Infrared and EPR Studies on Cyanide Binding to the Heme-Copper Binuclear Center of Cytochrome bo-type Ubiquinol Oxidase from Escherichia coli

RELEASE OF A CuB-CYANO COMPLEX IN THE PARTIALLY REDUCED STATE*

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Cyanide-binding to the heme-copper binuclear center of bo-type ubiquinol oxidase from Escherichia coli was investigated with Fourier transform-infrared and EPR spectroscopies. Upon treatment of the air-oxidized CN-inhibited enzyme with excess sodium dithionite, a 12C-stretching vibration at 2146 cm⁻¹ characteristic of the Fe=C=N-Cu bridge structure was quickly replaced with another stretching mode at 2034.5 cm⁻¹ derived from the Fe=C=N moieties. The presence of ubiquinone-8 or ubiquinone-1 caused a gradual autoreduction of the metal center(s) of the air-oxidized CN-inhibited enzyme and a concomitant appearance of a strong cyanide stretching band at 2169 cm⁻¹. This 2169 cm⁻¹ species could not be retained with a membrane filter (molecular weight cutoff = 10,000) and showed unusual cyanide isotope shifts and a D₂O shift. These observations together with metal content analyses indicate that the 2169 cm⁻¹ band is due to a CuCN complex released from the enzyme. The same species could be produced by anaerobic partial reduction of the CN-inhibited ubiquinol oxidase and, furthermore, of the CN-inhibited cytochrome c oxidase; but not at all from the fully reduced CN-inhibited enzymes. These findings suggest that there is a common intermediate structure at the binuclear center of heme-copper respiratory enzymes in the partially reduced state from which the CuB center can be easily released upon cyanide-binding.

Cytochrome bo-type ubiquinol oxidase in the aerobic respiratory chain of Escherichia coli catalyzes the two-electron oxidation of ubiquinol-8 (Q₈H₂)¹ and the four-electron reduction of dioxygen to water (1, 2). These redox reactions mechanistically couple with the formation of an electrochemical proton gradient across the cytoplasmic membrane not only by scalar protolytic reactions at the inner and outer surfaces of the membrane but also by a proton pumping mechanism (3–5). Based on the structural homologies of subunits I, II, and III, cytochrome c oxidases and some bacterial quinol oxidases including the E. coli cytochrome bo are classified in the heme-copper respiratory oxidase superfamily (6, 7). However, there is a notable difference in electron-donating substrates between the two enzymes, cytochrome c (a hydrophilic one-electron carrier) and quinol (hydrophobic two-electron and two-proton carriers) (8, 9). As a consequence, the CuA center is absent in subunit II of quinol oxidase (5, 10, 11).

Although the dioxygen reduction mechanism at the heme-copper binuclear center is thought to be identical in both enzymes (12–14), these differences raise the question whether the electron transfer reactions from the substrates to dioxygen and the proton pumping mechanism coupled to these redox reactions are alike or distinct. It is proposed that the last two steps of the four-electron transfer reactions to dioxygen are linked to proton pumping by cytochrome c oxidase (15–17). Thus, it becomes increasingly important to analyze the mixed-valence states of oxidase as a model for the intermediate species of the dioxygen reduction chemistry, since the understanding of the redox-linked structural change(s) at the metal center appears to be a key point to reveal the redox-linked proton pumping.

In a previous study, Tsubaki (18) analyzed cyanide binding to the Fe₃Cu₅-binuclear center of cytochrome c oxidase. In the resting (air-oxidized) state, a bound cyanide showed an infrared C–N stretching band at 2152 cm⁻¹, assignable to a bridging structure, Fe₃³⁺C=N–Cu⁵⁺. This assignment was confirmed recently by structural characterizations and infrared measurements on a series of model complexes containing the [Fe²⁺C=N–Cu²⁺] bridge unit (19). Upon partial reduction of the CN-inhibited cytochrome c oxidase an infrared band appeared at 2131 cm⁻¹, assignable to the Fe₃⁺C=N structure. Further reduction resulted in an appearance of two new infrared bands at 2056 and 2045 cm⁻¹, concomitantly, assignable to the Fe₃⁵⁺C=N species. These observations suggest three kinds of conformational change to occur at the Fe₃Cu₅-binuclear site as the reduction of the metal centers proceeds (18).

