# Spectroscopic Characterization of Carbon Monoxide Complexes Generated for Copper/Topa Quinone-Containing Amine Oxidases†

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ABSTRACT: Carbon monoxide complexes have been generated for copper/topa quinone (TPQ)-containing amine oxidases from *Arthrobactor globiformis* (AGAO) and *Aspergillus niger* (AO-I) and characterized by various spectroscopic measurements. Addition of CO to AGAO anaerobically reduced with its substrate 2-phenylethylamine led to a slight increase of absorption bands at 440 and 470 nm derived from the semiquinone form ($TPQ_{sq}$) of the TPQ cofactor, concomitantly giving rise to new CO-related absorption bands at 334 and 434 nm. The intensity of the $TPQ_{sq}$ radical EPR signal at $g = 2.004$ also increased in the presence of CO, while its hyperfine coupling structure was affected insignificantly. FT-IR measurements revealed C−O stretching bands ($\nu_{CO}$) at 2063 and 2079 $cm^{-1}$ for the CO complex of the substrate-reduced AGAO (at 2085 $cm^{-1}$ for AO-I), which shifted nearly 100 $cm^{-1}$ to lower frequencies upon using $^{13}C^{18}O$. Collectively, these results suggest that CO is bound to the Cu(I) ion in the Cu(I)/$TPQ_{sq}$ species formed in the reductive half-reaction of amine oxidation, thereby shifting the Cu(II)/aminoresorcinol ⇄ Cu(I)/semiquinone equilibrium toward the latter. When AGAO was reduced with dithionite, an intermediary form of the enzyme with Cu(II) reduced to Cu(I) but TPQ still in the oxidized state ($TPQ_{ox}$) was produced. Dithionite reduction of AGAO in the presence of CO resulted in the immediate formation of FT-IR bands at 2064 and 2083 $cm^{-1}$, which were assigned to the $\nu_{CO}$ bands of the CO bound to the $TPQ_{ox}$ enzyme. The intense 2083 $cm^{-1}$ band was then displaced by a new band at 2077 $cm^{-1}$, corresponding to the formation of the fully reduced topa. Significant variation of these $\nu_{CO}$ frequencies indicates that vibrational properties of CO bound to copper amine oxidases are sensitively influenced by the coordination structure of the Cu(I) ion, which may be modulated by the chemical and redox states of the TPQ cofactor.

Copper amine oxidases (EC 1.4.3.6) are ubiquitously distributed from bacteria to higher animals and catalyze the oxidation of various primary amines to their corresponding aldehydes in the net reaction:

$$RCH_2NH_3^+ + O_2 + H_2O \rightarrow RCHO + H_2O_2 + NH_4^+$$

The catalytic reaction proceeds by a ping-pong transamination mechanism, consisting of the initial two-electron oxidative deamination of the amine substrate (reductive half-reaction) and the subsequent two-electron reduction of molecular oxygen to hydrogen peroxide (oxidative half-reaction) (*1*, *2*). The enzymes are commonly homodimers of identical subunits with a molecular weight ranging from 70 000 to 95 000 and contain, in addition to the prosthetic copper ion, a redox-active organic cofactor, 2,4,5-trihydroxyphenylalanine quinone (topa quinone, TPQ[1]), within each subunit. The TPQ cofactor is linked to the polypeptide chain as a modified amino acid residue (*3*) and produced from a precursor tyrosine residue by the posttranslational modification that proceeds in a copper-dependent, self-processing reaction (*4*−*6*).

It has been well established that the TPQ cofactor participates in the reductive half-reaction by forming a covalent adduct with the amine substrate (substrate Schiff base complex) (*7*−*9*). The substrate Schiff base is then deprotonated by an active-site base, assigned to an invariant aspartate residue, to yield the reduced cofactor in a product Schiff base complex. Subsequent hydrolysis of the Schiff base releases the product aldehyde, leaving an aminoresor-

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[1] Abbreviations: AGAO, *Arthrobactor globiformis* phenylethylamine oxidase; AO-I, *Aspergillus niger* amine oxidase; TPQ, topa quinone; $TPQ_{red}$, topa quinone in a reduced form (aminoresorcinol or hydroxyquinol); $TPQ_{sq}$, topa semiquinone; $TPQ_{ox}$, topa quinone in the oxidized form; CO, carbon monoxide; RR, resonance Raman; FT-IR, Fourier transform-infrared; Hc, hemocyanin.

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cinol form of the reduced cofactor ($TPQ_{red}$). In the oxidative half-reaction, $TPQ_{red}$ (aminoresorcinol) is eventually reoxidized by dioxygen to the initial oxidized state ($TPQ_{ox}$), liberating ammonia. Although it has been proposed that the enzyme-bound copper ion is essential for the oxidative half-reaction (*10, 11*), the catalytic role of copper remains uncertain. Previous studies have shown that $TPQ_{red}$ is in a temperature-dependent, rapid equilibrium with the semiquinone form of the cofactor ($TPQ_{sq}$) by donating a single electron to Cu(II) (*12, 13*) and proposed the resulting Cu(I)/$TPQ_{sq}$ species to be a kinetically competent intermediate that directly reacts with dioxygen (*13*). The formation of the Cu(I)/$TPQ_{sq}$ species has been demonstrated with copper amine oxidases from various sources (*12*−*16*). However, a recent kinetic study on the oxidative half-reaction has claimed that the initial one-electron reduction of dioxygen occurs from the Cu(II)/$TPQ_{red}$ species and not from the Cu(I)/$TPQ_{sq}$ species (*17*).

