Fourier-Transform Infrared Study of Cyanide Binding to the Fe_{a3}-Cu_B Binuclear Site of Bovine Heart Cytochrome *c* Oxidase: Implication of the Redox-Linked Conformational Change at the Binuclear Site†

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ABSTRACT: Cyanide binding to the Fe_{a3}-Cu_B binuclear center of cytochrome *c* oxidase purified from bovine heart mitochondria was examined by Fourier-transform infrared spectroscopy. In the fully oxidized state, cyanide binding caused an appearance of a sharp infrared C–N stretching band at 2152 cm^{-1}. This 2152-cm^{-1} band was assigned to a bridging structure, Fe_{a3}^{3+}–C–N–Cu_B^{2+}, on the basis of the isotope replacement experiments. The bound cyanide giving the 2152-cm^{-1} band was hardly exchangeable with an exogenous ligand added afterward in the fully oxidized state and, upon partial reduction, was converted specifically to the 2132-cm^{-1} band species assignable to Fe_{a3}^{3+}–C–N. The reduction of the Fe_{a3} center resulted in appearances of two new infrared bands at 2058 and 2045 cm^{-1} concomitantly. At higher concentration of cyanide (>5 mM) an additional two infrared bands appeared at 2093 and 2037 cm^{-1}. The former two bands are assignable to the Fe_{a3}^{2+}-bound cyanides, whereas the latter two bands are possibly due to the Cu_B^{1+}-bound cyanides on the basis of the competition experiments using carbon monoxide. These observations suggest that there are three kinds of conformational change to occur at the Fe_{a3}-Cu_B binuclear site upon reduction of the metal centers. The first one occurs upon reduction of the Cu_B center, and the second one occurs upon reduction of the Fe_a and/or Cu_A centers. These are associated with the "closed" to "open" conformational transition characterized by the disappearance of the 2152-cm^{-1} band and the appearance of the 2132- and 2093-cm^{-1} bands. The third one can be induced upon reduction of the Fe_{a3} center, and this enables the binding of a second cyanide to the Cu_B^{1+}-CN center, at a higher concentration of cyanide, being oriented toward the Fe_{a3}^{2+} center to produce the 2037-cm^{-1} band. These structural changes at the Fe_{a3}-Cu_B binuclear site controlled by the redox levels of the metal centers may provide a functional role(s) for the cytochrome *c* oxidase-catalyzed reactions, such as the reduction of dioxygen to water and the vectorial proton pumping across the mitochondrial inner membrane.

Cytochrome *c* oxidase (CcO)[1] is an oligometic heme protein with an approximate molecular weight of 200 000 and works as a terminal oxidase of the electron-transfer system in the mitochondrial inner membrane. It can generate an electrochemical proton gradient across the membrane partly by the consumption of H^+ in the matrix side for the reduction of dioxygen and partly by the vectorial proton transport across the membrane coupled to the electron-transfer reaction (Capaldi, 1990; Malmström, 1990). CcO contains two heme A molecules and two copper ions as the prosthetic groups, besides zinc and magnesium ions and possibly an additional copper ion with unknown function (Einarsdóttir & Caughey, 1984, 1985; Steffens et al., 1987).

Among the various CcOs from different origins the CcO from bovine heart muscle, which has 13 different subunits (Azzi & Müller, 1990), is the most studied and well characterized enzyme. Three large subunits I–III are encoded by mitochondrial genes and, therefore, translated inside mitochondria. These subunits play major roles of the enzymatic functions (Palmer, 1987). Among the four redox centers of CcO, heme *a* (Fe_a), heme a_3 (Fe_{a3}), and Cu_B are expected to reside in subunit I and, in the fully oxidized resting state, the heme a_3 makes a spin–spin coupled binuclear center with the Cu_B, where the reduction of dioxygen to water takes place. Subunit II has a unique binding site for cytochrome *c* and a copper atom termed Cu_A and mediates an electron transfer from reduced cytochrome *c* to the metal centers in subunit I. As proposed by Kroneck et al. (1988, 1990), the EPR spectrum generally ascribed to Cu_A may actually originate from a binuclear Cu site with one unpaired electron delocalized between two equivalent Cu nuclei. Much less is known about the function of the subunit III; it has been suggested that this subunit has a specific role for the vectorial proton translocation (Casey et al., 1980; Puettner et al., 1985; Thelen et al., 1985), but contradictory results have been reported recently (Finel & Wikström, 1986, 1988; Haltia et al., 1991). Other small subunits, from IV to XIII, are encoded by the nuclear genes and are transported into mitochondria posttranslationally.

To explore the stereochemical structures and the electronic states of the redox centers, various techniques have been employed. These include optical, EPR, magnetic circular dichroism (MCD), resonance Raman, extended X-ray absorption fines structure (EXAFS), and Mössbauer spectroscopies together with magnetic susceptibility measurements [see Malmström (1990)]. Infrared spectroscopic studies can provide unique information concerning the microenvironment

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[1] Abbreviations: CcO, cytochrome *c* oxidase; FT-IR, Fourier-transform infrared; HRP, horseradish peroxidase.

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and electronic state of metal centers using sensitive infrared probes such as carbon monoxide (CO) and has been used for characterization of the Fe_{a3}-Cu_B binuclear site (Yoshikawa & Caughey, 1982; Einarsdóttir et al., 1988; Yoshikawa et al., 1977; Tsubaki et al., 1992).

In the present study the structural change at the Fe_{a3}-Cu_B reaction center (dioxygen reduction site) was investigated by Fourier-transform infrared (FT-IR) spectroscopy using cyanide, a very strong and specific inhibitor for CcO, as an infrared probe. However, the binding specificity of cyanide ions toward metals and their valence states is known to be less specific than that of carbon monoxide (Yoshikawa et al., 1985). The weaker dipole moment of the metal-bound cyanide, which gives weaker absorptivity, compared to that of the ferrous heme-bound carbon monoxide is an additional disadvantage. The cyanide binding to CcO has been recently studied by dispersive infrared spectroscopy (Yoshikawa & Caughey, 1990); however, their conclusions were apparently contradictory to the conventional view of the cyanide binding to the Fe_{a3}-Cu_B site (Thomson et al., 1981). The present work was done to solve this discrepancy based on the newly-collected data obtained by FT-IR spectroscopy. On the basis of the much higher quality of the FT-IR data and the new data analysis technique, we corrected some previous assignments and proposed new structural models for cyanide binding to the Fe_{a3}-Cu_B center of CcO.

MATERIALS AND METHODS

Preparation of CcO Sample. Cytochrome *c* oxidase (CcO) was isolated from bovine heart using the method of Yoshikawa et al. (1988) with some modifications. The crystalline CcO sample isolated was solubilized in 50 mM sodium phosphate buffer (pH 7.4) and treated with 10 mM EDTA in the same buffer at 4 °C followed by concentration with a Diaflow ultrafiltration apparatus (Amicon) using a Millipore membrane filter (PTTK04310; pore size 30 000 NMWL). The repeated washing of CcO (typically 5–6 times) with the EDTA buffer in the Diaflow apparatus removed nonintrinsic copper ions from CcO. This step is essential to obtain infrared spectra in good quality around the 2090-cm^{-1} region, since CN^- ion bound to the nonintrinsic copper ion gives a 2093-cm^{-1} band in D_2O (Yoshikawa & Caughey, 1990). Then, the sample was treated with 50 mM Tris-DCl (pD = 8.4) (D_2O buffer) and was concentrated with the Diaflow apparatus. This treatment was repeated several times for a complete exchange of the medium from H_2O to D_2O. This step is also essential since free cyanide in the neutral H_2O medium exists as HCN (pK_a = 9.22), which gives a 2093-cm^{-1} band, whereas DCN gives a 1887-cm^{-1} band. The sample in the D_2O buffer at the concentration ranging from 1.5 to 2.5 mM (on heme A basis) was used for the infrared spectroscopic measurements.

