# Structure of the Heme–Copper Binuclear Center of the Cytochrome *bo* Complex of *Escherichia coli*: EPR and Fourier Transform Infrared Spectroscopic Studies†

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ABSTRACT: The cytochrome *bo* complex is a terminal quinol oxidase in the aerobic respiratory chain of *Escherichia coli* and functions as a redox-coupled proton pump. To clarify the structural differences of the binuclear reaction center between the cytochrome *bo* complex and the mitochondrial cytochrome *c* oxidase, a combined study using EPR and Fourier transform infrared spectroscopies was carried out. The EPR spectrum of the highly purified cytochrome *bo* complex in the air-oxidized state showed a broad EPR signal (peak $g^* = 3.7$) from an integer spin system. This confirms the existence of the spin–spin exchange-coupled binuclear site, in which the $Fe_o^{3+}$ and $Cu_B^{2+}$ centers were bridged by an unknown ligand (X). Binding of azide at the binuclear site as an ionic modulator weakened the strength of the spin–spin exchange coupling and thus caused a narrowing of the broad EPR signal. Binding of another modulator, formate, at the binuclear site caused the formation of EPR signals at $g' = 12$ and 2.7, which are very similar to those observed for cytochrome *c* oxidase. Cyanide replaced the bridging ligand (X) to form an $Fe_o^{3+}$–C–N–$Cu_B^{2+}$ structure in which strong spin–spin exchange coupling is expected, leading to a complete EPR-invisible state. Infrared evidence (a 2146 $cm^{-1}$ C–N stretching band for the cyanide complex and a 2041 $cm^{-1}$ azide antisymmetric stretching band for the azide complex) supported the theory that these ligands form bridging structures at the binuclear center, as previously observed for cytochrome *c* oxidase. These observations suggest that a gross conformation at the binuclear site seems well-conserved among the heme–copper oxidase superfamily.

The cytochrome *bo* complex is a predominant terminal oxidase in the aerobic respiratory chain of *Escherichia coli* under conditions of high aeration, and it catalyzes the oxidation of ubiquinol-8 and the reduction of molecular oxygen to water (Anraku, 1988; Anraku & Gennis, 1987; Minagawa *et al.*, 1990). This redox reaction mechanistically couples with the formation of an electrochemical proton gradient across the cytoplasmic membrane *via* proton pumping and scalar protolytic reactions at the inner and outer surfaces of the cytoplasmic membrane (Kita *et al.*, 1982; Puustinen *et al.*, 1989, 1991).

The genes coding for the cytochrome *bo* complex have been cloned (Au *et al.*, 1985; Nakamura *et al.*, 1990) and sequenced (Chepuri *et al.*, 1990a; Minagawa *et al.*, 1990). DNA sequence analysis of the *cyoB* gene revealed homology with bacterial and mammalian cytochrome *c* oxidase subunit I, in which two hemes A and one of the coppers ($Cu_B$) are expected to bind (Chepuri *et al.*, 1990a), and its gene product was assigned to subunit I (Nakamura *et al.*, 1990). The deduced amino acid sequence of the *cyoA* gene product (assigned to subunit II; Nakamura *et al.*, 1990), however, does not contain amino acid residues purported to bind to $Cu_A$ ligands or to be involved in cytochrome *c* binding conserved in cytochrome *c* oxidase subunit II (Chepuri *et al.*, 1990a; Saraste, 1990). Nevertheless, its hydropathic profile is very similar to those of subunit II of cytochrome *c* oxidases (Chepuri *et al.*, 1990a,b).

Many biophysical studies have been carried out for the cytochrome *bo* complex to clarify the structures of the metal centers. Previous EPR and resonance Raman studies have revealed that the cytochrome *bo* complex contains a low-spin heme, a high-spin heme (Uno et al., 1985), and a copper atom ($Cu_B$) as the prosthetic groups (Kita *et al.*, 1984; Hata *et al.*, 1985). These heme prosthetic groups are heme B and heme O, which are different from heme A of cytochrome *c* oxidase (Puustinen & Wikström, 1991; Wu *et al.*, 1992). Recently, the high-spin heme binding site was found to be exclusively occupied by heme O (Saiki *et al.*, 1992) and the low-spin heme binding site to be promiscuous with respect to heme type (Puustinen *et al.*, 1992; Hill *et al.*, 1992). Infrared spectroscopy revealed that the C–O stretching bands of the CO-complexed enzyme and its photolyzed product at low temperature (30 K) in the glycerol-dehydrated *E. coli* membrane are very similar to those of the eukaryotic oxidases (Chepuri *et al.*, 1990b; Hill et al., 1992). Thus Chepuri *et al.* (1990b) have suggested that the high-spin heme (heme O) and the $Cu_B$ center may form a binuclear center, similar to that present in the $aa_3$-type cytochrome oxidases. Salerno *et al.* (1990) proposed that the binuclear center is magnetically coupled in the air-oxidized resting state on the basis of the EPR spectroscopic redox titration for the *E. coli* membrane samples. Very recently, Calhoun *et al.* (1992) showed that the formate complex of cytochrome *bo* quinol oxidase exhibits a similar EPR feature analogous to that of "slow" cytochrome oxidase. The Cu-EXAFS data for the oxidized cytochrome *bo* complex indicated the $Cu_B^{2+}$–$Fe_o^{3+}$ distance to be 2.92 Å (Ingledew & Bacon, 1991), close enough for this interaction.

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The similarities in the primary sequences of subunit I and in the biophysical properties between the cytochrome *bo* complex and the mitochondrial cytochrome *c* oxidases indicate that the reaction centers have a common molecular architecture and, therefore, a common molecular mechanism of redox-coupled proton pumping. Since the binuclear site is involved in the dioxygen reduction chemistry and is likely coupled directly to the proton pumping activity of this enzyme (Babcock & Wikström, 1992), an understanding of the more precise structural view of the heme–copper binuclear site is essential. Only a few studies, however, were done with the purified oxidase because of the difficulty in preparing the sample in large quantities (Uno *et al.*, 1985; Hata *et al.*, 1985; Minghetti *et al.*, 1992; Chessman *et al.*, 1993).

We have taken advantage of the use of molecular biology techniques in *E. coli* and have established a new purification procedure for the enzyme. Here we report a detailed study on the structure of the heme–copper binuclear center analyzed by EPR and Fourier transform infrared (FT-IR) spectroscopies using the highly purified cytochrome *bo* complex and various inhibitory ligands to the heme–copper binuclear site, such as carbon monoxide, cyanide, azide, and formate. The EPR spectroscopy suggested that, in the fully oxidized states, heme O and $Cu_B$ are close enough to each other to form the spin–spin exchange-coupled binuclear center *via* an unknown ligand and/or the exogenous ligand (azide, formate, and cyanide). The FT-IR data supported the theory that cyanide and azide form bridging structures between heme O and $Cu_B$, as previously observed for the mitochondrial cytochrome *c* oxidase (Tsubaki *et al.*, 1993a,b).

