Spectroscopic and Kinetic Investigation of the Fully Reduced and Mixed Valence States of ba₃-Cytochrome c Oxidase from Thermus thermophilus

A FOURIER TRANSFORM INFRARED (FTIR) AND TIME-RESOLVED STEP-SCAN FTIR STUDY

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background: Cytochrome c oxidase reduces O₂ to H₂O, a reaction coupled to proton translocation across the membrane.

results: Photolysis of CO from the mixed valence form of cytochrome ba₃-CO does not lead to a heme a₃³⁺-CuB¹⁺-CO binuclear center.

conclusion: The absence of reverse electron transfer between hemes a₃ and b is shown.

significance: Unique thermodynamic and kinetic properties of cytochrome ba₃ oxidase are presented.

The complete understanding of a molecular mechanism of action requires the thermodynamic and kinetic characterization of different states and intermediates. Cytochrome c oxidase reduces O₂ to H₂O, a reaction coupled to proton translocation across the membrane. Therefore, it is necessary to undertake a thorough characterization of the reduced form of the enzyme and the determination of the electron transfer processes and pathways between the redox-active centers. In this study Fourier transform infrared (FTIR) and time-resolved step-scan FTIR spectroscopy have been applied to study the fully reduced and mixed valence states of cytochrome ba₃ from Thermus thermophilus. We used as probe carbon monoxide (CO) to characterize both thermodynamically and kinetically the cytochrome ba₃-CO complex in the 5.25–10.10 pH/pD range and to study the reverse intramolecular electron transfer initiated by the photolysis of CO in the two-electron reduced form. The time-resolved step-scan FTIR data revealed no pH/pD dependence in both the decay of the transient CuB¹⁺-CO complex and rebinding to heme a₃ rates, suggesting that no structural change takes place in the vicinity of the binuclear center. Surprisingly, photodissociation of CO from the mixed valence form of the enzyme does not lead to reverse electron transfer from the reduced heme a₃ to the oxidized low-spin heme b, as observed in all the other aa₃ and ba₃ oxidases previously examined. The heme b-heme a₃ electron transfer is guaranteed, and therefore, there is no need for structural rearrangements and complex synchronized cooperativities. Comparison among the available structures of ba₃- and aa₃-cytochrome c oxidases identifies possible active pathways involved in the electron transfer processes and key structural elements that contribute to the different behavior observed in cytochrome ba₃.

Most biochemical redox reactions involve intramolecular delivery of electrons through a donor-acceptor pair (1–5). Determination of the pathways and dynamics of electron transfer (ET) is of crucial importance for understanding many catalytic reactions, especially when the redox-linked changes are coupled to other processes such as proton transfer and protonation/deprotonation events. Because both the electron transfer pathways and the conformational changes that lead to proton translocation are controlled by the protein structure, identification of both electron transport chains and proton labile residues is a necessary step toward the elucidation of the overall mechanism of function.

Cytochrome c oxidase (CcO) is the terminal membrane protein of cellular respiration in most aerobic organisms (6, 7). It catalyzes the reduction of molecular oxygen to water using electrons from cytochrome c in a process coupled with the pumping of protons across the membrane. The internal electron flow through the four redox centers (Scheme 1) (8–12) and the accompanied proton uptake to form H₂O is energetically and mechanistically linked to proton translocation generating the gradient that drives the synthesis of ATP. However, the mechanism of this linkage and the protein residue cofactors involved in the electron/proton transfer pathways remain unclear.

\[ \text{Cu}_{\text{a}} \rightarrow \text{low spin heme a/b} \rightarrow \text{heme a}_3/\text{o}_3-\text{Cu}_{\text{b}} \]

Scheme 1

The ba₃-type cytochrome c oxidase from the extremely thermophilic eubacterium Thermus thermophilus HB8 is expressed under elevated temperatures (47–85 °C) and limited oxygen supply (13, 14). As a bacterial terminal respiratory protein complex, it is located in the plasma membrane and catalyzes the reduction of molecular oxygen to water, a process coupled to

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Spectroscopic and Kinetic Properties of ba$_3$ Oxidase