Neutralized potassium cyanide solution (0.2–0.5 M) in the D_2O buffer was introduced with a Hamilton microsyringe to the concentrated CcO solution to convert the resting form of the enzyme to the cyanide-bound fully oxidized form. This conversion could be monitored spectroscopically by a shift of the Soret band peak from around 421 to 428 nm. For a complete conversion, 24 h of storing of CcO at room temperature was required; but about 70–80% of CcO was converted to the cyanide-bound fully oxidized form within the first 1 h. For preparation of the sample to be used for the cyanide isotope exchange experiments, the EDTA-treated CcO sample was treated first with 10 mM $K^{12}C^{14}N$ in 50 mM sodium phosphate buffer (pH 7.4) at 4 °C overnight and free cyanide ion or cyanic acid was removed by the repeated washing with 50 mM sodium phosphate buffer (pH 7.4) using the Amicon Diaflow apparatus. Then the medium was exchanged from H_2O to D_2O as described above.

The following potassium cyanide isotopes were used: $K^{12}C^{14}N$ (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, 99 atom % ^{13}C, ^{15}N, Icon). Other chemicals used were the highest quality available and were used without further purifications.

Measurements of Fourier-Transform Infrared Spectra. The CcO sample was introduced into an infrared cell having CaF_2 windows with a 51-μm path length. The path length was confirmed by the interference pattern generated with the empty infrared cell. The infrared spectra were recorded with a Perkin-Elmer Model 1850 Fourier-transform infrared spectrophotometer interfaced to a Perkin-Elmer 7700 computer, and this system was under control of the CDS-3 application software package for data acquisition and manipulation. The infrared spectrophotometer was operated in a double-beam mode. The reference infrared cell contained the CcO solution in the oxidized resting form at the same concentration. The temperature of the infrared cells was maintained at 10 °C by circulating water from a temperature-controlled water bath through cell holders. Nominal spectral resolution at 4.0 cm^{-1} was necessary to record the C–N infrared spectra in good signal to noise ratio due to the extremely low absorptivity of the C–N stretching band.

RESULTS

Fully Oxidized Cyanide-Bound Form. Upon addition of cyanide (5 mM) to the fully oxidized (resting state) cytochrome *c* oxidase (CcO), the Soret band maximum shifted from 421 to 428 nm and the α band intensity increased concomitantly, consistent with a formation of cyanide-bound CcO in the fully oxidized state. Most of this visible spectral change ceased within the first 1 h of incubation at room temperature (22 °C), but residual spectral change continued for the following 12–24 h. These observations are consistent with those of van Buuren et al. (1972) and Baker et al. (1987).

For the $^{12}C^{14}N$ complex a sharp infrared band was observed at 2152 cm^{-1} in the C–N stretching frequency region (Figure 1, upper spectrum). This infrared band showed characteristic isotope shifts upon changing cyanide isotope from $^{12}C^{14}N$ to $^{12}C^{15}N$ (2120 cm^{-1}), to $^{13}C^{14}N$ (2107 cm^{-1}) and to $^{13}C^{15}N$ (2073 cm^{-1}) (Figure 1). These shifts to lower frequencies upon changing with heavier cyanide isotopes are fully consistent with the notion that this band is due to the CcO-bound C–N stretching mode from the excellent agreement of these shifts to the expected values based on the C–N harmonic oscillator model (Table I).

Partially Reduced Cyanide-Bound Form. When the cyanide-bound form of the fully oxidized CcO was treated with excess of sodium dithionite, the visible absorption spectrum of this CcO complex showed a quick change, indicating the instantaneous reduction of the Fe_a and Cu_A centers and possibly the Cu_B center as well.[2] Only the Fe_{a3}–CN center remained in the oxidized state. This partially reduced state is relatively stable and this nature enabled us to collect infrared spectra, although a slow reduction of the cyanide-bound Fe_{a3}^{3+} center progressed steadily at room temperature.

The infrared spectra at this stage showed no 2152-cm^{-1} band characteristic to the cyanide ($^{12}C^{14}N$)-bound form of

[2] The so-called partially reduced cyanide derivative of CcO contains $Fe_a^{2+}/Cu_A^{1+}/Fe_{a3}^{3+}$–CN/$Cu_B^{1+}$ [see Johnson et al. (1981) and Nicholls and Chanady (1982)].

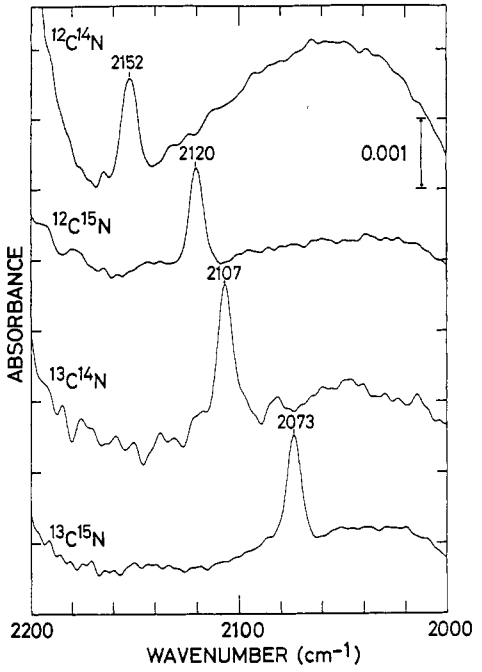

FIGURE 1: FT-IR spectra of cyanide bound form of the fully oxidized CcO. The spectra were arranged in the increasing order of the mass of cyanide isotopes from top to bottom. Sample concentration was 2.0 mM (on heme A basis) in 50 mM Tris-DCl (pD 8.4) buffer in D_2O in the IR cell (path length 51 μm). Cyanide concentration 5.0 mM. The spectra were sums of 400 cycles of spectral accumulation (40-min accumulation for each spectrum) with the normal spectral resolution at 4.0 cm^{-1}.

Table I: Effects of Cyanide Isotopes on the ν_{C-N} Stretching Frequencies of the CcO–Cyanide Complex

	ν_{C-N} (cm^{-1})			
modes[a]	$^{12}C^{14}N$	$^{12}C^{15}N$	$^{13}C^{14}N$	$^{13}C^{15}N$
1	2152	2120	2107	2073
(calcd)		(2119)	(2107)	(2073)
2	2132	2100	2086	2054
(calcd)		(2099)	(2087)	(2054)
3	2093	2060	2049	2016
(calcd)		(2061)	(2049)	(2016)
4	2058	2029	2015	1984
(calcd)		(2026)	(2015)	(1982)
5	2045	2014	2002	1970
(calcd)		(2013)	(2002)	(1970)
6	2037	2006	1995	1964
(calcd)		(2005)	(1994)	(1962)

[a] Modes 1–6 correspond to the modes in the structural models in Figure 8, respectively.

the fully oxidized CcO. Instead, there were two infrared bands at 2132 and 2093 cm^{-1} for the $^{12}C^{14}N$ complex (at $^{12}C^{14}N$ concentration of 5 mM). Both of the two infrared bands showed shifts upon substitution with a series of cyanide isotopes (Figure 2), being consistent with the notion that these are C–N stretching modes (Table I). The infrared spectroscopic results obtained as in Figures 1 and 2 are essentially the same as those of Yoshikawa and Caughey (1990) except the data for the $^{13}C^{15}N$ complex, which were not studied in the previous work.

Fully Reduced Cyanide-Bound Form. Incubation of the cyanide-bound form of the partially reduced CcO with sodium dithionite at room temperature caused a gradual intensification of the Soret band at 440 nm, which was accompanied by the absorbance increase at 585 nm (van Buuren et al., 1972). These visible spectral changes are consistent with the reduction of the cyanide-bound Fe_{a3}^{3+} center. During a course of the full reduction of CcO, the C–N stretching band at 2132 cm^{-1}

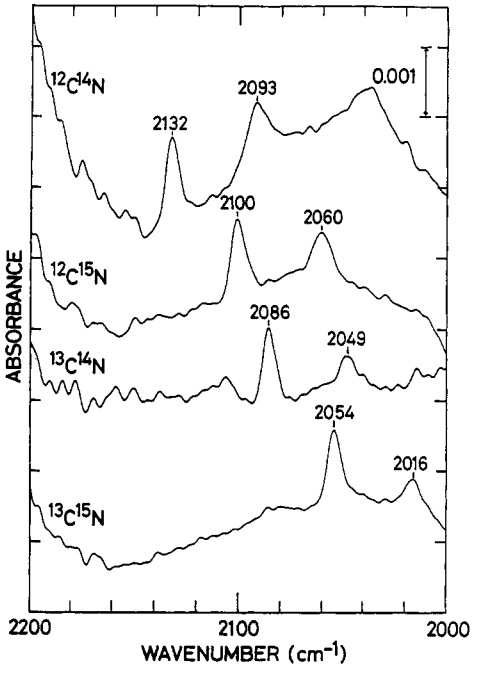

FIGURE 2: FT-IR spectra of cyanide-bound CcO in the partially reduced state. The spectra were arranged in the increasing order of the mass of cyanide isotopes from top to bottom. The Fe_a, Cu_A, and, presumably, Cu_B centers were in the reduced state, whereas the Fe_{a3} center was in the cyanide-bound oxidized state judged from visible absorption spectra. Other conditions are the same as in Figure 1.

