Observation of the Equilibrium Cu_B-CO Complex and Functional Implications of the Transient Heme $a_3$ Propionates in Cytochrome $ba_3$-CO from *Thermus thermophilus*

FOURIER TRANSFORM INFRARED (FTIR) AND TIME-RESOLVED STEP-SCAN FTIR STUDIES*

Received for publication, May 20, 2002, and in revised form, July 2, 2002 Published, JBC Papers in Press, July 3, 2002, DOI 10.1074/jbc.M204943200

Konstantinos Koutsoupakis‡, Stavros Stavrakis‡, Eftychia Pinakoulaki¶, Tewfik Soulimane§, and Constantinos Varotsis¶†

*From the ‡Department of Chemistry, University of Crete, 71409 Heraklion, Crete, Greece and §Paul Scherrer Institut, Life Sciences, OSRA/008, CH-5232 Villigen PSI, Switzerland

We report the first evidence for the existence of the equilibrium Cu_B$^{1+}$-CO species of CO-bound reduced cytochrome $ba_3$ from *Thermus thermophilus* at room temperature. The frequency of the C-O stretching mode of Cu_B$^{1+}$-CO is located at 2053 cm$^{-1}$ and remains unchanged in H$_2$O/D$_2$O exchanges and, between pH 5.5 and 9.7, indicating that the chemical environment does not alter the protonation state of the Cu_B histidine ligands. The data and conclusions reported here are in contrast to the changes in protonation state of Cu_P-His-290, reported recently (Das, T. K., Tomson, F. K., Gennis, R. B., Gordon, M., and Rousseau, D. L. (2001) *Biophys. J.* 80, 2039–2045 and Das, T. P., Gomes, C. M., Teixeira, M., and Rousseau, D. L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 9591–9596). The time-resolved step-scan FTIR difference spectra indicate that the rate of decay of the transient Cu_B$^{1+}$-CO complex is 34.5 s$^{-1}$ and rebinding to heme $a_3$ occurs with $k_3$ = 28.6 s$^{-1}$. The rate of decay of the transient Cu_B$^{1+}$-CO complex displays a similar time constant as the absorption changes at 1694(+)/1706(−), attributed to perturbation of the heme $a_3$ propionates (COOH). The $\nu$(C-O) of the transient Cu_B$^{1+}$-CO species is the same as that of the equilibrium Cu_B$^{1+}$-CO species and remains unchanged in the pH range 5.5–9.7 indicating that no structural change takes place at Cu_B between these states. The implications of these results with respect to proton pathways in heme-copper oxidases are discussed.

Cytochrome $ba_3$ from the thermophilic eubacterium *Thermus thermophilus* HB8 (ATCC27634; American Type Culture Collection, Manassas, VA) is expressed under limited O$_2$ supply and is composed of three subunits (1–3). The largest subunit (I) contains the ligands to a low spin heme $b$ and a binuclear center that consists of Cu$_B$ and heme $a_3$, and subunit II contains a mixed valence [Cu$_A$$^{1.5+}$–Cu$_B$$^{1.5+}$] homonuclear copper complex. Subunit IIa forms just one transmembrane helix. Based on the crystal structure, the binuclear center of cytochrome $ba_3$ is similar to those of the $aa_3$ type oxidases from Paracoccus denitrificans and bovine heart (1, 4, 5). In the oxidized form, heme $a_3$ is high spin five-coordinate, and Cu$_B$ is ligated with three histidines, His-290, His-291, and His-240 (if not stated otherwise, we adopt the residue numbering of the bovine heart enzyme). Tyrosine 244 is covalently linked to His-240. The A-type heme in cytochrome $ba_3$ contains a hydrophobic hydroxyethylglycanylgeranyl group, which is straight and reaches the cytoplasmic side without interfering with the proton pathways, instead of a hydroxyethylfarnesyl chain as seen in most bacterial and eucaryotic $aa_3$ oxidases (1, 4, 5).

Recently, it was reported that cytochrome $ba_3$ is capable of reducing nitric oxide to N$_2$O (7) in addition to reducing O$_2$ to H$_2$O and pumping protons, albeit with a relatively low efficiency of 0.5 H$^+/e^-$ (6).

The molecular mechanism of the $ba_3$ oxidase is expected to be similar to other distantly related heme-copper oxidases (*P. denitrificans*, mammalian, and $bo_3$ from *Escherichia coli*) with respect to the oxygen chemistry since the central features of the active site are similar, and the catalyzed chemical reaction is identical (1, 2). The proton pathway in $ba_3$ oxidase, as revealed from the crystal structure, starts at the cytoplasmic site and through the heme $a_3$ ligand H376, and the heme $a_3$ propionates lead directly to accumulation of water molecules (1, 2). The presence of water molecules near the heme $a_3$ propionates is conserved among all structurally known oxidases (*P. denitrificans*, mammalian, and $bo_3$ from *E. coli*), and their involvement in the proton exit channel has been supported by mutagenesis experiments (8, 9).