## EXPERIMENTAL PROCEDURES

*Construction of the Merodiploid Strain.* The cytochrome *bo* complex was purified from the *cyoABCDE*$^+$ merodiploid strain GO103/pMFO2 (*cyo*$^+$Δ*cyd*/*cyo*$^+$). It was essential to produce a large quantity of the heme BO-type wild-type oxidase. Overexpression of the cytochrome *bo* complex with multicopy vectors is known to result in the misincorporation of heme O into the low-spin heme binding site (Puustinen & Wikström, 1991; Puustinen *et al.*, 1992), because of the gene dosage effect of the *cyoE* gene that encodes a putative heme O synthase (Saiki *et al.*, 1992). The single copy expression vector pMFO2 that carries the entire *cyo* operon has been constructed by subcloning the 7.6-kb *Bam*HI–*Sph*I fragment of pHN3795 into pHNF2 (Nakamura, 1990) and then introducing it into the cytochrome *bd*-deficient strain GO103 (*cyo*$^+$Δ*cyd*::Km$^r$) (Oden *et al.*, 1990). In the strain, the expression level of the cytochrome *bo* complex reached 1.2 nmol/mg cytoplasmic membrane proteins.

*Large-Scale Preparation of Cytoplasmic Membranes.* One liter of overnight culture was inoculated in 10 L of minimal medium A (Davis & Mingioloi, 1959) with supplemetations of 1% Bacto tryptone (Difco), 0.5% Bacto casamino acids (Difco), 0.5% Bacto yeast extract (Difco), 1% glycerol, 20 μg/mL ampicillin sodium salt (Sigma), 50 μg/mL L-tryptophan, 50 μg/mL $FeSO_4 \cdot 7H_2O$, and 25 μg/mL $CuSO_4 \cdot 5H_2O$. The cells were grown exponentially at 37 °C using a Magnaferm jar fermentor (New Brunswick Scientific Co., Edison, NJ), with high agitation at 800 rpm and aeration at 12 L/min, and harvested when $OD_{650}$ of the culture reached 5–6 (i.e., 5–6 h after the inoculation). From about 250 g of the cells, spheroplasts were prepared on ice in 30 mM Tris-HCl (pH 8.0)/10% sucrose/20 mM EDTA[1] containing 1–2 mg/mL egg white lysozyme (Sigma) and disrupted by two passages through a French press at over 1000 kg/cm$^2$ in the presence of 1 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma). Unbroken cells and spheroplasts were removed by centrifugation at 5200*g* and 4 °C for 15 min. Crude membranes were recovered by ultracentrifugation using two Hitachi RP42 rotors (62 mL × 6) at 140000*g* and 4 °C for 2 h and then resuspended in 1–2 vol of 10% sucrose/3 mM EDTA/10 mM Tris-HCl (pH 8.0). Membrane suspensions were centrifuged at 140000*g* and 4 °C for 1 h to precipitate outer membranes selectively. The supernatant was diluted with 2 vol of 3 mM EDTA/10 mM Tris-HCl (pH 8.0) and recentrifuged at 140000*g* and 4 °C for 2 h to precipitate all of the membrane vesicles. Cytoplasmic membrane vesicles were enriched by repeating the differential centrifugation as described above. The cytoplasmic membranes were finally suspended in 10% sucrose/3 mM EDTA/10 mM Tris-HCl (pH 8.0) at a protein concentration of about 50 mg/mL and stored at −80 °C until use.

*Large-Scale Purification of the Cytochrome bo Complex.* The cytochrome *bo* complex was purified in sucrose monolaurate (SM) by anion-exchange HPLC (T. Mogi, and Y. Anraku, unpublished results). The membrane suspensions were solubilized at 4 °C by stirring with 2.5% SM-1200 (Mitsubishi-Kasei Food Corp., Tokyo) containing 100 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, and 1 mM PMSF at a protein concentration of 3 mg/mL. The extract was centrifuged at 140000*g* and 4 °C for 1 h. The supernatant was filtered through a 0.45-μm cellulose acetate filter (W-25–5; Tosoh Corp., Tokyo) and loaded on a preparative DEAE-5PW column (55 mm i.d. × 20 cm; Tosoh Corp., Tokyo) connected to a Model LC-8A HPLC system (Shimadzu Corp., Kyoto) at a flow rate of 25 mL/min. The column was washed thoroughly with 50 mM Tris-HCl (pH 7.4)/0.1% SM to elute any unbound proteins. The cytochrome *bo* complex was eluted by a linear NaCl gradient from 0.1 to 0.25 M washing buffer. The complex was separated completely from cytochrome $b_{556}$ that was eluted at higher salt concentrations. The peak fractions were collected, diluted 2-fold with 50 mM Tris-HCl (pH 7.4)/0.1% SM, and then reloaded on the same column. The purified cytochrome *bo* complex was obtained by rechromatography. From 7.5 g of cytoplasmic membrane proteins, 0.5 g of the purified oxidase can be obtained. For a small-scale purification (i.e., 1-g protein scale), a Model LC-9A HPLC system equipped with an SPD-M6A photodiode array detector (Shimadzu Corp., Kyoto) and a semipreparative DEAE-5PW column (21.5 mm i.d. × 15 cm) were used at a flow rate of 5 mL/min.

The purified cytochrome *bo* complex contained only stoichiometric amounts of five polypeptides, with apparent molecular masses of 75, 33.5, 28, 20.5, and 12 kDa, and showed a heme content of 12.1 nmol/mg protein, indicating that 1 mol each of protoheme IX (heme B) and heme O is present in the 169-kDa oxidase complex (T. Mogi, and Y. Ankaru, unpublished results). The heme content was estimated as the sum of heme B and heme O by the method of Berry and Trumpower (1987), since the structure of heme O is closely related to that of protoheme IX (Puustinen & Wikström, 1991; Wu *et al.*, 1992). On the basis of the heme content and the CO-binding spectra determined by the method of Kita *et al.* (1984), an extinction coefficient of the cytochrome *bo* complex for the CO binding (i.e., cytochrome *o*) was estimated to be 254 mM$^{-1}$ cm$^{-1}$ at a wavelength pair of 416–430 nm. Copper analysis by atomic absorption spectroscopy and reversed-phase HPLC analysis of the heme species (Saiki *et al.*, 1992) showed reproducibly that our wild-type five-subunit

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SM, sucrose monolaurate; HPLC, high-performance liquid chromatography.

preparation contains heme B, heme O, and $Cu_B$ at a molar ratio of 1:1:1. The extra copper(s) found in the two-subunit preparation (Kita *et al.*, 1984) could be attributable to nonspecific copper binding to a part of the oxidase that is normally embedded in the five-subunit preparation and to exhibition of the broad $g = 2$ EPR signal in the air-oxidized state (Hata *et al.*, 1985). Analysis of the Fe–CO stretching band by resonance Raman spectroscopy indicated that the lower extinction coefficient (145 $mM^{-1}$ $cm^{-1}$) of cytochrome *o* reported for the two-subunit preparation (Kita *et al.*, 1984) could be ascribed to loss of copper at the $Cu_B$ binding site (T. Uno, T. Mogi, and Y. Ankaru, unpublished results).

