Cyanide-binding Site of bd-type Ubiquinol Oxidase from Escherichia coli*

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We extended our investigation on the structure of the redox centers of bd-type ubiquinol oxidase from Escherichia coli using cyanide as a monitoring probe. We found that addition of cyanide to the air-oxidized O₂-bound enzyme caused appearance of an infrared C-N stretching band at 2161 cm⁻¹ and concomitant disappearance of the 647 nm absorption band of the cytochrome d (Fe⁺²)–O₂ species. Addition of cyanide to the air-oxidized CO-bound enzyme also resulted in disappearance of the 635 nm absorption band and the 1983.4 cm⁻¹ C–O infrared band of the cytochrome d (Fe⁺²)–CO species. The resulting species had a derivative-shaped electron paramagnetic resonance signal at g = 3.15. Upon partial reduction with sodium dithionite, this species was converted partly to a transient heme d (Fe⁺³)–C=N species having an electron paramagnetic resonance signal at g = 2.96 and a C–N infrared band at 2138 cm⁻¹. These observations suggest that the active site of the enzyme has a heme-heme binuclear metal center distinct from that of the heme-copper terminal oxidase and that the treatment of the air-oxidized enzyme with cyanide resulted in a cyanide-bridging species with "heme d(Fe⁺³)–C=N–heme bd₅₉₅(Fe⁺⁵)" structure.

Escherichia coli has two terminal ubiquinol oxidases in the aerobic electron transfer chain: bo-type ubiquinol oxidase, which is expressed under high oxygen tension, and bd-type ubiquinol oxidase, which predominates under low oxygen tension (1, 2). These oxidases are structurally unrelated, but both catalyze the two-electron oxidation of ubiquinol-8 and the four-electron reduction of dioxygen to produce water (1, 2). The E. coli bd-type ubiquinol oxidase has been isolated and was found to consist of two polypeptide chains: subunit I (58 kDa) and subunit II (43 kDa) (3, 4). The cydAB genes coding for both polypeptides have been cloned and sequenced (5). Within this enzyme, it has been claimed, there are three types of cytochrome heme species based on the optical spectra: cytochrome d, cytochrome bd₅₉₅, and cytochrome bd₅₅₈ (6, 7). Cytochrome d has a chlorin chromophore (heme D) (8) exhibiting a characteristic absorption maximum at 628 nm in the fully reduced state and is a primary binding site for exogenous ligands (3, 4, 9). The dioxygen molecule forms a very stable adduct with ferrous cytochrome d showing its Fe⁺²–O₂ stretching vibration at 568 cm⁻¹ (essentially identical to that of myoglobin) (10), and thus the enzyme in the air-oxidized state is actually an oxygenated form. The cytochrome d moiety also forms a remarkably stable oxoferryl (Fe⁺⁵–O) adduct (11). Subunit I contains cytochrome bd₅₉₅ that shows its α and β peaks at 562 and 532 nm, respectively, in the reduced state (12) and most likely forms the ubiquinol-8 binding site (13). Cytochrome bd₅₉₅ is an unusual b-type cytochrome. It was suggested that the optical spectrum of this cytochrome is very similar to that of high spin cytochrome c peroxidase, and its α and β bands are at 595 and 562 nm, respectively, in the reduced minus oxidized difference spectrum (7). Recent low temperature CO photolysis experiments monitored by the infrared C–O stretching absorption signal suggest that cytochrome d and cytochrome bd₅₉₅ form a binuclear center (14, 15).

In a previous study we analyzed the purified bd-type ubiquinol oxidase by resonance Raman, FT-IR, and EPR spectroscopies (16). We found that heme d-bound C–O and Fe⁺²–CO stretching frequencies were at 1807.4 and 471 cm⁻¹, respectively, in the fully reduced state. These values differ considerably from those of the heme-copper respiratory oxidases and the oxygen-carrying hemoproteins, both groups possessing a His residue as the heme axial ligand (17–20). EPR analyses on the air-oxidized O₂-bound and Na₂S₂O₄-reduced nitric oxide-bound forms revealed that there is no superhyperfine structure originating from the heme axial ¹⁴N ligand in the central resonance of the nitric oxide EPR signals (16). These results suggest strongly that the heme d axial ligand of bd-type ubiquinol oxidase is either a His residue in an anomalous condition or some other residue making the molecular structure around the oxygen-binding site different from those of the heme-copper respiratory oxidases. Indeed, electron nuclear double resonance spectroscopy has shown that the axial ligand to heme d is most probably not a histidine or other strong nitrogenous ligand (21). In the present study we extended our investigation on the redox centers of the E. coli bd-type ubiquinol oxidase using cyanide as a monitoring probe.

