We report the first evidence for the existence of the equilibrium CuB\(^{1+}\)-CO species of CO-bound reduced cytochrome \(b_\text{a}_3\) from \textit{Thermus thermophilus} at room temperature. The frequency of the C-O stretching mode of CuB\(^{1+}\)-CO is located at 2053 cm\(^{-1}\) and remains unchanged in H\(_2\)O/D\(_2\)O exchanges and, between pD 5.5 and 9.7, indicating that the chemical environment does not alter the protonation state of the CuB histidine ligands. The data and conclusions reported here are in contrast to changes in protonation state of CuB-His-290, reported recently (Das, T. K., Tomson, F. K., Gennis, R. B., Gordon, M., and Rousseau, D. L. (1999) \textit{Biophys. J.} 80, 2039–2045 and Das, T. P., Gomes, C. M., Teixeira, M., and Rousseau, D. L. (1999) \textit{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 CuB\(^{1+}\)-CO complex is 34.5 s\(^{-1}\) and rebinding to heme \(a_3\) occurs with \(k_\text{a}_3 = 28.6\) s\(^{-1}\). The rate of decay of the transient CuB\(^{1+}\)-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 CuB\(^{1+}\)-CO species is the same as that of the equilibrium CuB\(^{1+}\)-CO species and remains unchanged in the pD range 5.5–9.7 indicating that no structural change takes place at CuB between these states. The implications of these results with respect to proton pathways in heme-copper oxidases are discussed.

Cytochrome \(b_\text{a}_3\) from the thermophilic eubacterium \textit{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 \(a_3\). Subunit II contains a mixed valence \([\text{Cu}_{\text{A}}^{1.5+} - \text{Cu}_{\text{A}}^{1.5+}]\) homodinuclear copper complex. Subunit IIa forms just one transmembrane helix. Based on the crystal structure, the binuclear center of cytochrome \(b_\text{a}_3\) is similar to those of the \(aa_3\) type oxidases from \textit{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 \(b_\text{a}_3\) contains a hydrophobic hydroxyethylgeranylgeranyl group, which is straight and reaches the cytoplasmic side without interfering with the proton pathways, instead of a hydroxymethylfarnesyl chain as seen in most bacterial and eucaryotic \(aa_3\) oxidases (1, 4, 5). Recently, it was reported that cytochrome \(b_\text{a}_3\) 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 \(b_\text{a}_3\) oxidase is expected to be similar to other distantly related heme-copper oxidases (\textit{P. denitrificans}, mammalian, and \(b_\text{a}_3\) from \textit{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 \(b_\text{a}_3\) 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 (\textit{P. denitrificans}, mammalian, and \(b_\text{a}_3\) from \textit{E. coli}), and their involvement in the proton exit channel has been supported by mutagenesis experiments (8, 9).

Given that the \(b_\text{a}_3\) 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.
Several spectroscopic techniques have been used to show that \( ba_3 \) 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 \( ba_3 \) follows that found in all cytochrome \( c \) and quinol heme-copper oxidases, which proceeds according to Scheme 1.

\[
\begin{align*}
\text{Fe}^{2+} \text{Cu}_b^{-} + \text{CO} & \rightleftharpoons \text{Fe}^{2+}, \text{Cu}_b^{-} - \text{CO} \\
& \rightleftharpoons \text{Fe}^{2+}, \text{Cu}_b^{-}
\end{align*}
\]

**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 \( ba_3 \) 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\(^2\) difference (‘dark-minus-light’) approach has also been used to probe the structure and dynamics of the \( ba_3 \) 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\(_b\)\(^1\).

In this report, we have investigated the CO-bound cytochrome \( ba_3 \) 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 \( a \)-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\(_b\)\(^{1+}\)-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 pH 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\(_b\)\(^{1+}\)-CO complex is 34.5 s\(^{-1}\), and rebinding to heme \( a_3 \) occurs with an observed recombination rate constant \( k_2 = 28.6 \text{ s}^{-1} (t_{1/2} = 24.2 \text{ ms}) \). We also show that the transient Cu\(_b\)\(^{1+}\)-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\(_b\)\(^{1+}\)-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 re-actions at the binuclear center and the coupled protein response are discussed.