*Measurement of EPR Spectra.* EPR measurements were carried out at 5 or 15 K at X-band (9.23 GHz) microwave frequency with a home-built EPR spectrometer with 100-kHz field modulation by using a Varian X-band cavity. An Oxford flow cryostat (ESR-900) (from 4 to 80 K) was used for the measurements. The microwave frequency was calibrated with a microwave frequency counter (Takeda Riken Corp., Ltd., Model TR5212). The magnetic field strength was determined by nuclear magnetic resonance of protons in water.

*Measurement of Fourier Transform Infrared Spectra.* The cytochrome *bo* complex sample was introduced into an infrared cell having $CaF_2$ windows with a 51-$\mu$m 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 the control of a CDS-3 application software package for data acquisition and manipulation, as previously described (Tsubaki *et al.*, 1992). The infrared spectrophotometer was operated in a double-beam mode. The reference infrared cell contained the cytochrome *bo* complex solution in the oxidized resting form at the same concentration. The temperature of the infrared cells was maintained at 10 °C by circulating water through cell holders from a temperature-controlled water bath. A nominal spectral resolution of 4.0 $cm^{-1}$ was necessary to record the bound cyanide and azide infrared spectra in good signal to noise ratios, whereas a nominal spectral resolution of 0.5 $cm^{-1}$ was used for recording the CO infrared spectra. Absolute optical spectra of the cytochrome *bo* complex in the infrared cells were measured at room temperature with a UVIKON 860 UV–visible spectrophotometer (Kontron Instruments, Inc., Everett, MA) before and after FT-IR measurements.

*Miscellaneous.* Protein concentration was determined by using a BCA protein assay reagent (Pierce, Rockford, IL). Other chemicals were commercial products of analytical grade.

## RESULTS

### *Fourier Transform Infrared Spectra*

*CO Infrared Spectra.* When CO binds to heme O of the cytochrome *bo* complex in the fully reduced state, the major bound C–O stretching band is found at 1959.7 $cm^{-1}$ with a very narrow bandwidth ($\Delta\nu_{1/2}$ less than 4.0 $cm^{-1}$) (Figure 1), consistent with previous reports (Chepuri *et al.*, 1990b; Hill *et al.*, 1992) observed at cryogenic temperature (15–30 K) for the *E. coli* membrane preparations. This value is very similar to the corresponding C–O band of mitochondrial cytochrome *c* oxidase (1963.5 $cm^{-1}$, $\Delta\nu_{1/2} = 3.5$ $cm^{-1}$, for bovine heart cytochrome *c* oxidase; Tsubaki *et al.*, 1992). An additional minor bound C–O stretching band was seen at 1949 $cm^{-1}$ for all preparations examined.[2]

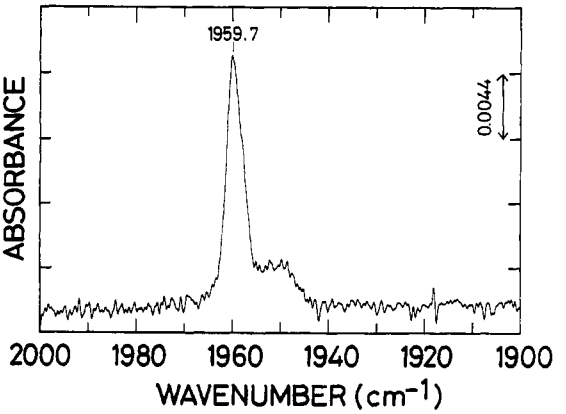


FIGURE 1: C–O stretching Fourier transform infrared spectrum of the cytochrome *bo* complex in the CO-reduced state: temperature, 10 °C; concentration of the cytochrome, ~400 $\mu$M in 50 mM Tris-HCl (pH 8.0) buffer containing 0.1% (w/v) sucrose monolaurate. Other conditions are described in the Experimental Procedures.

*CN Infrared Spectra.* When cyanide ($^{12}C^{14}N$) ion (0.6 mM) was added to the air-oxidized cytochrome *bo* complex, it reacted very slowly (more than 10 h to complete) to the active center of the enzyme on the basis of optical absorption spectroscopy. Such a slow process has also been observed in the reaction of cyanide with cytochrome *c* oxidase in the resting form (Baker *et al.*, 1987; Naqui *et al.*, 1984). As the Soret band peak shifts slowly from 410 to 415 nm with a concomitant increase in band intensity, suggesting conversion to a low-spin state, a clear C–N stretching band appears at 2146 $cm^{-1}$ in the infrared spectrum (Figure 2). This band showed a shift to 2100.5 $cm^{-1}$ upon isotopic substitution with $^{13}C^{14}N$ (Figure 2), which is very close to the value expected on the basis of a diatomic harmonic oscillator model (Nakamoto, 1986).

*$N_3$ Infrared Spectra.* When $^{14}N_3$ (0.3 mM) was added to the enzyme, a relatively sharp azide antisymmetric stretching band appeared at 2041 $cm^{-1}$ and a minor feature appeared around 2058 $cm^{-1}$, with both bands overlapping considerably with a broad free azide ion band centered at 2049 $cm^{-1}$ (Figure 3). Upon substitution with the terminally labeled azide ($^{15}N^{14}N^{14}N$, 0.3 mM), these two bound azide bands shifted to 2030.5 and 2043.5 $cm^{-1}$, respectively (Figure 3). Upon addition of cyanide (0.6 mM) to the preformed azide–cytochrome *bo* complex, the bound azide antisymmetric stretching bands disappeared quickly (within 30 min after the addition) and were replaced with the free azide band. A sharp bound C–N stretching band appeared concomitantly at 2146 $cm^{-1}$ in full intensity (Figure 3). However, during the ligand replacement reaction there was no intermediate species (a ternary complex where azide and cyanide ions bind to the binuclear site simultaneously) that had been observed for mitochondrial cytochrome *c* oxidase (Tsubaki & Yoshikawa, 1993b).

