Simultaneous Resonance Raman Detection of the Heme $a_3$-Fe-CO and Cu$_B$-CO Species in CO-bound $ba_3$-Cytochrome $c$ Oxidase from Thermus thermophilus

EVIDENCE FOR A CHARGE TRANSFER Cu$_B$-CO TRANSITION*

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Understanding of the chemical nature of the dioxygen and nitric oxide moiety of $ba_3$-cytochrome $c$ oxidase from Thermus thermophilus is crucial for elucidation of its physiological function. In the present work, direct resonance Raman (RR) observation of the Fe-$C$-O stretching and bending modes and the C-$O$ stretching mode of the Cu$_B$-CO complex unambiguously establishes the vibrational characteristics of the heme-copper moiety in $ba_3$-oxidase. We assigned the bands at 507 and 568 cm$^{-1}$ to the Fe-$C$-O stretching and Fe-$C$-$O$ bending modes, respectively. The frequencies of these modes in conjunction with the C-$O$ mode at 1973 cm$^{-1}$ showed, despite the extreme values of the Fe-$C$-O and C-$O$ stretching vibrations, the presence of the $a$-conformation in the catalytic center of the enzyme. These data, distinctly different from those observed for the $caaa_3$-oxidase, are discussed in terms of the proposed coupling of the $a$- and $\beta$-conformations that occur in the binuclear center of heme-copper oxidases with enzymatic activity. The Cu$_B$-CO complex was identified by its $\nu$(CO) at 2053 cm$^{-1}$ and was strongly enhanced with 413.1 nm excitation indicating the presence of a metal-to-ligand charge transfer transition state near 410 nm. These findings provide, for the first time, RR vibrational information on the EPR silent Cu$_B$(I) that is located at the O$_2$ delivery channel and has been proposed to play a crucial role in both the catalytic and proton pumping mechanisms of heme-copper oxidases.

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Cytochromes $ba_3$ and $caaa_3$ serve as the terminal heme-copper oxidases in the Gram-negative thermophilic eubacterium Thermus thermophilus (1). Both enzymes couple the reduction of dioxygen to proton translocation across the inner bacterial membrane, and in contrast to the eucaryotic heme-copper oxidases, catalyze the reduction of NO to N$_2$O (1, 2). Cytochrome $ba_3$ contains a homodinuclear copper complex (Cu$_A$), one low spin heme $b$, and a binuclear center that consists of Cu$_B$ and heme $a_3$ (1). The comparative kinetics data on CO photodissociation and rebinding of various heme-copper oxidases and the derived activation parameters have indicated that the CO-ligation/release mechanism in cytochrome $ba_3$ follows that found in other heme-copper oxidases (3–9) and proceeds according to the following scheme.

$$
\begin{align*}
&\text{Fe}^{2+}, \text{Cu}^{2+} + \text{CO} \rightarrow \text{Fe}^{3+}, \text{Cu}^{2+}-\text{CO} \rightarrow \text{Fe}^{2+}-\text{CO}, \text{Cu}^{3+} \\
&k_1 \quad \quad \quad k_2 \\
&k_{-1} \quad \quad \quad k_{-2} \\
&\text{A} \quad \quad \quad \text{B}
\end{align*}
$$

**Scheme 1**

In contrast to the bovine $aa_3$ oxidase, Cu$_B$ of cytochrome $ba_3$ has a relative high affinity for CO ($k_1 > 10^8$), whereas the transfer of CO to heme $a_3^{2+}$ is characterized by a small $k_2 = 8$ s$^{-1}$ and by a $k_{-2} = 0.8$ s$^{-1}$ that is 30-fold greater than that of the bovine $aa_3$ (3, 4).

