Resonance Raman, Infrared, and EPR Investigation on the Binuclear Site Structure of the Heme-Copper Ubiquinol Oxidases from *Acetobacter aceti*: Effect of the Heme Peripheral Formyl Group Substitution†

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ABSTRACT: *Acetobacter aceti* produces two different terminal ubiquinol oxidases (cytochromes a_1 and *o*) depending on the culture conditions. Two types of oxidases share a common protein moiety but with different heme components at the binuclear center (heme A for cytochrome a_1 and heme O for cytochrome *o*). We investigated the structure of the binuclear site of the two oxidases using resonance Raman, Fourier transform-infrared (FT-IR), and EPR spectroscopies to clarify the interactions of heme A formyl group with protein moiety. We found that the overall architecture and the electronic configuration at the binuclear center in the oxidized state seem to be well conserved irrespective of the heme peripheral group at position 8, except for the azide-inhibited state. In contrast, we observed great variations in the C−N stretching frequency and cyanide-binding affinity in the CN-reduced state, in addition to multiple C−O stretching bands in the CO-reduced state. Present and previous studies suggest that the conformational flexibility of the binuclear center in the reduced ligand-bound state may be a common feature among the heme-copper oxidase superfamily. In the CN-reduced state, a hydrogen bond network may be formed among the formyl group, water molecule(s), and the surrounding amino acid residue(s). This network may be very important to maintain proper orientations of the distal amino acid residues and/or the Cu_B^{1+} ion relative to the cyanide ion bound to the ferrous heme iron and could play a critical role for the high affinity in cyanide binding.

The heme A formyl group had been suggested to be indispensable for functions of mitochondrial cytochrome *c* oxidase (Babcock & Callahan, 1983; Babcock & Chang, 1979). However, the recent discovery of various types of heme-copper oxidases, such as bo_3-, ba_3-, and bb_3-types (García-Horsman et al., 1994; Gray et al., 1994), seems to indicate that the formyl group participates in neither proton pumping nor electron transfer. The prevalence of heme A in the heme-copper oxidase superfamily in higher organisms, particularly at the binuclear site (Lübben & Morand, 1994), however, suggests that heme A formyl group may have an unknown but important role.

Acetobacter aceti produces two different terminal oxidases depending on the culture conditions. Cells grown on shaking culture contain cytochrome a_1, while cytochrome *o* is present in cells grown on static culture (Matsushita et al., 1992b). Cytochrome a_1 was purified and characterized as a cytochrome *ba*-type ubiquinol oxidase (Matsushita et al., 1990) consisting of four subunits and containing 1 mol each of heme A, heme B, and copper ion (Matsushita et al., 1992a). The genes encoding subunits of cytochrome a_1 were cloned (Fukaya et al., 1993), and the deduced amino acid sequences of these subunits, especially subunit I, showed a great similarity to the *bo*-type ubiquinol oxidase from *Escherichia coli* (Chepuri et al., 1990), with 67% identity for subunit I. Cytochromes a_1 and *o* have a closely similar protein structure in subunit composition, immunoreactivity, peptide mapping, and NH_2-terminal amino acid sequences (Matsushita et al., 1992a). Furthermore, Southern hybridization analysis showed both cytochromes a_1 and *o* were derived from the same genes, indicating that the two oxidases share an identical protein moiety. Thus, differences between these two ubiquinol oxidases are solely due to a heme peripheral group of the heme at the binuclear site. Substitution of the methyl group at position 8 of heme O with a formyl group is likely an origin of the higher affinity for dioxygen and the lower sensitivity to KCN of cytochrome a_1 (Matsushita et al., 1992a,b). Thus, cytochrome a_1 and cytochrome *o* provide a unique opportunity to investigate interactions of the heme formyl group with the protein moiety and their effects on function. In the present study, we investigate the structure

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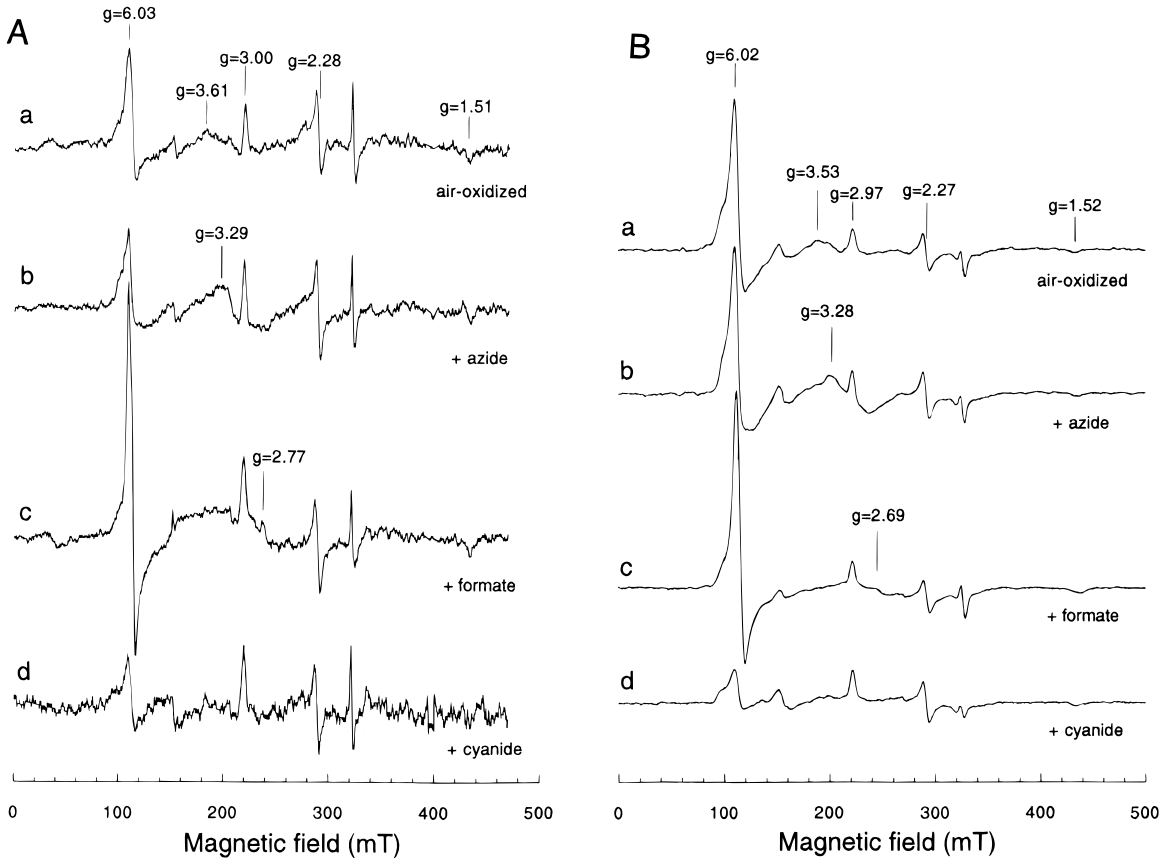