### *EPR Spectra*

*Air-Oxidized State.* Figure 4 shows the EPR spectra of the highly purified cytochrome *bo* complex in the air-oxidized

[2] The minor C–O band at 1949 $cm^{-1}$ was not present in the spectra of Hill *et al.* (1992). Possible reasons for this discrepancy are the measuring temperature (10 °C vs 15–30 K), hydration level ($H_2O$ vs glycerol-dehydrated), and protein environment (detergent vs membrane lipids). Existence of such a minor form ($\beta$ form) at the lower frequency side of the main band is seen for cytochrome *c* oxidases in bovine heart mitochondrial membrane (Fiamingo *et al.*, 1982) and in *Rhodobacter sphaeroides* membrane (Shapleigh *et al.*, 1992), as well as for the purified cytochrome *c* oxidase from bovine heart mitochondria (Tsubaki *et al.*, 1992).

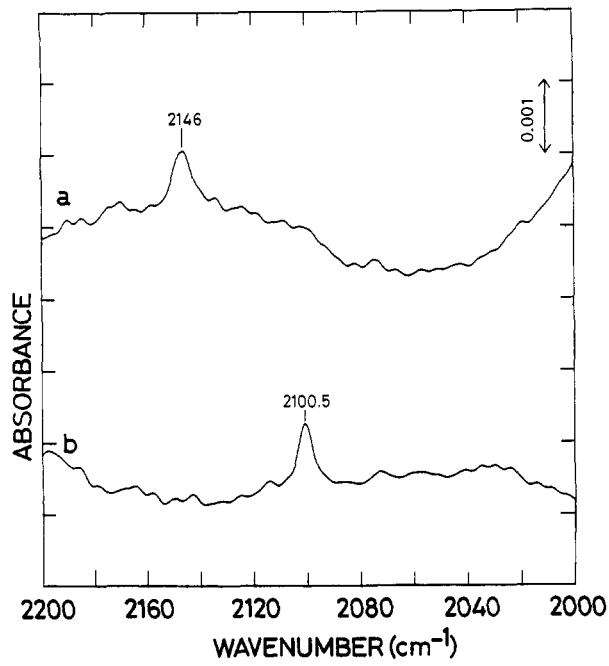


FIGURE 2: C–N stretching Fourier transform infrared spectra of the cytochrome *bo* complex in the cyanide-bound states: (a) cyanide ($^{12}C^{14}N$) (0.6 mM) bound air-oxidized state; (b) cyanide ($^{13}C^{14}N$) (0.6 mM) bound air-oxidized state. Temperature was 10 °C. Other conditions are the same as in Figure 1.

state measured at 5 (left) and 15 K (right), respectively. In the 5 K spectrum (line a), the high-spin signal with $g = 6.0$ and 2.0 and the low-spin signal with $g = 2.98$, 2.26, and 1.45 can be seen, as has been reported previously (Hata *et al.*, 1985; Salerno *et al.*, 1990; Salerno & Ingledew, 1991). A signal from the radical at $g = 2.0$, the broad signal around $g = 2.0$ previously ascribed to cupric ions by Hata *et al.* (1985), and an unknown feature at $g = 4.3$ were also detected. However, the broad EPR signal around $g = 2.0$ ascribable to cupric ions disappeared completely after repeated washings with the buffer containing 10 mM EDTA, suggesting that this signal is due to Cu contamination. Occasionally a minor high-spin component with a rhombic signal split about $g = 6.3$ and $g = 5.8$ was seen (spectrum not shown), which has also been noticed by Salerno and Ingledew (1991) in the membrane samples.

It must be stressed that the content of the high-spin g6 species observed in the EPR spectra was low compared to that of the low-spin species. A prominent difference in the EPR spectra of the air-oxidized states between the cytochrome *bo* complex and cytochrome *c* oxidase is the appearance of the broad EPR signal centered around $g' = 3$ (half line-width, ca. 55 mT; characterized by a peak at $g^* = 3.7$) (Figure 4, curves a and e). In the mitochondrial enzyme we could not observe any indication of this kind of signal in this region (spectrum not shown).

*Azide-Bound Air-Oxidized State.* Addition of 50–100 mM sodium azide to the air-oxidized cytochrome *bo* complex caused a narrowing of the peculiar broad EPR signal centered around $g' = 3$ (half-line-width, ca. 35 mT; characterized by a peak at $g^* = 3.2$) with a concomitant decrease in the high-spin (g6) signal intensity (Figure 4, curves b and f). The $Fe_B^{3+}$ low-spin (g3) signal ($g = 2.98$, 2.26, and 1.45) did not show any appreciable change upon this treatment. An increase of the azide concentration up to 200 mM did not further strengthen the broad EPR signal intensity around $g = 3$, suggesting that the azide binding site is already saturated at 50 mM azide (spectra not shown). An additional weak EPR signal with a peculiar band shape around $g' = 9$ developed simultaneously (Figure 4, curve b, indicated by a vertical broken line), suggesting that the $g' = 9$ and the broad $g' = 3$ signals might arise from the same population of the enzyme–azide complex. The microwave power saturation characteristics at 5 K for the $g = 2.98$ (low-spin), $g' = 3$, and $g' = 9$ signals of the cytochrome *bo* complex in the presence of 100 mM azide are shown in Figure 5. The g3 low-spin signal from the $Fe_B^{3+}$ center saturated readily, whereas the $g' = 3$ and $g' = 9$ signals were very difficult to saturate. The temperature dependence of the $g' = 3$ and $g' = 9$ signals was very similar each other. These properties bear a close resemblance to the $g' = 12$ and $g' = 2.95$ signals of cytochrome *c* oxidase in the resting form or in the formate-inhibited form (Cooper & Salerno, 1992). Since the $g' = 12$ and $g' = 2.95$ signals of cytochrome *c* oxidase come from the binuclear center having a similar integer spin system (Cooper & Salerno, 1992), it is reasonable to assume that these two ($g' = 3$, $g' = 9$) signals are also derived from the $Fe_o^{3+}$–$Cu_B^{2+}$ binuclear center of the cytochrome *bo* complex. Apart from the $g' = 3$ and $g' = 9$ broad EPR signals, there was a weak EPR signal with *g*-values at $g = 2.79$ and 1.73 in the azide-complexed state at 15 K (Figure 4, curve f, indicated by vertical broken lines).