Proton translocation from the cytoplasm to the periplasm. Furthermore, cytochrome ba$_3$ and its counterpart cytochrome caa$_3$ are capable of reducing NO to N$_2$O, although with a low activity (15–19). Despite the very low (<20%) overall sequence homology to other structurally known cytochrome $c$ oxidases, the four redox centers of cytochrome ba$_3$ display high similarity with those of other oxidases (20–23). The mixed valence (Cu$_A^{1.5+}$–Cu$_B^{1.5+}$) complex, bridged by Cys-149 and Cys-153 and coordinated by His-114, His-157, Met-160, and Gln-151 is located 19.0 Å away from the low-spin heme $b$, which is ligated by His-72 and His-386. The low-spin heme is connected to the high-spin heme $a_3$ of the binuclear center through His-386, Phe-385, and axially coordinated to heme $a_3$ His-384. The Fe$^{3+}$–to–Fe$^{3+}$ distance between the two hemes is 13.9 Å; however, they approach each other at a short edge-to-edge distance of 5.0 Å and an angle of 110°. The Cu$_B^{2+}$ atom of the binuclear center is located 4.4 Å from heme $a_3$ Fe$^{3+}$ and is coordinated by His-233, His-282, and His-283. In the reduced form of the enzyme this distance appears enlarged by 0.3 Å (23). The only unusual features in cytochrome ba$_3$ oxidase, compared with the other heme-copper oxidases, are the relatively short heme $a_3$ Fe$^{3+}$–Cu$_B^{2+}$ distance at 4.4 Å (20), (4.8–5.3 Å in caa$_3$, bo$_3$, and cbb$_2$-type oxidases) and the significantly larger heme $a_3$ Fe$^{3+}$–His-384 distance at 3.3 Å (2.0–2.2 Å in caa$_3$, bo$_3$, and cbb$_2$-type oxidases) (24–32).

Three distinct families of heme-copper oxidases have been identified. The type A oxidase superfamily is similar to the mitochondrial (aaa$_3$) enzyme. The type-B oxidase is related to the ba$_3$ from T. thermophilus whereas the type C are the cbb$_2$ type oxidases. The thermophilic Gram-negative eubacterium T. thermophilus HB8 (ATCC27634) expresses cytochromes caa$_3$ and ba$_3$, which serve as terminal oxidases for reducing oxygen to water. In contrast to the environment of the four metal centers, several amino acid residues that have been shown to be essential for proton transfer in other cytochrome $c$ oxidases are not conserved in cytochrome ba$_3$ oxidase (20, 21). Specifically, most of the amino acids forming the K and D channels, established for the mitochondrial enzymes, are replaced, and only the modified K-channel shows a possible functionality (20, 33). On this line, the axial heme $a_3$ ligand His-384 as well as Asn-366, Asp-372, and the propionate group of heme $a_3$ pyrrole ring A (20, 34, 35), leading to the conserved among all structurally known oxidases, water pool, which is connected to the bulk solvent, has been proposed to serve as the primary acceptor of both the pumped protons and the catalytically formed water molecules. Besides the significant structural differences observed in the proton pathways, a vectorial consumption of 1.0 H$^+/e^–$ was measured for the water formation along with a lowered proton pumping efficiency of 0.5 H$^+/e^–$ instead of 1.0 H$^+/e^–$ found in other oxidases (36, 37).

Several techniques have been employed to study the intraprotein electron transfer reactions during the enzyme catalysis, but it proved difficult to directly monitor the sequence of these non-light driven processes (38–43). However, the final heme-heme electron transfer step can be followed through the reverse electron flow between the two hemes, initiated by photolysis of CO from the reduced heme $a_3$ or $b_3/0_2$ under conditions where the low-spin heme $a$ or $b$ is initially oxidized. This form of the enzyme can be prepared by overnight exposure of the protein solution to CO gas and through the oxidation of CO to CO$_2$, leading to the so-called mixed valence state where the binuclear center is reduced but the other two electron donor sites (low-spin heme and Cu$_A^+$) remain oxidized (44). Binding of CO to heme $a_3$ raises the redox potential of the high-spin heme and traps the electrons at the active site. Photodissociation of CO from the high-spin heme causes the lowering of the heme redox midpoint potential and reverse electron transfer among the redox centers. Studies on aa$_3$ oxidases showed that fast electron equilibration between the two hemes occurs on the nanosecond time scale followed by additional redistribution with time constant of few μs (45, 46). At later times (30–50 μs) further equilibration involves the Cu$_A^+$ complex; however, its extent is small. At high pH values the overall process includes an additional ET phase between the hemes, in the millisecond time range, which is coupled to proton transfer through the K-pathway and release of a proton to the bulk medium. Rebinding of CO to high-spin heme induces the back-reactions of electron and proton transfer. This way the reverse ET in various CcOs was determined and found to be from 25 to 35% in Escherichia coli bo$_3$ (47) and bovine heart aa$_3$ (48) to 50–100% in Paracoccus denitrificans and Rhodobacter sphaeroides aa$_3$ oxidases (49–52).