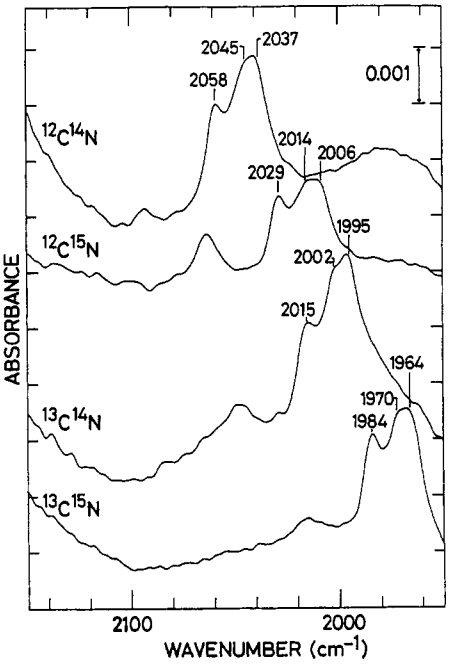

FIGURE 3: FT-IR spectra of cyanide bound CcO in the fully reduced state. The spectra were arranged in the increasing order of the mass of cyanide isotopes from top to bottom. Other conditions are the same as in Figure 1.

became weakened and the complex features around 2040 cm^{-1} developed (Figure 3). These features seem to consist of three bands at 2058, 2045, and 2037 cm^{-1}, from a comparison with the infrared spectra of the fully reduced CcO at the lower and higher cyanide concentration (see below). The same features could be produced, alternatively, by addition of potassium cyanide to the fully reduced CcO. Essentially there was no difference in the infrared spectra between these two preparations. All three infrared bands showed the expected shifts with cyanide isotopes as shown in Table I, indicating that

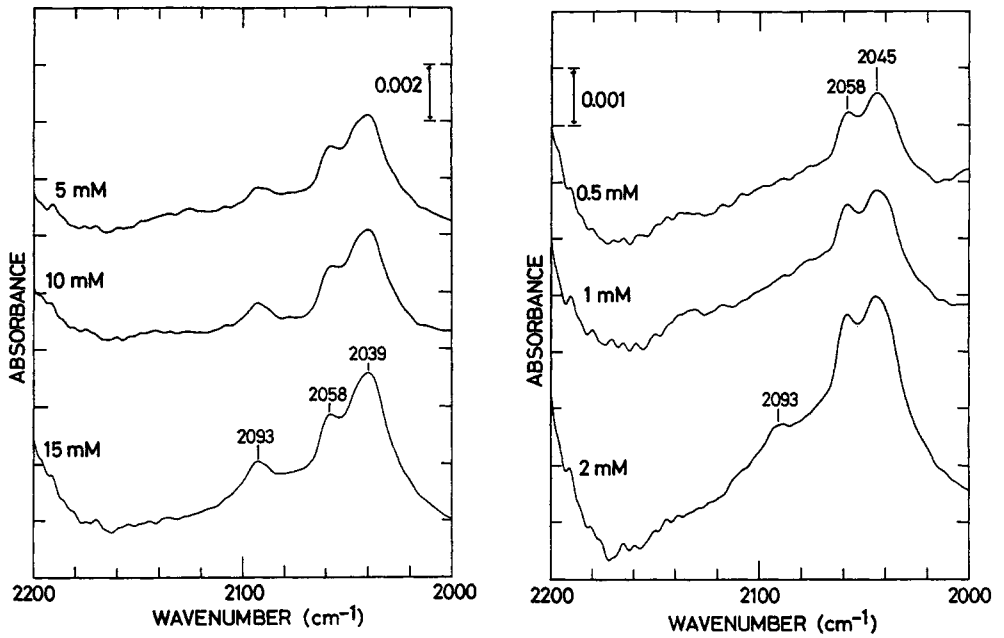

FIGURE 4: Infrared spectroscopic titration of the fully reduced CcO with potassium cyanide (right panel, 0.5–2.0 mM; left panel, 5–15 mM). Sample concentration 2.1 mM on heme A basis. Other conditions are the same as in Figure 1.

they are the C–N stretching modes bound to the fully reduced CcO.

Titration of Fully Reduced Form with Cyanide. A titration experiment of the fully reduced CcO with potassium cyanide was performed by FT-IR spectroscopy to characterize the three infrared bands at 2058, 2045, and 2037 cm^{-1}. At somewhat lower cyanide concentration (0.5–1.0 mM) there were only two infrared bands at 2058 and 2045 cm^{-1} (Figure 4). At 2.0 mM cyanide the 2093-cm^{-1} band began to appear (Figure 4). Increase of the concentration of cyanide (higher than 5.0 mM) caused a shift of the 2045-cm^{-1} band peak to 2039 cm^{-1} due to the development of the overlapping 2037-cm^{-1} band (Figure 4). These observations suggest that the binding site which gives the 2093-cm^{-1} band has a lower affinity for cyanide than those which give the 2058- and 2045-cm^{-1} bands and that, therefore, the 2058- and 2045-cm^{-1} bands have different origins from that of the 2037-cm^{-1} band.

Cyanide Binding at Lower Concentration. The fully oxidized CcO was treated first with 10 mM of EDTA in 50 mM sodium phosphate buffer (pH 7.4) overnight and concentrated by ultrafiltration using the Amicon Diaflow apparatus. This EDTA treatment was repeated several times, and then the sample was treated with 10 mM $K^{12}C^{14}N$ in 50 mM sodium phosphate buffer (pH 7.4) overnight at 4 °C. This condition leads to a nearly complete binding of cyanide to CcO as judged by the Soret spectral change. This solution was then diluted with 50 mM sodium phosphate buffer (pH 7.4) and concentrated by ultrafiltration. The repeated washing cycles caused depletion of free cyanide ion from the sample. Finally, the medium was replaced with the D_2O buffer as described in Materials and Methods. It was assumed that the sample at this stage was a "CcO–CN complex" on the basis of two spectroscopic characteristics: i.e., the sharp Soret band peak at 428 nm and the sharp infrared absorption at 2152 cm^{-1} (Figure 5, upper spectrum). Upon addition of sodium dithionite to the "CcO–CN complex", the sharp 2152-cm^{-1} band disappeared instantly and the 2132-cm^{-1} band was newly formed. Formation of the 2093-cm^{-1} band was only slight (Figure 5), indicating that a large part of the bound cyanide that gave the 2152-cm^{-1} band was selectively converted to the 2132-cm^{-1} species. As the reduction of the Fe_{a3}^{3+}–CN center proceeded, as judged from the visible absorption spectra, the

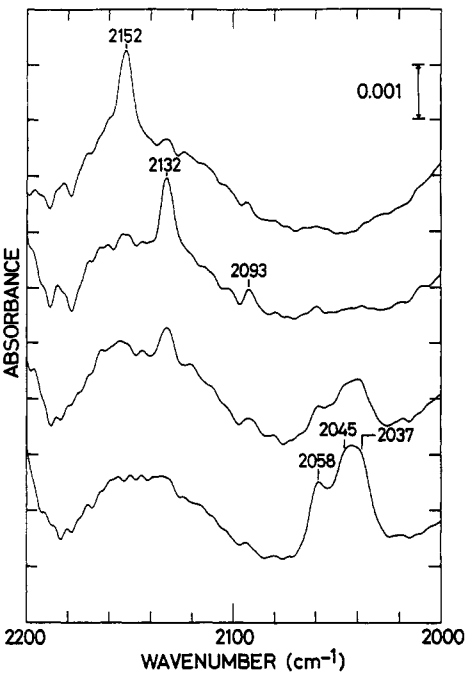

FIGURE 5: FT-IR spectra of the "CcO–cyanide complex" in various redox states. Upper line, in the fully reduced state; second line, just after the addition of sodium dithionite (27 min after) and, therefore, only heme a_3 remained in the cyanide-bound oxidized state; third line, 6 h 17 min after the addition of sodium dithionite and more than half of heme a_3 has been reduced; bottom line, all the redox centers were in the reduced state (26 h 19 min after the addition). Other conditions are the same as in Figure 1.