Introduction of cyanide (5 mM) to the preformed azide complex of the cytochrome *bo* complex removed the $g' = 3$ and $g' = 9$ (and even the $g = 2.79$) signals completely (spectrum not shown). Introduction of nitric oxide (NO) to the preformed azide–cytochrome *bo* complex caused no appreciable change in the EPR spectra (spectra not shown). This observation is in marked contrast to that observed for cytochrome *c* oxidase, in which simultaneous addition of azide and NO causes reduction of the $Fe_{a3}$ center followed by formation of a new complex that exhibits EPR signals ($g = 2.69$, 1.67) characteristic of a "triplet species" (Boelens *et al.*, 1984; Brudvig *et al.*, 1980; Stevens *et al.*, 1979).

*Formate-Bound Air-Oxidized State.* Addition of 50–100 mM sodium formate to the air-oxidized cytochrome *bo* complex caused formation of a new EPR signal[3] around $g' = 12$ (Figure 4, curves c and g, indicated by vertical broken lines). It is well-known that addition of formate to the fast form of cytochrome *c* oxidase (Hartzell–Beinert preparation or redox-cycled preparation of the slow enzyme) causes the development of an EPR signal around $g' = 12$ (Boelens & Wever, 1979; Cooper & Salerno, 1992; Schoonover & Palmer, 1991). The EPR signal very similar to this was previously noted for the resting form of cytochrome *c* oxidase (Greenaway *et al.*, 1977; Hagen, 1982). Apparently the present new EPR signal corresponds to those observed in the mitochondrial enzyme. Cooper and Salerno (1992) proposed that an additional EPR signal at $g' = 2.95$ arises from the same population of binuclear centers responsible for the $g' = 12$ signal of cytochrome *c* oxidase in the resting state. We noticed a new weak EPR signal around $g' = 2.7$ (Figure 4, curve c and g, indicated by vertical broken lines), analogous to the $g' = 2.95$ signal of the mitochondrial enzyme.

*Cyanide-Bound Air-Oxidized State.* Upon addition of 5 mM cyanide, the EPR signal intensity from the high-spin heme ($g = 6.0$) decreased dramatically without the formation of any new low-spin signal around $g = 3.5$ assignable to the $Fe_o^{3+}$–CN species (Johnson *et al.*, 1981; Goodman, 1984), whereas the low-spin signal of $Fe_B^{3+}$ at $g = 2.98$, 2.26, and 1.45 was essentially the same (Figure 4, curve h). Absence

[3] After completion of this work, a paper appeared describing the formation of a $g' = 12$ EPR signal upon addition of formate to the *E. coli* membranes containing the cytochrome *bo* complex but lacking the cytochrome *bd* complex (Calhoun *et al.*, 1992). The interpretation for their observation is essentially identical with ours.

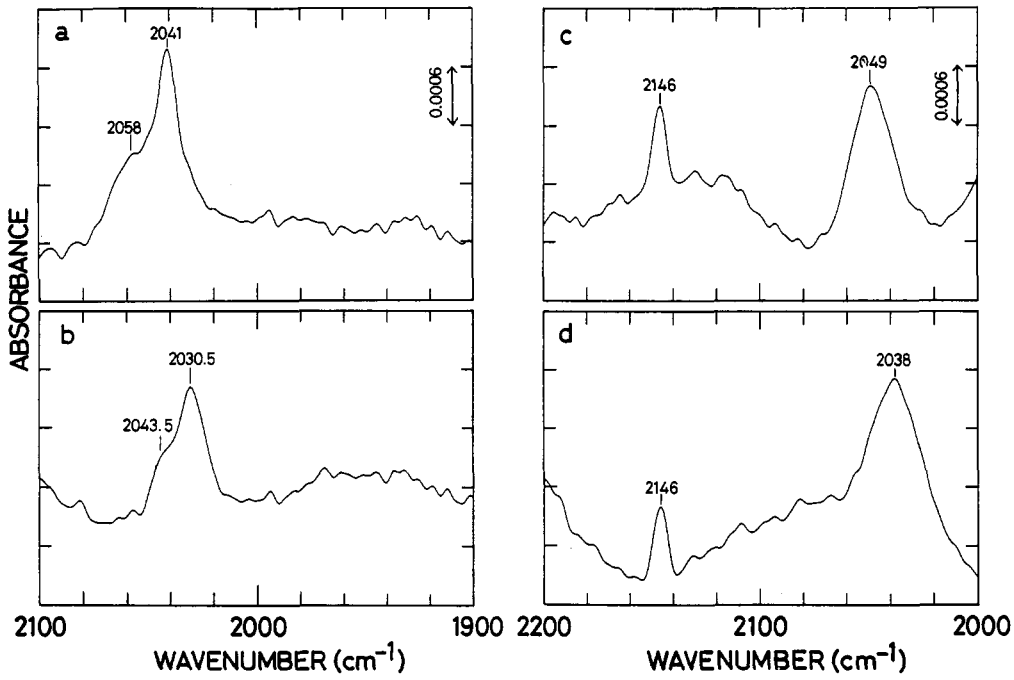


FIGURE 3: Azide antisymmetric stretching Fourier transform infrared spectra of the cytochrome *bo* complex in the azide-bound states and the effect of cyanide added to the azide-bound complexes: (a) azide ($^{14}N_3$) (0.3 mM) bound air-oxidized state; (b) azide ($^{15}N^{14}N^{14}N$) (0.3 mM) bound air-oxidized state; (c and d) replacements of bound azide ($^{14}N_3$ and $^{15}N^{14}N^{14}N$) with cyanide ($^{12}C^{14}N$, 0.6 mM), respectively. The bands at 2049 (c) and at 2038 $cm^{-1}$ (d) are due to free azide ions of $^{14}N_3$ and $^{15}N^{14}N^{14}N$, respectively. Other conditions are the same as in Figure 1.