In the case of fully reduced cytochrome ba$_3$, the heme $a_3$–Cu$_B$ binuclear center is subjected to peculiar properties (53–57) characterized by a high affinity of Cu$_B$ for CO ($K > 10^4$ M$^{-1}$) and a slow intramolecular ligand transfer to heme $a_3$ ($k = 8$ s$^{-1}$). As a result, cytochrome ba$_3$ is the only documented oxidase where the binding of CO to Cu$_B$ is exergonic and accounts for 25–30% of the total enzyme concentration (34). Time-resolved step-scan FTIR studies showed that after photolysis of CO from heme $a_3^{2+}$, the ligand is almost quantitatively transferred to Cu$_B^{1+}$, and the transiently formed Cu$_B^{1+}$–CO complex exhibits an unusual long lifetime of 20 ms. The midpoint redox potentials of hemes $b$ and $a_3$ have been reported by two groups and found them inverted, having values of 210/213 and 430/285 mV (versus Normal Hydrogen Electrode), respectively (58, 59). It was concluded that uncommon electron transfer properties should be expected relative to other oxidases. However, there is no experimental evidence about the active ET pathways that contribute to them and how the observed kinetic and thermodynamic deviations, relative to the mesophilic aa$_3$-type oxidases, are linked to protein structure.

This study focuses on two main subjects. The first is to fully characterize the reduced form of the enzyme through the binding of CO in a wide pH/pD range and follow the transient events occurred after the CO-photolysis from heme $a_3$. This way we will reach definite thermodynamic and kinetic conclusions about structural changes that may play a role under physiologically relevant conditions. A time-resolved step-scan FTIR technique is a sensitive probe able to monitor these transient and/or labile structures. The second is to use FTIR spectroscopy to follow the possible intramolecular electron transfer between the redox-active centers in ba$_3$-cytochrome $c$ oxidase from T. thermophilus.

The property of selectively reducing the heme $a_3$–Cu$_B$ binuclear site by the inhibitor CO permits the measurement of the
rate constants of the individual ET reactions through the extent of the reverse electron transfer induced by the photodissocia-
tion of CO from heme \(a_3\). Fe\(^{2+}\). Surprisingly, photolysis of CO from heme \(a_3\) does not result in reverse electron transfer from the heme \(a_3\) to the low spin heme \(b\), as it has been observed in all other heme-copper oxidases previously examined. Further-
more, comparison between the equilibrium fully-reduced and
-mixed valence spectra suggests that the proposed Cu\(_A\)-Cu\(_B\)
direct electron transfer pathway is not active in the reverse (back) direction. These results together with the already known inverted midpoint potentials of hemes \(b\) and \(a_3\) suggest that cytochrome \(ba_3\) uses a different mechanism of energy conver-
sion to achieve exergonicity during catalysis. Tracing the observed and calculated ET pathways found in other oxidases, in the cytochrome \(ba_3\) structure, allows us to identify the key structural differences that lead to this unusual behavior. Because the direct (redox-driven) or indirect (through confor-
mational changes) coupling of the electron transfer/oxygen
chemistry and proton pumping activity is generally accepted, the study presented here provides evidences for the overall mechanism of action. Finally, we discuss the conclusions in terms of how the proteins from thermophilic microorganisms are adapted to their extreme environment and what structural changes they use relative to the mesophilic ones to function effectively.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—Cytochrome \(ba_3\) was isolated from \(T.\ thermophilus\) HB8 cells according to previously published procedures (20). The samples used for the FTIR measurements had an enzyme concentration of 1.0–1.5 mM and were placed in a desired buffer (pH/pD 5.25–6.50, MES; pH/pD 6.50–8.50, Tris or HEPES; pH/pD 8.50–10.10, CHES). The solutions prepared in D\(_2\)O were measured assuming pD = pH + 0.4 (60). The fully reduced \([\text{Cu}_A\text{-Cu}_A\text{]}^{2+}, \text{Fe}^{2+}, \text{Fe}_{a_3}^{2+}, \text{Cu}_{b}^{1+}\] \(ba_3\)-CO complexes were formed by the exposure of sodium dithionite-reduced samples to 1 atm of CO (0.93 mM) and transferred to a tightly sealed FTIR cell composed of two CaF\(_2\) windows under anaerobic conditions. The path length was 14 and 21 \(\mu\)m for the H\(_2\)O and D\(_2\)O solutions, respectively. \(^{12}\)CO (99.5%) gas was obtained from Messer (Germany), and isotopic CO (\(^{12}\)CO) was purchased from Isotec (Miamisburg, OH). The mixed valence \([\text{Cu}_A\text{-Cu}_A\text{]}^{3+}, \text{Fe}_b^{3+}, \text{Fe}_{a_3}^{2+}, \text{Cu}_{b}^{1+}\] CO-bound form of the enzyme was prepared by overnight incuba-
tion of oxidized cytochrome \(ba_3\) with CO in the complete absence of O\(_2\) at room temperature. Upon oxidizing CO to CO\(_2\) (CO + H\(_2\)O \(\rightarrow\) CO\(_2\) + H\(_2\)), the enzyme becomes partially reduced and is stabilized by the binding of CO (44). To obtain the high concentration of the mixed valence \(ba_3\)-CO complex required for the FTIR measurements, a small amount of sodium dithionite Na\(_2\)S\(_2\)O\(_4\) (250 \(\mu\)M) was introduced in the \(ba_3\) sample to initiate the process. Under these conditions no significant trace of fully reduced cytochrome \(ba_3\) was detected as verified from the optical spectrum.

