EPR Study of NO Complex of bd-type Ubiquinol Oxidase from Escherichia coli

THE PROXIMAL AXIAL LIGAND OF HEME d IS A NITROGENOUS AMINO ACID RESIDUE*

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The heme axial ligands of bd-type ubiquinol oxidase of Escherichia coli were studied by EPR and optical spectroscopies using nitric oxide (NO) as a monitoring probe. We found that NO bound to ferrous heme d of the air-oxidized and fully reduced enzymes with very high affinity and to ferrous heme b955 of the fully reduced enzyme with low affinity. EPR spectrum of the 15NO complex of the reduced enzyme exhibited an axially symmetric signal with g-values at g1 = 2.004 and g2 = 1.993 and a clear triplet of triplet (or a triplet of doublet for the 14NO complex) superhyperfine structure originating from a nitrogenous proximal ligand trans to NO was observed. This EPR species was assigned to the ferrous heme d-NO complex. This suggests that the proximal axial ligand of heme d is a histidine residue in an anomalous condition or other nitrogenous amino acid residue. Furthermore, the EPR line shape of the ferrous heme d-NO was slightly influenced by the oxidation state of the heme b955. This indicates that heme d exists in close proximity to heme b955 forming a binuclear center. Another axially symmetric EPR signal with g-values at g1 = 2.108 and g2 = 2.020 appeared after prolonged incubation of the reduced enzyme with NO and was attributed to the ferrous heme b955-NO complex.

The bd-type ubiquinol oxidase is an alternative terminal oxidase in the aerobic respiratory chain of Escherichia coli and is expressed predominantly under low oxygen pressure (1). This enzyme catalyzes the oxidation of ubiquinol-8 and the reduction of molecular oxygen to water and forms a proton electrochemical gradient across the cytoplasmic membrane via scalar protonate reactions (2). It is composed of two subunits (I and II) (3, 4). Based on optical spectroscopic properties, the enzyme contains two hemes B and one heme D associated with cytochrome b558, cytochrome b959, and cytochrome d, respectively (5). Cytochrome d has a chloride chromophore, heme D (6–8), and is a primary exogenous ligand binding site (9). In the air-oxidized condition, heme d is actually in a reduced state and coordinates a molecular oxygen (10), as an FeO22+ dia magnetic EPR-inactive state. Heme b558 is claimed as a six co-ordinated ferric low-spin heme component of the oxidase which has been shown to be contained within subunit I (11, 12) and has been implicated in the oxidation of the substrate, ubiquinol-8, in the cytoplasmic membrane. Heme b955 is a ferric high-spin center (13, 14). EPR-active ferric heme b959 and heme b955 exhibit an intense g = 6.0 axial high-spin signal and, overlapped with this, rhombic high-spin signals (5, 13, 14). Additional two minor ferric low-spin species at g1 = 3.3 and g2 = 2.5 were also reported previously (5, 13, 15). However, the assignment for these EPR signals is still controversial. In our previous study, we have reported that anaerobic addition of nitric oxide (NO) to the air-oxidized enzyme caused an exchange of ferrous heme d-bound O2 with NO leading to an appearance of ferrous heme d-NO EPR signal around g = 2 region without eliminating the ferric high-spin signals. From a rough estimation of spin contents of these EPR-active species, we concluded that the ferrous heme-NO EPR signal and the ferric high-spin signals each corresponds to about 1 mol of heme/mol of the enzyme, and, thus, the bd-type ubiquinol oxidase contains only 1 mol each of cytochrome b and cytochrome d as the redox components (16). However, our recent results for the heme B and metal contents analyses of the purified bd-type ubiquinol oxidase indicated that there were two hemes B and one heme D in the oxidase (5). This result is distinctly incompatible with our previous EPR results. In addition, recent metal and heme content analyses as well as ligand binding titration experiments of bd-type ubiquinol oxidase from Azotobacter vinelandii indicated the stoichiometry of two B-type hemes and one heme D per molecule (17). Moreover, they reported that both CO and NO bind with high affinity to the ferrous heme d at a stoichiometry of 1 mol of ligand/mol of enzyme and with low affinity to the reduced heme b955 (17). Our previous EPR analyses on the NO-bound forms of the air-oxidized enzyme revealed that there is no superhyperfine structure originating from the heme axial 15N ligand trans to NO in the central resonance of the heme d-NO EPR signals (16). A recent electron nuclear double resonance study suggested that the proximal ligand to heme d is not a histidine residue or other strong nitrogenous ligand (18). In the present study, we have extended our EPR investigation on the NO complex of bd-type ubiquinol oxidase from E. coli in order to clarify the stereochemical and electronic structure of heme d as well as heme b955, active center. The present results clearly show the presence of a triplet of triplet superhyperfine structure in the EPR spectra originating from heme d nitrogenous proximal ligand trans to NO.