FIGURE 1: EPR spectra of cytochrome a_1 (A) and cytochrome *o* (B) in the air-oxidized states measured at 5 K. (a) Air-oxidized state as purified, (b) in the presence of 100 mM potassium azide, (c) in the presence of 100 mM of sodium formate, and (d) in the presence of 5 mM of potassium cyanide. Experimental conditions were modulation amplitude, 1 mT; modulation frequency, 100 kHz; microwave power, 5 mW; and microwave frequency, 9224.6 MHz. Sample concentration, 320 μM.

of the binuclear site of both types of oxidases using resonance Raman, FT-IR[1], and EPR spectroscopies.

EXPERIMENTAL PROCEDURES

Purification of Ubiquinol Oxidases from A. aceti. Cytochromes a_1 and *o* were purified from the membranes of *A. aceti* grown in shaking and static cultures, respectively. The enzyme was isolated first by solubilization with 1.25% β-octylglucoside and purified by DEAE-Toyopearl column chromatography in the presence of 1% β-octylglucoside as described previously (Matsushita et al., 1992a). The enzyme fraction thus obtained was diluted to a potassium phosphate concentration of 50 mM, and sucrose monolaurate and EDTA were added to final concentrations of 0.1% and 1 mM, respectively. Then, the enzyme solution was applied to a DEAE-Toyopearl column (about 1 mL bed volume per 2 mg of protein), which had been equilibrated with 50 mM potassium phosphate buffer (pH 6.5). After washing the column with the same buffer containing 0.1% sucrose monolaurate and 1 mM EDTA, the enzyme was eluted by a linear gradient of potassium phosphate (from 50 to 200 mM) in buffer (pH 6.5) that contained 0.1% sucrose monolaurate and 1 mM EDTA. Active fractions were pooled and concentrated by an ultrafiltration with UK200 membrane (Advantec, Tokyo) to about 300 μM.

Resonance Raman Spectroscopy. The purified oxidase (50 μM) in 50 mM potassium phosphate buffer (pH 6.5) containing 0.1% sucrose monolaurate was placed in an air-tight spinning cell. Resonance Raman scattering was obtained with the 441.6 nm line of a He/Cd laser (Model CD1805B, Kinmon Electrics) and with the 406.7 nm line of a Kr^+ ion laser (Model 2016, Spectra Physics). The scattering was detected with a cooled diode array (−20 °C; Model 1421HQ, PAR) attached to a 1 m single polychromator (Model DG-1000, Ritsu Oyo Kogaku). The slit width and slit height were set to be 200 μm and 10 mm, respectively. The excitation laser beam, about 50 μm in diameter, was focused to the sample, and the laser power (at the sample point) was adjusted as indicated in legends to the figures. All measurements were carried out at room temperature with a spinning cell (3500 rpm). The data accumulation time was 320 s. Raman shifts were calibrated with CCl_4, $CHCl_3$, and acetone, and accuracy of the peak positions of the Raman bands was ± 1 cm^{-1}. For reduction of the enzyme, a small volume of N_2-saturated dithionite solution was added anaerobically to a final concentration of 10 mM. Carbon monoxide (CO) adducts were obtained by similar ways to those of other heme proteins as described elsewhere (Hirota et al., 1994). $^{13}C^{18}O$ (99 atom % for ^{13}C and 98 atom % for ^{18}O, ICON, Mt. Marion, NY) was used for isotopic experiments. Cyanide adducts in the reduced state were obtained by anaerobic addition of cyanide solution to a final concentration of 10 mM.

Measurements of EPR, FT-IR, and Optical Spectra. EPR measurements were carried out at X-band (9.23 GHz) microwave frequency with a Varian E-12 EPR spectrometer equipped with an Oxford flow cryostat (ESR-900). Infrared spectra were recorded at 4 °C with a Perkin-Elmer FT-IR spectrophotometer (model 1850) as described previously

[1] Abbreviations: FT-IR, Fourier transform-infrared; EDTA, ethylenediaminetetraacetic acid.

(Tsubaki et al., 1993). Absolute optical spectra of the ubiquinol oxidases in the infrared cells with a 51 μm path length were measured at room temperature with a UVIKON 860 UV−visible spectrophotometer (Kontron Instruments, Inc.) before and after FT-IR measurements. The following potassium cyanide isotopes were used: $K^{12}C^{14}N$ (natural abundance, Nacalai Tesque); $K^{12}C^{15}N$ (99.4 atom % ^{15}N, Isotec Inc.); and $K^{13}C^{14}N$ (99 atom % ^{13}C, Isotec Inc.); $K^{13}C^{15}N$ (99 atom % ^{13}C, 99 atom % ^{15}N, Icon).