**FTIR Spectroscopy**—FTIR measurements were performed on a Bruker Equinox IFS55 spectrometer equipped with a mer-
cury cadmium telluride detector (Graseby D316). Its preampli-
fier (ac-coupled) allows us to record spectra with signal-to-
noise ratios higher than 40,000/1 in 1000 scans. All the spectra presented here are an average of 2000 scans. The instrument and the optical pathway were purged with N\(_2\), the spectral res-
olution was 4 cm\(^{-1}\), and the covered spectral range was 1200–
3000 cm\(^{-1}\). Power spectrum apodization with 32 cm\(^{-1}\) phase resolution and 4-point phase correction algorithm were used. The temperature was 20 °C. The 416-nm line of a continuous wave TuiOptics Diode Laser System (DL 100) was used as a pump light to photodissociate CO from cytochrome \(ba_3\)-CO complex. The incident power on the sample was 13 milliwatts, which corresponds to a photolysis yield of 35%. Care was taken with the mixed valence samples because prolonged irradiation leads to photoeduction to the fully reduced cytochrome \(ba_3\)-
CO. All the spectra shown are static light (laser-irradiated protein)-minus-dark (no irradiation) difference spectra reflecting the CO-dissociated form (positive peaks) and the CO-bound enzyme (negative peaks). The optical absorption spectra were recorded with a PerkinElmer Life Sciences Lambda 20 UV/visible spectrometer.

**Time-resolved Step-scan FTIR Spectroscopy**—The 532-nm pulses from a continuum Nd-YAG laser (7-ns wide, 3–7 Hz) were used as a pump light (10 mJ/pulse) to photolysse 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 exper-
iments, 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 start data collection before the laser fires allows 11 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 mercury cadmium telluride detector (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 \(\mu\)m was used to limit the free spectral range from 2.67 to 8 \(\mu\)m. This leads to a spectral range of 3900 cm\(^{-1}\), which is equal to an under-sam-
pling ratio of 4. Single-sided spectra were collected at 8 cm\(^{-1}\) spectral resolution, 5- or 100-\(\mu\)s time resolution, and 10 co-ad-
ditions per data point. Total accumulation time for each measure-
ment was 60 min, and 2–3 measurements were collected and averaged. Blackman-Harris three-term apodization with 32 cm\(^{-1}\) phase resolution and the Mertz phase correction algo-

**RESULTS**

**CO Binding in the Fully Reduced and Mixed Valence States of Cytochrome \(ba_3\)**—Fig. 1 displays the optical absorption spectra of the greatly reduced \([\text{Cu}_A\text{-Cu}_A\text{]}^{2+}, \text{Fe}^{2+}, \text{Fe}_{a_3}^{2+}, \text{Cu}_{b}^{1+}\] and mixed valence \([\text{Cu}_A\text{-Cu}_A\text{]}^{3+}, \text{Fe}_b^{3+}, \text{Fe}_{a_3}^{2+}, \text{Cu}_{b}^{1+}\] \(ba_3\)-CO complexes. The addition of CO to dithionite-reduced enzyme causes a shift of the Soret band of heme \(a_3\) from 444 to 427 nm and of the visible band from 614 to 592 nm, whereas the bands of the reduced heme \(b\) at 427 and 559 nm remain