The FTIR$^3$ and time-resolved FTIR studies of the CO-bound fully reduced form of $ba_3$ have revealed several unique characteristics of the enzyme including the formation of the equilibrium Cu$_B$-CO complex and the identification of a ligand docking site (4, 6, 7). It was reported that the rate of decay of the transient Cu$_B$-CO complex that is produced from the photolysis of complex B (Scheme 1) is 34.5 s$^{-1}$ and rebinding to heme $a_3$ occurs with $k_{-1} = 28.6$ s$^{-1}$ (4). The $\nu$(CO) of the transient Cu$_B$-CO species at 2053 cm$^{-1}$ is the same as that of the equilibrium Cu$_B$-CO species and remained unchanged in the pH and pD 5.5–10 ranges, indicating that no structural change takes place at Cu$_B$ between these states (4). Recently, the role of the ring A propionate of heme $a_3$-Asp$_{77}$-H$_2$O site as a proton carrier to the exit/output proton channel (H$_2$O pool) that is conserved among all structurally known heme-copper oxidases and is part of the Q-proton pathway in $ba_3$-cytochrome $c$ oxidase was reported (8). Further FTIR studies provided solid evidence that in cytochrome $ba_3$, the ligand delivery channel is located at the Cu$_B$ site, which is the ligand entry to the heme $a_3$ pocket (5). It was suggested that the properties of the O$_2$ channel are not limited to facilitating ligand diffusion to the active site but are extended in controlling the dynamics and reactivity of the reactions of $ba_3$ with O$_2$ and NO. Therefore, it is of particular interest to gain insight into the environment of the spectroscopic silent Cu$_B$.

Resonance Raman (RR) is a structure-sensitive technique, and thus, data of the CO-bound adducts of $ba_3$ can be interpreted to yield specific information concerning heme/Cu$_B$ geometric properties and heme/Cu$_B$-axial ligand bonding interactions (10–17). Additional information concerning the two different conformations of the heme-copper oxidases, termed $\alpha$ and $\beta$, have also been provided by FTIR and RR studies (10–

$^3$ The abbreviations used are: FTIR, Fourier transform infrared; RR, resonance Raman; MLCT, metal-to-ligand charge transfer; W, watt.
Although the presence of one or the other conformation has been implicated in the enzymatic activity of heme-copper oxidases, their functional significance remains to be determined (14). In the present work, we have studied the CO-bound ba$_3$ oxidase by RR with the aim to fully characterize complexes A and B and thus to explore the proposed role of CuB in the formation of either the $\alpha$- or the $\beta$-conformation and to determine the significance of the $\alpha$- and $\beta$-conformations in the enzymatic activity of heme-copper oxidases. Our data demonstrate, despite the extreme values of the Fe-CO and C–O stretching vibrations at 507 and 1973 cm$^{-1}$, respectively, the presence of the $\alpha$-conformation in the catalytic site of the enzyme. The analysis of the data also reveals that the environment of CuB does not control the strength of the Fe–C bond and thus the type of conformation of the binuclear center. The results presented here and those of caa$_3$, in contrast to previous reports, demonstrate that enzymatic activity is not controlled by the type of conformation ($\alpha$ or $\beta$) of the binuclear center. We have also made the novel RR characterization of the Cu-B-CO complex by its CO stretching frequency at 2053 cm$^{-1}$. In $\alpha$-Fe-heme–CuB the C–O mode of CuB-CO at 2053 cm$^{-1}$ provides for the first time an indication as to how to exploit the environment of the spectroscopic silent CuB center that has been implicated not only in the catalytic properties of heme-copper oxidases but also in the proton pumping mechanisms.

EXPERIMENTAL PROCEDURES

Cytochrome ba$_3$ from T. thermophilus was isolated by the procedure published previously (1, 4–6). Resonance Raman spectra were obtained from 40–50 $\mu$m samples, pH 7.5, in a cylindrical quartz spinning cell. The RR data were acquired as described elsewhere (10, 11, 13). FTIR spectra were recorded from 400 $\mu$m samples at 4 cm$^{-1}$ resolution with a Bruker Equinox 55 FTIR spectrometer equipped with a liquid nitrogen cooled mercury cadmium telluride detector. The fully reduced CO samples were anaerobically loaded into a cell with CaF$_2$ windows and a 0.025-mm spacer. An average of 2000 scans was used for each spectrum.