Given that the $ba_3$ oxidase is a member of the large heme-copper oxidase superfamily, it is essential to learn its function to understand possible alternative strategies in the dioxygen and nitric oxide reduction, and in the mechanism of proton translocation. In addition, the high affinity of Cu$_B$ for CO is unique among all heme-copper oxidases that allow spectroscopic measurements to be performed. This may lead to new insights concerning the functional mechanisms of heme-copper oxidases as well as how the properties of Cu$_B$ are related to proton translocation. Most proposed proton-pumping mechanisms involve Cu$_B$ and its histidine ligands (4, 9, 10). A histidine cycle (9), a histidine-cycle/shuttle (4), as well as the protonated/deprotonated state of His-290 (11) have been proposed as the crucial elements in the proton-pumping pathway. The existence and identity of such reorganization of the Cu$_B$ geometry caused by protonation/deprotonation and/or breakage of one of the Cu-N(His) bonds is a difficult matter to either prove or disprove since Cu$_B$ is spectrally silent and therefore no definite spectroscopic evidence has yet been observed.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Department of Chemistry, University of Crete, L. Knossou 300, P.O. Box 1470, Heraklion, Crete 71409, Greece. Tel.: 30-810393653; Fax: 30-810393601; E-mail: varotsis@edu.uoc.gr

*This paper is available on line at http://www.jbc.org*
Several spectroscopic techniques have been used to show that $b_{a_3}$ has peculiar ligand binding properties (12, for a review, see Ref. 13). However, there is a general consensus that the binding of CO to the binuclear center of $b_{a_3}$ follows that found in all cytochrome $c$ and quinol heme-copper oxidases, which proceeds according to Scheme 1.

$$\begin{align*}
\text{Fe}^{2+} \text{Cu}_b^{2+} + \text{CO} & \rightleftharpoons \text{Fe}^{2+}, \text{Cu}_b^{+}\cdot \text{CO} \\
& \rightleftharpoons \text{Fe}^{2+}\cdot \text{CO}, \text{Cu}_b^{+}
\end{align*}$$

**Scheme 1**

where $k_1$ and $k_{-1}$ represent the reversible binding of CO to Cu$_b$, and $k_2$ is the first-order transfer of CO from Cu$_b$ to the heme-Fe (12–20). The thermal dissociation rate of the heme-CO complex of bovine fully reduced cytochrome $c$ oxidase-CO is very slow (0.023 s$^{-1}$) and thus, $k_{-2}$ can be neglected (13). Recently, $k_{-2}$ was measured for the CO-bound reduced cytochrome $b_{a_3}$ of *T. thermophilus* (19) and found significantly faster (0.8 s$^{-1}$) than in the bovine $aa_3$ and $bo_3$ from *E. coli* enzymes (13, 15). Moreover, the FTIR difference ("dark-minus-light") approach has also been used to probe the structure and dynamics of the $b_{a_3}$ oxidase (12). It was reported that in the dark-minus-light FTIR spectra, the positive peaks at 1974 and 1983 cm$^{-1}$ represent CO bound to the ferrous heme $a_3$ whereas the asymmetric negative band centered at 2053 cm$^{-1}$ represents CO bound to Cu$^{2+}$.

In this report, we have investigated the CO-bound cytochrome $b_{a_3}$ complex at room temperature by FTIR and time-resolved step-scan FTIR (TR-FTIR) spectroscopy to probe the structure of the binuclear heme $a_3$-Cu$_b$ center and the coupled protein structures in response to the photodissociation/recombination of CO. The results indicate that in addition to the C-O stretching modes of the CO bound to heme $a_3$ at 1973 and 1982 cm$^{-1}$ reported earlier, an additional CO mode at 1967 cm$^{-1}$, representing the $\alpha$-form found in other heme-copper oxidases, is detected. Moreover, we make the unexpected observation of the C-O stretching mode of the equilibrium Cu$_b$[CO] complex (complex A in Scheme 1), which is detected at 2053 cm$^{-1}$ at room temperature and remained unchanged in H$_2$O/D$_2$O exchanges and between pD 5.5 and 9.7. Thus far, FTIR observation of the C-O stretching modes when bound to Cu$_b$ has been successful only by "light-minus-dark" difference FTIR spectra in which the photolyzed CO from the heme Fe migrates to Cu$_b$.

The TR-FTIR data indicate that the rate of decay of the transient Cu$_b$[CO] complex is 34.5 s$^{-1}$, and rebinding to heme $a_3$ occurs with an observed recombination rate constant $k_2 = 28.6$ s$^{-1}$ ($t_{1/2} = 24.2$ ms). We also show that the transient Cu$_b$[CO] complex is not pD-dependent (5.5–9.7). Our results indicate that Cu$_b$ maintains its associated ligands under different chemical conditions, and no deprotonation/protonation events occur to its ligands in the pD 5.5–9.7 range. Perturbation of the heme $a_3$ propionates (COOH) is observed upon CO photolysis. The rate of decay (43.3 s$^{-1}$) of the heme $a_3$ propionates (COOH) is on a time-scale coincident with the decay of the Cu$_b$[CO] complex suggesting that there is a coupling between ligation dynamics in the binuclear center and the environment sensed by the heme $a_3$ propionates. The dynamics of the ligation reactivities at the binuclear center and the coupled protein response are discussed.
Fig. 2. FTIR spectra of the CO-bound form of fully reduced cytochrome $b_{a_3}$ in $H_2O$ and $D_2O$ at 293 K. A, pH 7.5; B, pH 7.5; C, pH 5.5; D, pH 5.5, $^{13}CO$; E, pH 8.5; F, pH 8.5; and G, pH 9.7. Enzyme concentration was 1 mM and the path length 30 $\mu$m. The spectral resolution for all spectra was 4 cm$^{-1}$ except for the $^{13}CO$ derivative that was 0.5 cm$^{-1}$.