2132-cm^{-1} band became weaker and the complex features composed by the 2058-, 2045-, and 2037-cm^{-1} bands developed (Figure 5). It is noticeable that the weak 2093-cm^{-1} band also disappeared as the complex features around the 2058–2037-cm^{-1} region developed. This phenomenon is consistent with the notion that the site that gives the 2093-cm^{-1} band has a lower binding affinity toward cyanide than those that give the 2058- and 2045-cm^{-1} bands.

Cyanide Isotope Replacement Experiments. The "CcO–CN complex" prepared as in the previous section was subjected to the cyanide isotope replacement experiments to probe the

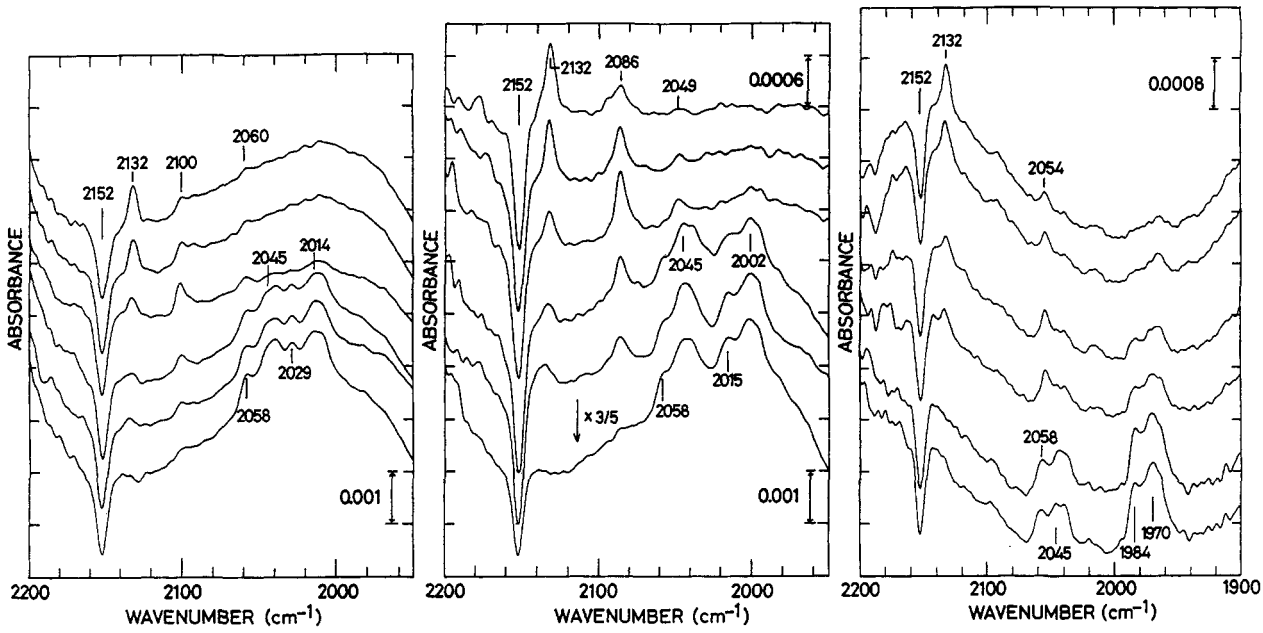

FIGURE 6: Replacement of the prebound cyanide ($^{12}C^{14}N$) with cyanide isotopes ($^{12}C^{15}N$, left panel; $^{13}C^{14}N$, middle panel; $^{13}C^{15}N$, right panel) upon reduction of CcO with sodium dithionite. Reference IR cell contains the preformed $^{12}C^{14}N$–*CcO* complex in the fully oxidized state. Measurements were performed as described in Figure 1, and the FT-IR spectra were arranged in the order of time elapsed. Left panel, 5 min, 55 min, 3 h 45 min, 18 h 47 min, 28 h 03 min, and 42 h 56 min after the addition of sodium dithionite, respectively; middle panel, 5 min, 1 h 48 min, 4 h, 16 h 46 min, 18 h 16 min, 23 h 47 min after the addition of sodium dithionite, respectively; right panel, 11 min, 1 h 01 min, 2 h 38 min, 4 h 59 min, 19 h 55 min, and 23 h 52 min after the addition of sodium dithionite, respectively.

cyanide-binding structure and the tightness of the cyanide binding. To the preformed "CcO–$^{12}C^{14}N$ complex" in the fully oxidized state (typically 2.3 mM on heme A basis) was added aerobically 2 mM (final concentration) cyanide isotope ($^{12}C^{15}N$, $^{13}C^{14}N$, or $^{13}C^{15}N$). The replacement of the prebound cyanide (indicated by the 2152-cm^{-1} infrared band) with the cyanide isotope added afterward in the fully oxidized state was monitored at 10 °C by FT-IR spectroscopy. Very slow replacement was observed for all the isotopes examined; typically an incubation of more than 24 h was required to observe a detectable amount of the isotopic shift under these conditions (spectra not shown), indicating that, in the fully oxidized state, cyanide ion binds to the Fe_{a3}-Cu_B moiety with a very high affinity.

Upon partial reduction of the redox centers of the "CcO–CN complex" with sodium dithionite in the presence of the cyanide isotope the isotope replacement occurred more easily. Most of the original $^{12}C^{14}N$ bound to CcO in the fully oxidized state (that gives the 2152-cm^{-1} band) was converted instantly into another environment that gives the 2132-cm^{-1} band (Figure 6, left panel) and, within the first 1 h after the addition of sodium dithionite, a considerable part of the bound $^{12}C^{14}N$ was replaced with $^{12}C^{15}N$ isotope added in the surrounding medium. The replacement of the bound cyanide was manifested as a sharp increase of the 2100-cm^{-1} band intensity and a concomitant intensity decrease of the 2132-cm^{-1} band (Figure 6, left panel). However, the intensity of the 2100-cm^{-1} band did not persist longer, since both of the 2132- and 2100-cm^{-1} band species were converted further to the fully reduced CcO–CN complexes. These final products gave the complex features around the 2060–2000-cm^{-1} region (Figure 6, left panel) as described above. Another interesting observation was the appearance of the 2060-cm^{-1} band only at early stage of the isotope replacement. The band intensity just after the addition of sodium dithionite is even stronger than the 2093-cm^{-1} band. This band is derived from the $^{12}C^{15}N$ complex and corresponds to the 2093-cm^{-1} band of the $^{12}C^{14}N$ complex. Very similar phenomena were also observed when other cyanide isotopes were used in the isotope replacement experiments, and such time-dependent infrared spectral changes were also displayed in Figure 6 for $^{13}C^{14}N$ (center panel) and for $^{13}C^{15}N$ (right panel).

Influence of Carbon Monoxide on the Cyanide Binding. Carbon monoxide (CO) is known to bind to both the Fe_{a3}^{2+} and Cu_B^{1+} centers, but it binds to the latter site only transiently after the photolysis of the heme-bound CO (Alben et al., 1981; Flamingo et al., 1982). Thus it is reasonable to expect that there will be a strong interaction between cyanide ion and carbon monoxide at the Fe_{a3}-Cu_B moiety in the fully reduced state. To test the existence of such an interaction, the fully reduced CcO–CO complex was prepared first in the presence of excess sodium dithionite. Then, 5 mM (final) or 20 mM (final) of a potassium cyanide ($^{12}C^{14}N$) was introduced to this preformed CcO–CO complex with minimum exposure of the sample solution to the air. The results are shown in Figure 7.