EXPERIMENTAL PROCEDURES

Purification of bd-type Ubiquinol Oxidase—The E. coli bd-type ubiquinol oxidase was isolated from the cytoplasmic membranes as described (22) with the slight modification that Tris-HCl buffer was replaced by sodium phosphate buffer and that magnesium chloride was omitted during solubilization. The strain GR84N (jcg2U, oyo' cydA2 recA1 cydAB7 Tef') (23), a kind gift from R. B. Gennis, which can overproduce bd-type ubiquinol oxidase, was used. The purified enzyme in 50 mM sodium phosphate (pH 7.4) containing 0.1% sucrose monolaurate SM-1200 (Mitsubishi-Kasei Food Corp., Tokyo) was stored at –80 °C until use.

Measurements of EPR, FT-IR, and Optical Spectra—EPR measure-

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§ The abbreviations used are: FT-IR, Fourier-transform infrared; EPR, electron paramagnetic resonance.
ments were carried out at X-band (9.23 GHz) microwave frequency with a home-built EPR spectrometer as described previously (16, 22) and a Varian E-12 EPR spectrometer equipped with an Oxford flow cryostat (ESR-900). Infrared spectra were recorded at 4 °C with a Perkin-Elmer (model 1850) FT-IR spectrophotometer. Absolute optical spectra of bd-type ubiquinol oxidase in the infrared cells with a 51-µL path length were measured at room temperature with a UVIKON 860 UV-visible spectrophotometer (Kontron Instruments, Inc.) before and after FT-IR measurements.

Miscellaneous—Protein concentration was determined using the BCA protein assay reagent (Pierce). Metal contents were determined by inductively coupled plasma atomic emission spectrophotometry with a SPS 1200VR plasma spectrometer (Seiko Instruments Inc., Tokyo). Heme B contents were analyzed as described previously (23, 25). The following potassium cyanide isotopes were used: K13C14N (natural abundance, Nacalai Tesque); K12C14N (99.4 atom % 13N, Isotec Inc.); K13C15N (99 atom % 13C, 99 atom % 15N, Icon). Other chemicals were commercial products of analytical grade.

RESULTS

Heme B, Metal Contents, and Optical Extinction Coefficients—The heme B and metal contents of the purified bd-type ubiquinol oxidase were examined. Heme B content was 18.2 ± 1.6 nmol/mg protein, whereas the heme B:Fe:Cu:Zn ratio (in mol basis) was 2.35 ± 0.60:0.084 ± 0.003:0.098 ± 0.026 (averages of three independent preparations). Theoretical value of the heme B content is 19.8 nmol/mg protein assuming the presence of 7 mol of heme B/mol of bd-type ubiquinol oxidase with a molecular mass of 101 kDa (3–5). It was reported that bd-type ubiquinol oxidase contains only 1 mol of cytochrome d/mol of the enzyme (14). Thus, previous and present heme B and metal content analyses of bd-type ubiquinol oxidase indicate that there is 1 mol of heme D and 2 mol of heme B and adventitious non-heme iron/mol of the enzyme and no other constitutive metals (including magnesium, calcium, chromium, manganese, cobalt, nickel, zinc, selenium, molybdenum, and tungsten) (4). On the basis of the present analyses, the optical extinction coefficients were calculated (Table I). The results show considerable deviations from those previously reported (3, 4, 7).

Optical Spectroscopy—The optical absorption spectral change upon addition of cyanide to the air-oxidized O2-bound enzyme was examined. The 647 nm band that is known to originate from the cytochrome d (Fe2+)–O2 species (10) disappeared in a time-dependent manner (Fig. 1), as previously reported (26, 27). Simultaneously a Soret band spectral change (a peak at 431 nm and a trough at 411 nm in the air-oxidized cyanide-treated minus air-oxidized O2-bound difference spectrum) was observed (Fig. 1) (28). Exposure of the air-oxidized O2-bound sample to a CO atmosphere caused a shift of the 647 nm peak to 635 nm, consistent with the formation of the cytochrome d (Fe2+)–CO species (3, 4, 16). The addition of cyanide to this air-oxidized CO-bound enzyme also caused the characteristic 635 nm band to disappear. The resulting species in both cases had a very broad absorption band around 630 nm, indicative of the oxidation of cytochrome d. Pretreatment of the air-oxidized O2-bound (or air-oxidized CO-bound) enzyme with cyanide retards the appearance of the reduced cytochrome d absorption band at 629 nm upon the addition of Na2S2O4 (spectra not shown), consistent with the previous results (29). On the other hand, the addition of cyanide (10 mM) to the fully reduced (with Na2S2O4) form of bd-type ubiquinol oxidase did not affect the visible absorption spectrum (data not shown) (29, 30).