RESULTS

EPR Spectroscopy. EPR spectra of the air-oxidized cytochromes a_1 and *o* in the presence or absence of various inhibitors were measured at 5 K (Figure 1, panels A and B, respectively). The spectra of both types of enzymes are very similar each other and to those of the corresponding species of the *E. coli bo*-type ubiquinol oxidase (Tsubaki et al., 1993). In the air-oxidized state (a), the spectra are characterized by a high-spin signal ($g = 6.03$ for cytochrome a_1 and $g = 6.02$ for cytochrome *o*) and a low-spin signal ($g = 3.00$, 2.28, and 1.51 for cytochrome a_1 and $g = 2.97$, 2.27, and 1.52 for cytochrome *o*). The weak intensity of the $g = 6$ high-spin signal relative to that of the $g = 3$ low-spin signal indicates that a large part of the heme-copper binuclear center is in a spin−spin coupled EPR-invisible state for both types of the oxidase. In addition, there are broad signals around $g = 3.6$ ($g = 3.61$ for cytochrome a_1 and $g = 3.53$ for cytochrome *o*) characteristic of another form of the spin−spin coupled species (Tsubaki et al., 1993). Addition of formate (100 mM) to the air-oxidized enzymes resulted in the disappearance of these species, the appearance of new EPR species around $g = 2.7$ ($g = 2.77$ for cytochrome a_1 and $g = 2.69$ for cytochrome *o*) and the intensification of the $g = 6$ high-spin signals (c). Addition of azide (100 mM) to the air-oxidized enzymes caused a shift of the broad $g = 3.6$ signal to around 3.3 (b) in both types of the oxidase. Addition of cyanide (5 mM) to the air-oxidized state caused a decrease of the $g = 6$ high-spin signals, but there was neither a low-spin signal corresponding to the ferric heme-cyanide species nor a spin-coupled signal (d) indicating the formation of the cyanide-bridging EPR-invisible species (Tsubaki et al., 1993, 1996). All these unusual EPR characters are derived from the spin−spin coupling interactions at the binuclear center (Calhoun et al., 1994; Tsubaki et al., 1993).

Resonance Raman Spectroscopy. Resonance Raman spectra of both types of the enzyme in the air-oxidized, reduced, CO-reduced, and CN-reduced states were examined in the higher frequency region (1000−1800 cm^{-1}; spectra not shown). The spectra of corresponding states showed remarkable similarities to each other despite the difference in the heme macrocyclic structure at the binuclear site. The only exceptions were the appearance of a band arising from the heme A peripheral formyl group around 1670 cm^{-1} in the spectra of cytochrome a_1 (Figure 2). The formyl C═O stretching bands appeared at 1671 cm^{-1} in the air-oxidized state (a), at 1668 cm^{-1} in the reduced state (b), at 1667 cm^{-1} in the CO-reduced state (c), and at 1651 cm^{-1} in the CN-reduced state (d). Corresponding bands had been assigned for heme a_3 of bovine heart cytochrome *c* oxidase (Babcock & Callahan, 1983; Callahan & Babcock, 1983; Salmeen et al., 1978) (Table 1). These frequencies are also very similar to those of the corresponding bands of other heme-copper oxidases (Table 1). The formyl group of heme a_3 in the air-oxidized and reduced states of cytochrome *c* oxidase had been considered to be an unaltered state (*i.e.*, in a non-hydrogen-bonding environment) on the basis of the data on ferrous heme A model compounds (Van Steelandt-Frentrup et al., 1981). Thus, the immediate vicinity of the heme a_3 formyl group of cytochrome a_1 in both the air-oxidized and reduced states is also likely to be very hydrophobic.

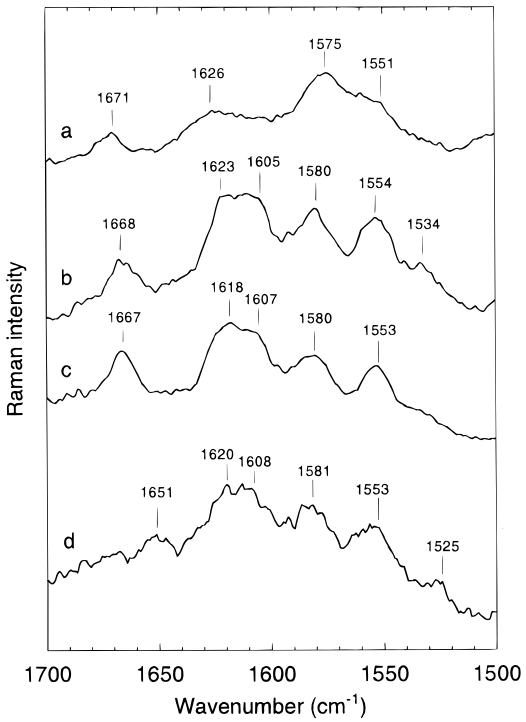

FIGURE 2: Resonance Raman spectra in the 1500−1700 cm^{-1} region of cytochrome a_1 in the (a) air-oxidized, (b) $Na_2S_2O_4$-reduced, (c) CO-reduced, and (d) CN-reduced states. Experimental conditions were excitation wavelength, 406.7 nm; laser power at the sample point, 4 mW; at room temperature; sample concentration, 50 μM.

As shown in Figure 3, an Fe^{2+}−His stretching vibration was observed at 211 cm^{-1} in the reduced state of both cytochromes a_1 (a) and *o* (b). Although we did not carry out an isotopic substitution experiment for the identification of this band, the remarkable similarity of the spectrum of cytochrome a_1 (a) to that of the *E. coli bo*-type ubiquinol oxidase [in which the Fe^{2+}−His stretching band was identified at 208 cm^{-1} with iron isotopic substitution (Tsubaki et al., 1994)] suggests that the 211 cm^{-1} band is from the Fe^{2+}−His stretch. This frequency is very close to those of other heme-copper oxidases (Lynch et al., 1993; Ogura et al., 1983; Shapleigh et al., 1992b; Sone et al., 1994), but is distinctly lower than those of His-coordinated oxygen carriers.

The Fe^{2+}−CO stretching vibrations were identified at 525 cm^{-1} for both types of the enzyme as shown in Figure 4 and Table 2. For cytochrome *o* (right panel), the 522 cm^{-1} band for the $^{12}C^{16}O$ species (a) showed a 14 cm^{-1} down shift upon substitution with $^{13}C^{18}O$ isotope (b). Similarly for cytochrome a_1 (left panel), the 522 cm^{-1} band showed an identical shift to 508 cm^{-1} upon $^{13}C^{18}O$ substitution (curves a and b). To confirm these observations, isotope-difference spectra are shown at the bottom of each panel. The difference spectra showed, in each type of the enzyme, a peak at 525 cm^{-1} and a trough at 508 cm^{-1}. The shift of the peak to 525 cm^{-1} in the difference spectra from 522 cm^{-1} of the absolute spectra might be due to a porphyrin band near 522 cm^{-1} whose effects should be completely removed

Table 1: Formyl C=O Stretching Frequencies of Heme a_3 in Heme-Copper Oxidases[a]