According to the X-ray crystallographic structures determined so far for the copper amine oxidases from pea seedling (*18*), *Escherichia coli* (*19, 20*), *Arthrobacter globiformis* (*21*), and a yeast *Hansenula polymorpha* (*22*), they all have very similar folding structures, even though the overall sequence identities between these enzymes are as low as 30−40%. Furthermore, the coordination structures of the Cu(II) ion are virtually identical, having four equatorial ligands (three conserved histidine residues and one water molecule) and one axial ligand (another water molecule), positioned in a distorted square-pyramidal geometry. The TPQ cofactor is located close to the copper site, being connected indirectly to the Cu(II) ion through a hydrogen-bonding network involving several active-site water molecules (*18*−*22*). However, the orientation of the TPQ ring relative to the copper atom is considerably different among the four enzymes. In the *H. polymorpha* enzyme, the TPQ ring is in an 'active' conformation poised for catalysis, where the substrate-binding carbonyl O5 atom of TPQ is on the opposite side from the copper center and in proximity to the catalytic base Asp319 (*22*), similar to the orientation in the inhibitor-bound form of the *E. coli* enzyme (*20*). In contrast, the TPQ ring in the native *E. coli* and *A. globiformis* enzymes is rotationally disordered (*19, 21*), and the TPQ orientation in the pea seedling enzyme is unfavorable for catalysis with its O5 atom facing the copper site (*18*). The nearly 180° difference in the TPQ orientation supports a facile flipping of the TPQ ring as proposed from spectroscopic studies of the reaction intermediates formed in the wild-type as well as mutant copper amine oxidases (*23*−*25*) and also suggests variations in the copper−TPQ interactions among the enzymes.

To obtain further insight into the copper−TPQ interactions in copper amine oxidases, we have used carbon monoxide (CO) that can bind to the Cu(I) centers in a variety of copper proteins (*26*−*32*). We here demonstrate for the first time that CO does bind to the copper amine oxidases from *A. globiformis* (AGAO) (*33*) and a fungus *Aspergillus niger* (AO-I) (*34, 35*) and provide evidence that vibrational properties of CO bound to the Cu(I) ion are sensitively affected by the coordination structure of the Cu atom, which may be modulated by the chemical and redox states of the TPQ cofactor.

## EXPERIMENTAL PROCEDURES

*Enzyme Purification and Assay*. Recombinant AGAO overproduced in *E. coli* cells was purified in the Cu(II)/TPQ-containing active form by the published methods (*4, 36*) except that 100 $\mu$M (final concentration) $CuSO_4$ was added to the buffers used for cell disruption and subsequent dialysis. The purification was performed with particular cautions to obtain spectrophotometrically pure preparations thoroughly devoid of impurity proteins that had strong absorption around 400 nm likely due to the bound heme in catalase (Kishishita and Tanizawa, unpublished observations). For each fraction eluted from the chromatography columns, the enzyme activity was assayed as described below, and the purity was checked by SDS−polyacrylamide gel electrophoresis. Fractions containing the enzyme with a specific activity of >50 units/mg of protein were combined and concentrated by ultrafiltration to the desired concentrations. Cultivation of *Aspergillus niger* AKU3302 mycelia, induction of AO-I by addition of *n*-butylamine, and enzyme purification were performed by the published methods (*34, 35*). Activities of AGAO and AO-I were determined with 2-phenylethylamine and benzylamine, respectively, as a substrate, as reported previously (*4, 35*). Protein concentrations of AGAO and AO-I were determined from the molecular absorption coefficient at 280 nm (*4*) and by Lowry's method (*37*) with bovine serum albumin as the standard, respectively, and calculated as subunit concentrations.

*Spectroscopic Measurements*. Optical absorption spectra were recorded at 20 °C with a Shimadzu UV 3101PC spectrophotometer. EPR spectra were measured at ambient temperature with a JES-RE1X EPR spectrophotometer (JEOL, Tokyo, Japan). The frequency and microwave power for the EPR measurements were 9.449 GHz and 3 mW, respectively, and the modulations were 0.1 and 0.63 mT for measurements of the $TPQ_{sq}$ radical and Cu(II), respectively. Resonance Raman (RR) scattering was excited at 514.5 nm with an $Ar^+$ ion laser (Spectra Physics, 2017) and detected with a triple polychromator (JASCO, NR-1800) equipped with a CCD detector (Princeton Instruments). RR spectra were measured at ambient temperature in a spinning cell (3000 rpm). Raman shifts were calibrated with acetone and toluene, and the accuracy of the peak positions of the Raman bands was $\pm 1$ $cm^{-1}$. Fourier transform-infrared (FT-IR) spectra of the CO complexes of AGAO and AO-I were obtained at ambient temperature with a Perkin-Elmer 2000 FT-IR spectrometer. Resolution for the FT-IR measurements was 2 $cm^{-1}$. The frequencies, extinction coefficients, and relative areas of the FT-IR bands were obtained by Gaussian fitting of the observed spectra. For establishing fully anaerobic conditions in these spectroscopic measurements, all sample solutions prepared in 100 mM potassium phosphate buffer, pH 7.2, were thoroughly deaerated in a glovebag filled with $N_2$ gas, and all the spectrometer cells and sample tubes were tightly sealed inside the bag. Substrate-reduced amine oxidases were prepared by mixing anaerobically the enzyme solution with excess substrate, and to achieve the complete consumption of the remaining oxygen, the amine oxidase reaction was allowed to proceed for at least 1 min prior to the measurements. CO gas (99.95%) was introduced to the enzyme solution by gently bubbling for 3 min in most cases before or after the addition of substrate, depending on the