Upon addition of 5 mM (or less) of potassium cyanide to the CcO–CO complex there was only one C–N stretching band at 2093 cm^{-1} and no features around the 2050–2030-cm^{-1} region (Figure 7, upper panel). On the other hand, the C–O stretching band of Fe_{a3}^{2+}–CO at 1963.4 cm^{-1} seemed not affected by the presence of 5 mM cyanide (Figure 7, upper panel). The increase of the cyanide concentration up to 20 mM restored the 2037-cm^{-1} band and the intensity of the 2093-cm^{-1} band increased concomitantly (Figure 7, lower panel); however, there were still no features at 2058 and 2045 cm^{-1} that were unique in the absence of carbon monoxide at lower cyanide concentration (Figure 4, left panel). In addition, the C–O stretching band intensity of Fe_{a3}^{2+}–CO at 1963.4 cm^{-1} became weaker. The higher cyanide concentration affected the visible absorption spectrum of the fully reduced CcO–CO as well; i.e., the higher cyanide concentration diminished the Soret band intensity at 425 nm considerably (spectra not shown). However, these spectral changes were not due to formation of the partially oxidized species during the experiment as judged from FT-IR or visible absorption spectroscopies.

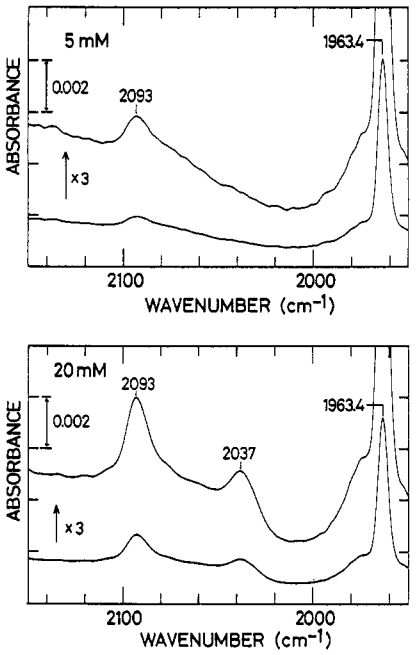

FIGURE 7: Effect of carbon monoxide binding to the Fe_{a3}^{2+} center upon cyanide binding to the fully reduced CcO. Upper panel, cyanide concentration 5 mM; lower panel, cyanide concentration 20 mM. Measurements of the FT-IR spectra were performed as described in Figure 1.

As a control experiment, the fully reduced CcO with excess sodium dithionite was mixed first with 5 or 20 mM (final) of potassium cyanide, and then carbon monoxide gas was introduced to a small tube containing the CcO–CN sample. When these samples were examined by FT-IR spectroscopy, essentially similar results with Figure 7 were obtained. The actual effect of cyanide at a given concentration was smaller than the corresponding concentration in Figure 7 possibly due to the partial evaporation of cyanide during the introduction of carbon monoxide gas (although the gas flow rate through the test tube containing the sample was maintained at a minimum).

Thus there are total of, at least, 6 cyanide species bound to CcO giving different ν_{C-N} stretching frequencies. We had expected to detect small but distinctive differences in the isotope shift patterns enough to assign a possible binding structure at the binuclear site for each C–N stretching mode; but within the accuracy of the frequency shifts upon isotopic substitution there was no systematic difference among these C–N stretching modes (Table I). This fact indicates that the C–N bond is so strong that the coupling of the C–N stretching with other vibrations (such as Fe_{a3}–C stretching, Fe_{a3}–C–N bending, etc.) is extremely weak.

DISCUSSION

Assignments of the Observed C–N Stretching Bands. It is known that free cyanide ion gives a stretching band at 2079 cm^{-1}. But its molecular extinction coefficient is only 0.03 mM^{-1} cm^{-1} (Yoshikawa & Caughey, 1985; Nakamoto, 1986). The observed C–N stretching bands in the present study have one order of higher molecular extinction coefficients (the 2153-cm^{-1} band, 0.31–0.38 mM^{-1} cm^{-1}; the 2132-cm^{-1} band, 0.23–0.25 mM^{-1} cm^{-1}), indicating that all of these bands are from metal-bound cyanide species and are not derived from free cyanide ion trapped in the protein matrix.

The minimum functional unit of CcO can be viewed as containing two heme irons (Fe_a and Fe_{a3}) and two copper atoms (Cu_A and Cu_B) with only two metal centers (Fe_{a3} and Cu_B) potentially able to bind exogenous ligands. However, the possibility of cyanide binding to Zn, Mg, and Cu_x (the third copper in excess of Fe) must be also considered (Einarsdóttir & Caughey, 1985; Steffens et al., 1987). From X-ray absorption measurements it has been concluded that the zinc ion is bound to three sulfur atoms, probably in subunit VIa (Naqui et al., 1988). Thus the zinc ion seems to have only a structural role and, therefore, to have no redox-linked functional role. In addition, a purified sample of CcO of *Paracoccus denitrificans* contained only substoichiometric amounts of zinc and magnesium ions, suggesting that these ions are not likely to be functionally involved (Steffens et al., 1987). On the other hand, there has been no report for the magnesium–cyano complex so far, indicating that the cyanide binding affinity for Mg is very low (Nakamoto, 1986). The third copper (Cu_x) is likely not to be a redox-linked functional component. Even if the Cu_x acts as another component of the Cu_A center as proposed by Kroneck et al. (1988, 1990), the cyanide binding to the Cu_A center seems unlikely. Thus the possibilities that Zn, Mg, and Cu_x act as the cyanide binding sites are very low as previously discussed by Yoshikawa and Caughey (1990), and therefore, only cyanide binding to the Fe_{a3}-Cu_B binuclear site was considered in the present study. Following are new assignments and their justifications based on the present study.

(A) 2132-cm^{-1} Band. The 2132-cm^{-1} band that appeared at the partially reduced state can be assigned to Fe_{a3}^{3+}–CN based on the similarity in ν_{C-N} values for Fe^{3+}–CN bands of hemin cyanides and other oxidized hemoprotein–cyanide complexes as suggested first by Yoshikawa and Caughey (1990). The authenticity of this assignment seems clear.

(B) 2152-cm^{-1} Band. Yoshikawa and Caughey (1990) proposed that this band and a somewhat variable band appearing at 2165 cm^{-1} were due to the cyanide bound to cupric copper (possibly Cu_B^{2+}) on the basis of the following observations: (1) When the resting oxidized CcO–CN complex was reduced anaerobically with β-NADH in the presence of catalytic amount of phenazine methosulfate, the 2152-cm^{-1} band was weakened concomitantly with appearance of the 2093-cm^{-1} band which was assigned reasonably to Cu_B^{1+}–CN. (2) The 2165-cm^{-1} band appearing at the one electron reduced state also disappeared at the oxidation state lower than the two electron reduced state. They suggested further that the binding of cyanide to Cu_B^{2+} might alter the bonding of a bridging ligand X (e.g., S or Cl) between Cu_B^{2+} and Fe_{a3}^{3+}, causing the Soret peak shift from 418 to 428 nm. However, this assignment is apparently inconsistent with the classical view that cyanide binds to the Fe_{a3}-Cu_B binuclear site of the resting CcO, forming a μ-cyano complex, Fe_{a3}^{3+}–C–N–Cu_B^{2+} (Thomson et al., 1981; Boelens et al., 1983).