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unchanged (34). Overnight incubation of oxidized cytochrome $b_{a_3}$ with CO results in the reduction of the binuclear center, whereas the heme $b$ and CuA centers remain oxidized. This form of the enzyme is finally stabilized by the binding of CO to heme $a_3\text{Fe}^{2+}/\text{H}^{11001}$ and CuB$_1\text{Fe}^{2+}/\text{H}^{11001}$. The optical spectrum shown in Fig. 1 confirms the mixed valence state of the $b_{a_3}$-CO complex as it exhibits band characteristics of the heme $a_3\text{Fe}^{2+}/\text{H}^{11001}$-CO complex at 427 and 592 nm, of oxidized heme $b$ at 415 nm, and of reduced heme $a_3$ at 444 and 614 nm. The fraction of the unligated heme $a_3\text{Fe}^{2+}$ represents the equilibrium complex A (Scheme 2) (34), where the CO is bound to CuB and is calculated to the same extent as in the fully reduced state of the enzyme (25%). Based on the small 559-nm band that appeared in the spectrum of the mixed valence cytochrome $b_{a_3}$-CO, we also conclude that 5–8% of heme $b$ is reduced.

Fig. 2 presents the equilibrium FTIR spectra of the fully reduced cytochrome $b_{a_3}$-CO complex at the indicated pH and pD values. The spectra at pH 5.25 and 9.60 and pD 5.50 and 7.50 represent the $b_{a_3}$-CO complex. In the $^{12}$C$^{16}$O derivatives we observe four CO-sensitive modes at 1967, 1971, 1973, and 2053 cm$^{-1}$, which shift to 1923, 1927, 1929, and 2007 cm$^{-1}$, respectively, upon replacement of $^{12}$CO with $^{13}$CO. The first three peaks are assigned to the C-O stretching vibration of heme $a_3\text{Fe}^{2+}$-CO complex (equilibrium complex B, Scheme 2), whereas the peak at 2053 cm$^{-1}$ represents the equilibrium CuB$_1\text{Fe}^{2+}$-CO complex (equilibrium complex A, Scheme 2), which accounts for almost 25% of the enzyme (34). The frequencies and bandwidths of these C-O modes remained unchanged between $H_2O$ and $D_2O$ and also in the 5.25–10.10 pH/pD range.

Fig. 3A shows the double difference equilibrium FTIR spectrum of the fully reduced (FR) cytochrome $b_{a_3}$-CO complex at pH 8.5. The solid line represents the MV form ([Cu$_8$-Cu$_7$]$^{2+}$, Fe$_b^{3+}$, Fe$_a^{2+}$, Cu$_b^{1+}$], and the dotted line represents the FR form ([Cu$_8$-Cu$_7$]$^{2+}$, Fe$_b^{3+}$, Fe$_a^{2+}$, Cu$_b^{1+}$]) of the $b_{a_3}$-CO complex.

**SCHEME 2**

\[
\begin{align*}
\text{Fe}^{2+}\text{CuB}^+ + \text{CO} & \xrightleftharpoons{\kappa_1} \text{Fe}^{2+},\text{CuB}^+-\text{CO} \\
\text{Fe}^{2+},\text{CO},\text{CuB}^+ & \xrightleftharpoons{\kappa_2} \text{Complex A} \\
\text{Complex B} & \end{align*}
\]
pD 8.5. Three major heme $a_3$ $Fe^{2+}$-CO conformers are evident from the presence of CO-sensitive modes at 1967.5, 1973.0, and 1981.5 cm$^{-1}$, whereas the Cu$_B$ $^{1+}$-CO complex (2053.5 cm$^{-1}$) adopts a single conformation. The ratio of the Fe-CO/Cu-CO relative areas is 5.2 and considering the integrated absorptivities expected for the observed heme $a_3^{2+}$-CO and Cu$_B$$^{1+}$-CO complexes ($\epsilon_{Fe-CO}/\epsilon_{Cu-CO} = 1.68$) (61), we conclude that 73% of the enzyme has the CO bound in heme $a_3$ and 27% in the Cu$_B$. This ratio corresponds to an equilibrium constant of 2.7, or $\Delta G^e = -0.55$ kcal/mol, for the transfer of CO from Cu$_B$ to heme $a_3$ $Fe^{2+}$ at 293 K. The three major modes at 1967.5, 1973.0, and 1981.5 cm$^{-1}$ represent the 93.5% of the total heme $a_3$ $Fe^{2+}$-CO complexes, and their relative ratios reflect an energy difference $\Delta G^e$ of $-0.15$ and $-0.70$ kcal/mol between the 1981.5–1973.0 cm$^{-1}$ and 1967.5–1973.0 cm$^{-1}$ conformers, respectively, at 293 K. None of the observed modes showed sensitivity to H/D exchange and in the 5.25–10.10 pH/pD range, suggesting a highly rigid and hydrophobic environment for the binuclear center (34, 62). The adoption of a single geometry for the Cu$_B$$^{1+}$-CO complex reflects the stability of the Cu$_B$ environment (His-282, His-283, His-233–Tyr-237), which is not subjected to protonation/deprotonation changes.