	$\nu_{C=O}$ (cm^{-1})	state
Cytochrome *c* Oxidase		
cytochrome aa_3		
Rhodobacter sphaeroides		
(Hosler et al., 1992; Shapleigh et al., 1992b)	1662	reduced
Paracoccus denitrificans		
(Heibel et al., 1993b)	1662.9	reduced
	1671.6	oxidized
Bovine heart		
(Callahan & Babcock, 1983)	1665	reduced
	1676	oxidized
(Argade et al., 1986; Ching et al., 1985)	1664	reduced
	1666	CO-reduced
	1644	CN-reduced
	1671	oxidized
	1671	CN-oxidized
(Rousseau et al., 1988)	1666	NO-reduced
(Heibel et al., 1993b)	1664.7	reduced
	1673.6	oxidized
Sulfolobus acidocaldarius		
(Heibel et al., 1993a)	1668	reduced
	1672	reduced 5cHS
	1673	oxidized 6cHS
	1666	oxidized 5cHS
cytochrome ba_3		
Thermus thermophilus		
(Oertling et al., 1994)	1669	reduced
	1657	CN-reduced
	1676	oxidized
cytochrome caa_3		
Bacillus PS3		
(Ogura et al., 1984)	1667	reduced
(Sone et al., 1986)	1665	reduced
Heme-Copper Quinol Oxidase		
cytochrome aa_3		
Bacillus subtilis		
(Lauraeus et al., 1992)	1666	reduced
	1644	CN-reduced
	1674	oxidized
	1671	CN-oxidized
cytochrome ba_3(*ba*-type)		
Acetobacter aceti		
(this article)	1668	reduced
	1667	CO-reduced
	1671	oxidized
	1651	CN-reduced

[a] Frequencies for the CN-reduced states are indicated in boldface. 5cHS, 5-coordinated high-spin; 6cHS, 6-coordinated high-spin.

in the difference spectra. The larger isotopic shift of the $Fe^{2+}-CO$ stretching band upon $^{13}C^{18}O$ substitution (17 cm^{-1}) than that of other heme proteins (12−15 cm^{-1}) (Hirota et al., 1994) suggests that the band at 525 cm^{-1} is overlapped with at least two $Fe^{2+}-CO$ stretching bands indicating the presence of more than two Fe−CO conformations for the CO-reduced form, which is consistent with the FT-IR results (see text below).

FT-IR Spectroscopy. The bound C−O stretching band was investigated in the CO-reduced form (Figure 5, panel A). For cytochrome a_1(trace a), there were two overlapping bands, a major band centered at 1967 cm^{-1} and a minor band at 1972 cm^{-1}. For cytochrome *o* (trace b), there were three bands; the major band at 1960.5 cm^{-1} and two minor bands at 1966 and 1973 cm^{-1}, although no precise band deconvolution was performed due to the low quality of the spectral data. The C−O stretching frequencies for various heme-copper oxidases are summarized in Table 2 together with the corresponding $Fe^{2+}-CO$ stretching frequencies.

In Figure 5 (panel B), we present the C−N stretching bands of the CN adducts of air-oxidized cytochrome a_1 (2147 cm^{-1}) (a) and cytochrome *o* (2146 cm^{-1}) (b). These frequencies are quite close to that observed for the *E. coli bo*-type ubiquinol oxidase (2146 cm^{-1}) indicating a cyanide-bridging configuration, as reported previously (Tsubaki et al., 1993, 1996). In the CN-reduced form of cytochrome a_1, we could observe a clear infrared C−N stretching band for a ferrous heme-CN species at 2051 cm^{-1} (not shown). However, we could not detect any corresponding C−N stretching band for cytochrome *o* at the same cyanide concentration (5 mM). Corresponding bands for the *E. coli bo*-type ubiquinol oxidase was observed at 2034.5 cm^{-1} (Tsubaki et al., 1993, 1996); whereas for cytochrome *c* oxidase it was observed at 2045 cm^{-1} with a shoulder at 2058 cm^{-1} (Tsubaki, 1993b). The effect of cyanide isotopic substitution on these cyanide bands together with their assignments are summarized in Table 3.

The azide stretching vibration of the azide-bound air-oxidized enzyme was analyzed with both types of oxidase and the results are shown in Figure 5 (panel C). The internal antisymmetric stretching band of the bound azide was observed at 2042 (major) and 2056 cm^{-1} (minor) for

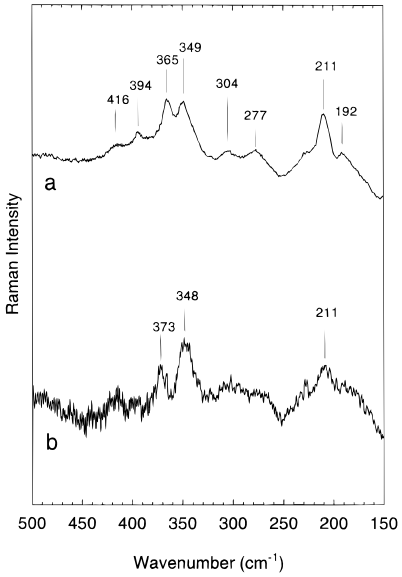

FIGURE 3: Resonance Raman spectra of the reduced form of cytochrome a_1 (a) and cytochrome *o* (b) in the 150−500 cm^{-1} region. Excitation wavelength, 441.6 nm; laser power at sample point, 14 mW. Other conditions were the same as in Figure 2.

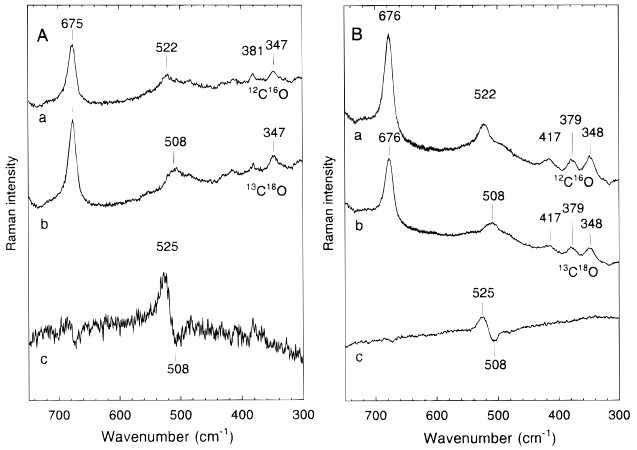

FIGURE 4: Resonance Raman spectra of the CO-reduced form of cytochrome a_1 (A) and cytochrome *o* (B) in the 300−700 cm^{-1} region. The Fe^{2+}−CO stretching band was identified using natural abundant $^{12}C^{16}O$ (a) and isotopically labeled $^{13}C^{18}O$ (b). Difference spectra ($^{12}C^{16}O - {}^{13}C^{18}O$) (c) are also presented. In the right panel, the ordinate of spectrum (c) was expanded 5-fold for clarification. Experimental conditions were excitation wavelength, 406.7 nm; laser power at the sample point, 12 mW. Other conditions were the same as in Figure 2.