The present findings, however, indicate that the 2152-cm^{-1} band is due to the bridging structure, Fe_{a3}^{3+}–C–N–Cu_B^{2+}. It was shown in the cyanide isotope replacement experiments that the cyanide that gave the 2152-cm^{-1} band remained bound to the Fe_{a3}^{3+} center upon reduction of other three redox centers and showed the transient 2132-cm^{-1} band. Another evidence is the appearance of the 2060-cm^{-1} band just after the addition of sodium dithionite in the isotope replacement experiment with $^{12}C^{15}N$ (Figure 6, left panel). The 2060-cm^{-1} band was shown to be derived from the Cu_B^{1+}–$^{12}C^{15}N$ complex (Table I and Figure 8), thus it is very hard to explain why the 2060-cm^{-1} band has such a stronger intensity than the 2093-cm^{-1} band (due to Cu_B^{1+}–$^{12}C^{14}N$) just after the reduction of the Cu_B center occurred if the 2152-cm^{-1} band is due to Cu_B^{2+}–$^{12}C^{14}N$. For the other two sets of the cyanide isotope replacement experiments (Figure 6, center and right panels) very similar arguments can be made. If the 2152-cm^{-1} band

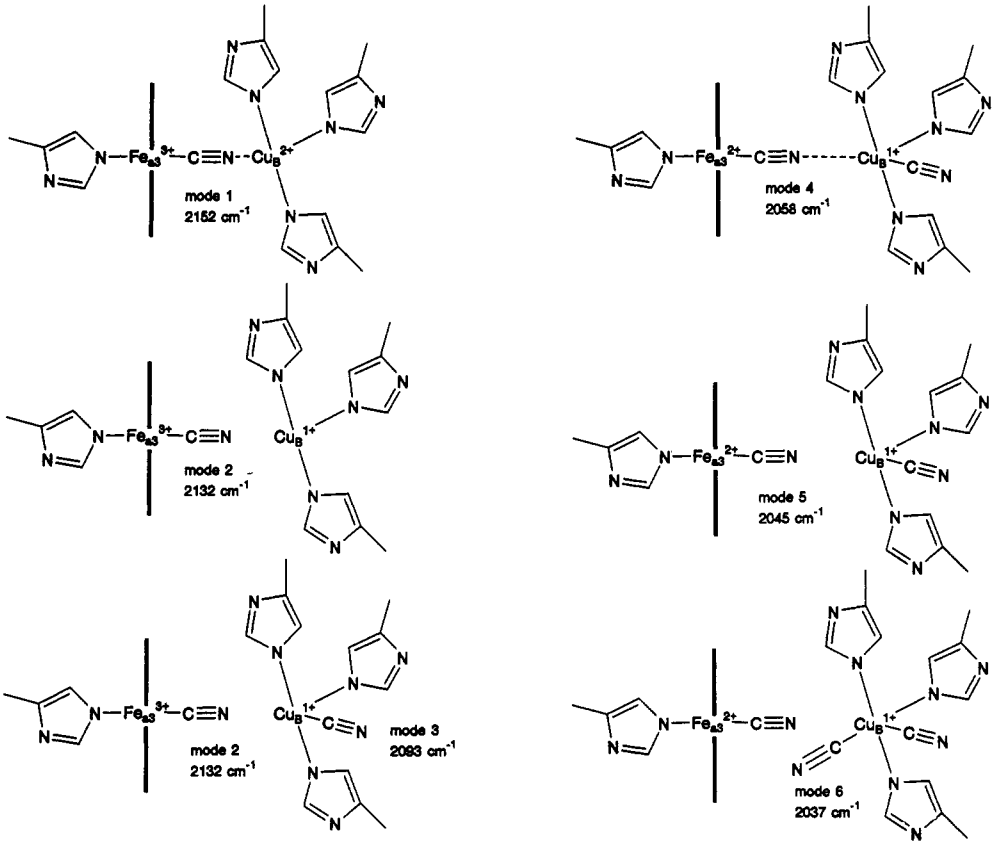

FIGURE 8: Summary of present assignments on the ν_{C-N} stretching frequencies of the CcO–cyanide complex.

is due to the Fe_{a3}^{3+}–X–Cu_B^{2+}–$^{12}C^{14}N$ complex, this cyanide ion must be first liberated and then selectively transferred to the Fe_{a3}^{3+} center to form the Fe_{a3}^{3+}–CN complex without exchange with surrounding free cyanide ion. Although such a specific possibility cannot be neglected, at this stage, the assignment of the 2152-cm^{-1} band to the Fe_{a3}^{3+}–C–N–Cu_B^{2+} bridging structure (the classical view) seems favorable on the basis of the following evidence. (1) Recent reexaminations of the cyanide binding at the partially reduced states under improved experimental conditions showed the reciprocal relationship of the band intensities between the 2152- and 2132-cm^{-1} bands, being inconsistent with the previous observations (Yoshikawa et al., unpublished). (2) The unusually slow replacement of the bound cyanide that gives the 2152-cm^{-1} band with free cyanide isotopes is consistent with the reported values of cyanide binding parameters to the fully oxidized CcO (van Buuren et al., 1972; $K_d = 1.0 \times 10^{-6}$ M, $k_{on} = 1.8$ M^{-1} s^{-1}, and $k_{off} = 1.8 \times 10^{-6}$ s^{-1}) and to the partially reduced enzyme (Jones et al., 1984; $K_d = 4.8 \times 10^{-6}$ M, $k_{on} > 10^6$ M^{-1} s^{-1}, and $k_{off} < 4.8$ s^{-1}), and these observations can be easily understood on the basis of the bridging structure. On the other hand, it was reported that cyanide binds to the copper binuclear site (Cu^{2+}) of hemocyanin with a carbon end-on-bonded geometry with $k_d = 0.14$ M, considerably lower binding affinity compared to the binding of cyanide to CcO (Pavlosky & Laraabee, 1988). (3) The higher frequency of the bridging C–N structure by 20 cm^{-1} is consistent with the view that such a structure has a higher ν_{C-N} stretching frequency than the terminally coordinated C–N as shown in various model complexes (Nakamoto, 1986). (4) A model study suggested a ν_{C-N} value for a Fe^{3+}–porphyrin–Cu^{2+} cyanide complex in the Fe^{3+}–C–N–Cu^{2+} bridging structure to be 2150 cm^{-1} (Gunter et al., 1984). (5) The appearance of the low-spin EPR signal of the Fe_{a3}^{3+}–CN ($g = 2.42$) produced by addition of NO to the CcO–cyanide complex indicates that the NO binding is specific to the Cu_B^{2+} center and does not affect a bond between the Fe_{a3}^{3+} center and cyanide (Stevens et al., 1979; Brudvig et al., 1980; Boelens et al., 1983).[3] Otherwise the NO binding to the CcO–cyanide complex must facilitate a transfer of the bound cyanide from the Cu_B^{2+} center to the Fe_{a3}^{3+} center. (6) Upon addition of cyanide to the preformed CcO–azide complex in the fully oxidized state, a "CcO–azide–cyanide ternary complex" is expected to be formed. In this ternary complex the cyanide is presumably bound to the Cu_B^{2+} center. However, we could not detect any C–N stretching infrared band corresponding to the Cu_B^{2+}–C–N structure, indicating a possibility that the intrinsic infrared absorptivity of the Cu_B^{2+}–C–N moiety is extremely weak (Tsubaki & Yoshikawa, 1993; following paper in this issue).

All these observations seem to favor the classical view that cyanide ion forms the bridging structure (Fe_{a3}^{3+}–C–N–Cu_B^{2+}) in the fully oxidized state. This bridging structure can be destroyed upon the reduction of other redox centers, and the cyanide ion remains bound to the Fe_{a3}^{3+} center to give the transient 2132-cm^{-1} band after this redox-linked conformational change.

The nature of the 2165-cm^{-1} band reported by Yoshikawa and Caughey (1990) is not clear at this stage since the 2165-cm^{-1} band could not be reproduced in the present study using sodium dithionite as a reducing agent. This species seems to be specific for the early stage of the partially reduced state.

