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Decay of the Transient Cu_B−CO Complex Is Accompanied by Formation of the Heme Fe−CO Complex of Cytochrome *cbb*$_3$−CO at Ambient Temperature: Evidence from Time-Resolved Fourier Transform Infrared Spectroscopy

Stavros Stavrakis, Konstantinos Koutsoupakis, Eftychia Pinakoulaki, Andrea Urbani,[†,‡] Matti Saraste,[†] and Constantinos Varotsis*

Department of Chemistry, University of Crete, 71409 Heraklion, Crete, Greece

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The microaerobic *cbb*$_3$-type cytochrome *c* oxidase isolated from *Pseudomonas stutzeri* is an isoenzyme in the family of cytochrome *c* oxidases (CcO).[1a,b] It contains three *c*-type low-spin hemes, one low-spin *b*-type heme, and a heme b_3−Cu_B binuclear center.[2a] The structural characteristics of the binuclear center in *cbb*$_3$ is unique when compared to those of *aa*$_3$-type oxidases; it lacks the hydroxyethylfarnesyl side chain and the highly conserved among the heme-copper oxidases tyrosine 244, both of which have been proposed to play a crucial role in the properties of the binuclear center.[2a] The complex catalyzes the reduction of molecular oxygen by oxidation of cytochrome *c* and contributes to the proton gradient across the cytoplasmic membrane. The resonance Raman studies of the fully reduced[2b] and CO-bound[3] form of *cbb*$_3$ oxidase have revealed several structural characteristics of the heme pocket, including an indication, from the frequencies of the Fe−CO and Fe−C−O that the binuclear center has a unique active site with a pocket more open than that in other heme-copper oxidases. Therefore, data on the photodynamics of the CO-bound adduct of cytochrome *cbb*$_3$ in conjunction with those of the *aa*$_3$-type oxidase can be interpreted to yield specific information concerning electronic and heme/Cu_B geometric properties, and heme/Cu_B-axial ligand bonding interactions. The time-resolved FTIR data reveal that in *cbb*$_3$-type oxidase the decay of the transient Cu_B−CO complex is concurrent with the formation of the heme b_3−CO complex, and the ν(CO) of Cu_B at 2065 cm^{-1}, despite the lack of the cross-link tyrosine 244, is similar to that observed in cytochrome *aa*$_3$[4,5] and cytochrome *bo*$_3$.[6] The former observation contrasts sharply with the well-known behavior of CO recombination to the heme Fe of cytochrome *aa*$_3$- and *bo*$_3$-type oxidases.[7−10]

We detect a single band in the FTIR spectrum of the CO-bound *cbb*$_3$ which has carbon isotopic sensitivity (Figure 1A). We assign the band at 1956 cm^{-1} to the C−O stretching mode of heme b_3−CO. In the ^{13}CO-bound derivative this peak shifts to 1913 cm^{-1} (Figure 1B). The frequency of this CO mode is independent of H_2O/D_2O exchanges (Figure 1C) and very close to those that have been reported for the CO adducts of the *aa*$_3$-type oxidases[4,5,7,8,10−14] and *bo*$_3$-type,[6] but 6 cm^{-1} higher than that previously reported for the CO adduct of *cbb*$_3$ from *Rh. capsulatus* at 15 K.[2a] The FTIR results along with the resonance Raman (RR) data[3] establish that the β-form is the stable conformation. The broad bandwidth of the CO mode in *cbb*$_3$ (fwhm = 13 cm^{-1}) is an indication of the wide distribution of allowed CO conformations. Similar observations were detected in the β-form of the *aa*$_3$-type oxidases.[13]

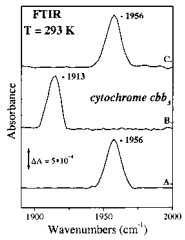

Figure 1. FTIR spectra of the CO bound form of fully reduced cytochrome *cbb*$_3$ at 293 K. (A) pD 7.5, (B) *cbb*$_3$−^{13}CO, pD 7.5, (C) pH 7.5. Enzyme concentration was 1 mM, the path length, 30 μm, and spectral resolution, 4 cm^{-1}.