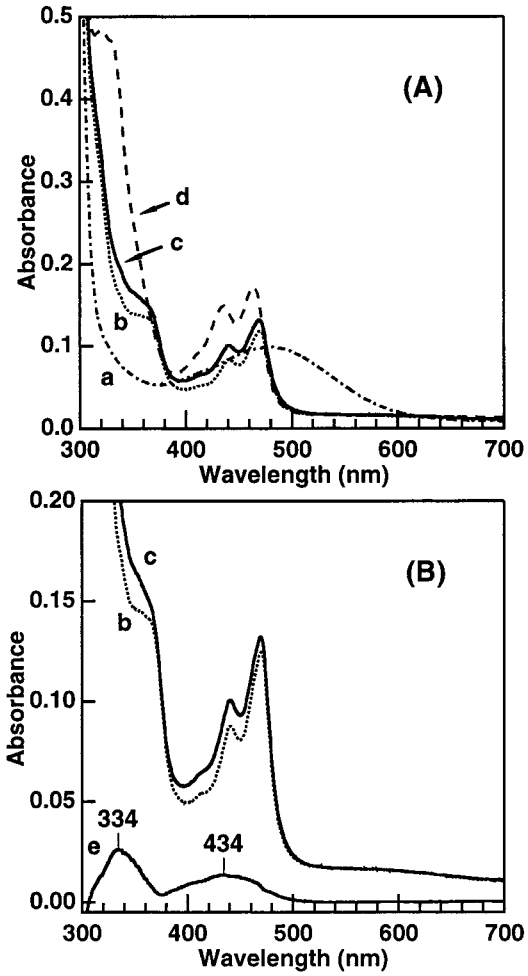


FIGURE 1: Optical absorption spectra of AGAO. (A) Absorption spectra were measured at 20 °C for the initial enzyme with $TPQ_{ox}$ (a), the enzyme anaerobically reduced with substrate (1.2 mM 2-phenylethylamine) (b), the enzyme reduced with substrate in the presence of dissolved CO (c), and the enzyme reduced with substrate in the presence of 1.2 mM $CN^-$ (d); the concentration of AGAO was 60 $\mu$M subunit. (B) The difference spectrum (e) was obtained by subtracting the spectrum of $TPQ_{sq}$ measured in the absence of CO (b, spectrum b in panel A was multiplied by a factor of 1.05 to cancel out the increase in the absorption peaks of $TPQ_{sq}$) from that measured in the presence of CO (c, same as c in panel A).

convenience of sample handling. $^{13}C^{18}O$ gas (99 atom % for $^{13}C$ and 95 atom % for $^{18}O$) was obtained from ICON. Reduction of AGAO with dithionite was performed by adding a small volume of 50, 120, and 200 mM sodium dithionite solutions dissolved in $N_2$-saturated 100 mM potassium phosphate buffer, pH 7.2, for absorption, RR, and FT-IR measurements, respectively.

## RESULTS AND DISCUSSION

*Effect of CO on the Absorption Spectrum of Substrate-Reduced AGAO.* Anaerobic incubation of the purified AGAO, containing the oxidized cofactor ($TPQ_{ox}$) having a $\lambda_{max}$ at about 480 nm (*4*), with its substrate 2-phenylethylamine resulted in a marked change of the UV−vis absorption spectrum (Figure 1A, curve a→b). Two peaks at about 440 and 470 nm and a shoulder band in the 350−370 nm region are readily assigned to the absorption of the $TPQ_{sq}$ species demonstrated previously to be formed in various copper amine oxidases upon anaerobic reduction with substrate (*12*).

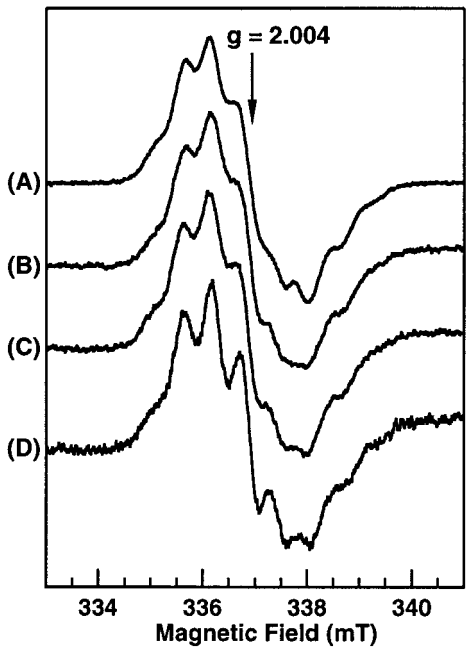


FIGURE 2: EPR spectra of the $TPQ_{sq}$ species of AGAO. $TPQ_{sq}$ was produced by reduction with substrate (5 mM 2-phenylethylamine) in the presence of 7.5 mM $CN^-$ (A), dissolved $^{12}C^{16}O$ (B), and dissolved $^{13}C^{18}O$ (C), and in the absence of $CN^-$ and CO (D); the concentration of AGAO was 390 $\mu$M subunit. Spectra B, C, and D were multiplied by 6.4, 7.2, and 10, respectively, to adjust the intensity of the radical signals and were offset for clarity. Instrumental conditions: microwave power, 3 mW; frequency, 9.449 GHz; modulation, 0.1 mT; measured at ambient temperature.

The intensities of these absorption bands increased by about 5% when the reductive half-reaction was carried out in the presence of dissolved CO (Figure 1A, curve c), although the increase was more extensive in the reaction in the presence of $CN^-$ (Figure 1A, curve d), whose promotive effect on $TPQ_{sq}$ formation had already been reported (*12*). The difference spectrum obtained by subtracting the spectrum of $TPQ_{sq}$ measured in the absence of CO from that measured in the presence of CO revealed new broad absorption bands at 334 and 434 nm (Figure 1B). These bands are probably due to the CO bound to the substrate-reduced AGAO. Because such absorption bands are not observed in the ±CO difference spectrum of the native $TPQ_{ox}$ enzyme (data not shown), CO can bind only to the enzyme in a reduced form. Similar absorption bands have been reported previously for the CO complex of hemocyanin (Hc) with $\lambda_{max}$ at about 310 and 440 nm, the former band having an extinction coefficient much larger than the latter (*38, 39*). The similarity in their wavelengths suggests that CO is bound to the Cu atom in AGAO, as in Hc. However, there is a notable difference in the relative intensities of the two CO-related absorption bands in AGAO and Hc, which may be due to the difference in the structures of the Cu site; AGAO has a mononuclear Cu site (*21*), whereas Hc possesses a binuclear Cu site (*40*).