EXPERIMENTAL PROCEDURES

Purification of bd-type Ubiquinol Oxidase—The bd-type ubiquinol oxidase was isolated from cytoplasmic membranes of E. coli as described previously (19). The strain GR9/NJG2 (cyo' cydA2 recA1 cydAB- Tet' (20)), a kind gift from Dr. R. B. Gennis, which can over-
produce 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 and Optical Spectra—EPR measurements were carried out at X-band (9.23 GHz) microwave frequency with a Varian E-120 EPR spectrometer with 100-kHz field modulation. An immersion Dewar flask was used for measurements at 77 K, whereas an Oxford flow cryostat (ESR-900) was used for the measurements at 5 K and/or 15 K. The combination of NO with the air-oxidized bd-type ubiquinol oxidase was carried out anaerobically as described previously (16). The combination of NO with the fully reduced enzyme was carried out as follows. The samples in EPR tubes with screw-cap septums were degassed by repeated evacuation and flushing with oxygen-free N2 gas. Solid sodium dithionite was anaerobically introduced to reduce the air-oxidized enzyme and, then NO gas, previously washed with 1 N NaOH, was introduced with a gas-tight syringe. The alternative NO complex of the fully reduced form of bd-type ubiquinol oxidase was prepared as follows; an appropriate amount of solid Na15NO2 and/or Na14NO2 placed inside the EPR tube before degassing was mixed with the sample solution, and then solid sodium dithionite was added anaerobically to form the ferrous-NO complex. Optical spectra were recorded on a Shimadzu UV-2200A spectrometer (Shimadzu Corp., Kyoto) at room temperature. A cuvette with screw-cap septum was used for anaerobic measurements.

RESULTS AND DISCUSSION

EPR of Cytochrome d-NO Complex—The air-oxidized bd-type ubiquinol oxidase exhibited two kinds of ferric high-spin EPR species, one with an intense axial g = 6.0 signal and the other with rhombic signals at g = 6.15 and 5.7, and additional minor ferric low spin species at g = 2.46, 2.32, and 1.83 at 15 K (spectra not shown) as reported previously (5, 16). Other peculiar absorption was also observed at g = 3.31 at 5 K (5, 15).

An aerobic addition of nitric oxide (15NO) to the air-oxidized enzyme caused an exchange of ferrous heme d-bound O2 with NO leading to an appearance of axially symmetric heme d-bound NO EPR signals at g = 2.041 and g = 1.993 at 77 K as shown in Fig. 1A, upper trace. The addition of NO to the air-oxidized enzyme did not eliminate the ferric high-spin signals, neither the axial nor the rhombic signal, at 15 K as reported previously (16). If NO binds to the ferric high-spin cytochromes b, their nitrosyl complexes will become EPR-inactive due to spin-pairing. Thus, it is concluded that NO does not bind to the ferric high-spin cytochromes b species.

A careful observation revealed a hyperfine structure in the g region of the second derivative spectrum of the ferrous heme d-bound NO EPR signals, which might be ascribed to a superhyperfine interaction with another 14N nucleus trans to NO (lower trace in Fig. 1A). In order to clarify whether this is really a superhyperfine splitting due to 14N nucleus trans to NO or not, the EPR spectrum of the 14NO complex was compared with that of the 15NO complex. Within a few minutes after addition of solid sodium dithionite to the nitrite (Na14NO2 or Na15NO2)-treated enzyme, axially symmetric NO EPR signals similar but much more intense than that of the NO-bound form of the air-oxidized enzyme appeared. The increment in the signal intensity of the nitrite-treated dithionite-reduced enzyme indicated that the minor ferric low-spin heme d in the air-oxidized enzyme was also converted to the ferrous heme d-NO species. In addition, a clear triplet of triplet (for the 14NO complex) or a triplet of doublet (for the 15NO complex) superhyperfine splitting appeared in the g region as shown in Fig. 1, B and C (the upper and lower traces in Fig. 1 indicated 1st and 2nd derivative EPR spectra, respectively). The principal g-values were estimated to be g∥ = 2.041 and g⊥ = 1.993, and the superhyperfine constants were NOA|| = 1.5 mT, NOA⊥ = 1.4 mT, NAs = 0.67 mT for the 14NO complex and NOA|| = 2.1 mT, NOA⊥ = 1.9 mT, NAs = 0.67 mT for the 15NO complex, respectively. The triplet of triplet and/or triplet of doublet splittings in the g signal can reasonably be ascribed to the superhyperfine interaction with another 14N nucleus trans to NO. This result is inconsistent with electron nuclear double resonance studies in which the proximal axial ligand to heme d was concluded to be not a histidine residue or other nitrogenous ligand (18). However, the electron nuclear double resonance study was done for the air-oxidized form assuming that the axial high-spin signal (g = 5.92, 2.0) was derived from heme d (18) and, therefore, not compatible with the conditions of the present study.

