectroscopic studies suggested the formation of the oxy intermediate [11,12].

Subunit I of UQO binds a low-spin heme *b*, a high-spin heme *o* and Cu_B and plays a central role in reduction of dioxygen [5,6]. Site-directed mutagenesis studies identified that His^{333} and His^{334} of a transmembrane helix VII are obligatory ligands for Cu_B [13–18]. His^{284} of helix VI may also serve as a third ligand. Vibrational spectroscopic studies with the Cu_B-deficient mutant oxidase suggest that Cu_B determines the geometry of bound ligands at the heme-copper binuclear center [14,15,19]. Based on detailed time-resolved spectroscopic studies on cytochrome *c* oxidase, Woodruff and his colleagues [20] postulated that Cu_B is involved in a ligand shuttle mechanism.

In this article, we examined a defect of dioxygen reduction in the Cu_B-deficient mutant oxidase by time-resolved electronic spectroscopy. Kinetic analyses of CO recombination and dioxygen reduction on the mutant UQO suggest that Cu_B enhances preferential binding of dioxygen at the heme-copper binuclear center and facilitates dioxygen reduction by promoting the heme *o*-to-ligand electron transfer.

2. Materials and methods

2.1. Construction of bo-type UQO overproducing strain

Strain ST4683 (*Δcyo Δcyd*; ref. [21]) was anaerobically transformed with pHN3795 [22], a pBR322 derivative, which carries the entire cytochrome *bo* operon (*cyoABCDE*) on an 10.2 kb *Bam*HI-*Pvu*II chromosomal fragment. To avoid a lethal gene dosage effect, the expression of the wild-type UQO was suppressed under these conditions [22]. The resultant small colonies on L-broth plates containing 0.2% glucose and 50 μg/ml ampicillin were further allowed to grow aerobically for two days. One of large reddish-brown colonies was picked up and the isolated plasmid was designated as pHN3795-1 which confers a 3-fold overproduction of the wild-type UQO. A G^{-36} to T^{-36} mutation at the −35 promoter region resulted in a decrease of the expression level of the enzyme (K. Saiki and Y. Anraku, unpublished results), thus allowing the aerobic growth of the transformant. Spectroscopic properties, enzyme activity and heme composition of the wild-type UQOs isolated from strain GO103 (*cyo*$^+$ *Δcyd*; ref. [23]) harboring a multicopy plasmid pHN3795-1 and from GO103 harboring a single copy plasmid pMFO2 (*cyo*$^+$; refs. [19,21]) were found to be indistinguishable from each other.

For overproduction of the H333A mutant UQO, pHN3795-H333A was constructed by replacement of an 0.8 kb *Bgl*II fragment of pHN3795-1 with the counterpart of pCYOF2-H333A in which His^{333} in subunit I had been substituted with alanine [13]. After double digestion of candidate plasmids with *Bgl*II and *Hae*III, recombination was confirmed by Southern blotting analysis using an ECL direct nucleic acid labelling and detection system (Amersham). Due to the H333A mutation (i.e. CAC-to-GCC codon change), *Hae*III cuts an 270 bp *Bgl*II-*Hae*III fragment into 149 and 121 bp fragments.

2.2. Purification of bo-type ubiquinol oxidase

The wild-type UQO and the Cu_B-deficient mutant UQO (H333A) were isolated from *E. coli* strains GO103/pMFO2 and ST4533 (*Δcyo cyd*$^+$; ref. [24])/pHN3795-H333A, respectively, as described previously [19]. The enzymes in 50 mM Tris-HCl (pH 7.4) containing 0.1% (w/v) sucrose monolaurate SM-1200 (Mitsubishi-Kasei Food Co., Tokyo) were stored at −80°C until use. Enzyme concentration was determined from heme content [19]. The absence of Cu_B in the H333A mutant UQO was confirmed by resonance Raman spectroscopy (S. Hirota, T. Hirano, T. Mogi, Y. Anraku, and T. Kitagawa, unpublished results). The $\nu_{Fe\text{-}CO}$ stretching vibration mode of the mutant UQO shifted from 521 cm^{-1} of the wild-type UQO to 500 cm^{-1}, as reported previously [14].

2.3. Enzyme assay

Quinol oxidase activity was determined spectrophotometrically at 25°C [24]. Enzyme concentrations of the wild-type and the H333A

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**This is paper XVII in the series of 'Structure–function studies on the *E. coli* cytochrome *bo* complex'.