*Effect of CO on the EPR Spectrum of Substrate-Reduced AGAO.* EPR spectra of the $TPQ_{sq}$ radicals generated in the substrate-reduced AGAO under various conditions were measured at room temperature. As reported previously (*41*), the radical EPR signal at $g = 2.004$ showed a characteristic hyperfine structure with multiple spin splittings (Figure 2), presumably caused by coupling with one TPQ ring-attached $^{14}N$ nucleus ($I = 1$, derived from substrate) and two $^1H$ ($I =$

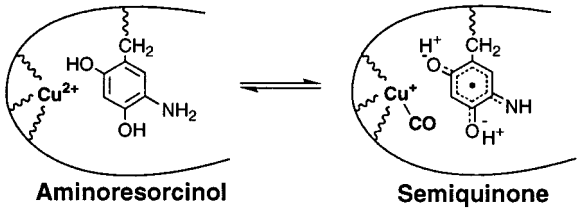


FIGURE 3: Schematic representation of the Cu(II)/aminoresorcinol ⇄ Cu(I)/semiquinone equilibrium and CO binding to Cu(I).

1/2) nuclei (*42*). The EPR hyperfine structure was essentially the same in the spectra measured in the presence or absence of CO ($^{12}C^{16}O$ and $^{13}C^{18}O$) and $CN^-$, although minuscule differences were observed among the four spectra, e.g., for the peaks around 336.7, 337.1, and 337.3 mT. The similarity in the hyperfine structures of $TPQ_{sq}$ radical produced under different conditions again suggests that CO is bound to the Cu atom, most likely being reduced to the Cu(I) ion, but not to the TPQ cofactor. This suggestion is also supported by the virtually identical hyperfine structures of $TPQ_{sq}$ radical in the $^{12}C^{16}O$- and $^{13}C^{18}O$-bound enzymes; perturbation by the $^{13}C$ ($I = 1/2$) isotope effect, predicted to occur if CO was covalently bound to $TPQ_{sq}$, was unobservable in the hyperfine structures. The identity of the EPR hyperfine structures has also been noted previously for the $^{12}CN^-$ and $^{13}CN^-$ complexes of $TPQ_{sq}$ radical generated in various copper amine oxidases (*43*), in which the $CN^-$ ion is assumed to occupy an equatorial coordination site of the Cu atom (*44*). Thus, CO as well as $CN^-$ is bound probably via coordination to Cu(I) but not covalently to $TPQ_{sq}$. Nevertheless, the CO bound to Cu(I) appears to also affect the electronic state of the $TPQ_{sq}$ radical, as judged from the slight signal broadening in the EPR spectra measured in the presence of CO and $CN^-$ (see Figure 2).

Intensities of the EPR signal of $TPQ_{sq}$ radical at $g = 2.004$ increased by about 1.5- and 10-fold when the spectra were measured in the presence of CO and $CN^-$, respectively, with concomitant decreases in the intensities of Cu(II) EPR signals (about 10% decrease in the presence of CO) (data not shown). Combined with the slight increases of UV−vis absorption peaks of $TPQ_{sq}$ radical, these results show that the Cu(II)/$TPQ_{red}$ (aminoresorcinol) ⇄ Cu(I)/$TPQ_{sq}$ equilibrium is shifted toward the latter by CO binding to Cu(I) (Figure 3). If the Cu(I)/$TPQ_{sq}$ species is a kinetically competent intermediate in the catalytic reaction of amine oxidation (*13*), CO may inhibit the reaction by trapping the Cu(I)/$TPQ_{sq}$ species, like the $CN^-$ ion inhibiting the reactions of various copper amine oxidases (*44*). However, no significant inhibition was observed in the reaction performed under a 50% CO atmosphere [$O_2$:CO:$N_2$ (v/v/v) = 2:5:3] (data not shown), suggesting that the oxidative half-reaction by dioxygen is sufficiently rapid, exceeding by far the CO binding, or that the affinity of Cu(I)/$TPQ_{sq}$ species for $O_2$ is several orders of magnitude higher than that for CO.

*FT-IR Characterization of CO Bound to Substrate-Reduced Amine Oxidases.* More direct evidence for CO binding to the substrate-reduced AGAO was obtained by FT-IR measurements. FT-IR spectra measured in the 1930−2150 $cm^{-1}$ region for AGAO reduced with substrate in the presence of $^{12}C^{16}O$ exhibited two incompletely resolved bands at 2063 and 2079 $cm^{-1}$, which shifted to lower frequencies at 1965 and 1983 $cm^{-1}$, respectively, upon using

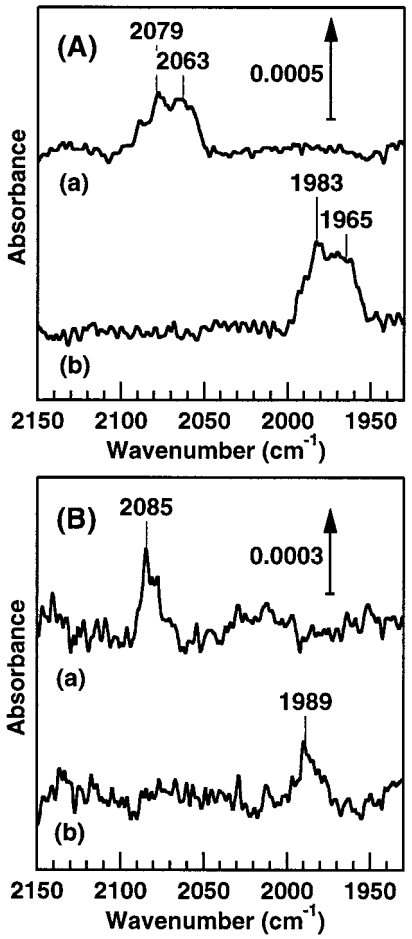


FIGURE 4: FT-IR spectra of the $^{12}C^{16}O$ (a) and $^{13}C^{18}O$ (b) complexes of the substrate-reduced AGAO (A) and AO-I (B). Measuring time: 25−350 min (A, a), 25−360 min (A, b), 30−660 min (B, a), and 30−600 min (B, b) after addition of substrate (10 mM 2-phenylethylamine for AGAO, 5 mM 1,4-diaminobutane for AO−I); the subunit concentrations were 1.3 mM AGAO and 1.2 mM AO-I. Instrumental conditions: resolution, 2 $cm^{-1}$; cell path length, 0.0046 cm; measured at ambient temperature. After measurements, each enzyme was air-oxidized, and its IR spectrum was subtracted from that of the corresponding enzyme with the bound CO.