Subsequently Tsubaki et al. (11) carried out a combined study using EPR and FT-IR spectroscopies to clarify the structural differences of the binuclear center between bo-type ubiquinol oxidase and cytochrome c oxidase. EPR spectra of bo-type ubiquinol oxidase in the air-oxidized state showed EPR signals from an integer spin system confirming the existence of...
the spin-spin exchange-coupled binuclear site (20–23). EPR spectra of the cyanide, azide, and formate complexes in the air-oxidized state indicated that a gross conformation at the binuclear site seems well conserved among the heme-copper oxidase superfamily (11). FT-IR spectroscopy confirmed these observations: the cyanide that binds to the air-oxidized enzyme exhibits an infrared band at 2146 cm\(^{-1}\) characteristic to the Fe\(^{2+}\)-C=N–Cu\(^{2+}\) structure (11).

In the present study we extended the FT-IR and EPR spectroscopic studies to clarify the structure at the heme-copper binuclear center using cyanide as a monitoring probe.

**EXPERIMENTAL PROCEDURES**

**Purification of Cytochrome bo-type Ubiquinol Oxidase—**The wild-type (11) and the subunit I binuclear center mutant oxidases (H284A, H333A (24), and Y288L) \(^1\) were purified as described previously. Loosely bound quinones were removed from the wild-type oxidase by precipitation with PEG 4000 (25). The bound Q\(_b\)-free wild-type oxidase was isolated from E. coli strain MU1227/pMF02 (cyo\(^{-}\)/cyt\(_b\)A\(^{+}\)/cyo\(^{-}\)) (25).

**Purification of Cytochrome c Oxidase—**Cytochrome c oxidase was isolated from bovine heart using the method of Yoshikawa et al. (26). The crude mitochondria sample was solubilized in 50 m\(\text{M}\) sodium phosphate buffer (pH 7.4) and was treated with 10 m\(\text{M}\) EDTA as described previously (18). The sample was then, diluted with 50 m\(\text{M}\) Tris-DCl (pD = 8.0) (D\(_2\)O buffer) and was concentrated with a Diaflow apparatus. This treatment was repeated several times for the complete exchange of the medium from H\(_2\)O to D\(_2\)O.

**Measurement of Fourier Transform Infrared and Optical Spectra—**FT-IR spectra of the purified bo-type ubiquinol oxidase were measured at 10 °C as described previously (11, 27). A nominal spectral resolution of 4.0 cm\(^{-1}\) was chosen. Absolute optical spectra of the oxidase in the infrared cells were measured at room temperature with a UVIKON 860 UV-visible spectrophotometer (Contron Instr.) before and after FT-IR measurements. Partially reduced CN-inhibited enzymes were prepared according to Yoshikawa ad Caughey (28) with slight modifications as described previously (29).

**Measurement of EPR Spectra—**EPR spectra were measured at 5 or 15 K at X-band (9.23 GHz) microwave frequency with a home-built EPR spectrometer as described previously (11) and a Varian E-12 EPR spectrometer equipped with an Oxford flow cryostat (ESR-900).

**Miscellaneous—**Metal contents were determined by inductively coupled plasma atomic emission spectrophotometry with an SPS 1200VR plasma spectrometer (Seiko Instruments Inc., Tokyo). Heme content can be analyzed as described previously (30). Bound Q\(_b\) in the purified oxidase was estimated on reverse-phase high performance liquid chromatography analysis as described previously (25). The following potassium cyanide isotopes were used: K\(^{12}\)C\(^{12}\)N (natural abundant, Nacalai Tesque); K\(^{12}\)C\(^{15}\)N (99.4 atom %\(^{15}\)N, Isotec Inc.); K\(^{13}\)C\(^{14}\)N (99 atom %\(^{13}\)C, Isotec Inc.); K\(^{13}\)C\(^{15}\)N (99 atom %\(^{13}\)C, 99 atom %\(^{15}\)N, Icron). Other chemicals were commercial products of analytical grade.