Abbreviations: UQO, the *E. coli bo*-type ubiquinol oxidase; H333A, subunit I mutant where His^{333} had been substituted with alanine.

SSDI 0014-5793(95)00852-7

mutant oxidases were 0.5 nM and 0.5 μM, respectively, and ubiquinol-1 was used at 0.45 mM.

2.4. Spectroscopic analysis

The reaction of the dithionite-reduced UQO with dioxygen was studied in 50 mM Tris-HCl (pH 7.4) containing 0.1% sucrose monolaurate at 20°C by using a flow-flash technique in combination with either rapid scanning or double-flash spectrophotometry [25,26].

3. Results and discussion

3.1. Reaction of Cu_B-deficient mutant UQO with carbon monoxide

The formation of a heme *o*-CO adduct of the H333A mutant UQO was investigated by following the recombination of photodissociated CO to heme *o*. The absorbance change at 428.5 nm was biphasic and the observed rate constants for both phases increased linearly with CO concentration as shown in Fig. 1. The relative amplitude of the fast phase to the slow phase was almost independent of CO concentration and was 1.76 ± 0.06 on the average. These results suggest most simply the existence of two conformers in the sample. No such biphasic behavior has been reported for the membrane preparations of the H333L and H334L mutant UQOs [27,28]. Therefore, it is likely that the purification procedures have altered the molecular environment of heme *o* in some way, as suggested for CN binding to the air-oxidized wild-type UQO [29], though its fundamental properties are retained as shown below. As the heme analysis of the H333A mutant UQO by reverse-phase high performance liquid chromatography indicated a ratio of 4:1 for BO- and BB-types, this heterogeneity of the preparation, may be another alternative to explain the biphasic behavior. At present, we would like to focus our attention on the major component. The slopes in Fig. 1 gave the bimolecular rate constants of 3.2×10^5 $M^{-1}s^{-1}$ and 6.0×10^4 $M^{-1}s^{-1}$ for the fast and slow phases of CO binding, respectively. As the *off* rate constant was too small to be estimated from the intercept on the ordinate, it was determined by replacing the bound CO in the presence of 0.5 mM free CO with 0.1, 0.2, and 0.4 mM nitric

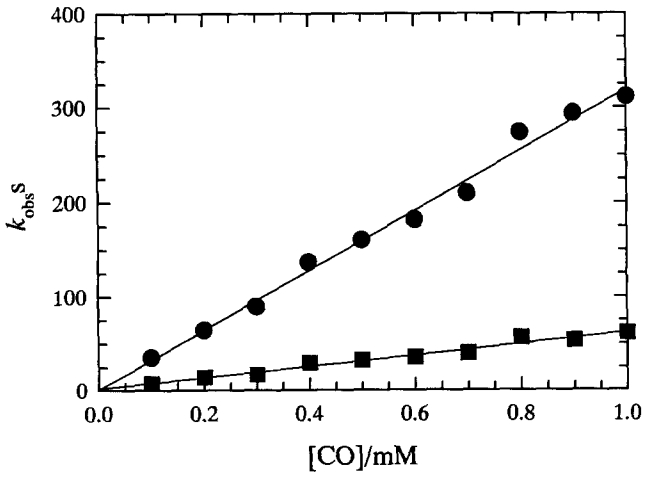

Fig. 1. Effect of CO concentration on reaction of the Cu_B-deficient mutant UQO with CO. Concentration of the H333A mutant UQO was 1.25 μM in 50 mM Tris-HCl (pH 7.4) containing 0.1% sucrose monolaurate. The absorbance change was followed at 428.5 nm upon flash photolysis of the CO adduct, and 20 traces were accumulated in each case. The observed rate constants for the fast (●) and slow (■) phases were calculated as described previously [9,10].

