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Cytochrome *d* axial ligand of the *bd*-type terminal quinol oxidase from *Escherichia coli*

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Using various spectroscopic techniques, we studied the structure of the dioxygen reduction site of the *bd*-type terminal quinol oxidase in the aerobic respiratory chain of *Escherichia coli*. Resonance Raman and FT-IR spectroscopies identified the $\nu(Fe^{2+}–CO)$ and $\nu(C–O)$ stretching frequencies at 471 and 1980.7 cm^{-1}, respectively, at the cytochrome *d* center of the dithionite-reduced CO-bound enzyme. The CO ligation in the cytochrome *bd* complex is considerably different from those of the heme-copper terminal oxidases. Anaerobic addition of NO to the air-oxidized enzyme caused an exchange of cytochrome *d*-bound dioxygen with NO leading to an appearance of cytochrome *d*–NO EPR signal. But there is no superhyperfine structure originating from the cytochrome *d* proximal ^{14}N ligand in the central resonance of the NO EPR signal. These results suggest that cytochrome *d* axial ligand of the cytochrome *bd* complex is likely a histidine residue in an anomalous condition or other than a histidine residue and, therefore, the molecular structure around the dioxygen-binding site is different from that of the heme-copper terminal oxidases.

Cytochrome *bd* complex; Cytochrome *d* axial ligand; Resonance Raman; Fe–CO bond; EPR; Nitric oxide

1. INTRODUCTION

The cytochrome *bd* complex is one of terminal quinol oxidases in the aerobic respiratory chain of *Escherichia coli* and expressed predominantly under low oxygen pressure. The cytochrome *bd* complex has a higher affinity for molecular oxygen and more resistant to respiratory inhibitors such as cyanide and azide than the cytochrome *bo* complex, an alternative quinol oxidase comprising the heme-copper binuclear center for the dioxygen reduction [1]. This enzyme is encoded by the *cydAB* genes [2] and consist of two polypeptides; subunit I (58 kDa) and subunit II (43 kDa) [3,4]. Based on optical spectroscopic properties, it is claimed, there are three types of cytochrome species associated with this complex; cytochrome b_{558}, cytochrome b_{595}, and cytochrome *d* [5,6]. Subunit I contains cytochrome b_{558} that shows the α and β peaks at 562 and 532 nm, respectively, in the reduced state at room temperature [7] and is most likely the ubiquinol-8 oxidation site [8]. Cytochrome b_{595} is an unusual *b*-type cytochrome exhibiting its α and β bands at 595 and 562 nm, respectively, in the reduced *minus* oxidized difference spectrum [6]. Cytochrome *d* has a chlorin chromophore (heme D) [9], exhibiting a characteristic absorption maximum at 628 nm in the fully reduced state and is a primary exogenous ligand binding site [3,10]. In the air-oxidized condition, cytochrome *d* is actually in the reduced state and coordinates a molecular oxygen [11]. On the other hand, EPR studies on the cytochrome *bd* complex revealed that there are a total of four heme species in the air-oxidized state besides cytochrome *d* which exists as a $Fe^{2+}–O_2$ diamagnetic EPR-invisible state: two high-spin heme components (one axial and one rhombic species) and two minor low-spin heme components at $g_z = 3.3$ and $g_z = 2.5$. Although the assignment for these EPR signal is controversial, there seems a consensus that the $g_z = 2.5$ species represents a subpopulation of cytochrome *d* [10,12–14].

Here we report results of a combined study using resonance Raman, FT-IR and EPR spectroscopies for the purified cytochrome *bd* complex from *E. coli*. We present a line of evidence to suggest that the proximal ligand of cytochrome *d* component of the cytochrome *bd* complex is likely different from a usual histidine ligand.