Fig. 3. Step-scan TR-FTIR difference spectra. A, step-scan TR-FTIR difference spectra of the CO-bound form of fully reduced cytochrome $b_{a_3}$ (pH 8.5) at 0.15, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 21, 22, 23, 25, 30, 35, 40, 45, 50, 55, 60, 64, 70, and 78.5 ms subsequent to CO photolysis. The spectral resolution was 8 cm$^{-1}$, and the time resolution was 100 $\mu$s. A 532 nm, 3–10 mJ/pulse pump beam was used for photolysis. B, step-scan TR-FTIR difference spectrum of the CO-bound form of fully reduced cytochrome $b_{a_3}$ (pH 8.5) from 0 to 1 ms subsequent to CO photolysis. The spectrum is the average of 200 individual spectra. The TR-FTIR spectra in the inset were obtained at 0.1, 0.2, 0.5, 4, 6, 10, 12, 15, 25, and 40 ms subsequent to CO photolysis.

The FTIR spectrum of the CO-bound cytochrome $b_{a_3}$ complex at neutral pH exhibits peaks at 1967, 1973, 1982, and 2053 cm$^{-1}$ (Fig. 2, trace A). In the $^{13}CO$ derivative these peaks shift to 1923, 1928, 1937, and 2007 cm$^{-1}$, respectively (Fig. 2, trace D). We assign the peaks at 1967, 1973, and 1982 cm$^{-1}$ to the C-O stretching modes of heme $a_3$-CO (complex B), and the peak at 2053 cm$^{-1}$ to the C-O stretching mode of Cu$_{a_3}$-CO (complex A). The mode at 1967 cm$^{-1}$ is similar to that found for the $a$-form in the CO adducts of $aa_3$ oxidases from bovine heart, Rhodobacter sphaeroides, $aa_3$-600, and P. denitrificans (21–28), and the 1973 cm$^{-1}$ mode is 2 cm$^{-1}$ lower than the $\gamma$-form found in P. denitrificans (28). The frequency of the 1982 cm$^{-1}$ mode, however, does not coincide with the $\beta$-form found in other heme-copper oxidases (20–23), and thus, we suggest that it represents a structure of the active site in which Cu$_{a_3}$ exerts a strong steric effect on the heme $a_3$-bound CO. The $\alpha$-form represents a conformation of the enzyme in which the frequencies of $\nu$(Fe-CO) and $\nu$(CO) lie off the correlation curve for heme proteins with a trans-histidine ligand (22). In the $\beta$-form, the frequencies are placed on the correlation curve (22), and in the $\gamma$-conformation, Cu$_{a_3}$ is moved closer to the CO-bound heme $a_3$, thereby the Fe-C-O moiety is further distorted from its preferred symmetry in the $a$-form (28). The frequency of the CO mode of the Cu$_{a_3}$-CO complex at 2053 cm$^{-1}$, however, is 10–12 cm$^{-1}$ higher and 8–12 cm$^{-1}$ lower than the corresponding frequencies of the $\beta$- and $\alpha$-forms found in other heme-copper oxidases (23, 25), and thus, we are unable to assign it to either the $\alpha$- or the $\beta$-form at present.