(C) 2093-cm^{-1} Band. The 2093-cm^{-1} band appearing both in the partially and in the fully reduced states is at a frequency

[3] The $g = 3.42$ value is clearly different from the corresponding value of $g = 3.58$ (Johnson et al., 1981) or $g = 3.63$ (Goodman, 1984) for the partially reduced cyanide-inhibited derivative of CcO. This fact may indicate that the cyanide-bound species giving the $g = 3.42$ signal is still in the bridging state but only the magnetic coupling is removed upon NO binding to the Cu_B^{2+} center.

appropriate for cuprous cyanide (Cu^{1+}) (Nakamoto, 1986). Another interesting property of this 2093-cm^{-1} band is the insensitivity toward carbon monoxide. Carbon monoxide binds very strongly to the Fe_{a3}^{2+} center and only transiently to the Cu_B^{1+} center after the photolysis of Fe_{a3}^{2+}–CO (Dyer et al., 1989a,b). These observations are consistent with the notion that the 2093-cm^{-1} band is due to Cu_B^{1+}–CN as suggested first by Yoshikawa and Caughey (1990). The assignment of the 2093-cm^{-1} band to the Cu_B^{1+} species demands the stereochemistry of the cyanide binding to the Cu_B^{1+} center away from Fe_{a3}^{2+} center, or the Cu_B^{1+} center itself being moved far away from the Fe_{a3}^{2+} to make enough space. This 2093-cm^{-1} band intensity is very weak at lower cyanide concentration as indicated in the cyanide titration experiment in the fully reduced state (Figure 4). This fact suggests that the cyanide-binding affinity by this Cu_B^{1+} site is much weaker than those by the sites that give the 2058- and 2045-cm^{-1} bands (Fe_{a3}^{2+}–CN species, see next section). This interpretation is consistent with a recent cyanide binding study in the fully reduced state (Hill & Marmor, 1991). This 2093-cm^{-1} band appears weakly and only transiently when the "CcO–CN complex" was reduced with sodium dithionite (Figure 5). This may be explained as due to the aberrant partial release from the Fe_{a3}^{3+} center or due to the cyanide trapped in the protein matrix in the fully oxidized state.

(*D*) *2058- and 2045-cm^{-1} Bands.* The 2058- and 2045-cm^{-1} bands behaved very similarly each other. The complete suppression of these bands by carbon monoxide and the concomitant appearance of the 1963.4-cm^{-1} C–O stretching band strongly suggest that these two bands are due to Fe_{a3}^{2+}–CN. The shift to lower frequency by as large as 87 (or 76) cm^{-1} from 2132 cm^{-1} (Fe_{a3}^{3+}–CN) to 2045 (or to 2058) cm^{-1} (Fe_{a3}^{2+}–CN) is consistent with Fe^{2+} being a less-effective σ-acceptor and an effective $d\pi$-donor than Fe^{3+} (Nakamoto, 1986). Cyanide acts as a σ-donor by donating electrons to the metal and also as a π-acceptor by accepting electrons from the metal. The σ-donation tends to raise the ν_{C-N} since electrons are removed from the 5σ orbital, which is weakly antibonding, while π-back-bonding tends to decrease the ν_{C-N} because the electrons enter the antibonding $2p\pi^*$ orbital (Nakamoto, 1986). The present assignment is fully consistent with this view.

Horseradish peroxidase (HRP) is known to show the bound C–N stretching modes both in the oxidized (2131 cm^{-1} in both D_2O and H_2O) and in the reduced (2037 cm^{-1} in D_2O and 2029 cm^{-1} in H_2O) states (Yoshikawa et al., 1985). HRP and CcO are unique for the cyanide binding in the reduced form, since other hemoproteins such as hemoglobin and myoglobin do not bind cyanide in the reduced state when the concentration of the cyanide is not so high. Teraoka and Kitagawa (1980) suggested that CN^- binds to reduced HRP in a σ-type bonding, whereas reduced hemoglobin and myoglobin bind to CN^- in π-type bonding on the basis of resonance Raman data (Kitagawa et al., 1976). Such modulation of the bonding type may be most likely regulated by the imdiazole group of the distal histidine residue that seems essential for the catalytic reaction of HRP. The imidazole group must interact with the Fe^{2+}–CN moeity via hydrogen bonding interaction leading to the unusually high binding affinity of the reduced HRP to CN^-. Indeed the existence of a hydrogen bonding(s) to the bound cyanide in HRP in the reduced state is clearly demonstrated by a large D_2O shift on the bound C–N stretching frequency (from 2029 to 2037 cm^{-1}) (Yoshikawa et al., 1985). Is it possible that the unusually high binding affinity of CN^- to the Fe_{a3}^{2+} center could be attributable to the existence of distal amino acid residue(s) (His residue?) located at very vicinity to the Fe_{a3}^{2+} center? Existence of several His residues (possibly three) ligated to the Cu_B center has been proposed from EXAFS studies [Reinhammar et al., 1980; Cline et al., 1983; see Scott (1989)]. However, we could not detect a large D_2O shift on the bound C–N stretching frequencies for the fully reduced state [from 2058 (D_2O) to 2059 (H_2O) cm^{-1} and from 2045 (D_2O) to 2044 (H_2O) cm^{-1}]. This observation may indicate the absence of strong hydrogen bonding(s) to the bound cyanide or the absence of exchangeable hydrogen atom(s) at the His residue(s) in the CcO binuclear site. As an alternative possibility the stabilizing mechanism of the Fe_{a3}^{2+}–CN structure in CcO may be very different from that of HRP. The Cu_B^{1+} center may have the stabilizing power (probably by a Coulomb type interaction) on the Fe_{a3}^{2+}-bound cyanide. In this case the 13-cm^{-1} difference between the 2058- and 2045-cm^{-1} bands may be ascribed to the conformational heterogeneity at the Fe_{a3}^{2+}–CN···Cu_B^{1+} site, most likely to the difference in the extent of the interaction between bound CN and Cu_B^{1+}. The 2058-cm^{-1} band has a 13 cm^{-1} higher frequency than the 2045-cm^{-1} band and, thus, may have a weakly-bridged structure like Fe_{a3}^{2+}–C–N···Cu_B^{1+} since the bridging structure tends to have a higher C–N stretching frequency than those in nonbridging structures (Nakamoto, 1986) as found in the oxidized state.

(*E*) *2037-cm^{-1} Band.* The 2037-cm^{-1} band is very peculiar. This species appears only in the fully reduced state and is not obvious at lower cyanide concentrations (lower than 2 mM). More interestingly, the formation of this species is inhibited by the presence of carbon monoxide at lower cyanide concentrations. However, addition of a higher cyanide concentration causes the restoration of this 2037-cm^{-1} band (but not for the 2058- and 2045-cm^{-1} bands) with concomitant decreases of the C–O stretching band intensity at 1963.4 cm^{-1} and the Soret band intensity at 440 nm. These observations suggest that the cyanide ion bound to this site has a weak competitive interaction with the carbon monoxide bound to the Fe_{a3}^{2+} center. This consideration leads to the suggestion that a second C–N ion may bind to the Cu_B^{1+}–CN center, besides the 2093 cm^{-1} species, being oriented toward the Fe_{a3}^{2+} center. As an alternative possibility, the second cyanide bound to Cu_B^{1+} may interact with Fe_{a3}^{2+}–CO indirectly via the "endogenous transferable ligand (L) between Fe_{a3} and Cu_B" as proposed by Woodruff et al. (1991). As another possibility the second cyanide may bind to the metal through the nitrogen atom instead of the carbon atom; this assumption may explain the large frequency difference (2093 and 2037 cm^{-1}) and the binding affinity difference. The absence of the competitive interaction between the cyanides bound to the Fe_{a3}^{2+} and Cu_B^{1+} centers [i.e., between the 2058- (or 2045-) and 2037-cm^{-1} bands] indicates that the binding geometry of cyanide toward the Fe_{a3}^{2+} center is slightly different from that of carbon monoxide.

A multiple binding of cyanide ions to the Fe_{a3}^{2+} center for the cause of the 2037-cm^{-1} band seems unlikely. This model cannot explain the weak competitive interaction between the 2037-cm^{-1} species and the Fe_{a3}^{2+}–CO moeity. Further, the binding of a second C–N to the Fe_{a3}^{2+}–CN center either from the proximal side or from the distal side of the heme iron should affect the preexisting C–N stretching frequency considerably, but actually there seemed no influence at all for both of the 2058- and 2045-cm^{-1} bands upon appearance of the 2037-cm^{-1} band (Figure 4). Another possibility that the 2037-cm^{-1} band is due to the denatured form of CcO–CN caused by a copper depletion is also unlikely, since this band begins to appear at cyanide concentrations of 2 mM, much

lower than that used for removal of copper ion (0.2–1.0 M) (Weintraub et al., 1982).

In Figure 8, a summary of the present assignments on the various C–N stretching frequencies of CcO–cyanide complex is shown schematically (cf. Table I).