2. MATERIALS AND METHODS

2.1. E. coli *strain and growth conditions*

E. coli strain ST4533 (W3092 *Δcyo*-Km^r *cyd*$^+$ *recA srlA*::Tn10) which lacks the entire cytochrome *bo* operon was used in this study. Cells were grown at 37°C in a rich medium [15] supplemented with

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50 μg/ml $FeSO_4 \cdot 7H_2O$ and 50 μg/ml kanamycin sulfate (Sigma). One liter of the overnight culture was inoculated into 10 liters of fresh medium in a Magnaferm jar fermentor (New Brunswick Scientific, NJ). Cells were grown aerobically with agitation at 800 rpm and aeration at 12.5 liters/min for about 8 h until OD_{650} exceeds 10, and then continued to grow less aerobically at 200 rpm and 2 liters/min for 40 h to induce the expression of the cytochrome *bd* complex.

2.2. *Purification of the cytochrome* bd *complex*

The cytochrome *bd* complex was purified by the method described for a large scale preparation of the cytochrome *bo* complex [15], except that solubilization of the cytochromes was carried out in the presence of 10 mM Tris-HCl (pH 7.4) instead of 100 mM buffer.

2.3. *Vibrational spectroscopies*

Dithionite-reduced CO-bound *minus* air oxidized and air-oxidized CO-bound *minus* air-oxidized FT-IR difference spectra were recorded at 10°C with a nominal spectral resolution at 2.0 cm^{-1} as previously described [16]. Absolute optical spectra of the cytochrome *bd* complex in the infrared cells were measured at room temperature.

Resonance Raman spectra of the dithionite-reduced CO-bound enzyme were obtained with excitation at 406.7 nm using a Kr^+ ion laser as described previously [17,18]. The *ν*(Fe–CO) stretching mode was assigned by an isotope shift with $^{13}C^{18}O$ (99 atom % of ^{13}C, 98 atom % of ^{18}O; Shoko Tsusho Co. Ltd., Tokyo).

2.4. *EPR spectroscopy*

EPR measurements were carried out at X-band (9.23 GHz) microwave frequency with a home-built EPR spectrometer with 100 kHz field modulation by using a Varian X-band cavity as described previously [15]. The combination of nitric oxide (NO) with the air-oxidized cytochrome *bd* complex was carried out anaerobically as follows. After the repeated evacuation and flushing of pure nitrogen, NO gas passed through 1 N KOH solution was introduced into an EPR tube sealed with a rubber septum.

2.5. *Biochemical analysis*

Protein concentration was determined by using BCA protein assay reagent (Pierce, Rockford). Heme B content was determined by the pyridine hemochromogen method [19], whereas cytochrome *d* content was estimated from the reduced *minus* oxidized difference spectrum using an extinction coefficient (10.7 $mM^{-1} \cdot cm^{-1}$ at the wavelength pair of 627–650 nm) of the cytochrome b_{560}–*d* complex from *Photobacterium phosphoreum* [20].

3. RESULTS AND DISCUSSION

3.1. *Vibrational spectra*

FT-IR spectrum of the dithionite-reduced $^{12}C^{16}O$-bound enzyme showed an unusually high *ν*(C–O) stretching frequency at 1980.7 cm^{-1} with a relatively narrow band width ($\Delta_{1/2}$ = 4.5 cm^{-1}) (Fig. 1A, upper). This frequency is very close to the value of 1984 cm^{-1} at low temperature (e.g. 12–20K) reported for membrane vesicles prepared from the *E. coli* cytochrome *bd* complex-overproducing strain [21]. The binding of CO to the ferrous cytochrome *d* was confirmed by a shift of a sharp 629.5 nm peak originated from ferrous cytochrome *d* of the dithionite-reduced enzyme toward higher wavelength to 635 nm (data not shown).

Carbon monoxide (CO) can bind to cytochrome *d* of the cytochrome *bd* complex even in the air-oxidized form [3], since the enzyme in the air-oxidized state is actually an oxygenated state [11] containing about one molecule of dioxygen bound to ferrous cytochrome *d*