It has been reported that there are three invariant histidine residues (His-19 and His-186 in subunit I and His-56 in subunit II) in bd-type ubiquinol oxidases from E. coli (20, 21) and A. vindendii (22, 23). A site-directed mutagenesis study on bd-type ubiquinol oxidase from E. coli enzyme showed that only two His residues appeared to function as heme axial ligands (24). In subunit I, His-186 is most likely a heme axial ligand to cytochrome b999 and His-19 is likely an axial ligand to either cytochrome b999 or cytochrome d (24). Although it is uncertain...
at this moment whether His-19 is the proximal axial ligand of cytochrome d or not, our present EPR results strongly suggest that the proximal axial ligand of cytochrome d is a histidine residue in an anomalous condition or other nitrogenous amino acid residue. The appearance of the clear superhyperfine splitting in the nitrite-treated dithionite-reduced enzyme (but not so obvious in the NO-complex of the air-oxidized enzyme) indicates that the coordination structure around the axial ligand binding site of heme d may be altered slightly upon reduction of the other redox center (i.e. heme b$_{595}$) or direct electrochemical interaction between oxidized heme b$_{595}$ and heme d-NO may be changed. This observation is consistent with the notion that heme d exists in close proximity to heme b$_{595}$, which might form a binuclear center (5, 25).

EPR of NO Complex of Cytochrome b$_{595}$—It has been reported that NO binds to the fully reduced cytochrome b$_{595}$ in bd-type ubiquinol oxidase from A. vinelandii at higher ligand concentration (17). In order to confirm whether the fully reduced cytochrome b$_{595}$ in the E. coli enzyme also binds NO or not, the nitrite-treated dithionite-reduced enzyme was allowed to stand on ice for several hours. New intense axial symmetric EPR signals appeared at $g_1 = 2.108$ and $g_2 = 2.020$ without eliminating the EPR signals of heme d-NO. Upon further prolonged treatment on ice, the new EPR signals grew gradually in intensity. After 6 h of incubation, fresh NO gas was introduced into the EPR sample tube, leading to a further (~20–30%) increment in their EPR signal intensities (spectrum not shown). The EPR signals did not change any more upon further incubation. The identical EPR spectrum was obtained from the alternatively prepared fully dithionite-reduced enzyme after prolonged incubation with NO gas without treatment of nitrite (Fig. 2B). Spectrum C in Fig. 2 was obtained by subtracting the spectrum of the nitrite-dithionite-reduced-dioxygen form (Fig. 2A) from that of the dithionite-reduced NO-treated form (Fig. 2B). We estimated the total spin contents of these EPR signals by double integration of the spectra as shown in Fig. 3. The time course of the formation of NO complexes indicates that NO binds to the fully reduced bd-type ubiquinol oxidase in biphasic as illustrated in the inset of Fig. 3. The fast phase is due to a formation of the ferrous heme d-NO, and the slow phase can be ascribed to a formation of the ferrous heme b$_{595}$-NO complex. The ratio of the total spin contents of the fully reduced NO form (i.e. dithionite-reduced NO-treated form) versus the nitrite-dithionite-treated reduced form (within a few minutes after addition of solid sodium dithionite) is approximately 2:1. The ferrous heme d-NO signal and the residual fully reduced heme-NO signal, thus, each corresponds to about 1 mol of heme/mol of the enzyme. The present results from the E. coli enzyme are in agreement with those of bd-type ubiquinol oxidase from A. vinelandii as reported recently (17). However, our present EPR results alone can not eliminate a possibility that NO binds to the fully reduced cytochrome b$_{595}$.

Optical Spectra of NO-treated bd-type Ubiquinol Oxidase—In order to confirm whether NO actually binds to the fully reduced cytochrome b$_{595}$ or not, the NO binding to the reduced bd-type ubiquinol oxidase was analyzed by optical absorption spectroscopy at room temperature as shown in Fig. 4. The absolute optical spectra of the air-oxidized and the fully dithionite-reduced complex are similar to those reported previously (3, 4, 17). Upon anaerobic addition of NO to the air-oxidized enzyme, the $\alpha$ absorption band corresponding to the air-oxidized cytochrome d shifted from 646 to 641 nm, and the Soret peak shifted slightly from 413 to 414 nm. Other absorption bands did not show any change. These observations are consistent with the notion that anaerobic addition of NO to the air-oxidized enzyme causes an exchange of the heme d-NO complex for the NO complex. The ratio of the total spin contents of the NO-complex of the air-oxidized enzyme versus incubation time.