$^{13}C^{18}O$ (Figure 4A). The observed isotopic wavenumber shifts of nearly 100 $cm^{-1}$ are in good agreement with the calculated values for a carbon−oxygen two-atom model. Therefore, we assign these bands to the C−O stretching ($\nu_{CO}$) bands of the CO bound to AGAO. Emergence of two bands at slightly different wavenumbers suggests the existence of at least two different states (environments) for the bound CO. FT-IR bands with similar frequency were observed at 2085 and 1989 $cm^{-1}$ for the $^{12}C^{16}O$ and $^{13}C^{18}O$ complexes, respectively, of the substrate-reduced AO-I, though apparently in a single band for each isotopic CO (Figure 4B). It is thus conceivable that the ability to form the CO complex is a common property of copper amine oxidases. Previous studies using synthetic Cu complexes (*45*) have shown that CO terminally coordinated to the single Cu atom has a $\nu_{CO}$ frequency ranging 2000 to 2130 $cm^{-1}$, while that bridging two Cu atoms has a $\nu_{CO}$ frequency around 1925 $cm^{-1}$. The $\nu_{CO}$ frequencies at 2063 and 2079 $cm^{-1}$ in AGAO and at 2085 $cm^{-1}$ in AO-I are therefore consistent with the suggestion that CO is bound at a position equatorially coordinating to the mononuclear Cu atom in the two copper amine oxidases.

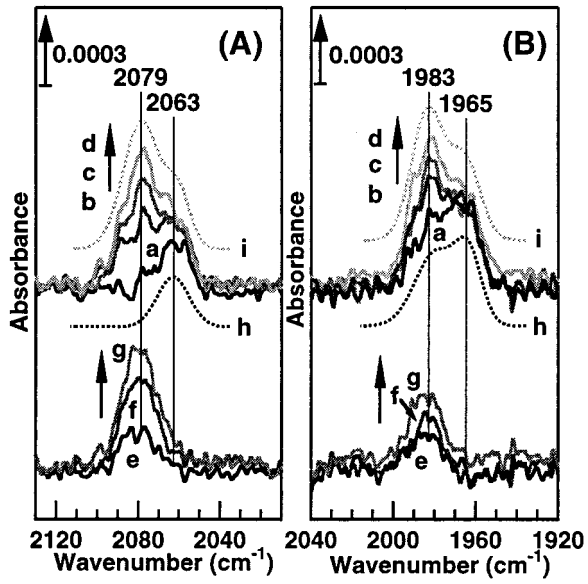


FIGURE 5: FT-IR spectra of the $^{12}C^{16}O$ (A) and $^{13}C^{18}O$ (B) complexes of the substrate-reduced AGAO measured at different time intervals after addition of substrate (upper) and their difference spectra (lower). Measuring time: 25−70 min (A, a), 115−175 min (A, b), 290−350 min (A, c), and 460−520 min (A, d) after addition of substrate; and 25−70 min (B, a), 130−190 min (B, b), 300−360 min (B, c), and 420−480 min (B, d) after addition of substrate; other conditions were the same as those in Figure 4. Difference spectra were obtained by b − a (e), c − a (f), and d − a (g). Dotted lines h and i were obtained by Gaussian fitting of a and d, respectively.

The lower wavenumber band for the CO complex of substrate-reduced AGAO (at 2063 $cm^{-1}$ with $^{12}C^{16}O$, at 1965 $cm^{-1}$ with $^{13}C^{18}O$) was observable even in the first FT-IR spectrum immediately recorded after reduction with substrate (within 30 min), which approximately corresponded to the appearance of UV−vis absorption peaks of the $TPQ_{sq}$ radical (Figure 1A), and its intensity did not change significantly during further FT-IR measurements (Figure 5). Based on the peak areas of $\nu_{CO}$ bands in the dithionite-reduced enzyme described below [Cu(I)/$TPQ_{ox}$ or Cu(I)/$TPQ_{red}$ species], which was presumed to be fully bound with CO, the amount of CO-bound species in the substrate-reduced enzyme, having an approximate extinction coefficient of 40 $M^{-1}$ $cm^{-1}$ at 2063 $cm^{-1}$, was estimated to be about 10% of the total enzyme species. In contrast, the intensities of the higher wavenumber band (at 2079 $cm^{-1}$ with $^{12}C^{16}O$, at 1983 $cm^{-1}$ with $^{13}C^{18}O$) increased gradually on continued measurements for several hours, the increase being more perspicuous in the difference spectra exhibiting peaks at 2079 $cm^{-1}$ with $^{12}C^{16}O$ and at 1983 $cm^{-1}$ with $^{13}C^{18}O$ (Figure 5). As discussed later, the increase of the $\nu_{CO}$ band at 2079 $cm^{-1}$ may reflect changes in the coordination structure of the Cu site in the Cu(I)/$TPQ_{sq}$ species.

*Reduction with Dithionite.* A chemical reducing agent, sodium dithionite, has been known to reduce both the Cu(II) ion and the $TPQ_{ox}$ cofactor in copper amine oxidases to Cu(I) and $TPQ_{red}$ (hydroxyquinol form), respectively, and often used to deplete the Cu atom from the proteins (*11, 46*). In the present studies, we have investigated the time dependence of dithionite reduction of AGAO and found that Cu(II) is reduced much faster than $TPQ_{ox}$. As judged from the immediate appearance of FT-IR $\nu_{CO}$ bands (vide infra), it is evident that Cu(II) is reduced to Cu(I) within about 20 min after addition of dithionite. However, in the similar time range (10−20 min), the TPQ cofactor is still in an oxidized state with an absorption peak at 492 nm (Figure 6A, curve b), which then gradually decreases during the following measurements (Figure 6A, curve c), representing full reduction to $TPQ_{red}$ (hydroxyquinol) that should have a $\lambda_{max}$ below 400 nm (*43, 47*). The rates of reduction of Cu(II) and TPQ depended on the concentrations of the enzyme and dithionite (data not shown). Furthermore, RR spectra obtained by excitation at a wavelength close to the absorption peak (514.5 nm) show unequivocally that the enzyme exhibits Raman bands almost identical with those characteristic of $TPQ_{ox}$ (Figure 6B). Thus, the enzyme in an early stage of dithionite reduction can be regarded as a 'half'-reduced form with Cu(II) reduced to Cu(I) but TPQ still in the oxidized state ($TPQ_{ox}$). Because the Raman spectrum of the fully dithionite-reduced enzyme with $TPQ_{red}$ (hydroxyquinol) excited at this