Although the species we detect in the FTIR data could arise from a heme $a_3$-CO/Cu$_{a_3}$-CO complex, consideration of $k_{-2}$ (0.8 s$^{-1}$) suggests that the peaks we detect are associated with the formation of complexes A and B (Scheme 1). The frequencies of these C-O modes are all very close to those that have been previously reported for the reduced CO-bound enzyme in the photo-steady-state (12) and their frequencies and bandwidths remained unchanged between $H_2O$ and $D_2O$ and also between pH 5.5 and 9.7 (Fig. 2, traces A—B and B—G). The ratio of the relative areas of the Fe-CO/Cu-CO is 4:1 in the absolute spectra, and considering the integrated absorptivities expected of CO complexes of heme and copper proteins (26), the ratio of $\varepsilon_{Fe-CO}/\varepsilon_{Cu-CO}$ is $\sim$ 1:5:1 suggesting $\sim$ 30% of unligated heme $a_3$, in agreement with the magnetic circular dichroism (17) and optical data (19).
Fig. 3A shows the step-scan time-resolved FTIR difference spectra ($t_d = 150 \mu s$–78.5 ms, 8 cm$^{-1}$ spectral resolution) of fully reduced $b_a$-CO subsequent to CO photolysis by a nanosecond laser pulse (532 nm). Under our experimental conditions (8 cm$^{-1}$ spectral resolution), the 1977 cm$^{-1}$ band is observed at 1977 cm$^{-1}$. The $4 \times 10^{-2}$ resolution experiment (data not shown) clearly demonstrates the presence of the three bands but the signal-to-noise is lower than that observed with 8 cm$^{-1}$ resolution. The negative peak at 1977 cm$^{-1}$ arises from the photolyzed heme $a_3$-CO. The positive peak that appears at 2053 cm$^{-1}$ is attributed to the C-O stretch ($v_{C=O}$) of Cu$_B$ as found under continuous light illumination by Einarsdóttir et al. (12), and its intensity is diminished near 70 ms. The frequency of the C-O mode in the transient Cu$_B$ has been assigned to that of the equilibrium Cu$_B$-CO complex (complex A). This observation suggests that no structural change at Cu$_B$ occurs in association with CO binding to and dissociation from heme $a_3$. No significant intensity variations are detected in the transient difference spectra ($t_d = 5$–3000 $\mu$s) for either the 2053 and 1977 cm$^{-1}$ modes. At later times, however, the decreased intensity of the transient 2053 cm$^{-1}$ mode (3–80 ms) is accompanied by an increased intensity at 1977 cm$^{-1}$. The intensity ratio ($\lambda$) for either the 2053 and 1977 cm$^{-1}$ is indicative of the C-O stretching vibration of protonated carboxylic groups of the heme $a_3$ propionates because in the reduced-minus-oxidized FTIR difference spectrum of cytochrome $b_a$-Cu$_B$ the band at 1706 cm$^{-1}$ has been assigned to the C-O mode of a protonated heme $a_3$ propionate (29). The propionate C=O stretching band is seen as a derivative-shaped feature in the TR-FTIR difference spectrum with the trough/peak at 1706/1694 cm$^{-1}$. The positive features at 1642 and 1665 cm$^{-1}$ are due to amide I (a-helical). Therefore, it appears that upon photodissociation of CO from heme Fe$_{a_3}$ and binding to Cu$_B$ (2), the protein conformation changes near the heme $a_3$ propionates. The time evolution of the trough/peak at 1706/1694 cm$^{-1}$ is depicted in Fig. 5, inset, demonstrates that these transient C-O stretches decay on a time-scale coincident with the decay of the transient Cu$_B$-CO complex.

Fig. 4 continues the study of the transient species subsequent to CO photolysis in the pD 5.5–9.7 range but using 355 nm laser pulse rather than 532 nm. The data show that photodissociation of CO from heme $a_3$ produces the same transient photoproduct as that obtained with 532 nm photolysis pulse and without changes in the intensity ratio of the 1977/2053 cm$^{-1}$ modes. The 5-$\mu$s transient difference spectra (Fig. 4, traces A–F) show that minimal changes are observed in the coordinate site of Cu$_B$ over a pD range of 5.5–9.7.

The continuous variability in intensity of the CO modes associated with heme $a_3$ and Cu$_B$ over a 0.005–80-ms time-scale is the most quantified aspect of ligand dissociation from Cu$_B$ and ligand red-binding to heme $a_3$ in heme-copper oxidases. The total area of the Cu$_B$-CO and Fe$_{a_3}$-CO bands shown in Fig. 3A were measured as a function of time to determine the rate of the decay of the transient Cu$_B$-CO complex (34.5 s$^{-1}$) and, since it is known (13–14,19) that $k_{-2} \ll k_2$ then $k_{diss} = k_2$.

The total area of the Cu$_B$-CO and Fe$_{a_3}$-CO bands shown in Fig. 3A were measured as a function of time to determine the rate of the decay of the transient Cu$_B$-CO complex (34.5 s$^{-1}$) and, since it is known (13–14,19) that $k_{-2} \ll k_2$ then $k_{diss} = k_2$.

The rate of recombination of CO to heme $a_3$ ($k_3 = 28.6$ s$^{-1}$) at room temperature. The curves are three-parameter fits to the experimental data according to first-order kinetics. Fig. 5, inset, shows the decay of the 1694 and 1706 cm$^{-1}$ modes (43.3 s$^{-1}$) as measured by the intensity of the corresponding modes shown in Fig. 3B (inset). Table I summarizes the CO kinetic properties in heme-copper oxidases.
Kinetic parameters for CO binding from T. thermophilus according to Scheme 1.

|  | \(k_2\) \(10^{-2}\) | \(k_1\) \(10^{-4}\) | Reference |
|---|---|---|---|
| \(aa_3\) | 1030 | \(7 \times 10^4\) | 13 |
| \(cya_3\) | 50 | \(2 \times 10^4\) | 13 |
| \(ba_3\) | 8 | ? | 13 |
| \(ba_3\) | 28.6 | ? | This work |