FT-IR Spectroscopy—Just after the addition of cyanide (5 mM) to the air-oxidized O2-bound enzyme, there was no infrared band assignable to a bound cyanide species, except for a band of free H12C14N at 2093 cm−1 (31). A weak feature at 2161 cm−1 developed in a time-dependent manner (spectra not shown). This band showed cyanide isotope-sensitive shifts to 2129 cm−1 (12C15N), to 2114 cm−1 (13C14N), and to 2082 cm−1 (13C15N). The identical cyanide band developed, but more quickly, upon the addition of cyanide (5 mM) to the air-oxidized CO-bound enzyme, with a concomitant disappearance of the cytochrome d(Fe2+)–C–O stretching band at 1983.4 cm−1 (Fig. 2, A and B). The addition of Na5S4O4 to the cyanide-pretreated enzyme in the CO atmosphere reversed the spectral change. The cytochrome d(Fe2+)–C–O stretching band at 1980.7 cm−1 developed in a time-dependent manner with a concomitant decrease of the 2161 cm−1 band intensity (Fig. 2, C, D, and E). A shoulder around 1975 cm−1 was observed in the early stage of the spectral change (Fig. 2C), and a weak C–N infrared band at 2138 cm−1 was observed during the spectral change (Fig. 2C). Pretreatment of the air-oxidized O2-bound enzyme with Na5S4O4 and CO before the addition of cyanide produced only one infrared band of the heme d(Fe2+)–bound C–O stretching mode at 1980.7 cm−1 (16). The 2161 cm−1 species was not observed at all under the fully reduced conditions.

EPR Spectroscopy—The addition of cyanide (5 mM) to the air-oxidized O2-bound enzyme just before the EPR measurement caused changes in the spectral line shape of the g = 6 high spin signal region (spectra not shown). The line shapes of both the rhombic and axial components are altered, with the axial component being slightly broader and the rhombic signal being further distorted. These observations are essentially consistent with those reported for the membrane-bound bd-type ubiquinol oxidase (32). However, the intensity of the high spin

| Sample | Wavelength pair | ε | cm−1 | pmol/mg
|---|---|---|---|---|
| Air-oxidized (oxygenated) | 414/700 | 223,000 (3) |
| Redox difference | (air-oxidized O2-bound minus reduced) | 628/651 | 27,900 (3) |
| Heme B | 561/580 | 21,000 (3) |
| Metal contents | 599/606.5 | 1,900 (3) |
| Na5S4O4-reduced (–O2) | 437/465 | 187,000 (3) |
| Reduced CO-bound (–O2) | 429/700 | 303,000 (1) |
| Air-oxidized CN-bound | 416/700 | 185,000 (1) |
Figure 2. FT-IR spectra of bd-type ubiquinol oxidase in the C-O and C-N stretching vibration region. The air-oxidized O₂-bound enzyme was exposed first to carbon monoxide (¹²C¹⁶O) atmosphere (A); then cyanide (¹²C¹⁴N; 5 mM) was added anaerobically to the air-oxidized CO-exposed enzyme, (B, taken 40 min after the addition of cyanide). Then Na₂S₂O₄ was added anaerobically to the air-oxidized CO-exposed cyanide-treated enzyme, and the development of the 1980.7 cm⁻¹ band and decay of the 2161 cm⁻¹ band were recorded (C, D, and E; taken just after, 100 min after, and 16 h after the addition of Na₂S₂O₄, respectively). Sample concentration was 0.60 mM (1.21 mM in heme B concentration). The band at 2093 cm⁻¹ is due to free H¹²C¹⁴N.

signals did not change appreciably compared with that of the air-oxidized O₂-bound state before addition of cyanide. Prolonged incubation of the enzyme with cyanide caused diminution of the g = 6 rhombic signal, whereas the axial high spin signal was little changed during the incubation (spectra not shown).

Cyanide also caused disappearance of ferric low spin signals at gₓ = 2.46, gᵧ = 2.32 (Fig. 3, A and B), and gᵧ = 1.83 (not shown) at 15 K. Those were assigned to a minor form of ferric cytochrome bd (9). Concomitantly, a derivative-shaped EPR signal at gₓ = 3.15 developed (Fig. 3B) together with another low spin signal at gᵧ = 2.82. Prolonged incubation of the sample at 4°C in the dark caused an increase in intensity of these EPR signals (Fig. 3C). The EPR signal at gₓ = 3.15 is distinct from the gₓ = 3.3 signal previously assigned as ferric low spin cytochrome b₅₅₃ (32–34), although both signals overlapped.