Fig. 3B presents the double difference equilibrium FTIR spectrum of the mixed valence (MV) $ba_3$-CO complex at pH 8.5. All the heme $a_1$ $Fe^{3+}$-CO modes detected for the FR state are slightly (1–3 cm$^{-1}$) upshifted, and the Cu$_B$-CO mode is downshifted by 2.5 cm$^{-1}$, in agreement with previous observations for the E. coli $bo_3$ and bovine $aa_3$ oxidases (47, 48, 63). The three major peaks at 1971.0, 1975.5, and 1982.5 cm$^{-1}$ represent again the 93.0% of the total heme $a_3$ $Fe^{2+}$-CO complexes; however, the high energy conformer becomes dominant in expense of the 1973.0 cm$^{-1}$ (FR state) conformer. No difference in the 6.50–8.50 pH/pD range was detected, in agreement with the observations made for the FR state (data not shown). The relative Fe-CO/Cu$_B$-CO area ratio is reduced from 4.7 to 5.2 in the FR state; considering the change of the integrated absorptivities for the shifted heme $a_3^{2+}$-CO and Cu$_B$$^{1+}$-CO bands ($\epsilon_{Fe-CO}/\epsilon_{Cu-CO} = 1.62$), we calculate a 71–29% equilibrium ratio of the bound CO between the heme $a_3$ $Fe^{2+}$ and Cu$_B$ centers. The observed frequency shifts of the CO-bound modes and the inverted population ratio between the 1973.0 and 1981.5 cm$^{-1}$ conformers are attributed to the presence of CO$_2$/H$_2$CO$_3$ molecules, catalytically formed during the reduction of the binuclear center by the CO. The altered electrostatic interactions induced by the presence of the CO$_2$/H$_2$CO$_3$ molecules in the binuclear center appears to shift the fragile (0.15 kcal/mol) equilibrium toward the 1981.5 cm$^{-1}$ conformer. However, these changes do not seem enough to drive the 1967.5–1973.0 cm$^{-1}$ modes equilibrium (0.70 kcal/mol), and their magnitude is calculated to 0.50 kcal/mol, as evidenced from the slight equilibrium shift in the heme $a_3$ to Cu$_B$ CO transfer. Details about this effect will be given elsewhere.

Comparison between the spectra presented in Fig. 3 reveals that no extra CO-sensitive modes relative to the FR, is detected in the MV state of cytochrome $ba_3$-CO complex. Through the crystal structure it was proposed that Cu$_B$ might be involved in a direct electron transfer pathway between the Cu$_A$ site and the binuclear center. Our data clearly show that Cu$_B$ remains reduced when CO is bound to heme $a_3$, and no reverse electron transfer takes place between Cu$_B$ and Cu$_A$. If a (Cu$_A$-Cu$_A$)$^{2+}$, Fe$_{a_3}$$^{2+}$-CO, Cu$_B$$^{2+}$ complex would be generated instead of the ordinary (Cu$_A$-Cu$_A$)$^{3+}$, Fe$_{a_3}$$^{2+}$-CO, Cu$_B$$^{+}$, the heme $a_3^{2+}$-CO complex would exhibit different O stretching frequencies. In particular, the C-O modes would be shifted to lower frequencies, as the more polar Cu$_B$$^{2+}$ would stabilize the C$^{6+}$-O$^{6-}$ resonance form. However, these results do not exclude the possibility of the forward ET between the two Cu sites.