### DISCUSSION

**The Equilibrium (Complex A) and Transient CuB\(^{1+}\)-CO Complexes**—As shown in Fig. 2 (traces A–G) the frequency of the CO mode associated with CuB is invariant between pD 5.5 and 9.7 and unaffected by H/D exchange, consistent with no change in the protonation of groups in close proximity to CO under our experimental conditions. The frequency of \(v(\text{CO})\) that we observe is similar to those of Limulus hemocyanin \((v_{\text{CO}} = 2053\ \text{cm}^{-1})\) (27) and nitrite reductase from Alcaligenes faecalis \((v_{\text{CO}} = 2050\ \text{cm}^{-1})\) (30) but significantly different from that found in other CuB-containing oxidases (14, 21, 23, 26, 31, 32). In the absence of steric constraints, CO binds to CuB in a linear fashion \((\text{M–C–O})\), as required for optimal bonding between the \(d\) orbitals of Cu and the antibonding \(\pi^*\) of CO. Back-donation of electron density from the \(d\) orbitals to the antibonding \(\pi^*\) orbitals strengthens the Cu–C bond and weakens the C–O bond. Thus, back-donation shifts the \(v(\text{CO})\) to lower frequency. Similarly, back donation from one of the CuB-His is expected to affect \(v(\text{CO})\). For example, the high value for \(v(\text{CO})\) found in peptidylglycine monoxygenase \((2093\ \text{cm}^{-1})\) has been attributed to weaker back-donation of the methionine ligand compared with the histidine (His) ligand to the copper (33). Therefore, we can derive structural information for the CuB-N(His) ligands from the \(v(\text{CO})\) frequency of the CuB\(^{1+}\)-CO complex. The insensitivity of the \(v(\text{CO})\) of CuB frequency to \(\text{H}_2\text{O}/\text{D}_2\text{O}\) exchange and to pD 5.5–9.7 range indicate that the degree of back-donation of electron density from the \(d\) orbitals to the antibonding \(\pi^*\) orbitals is not altered under these conditions. If one of the CuB-His ligands is capable of cycling through the imidazolate, imidazole, and imidazolium states then \(v(\text{CO})\) is expected to vary. A change in the protonation state of one of the His ligands would have significant changes in the back donation and thus on the frequency of \(v(\text{CO})\). However, we see no change in the frequency and bandwidth of the CO mode and conclude that the CuB-His environment is very rigid and not subject to conformational transitions that are associated with protonation/deprotonation events of the CuB-His ligands.

It is generally believed that the ligands to CuB are critically involved in the catalytic function of cytochrome c oxidase because of their close proximity to the heme \(a_3\) center. Evidence supporting their role as \(H^+\) donors during the catalytic cycle and in structural changes that modulate the position of CuB with respect to CO was recently reported (34–36). Although transient motions of the protein are required to allow ligand access to the binuclear center and net dissociation to the surroundings, the similarity in the C–O frequency of the equilibrium and transient CuB\(^{1+}\)-CO complex argues against ligand reorganization at CuB or transient motion near the vicinity of the CuB site.

Information concerning the active site of heme-copper oxidases has been deduced from resonance Raman studies of the CO-bound form of the enzymes (22, 24, 28, 34). In one of these studies, Rousseau and co-workers (34), based upon pH dependent changes seen in the heme \(a_3\) Fe-CO stretching frequency, proposed that structural changes that modulate the position of CuB with respect to the heme-CO are coupled to protonation/deprotonation events of one or more residues. They suggested that these residues are the His-290 and His-291 that are coordinated to CuB and excluded His-240 because it is covalently linked through its nitrogen to Tyr-244. Additional involvement of His-290 protonation/deprotonation events was suggested from resonance Raman experiments in an \(aa_3\) quinol oxidase from an acidophilic arhaeon (11). Das et al. (11) reported that there are changes in the heme \(a_3\) formyl C–O stretching mode upon heme reduction that indicated change in H-bonding to the formyl group. His-290 is the closest residue to the formyl oxygen, and thus, they suggested that the proton on the His-290 is very labile and proposed a model for proton pumping. The involvement of His-290 as the crucial element in the proton-pumping pathway was also suggested by Michel and co-workers (4) whereas in Wikström’s model (10) a different histidine residue, His-291, was proposed to be involved in the proton pumping pathway. It is important to note that the results presented here provide spectroscopic evidence for the first time for the equilibrium CuB environment under different chemical conditions and demonstrate that there is no structural change of the CuB-His ligands in the pD 5.5–9.7 range.

**Transient Photoproducts and Determination of \(k_2\)**—Several FTIR spectroscopic studies have used photodissociation of heme-CO to probe the transient binding of CO to CuB (12–15, 20, 32). This way, the role of CuB in the proton pathway has been implicated because protonation/deprotonation processes of carboxylic groups of amino acid residues occur near CuB subsequent to CO photolysis from heme \(a_3\) and transient binding to CuB (32). The time-resolved FTIR measurements reported here were made by following the FTIR transient absorbances associated with the CO stretching frequencies of the heme \(a_3\) and CuB\(^{1+}\)-CO complexes. Consequently, the precise chemical nature of the kinetics can be understood without ambiguities, as there often is in kinetic optical absorption spectroscopy.