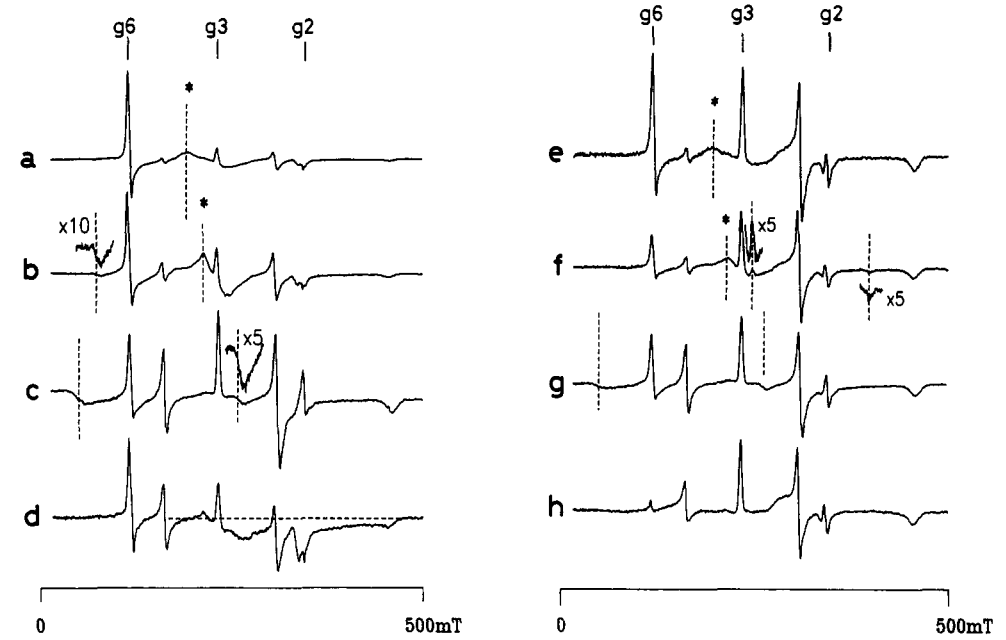


FIGURE 4: EPR spectra of the cytochrome *bo* complex in the air-oxidized state (a and e), in the azide (100 mM) bound air-oxidized state (b and f), in the formate (50 mM) bound air-oxidized state (c and g), and in the cyanide (5 mM) bound air-oxidized state (d and h). Vertical broken lines marked by an asterisk indicate EPR signal peaks at $g^* = 3.7$ (curves a and e) and $g^* = 3.2$ (curves b and f). Other vertical broken lines in curves b, c, f, and g are specified in the text. Insets show enlargements of the spectra with specified magnifications for the $g' = 9$ signal (curve b), the $g' = 2.7$ signal (curve c), and the signal at $g = 2.79$ and 1.73 (curve f). A horizontal broken line in curve d indicates a base line. Spectrometer conditions were as follows: temperature, 5 K for curves a–d (left) and 15 K for curves e–h (right); microwave power, 5 mW; microwave frequency, 9226.0 MHz; modulation amplitude, 1.0 mT; modulation frequency, 100 kHz. Concentration of the enzyme was ~550 $\mu$M in 50 mM Tris-HCl (pH 8.0) buffer containing 0.1% (w/v) sucrose monolaurate.

of the EPR signal assignable to the $Fe_o^{3+}$–CN species could be interpreted as due to the ferromagnetic or antiferromagnetic coupling of $Fe_o^{3+}$–CN with the $Cu_B^{2+}$ center, where the bridging cyanide mediates the spin–spin exchange coupling as previously suggested for the cyanide-bound cytochrome *c* oxidase (Thomson *et al.*, 1981). A more interesting result was the complete disappearance of the broad EPR signal around $g' = 3$ (peak at $g^* = 3.7$) observed in the air-oxidized state upon addition of cyanide (spectrum not shown). Occasionally we noticed the appearance of a very broad and weak feature centered around $g = 2$ in the 5 K spectrum (Figure 4, curve d).

Another important observation we made was the absence of the EPR signal attributable to $Fe_o^{3+}$–CN upon addition of nitric oxide (NO) to the cyanide-bound cytochrome *bo* complex (spectra not shown). In cytochrome *c* oxidase, anaerobic treatment of the cyanide-bound enzyme with NO causes the formation of a $g = 3.4$ EPR signal attributable to the $Fe_{a3}^{3+}$–

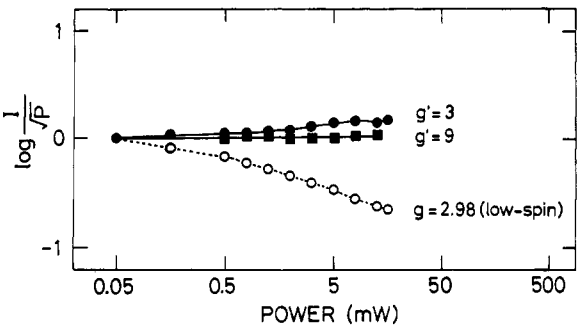


FIGURE 5: Microwave power saturation properties of EPR signals from the cytochrome *bo* complex in the azide (100 mM) bound air-oxidized state. The $g' = 3$, $g' = 9$, and $g = 2.98$ signals were studied in the same sample. Signal amplitudes were determined as follows. The peak *minus* trough height in the first derivative spectra was used for the $g' = 3$ and $g' = 9$ signals, whereas the peak height after subtraction of the $g' = 3$ signal was used for the $g = 2.98$ low-spin signal. The signal sizes were normalized so that at low microwave powers they had identical values of $S/P^{1/2}$. Spectrometer conditions were as follows: temperature, 5 K; microwave frequency, 9226.1 MHz; modulation amplitude, 1.0 mT; modulation frequency, 100 kHz; response, 125 ms. Other conditions are the same as in Figure 4.

CN species being spin-decoupled from the NO-bound $Cu_B^{2+}$ center (Boelens *et al.*, 1983; Stevens *et al.*, 1979).

## DISCUSSION

*FT-IR Analyses.* In the fully reduced CO-bound state, a clear resemblance in spectral properties between the cytochrome *bo* complex and cytochrome *c* oxidase was demonstrated. The extremely narrow bandwidth of the C–O stretching band is consistent with the reports of Chepuri *et al.* (1990b) and Hill *et al.* (1992) (although observed at cryogenic temperature for the membrane samples). It indicates that the active-site structures are very similar each other and that an ordered and nonpolar environment of the active site must be maintained in both enzymes (Yoshikawa *et al.*, 1977; Yoshikawa & Caughey, 1982). The 3.8 $cm^{-1}$ difference in the C–O stretching frequencies between the cytochrome *bo* complex and the mitochondrial enzyme can be attributed to the absence of the formyl group at pyrrole ring D in heme O (Wu *et al.*, 1992). It was shown that the presence of the strongly electron-withdrawing formyl group at the heme periphery in the heme-reconstituted myoglobin caused increases in the bound C–O stretching frequency of 4–10 $cm^{-1}$, depending on the number and location of the formyl group (Tsubaki *et al.*, 1980).

A close resemblance was also seen for cyanide binding to the binuclear center of the air-oxidized enzyme. The slow reactivity toward cyanide based on the visible absorption spectral change and the resultant formation of the 2146 $cm^{-1}$ infrared band (apparently corresponding to the 2152 $cm^{-1}$ band of the cyanide-inhibited cytochrome *c* oxidase in the oxidized form) in the infrared spectra (Yoshikawa & Caughey, 1990; Tsubaki & Yoshikawa, 1993a) indicate that the active-center structures of the air-oxidized cytochrome *bo* complex and its cyanide-bound form are very similar to those of the mitochondrial enzyme. Thus, it is very likely that the cyanide ligand has a bridging structure between two metal centers, i.e., $Fe_o^{3+}$–C–N–$Cu_B^{2+}$, as has been proposed for the mitochondrial enzyme (Thomson *et al.*, 1982; Tsubaki & Yoshikawa, 1993a).