**RESULTS**

FT-IR and EPR Spectra of the CN-inhibited bo-type Ubiquinol Oxidase—Addition of cyanide (5 m\(\text{M}\)) to the air-oxidized ubiquinol oxidase (in H\(_2\)O buffer) showed a cyanide stretching infrared band at 2146 cm\(^{-1}\) (Fig. 2, b1). CO forms a stable Fe\(^{2+}\)-CO adduct in the fully reduced state showing a sharp infrared band at 1959.7 cm\(^{-1}\) (11, 31) and can suppress the formation of the Fe\(^{2+}\)-C=N–Cu\(^{2+}\) species. We could not detect any infrared species representing the Fe\(^{2+}\)-C=N structure after the addition of sodium dithionite. In contrast, a relatively stable intermediate of the Fe\(^{2+}\)-C=N–Cu\(^{2+}\) structure showing a 2131 cm\(^{-1}\)-infrared band was observed for cytochrome c oxidase (18).

**EPR spectra of the air-oxidized CN-inhibited ubiquinol oxido-
Cyanide Binding to the Heme-Copper Center

Fig. 2. Cyanide (12C14N) binding to the fully reduced ubiquinol oxidase (a) and the effect of carbon monoxide on the CN binding (b and c). a, cyanide (12C14N) was anaerobically added to a final concentration of 5 mM to the fully reduced enzyme (0.39 mM) with excess sodium dithionite. b, the enzyme was first fully reduced with excess sodium dithionite in the presence of carbon monoxide and then 12C14N was anaerobically added to the enzyme at a final concentration of 5 mM. b, the ordinate of b, is reduced by one-fourth to clarify the CO binding to the enzyme which shows the 1599.7 cm⁻¹ band. Other conditions are the same as described in the legend to Fig. 1.

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binuclear center mutant oxidases, H284A, H333A (24, 30, 33), and Y288L,2 were examined by FT-IR spectroscopy. When cyanide (5 mM) was added to the air-oxidized H284A mutant oxidase, there was a weak infrared band at 2124 cm⁻¹ but no band around 2146 cm⁻¹. Simultaneous addition of 4-fold excess Q₁ to the mutant oxidase did not cause any infrared spectral change. Addition of cyanide to the Cu₉-deficient H333A oxidase caused the appearance of weak infrared bands at 2160 and 2121 cm⁻¹, but not at 2169 cm⁻¹ (spectra not shown). Addition of cyanide to the air-oxidized Y288L mutant oxidase showed no bound cyanide band (spectra not shown). In the dithionite-reduced states, these mutant oxidases did not show any clear cyanide band associated with the ferrous heme (spectra not shown). Addition of cyanide to the bound Q₈-free wild-type oxidase showed a weak infrared band at 2146 cm⁻¹ together with multiple bands around 2126 cm⁻¹ region (Fig. 4a). Simultaneous addition of 4-fold excess Q₁ with cyanide gave the same result. Prolonged incubation produced the 2169 cm⁻¹ band, but it was very weak (Fig. 4b).

Partial Reduction of the CN-inhibited Cytochrome c Oxidase—Upon addition of cyanide (5 mM) to the resting (air-oxidized) cytochrome c oxidase in a D₂O buffer, a cyanide band appeared at 2152 cm⁻¹ (Fig. 5a), which has been assigned to the FeE₅₃-Cₐ-N=C₃₃₈₉ species (18). Introduction of a first and a second electron equivalent to the air-oxidized CN-inhibited enzyme caused a dramatic decrease of the 2152 cm⁻¹ band intensity with a concomitant increase in intensity of a band at 2131 cm⁻¹ (Fig. 5, b and c), which has been assigned as the FeE₅₃-Cₐ-N= species (18). Introduction of a third electron equivalent removed the 2151 cm⁻¹ band completely while the 2131 cm⁻¹ band became further intensified (Fig. 5d).