Our data clearly show that upon CO photolysis, protein conformational changes near the heme \(a_3\) propionic acids perturb the C=O bond. The change in the relationship between the C=O of the propionic acid and the proton side chain of a residue or its interaction with a water molecule is most readily seen by the positive feature at 1694 cm\(^{-1}\). The lower frequency at 1694 cm\(^{-1}\) in the transient spectra means weaker C=O bond and therefore stronger H-bonding to surrounding groups or structural water. This way, hydrogen bonding to C=O is large enough to cause perturbation of the group. There is only one possibility that meets these conditions based on inspection of the crystal structure of the enzyme, namely Asp-372 \((T. ther- mosphilus\) numbering). This residue is part of a possible proton channel proposed by Soulimane et al. (1, 2), who has suggested that this residue leads to the end of the proton pathway. The well developed pathways leading from Asp-372 and nearby water molecules to the periplasmic side of the membrane consist of several hydrogen bonded polar amino acid chains and additional water molecules. The water molecules are connected to the bulk solvent on the periplasmic side of the membrane, which may result in a fast equilibrium between these water molecules and the bulk solvent.

The evidence presented above makes plausible mechanisms of proton pathway(s) directly associated with the propionates of the heme \(a_3\) redox center and the proton-labile side chain of Asp-372. A protein conformational change has been suggested as the molecular switch for proton delivery (36). Therefore, the molecular switch such as the one described above could be triggered by the ligation reactions of the binuclear center observed in the current work. It becomes intriguing to speculate...
that the water molecules near the top of the propionic acids that are conserved in all structurally known oxidases (1, 4, 5, 38) is not accidental but rather play a vital role in the biological function of the heme-copper oxidases.

The observed rates for the decay of the transient Cu\(_3\)\(^{1+}\)-CO complex and the heme \(a_3\)\(^{2+}\)-CO recombination (\(k_2\)) that we have determined in this study suggest that the rate-limiting step for the heme \(a_3\) recombination of CO is the decay of the Cu\(_3\)\(^{1+}\)-CO complex. The observed rate (34.5 s\(^{-1}\)) for the decay of the transient Cu\(_3\)\(^{1+}\)-CO complex is much smaller than that of bovine aa\(_3\) (13). This indicates that the flow-flash experimental approach, in which the loss of CO is the rate-determining step in the formation of the heme-O\(_2\) adduct and thus requires the photodissociated CO not to interfere with the reaction with O\(_2\), is not the appropriate method for the identification of oxygen intermediates during the catalytic turnover of the bovine enzyme (39). It has been reported that Cu\(_b\) in bovine, in contrast to bovine aa\(_3\), has a relative high affinity for CO (\(K_1 > 10^4\)) indicating unusual kinetics of electron transfer and ligand binding (17, 19). The kinetic properties of CO and CN binding were interpreted, prior to the determination of the three-dimensional structure of the enzyme to peculiar features in the binuclear center (19). The recently reported crystal structure of aa\(_3\) (1) does not support the concept of unique features in the binuclear center that will influence the thermodynamic and kinetic properties of exogenous ligands. The value of \(k_2\) that we have measured in aa\(_3\) is very similar to that reported for its counterpart caa\(_3\) (13).

Complete CO rebinding to heme \(a_3\) is no doubt coupled to a relaxation of the protein as well. At this point the binuclear center has all of the spectroscopic characteristics of that presented in Scheme 1. At higher temperatures, there is additional protein reorganization, which influences the CO rebinding. The kinetics of these latter processes have been studied in detail by Brunori and co-workers (19) and have been reported on recently.

**Heme Pocket Dynamics in Cytochrome ba\(_3\) Oxidase**—Until recently, a substantial barrier erected by the protein to the re-binding of CO to the heme subsequent to the photoinitiated transfer of CO to Cu\(_b\), which was suggested to be the binding of an endogenous ligand L to heme \(a_3\), was interpreted as the rate-limiting step to ligand rebinding (13). However, most recent picosecond resonance Raman data indicated that the proximal ligation survives photolysis and that the control of the access of exogenous ligands to the heme \(a_3\) site by means of a ligand exchange process can be ruled out (18). If the dissociation of CO from Cu\(_b\)\(^{1+}\) is the rate-limiting step for recombination, the slow recombination time observed is explained in Scheme 2 as a product of the slow thermal rate of equilibration of Cu\(_b\)\(^{1+}\)-CO with CO. To present a complete view of the ligand photodynamics, we include two branches: the thermal and the photochemical for CO ligation dynamics at the heme \(a_3\) site.