Photodissociation of CO from the Fully Reduced and Mixed Valence States of Cytochrome $ba_3$—Fig. 4 shows the step-scan time-resolved FTIR difference spectra ($t_d$ = 150 µs–78.5 ms, 8-cm$^{-1}$ spectral resolution) of fully reduced $ba_3$-CO (pH 6.50) subsequent to CO photolysis by a nanosecond laser pulse (532 nm). Under these experimental conditions, the 1967, 1973, and 1982 cm$^{-1}$ peaks cannot be resolved, and thus a single negative peak was observed at 1977 cm$^{-1}$. The negative peak at 1977 cm$^{-1}$ arises from the photolysed heme $a_3$-CO. The positive peak that appears at 2053 cm$^{-1}$ is attributed to the C-O stretch ($v_{C-O}$) of the transiently formed Cu$_B$$^{1+}$-CO complex, as found under continuous light illumination (53), and arises from the near ballistic transfer of the photolysed CO to Cu$_B$. Its intensity persists until almost 70 ms. The frequency of the C-O mode in the transient Cu$_B$$^{1+}$-CO complex is the same as that of the equilibrium Cu$_B$$^{1+}$-CO (complex A). This observation suggests that no structural change at Cu$_B$ occurs in association with CO binding to and dissociation from heme $a_3$. No significant intensity variations are detected in the transient difference spectra ($t_d$ = 5–3000 µs) for either the 2053 and 1977 cm$^{-1}$ modes. At later times, however, the decreased intensity of the transient 2053 cm$^{-1}$ mode (3–70 ms) is accompanied by an increased intensity at 1977 cm$^{-1}$. The intensity ratio (1.5) of the Fe-CO/Cu$_B$-CO remains constant for all data points, and thus, we conclude that no significant fraction of CO escapes the binuclear center at 293 K (34). This is consistent with the low temperature experiments in which it was demonstrated that the Cu$_B$$^{1+}$-CO intermediate is fully formed and that no significant fraction of CO escapes the metal centers below 300 K (53).
Figs. 5 and 6 display the kinetic evolution of the 1977 (pho-
tolysed heme $a_3^{2+}$/CO complex) and 2053 cm$^{-1}$ (transiently formed CuB$^1$/CO complex) modes at the indicated pH and pD values. The data showed no significant pH dependence of both rates. The rate of the transiently formed CuB$^1$/CO complex exhibited a decay with $t_{1/2} = 23.6$ ms, which corresponds to a $k_{1\text{obs}}$ value of 29.4 s$^{-1}$ at pH 7.50, and this rate became slightly ($\approx 15\%$) bigger at pH 6.00 and smaller ($\approx 20\%$) at pH 9.00. Rebinding of CO to heme $a_3$ Fe$^{2+}$ occurred with $t_{1/2} = 28.0$ ms ($k_{2\text{obs}} = 24.8$ s$^{-1}$), and this rate was also slightly pH-dependent in the same trend as the decay of the CuB$^1$/CO complex. The same behavior was observed in the experiments performed in D$_2$O (Fig. 6).

Fig. 7A shows the static light-minus-dark FTIR spectrum of fully reduced $ba_3$-CO complex. Photolysis of the heme $a_3$-CO complex by 416 nm c.w. laser light results in the migration of CO to Cu$\beta$. The ratios of the negative Fe-CO bands were the same with those observed in the absolute FTIR spectrum, and the frequency of the transient Cu$\beta$-CO complex was the same (2053.5 cm$^{-1}$) with that of the equilibrium complex. Besides the small Cu$\beta$-13CO band at 2007.5 cm$^{-1}$, which represents the 1.1% natural abundance of 13CO, no other CuB-CO mode was detected in the 2000–2100 cm$^{-1}$ spectral region (Fig. 7A, spectrum b), even at a ratio of 0.5% of the 2053.5 cm$^{-1}$ peak. This is in agreement with the absolute FTIR spectrum where only a single Cu$\beta$-CO mode was detected in contrast to the multiple heme $a_3$-Fe-CO modes. The ratio of the Fe-CO/Cu$\beta$-CO integrated areas is 2.32. Using Alben's correlation of vibrational frequencies with the integrated absorptivities for CO complexes, we conclude that 75% of the photolysed CO is transferred to Cu$\beta$. This value is somewhat lower than the 100% reported earlier in the time-resolved step-scan FTIR study of

FIGURE 5. Kinetic analysis of the heme $a_3^{2+}$/CO and CuB$^1$/CO modes at indicated pH values. Shown is a plot of the 2053 cm$^{-1}$ (squares) and 1977 cm$^{-1}$ (circles) modes versus time subsequent to CO photolysis. $\Delta A$ was measured from the bands area at times between 0 and 78.5 ms subsequent to CO photolysis from heme $a_3$. The curves are three-parameter exponential fits to the experimental data according to first-order kinetics.
However, the difference is attributed to the limited spectral resolution (8 cm\(^{-1}\)) of the step-scan measurements, under which the absolute intensities of the multiple heme \(a_3\)-CO bands could not be resolved.