cytochrome a_1, whereas the corresponding bands were observed at 2041 (major) and 2055 cm^{-1} (minor), respectively, for cytochrome *o*. These frequencies were very close to those observed for the corresponding vibrations of the *E. coli bo*-type ubiquinol oxidase [2041 (major) and 2051 cm^{-1} (minor)] (Tsubaki et al., 1993) and of cytochrome *c* oxidase [2039.5 (minor) and 2051 cm^{-1} (major)] (Tsubaki, 1993a).

Titration of Cyanide-Binding to Cytochromes a_1 and o. Cyanide-binding to cytochromes a_1 and *o* in the reduced state was investigated by titration with neutralized potassium cyanide solution and analyzed with visible absorption spectroscopy. The spectral change upon binding of cyanide to cytochrome a_1 (spectrum not shown) was very similar to that of cytochrome *c* oxidase from bovine heart (Hill & Marmor, 1991). The extent of the spectral change in the visible region at 590−602 nm was titrated by stepwise addition of cyanide to a dithionite-reduced sample at pH 7.0. A Hill plot of absorptions gave a dissociation constant (K_d) of 0.35 mM. This value is very close to those [0.1 mM (van Buuren et al., 1972), 0.555 mM (Antonini et al., 1971), and 0.23 mM (Hill & Marmor, 1991)] obtained previously for cytochrome *c* oxidase from bovine heart. We could not obtain a definite K_d value for cytochrome *o* due to a considerable release of Cu_B at higher cyanide concentration (>10 mM). However, assuming a similar spectral change to occur in the CN-reduced state to that of the *E. coli bo*-type ubiquinol oxidase, we extrapolated the K_d value to be around 20 mM, slightly higher than the value [7 mM (Mitchell et al., 1995)] for the *E. coli bo*-type ubiquinol oxidase.

DISCUSSION

Implications on the Binuclear Site Structures of Cytochromes a_1 and o Based on the X-ray Structures of Cytochrome c Oxidase. Although there is no X-ray structure of ubiquinol oxidase subfamily available at this moment, their primary structures are highly homologous to those of cytochrome *c* oxidases, and therefore, the tertiary structure is expected to be very similar to those of cytochrome *c* oxidases. Recent X-ray crystallographic studies on cytochrome *c* oxidases [from bovine heart mitochondria (Tsukihara et al., 1995; Tsukihara et al., 1996) and *Paracoccus denitrificans* (Iwata et al., 1995)] in the oxidized state showed that the binuclear site structure proposed previously on the basis of site-directed mutagenesis studies on the *E. coli bo*-type ubiquinol oxidase (Calhoun et al., 1993; Minagawa et al., 1992; Tsubaki et al., 1994), in which high-spin heme a_3 is coordinated with His421[2] and Cu_B is coordinated with three totally conserved histidyl residues (His284, His333, and His334), is correct. In both the bovine and *Paracoccus* oxidases, the formyl group of heme a_3 is located very close to the imidazole ring of His333 (His290 of the bovine oxidase) (Figure 6) (Iwata et al., 1995; Tsukihara et al., 1995). Other two imidazole rings of the histidyl residues [His284 (His240 of the bovine oxidase) and His334 (His291 of the bovine oxidase)] are also located near the heme plane. The distance between heme iron and Cu_B is about 4.5 Å for the oxidized bovine oxidase (Figure 6) (Tsukihara et al., 1995), but it increases slightly (about 5.2 Å) for the azide-bound oxidized *Paracoccus* oxidase (Iwata et al., 1995) probably to accommodate an azide ion between heme iron and Cu_B. Of particular interest is that electron density for the side chain of His333 (His290 of the bovine oxidase) was not clear in the X-ray structure of the *Paracoccus* oxidase. This indicates that the binding of azide at the binuclear site may cause a change in the coordination of His333 structure, resulting in multiple orientations of the imidazole ring (see later).

Close Similarities of Cytochrome a_1 and o to the E. coli bo-Type Ubiquinol Oxidase and to Cytochrome c Oxidase in the Air-Oxidized States. In the air-oxidized state, in the presence or absence of various inhibitors, except for N_3, we could not observe any significant differences between cytochrome a_1 and cytochrome *o* in resonance Raman, FT-IR and EPR spectra (Figures 1,2 and 5). Furthermore, the spectra of cytochromes a_1 and *o* were remarkably similar to

[2] The numbering is based on the *E. coli bo*-type ubiquinol oxidase sequence (Chepuri et al., 1990), which is also applicable to the sequence of *A. aceti* ubiquinol oxidase, since these two have end-to-end similarity in the deduced amino acid sequences (Fukaya et al., 1993).

Table 2: Bound C−O and Fe^{2+}−CO Stretching Frequencies of Heme-Copper Oxidases

	ν_{C-O} (cm^{-1})	ν_{Fe-CO} (cm^{-1})	ref
	Mitochondria		
cytochrome *c* oxidase			
(bovine heart)	1963.3	520	Argade et al., 1984 Yoshikawa & Caughey, 1982
		516	Hirota et al., 1994
(porcine heart)	1963.7		Young et al., 1984
(rat kidney)	1965.1		Young et al., 1984
(blowfly flight muscle)	1963.2		Young et al., 1984
	Bacteria		
cytochrome *c* oxidase			
cytochrome ba_3			
(*Thermus thermophilus*)	1974 (1983)		Einarsdóttir et al., 1989
cytochrome c_1aa_3			
(*Thermus thermophilus*)	1947 (1953)		Einarsdóttir et al., 1989
cytochrome aa_3			
(*Rb. sphaeroides*)	1964		Shapleigh et al., 1992a
	1966 (1955)	519 (493)	Wang et al., 1995b
cytochrome cbb_3			
(*Rb. sphaeroides* JS100)	1950	495	García-Horsman et al., 1994 Wang et al., 1995a
cytochrome *cao*			
(*Bacillus* PS3)		522	Sone et al., 1994
heme-copper quinol oxidase			
cytochrome *bo*			
(*Escherichia coli*)	1959.7	523	Tsubaki et al., 1993 Uno et al., 1985
		521	Hirota, et al., unpublished experiment
cytochrome a_1(*ba*)			
(*Acetobacter aceti*)	1967 (1972)	525	this article
cytochrome *o* (*bo*)			
(*Acetobacter aceti*)	1960.5	525	this article