**EXPERIMENTAL PROCEDURES**

Cytochrome \( ba_3 \) was isolated from *T. thermophilus* HB8 cells according to previously published procedures (3). The samples used for the FTIR measurements had an enzyme concentration of 1–10 mM and were placed in a desired buffer (pD 5.5–6.5, MES; pD 7.5, HEPES; pD 8.5–9.5 CHES). The sample solutions prepared in D\(_2\)O buffers were measured by using a pH meter and assuming pD = pH (observed) + 0.4. Dithionite-reduced samples were exposed to 1 atm of CO (1 mM) in an anaerobic cell to prepare the carbonmonoxy adduct and transferred to a tightly sealed FTIR cell under anaerobic conditions (1–30 µm). CO gas was obtained from Messer (Frankfurt, Germany), and isotopic CO (\(^{13}\)CO) was purchased from Isotec (Miamisburg, OH). The 532 and 355 nm pulses from a continuum Nd:YAG laser (7-ns wide, 3–7 Hz) were used as a pump light (3–10 mJ/pulse) to photolyze the \( ba_3 \)-CO oxidase. FTIR measurements were performed on a Bruker Equinox IFS 55 spectrometer equipped with the step-scan option (Bruker, Newark, DE). For the time-resolved experiments, a TTL pulse (transistor transistor logic) provided by a digital delay pulse generator (Quantum Composers, 9314T) triggers, in order, the flashlamps, Q-switch, and the FTIR spectrometer. Pretriggering the FTIR spectrometer to begin data collection before the laser fires allows eleven fixed reference points to be collected at each mirror position, which are used as the reference spectrum in the calculation of the difference spectra. Changes in intensity were recorded with an MCT detector (mercury cadmium telluride, Graseby infrared D316, response limit 600 cm\(^{-1}\)) amplified in the DC-coupled mode and digitized with a 200–kHz, 16-bit, analog-to-digital converter. A broadband interference optical filter (Optical Coating Laboratory, Santa Rosa, CA) with short wavelength cut off at 2.67 µm was used to limit the free spectral range from 2.67 to 8 µm. This leads to a spectral range of 3900 cm\(^{-1}\), which is equal to an under-sampling ratio of 4. Single-sided spectra were collected at 8 cm\(^{-1}\) spectral resolution, 5 or 100 µs time resolution, and 10 co-additions per data point. Total accumulation time for each measurement was 60 min, and 2–5 measurements were collected and averaged. Blackman-Harris three-term apodization with 32 cm\(^{-1}\) phase resolution and the Mertz phase correction algorithm were used. Difference spectra were calculated as \( \Delta A = -\log(I/I_0) \). Optical absorption spectra were recorded with a Perkin-Elmer Lambda 20 UV-visible spectrometer before and after the FTIR measurements to ensure the formation and stability of the CO adducts.

**RESULTS**

The optical absorption spectrum of the dithionite-reduced \( ba_3 \) enzyme displays Soret maxima at 427 (heme \( b^{2+} \)) and 444
(heme $a_3^{2+}$) nm and visible maxima at 559 (heme $b^{2+}$) and 614 (heme $a_3^{2+}$) nm (Fig. 1, trace $A$). Flushing CO over the reduced enzyme shifts the visible band of heme $a_3^{2+}$ by 22 to 592 nm and the Soret to 427 nm (Fig. 1, trace $B$) whereas the bands due to heme $b^{2+}$ remained, as expected, unchanged. The difference spectrum (Fig. 1, trace $C$) confirms the coordination of CO to the reduced enzyme.

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 $^{13}$C$^{16}$O 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 $y$-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 (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 $k_{\text{eq}}$ (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$_2$O and D$_2$O 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 optical data (19).

The TR-FTIR spectra in the inset were obtained at 0.1, 0.2, 0.5, 4, 6, 10, 12, 15, 20, 21, 22, 23, 25, 30, 35, 40, 45, 50, 55, 60, 64, 70, and 78 ms subsequent to CO photolysis. The spectral resolution was 8 cm$^{-1}$, and the time resolution was 2 cm$^{-1}$, respectively (Fig. 2, trace $E$). The frequency of the CO stretching mode of Cu B in the Fe-CO/Cu-CO complex at neutral pH exhibits peaks at 1967, 1973, 1982, and 2053 cm$^{-1}$, whereas the bands due to heme $b$-CO are further distorted from its preferred symmetry in the $a$-form (28). The frequency of the CO mode of the Cu$_B$-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.
Fig. 3A shows the step-scan time-resolved FTIR difference spectra \( t_d = 150 \mu s \sim 78.5 \text{ ms} \), 8 cm\(^{-1}\) spectral resolution) of fully reduced \( a_3\)-CO subsequent to CO photolysis by a nanosecond laser pulse (532 nm). Under our experimental conditions (8 cm\(^{-1}\) spectral resolution), the 1977 cm\(^{-1}\), and 1982 cm\(^{-1}\) peaks are not resolved, and thus a single negative peak is observed at 1977 cm\(^{-1}\). The 4 cm\(^{-1}\) resolution experiment (data not shown) clearly demonstrates the presence of the three bands but the signal-to-noise is lower than that obtained 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 \((\nu_{\text{CO}})\) of CuB as found under continuous light illumination by Einarsdottir et al. (12), and its intensity is diminished near 70 ms. The frequency of the C-O mode in the transient CuB\(^{1-}\)-CO complex is the same as that of the equilibrium CuB\(^{1-}\)-CO (complex A). This observation suggests that no structural change at CuB occurs in association with CO binding to and dissociation from heme \( a_3\). No significant intensity variations are detected in the transient difference spectra \( t_d = 5 \sim 3000 \mu s \) 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 \((-1.5\) \) of the Fe-CO/CuB-CO remains constant for all data points, and thus, we conclude that no fraction of CO escapes the binuclear center at 293 K. This is consistent with the low temperature experiments in which it was demonstrated that the CuB\(^{1-}\)-CO intermediate is fully formed and that no significant fraction of CO escapes the metal centers below 300 K (12).