During these spectral changes we observed an appearance of a 2162 cm⁻¹ band in a D₂O buffer (Fig. 5b and c). In a similar experiment carried out in an H₂O buffer, the 2162 cm⁻¹ band in the partially reduced states (one-fourth and one-half) shifted to 2169 cm⁻¹ without affecting the 2152 and 2131 cm⁻¹ bands (spectra not shown). These observations strongly suggest that cyanide species very similar to that found in the CN-inhibited bo-type ubiquinol oxidase was produced in the partially reduced state(s) of the CN-inhibited cytochrome c oxidase. The intensity of the 2169 cm⁻¹ band (in the D₂O buffer, or the 2162 cm⁻¹ band in the H₂O buffer) changed in a dose- and time-dependent manner. A higher cyanide concentration (20 mM) and a longer incubation time (70 h) in the partially reduced state (one-fourth-reduced) caused a stronger intensity of the 2169 cm⁻¹ band (spectra not shown). The 2165 cm⁻¹ band (in a D₂O buffer) observed by Yoshikawa and Cauhey (28, 34) is likely due to the same species observed in the present study.

Metal Content Analysis—The filtrate that passed through a membrane filter (MWCO = 10,000) after the cyanide treatment of ubiquinol oxidase had no color indicative of a heme species. Therefore, the most reasonable candidate for the 2169 cm⁻¹ species is a copper-cyano species. A preliminary metal content analysis of the filtrate after the cyanide treatment showed a substantial amount of copper ions; whereas the filtrate without the cyanide treatment shows a trace amount of copper ions (data not shown). To establish a loss of the Cu₉ center from bo-type ubiquinol oxidase by the cyanide treatment in the presence of ubiquinone, we carried out metal content analyses (Table II). The wild-type oxidase that contained 2.21 mol of Q₈/mol of the enzyme lost about 30% of the Cu₉ center upon cyanide treatment, whereas the bound Q₈-free oxidase retained copper completely.

**DISCUSSION**

Implication for the Cyanide Coordination Structure at the Binuclear Center—The CN-bridging band at 2146 cm⁻¹ for the air-oxidized CN-inhibited enzyme (11) was 6 cm⁻¹ lower than that of cytochrome c oxidase (18), probably reflecting a slight increase in a Cu–NC bond and decrease in a Cu–N–C bond angle (19). But the FeE₅₃-Cₐ-N stretching frequency at 2034.5 cm⁻¹ in the fully reduced state was much lower (23.5 or 11 cm⁻¹) than the corresponding frequencies of cytochrome c oxidase (2058 and 2045 cm⁻¹) (18). The C–O stretching frequency of bo-type ubiquinol oxidase (1959.7 cm⁻¹) (11, 24) was, however, only 3.8 cm⁻¹ lower than the corresponding frequency of cytochrome c oxidase (1963.5 cm⁻¹) (35). This small change is...
attributable mostly to the absence of a formyl group in heme O, as suggested before (11).

The large difference of the bound C–N stretching vibration between bo-tyop ubiquinol oxidase and cytochrome c oxidase in the reduced state is likely due to a specific character of the cyanide-binding to the binuclear center (36). Cyanide binding to other typical ferrous hemoproteins is extremely weak, except for horseradish peroxidase in which the electrostatic interaction (or hydrogen bond) between a protonated distal His residue and a ferrous heme-bound cyanide plays a substantial role in the stabilization (37). Thus, it is possible that the ferrous heme-bound cyanide at the binuclear center is stabilized by a protonated His residue in the vicinity of the heme in the fully reduced state. The protonation of the His residue may be directly coupled to the uptake of a proton upon binding of cyanide to the reduced oxidase (38). Among three invariant His residues (His-284, His-333, and His-334) on the distal side of the high spin heme (6, 7), His-284 is likely to have such a role since it is probably not an obligatory ligand to CuB unlike His-333 and His-334 (12, 24, 30). Alternatively, His-333 may perform such a part in cytochrome c oxidase, since it seems to be disordered or to have multiple conformations in an x-ray crystal structure for the azide-inhibited air-oxidized cytochrome c oxidase from Paracoccus denitrificans (39), but not for the air-oxidized cytochrome c oxidase from bovine heart mitochondria (40). The x-ray crystal structures revealed also that His-284 can form hydrogen bond with Tyr-288 and Trp-280 and His-333 with Thr-352 or the carbonyl oxygen of Phe-348 (39, 40). Thus the greater difference in the Fe2+–C=N stretching vibration is likely due to the difference in the interaction between the protonated distal His residue and the Fe2+–C=N moiety.