RESULTS

Fig. 1 shows the low frequency RR spectra of CO-bound ba$_3$ at neutral pH obtained with 428.7 nm excitation, in which resonances from heme b$_{2+}$ and heme a$_5$-CO are enhanced (4). We detect two modes that have carbon and oxygen isotopic sensitivity. The shifts are more evident in the difference spectrum (trace c) between the $^{12}$C$_{16}$O- and $^{13}$C$_{18}$O-bound states. The $\nu$(Fe-CO) at 507 cm$^{-1}$ is 12–15 cm$^{-1}$ lower than those of the a$_5$-type oxidases from P. denitificans (10), a$_5$-CO–600 from B. subtilis (11) and bovine heart (12), and Rhodobacter capsulatus (14, 15). In addition, the shift of the weak mode at 568 cm$^{-1}$ (trace a) to 550 cm$^{-1}$ (trace b) appears in the isotope difference spectrum (trace c). This mode is attributed to the Fe–C–O bending mode and is 5–10 cm$^{-1}$ lower than that of the a$_5$-type oxidases (10–15).

In Fig. 2A we present the high frequency region of the RR spectra. The difference spectrum (trace a) between $^{12}$C$_{16}$O- and $^{13}$C$_{18}$O-bound states demonstrates the presence of modes at 2053 cm$^{-1}$ (15) and 1973 cm$^{-1}$ (12). We assign the 1973 cm$^{-1}$ mode to the CO stretching vibration of Fe-CO (complex B). In trace b we display the difference spectrum obtained at higher laser power (500 $\mu$W). Under these conditions partial photodissociation of Fe-CO (complex B) is observed. This effect causes the migration of CO to CuB, and in conjunction with 1) the unusual long lifetime (4) of the CuB-CO species ($t_{1/2} = 20.1$ ms) and 2) the presence of the equilibrium complex A, we observe the C–O mode of CuB-CO at 2053 cm$^{-1}$. For $^{13}$C$_{18}$O this mode shifts to 1959 cm$^{-1}$, confirming the assignment. The observed CO stretching vibrations are very similar to those obtained by FTIR (trace c). The 413.1 nm excitation RR data shown in Fig. 2B were obtained under the same conditions as those in Fig. 2A, trace b. Comparison of trace b, Fig. 2A with trace c, Fig. 2B shows that the band pair 2053/1959 cm$^{-1}$ is more enhanced with 413.1 nm excitation. The opposite occurs for the 1973/1884 cm$^{-1}$ band pair. It is readily observed with 428.7 nm excitation, but it has lost most of its intensity in the 413.1 nm excitation spectra. This is consistent with the optical absorption spectrum of the CO-bound heme a$_5$ at $\lambda_{max} = 430$ nm (4). We attribute the increased intensity of the 2053/1953 cm$^{-1}$ band pair to the presence of a CuB–MLCT transition near 410 nm. We have been unable, however, to detect it by absorption spectroscopy because it is obscured by the strong Soret transition. Our assignment finds support from the absorption spectrum of the CO-bound form of copper/tona quinone-containing amine oxidase from Arthrobacter globiformis where two transitions with maximum absorbance at 334 and 434 nm were observed (20).

DISCUSSION

The His$_{384}$-Heme a$_5$Fe...Cu$_B$ Moiety...—The properties of the His-heme a$_5$-CO...CuB unit of heme-copper oxidase have been determined from the frequencies and intensities of the $\nu$(Fe$_{2+}$-CO), $\nu$(C–O), and $\delta$(Fe–C–O) modes, as well as from the correlation between the $\nu$(Fe$_{2+}$-CO) and $\nu$(C–O) frequencies (10–19). The molecular origin for the heme Fe-CuB binuclear center of heme-copper oxidases that is responsible for the unique Fe-CuB-CO modes is of significant interest. The frequencies of the Fe-CO and C–O modes of CO-bound ba$_3$ oxidase are...
The Heme $\alpha_2$-Cu$^+$ Moiety of ba$_3$ Cytochrome $c$ Oxidase