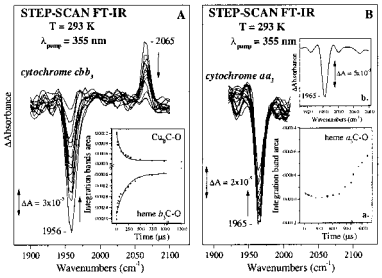

Figure 2. (A) Time-resolved step-scan FTIR difference spectra of the CO-bound form of fully reduced cytochrome *cbb*$_3$ (pD 7.5) at 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 750, 1000, and 1300 μs after CO photolysis, obtained with spectral resolution of 8 cm^{-1} and 5 μs time resolution. Total number of slices was 800, with 40 slices before and 760 after the laser firing. A total of 10 co-additions per retardation data point was collected. The 355-nm pulse from a Nd:YAG laser (7-ns width, 7 Hz) was used as a pump light (3 mJ/pulse) to photolyze the *cbb*$_3$−CO oxidase. Inset: Kinetic analysis of the 2065 cm^{-1} ($\nu_{CO(CuB)}$, squares) and 1956 cm^{-1} ($\nu_{CO(Fe)}$, circles) modes vs time after CO photolysis. The curves are three parameter fits to the experimental data according to first-order kinetics. (B) Time-resolved step-scan FTIR difference spectra of the photolyzed CcO−CO complex at 30, 40, 50, 75, 100, 150, 250, 375, 500, 625, 750, 875, 1000, 1750, 2500, 3000, and 3765 μs. Insets: (a) Plot of the 1965 cm^{-1} ($\nu_{CO\ (Fe)}$, squares) mode vs time after CO photolysis. (b) Step-scan difference spectra of the photolyzed CcO−CO complex in H_2O (solid line) and D_2O (dot line) at 5 μs.

Figure 2A shows the time-resolved FTIR spectra (t_d = 5 μs to 1.3 ms, 8 cm^{-1} spectral resolution) of fully reduced *cbb*$_3$−CO after CO photolysis by a nanosecond laser pulse (355 nm). Upon photolysis, CO is transferred from heme b_3 to Cu_B. The negative peak at 1956 cm^{-1} arises from the photolyzed heme b_3−CO complex, and the positive peak that appears at 2065 cm^{-1} (t_d = 5 μs) is attributed to the C−O stretch (ν_{CO}) of Cu_B as found under white light illumination at low-temperatures,[2a] and its intensity persists up to 1 ms. The frequencies of the C−O modes of Cu_B at 2064 and 2042 cm^{-1} in the *aa*$_3$-type oxidases are associated with

* To whom correspondence should be addressed: E-mail: varotsis@edu.uoc.gr. Fax: 30-810-393601.
† European Molecular Biology Laboratory, Meyerhofstrasse 1, Postfach 102209, D-69012 Heidelberg, Germany.
‡ Clinical Biochemistry Laboratory, Ospedale del Bambino Gesu' IRCCS, Piazza S. Onofrio 4, 00165 Rome, Italy.

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the α- and β-forms, respectively.[11,13] Thus, the frequencies of the modes involving CO in cbb_3 allows the protein to fall on the ν(Fe−CO) versus ν(C−O) correlation curve for the β-form but with a corresponding ν(CO) of Cu_B of the α-form. Moreover, these data suggest that despite the absence of the cross-link in cbb_3, the frequencies of the ν(CO) we detect are similar to the corresponding frequencies found in CO-bound aa_3-[4,5,7,11] and bo_3-type[6,9] oxidases. The decay of the 2065 cm^{-1} peak ($t_{1/2}$ = 120 ± 16 μs) and the development of the 1956 cm^{-1} peak ($t_{1/2}$= 144 ± 28 μs) suggest that the formation of the Fe−CO complex is concurrent with the decay of the Cu_B−CO complex. Moreover, the intensity ratio of Fe−CO/Cu_B−CO (2.15) remains constant for all data points, and thus we conclude that no fraction of CO escapes the binuclear center at 293 K.