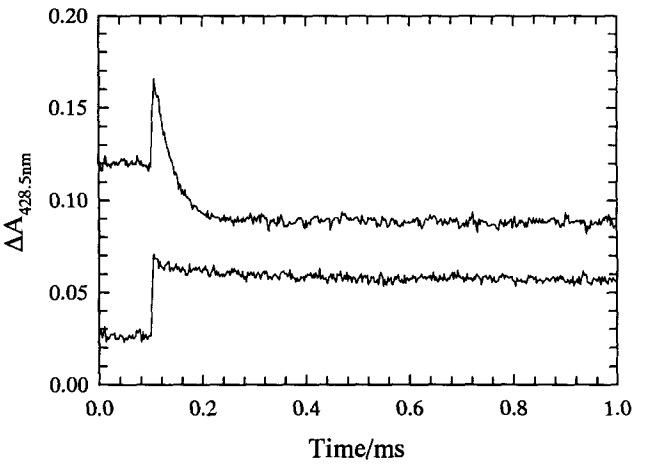

Fig. 2. Time courses for reaction of the fully-reduced wild-type and Cu_B-deficient mutant UQOs with dioxygen followed at 428.5 nm. Concentrations of dioxygen and CO were 0.7 mM and 50 μM, respectively. Accumulations for the wild-type (top) and mutant (bottom) UQOs, 1.25 μM each, were 4 and 7, respectively. The abrupt absorption increase represents a release of the reduced enzyme upon CO photolysis. Other conditions are the same as in the legend to Fig. 1.

oxide. The exchange reaction was also biphasic. Independently of nitric oxide concentrations, the relative amplitude was 3.2 ± 0.2 ($n = 6$), and the two rate constants, that can be equated to the dissociation rate constants of CO from the mutant-CO complex, were 2.57 ± 0.02 s^{-1} and 0.11 ± 0.01 s^{-1}, respectively. In combination with the *on* rate constants as obtained above, the dissociation constants of 8.0×10^{-6} M and 1.8×10^{-6} M were calculated for the two kinds of the CO complex. Comparison of the kinetic parameters and the dissociation constants with those determined for the wild-type UQO either in the chloride- or sulfate-form [10] manifest characteristic features of the Cu_B-deficient enzyme as follows. One of the *on* rate constants, 3.2×10^5 $M^{-1}s^{-1}$, is about 5 times larger than that for both the chloride- and sulfate-type wild-type UQOs (6.5 and 6.4×10^4 $M^{-1}s^{-1}$, respectively) [10], and lies between 4.4×10^5 $M^{-1}s^{-1}$ for the H334L and 2.3×10^5 $M^{-1}s^{-1}$ for the H333L mutant enzymes as estimated by Lemon et al. [27]. Thus, boosted by a smaller *off* rate constant, the dissociation constant of the H333A-CO complex, 8.0×10^{-6} M, is more than 10 times smaller than that for the wild-type enzymes [10]. These results suggest that removal of Cu_B from the wild-type UQO increases the accessibility of heme *o* towards CO as proposed previously [27] but also stabilizes the CO adduct. However, these features are only specific to the CO adduct formation and do not apply to the dioxygen binding kinetics.

3.2. Reaction of Cu_B-deficient mutant UQO with dioxygen

Fig. 2 illustrates the time traces at 428.5 nm for the wild-type and the H333A mutant UQOs upon initiation of the reaction with dioxygen by the flow-flash method. The absorbance change for the wild-type UQO was monophasic as reported previously [9,10], and the observed rate constant was 2.6×10^4 s^{-1} in the presence of 0.7 mM dioxygen at 20°C. On the other hand, the trace for the H333A mutant showed a much slower decrease. These results are in accord with the very low oxidase activity of the Cu_B-deficient UQO, 1.4 e^- s^{-1}, compared to 1,030 e^- s^{-1} for the wild-type enzyme in the presence of 0.45 mM

ubiquinol-1. Subsequently, the spectral changes during the oxidation of the H333A mutant UQO were recorded by rapid-scanning in combination with the flow-flash method in a time range from 1 ms to 600 s (Fig. 3A). It should be noted that a distinct shoulder appeared transiently around 418 nm and finally was replaced by a spectrum with a maximum absorption around 416 nm after 10 min. A broad absorption around 428 nm in the last spectrum indicates that the oxidation of heme *b* was not completed even after 10 min. The 418-nm shoulder as well as the 416-nm peak disappeared upon flash photolysis but it returned to the original state immediately. The spectral profiles during the recovery were exactly the same as those for the CO compound of the mutant UQO. No such spectral species was observed when the oxidation was initiated by mixing the dithionite-reduced mutant with dioxygen (Fig. 3B). Therefore, we conclude that the 418-nm shoulder as well as the 416-nm band is ascribed mostly to the heme *o*-CO complex in which CO derives from that used to block the reduced mutant enzyme. The appearance of the CO adduct during the oxidation of the H333A mutant clearly indicates that CO competes favorably with dioxygen towards heme *o*, and suggests coexistence of a putative primary oxygen complex with the CO adduct.