The binuclear center mutant oxidases showed neither CN-bridging infrared band in the air-oxidized state nor Fe2+–C=N infrared band in the fully reduced state, although several Fe3+-C=N species seemed to be formed. It is clear that presence of the CuB center is essential for the binding of cyanide to the ferrous heme since these mutant oxidases did somehow bind CO (although with a very broad infrared band around 1970 cm⁻¹ (His333Ala (24) and Y288L) or with very weak affinity (H284A)). However, we could not evaluate the specific role of the imidazole group of His-284 and His-333 and the phenol group of Tyr-288 in the present study.

Structural Implication on the 2169 cm⁻¹ Species—We found that the presence of excess Q₈ or Q₃ in the wild-type ubiquinol oxidase preparation caused a gradual development of a new cytochrome c band at 2169 cm⁻¹ associated with the autoreduction of the metal center(s) of the air-oxidized CN-inhibited enzyme. The 2169 cm⁻¹ band could not be observed for the binuclear center mutant oxidases (H284A, Y288L, and H333A) in which the CuB center was greatly perturbed or almost eliminated (30, 33). This 2169 cm⁻¹ species showed unusual cyanide isotope shifts and a D₂O shift (Table I) and was able to pass through a membrane filter (MWCO = 10,000). Metal content analyses of the enzyme before and after the anaerobic cyanide treatment in the presence of excess Q₈ revealed that a substantial amount (30%) of copper ions was released in the filtrate after the treatment (Table I). These observations suggest that the 2169 cm⁻¹ band arose from a low molecular weight copper-cyano species (i.e. a Cu₁-CN complex) released from bo-type ubiquinol oxidase. EPR analysis revealed no indication of a Cu²⁺ ion in the enzyme preparation that showed the 2169 cm⁻¹ band, suggesting that this Cu₉-CN complex was in reduced state (i.e. Cu¹⁻ state).

There are several reports describing the release of copper ions from copper proteins in the presence of cyanide. For cytochrome c oxidase both the Cu₉ and CuB centers of the air-oxidized enzyme could be released by dialysis against a CN-containing solution (41, 42). It must be noted, however, that the conditions are very different from the one in the present study. The dialysis was done at alkaline pH (i.e. pH 10), and a much higher concentration (50 mM – 1.0 M) of cyanide was required (41, 42). Among the previous reports, our particular interest is the cyanide binding study for CuZn-superoxide dismutase from bovine erythrocyte (43). Raman and FT-IR spectroscopic studies revealed that the native Cu²⁺-superoxide dismutase binds one cyanide showing a band at 2137 cm⁻¹. With increased concentration of cyanide the 2137 cm⁻¹ band became weaker, and strong vibrational modes developed concomitantly. These bands were due to di-, tri-, and tetracyano Cu²⁺ complexes, respectively, arising from copper removal from the protein. Simultaneously a new band appeared at 2169 cm⁻¹ having an abnormally large δ¹³CN shift of ~60 cm⁻¹ (43). This 2169 cm⁻¹ species was also observed in the filtrate through a membrane filter (43). All of these observations strongly suggest that the 2169 cm⁻¹ species found for the superoxide dismutase-CN system is identical with the 2169 cm⁻¹ species in the present study. Han et al. (43) concluded that the 2169 cm⁻¹ species was neither a protein species nor a microcell of CuCN solid because of its abnormal cyanide isotopic shifts.