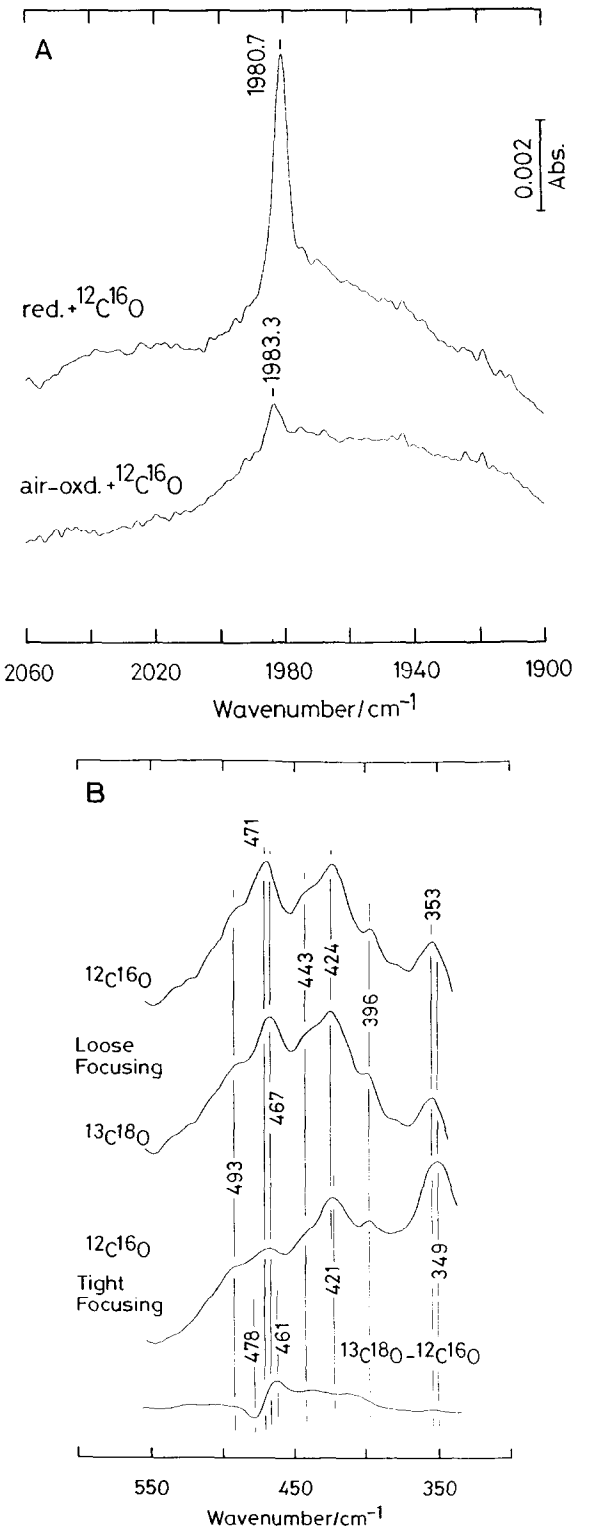

Fig. 1. (A) FT-IR spectra in the C–O stretching region of the cytochrome *bd* complex in the dithionite-reduced CO-bound form (upper) and in the air-oxidized CO-bound form (lower) at 10°C. (B) Resonance Raman spectra in the Fe–CO stretching region of the cytochrome *bd* complex in the dithionite-reduced CO-bound form (in the order of $^{12}C^{16}O$, $^{13}C^{18}O$, both with loose focusing, $^{12}C^{16}O$ with tight focusing, and the difference of $^{13}C^{18}O$ *minus* $^{12}C^{16}O$, from upper to bottom) at room temperature. Laser excitation at 406.7 nm. Sample concentration: ~ 200 μM for FT-IR and ~ 50 μM for resonance Raman measurements in 50 mM Tris-HCl (pH 7.4) containing 0.1% sucrose monolaurate.