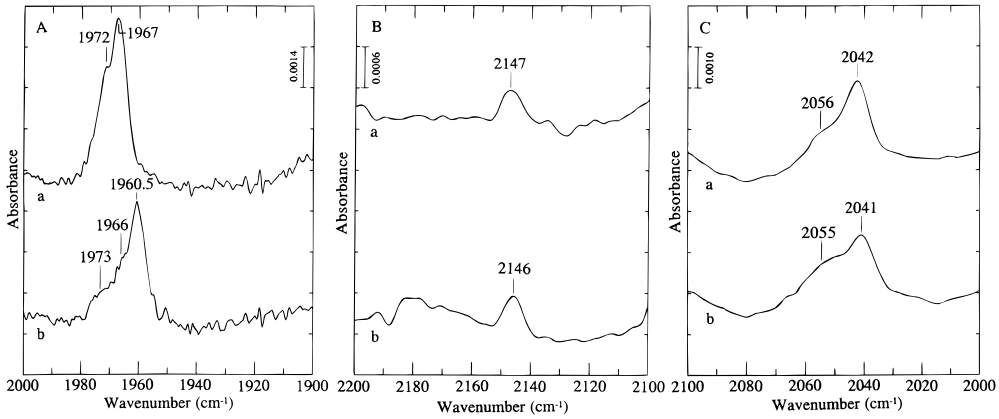

FIGURE 5: (Panel A) FT-IR spectra of the bound C−O stretching band of cytochrome a_1 (a) and cytochrome *o* (b) in the CO-reduced form (natural abundant $^{12}C^{16}O$ was used). (Panel B) FT-IR spectra of the bridging cyanide stretching band of cytochrome a_1 (a) and cytochrome *o* (b) in the air-oxidized CN-inhibited form ($^{12}C^{14}N$). (Panel C) FT-IR spectra of the bound azide internal antisymmetric stretching band of cytochrome a_1 (a) and cytochrome *o* (b) in the air-oxidized azide-inhibited form ($^{14}N_3$). Experimental conditions were sample concentration, 320 μM; temperature 4 °C; spectral data accumulation, 400 cycles (2 h); nominal spectral resolution, 1.0 cm^{-1} for the CO-reduced form and 4.0 cm^{-1} for the CN-inhibited and azide-inhibited forms.

Table 3. Assignments of cyanide C−N stretching vibrations of the cyanide complexes of cytochrome a_1*(ba-)* and cytochrome *o* (*bo*-type) (*Acetobacter aceti*)

assignments	ν_{C-N} (cm^{-1})			
	$^{12}C^{14}N$	$^{12}C^{15}N$	$^{13}C^{14}N$	$^{13}C^{15}N$
	cytochrome a_1 (*ba*)			
$Fe_a^{3+}-C{=}N-Cu_B^{2+}$	2147	2114.5	2102	2068
$Fe_a^{2+}-C{=}N$	2051	2021	2008	1975
	cytochrome *o* (*bo*)			
$Fe_o^{3+}-C{=}N-Cu_B^{2+}$	2146	2114	2101	2067
$Fe_o^{2+}-C{=}N$	ND[a]	ND	ND	ND

[a] ND, not detected.

those of the *E. coli bo*-type ubiquinol oxidase (Moody et al., 1995; Tsubaki et al., 1993; Watmough et al., 1993) and showed some resemblance to those of cytochrome *c* oxidase (Cooper & Salerno, 1992; Moody et al., 1991; Schoonover & Palmer, 1991). The binuclear center is probably tightly maintained in the air-oxidized state and, therefore, the overall architecture and even the electronic configuration are also unaltered among the heme-copper oxidase superfamily irrespective of the heme peripheral group at position 8.

Azide-Inhibited Air-Oxidized State. Although there was no significant difference in FT-IR and EPR spectra for the azide-inhibited, air-oxidized state between cytochromes a_1 and *o*, the azide-inhibited, air-oxidized state may be an exception to the tight nature of the binuclear center in the air-oxidized state, as evoked by X-ray crystallography (Iwata et al., 1995). It is not clear whether a similar coordination-structural change occurs in the ubiquinol oxidases upon azide-binding. The presence of two azide infrared bands for both types of ubiquinol oxidase in the present study and for the *E. coli bo*-type ubiquinol oxidase and cytochrome *c*