Anaerobic addition of Na₂S₂O₄ to this cyanide-preincubated form caused a rapid disappearance of the high spin signals together with the low spin signal at gₓ = 2.82. The derivative-shaped EPR signal at gₓ = 3.15 persisted and a transient EPR species with gₓ = 2.96 appeared (Fig. 3D). The gₓ = 2.96 signal can be ascribed to a cyanide adduct of cytochrome d(Fe³⁺⁻CO) (32, 35). Prolonged incubation of the Na₂S₂O₄-treated sample on ice removed the derivative-shaped EPR signal and the transient EPR signal completely.

DISCUSSION

Number of Metal Centers in bd-type Ubiquinol Oxidase—The heme B and iron contents of the purified enzyme suggest that there are only three cytochromes present. Thus, we conclude that bd-type ubiquinol oxidase is a cytochrome bbd-type enzyme as reported previously (4, 6, 7). Assuming this structure, we recalculated the extinction coefficients for various wavelength pairs (Table I). The new set gave us reasonable estimation of the number of metal centers in the oxidase. We found that the preparations of Kita et al. (3), Konishi et al. (36), and Lorenz et al. (7) all contain two cytochromes b and one cytochrome d in the oxidase (i.e. 2:1.05, 2:0.76, 2:1.08 (b₅₅₃:b₅₅₃:d = 1:1:02:1.09, respectively).

FT-IR Spectra—Only one bound cyanide infrared band (2161 cm⁻¹) was observed upon the addition of cyanide to the air-oxidized O₂-bound enzyme. The increase of the band intensity was in parallel to the intensity decrease of the optical absorption band at 647 nm (Fig. 1). The same infrared band was formed upon the addition of cyanide to the air-oxidized CO-bound enzyme with concomitant disappearances of the 1983.4 cm⁻¹ C-O bound infrared band and the 635 nm band of cytochrome d (Fig. 2). Thus, it is quite certain that cyanide binds to the cytochrome d(Fe³⁺⁻CO) center. The frequency is in the region of the so-called “bridging cyanide” band to metals in the oxidized state (i.e. 2181 – 2141 cm⁻¹) (22, 37, 38). Therefore, it is very likely that the 2161 cm⁻¹ band is due to a species having a cyanide-bridging structure between heme d(Fe³⁺) and one of the other two ferric heme irons.

EPR Spectra—The postulated cyanide-bridging species is expected to be either EPR-inactive or to have an unusual EPR character because the spin-spin coupling depends on the relative geometry and the spin state of the participating metal centers. The derivative-shaped EPR signal at gₓ = 3.15 is likely derived from the cyanide-bridging species (Fig. 3). The ferric low spin EPR signals at gₓ = 2.46, gᵧ = 2.32, and gᵧ = 1.83 that are assignable to a minor form of ferric cytochrome d (9) seem at least partially responsible for the appearance of the peculiar

Recently, R. B. Gennis recalculated and obtained essentially the same extinction coefficients for the E. coli oxidase (personal communication).
signal at g = 3.15 and the low spin signal at g = 2.82, because these EPR signals appeared just after the addition of cyanide. However, a major part of the g = 3.15 species developed as the oxidation of the oxygenated form of cytochrome d(Fe$^{3+}$) proceeded after the addition of cyanide.

On the other hand, the g = 6 high spin rhombic EPR signal diminished appreciably upon prolonged incubation with cyanide, whereas the axial high spin signal changed little during the incubation. Although the assignment for the g = 6 high spin axial signal is still controversial, there seems to be a consensus that the g = 6 high spin rhombic signal is due to cytochrome b$_{595}$ (32, 33, 39). From these pieces of evidence we conclude that the cyanide-bridging structure at the active site is a kind of “heme d(Fe$^{3+}$)-C–N–heme b$_{595}$(Fe$^{3+}$).”

The nature of the EPR signal at g = 2.82 is not clear because the intensity of this signal was preparation-dependent. This may be due to a minor form of heme d(Fe$^{3+}$)-CN derived from the cyanide-bridging species in which the bridging structure is partially destroyed upon freezing. This scenario is consistent with the observation of only one C–N stretching infrared band (i.e. the 2161 cm$^{-1}$ band) at room temperature. However, other possibilities (e.g. heme b$_{595}$(Fe$^{3+}$)-CN or heme b$_{595}$(Fe$^{6+}$)-NC species) cannot be completely excluded.