Infrared and UV−vis experiments of CO-bound aa_3 have shown that CO dissociates from Cu_B on a microsecond time scale (k_{-1} = 5×10^5 s^{-1}), and a thermal equilibrium between Cu_B-bound and "free" CO in solution (K = 87 M^{-1}) is established.[7,8,10] The CO, however, does not return to heme a_3 for milliseconds (k_2 = 1030 s^{-1}).[7,8,10] Figure 2B shows the time-resolved FTIR spectra (t_d = 5 μs to 4 ms, 8 cm^{-1} spectral resolution) of mammalian fully reduced aa_3−CO after CO photolysis in D_2O. We detect no intensity changes of the 1965 cm^{-1} peak from t_d = 5−750 μs which implies the lack of CO rebinding to heme a_3 (Figure 2B, inset a). At times longer than 750 μs the intensity increase of the 1965 cm^{-1} peak signals the onset of ligand rebinding to heme a_3. This behavior is analogous to that previously observed by Dyer et al.[7,8] and Ondrias et al.[16] The former authors proposed that on the time scale CO leaves Cu_B ($t_{1/2}$ = 1.5 μs) there are no intrinsic heme barriers to recombination with heme a_3 because typical geminate recombination rates are nanoseconds. Thus, another barrier must form which prevents CO recombination prior to 1 ms, and either a local protein conformational change or the binding of an endogenous ligand which blocks the distal ligation site were suggested as possible candidates. Time-resolved ps RR experiments[17] have demonstrated that heme a_3^{2+} is five-coordinate, high-spin, with proximal histidine ligation to the heme at the earliest times following CO photodissociation, and thus, no endogenous ligand blocks the distal binding site of heme a_3. The 5 μs spectra in H_2O and D_2O, shown in inset b, demonstrate the absence of peaks associated with C−O modes of Cu_B (~2065 cm^{-1}). Thus, the $t_{1/2}$ of Cu_B−CO in CcO, in contrast to that reported from the photostationary experiments in D_2O,[5] is less than 5 μs in both H_2O and D_2O.[7,10]

The mechanism proposed for the dynamics of CcO proceeds according to the following scheme:

$$\text{His}-\text{Fe}^{2+}-\text{CO},\ \text{Cu}_\text{B}{}^{+} \xrightarrow{h\nu} \{\text{His}-\text{Fe}^{2+}\}^*,\ \text{Cu}_\text{B}{}^{+}-\text{CO} \rightarrow \text{CO (s)},\ \text{Cu}_\text{B}{}^{+},\ \text{His}-\text{Fe}^{2+} \rightarrow \text{Fe}^{2+}-\text{CO},\ \text{Cu}_\text{B}{}^{+}$$

It involves a state denoted by an asterisk that represents a nonequilibrium state of the heme a_3 state characterized by an upshifted Fe−his stretching vibration.[16] The transient heme a_3 state species relaxes to the equilibrium-reduced species at times >10 μs.[16] The Cu_B−CO complex is fully developed within 1 ps after photolysis from heme a_3 demonstrating the absence of activation barriers to the CO transfer from heme a_3 to Cu_B.[8] The decay of the Cu_B−CO complex (~1.5 μs) is followed by the equilibration of Cu_B−CO with CO in solution which occurs with a rate constant of ~7×10^5 s^{-1}, and is accompanied by heme a_3 relaxation but not by CO rebinding to heme a_3.[7−8,10] Rapid recombination to heme a_3 can be enhanced by inhibiting ligand movement away from the copper atom by placing diffusional barriers and by removing steric restrictions directly adjacent to the heme a_3 Fe atom. It is also expected that transient motions of the protein are required to allow ligand access to the heme a_3 Fe and net dissociation from Cu_B to the surroundings. On the basis of the above analysis we postulate that the local protein environment around Cu_B atom directs the thermal dissociated ligand from 5 to 750 μs away from the heme a_3−Cu_B pocket, thus preventing it from recombining to the heme a_3 site concurrently with its release from Cu_B (see below). Thus, the heme a_3−Cu_B pocket is constructed in such a manner that facilitates rapid migration of the photodissociated CO from heme a_3 to Cu_B and slow recombination of CO to heme a_3.

The cbb_3 is the first heme-copper oxidase reported in which the decay of the Cu_B−CO complex is accompanied by the formation of the Fe−CO complex at room temperature. It is also the only oxidase with an open heme−copper structure lacking the α-form of the heme, thus the distal interactions due to Cu_B and its local environment, and also lacking the hydrogen bonding between the farnesyl hydroxyl and the tyrosine hydroxyl which couples together the heme a_3 and Cu_B. The rates for the Cu_B−CO dissociation and heme b_3−CO recombination we have determined in this study suggest that the rate-limiting step for the heme b_3 recombination of CO, in contrast to that for aa_3 and bo_3, is the $t_{1/2}$ of the Cu_B−CO complex. Although the heme b_3−Cu_B pocket is more open than the heme a_3−Cu_B, the recombination process is faster and is triggered by the decay of the Cu_B−CO complex and without a lag phase. If the structural differences in the binuclear center of cbb_3 are a reflection of the kinetic data we have observed, then data on the photodynamics of cbb_3−CO at earlier times (t_d < 5 μs) could be important for elucidating the mechanism for ligand entry/exit in heme-copper oxidases. Experiments on this point are in progress.

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