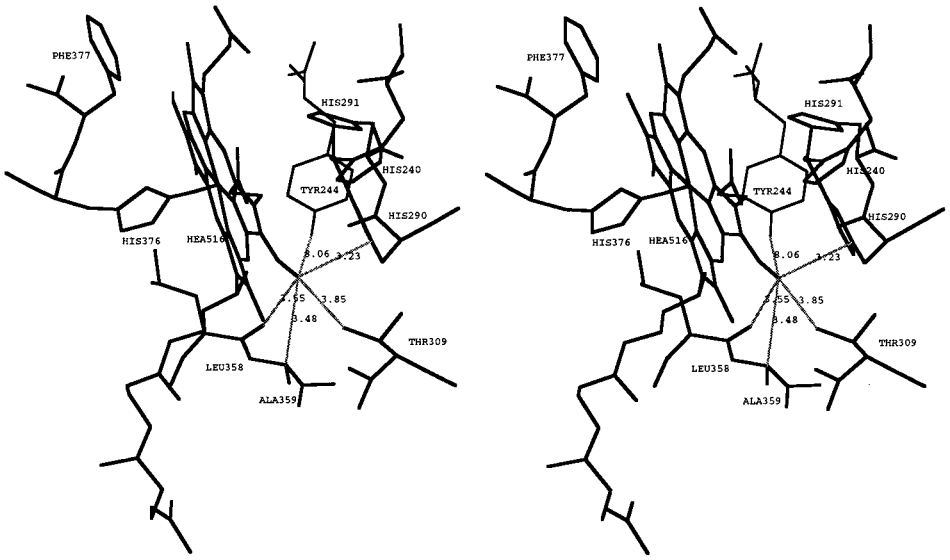

FIGURE 6: Stereo view of the heme-copper binuclear site of oxidized cytochrome *c* oxidase (bovine heart) (Tsukihara et al., 1995, 1996). The heme a_3 was coordinated with His376 (numbering for the bovine enzyme; corresponding to His421 of the ubiquinol oxidases) and Cu_B is coordinated with His240, His290, and His291 (His284, His333, and His334, respectively, of the ubiquinol oxidases). Tyr244 (Tyr288 of the ubiquinol oxidases) is also very close to the heme plane. The closest distances (in angstroms) from surrounding amino acid residues to the formyl oxygen atom were indicated. The residues close to the formyl oxygen atom are His290, Thr309, Leu358, and Ala359 (His333, Thr352, Leu401, and Ala402, respectively, of the ubiquinol oxidases). The coordinates were obtained from Brookhaven Protein Data Bank (number, 1occ).

oxidase in the previous studies (Tsubaki, 1993a; Tsubaki et al., 1993), but with a different ratio in intensity for cytochrome *c* oxidase (Tsubaki, 1993a), suggests that this phenomenon is universal and that the conformational heterogeneity exists to some extent even in the air-oxidized state. The appearance of the $g = 3.3$ EPR signal derived from the spin−spin coupling interactions at the binuclear center in all the azide-inhibited ubiquinol oxidases in the present and previous studies (Tsubaki et al., 1993), but not for the azide-inhibited cytochrome *c* oxidase (Tsubaki et al., 1993), may be related to this nature.

Variations in the Reduced Ligand-Bound States. In the reduced state without any exogenous ligand, we could not detect any significant difference between cytochromes a_1 and *o* caused by the heme peripheral substitution at position 8. However, when CO and CN ligated to the ferrous high-spin heme, there appeared to be significant variations in the ligand internal vibrations, although the Fe^{2+}−CO bond seemed to be perturbed little (see Table 2).

The bound C−O stretching bands for both types of the oxidase showed multiple bands and their peak frequencies differed by 6.5 cm^{-1}. In contrast, the *E. coli bo*-type ubiquinol oxidase showed a narrow C−O stretching band at 1959.7 cm^{-1} (Tsubaki et al., 1993). Cytochrome *c* oxidase showed it at 1963.3 cm^{-1} with a very narrow band width (about 3.5 cm^{-1}). Some members of the heme-copper oxidase family have extremely different C−O stretching frequencies (from 1947 to 1983 cm^{-1}) and multiple C−O bands (Table 2). Even for cytochrome *c* oxidase, there are several minor bands that become apparent at the low temperatures as "β-forms" (Fiamingo et al., 1986; Fiamingo et al., 1982). Both cytochromes a_1 and *o* showed only α-forms, but the present result suggests that even within the α-forms there are conformational variations. The recent resonance Raman studies by Rousseau and co-workers on aa_3-type (Wang et al., 1995b) and cbb_3-type (Wang et al., 1995a) cytochrome *c* oxidases from *Rhodobacter capsulatus* are of interest. The former showed two distinct Fe^{2+}−CO stretching modes (519 and 493 cm^{-1}), and each of them gave C−O stretching frequencies at 1966 (α-form) and 1955 (β-form) cm^{-1}, respectively (Wang et al., 1995b). The cbb_3-type oxidase showed only the β-form with Fe^{2+}−CO stretching band at 495 cm^{-1} and C−O stretching band at 1950 cm^{-1} (Wang et al., 1995a). Thus, the multiple C−O bands may be more or less a common feature of the binuclear site of various heme-copper oxidases in the reduced state. Caughey and co-workers first pointed out that the CO adducts of myoglobin and hemoglobin show several discrete C−O stretching bands (Caughey et al., 1981; Makinen et al., 1979) and have assigned them to discrete, rapidly interconverting conformers (Potter et al., 1990). Later, it was found that the internal stretching vibrations of heme-bound ligand are sensitive to the polarity of the environments determined by orientations of the distal residues (Oldfield et al., 1991). Thus the heme-bound C−O stretching frequency can reflect the combined effects of the electronic character of the heme iron determined by the orientation of the proximal ligand (*i.e.*, largely His residue in hemoproteins) and the electrostatic interactions of CO with the distal residues (Jewsbury et al., 1994; Li et al., 1994). A similar mechanism must be operative for the appearance of the multiple C−O stretching bands in the heme-copper oxidases. The observed multiplicity of the C−O stretching band for both types of ubiquinol oxidase from *A. aceti* could be ascribed to the electrostatic effects from the surrounding amino acid residues. At the binuclear site there are three imidazole rings and one phenol rings [His284, His333, His334, and Tyr288 of the ubiquinol oxidases (see Figure 6)] in addition to the Cu_B ion as possible effectors to control the electrostatic potential around the heme-bound CO moiety. The subtle difference at position 8 of the heme periphery between cytochromes a_1 and *o* can be sensed by the closest distal residue and propagated to the coordination sphere. Structural reorganization at the binuclear site results in different orientations of the distal amino acid residues, which, then, might determine a prevailing C−O infrared band.

Variations in the C−N stretching infrared band in the CN-reduced state were more drastic (Table 3). For cytochrome

a_1, there was a clear C−N stretching band at 2051 cm^{-1}, but we could not detect a corresponding band for cytochrome *o*. For the *E. coli bo*-type ubiquinol oxidase, we observed the C−N stretching band at 2034.5 cm^{-1} (Tsubaki et al., 1993, 1996); whereas for cytochrome *c* oxidase, it was at 2045 cm^{-1} with a minor band at 2058 cm^{-1} (Tsubaki, 1993b). The absence of the C−N stretching infrared band for cytochrome *o* is consistent with its extremely low binding affinity for cyanide. Partial release of the Cu_B ion from cytochrome *o* during the cyanide treatment in the reduced state may also serve as one of the reasons (M. Tsubaki, et al., unpublished experiment), since it is well established that presence of the Cu_B^{1+} ion is essential for the cyanide binding (but not for CO-binding) to the reduced high-spin heme iron due to its charge effect (Mitchell et al., 1995).