Conformational Change at the Fe_{a3}^{3+}-Cu_B^{2+} Binuclear Site upon Cyanide Binding. It has long been known that CcO in the fully oxidized state can exist in more than one conformation, for example, the forms reacting with cyanide at different rates, one reacting slowly and the other reacting rapidly. The origin of the heterogeneity is mainly due to the existence of different structures at the Fe_{a3}-Cu_B binuclear site (Brudvig et al., 1981; Baker et al., 1987; Schoonover et al., 1988). An important point to note is that the final form after the reaction of cyanide with the fully oxidized CcO is homogeneous, exhibiting only one C–N stretching frequency at 2152 cm^{-1}. This observation is not consistent with the recent magnetic susceptibility data for the cyanide-bound fully oxidized enzyme that exhibits magnetic heterogeneity, with a major fraction (78%) having a weak magnetic coupling ($-J = 1.3$ cm^{-1}) and a minor fraction (22%) having an intermediate value ($-J = 44$ cm^{-1}) (Barnes et al., 1991). But the latter coupling constant is close to the value of 38.5 cm^{-1} obtained for the cyanide-bound enzyme found to be homogeneous by Tweedle et al. (1978). The conformational homogeneity (but may not be magnetically) of the cyanide-bound form of the enzyme indicates that the degree of the conformational heterogeneity at the binuclear site in the resting state may be relatively minor and this heterogeneity can be easily removed by formation of the strong bridge structure with cyanide between the Fe_{a3}^{3+} and the Cu_B^{2+} centers.

Conformational Change at the Fe_{a3}-Cu_B Binuclear Site upon Partial Reduction. The treatment of the fully oxidized CcO–CN complex with sodium dithionite caused a rapid reduction of the Fe_a, Cu_A, and Cu_B centers and a concomitant production of the 2132-cm^{-1} band, suggesting a conformational change(s) at the binuclear site. This frequency is in the range for usual ferric hemoprotein–cyanide complexes (Yoshikawa et al., 1985). The 2132-cm^{-1} band also can be produced by the addition of cyanide to the partially reduced form of CcO (Yoshikawa & Caughey, 1990). It was shown that the formation of the 2132-cm^{-1} species could be induced even at the one electron reduced state of CcO, in which the added electron resides mainly at the Cu_B center and there is no indication of the 2093-cm^{-1} band at this stage (Yoshikawa & Caughey, 1990). Therefore, reduction of the Cu_B center is the sole requirement for the formation of the 2132-cm^{-1} species, and the further reduction of the Fe_a and/or Cu_A center causes the appearance of the 2093-cm^{-1} band. It must destroy the bridging structure at the binuclear site, leaving the cyanide at the Fe_{a3}^{3+} center and the further reduction of Fe_a and/or Cu_A results in the binding of cyanide to the Cu_B^{1+} center. Indication of the additional conformational change at the binuclear site upon the Fe_a and/or Cu_A reduction has been suggested by the shift of the Fe_{a3}^{3+}–CN low-spin EPR signal from $g = 3.61$ to 3.63 (Goodman, 1984). Such a series of conformational change at the binuclear site associated with the partial reduction of the redox centers is known as the "closed" to "open" form transition (Jones et al., 1984; Jensen et al., 1984). Baker et al. (1987) have pointed out that both the slow and the rapid reacting forms toward cyanide ion exist in the "closed" state, since even the rapid reacting form binds cyanide very slowly compared to the "open" conformation formed upon partial reduction. This argument leads to the proposal that the 2152-cm^{-1} band is derived from the "closed" form whereas the 2132- and 2093-cm^{-1} bands are indicative of the "open" form.

Cyanide Binding to the Cu_B Center. The absence of any ν_{C-N} stretching band corresponding to the Cu_B^{2+}–CN structure in the fully oxidized state seems to indicate that either (1) cyanide does not bind to the Cu_B^{2+} center or (2) the Cu_B^{2+}–CN could not be observed by infrared spectroscopy due to weak infrared absorptivity of this moeity (see previous discussion for the 2152-cm^{-1} band). In the former case, a series of drastic conformational change is expected to occur upon the reductions of the Cu_B and Fe_a and/or Cu_A centers as discussed above. It enables the binding of cyanide to the Cu_B^{1+} center oriented away from the Fe_{a3} center to give the 2093-cm^{-1} band. There is no compelling reason to consider that the intrinsic binding affinity of cyanide to the Cu_B^{2+} is much weaker than toward the Cu_B^{1+}, since the Cu^{2+}–CN complex has been formed in laccase, ascorbate oxidase, and Cu^{2+}–Leu-Leu-Leu complex exhibiting ν_{C-N} bands near 2168 cm^{-1} (Yoshikawa et al., 1984), although there is a tendency that Cu^{1+} has a higher affinity for cyanide than does Cu^{2+} (Cotton & Wilkinson, 1972). In the latter case a second cyanide may be actually bound to the Cu_B^{2+} center even in the fully oxidized state, leading to a structure like Fe_{a3}^{3+}–C–N–Cu_B^{2+}–C–N. Indeed, it has been indicated by a decrease of the Fe_{a3}^{3+} EPR signal intensity upon addition of cyanide to the oxidized CcO–NO complex via displacement of NO molecules with cyanides (Stevens et al., 1979). But this model does not seem compatible with the absence of the 2093-cm^{-1} band in the one electron reduced state of CcO (Yoshikawa & Caughey, 1990). Recently, a very interesting study concerning the reaction of cyanide with cytochrome ba_3 from *Thermus thermophilus* appeared (Surerus et al., 1992). In this peculiar cyanide complex, Cu_A, cytochrome *b*, and Cu_B remain oxidized, while cytochrome a_3 is reduced and cyanide is bound as the Fe_{a3}^{2+}–CN Cu_B^{2+}–CN complex. Infrared measurement of this cyanide complex may clarify the cyanide binding to the Cu_B^{2+} center.

Further Conformational Change at the Fe_{a3}-Cu_B Binuclear Site upon Reduction of Fe_{a3}. The 2037-cm^{-1} band has been assigned to the cyanide bound to the Cu_B^{1+} center oriented toward Fe_{a3}^{2+}–CN (or Fe_{a3}^{2+}–CO). For the multiple ligand binding, the Fe_{a3}^{2+} center and the Cu_B^{1+} center must be separated further from each other to fit two molecules of cyanide ions (or cyanide and carbon monoxide). Therefore, a further drastic conformational change at the binuclear site (a further increase of the distance between the Fe_{a3} and the Cu_B centers) must occur upon the Fe_{a3} reduction. Indeed, most of the 2037-cm^{-1} band is associated with the 2058- and 2045-cm^{-1} bands (Fe_{a3}^{2+}–CN) and the 2093-cm^{-1} band (Cu_B^{1+}–CN), indicating that the reduction of both the Fe_{a3} and Cu_B centers is necessary to cause the large separation between these two centers. But, in some experiments, particularly at a higher cyanide concentration, the 2037-cm^{-1} band developed considerably, showing that a large part of the Fe_{a3} center is still in the oxidized state (i.e., Fe_{a3}^{3+}–CN) as shown in Figure 2 (upper spectrum). A more elegant experiment is necessary to answer this question.

Implication to the Coformational Change at the Binuclear Site during the Physiological Turnover. The present study shows clearly that the redox level of the metal centers controls the three kinds of structural change around the binuclear site very accurately. Such an indication has been reported for the carbon monoxide binding to the Fe_{a3} center (Yoshikawa et al., 1982; Einarsodóttir et al., 1989). The C–O stretching frequency of Fe_{a3}^{2+}–CO is controlled by the overall redox level of CcO; in the fully reduced state [i.e., CcO(VI)] the

C–O stretching frequency is at 1963.3 cm^{-1}, whereas in the partially reduced state, like CcO(I) being reduced with only one electron equivalent, the C–O stretching frequency is at 1965.4 cm^{-1} (Yoshikawa et al., 1982, Einarsdóttir et al., 1989). Thus there is a 2.1-cm^{-1} shift upon the overall redox level change.

In the following paper in this issue we will show that, when azide is bound to the binuclear center, similar redox-linked conformational changes at the binuclear center to that observed in the present study are also operative (Tsubaki & Yoshikawa, 1993; following paper in this issue). Thus it is very likely that the tertiary structural changes around the Fe_{a3}-Cu_B binuclear site upon reduction of the metal centers play important roles during the catalysis of dioxygen to water using 4 electrons from reduced cytochrome *c*.

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