The model is similar to that of Schelvis et al. (18) except we have included the equilibrium Cu\(_b\)\(^{1+}\)-CO complex (complex A). The model consists of two branches for CO ligation dynamics at the heme \(a_3\) site, one thermal and the other photochemical. The thermal branch describes the equilibrium kinetics of (re)binding and dissociation of CO to and from Cu\(_b\) and heme \(a_3\). We do not have evidence of the photochemical properties of Cu\(_b\)\(^{1+}\)-CO (complex A); however, we have retained the interpretation of Einarsdóttir et al. (40) who showed no electronic transitions originated from the transient Cu\(_b\)\(^{1+}\)-CO of bovine aa\(_3\) in the transient optical data, and thus, we presume that the Cu\(_b\)\(^{1+}\)-CO complex (complex A) is not photolabile under 532 or 355 nm excitation. In the scheme, we discriminate between two different Cu\(_b\)\(^{1+}\)-CO complexes: one that is part of the photochemical branch (state 5) in which the pocket is not at thermal equilibrium, and the other with the heme \(a_3\) pocket relaxed (state 2). Fast dynamic experiments of bovine aa\(_3\) have shown that photodissociation of CO from heme \(a_3\) occurs within 150 fs (state 4) and the transient Cu\(_b\)\(^{1+}\)-CO (state 5) is fully formed within 1 ps (13). We expect the same time-scales for the initial events in the photodynamics of cytochrome ba\(_3\). Thus, in the photochemical branch, His-heme \(a_3\) is in a non-equilibrium state (state 5) and it relaxes to its equilibrium conformation and CO dissociates from Cu\(_b\) and equilibrates into the surrounding solvent (state 1). Geminate recombination is not observed after CO photodissociation, therefore neither 4 nor 5 are the geminate dissociative states formed after photolysis. In states 4 and 5, the photolyzed CO and the heme pocket contain excess energy resulting from the photolysis. The activated CO binds to Cu\(_b\) before the binuclear center is thermalized. The exceptionally high affinity for CO binding to Cu\(_b\) (\(K_1 > 10^4\) M\(^{-1}\)) prevents geminate recombination. Nongeminate recombination of CO occurs through the thermal branch from state 2 with an observed rate constant \(k_2 = 28.6 \pm 1\) (\(t_{1/2} = 24.2\) ms).

The kinetics of CO rebinding in cytochrome ba\(_3\) are considerably slower than those of bovine cytochrome aa\(_3\) and cytochrome caa\(_3\) from T. thermophilus (12–13, 19). It is apparent that the dynamics of the heme \(a_3\) proximal pocket is not a possible explanation for the observed ligand rebinding behavior and that distal pocket effects play the dominant role in the recombination process. The efficiency of CO to escape from the heme pocket is predicted upon the relative activation barriers to rebinding versus escape and/or diffusivity through the protein. Our room temperature results are consistent with no barrier, as opposed to cytochrome aa\(_3\), to ligand rebinding to heme \(a_3\), and ligand removal from Cu\(_b\) occurs via escape into the surrounding solvent before binding to the heme \(a_3\).

**CONCLUSIONS**

The equilibrium Cu\(_b\)\(^{1+}\)-CO complex and the protein response subsequent to CO photolysis from heme \(a_3\), detected here at room temperature form a foundation for the understanding of the Cu\(_b\) environment in heme-copper oxidases and the molecular relaxation pathway of photodissociated \(ba_3\). The importance of the protein in the relaxation process is pronounced. Indeed from the transient FTIR measurements on photodissociated carbon monoxide heme-copper oxidases, we infer that the propionic acids of heme \(a_3\) undergo significant vibrational changes upon CO photodissociation, either by protonation/deprotonation or by environmental changes. These features decay on a time-scale (43.3 s\(^{-1}\)) coincident with the...
Equilibrium Cu$_{B}$-CO Complex in Cytochrome Oxidase

Decay of the transient Cu$_{B}$-CO complex (34.5 s$^{-1}$) suggesting that there is a coupling between ligation dynamics in the binuclear center and the environment sensed by the heme a$_{3}$ proximates. The complexity of the reorganization of ba$_{3}$ oxidase after photodissociation of CO should serve as a basis for the study of other heme-copper oxidases with similar biologically active centers. To uncover the relaxation pathway it will be necessary to study the transient species by different techniques and to derive information from their time evolution at physiological temperatures. Such studies will lead to an advanced understanding of the dynamic processes that occur in heme-copper oxidases.