The 8 cm⁻¹ D₂O downshift observed for the 2169 cm⁻¹ species is not likely due to a direct hydrogen bonding of an H₂O (or D₂O) molecule in medium to the CN moiety of the Cu²⁺-CN complex, as hydrogen bonding becomes weakened by an H-D exchange (44). For the horseradish peroxidase (Fe²⁺)-CN system, an 8 cm⁻¹ D₂O upshift (from 2029 to 2037 cm⁻¹) of the C-N stretching frequency has been attributed to the formation of a hydrogen bond between the heme-coordinated cyanide anion and a protonated (or deuterated) distal His residue (37). It is more likely, therefore, that an H₂O (or D₂O) molecule itself also participates in forming the Cu²⁺-CN complex and the resulting intramolecular interactions between cyanide(s) and a water ligand(s) may be essential for the appearance of the 2169 cm⁻¹ band and its unusual vibrational mode pattern.

We have tried to prepare the Cu¹⁻-CN complex by mixing copper(I) chloride and/or copper(I) cyanide with varying amounts of cyanide in aqueous solution, but without success. It was reported that a reaction involving copper(I), cyanide, and
water under conditions of high temperature and pressure gave a by-product species with $\text{Cu}_2(\text{CN})_2(\text{H}_2\text{O})_2$, structure, comprising a two-dimensional polymer (45). We propose that this kind of species may be responsible for the 2169 cm$^{-1}$ band.

Mechanism of the Formation of the Cu$_{12}^-$CN Complex—The partially reduced conditions seem essential for the formation and release of the Cu$_{12}^-$CN complex. Indeed the 2169 cm$^{-1}$ species could be quickly produced by anaerobic partial reduction of the CN-inhibited enzyme but not at all from the fully reduced form. The absence of the 2034.5 cm$^{-1}$ band that is characteristic to the Fe$_{12}^2-$C=N adduct may be noticed when the 2169 cm$^{-1}$ species developed fully by the autoreduction of the CN-inhibited enzyme. This observation is consistent with the notion that the presence of the Cu$_1$ center is essential for the binding of cyanide to the reduced enzyme as discussed in the previous section.

The CN-inhibited bound Q$_8$-free oxidase resulted in neither the development of the 2169 cm$^{-1}$ band nor the release of the Cu$_1$ center. A simultaneous addition of excess Q$_1$ with cyanide to the bound Q$_8$-free oxidase was not so effective. These observations suggest that the precise structure around the binuclear site or the quinone binding site(s) may be essential for the formation of the Cu$_1$CN complex. Indeed the 2169 cm$^{-1}$ species resulted in neither formation of the 2169 cm$^{-1}$ adduct may be noticed when the 2169 cm$^{-1}$ species developed fully by the autoreduction of the CN-inhibited enzyme. This observation is consistent with the notion that the presence of the Cu$_1$ center is essential for the binding of cyanide to the reduced enzyme as discussed in the previous section.

It is of great importance that the same 2169 cm$^{-1}$ species could be produced by anaerobic partial reduction of the CN-inhibited cytochrome c oxidase in which no ubiquinone molecule is bound. This observation suggests that there is a common intermediate structure at the binuclear center of the heme-copper respiratory oxidases in the partially reduced CN-inhibited state which is very susceptible to the cyanide binding(s) and release of the Cu$_1$ center. The greater formation of the 2169 cm$^{-1}$ species for bo-type ubiquinol oxidase than cytochrome c oxidase may be related to the instability of the Fe$^{3+}-C=N$ species (although its presence was confirmed with the g$_z$ = 3.24 EPR signal) in the partially reduced CN-inhibited enzyme. This difference is also likely due to a slight difference(s) of the cyanide coordination structure (including distal His residues) at the binuclear center. The unique property of the heme-copper oxidase revealed in the present study may provide a clue for understanding a mechanism of the dioxygen reduction chemistry and the redox-linked proton pumping.

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