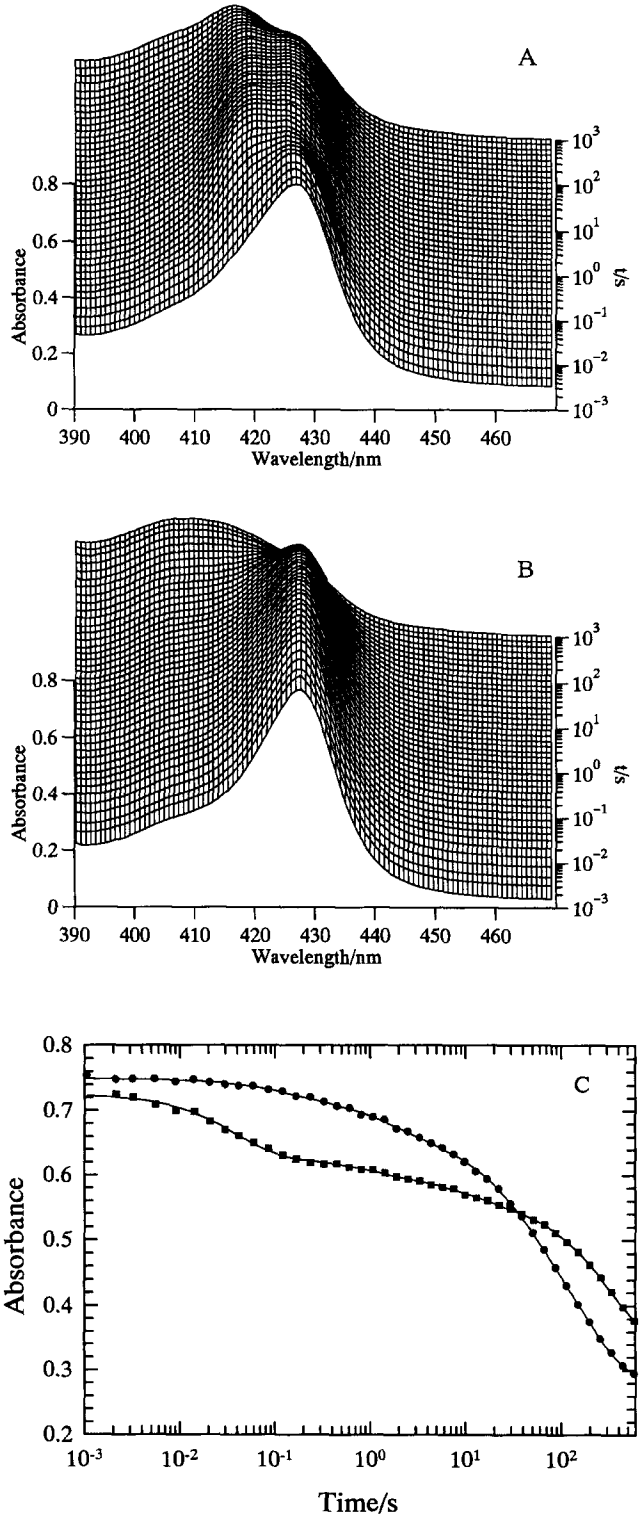

Fig. 3C illustrates the absorbance changes at 428.5 nm extracted from the data in Fig. 3A and 3B. In each case an equation with 4-exponential terms was fitted satisfactorily to the respective data, giving kinetic parameters as summarized in the legend to Fig. 3C. The largest rate constant obtained in the flow-flash measurement, $k_{obs1} = 25.4\ s^{-1}$, is related to the generation of the CO adduct as an intermediate because this value is comparable to a rate constant of $16\ s^{-1}$ ($3.2 \times 10^5\ M^{-1}s^{-1}$ and 5×10^{-5} M for CO concentration) for CO binding. In analogy with this, k_{obs1} of $8.0\ s^{-1}$ obtained in the mixing experiment may represent the binding of dioxygen to heme *o* as the obligatory step of the oxidation. Thus, the kinetic parameters for each step were determined at different concentrations of dioxygen in an attempt to relate the rate constant of the initial phase to dioxygen concentration. However, the rate constants derived varied between 2 and $12\ s^{-1}$ at dioxygen concentrations between 0.175 and 0.7 mM, and there was no definitive relationship between them (data not shown). As the amplitude of the initial phase was around 5% of the total at most, the parameters relating to this phase are very vulnerable to method of data fitting. This situation renders the derived results less reliable. If we take the value of $8.0\ s^{-1}$ to represent the binding rate constant of dioxygen to heme *o*, we recognize that this is 3,000 times smaller than that of the wild-type enzyme, and that this is comparable to the binding rate constant of CO to the H333A mutant.