The variation of $\nu$(CO) in mononuclear Cu-containing proteins has been attributed to changes in the coordination structure of the metal (20). The insensitivities of the $\nu$(CO) of Cu$_b$ to the frequency to $H_2/O/D_2$O exchange and to pH/pD 5.5–9.7 range indicated that the degree of back-donation of electron density from the $d$ orbitals to the antibonding $\pi^*$ orbitals is not altered under these conditions (5, 8). In the presence of small amounts of O$_2$, however, the $\nu$(C–O) of Cu$_b$ at 2053 cm$^{-1}$ (complex A) shifts to 2045 cm$^{-1}$ and remains unchanged in H$_2/O/D_2$O exchanges and in the pH 6.5–9.0 range (5). This observation was attributed to an increased electron density in the CO antibonding orbitals that results in weakening the C–O bond strength (lower $\nu_{OC}$). It was concluded that the change in the $\nu$(CO) of complex A results in an increase of $k_{-2}$ and thus to a higher affinity of Cu$_b$ for exogenous ligands. The nature of ligands coordinated to the metal in copper proteins has been of particular interest but difficult to determine, since conformational and/or chemical changes in the proteins may lead to ligand exchanges by the EPR silent

The Cu$^+$-CO Complex—The variation of $\nu$(CO) in mononuclear Cu-containing proteins has been attributed to changes in the coordination structure of the metal (20). The insensitivities of the $\nu$(CO) of Cu$_b$ to the frequency to $H_2/O/D_2$O exchange and to pH/pD 5.5–9.7 range indicated that the degree of back-donation of electron density from the $d$ orbitals to the antibonding $\pi^*$ orbitals is not altered under these conditions (5, 8). In the presence of small amounts of O$_2$, however, the $\nu$(C–O) of Cu$_b$ at 2053 cm$^{-1}$ (complex A) shifts to 2045 cm$^{-1}$ and remains unchanged in H$_2/O/D_2$O exchanges and in the pH 6.5–9.0 range (5). This observation was attributed to an increased electron density in the CO antibonding orbitals that results in weakening the C–O bond strength (lower $\nu_{OC}$). It was concluded that the change in the $\nu$(CO) of complex A results in an increase of $k_{-2}$ and thus to a higher affinity of Cu$_b$ for exogenous ligands. The nature of ligands coordinated to the metal in copper proteins has been of particular interest but difficult to determine, since conformational and/or chemical changes in the proteins may lead to ligand exchanges by the EPR silent
copper (I). Although we have identified additional vibrations in the RR spectra (data not shown) that can be attributed to the internal vibrations of CuHr-(N-His), the insufficient amounts, so far, of 15N-enriched enzyme have prevented us from collecting high quality RR data of the 15N-enriched enzyme. Therefore, it remains to be established whether internal ligand motions are resonance-enhanced, either because the ligand coordinates themselves are displaced upon MLCT excitation or the ligand coordinates contribute to ground-state normal modes that involve CuHr-L(N) stretching coordinates.

In summary, the unique CO binding properties of ba3 oxidase have allowed us, first, to investigate from the frequencies and intensities of Fe–C–O the role of CuB in the structure of this coordinated ligand. We suggest that CuB maintains the same role in the structure of the physiological coordinated ligands, such as NO and O2. This way, the role of CuB in the structure-function relationship has been established. Second, we have identified the CuB-CO complex by its $\nu$(CO) at 2053 cm$^{-1}$. The $\nu$(CO) is strongly enhanced with 413.1 nm excitation indicating the presence of a metal-to-ligand charge transfer transition state near 410 nm. The RR identification of the CuB-CO complex provides us direct access, for the first time, to spectroscopic silent CuB site under steady state conditions. This way, the environment of CuB that has been implicated in both the O2 delivery channel and proton translocation mechanisms can now be explored (5, 8).

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