For azide binding to the air-oxidized enzyme, a close similarity between cytochrome *bo* complex and cytochrome *c* oxidase was also demonstrated. The major and minor bound azide species showed their antisymmetric stretching bands at 2041 and 2058 $cm^{-1}$, respectively, both very close to the value of the corresponding band (2051 $cm^{-1}$) for cytochrome *c* oxidase (Tsubaki & Yoshikawa, 1993b). Further, it could be concluded that a large part of the bound azide forms a bridging structure between the $Fe_o^{3+}$ and $Cu_B^{2+}$ centers on the basis of the absence of splitting for the major band when a terminally labeled azide ($^{15}N^{14}N^{14}N$) was used, as previously suggested for the cytochrome *c* oxidase–azide complex (Tsubaki & Yoshikawa, 1993b; Yoshikawa & Caughey, 1992). If azide binds to the metal in an end-on fashion, a 10–17 $cm^{-1}$ splitting is expected for the $^{15}N^{14}N^{14}N$ species, and such a separation should be observable even at 4.0 $cm^{-1}$ spectral resolution (Tsubaki & Yoshikawa, 1993b).

*EPR Analyses.* van Gelder and Beinert (1969) first proposed an antiferromagnetic spin–spin exchange coupling within a {$Fe_{a3}^{3+}$–$Cu_B^{2+}$} pair, resulting in an $S = 2$ integer spin system, to be the nature that causes the lack of expected EPR signals and the diminished paramagnetism of cytochrome *c* oxidase in the resting state. Magnetic susceptibility measurements clearly show the presence of an $S = 2$ system (Barnes *et al.*, 1991; Moss *et al.*, 1978; Tweedle *et al.*, 1978) in the resting form of cytochrome *c* oxidase. Possible candidates for a bridging ligand (X) between $Fe_{a3}^{3+}$ and $Cu_B^{2+}$ that mediate the spin–spin exchange coupling are water, hydroxyl, carboxylate, tyrosinate (Brudvig *et al.*, 1981), S, or Cl (Li *et al.*, 1987; Scott, 1989).

It is proposed that the catalytic site of the cytochrome *bo* complex is also a tightly coupled heme–copper binuclear center by the EPR spectroscopic redox titration of the membrane samples (Salerno *et al.*, 1990). The spin–spin exchange-coupled interaction requires close proximity of the two paramagnetic metal centers, and indeed, the Cu-EXAFS data for the oxidized cytochrome *bo* complex indicated the $Fe_o^{3+}$–$Cu_B^{2+}$ distance to be only 2.92 Å (Ingledew & Bacon, 1991). The absence of the EPR signal attributable to $Cu_B^{2+}$ and the presence of the weak $Fe_o^{3+}$ high-spin signal for the highly purified enzyme in the air-oxidized state support the notion that the cytochrome *bo* complex contains only one copper ion ($Cu_B^{2+}$) that is spin–spin exchange-coupled to the high-spin $Fe_o^{3+}$ antiferromagnetically. In this context, the high-spin signal at $g = 6$ of the air-oxidized enzyme is likely from (1) the $Fe_o^{3+}$ center of the partially reduced form with the $Cu_B$ center being cuprous (Palmer, 1987), (2) the $Fe_o^{3+}$ center of the partially denatured form with the $Cu_B$ center being removed adventitiously, or (3) the (denatured?) high-spin $Fe_B^{3+}$ species.

The broad $g' = 3$ (peak at $g^* = 3.7$) EPR signal in the air-oxidized cytochrome *bo* complex is most likely derived from an $S = 2$ integer spin system produced by spin–spin exchange coupling at the binuclear site. Since the quantitation of the spin content is very difficult for a broad signal such as this, it cannot be concluded whether or not all of the spin–spin-coupled binuclear center exists in the same $S = 2$ spin system producing the broad $g' = 3$ signal. We could not detect the low-field $g' = 12$ EPR signal[4] that often appears in the resting form of cytochrome *c* oxidase.

The $g' = 12$ and $g' = 2.7$ EPR signals of the air-oxidized formate-bound form (and the $g' = 3$ and $g' = 9$ signals of the air-oxidized azide-bound form as well) of the cytochrome *bo* complex (Figure 5) are quite analogous to the $g' = 2.95$ and $g' = 12$ signals of cytochrome *c* oxidase in the resting form. It indicates that these signals originate from very similar electronic structures of the binuclear site, having the $S = 2$ integer spin system. The most plausible explanation for the formation of the $g' = 12$ EPR signal of the cytochrome *bo* complex upon addition of formate may be an exchange of the bridging ligand (X) between $Fe_{a3}^{3+}$ and $Cu_B^{2+}$ with formate.

However, Schoonover and Palmer (1991) have proposed that formate may bind to $Cu_B^{2+}$ away from the $Fe_{a3}^{3+}$; i.e., the binding of formate to $Cu_B^{2+}$ leads to a change in the nature of the bonding between X (bridging ligand) and $Fe_{a3}^{3+}$, and this change is responsible for the alterations in the optical spectrum and the appearance of the $g' = 12$ (and $g' = 2.95$) signal. Boelens *et al.* (1983) suggested that formate bridges between $Fe_{a3}^{3+}$ and $Cu_B^{2+}$, on the basis of EPR spectra of the formate-inhibited oxidized cytochrome *c* oxidase in the presence of NO. Thus, formate is not necessarily bound to the $Cu_B^{2+}$ center away from the $Fe_{a3}^{3+}$ center in the resting state to produce the $g' = 12$ signal.

For the EPR spectra of the azide-bound air-oxidized cytochrome *bo* complex, arguments very similar to those for formate binding can be made. Since a major part of the bound azide (even at lower azide concentration) forms a bridging structure between $Fe_o^{3+}$ and $Cu_B^{2+}$, according to the infrared spectroscopic data (Figure 3), the most reasonable explanation of the broad $g' = 3$ and $g' = 9$ signals is an altered integer spin ($S = 2$) system within the binuclear center caused by the azide binding in a bridging configuration. There is still the question of why azide binding to the binuclear center causes peculiar EPR signals in the cytochrome *bo* complex but not in cytochrome *c* oxidase, in which the binuclear site largely seems to be in an EPR-undetectable state (M. Tsubaki, and H. Hori, unpublished results).