Fig. 3B shows a single TR-FTIR difference spectrum averaged from 5–1000 \( \mu \)s in the 1500–2200 cm\(^{-1}\) region. It is evident that there are features in the 1600–1710 cm\(^{-1}\) region whose absorbances (positive or negative) are on the order of \( 3 \times 10^{-2} \) absorbance units or less. The frequency of the band at 1706 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 \( b_a \), the band at 1706 cm\(^{-1}\) has been assigned to the C-O mode of a protonated heme \( a_3 \) propionate (29). The protonate 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 \((\alpha\)-helical\). Therefore, it appears that upon photodissociation of CO from heme Fe\(^{2+}\)-CO \(^{1-}\) and binding to CuB\(^{1-}\), the protein conformation changes near the heme \( a_3 \) propionates. The time evolution of the trough/peak at 1706/1694 cm\(^{-1}\) that 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 CuB\(^{1-}\)-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 pulses 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 CuB over a pD range of 5.5–9.7.

The continuous variability in intensity of the CO modes associated with heme \( a_3 \) and CuB over a 0.005–80-ms time-scale is the most quantified aspect of ligand dissociation from CuB and ligand re-binding to heme \( a_3 \) and is depicted in Fig. 5. The total area of the CuB\(^{1-}\)-CO and Fe\(^{2+}\)-CO bands shown in Fig. 3A were measured as a function of time to determine the rate of the decay of the transient CuB\(^{1-}\)-CO complex \((34.5 \text{s}^{-1})\) and, since it is known \((13–14,19)\) that \( k_{-1} \ll k_2 \) then \( k_{\text{obs}} = k_2 \), the rate of recombination of CO to heme \( a_3 \) \((k_2 = 28.6 \text{s}^{-1})\) 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 \((43.3 \text{s}^{-1})\) 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.
**Equilibrium CuB-CO Complex in Cytochrome Oxidase**

![Image of a page from a scientific document](https://via.placeholder.com/150)

**Table I**

| Kinetic parameters for CO binding |  |
|----------------------------------|--|
| from T. thermophilus according to Scheme 1. |  |
| $k_2$ | $k_1$ | Reference |
| 1030 | $7 \times 10^6$ | 13 |
| 50 | $2 \times 10^6$ | 13 |
| 8 | ? | 13 |
| 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 pH 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 $v$(CO) that we observe is similar to those of *Limulus* hemocyanin (CO = 2053 cm\(^{-1}\)) (27) and nitrite reductase from *Alcaligenes faecalis* (CO = 2050 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 (M–C–O), as required for optimal bonding between the $\sigma^*$ orbitals of Cu and the antibonding $\pi^*$ orbitals 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 $v$(CO) to lower frequency. Similarly, back donation from one of the CuB-His is expected to affect $\nu$(CO). For example, the high value for $\nu$(CO) found in peptidylglycine monoxygenase (2093 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 $\nu$(CO) frequency of the CuB\(^{1+}\)-CO complex. The insensitivity of the $\nu$(CO) of CuB frequency to H\(_2\)O/D\(_2\)O exchange and to pH 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 imidazole, imidazole, and imidazolium states then $v$(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 $v$(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 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 $a_3$ quinol oxidase from an acidophilic archeaeon (11). Das et al. (11) reported that there are changes in the heme $a_2$ 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 pH 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. thermophilus* numbering). This residue is part of a possible proton channel proposed by Soulime 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 ba$_3$ enzyme (39). It has been reported that Cu$_b$ in ba$_3$ is in contrast to bovine aa$_3$, has a relative high affinity for CO ($K > 10^4$) 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 ba$_3$ (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 ba$_3$ 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 photoinitiating 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 re-binding (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 disso-
Equilibrium Cu_{H}^{1-}CO Complex in Cytochrome Oxidase

decay of the transient Cu_{H}^{1-}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} propionates. 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.

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