The formation of a photodissociable species, attributable to the oxy complex [11], was examined by flash photolysis studies. The reaction was initiated by mixing the dithionite-reduced H333A mutant with dioxygen, and the mixture was exposed to a laser flash. Fig. 4 illustrates a difference spectrum with a peak at 412–413 nm and a trough at 430 nm that was obtained by applying the flow-double flash method [26]. This spectrum is

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Fig. 3. Spectral changes during reaction of the fully-reduced Cu_B-deficient mutant UQO with dioxygen. Concentrations of the mutant UQO and dioxygen were 2.5 μM and 0.7 mM, respectively. (A) The reaction was initiated by the flow-flash method in the presence of 50 μM CO. Out of 512 absorption spectra, 41 spectra arranged equal-spatially on the logarithmic time scale are illustrated starting from 2.1 ms. (B) The reaction was initiated by mixing the dithionite-reduced mutant UQO with the medium saturated with dioxygen. Forty two absorption spectra are shown starting from 1 ms. (C) The absorbance changes at 428.5 nm were extracted from curves (A) (■) and (B) (●), and the equation, $A_{428.5} = a + \sum_{i=1}^{4} b_i \cdot \exp(-k_{app,i}\, t)$, was fitted to each data set to obtain the following parameters. The solid lines were obtained by using these parameters and the equation. (■), $a = 0.3375$, $b_i = 0.09667$, 0.02616, 0.03946, 0.2261; $k_{app,i} = 25.4$, 1.12, 0.098, 0.003; (●), $a = 0.2838$; $b_i = 0.02658$, 0.05664, 0.1112, 0.2712; $k_{app,i} = 7.98$, 0.603, 0.0322, 0.0055.

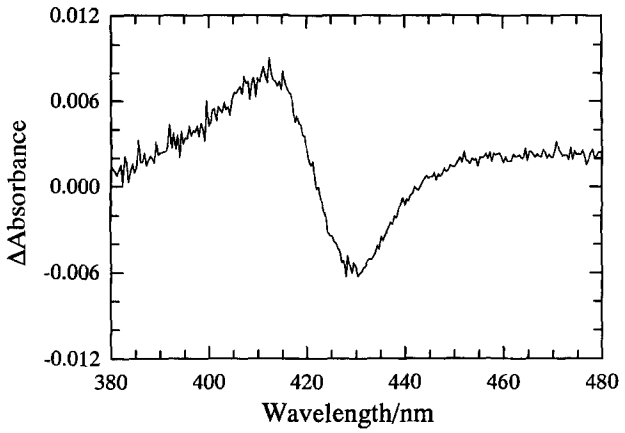

Fig. 4. Generation of a photodissociable species during reaction of the fully-reduced Cu_B-deficient mutant UQO with dioxygen. The dithionite-reduced mutant enzyme (2.5 μM) was allowed to react with 0.7 mM dioxygen on the flow-flash, rapid-scan apparatus, the photolyzing laser was flashed on the reaction mixture, with and without a mask, at 150 ms after initiation of the reaction, and the absorption spectra were recorded by the flow-double flash method.

quite similar to that of the CO complex in reference to the reduced UQO. However, the present photodissociable species occurred in the complete absence of CO and persisted for a long time period to some hundred milliseconds after initiation of the reaction (data not shown). So it most probably can be ascribed to the heme o-O_2 adduct, or to its reduction intermediate as will be discussed below.