The addition of Na$_2$S$_2$O$_4$ to the preformed cyanide complex produced a new, but transient, low spin heme species having with the g$_∥$ = 2.96 EPR signal (Fig. 3D) and the 2128 cm$^{-1}$ low spin cyanide infrared band (Fig. 2). This species is assignable to heme d(Fe$^{3+}$)-CN unambiguously (32, 35). The observed spectral changes are most likely due to a breakage of the cyanide-bridging structure caused by the reduction of cytochrome b$_{595}$ heme itself or by the conformational change around the binuclear center upon reduction of cytochrome b$_{595}$ heme. Such influence of the redox states of other metal centers on the heme d has been observed for the bound C–O stretching frequencies, i.e. the C–O band appears at 1980.7 cm$^{-1}$ in the fully reduced state, whereas it is at 1983.4 cm$^{-1}$ in the partially reduced state (Fig. 2) (16). These spectral characteristics are indicative of the heme-heme interactions and have some similarities to those of the heme-copper oxidase (22, 37, 40).

The lower g$_∥$ value (2.96 for the partially reduced state and 2.82 for the air-oxidized state) and the higher C–N stretching frequency (2128 cm$^{-1}$) of the heme d(Fe$^{3+}$)-CN moiety compared with those of corresponding species of the heme-copper oxidase (g$_∥$ = 3.24 (41) and 2123 cm$^{-1}$ for the E. coli bo-type ubiquinol oxidase and g$_∥$ = 3.58 (42) and 2132 cm$^{-1}$ (37) for bovine cytochrome c oxidase) might be ascribed, however, mainly to the chlorin macrocycle of heme d. It was reported that cyanide adducts of ferric hemoproteins containing chlorin macrocycle exhibited a g$_∥$ value as low as 2.6 (43). It is also known that presence of electrophorating groups (such as hydroxyl groups) on iron porphyrin macrocycle leads to an increase of the bound C–N stretching vibration (31).

Structure of the Heme-Heme Binuclear Metal Center—Hill et al. (14, 15) claimed that there is a heme d-heme b$_{595}$ binuclear center at the catalytic site of bd-type ubiquinol oxidase on the basis of the low temperature FT-IR study. Heme b$_{595}$ traps the photolyzed CO from cytochrome d and exhibits the 1974 cm$^{-1}$ band at cryogenic temperature (14). Later, Krasnoselskaya et al. (28) proposed that cyanide reacts with the air-oxidized O$_2$-bound bd-type ubiquinol oxidase to cause a decomposition of the cytochrome d-O$_2$ complex and an oxidation of heme d resulting in a formation of a cytochrome d(Fe$^{3+}$)-CN. As one possibility, they proposed a structure like “heme d(Fe$^{3+}$)-C–N–heme b$_{595}$(Fe$^{3+}$).” This is consistent with our present conclusion.

We should consider, at this point, a possible structure of the heme-heme binuclear center to obtain detailed insight into the dioxygen reduction mechanism of bd-type quinol oxidase. In the cyanide-bridging structure, the C atom of the cyanide is likely bound to the Fe atom of heme d with the Fe–C bond being perpendicular to the heme plane (with the Fe–C and C–N bond lengths as 1.9 and 1.1 Å, respectively (38)). The Fe(b$_{595}$)-NC distance and Fe(b$_{595}$)-N–C angle would vary markedly with the relative distance and orientation of the two metal centers (38). The orientations of heme d and heme b$_{595}$ have been determined by EPR studies on oriented multilayer preparations of cytoplasmic membrane fragments (44). Both ferric high and low spin heme d are oriented with their heme planes perpendicular to the membrane plane. Ferric high spin heme b$_{595}$ is oriented with its heme plane at approximately 55° to the membrane plane. Thus, heme d and heme b$_{595}$ planes are facing each other with 35° orientation. Assuming the Fe(b$_{595}$)-N bond to be perpendicular to the heme plane with the bond length of 2.2 Å (38), the distance of the two metal center was calculated as 5.1 Å. In this case, peripheral group(s) of the two hemes must be in a close contact each other, the access of an amino acid residue(s) into the ligand binding pocket (i.e. the space between the two metal centers) must be highly restricted, and the ligand-binding pocket itself would be very hydrophobic. The hydrophobic nature of the ligand-binding pocket is consistent with the unusual stability of the oxygenated species as well as the oxoferryl (Fe$^{IV}=O$) adduct (10, 11). This might be one of the reasons why bd-type ubiquinol oxidase is more resistant to anionic inhibitors such as cyanide and azide than bo-type ubiquinol oxidase (2, 3).

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