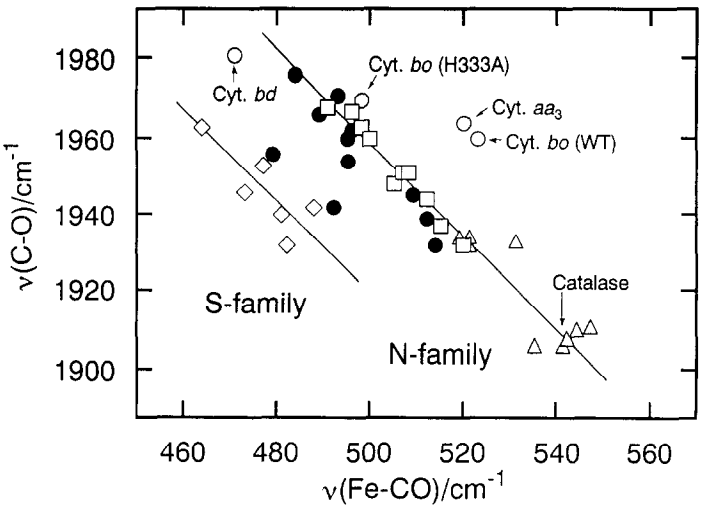

Fig. 2. The ν(Fe–CO) vs. ν(C–O) plot analysis of various ferrous hemoprotein carbonyls and their model complexes. Data points are adopted from Uno et al. (unpublished).

[10,12,22]. This was confirmed in the visible absorption spectra as a slight shift of a peak at 645.5 nm derived from ferrous cytochrome *d*–O_2 of the air-oxidized enzyme to 636 nm upon exposure to CO (data not shown). The FT-IR spectrum of the same sample showed the ν(C–O) stretching band at 1983.3 cm^{-1}, but with a much lower intensity than that of the dithionite-reduced CO-bound form, indicating that CO can bind to only a part of ferrous cytochrome *d* in the air-oxidized enzyme (Fig. 1A, lower). Comparison of the integrated areas of the ν(C–O) stretching band showed that only 15–20% of the total cytochrome *d* exchanged the bound molecular oxygen with exogenous CO. This can be ascribed to an extremely high binding affinity of the ferrous cytochrome *d* for molecular oxygen (K_m = 0.38 μM) [4].

Resonance Raman spectra of the dithionite-reduced CO-bound form showed that a relatively sharp band at 471 cm^{-1} with $^{12}C^{16}O$ and at 467 cm^{-1} with $^{13}C^{18}O$ (Fig. 1B). Tight focusing of the laser beam caused the weakening of the band intensity. The difference spectrum of $^{13}C^{18}O$ *minus* $^{12}C^{16}O$ showed a peak at 461 cm^{-1} and a trough at 478 cm^{-1}. These confirm the band as a ν(Fe–CO) stretching mode, although the observed 4 cm^{-1} downshift upon isotope replacement from $^{12}C^{16}O$ to $^{13}C^{18}O$ is rather unusual [23]. The observed ν(Fe–CO) stretching frequency is extremely low compared with that of the cytochrome *bo* complex (523 cm^{-1} with $^{12}C^{16}O$), the heme-copper terminal oxidase in the *E. coli* aerobic respiratory chain [24,25] and is very similar to those of the cytochrome P_{450} carbonyl complexes [26–28] which are known to have a thiolate axial ligand *trans* to bound carbon monoxide. The ν(Fe–CO) stretching mode was not detected for the CO-bound air-oxidized form, probably due to a low population of the CO-bound cytochrome *d* component.

To evaluate the nature of the Fe–CO bonding of the cytochrome *d*–CO complex, we conducted the ν(Fe–CO) vs. ν(C–O) plot analysis [18,29–31] (Fig. 2). It is known that the ν(Fe–CO) and ν(C–O) stretching frequencies of a wide variety of hemoproteins (and model complexes) show an inverse-linear correlation, and two sets of lines (N-family and S-family) can be drawn to be parallel with each other corresponding to the proximal ligands *trans* to CO. N-family has a nitrogenous imidazole ligand derived from a His residue and S-family has a cysteinyl thiolate ligand. (It is not known whether such a correlation still holds for chlorin-carbonyls, but it was reported that the effect of the change from porphyrin to chlorin skeleton on the axial ligand vibrations is unexpectedly small [32].) The data point for the cytochrome *bd*–CO complex locates near the left-upper portion of the line for N-family, but deviates in the direction toward the line for S-family [28,29]. Such a deviation is known to occur with stronger donor axial ligands, such as, imidazolate and strongly H-bonded imidazole [30]. These unusual bound CO vibrations suggest that cytochrome *d* axial ligand is not a usual His ligand but either a His residue in an anomalous condition for a different amino acid residue.