We should pay attention to the narrowing of the broad $g' = 3$ EPR signal for the air-oxidized state (half-line-width, ca. 55 mT; peak at $g^* = 3.7$) upon azide binding (half line-width, ca. 35 mT; peak at $g^* = 3.2$) and the shift of the signal to $g' = 2.7$ and 12.0 upon formate binding. These changes in the broad EPR signal derived from the $S = 2$ integer spin system upon ligand bindings must be direct indications of changes in the strength of the spin–spin exchange coupling. In this context we propose that the bridging ligand (X) still exists to mediate the spin–spin exchange coupling when azide or formate bridges the $Fe_o^{3+}$ and $Cu_B^{2+}$ centers. Therefore, azide and formate may be considered as ionic effectors to modulate the strength of the coupling between $Fe_o^{3+}$ and $Cu_B^{2+}$.

The existence of the weak EPR signal at $g = 2.79$ and 1.73 in the azide-bound state is additional evidence supporting the close resemblance between the cytochrome *bo* complex and the mitochondrial cytochrome *c* oxidase. In the cytochrome *c* oxidase system, an EPR signal with *g*-values at 2.78, 2.2, and 1.75 was observed during partial reduction in the presence of azide (Goodman, 1984; Shaw *et al.*, 1978; Wever & van Gelder, 1974). This EPR signal has been assigned to be derived from the $Fe_{a3}^{3+}$–azide center of the partially reduced enzyme in which the $Fe_a$ and $Cu_A$ centers are oxidized while the $Cu_B$ center is reduced, on the basis of potentiometric titrations (Goodman, 1984). Thus the EPR signal at $g = 2.79$ and 1.73 of the azide-bound cytochrome *bo* complex is most likely due to the $Fe_o^{3+}$–azide center of the partially reduced enzyme in which only the $Cu_B$ center is reduced. The presence of a fair amount of the cuprous state for the $Cu_B$ center in the air-oxidized cytochrome *bo* complex may explain the relatively strong intensity of the $Fe_o^{3+}$ high-spin (g6) EPR signal in the air-oxidized state compared to that of the mitochondrial enzyme.

The situation is much clear for the EPR spectra of the cyanide-bound air-oxidized cytochrome *bo* complex. It is concluded in the present study that cyanide binds to the binuclear site in a bridging conformation, as is found for the cyanide-bound cytochrome *c* oxidase (Tsubaki & Yoshikawa, 1993a) by FT-IR spectroscopy. Cyanide is believed to replace the pre-existing bridging ligand (X) upon binding in the air-oxidized state; therefore, the spin–spin exchange coupling must be mediated by bridging cyanide, resulting in an EPR-silent species. The spin–spin exchange coupling may be ferromagnetic in its electronic ground state with $S = 1$ (Johnson *et al.*, 1981; Kent *et al.*, 1982; Thomson *et al.*, 1981, 1982) or antiferromagnetic in its ground state with $S = 0$ (Tweedle *et al.*, 1978), as proposed for cytochrome *c* oxidase.

Although the gross conformations at the binuclear site seem very similar to each other as discussed above, there are some EPR spectroscopic indications suggesting the minor differences in the local structure around the $Cu_B^{2+}$ center between the cytochrome *bo* complex and cytochrome *c* oxidase. First, the simultaneous binding of cyanide (the bridging ligand between $Fe_o^{3+}$ and $Cu_B^{2+}$) and NO to the $Cu_B^{2+}$ center does not occur in the cytochrome *bo* complex. Second, the simultaneous binding of azide (the bridging ligand between $Fe_o^{3+}$ and $Cu_B^{2+}$) and NO to the $Cu_B^{2+}$ center also does not take place. Such inaccessibility of the exogenous ligand to the $Cu_B^{2+}$ center when a bridging ligand is present between $Fe_o^{3+}$ and $Cu_B^{2+}$ may explain the absence of the intermediate species in cytochrome *bo* complex during the ligand replacement reaction from azide to cyanide as observed by FT-IR spectroscopy. For cytochrome *c* oxidase, we can observe a somewhat stable ternary complex in which azide and cyanide are postulated to bind to the binuclear site simultaneously (Tsubaki & Yoshikawa, 1993b).

In conclusion, the present study clearly proved that there is a heme–copper binuclear center in the highly purified cytochrome *bo* complex. Close similarity of the binuclear site structure between the cytochrome *bo* complex and the cytochrome *c* oxidase is evident from the EPR and FT-IR spectroscopic data. EPR spectroscopy suggested that, in the fully oxidized state, heme O and $Cu_B$ are close enough to each other to form the spin–spin exchange-coupled binuclear center *via* an unknown ligand (X) and/or the exogenous ligand (azide, formate, and cyanide), very similar to those formed by heme A and $Cu_B$ in the mitochondrial cytochrome *c* oxidase. FT-IR data supported the theory that cyanide and azide form bridging structures between heme O and $Cu_B$ (characterized by the 2146 $cm^{-1}$ band for the cyanide complex and the 2041 $cm^{-1}$ band for the azide complex), as previously observed for the mitochondrial oxidase. The well-conserved binuclear structure is apparently the key feature of the reaction center of the heme–copper oxidase superfamily and may play an important role in the proton pumping mechanism. To clarify the mechanisms of the dioxygen reduction and the proton pumping, more elegant studies targeting the "fast form" and the partially reduced states are highly necessary.

[4] The enigmatic $g' = 12$ EPR signal is considered to be due to a conformational form of the mitochondrial enzyme in which the binuclear center reacts sluggishly with cyanide; but this signal could be abolished completely by cyanide without affecting the $Fe_a^{3+}$ and $Cu_A^{2+}$ EPR signals (Palmer, 1987). Thus the $g' = 12$ signal is a property of a form of the binuclear center. Brudvig *et al.* (1981) suggested that the $g' = 12$ signal could arise from high-spin $Fe_{a3}^{3+}$ ($S = {}^5/_2$) coupled *via* a strong-field ligand (a $\mu$-oxo ligand, for example) to a $Cu_B^{2+}$ ($S = {}^1/_2$) site. However, Hagen (1982) proposed that a high-spin $Fe_{a3}^{4+}$ ($S = 2$) center must be coupled with an EPR-silent cuprous $Cu_B$ ($Cu_B^+$) ($S = 0$). Cooper and Salerno (1992) favored the latter model on the basis that the $g' = 2.95$ signal arose from the same population of binuclear centers responsible for the $g' = 12$ signal. But Mössbauer data on mitochondrial and bacterial enzymes have indicated the presence of a high-spin ferric heme and ruled out the $Fe_{a3}^{4+}$ assignment (Kent *et al.*, 1982, 1983).

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