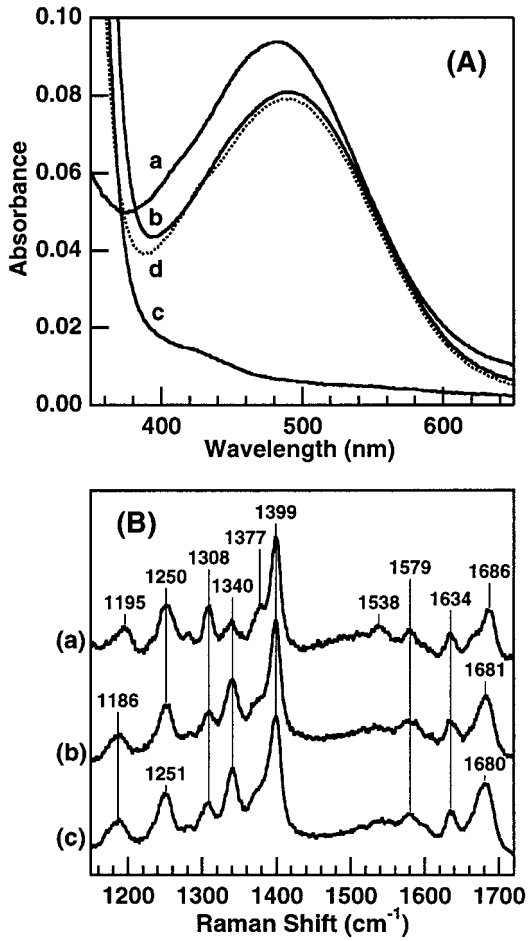


FIGURE 6: Optical absorption and RR spectra of dithionite-reduced AGAO. (A) Absorption spectra were measured at 20 °C for the initial oxidized enzyme (60 $\mu$M subunit) (a), the enzyme anaerobically reduced with 1.2 mM dithionite (b, after 8 min; c, after 60 min), and the enzyme reduced with dithionite in the presence of dissolved CO (d, after 15 min). (B) RR spectra were measured at ambient temperature for the initial oxidized enzyme (650 $\mu$M subunit) (a), the enzyme anaerobically reduced with 5.1 mM dithionite (b, after 30−330 s), and the enzyme reduced with dithionite in the presence of dissolved CO (c, after 30−330 s). The RR spectrum of the fully dithionite-reduced enzyme with $TPQ_{red}$ (hydroxyquinol) was subtracted from the spectrum of each sample. The ordinate scales were normalized with the intensity of the 1399 $cm^{-1}$ band, and the spectra were offset for clarity. Instrumental conditions: excitation wavelength, 514.5 nm: slit height, 10 mm; slit width, 200 $\mu$m; laser power at the sample point, 40 mW.

wavelength shows only nonresonant protein-derived Raman bands (data not shown), the difference spectra obtained by subtracting the Raman spectrum of the fully dithionite-reduced enzyme from those of the native $TPQ_{ox}$/Cu(II) enzyme, the $TPQ_{ox}$/Cu(I) enzyme (half-reduced with dithionite), and the $TPQ_{ox}$/Cu(I)−CO enzyme (half-reduced with dithionite in the presence of CO) were proven to present much clear pictures of the resonance-enhanced modes of the $TPQ_{ox}$ cofactor in the enzyme with different states of the Cu ion. A similar subtraction procedure, in which the Raman spectrum of copper-free precursor protein is subtracted, has been previously developed to obtain RR spectra of the underivatized TPQ cofactor without protein-derived vibrational modes (*24, 48*). The RR spectrum of $TPQ_{ox}$ obtained in the present study by subtracting the spectrum of the fully dithionite-reduced enzyme was identical with that obtained by subtracting the copper-free precursor enzyme (*24, 48*).

Among almost identical vibrational modes observed between 1150 and 1720 $cm^{-1}$, only two bands at 1195 and 1686 $cm^{-1}$ underwent a shift of −9 and −5 $cm^{-1}$, respectively, in the early stage of dithionite reduction of AGAO (Figure 6B). Although the 1195 $cm^{-1}$ band remains to be assigned, the 1686 $cm^{-1}$ band has been assigned to the C═O stretching mode of the C-5 carbonyl of the TPQ ring (*48*). According to the previous studies, the carbonyl at the C-5 position of TPQ has a double bond character greater than the carbonyl at the C-2 position (in the canonical *p*-quinone form) assigned as showing the 1579 $cm^{-1}$ band (*24*). Hence, the C-5 position of TPQ has been thought to be the site of nucleophilic attack by substrates, inhibitors such as phenylhydrazine, and solvent water (*24, 48*). The 5 $cm^{-1}$ shift of the 1686 $cm^{-1}$ band to a lower frequency in the half-reduced, $TPQ_{ox}$/Cu(I) enzyme therefore suggests that the electron density of the C-5 carbonyl is slightly more extended toward the ring $\pi$-electron system than in the $TPQ_{ox}$/Cu(II) enzyme. Conversely, a weak electronic interaction may exist between the TPQ cofactor and the Cu site in the native $TPQ_{ox}$/Cu(II) enzyme. In the crystal structure of holo-AGAO, although the insufficient electron density around TPQ suggests the TPQ ring is rotationally disordered, one possible TPQ orientation is that its C-5 carbonyl oxygen atom and the Cu atom are connected to each other through hydrogen bonds mediated by two water molecules (*21*). Reduction of Cu(II) to Cu(I) with dithionite could lead to the change in the Cu coordination structure, losing the water molecules and/or histidine ligands, and thereby may eliminate the interaction with TPQ. The small red-shift of the absorption peak of $TPQ_{ox}$ from about 480 to 492 nm (Figure 5A) is also compatible with our current interpretation that the electron density of the C-5 carbonyl in the $TPQ_{ox}$/Cu(I) enzyme is more delocalized than in the $TPQ_{ox}$/Cu(II) enzyme. In this regard, it is interesting to note that the Cu(II)-containing histamine oxidase from *A. globiformis* exhibits an absorption maximum at nearly 500 nm due to $TPQ_{ox}$ (*6*) and the C═O stretching mode of the C-5 carbonyl at 1679 $cm^{-1}$ (*48*), comparable to that in the $TPQ_{ox}$/Cu(I) AGAO. These findings suggest variations of the Cu(II)−$TPQ_{ox}$ interaction among various copper amine oxidases.