3.3. Physiological role of Cu_B

The present results indicate that the heme o-O_2 adduct is actually formed in the H333A mutant UQO that lacks Cu_B. Although the dissociation constant of this adduct has not yet been determined, this may be comparable to that of the heme o-CO adduct having the dissociation constant of 8.0×10^{-6} M because both adducts coexist as long as the dioxygen reduction by the mutant enzyme proceeds under a mixed atmosphere of dioxygen and CO. As the CO adduct is more abundant even when the dioxygen concentration is 19 times higher than that of CO (data not shown), a partition coefficient between both ligands seems to be in favor of the CO adduct formation. This presumed property of the H333A mutant is quite similar to that of myoglobins for which the partition coefficients of 19 to 167 have been reported [30]. Further, resonance Raman and Fourier-transform infrared spectroscopies revealed that CO binding properties of the H333A mutant UQO had a great resemblance to oxygen carrier proteins [14].

Apparent stabilization of the heme o-O_2 complex or its reduction intermediate of the mutant enzyme is aided by a drastic decrease in the rate of intramolecular electron transfer. In the wild-type UQO the rate constant for the heme b-heme o electron transfer was assumed to be higher than 5×10^4 s^{-1} at pH 7.4 and 20°C [9]. On the contrary, the oxidation of the hemes in the mutant UQO takes several hundreds of seconds for completion. In reverse electron transfer experiments using a mixed-valence enzyme, Brown et al. have found that the absence of Cu_B in the H333L mutant does not have a dramatic effect on the heme o-heme b electron transfer proceeding with a minimum rate constant of 1×10^4 s^{-1} [28]. Accordingly, Cu_B does not partcipate in the heme-to-heme long range electron transfer. It may be mediated via super conduction mechanism through a covalent bond system consisting of side-chains of the axial ligands and its connecting peptide bond (His^{421}-Phe^{420}-His^{419}) [5]. If the rate constant of Brown et al. [28] holds for the forward heme b-to-heme o electron transfer, the apparent decrease in the rate constant for the heme b-to-ligand intramolecular electron transfer would be ascribed to a decrease rate constant for heme o-to-ligand electron transfer that should proceed in the dioxygen reduction.

An appreciable decrease in the heme b-to-ligand electron transfer and of dioxygen binding in the present study strongly suggests that Cu_B in the wild-type UQO plays a key role not only in promoting the preferential trapping of dioxygen at the heme-copper binuclear center but also in facilitating electron transfer from heme o to the bound dioxygen or its reaction intermediate(s) by tuning electronic states of the metal center. Cu_B may impose configurational constraints on the bound dioxygen as well as the reaction intermediates in favor of the forward reactions, and thus controls a reaction field for dioxygen reduction together with a bound chloride ion [10]. This mechanism renders a continuous electron delivery to the bound ligand through Cu_B [31] less likely, since Cu_B should be placed at the distal end of the heme b-heme o-Cu_B electron transfer pathway [5]. Structural as well as mechanistic significance of Cu_B in conferring these fundamental properties on UQO will be clarified by further studies.

Although the true binding rate constant of dioxygen to heme o in the mutant enzyme remains to be determined, a value of 8.0 s^{-1} in the presence of 0.7 mM dioxygen has been assumed tentatively. Again this is more than 3,000 times smaller than 2.6×10^4 s^{-1} determined for the wild-type UQO. Such a marked decrease in the binding rate constant for dioxygen clearly contrasts with the increased binding rate constant for CO that occurs as Cu_B is depleted of the wild-type enzyme [27,28]. Our finding poses a case that dioxygen behaves quite differently from CO and warns against a careless use of CO as a ligand model of dioxygen.

Recent molecular biological and biochemical studies indicate that a microaerobic cbb_3-type cytochrome c oxidase of leguminous bacteria is the most distant member of the heme-copper respiratory oxidase and subunit I of this primordial oxidase is evolved from the NorB subunit of nitric oxide reductase of the anaerobic, denitifying bacteria [32,33]. It should be noted that the NorB subunit contains both low-spin and high-spin heme bs but lacks Cu_B [33,34]. These findings support our proposal that Cu_B is a specific device engineered for a consecutive four-electron reduction of dioxygen, thereby suppressing release of reaction intermediates of dioxygen that are toxic for the aerobic growth of an ancestral aerobic bacterium.

Acknowledgements: We thank R.B. Gennis of University of Illinois for *E. coli* strain GO103, S. Hirota and T. Kitagawa for measurement of resonance Raman spectra, M. Ohno of Eisai Co. Ltd. for ubiquinone-1, and H. Qadota of University of Tokyo for technical comments. This work was supported in part by Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture, Japan.

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