The imidazolate or strongly H-bonded imidazole axial ligand *trans* to CO is highly possible as a cause of the deviation from the line for N-family toward S-family. But all the known hemoprotein species having with these proximal ligands have a tendency to show a much lower bound ν(C–O) stretching frequency [30].

Other candidate residues for the axial ligand of cytochrome *d* are tyrosine (as catalase), methionine (as cytochrome *c*) and cysteine (as cytochrome P_{450}, chloroperoxidase, and NO synthase). However, in case of phenolate coordination *trans* to CO, the ν(Fe–CO) mode is unusually high at 542 cm^{-1} while the ν(C–O) mode is low at 1908 cm^{-1} [33]. So the data point for catalase is placed at just opposite side in the plot (Fig. 2). It should be noted that Cys-214 in the middle of the transmem-

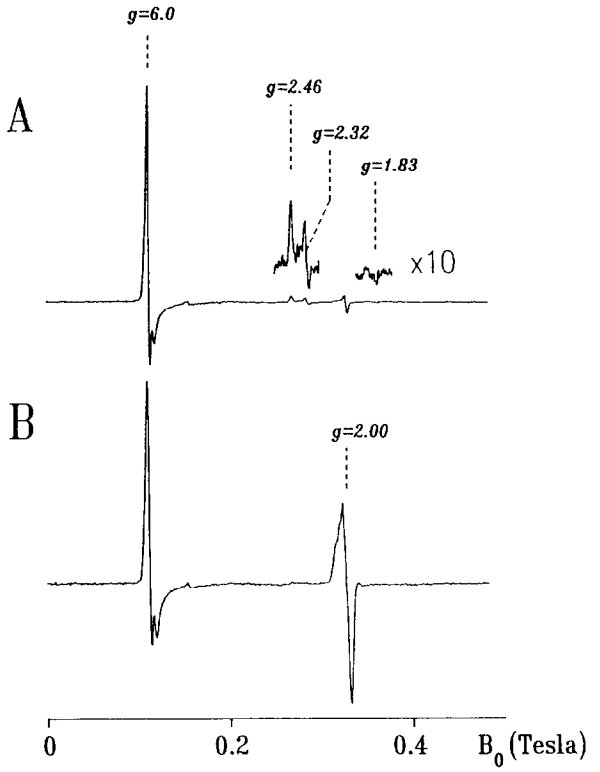

Fig. 3. EPR spectra of the purified cytochrome *bd* complex at 15K. (A) In the air-oxidized state. (B) In the presence of nitric oxide (^{14}NO) under anaerobic condition. Sample conditions are the same as for the FT-IR measurements.

brane helix V of subunit II is only one cysteine residue totally conserved in the three known sequences of the cytochrome *bd* complex [2,34–36]. Difficulty in the assignment of a cysteinyl thiolate ligation is the absence of a ν(O–O) stretching band in the resonance Raman spectrum of the oxygenated form of the cytochrome *bd* complex [11]. It is well known that a thiolate axial ligation *trans* to O_2 causes a strong resonance enhancement of the heme bound ν(O–O) stretching Raman band [37,38].

3.2. *EPR spectra*

The air-oxidized cytochrome *bd* complex exhibited an intense $g = 6.0$ ($g_{x,y}$) axial high-spin signal and, overlapped with this, rhombic high-spin signals at $g = 6.15$ (g_x) and 5.70 (g_y) (Fig. 3A) at 15K as previously reported [10,14]. The minor ferric low-spin signal observed in [10] could be seen at $g = 2.46$ (g_z), 2.32 (g_y), and 1.83 (g_x). The other ferric low-spin signal also could be observed at $g = 3.31$ at 5K as previously reported [13,14], but not at 15K.

Anaerobic addition of nitric oxide (^{14}NO) to the air-oxidized form caused an appearance of an axial NO EPR signal around $g = 2$ region without eliminating the $g = 6$ high-spin signals (neither the axial nor rhombic signal) at 15K, although the line shapes of the high-spin EPR signals changed considerably (Fig. 3B; detail not shown). The weak ferric low-spin signals at $g = 2.46$, 2.32, and 1.83 disappeared completely indicating a formation of the diamagnetic ferric cytochrome *d*–NO complex. The other EPR signal at $g = 3.31$ showed an upfield shift to $g = 3.02$ at 5K.