Although addition of CO in the dithionite reduction of AGAO affected neither the 492 nm absorption peak nor the RR spectrum (Figure 6), binding of CO to Cu(I) was observed by FT-IR measurements within 10−20 min of dithionite reduction (Figure 7). An intense FT-IR band at 2083 $cm^{-1}$ with an extinction coefficient of about 490 $M^{-1}$ $cm^{-1}$ and a minor one at 2064 $cm^{-1}$ with an extinction coefficient of about 220 $M^{-1}$ $cm^{-1}$ immediately appeared after addition of dithionite to AGAO in the presence of CO. The sum of these extinction coefficients is close to the extinction coefficient of the $\nu_{CO}$ band reported for the CO adduct of dopamine $\beta$-hydroxylase (*31*), suggesting that the dithionite-reduced AGAO is fully bound with CO. The intensity of the 2083 $cm^{-1}$ band then decreased gradually, being displaced by a new band at 2077 $cm^{-1}$ (about 220 $M^{-1}$ $cm^{-1}$) and accompanied by a slight increase in the intensity of a 2061 $cm^{-1}$ band (about 240 $M^{-1}$ $cm^{-1}$). The 2061 and 2077 $cm^{-1}$ bands probably correspond to the 2063 and 2079 $cm^{-1}$ bands, respectively, observed for CO bound to the substrate-reduced AGAO (Figure 5). The time scale for the band shift from 2083 to 2077 $cm^{-1}$ was roughly comparable with that for the reduction of $TPQ_{ox}$ to $TPQ_{red}$ (hydroxyquinol) (Figure 6). Because the enzyme contains the cofactor still in the $TPQ_{ox}$ state in the early stage of dithionite reduction as described above, the 2083 and 2077 $cm^{-1}$ bands are assigned to the $\nu_{CO}$ bands of CO bound to the Cu(I)/$TPQ_{ox}$ and Cu(I)/$TPQ_{red}$ (hydroxyquinol) species, respectively. The $\nu_{CO}$ frequency difference, very small though, demonstrates that the $\nu_{CO}$ frequency is affected by the redox state of the TPQ cofactor.

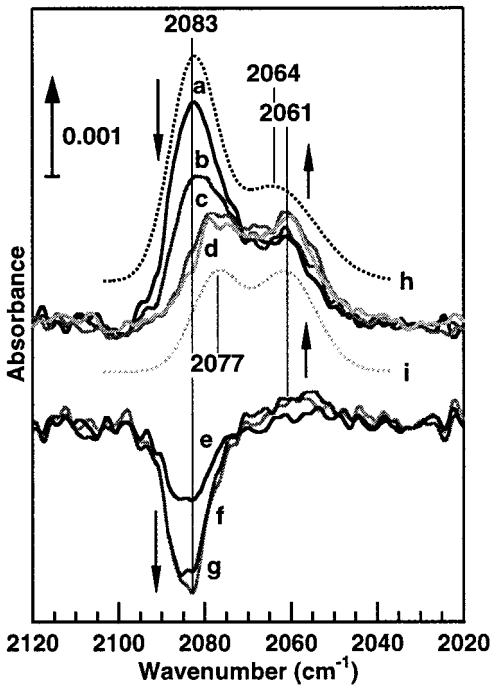


FIGURE 7: FT-IR spectra of the CO complex of the dithionite-reduced AGAO measured at different time intervals after addition of dithionite (upper) and their difference spectra (lower). Measuring time: 10−20 min (a), 25−40 min (b), 85−100 min (c), and 160−175 min (d) after addition of dithionite; 0.94 mM AGAO (subunit) and 10 mM dithionite; other conditions were the same as those in Figure 4. Difference spectra were obtained by b − a (e), c − a (f), and d − a (g). Dotted lines h and i were obtained by Gaussian fitting of a and d, respectively.

*Comparison of $\nu_{CO}$ Frequencies of CO Bound to Various Copper Proteins.* In Table 1, the observed $\nu_{CO}$ frequencies of CO bound to AGAO and AO-I are compared with those reported for the CO complexes of other copper proteins. The comparison suggests that the bond character of CO bound

Table 1: Stretching Frequencies of CO Bound to Various Copper Proteins

| protein | $\nu_{CO}$ ($cm^{-1}$) | ref |
|---|---|---|
| molluscan Hc | 2063 | *26* |
| crustacean Hc | 2043 | *26* |
| cytochrome *c* oxidase | 2062[a] | *27* |
| cytochrome *bo* | 2063[a] | *28* |
| cytochrome $ba_3$ | 2053 | *29* |
| dopamine $\beta$-hydroxylase | 2089 | *30* |
| peptidylglycine α-amidating enzyme | 2093 | *32* |
| AGAO (Cu(I)/$TPQ_{ox}$) | 2064/2083 | this paper |
| AGAO (Cu(I)/$TPQ_{sq}$) | 2063/2079 | this paper |
| AGAO (Cu(I)/$TPQ_{red}$ (hydroxyquinol)) | 2061/2077 | this paper |
| AO-I | 2085 | this paper |

[a] Only major peaks are listed.