bound O$_2$ with NO and that NO does not bind to the ferric cytochromes b$_{558}$ or b$_{595}$, as confirmed by present EPR spectroscopy. The NO complex of the air-oxidized enzyme was then reduced by anaerobic addition of a small quantity of solid sodium dithionite. In addition to the $\alpha$ band absorption of heme...
d-NO at 641 nm, new absorption bands that correspond to the α and β bands of the fully reduced cytochrome b558 appeared at 560 and 530 nm, respectively, and the Soret peak shifted to 428 nm. The identical spectrum was obtained by anaerobic addition of NO gas to the fully reduced enzyme. In the latter case, upon addition of NO, the Soret band at 429 nm with the shoulder around at 438 nm of the fully reduced form decreased gradually in its intensity and their peaks shifted to 428 nm, and the absorption around 400 nm increased in intensity concomitantly. The absorption bands at 560 and 530 nm and the Soret peak at 428 nm correspond to the fully reduced cytochrome b558, and the absorption in the Soret region around 438 nm is ascribable to the fully reduced cytochrome b595 (26, 27). Thus, NO does not bind to the reduced cytochrome b558 but binds to the reduced cytochrome b595 with low affinity. The same conclusion was obtained from the NO complexes prepared by the dithionite reduction of the nitrite-treated enzyme (spectra not shown). Upon anaerobic addition of a small quantity of solid sodium dithionite to the nitrite-treated air-oxidized enzyme, the NO-bound reduced cytochrome d (641 nm) and both the fully reduced cytochrome b595 (560, 530, and 429 nm) and cytochrome b595 (595 and 438 nm) were formed quickly. The Soret shoulder at 438 nm of the reduced cytochrome b595 decreased gradually and new broad absorption band around 400 nm increased, without eliminating the absorptions (560, 530, and 429 nm) corresponding to the reduced cytochrome b558, although the Soret peak at 429 nm shifted slightly to 428 nm. These optical spectroscopic results indicate that the reduced cytochrome b595-NO complex is formed slowly but NO does not bind to the reduced cytochrome b558, being consistent with the EPR results. Therefore, the obtained EPR spectrum (spectrum C in Fig. 2) by subtracting the spectrum of the nitrite-treated dithionite-reduced form from that of the dithionite-reduced NO-treated form is attributed to the spectrum of the pure b595-NO complex.

In our previous study on bd-type ubiquinol oxidase, we proposed that this enzyme contains only 1 mol each of the cytochrome b and the cytochrome d as the redox components (16). However, we estimated the spin contents of the ferrous heme-NO and the ferric high-spin species by double integration method at 15 K without considering the effects of temperature-dependent relaxation time on the shape and the intensity of the EPR signals, thus leading to an incorrect conclusion.

A consistent heme stoichiometry that bd-type ubiquinol oxidase contains two hemes B and one heme D was supported by pyridine hemochrome spectra of the purified enzyme in the present study. As the ferrous pyridine hemochrome of heme D was reported to be unstable (6, 26), the spectrum was measured in aqueous alkaline pyridine solution immediately after reduction with solid sodium dithionite. The spectrum of freshly prepared pyridine hemochrome of bd-type ubiquinol oxidase is shown in Fig. 5. Subtracting a pyridine hemochrome spectrum of heme B (obtained from 2 equimolar sperm whale myoglobin) from the pyridine hemochrome spectrum of bd-type ubiquinol oxidase, a reliable spectrum of the pyridine hemochrome of heme D was obtained as shown in Fig. 5. For the α peak of cytochrome d in the reduced minus air-oxidized difference spectrum (inset in Fig. 5), a molar extinction coefficient for bd-type ubiquinol oxidase has been reported (5). Using our recent molar extinction coefficient of 27.9 mM \(^{-1}\) cm\(^{-1}\) for the redox pair 628 nm (for reduced state) minus 651 nm (for air-oxidized state) (5), a reliable extinction coefficient at 608 nm of the pyridine hemochrome of heme D was estimated to be 23.4 mM \(^{-1}\) cm\(^{-1}\). In conclusion, bd-type ubiquinol oxidase from E. coli is a cytochrome b/dl-type enzyme as reported previously (5, 13, 14, 17). Optical and EPR results indicate that NO binds to the reduced heme d with very high affinity and to the fully reduced heme b595 with low affinity. Two kinds of EPR signals correspond to both NO complexes of fully reduced cytochrome d and b595 with 1:1 spin contents. A clear superhyperfine interaction with \(^{14}\)N nucleus trans to NO in the EPR spectrum of the NO complex of cytochrome d indicates that the proximal axial ligand of heme d is a histidine residue or other nitrogenous amino acid residue. Further detailed studies are desired to reveal the precise structure of the active center of this enzyme.

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