A rough estimation of spin contents of these EPR-visible species at 15K by double integration indicated that the ferrous-heme-NO EPR signal and the $g = 6$ high-spin signals each corresponds to about 1 mol of heme/1 mol of the cytochrome *bd* complex. The ferric low-spin signals at $g = 2.46$, 2.32 and 1.83 in the air-oxidized state account for only less than 10% of the total EPR signal. The formation of the ferrous cytochrome *d*–NO species in the air-oxidized state is consistent with an exchange of the ferrous cytochrome *d*-bound dioxygen with exogenous NO. Present results indicate further that there is no direct binding of NO to the ferric high-spin heme(s) but there may be conformational interactions between the NO binding site (i.e. cytochrome *d*) and the ferric high-spin heme(s).

There was no small triplet splittings in the central triplet of the NO EPR signal being ascribed to the superhyperfine interaction of ^{14}NO with another axially bound ^{14}N nuclei (i.e. ^{14}N of a proximal His ligand) [39] from 5 to 35K. Second derivative spectra confirmed the absence of such a superhyperfine interaction for the ferrous cytochrome *d*–NO complex (data not shown). The central resonance of the EPR signal of the ferrous heme–NO complex has been assigned to the axial or z-absorption (g_z) and small triplet hyperfine splittings in the g_z-signal can be reasonably ascribed to the superhyperfine interaction with another ^{14}N nucleus *trans* to NO [39]. But the present result does not mean necessarily that the axial ligand of cytochrome *d* is other than a His residue. Absence of the superhyperfine splitting may indicate that the unpaired electron of NO is hardly delocalized toward the *trans* axial base ligand [40,41], although it was stated that "preliminary ENDOR studies suggest that the proximal ligand of chlorin *d* is not a histidine" (T.M. Zuberi, R.B. Gennis, F. Jiang and R.L. Belford, unpublished results) (cited in [42]).

There are three invariant histidine residues (His-19 and His-186 in subunit I and His-56 in subunit II) in the cytochrome *bd* complex of *E. coli* [2,36] and *Azotobactor vinelandii* [35]. A site-directed mutagenesis study on the *E. coli* cytochrome *bd* complex [34] showed that only two His residues appeared to function as heme axial ligands. His-186 in subunit I is most likely a heme axial ligand to cytochrome b_{558} and His-19 in subunit I is likely an axial ligand to either cytochrome *d* or cytochrome b_{595} [34]. Cytochrome b_{595} is more likely to have an axial histidine ligand according to the suggestion of the optical spectroscopic similarities to cytochrome *c* peroxidase [6] which contains an axial histidine ligand. This argument leaves no available histidine residue for cytochrome *d* axial ligand.

However present biochemical analysis of the purified cytochrome *bd* complex showed that the average contents of heme B and cytochrome *d* of four preparations were 9.2 and 10.5 nmol/mg protein, respectively. This suggests that the cytochrome *bd* complex contains only one mole each of cytochrome *b* and cytochrome *d* as the redox components, as reported for the cytochrome b_{560}–*d* complex in *Photobacterium phosphoreum* which shows very similar optical absorption spectra with those of the cytochrome *bd* complex [20]. Therefore the $g = 6$ high-spin (both the axial and rhombic) signals and the $g = 3.31$ signal are likely derived from the same one cytochrome *b* component but in different conditions. The present spin content analysis seems to support this view. In this case, the possibility of the His axial ligation to cytochrome *d* cannot be ruled out.

In conclusion the molecular structure around the oxygen binding site of the cytochrome *bd* complex seems very different from that of the heme-copper terminal oxidase family and other dioxygen-carrying hemoproteins, even if a His residue is actually the axial ligand of cytochrome *d*. Therefore, a reliable assignment of the axial ligands of the heme prosthetic groups is highly desired for further understanding of the dioxygen reduction mechanism of this unique terminal oxidase, the cytochrome *bd* complex.

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