Cyanide-Binding in the Reduced State and the Formyl C═O Stretching Frequency. In considering the reason for the significant difference in the C−N stretching frequency between heme A-bound cyanide (cytochrome *c* oxidase and cytochrome a_1) and heme O-bound cyanide (the *E. coli bo*-type ubiquinol oxidase) and the considerable lowering of the binding-affinity of cyanide to the ferrous high-spin heme in cytochrome *o* (and the *E. coli bo*-type ubiquinol oxidase), we noticed interesting results from resonance Raman spectroscopy. In the reduced or CO-reduced states, cytochrome a_1 formyl C═O stretching band appeared around 1670 cm^{-1} being consistent with previous results obtained for cytochrome *c* oxidase from bovine heart (Table 1). In the CN-reduced state, however, the formyl C═O band appeared at 1651 cm^{-1}; that is also within the region of the previously reported values for the CN-reduced form of cytochrome *c* oxidase (1644−1657 cm^{-1}) (Table 1). The lowering of the formyl C═O stretching frequency has been noticed by previous investigators (Argade et al., 1986; Ching et al., 1985). Although they ascribed this phenomenon as a high- to low-spin state change upon binding of cyanide to the ferrous high-spin heme (Ching et al., 1985), the significant lowering of the frequency [as much as 25 cm^{-1}, compared to those of the CO-reduced and NO-reduced states (Table 1)] is more reasonably ascribed to a putative hydrogen bond formation of the formyl C═O group with water molecule(s) fixed with side-chain group(s) of nearby amino acid residue(s), most likely with His333 or Thr352 (Figure 6).[3] Upon the formation of the hydrogen bond network, the orientations of the imidazole and phenol rings of His284, His333, His334, and Tyr288 were reorganized. The new electrostatic potential formed by these distal residues and the Cu_B^{1+} center must be essential to render the higher C−N stretching vibration (15−20 cm^{-1} higher) than heme O-bound cyanide and the higher affinity (∼60-fold) of cyanide-binding to the binuclear site in the reduced state.

The greater lowering of the formyl C═O stretching frequency in the CN-reduced cytochrome *c* oxidase (about 30 cm^{-1} lowering from the air-oxidized state) compared to that of the *ba*-type ubiquinol oxidase (about 20 cm^{-1} lowering) (Table 1) may indicate a stronger hydrogen bonding to the formyl group and, therefore, a tighter hydrogen bond network, in the CN-reduced cytochrome *c* oxidase.

It is known that, in the reduced state of cytochrome *c* oxidase, only cyanide ion binding can cause a charge compensation by uptake of one proton (Mitchell & Rich, 1994; Rich et al., 1996). For the *bo*-type ubiquinol oxidase, it is also reported that a proton is bound together with a cyanide ion (Mitchell et al., 1995). Although it is not clear where the proton-trapping site is, it must be close enough to the binuclear site to compensate the charged ligand. Such a protonatable group may be also participating in the formation of the hydrogen bond network.

We noticed further that the putative hydrogen bond network is also important for the stabilization of the Cu_B center in the fully reduced or partially reduced states. In the partially reduced state, the Cu_B center of cytochrome *o* from *A. aceti* (M. Tsubaki, et al., unpublished experiment) and the *bo*-type ubiquinol oxidase from *E. coli* (Tsubaki et al., 1996) can be easily removed by a cyanide-treatment. Thus, the hydrogen bond network participated by the heme a_3 formyl group seems to play a very important role for maintaining the structure of the binuclear center.

As an origin of the higher C−N stretching vibration in the reduced heme A-bound cyanide than in heme O-bound cyanide, one may propose that it is due to the intrinsic nature of heme A caused by the presence of the electron-withdrawing formyl group. However, the electron-withdrawing effect of the formyl group, if any, should contribute to the increase in the bound C−N stretching frequency only slightly, since the formyl substitution at the heme periphery caused the increase of the heme-bound C−O frequency by, at most, 3−4 cm^{-1}, whereas, for the heme-bound NO and O_2, it seems negligible (Caughey et al., 1977; Zhao et al., 1994). A similar argument may be made for the lower binding affinity of cytochrome *o* toward cyanide than cytochrome a_1. Presence of the electron-withdrawing formyl group at the heme periphery in the heme-reconstituted myoglobins reduced the binding affinity toward O_2 but increased the affinity toward CO (Sono & Asakura, 1975; Sono et al., 1976). However, the magnitude of the differences was only slight in both cases (2−3-fold) (Sono & Asakura, 1975; Sono et al., 1976). Thus, the participation of electron-withdrawing ability of the formyl group in the lowering of the cyanide binding affinity seems marginal.

In conclusion, comparison of the binuclear site structures of the *ba*-type and *bo*-type of ubiquinol oxidases from *A. aceti* using resonance Raman, FT-IR, and EPR spectroscopies showed that, in the air-oxidized state, the overall architecture and the electronic configurations of the binuclear center seemed well conserved irrespective of the heme peripheral group at position 8, except for the azide-inhibited state. In contrast, we observed multiple C−O stretching bands with different peak positions and variation in the C−N stretching band caused by substituting the heme periphery in the reduced state. These observations may be regarded as a direct evidence of the conformational flexibility or heterogeneity at the binuclear site in the reduced state. In addition, Raman data suggest that the formyl C═O group may make a putative hydrogen bond network with water molecule(s) and conserved distal amino acid residues, and this network

[3] A possibility of the direct hydrogen bonding of His333 imidazole group to the formyl oxygen atom should not be neglected. In this case, the imidazole plane may rotate ∼90° along the Cu_B-N_ϵ bond axis to afford the closest access of N_δ atom to the formyl C═O group (see Figure 6). Thr352 (Thr309 of the bovine oxidase) may have some roles for the formation of the hydrogen bond. Such a direct hydrogen bond from Arg54 of the *Paracoccus* oxidase (Arg38 of the bovine oxidase) to the formyl oxygen at the heme *a* site was confirmed (Iwata et al., 1995). The formyl C═O stretching frequency of heme *a* in oxidized cytochrome *c* oxidase was observed at 1650 cm^{-1} (20−25 cm^{-1} lower than the corresponding frequency of heme a_3) (Babcock & Callahan, 1983).

may be very important for the cyanide binding at the binuclear site in the reduced state.

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