REFERENCES

1. Soulimane, T., Buse, G., Bourenkov, G. P., Bartunik, H. D., Huber, R., and Than, M. E. (2000) EMBO (Eur. Mol. Biol. Organ) J. 19, 1766–1776
2. Than, M. E., and Soulimane, T. (2000) in Handbook of Metalloproteins (Messerschmidt, A., Huber, R., Poulos, T., and Wieghardt, K., eds) pp. 363–378, John Wiley and Sons Ltd., Chichester, UK
3. Soulimane, T., and Buse, G. (1995) Eur. J. Biochem. 227, 588–595
4. Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995) Nature (Lond.) 376, 660–669
5. Yoshikawa, S., Shinzawa-Itoh, K., Nakashima, R., Yaono, R., Yamashita, E., Inoue, N., Yao, M., Fei, M. J, Libeu, C. P., Mizushima, T., Yamaguchi, H., Tomizaki, T., and Tsukihara, T. (1998) Science (Wash. D. C.) 280, 1723–1729
6. Kannt, A., Soulimane, T., Buse, G., Becker, A., Bamberg, E., and Michel, H. (1998) FEBS Lett. 434, 17–22
7. Giuffrè, A., Stubauer, G., Stari, P., Brunori, M., Zumft, W. G., Buse, G., and Soulimane, T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14718–14723
8. Ostermeier, C., Harrenga, A., Ermel, U., and Michel, H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10547–10553
9. Puustinen, A., and Wikström, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 35–37
10. Morgan, J. E., Verkhoysky, M. I., and Wikström, M. (1994) J. Bioenerg. Biomembr. 26, 599–608
11. Das, T. P., Gomes, C. M., Teixeira, M., and Rousseau, D. L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9591–9596
12. Einarsdóttir, O., Killough, P. M., Fee, J. A., and Woodruff, W. H. (1989) J. Biol. Chem. 264, 2405–2408
13. Woodruff, W. H. (1993) J. Bioenerg. Biomembr. 25, 177–188
14. Einarsdóttir, O., Dyer, R. B., Lemon, D. D., Killough, P. M., Hubig, S. M., Atherton, S. J., López-Garriga, J. J., Palmer, G., and Woodruff, W. H. (1993) Biochemistry 32, 12013–12024
15. Lemon, D. D., Calhoun, M. W., Gennis, R. B., and Woodruff, W. H. (1993) Biochemistry 32, 11953–11956
16. Varotsis, C., Kreszowski, D. H., and Babcock, G. T. (1996) Biospectroscopy 2, 331–338
17. Goldbeck, R. A., Einarsdóttir, O., Dawes, T. D., O’Connor, D. B., Sururer, K. K, Fee, J. A., and Kliger, D. S. (1992) Biochemistry 31, 9376–9387
18. Schellis, J. P. M., Deinum, G., Varotsis, C. A., Ferguson-Miller, S., and Babcock, G. T. (1997) J. Am. Chem. Soc. 119, 8409–8416
19. Giuffrè, A., Forte, E., Antonini, G., D’Itti, E., Brunori, M., Soulimane, T., and Buse, G. (1999) Biochemistry 38, 1057–1065
20. Stavvakis, S., Koutsoupakis, K., Pinkoulaiki, E., Urbani, A., Saraste, M., and Varotsis, G. T. (2002) J. Am. Chem. Soc. 124, 3814–3815
21. Mitchell, D. M., Shapleigh, J. P., Archer, A. M., Alben, J. O., and Gennis, R. B. (1996) Biochemistry 35, 9446–9450
22. Wang, J. W., Takahashi, S., Hofer, J., Mitchell, D. M., Ferguson-Miller, S., Gennis, R. B., and Rousseau, D. L. (1995) Biochemistry 34, 9819–9825
23. Stavrakis, S., Itri, E., Brunori, M., Soulimane, T., and Mantele, W. (1999) Biochemistry 38, 7565–7571
24. Varotsis, C., and Yamvouka, M. (1998) J. Am. Chem. Soc. 210, 6760–6763
25. Park, S., Pan, L.-P., Chan, S. I., and Alben, J. (1996) Biochem. J. 310, 1036–1047
26. Alben, J. O., Moh, P. P., Fiamingo, F. G., and Altschuld, R. A. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 234–237
27. Fager, L. Y., and Alben, J. O. (1994) Biochemistry 33, 1433–1446
28. Pinkoulaiki, E., Pitzner, U., Ludwig, B., and Varotsis, C. (2002) J. Biol. Chem., 277, 13563–13568
29. Hellwig, P., Soulimane, T., Buse, G., and Mantele, W. (1999) Biochemistry 38, 9648–9655
30. Zhang, H., Loubanger, M. J., Mauk, A. G., and Murphy, M. E. P. (2000) J. Phys. Chem. B 104, 10738–10742
31. Puustinen, A., Bailey, J. A., Dyer, R. B., Mecklenberg, S. L., Wikström, M., and Woodruff, W. (1997) Biochemistry 36, 13195–13200
32. Iwase, T., Varotsis, C., Shinzawa-Itoh, K., Yoshikawa, S., and Kitagawa, T. (1999) J. Am. Chem. Soc. 121, 1415–1416
33. Konstantinov, A. A., Siletsky, S., Mitchell, D., Kaulen, A., and Gennis, R. B. (1993) Biochemistry 32, 9085–9090
34. Das, T. K., Tomson, F. K., Gennis, R. B., Gordon, M., and Rousseau, D. L. (2001) Biophys. J. 80, 2039–2045
35. Proshlyakov, D. A., Pressley, M. A. DeMaso, C., Leykam, J. F., DeWitt, D. L., and Babcock G. T. (2006) Science (Wash. D. C.) 299, 1588–1591
36. MacMillan, F., Kannt, A., Behr, J., Prinsen, T., and Michel, H. (1999) Biochemistry 38, 9179–91884
37. Konstantinov, A. A., Siletsky, S., Mitchell, D., Kaulen, A., and Gennis, R. B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9085–9090
38. Abrahamson, J., Riistama, S., Larsson, G., Jasaitis, A., Svensson-Ek, M., Laukkonen, L., Puustinen, A., Iwata, S., and Wikström, M. (2000) Nat. Struct. Biol. 7, 910–917
39. Varotsis, C., Zhang, Y., Appelman, E. H., and G. T. Babcock (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 237–241
40. Einarsdóttir, O., Dawes, T. D., and Georgiadis, K. R. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6934–6937