to AGAO with lower $\nu_{CO}$ frequencies (at 2061−2064 $cm^{-1}$) is similar to those bound to hemocyanins (Hc), cytochrome *c* oxidase, and other proteins belonging to the heme−copper superfamily. Likewise, the bond character of CO bound to AGAO and AO-I with higher $\nu_{CO}$ frequencies (at 2077−2083 $cm^{-1}$) may be similar to those bound to dopamine $\beta$-hydroxylase and peptidylglycine α-amidating enzyme. In general, if the electron density of a metal ion is elevated by an increase in the electron-donating ability of a ligand, then the electron density of the $\pi$ antibonding orbital ($\pi^*$) of the bound CO is increased through the $\pi$ back-bonding from the filled d orbital of the metal, which further results in a decrease in the C−O bond strength and consequently the $\nu_{CO}$ frequency. Indeed, the $\nu_{CO}$ frequencies of Cu−CO model compounds have been reported to decrease from 2088−2096 to 2069−2073 $cm^{-1}$ by an increase in the extent of coordination of imidazoles to the Cu atom (*49*). In contrast, the relatively high $\nu_{CO}$ frequency of CO bound to dopamine $\beta$-hydroxylase has been suggested to be due to a decrease in either the coordination number of Cu or the basicity of the coordinating ligands (*30, 31*). Based on the considerably high $\nu_{CO}$ frequency of CO bound to peptidylglycine α-amidating enzyme, the active-site Cu atom has been proposed to have a poor donor ligand such as methionine (*32*); a recent X-ray crystallographic study has revealed that one (CuB) of the two catalytic Cu atoms in this enzyme, involved in binding of molecular oxygen, is coordinated with two histidine $\epsilon$-nitrogen atoms, one methionine sulfur atom, and one water molecule (*50*). On the other hand, Hc and cytochrome *c* oxidase utilize three histidine residues in binding the Cu atom (*40, 51, 52*) and therefore exhibit relatively low $\nu_{CO}$ frequencies of CO bound to their Cu centers (*26, 27*) (Table 1).

*Implications for Copper Coordination Change and Its Mechanistic Relevance.* Since the stretching vibration frequencies of CO bound to metalloproteins reflect the coordination structure of the metal center as described above, appearance of the dual modes of the C−O bond character in AGAO suggests the presence of a mixed state of the coordination structure of the Cu(I) ion in the enzymes containing $TPQ_{ox}$, $TPQ_{sq}$, and $TPQ_{red}$ (hydroxyquinol). According to the recent X-ray absorption study on dithionite-reduced copper amine oxidases from various species (*53*), the coordination number of the Cu atom decreases, upon reduction of Cu(II) to Cu(I) with dithionite, from 5 (or possibly 6) to 3 (including at least two imidazoles) for all the enzymes examined. The lower $\nu_{CO}$ frequency at 2061−2064 $cm^{-1}$ of CO bound to AGAO, which emerges nearly constantly in the early stage of anaerobic reduction with substrate or dithionite, therefore appears to represent the CO replacing the equatorial water and being bound to Cu(I) with three histidine coordination as in the native Cu(II)/$TPQ_{ox}$ enzyme (*21*), whereas the higher $\nu_{CO}$ frequencies at 2077−2083 $cm^{-1}$ may represent CO bound to Cu(I) coordinated with only two histidine residues. In particular, the initial emergence of the strong 2083 $cm^{-1}$ $\nu_{CO}$ band in the half-reduced Cu(I)/$TPQ_{ox}$ state implies that the Cu(I) must have lost a histidine ligand, which appears to be re-coordinated on further reduction of TPQ to the fully reduced Cu(I)/$TPQ_{red}$ (hydroxyquinol) state, as suggested from the gradual decrease of the 2083 $cm^{-1}$ band (Figure 7). Similarly, the slowly emerging 2079 $cm^{-1}$ band in the substrate-reduced Cu(I)/$TPQ_{sq}$ state suggests the decrease in the coordination number of Cu(I).

In addition to the coordination structure of a metal ion, $\nu_{CO}$ frequencies of CO bound to metalloproteins may be affected by the protein environment surrounding the bound CO. For example, electrostatic fields of the active sites of heme proteins have been shown to influence significantly the $\nu_{CO}$ frequency of the bound CO (*54, 55*). The existence of positive charges from proton-donating residues close to the bound CO causes the electron density of the $\pi^*$ orbital of the bound CO to increase and thereby the C−O bond strength and the $\nu_{CO}$ frequency to decrease. However, in the immediate vicinity of the Cu site of AGAO, there is neither positively charged nor negatively charged residues besides TPQ (*21*). Furthermore, two CO-binding modes with $\nu_{CO}$ frequencies at 2061−2064 and 2077−2083 $cm^{-1}$ are observed for CO bound to all AGAO proteins in the $TPQ_{ox}$, $TPQ_{sq}$, and $TPQ_{red}$ (hydroxyquinol) states. Therefore, the variation of $\nu_{CO}$ frequencies of CO bound to AGAO is most likely caused by the change in the coordination structure of the Cu(I) ion.

Collectively, the results obtained in the present studies show that the coordination structure of the Cu atom in AGAO is sensitively modulated by the chemical and redox states of TPQ, which include the hydrogen bond interaction with water molecules (*21*), and are probably associated with the orientation of TPQ ring (*23*−*25*). The 12 nm red-shift of the absorption peak and the −5 $cm^{-1}$ frequency shift of the RR band of $TPQ_{ox}$ observed during the early stage of dithionite reduction are also likely attributed to the loss of water molecules hydrogen-bonding between TPQ and Cu(II) and/or directly coordinating to Cu. Although it remains unknown whether the coordination structure of the Cu atom changes during the catalytic cycle of copper amine oxidases, significant differences in the geometry of the Cu site have been indeed noted in the crystal structures of the Cu/TPQ-less precursor and Cu/TPQ-containing mature forms of AGAO, and the change in the coordination structure of the Cu atom has been proposed to be an important factor in the biogenesis of the TPQ cofactor (*21*).

In summary, we have generated the CO complexes of copper amine oxidases for the first time and characterized them by various spectroscopic measurements. It has been demonstrated that the vibrational spectrum of the bound CO can be a sensitive gauge of the coordination structure of the Cu atom and the chemical and redox states of the TPQ cofactor, both contained in this class of enzymes.

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