Fig. 7B displays the light-minus-dark difference FTIR spectrum of mixed valence cytochrome \(ba_3\)-CO complex subsequent to a 416-nm laser photolysis. In agreement with the FR complex, the transient CuB-CO complex in the MV form absorbed at the same frequency as the equilibrium one at 2051.0 cm\(^{-1}\), and no changes were observed after H\(_2\)O/D\(_2\)O exchange and in the 6.5–8.5 pH range (data not shown). However, the ratio of the Fe-CO/CuB-CO integrated areas was reduced to 2.06 compared with the 2.32 value in the FR \(ba_3\)-CO, leading to an 80% transfer of the photolysed CO to CuB. The increased compared with FR, transfer of CO to CuB is attributed to structural changes in the binuclear center due to the presence of the CO\(_2\)/H\(_2\)CO\(_3\) molecules, byproducts of the MV \(ba_3\)-CO complex formation. We suggest that carbon dioxide occupies the same secondary binding site, which has been previously shown to temporally host photolysed CO molecules preventing this way the escape of CO to the solvent (64, 65).

As shown in the 10-fold expanded 2000–2100 cm\(^{-1}\) spectral region (Fig. 7B, spectrum b), no other CuB-CO mode was detected even at a ratio of 0.5% of the 2051.0 cm\(^{-1}\) band. The absence of any other than the 2051.0 cm\(^{-1}\) CuB-CO mode in a spectral region with a very high signal-to-noise ratio > 10\(^6\) reflects the complete absence of reverse electron transfer from the \(ba_3\)-CO (34). However, the difference is attributed to the limited spectral resolution (8 cm\(^{-1}\)) of the step-scan measurements, under which the absolute intensities of the multiple heme \(a_3\)-CO bands could not be resolved.
**Spectroscopic and Kinetic Properties of ba$_3$ Oxidase**

### TABLE 1

| Enzyme                   | Reverse electron transfer (a$_3$/o$_3$)$^{3+}$ $\rightarrow$ (a/b)$_{3+}$ | Reference | Midpoint redox potential (vs. SHE) | Reference |
|--------------------------|-------------------------------------------------|-----------|-----------------------------------|-----------|
| *P. denitrificans* aa$_3$| 50% at pH 7.0 100% at pH 11.0                    | 49        | pH 8.0                           | 69        |
| Bovine heart aa$_3$      | 21 and 17 cm$^{-1}$                              | 48        | ND                               | 58        |
| *R. sphaeroides* aa$_3$  | 2046                                              | 47        | pH 7.6                           | 59        |
| *E. coli* bo$_3$         | 0% at pH 6.5–8.5                                 | This work | Heme $b$: 210 mV                |           |
| *T. thermophilus* ba$_3$ | 4.2% at pH 7.4                                    |           | Heme $a$: 430 mV                |           |

The high spin heme a$_3$$^{3+}$ to [(Cu$_{a}$/Cu$_{b}$)$_{3+}$, heme b$^{3+}$] upon dissociation of CO from heme a$_3$. If a [(Cu$_{a}$/Cu$_{b}$), Fe$_3$$^{5+}$, Fe$_{a3}$$^{3+}$, Cu$_b$$_{1+}$-CO complex would be generated instead of the ordinary [(Cu$_{a}$/Cu$_{b}$), Fe$_b$$^{6+}$, Fe$_{a3}$$^{2+}$, Cu$_b$$_{1+}$-CO, the Cu$_b$$_{1+}$-CO complex would exhibit a clear different C-O stretching frequency.

In particular, the C-O mode would be shifted to lower frequencies ($\approx 2030$ cm$^{-1}$), as the more polar heme a$_3$.Fe$^{3+}$ would stabilize the C$^{b+}$-O$^{b-}$ resonance form of the CO. This result is in contrast to all the other previously examined oxidases, which showed a significant fraction (25–100%) of back electron transfer from heme a$_3$/o$_3$ to the low-spin heme a/b (Table 1).

FTIR studies on the MV complex of *E. coli* bo$_3$ and bovine heart aa$_3$ oxidases have shown that c.w. laser photolysis of the CO-bound form of the enzyme results in an additional transient Cu$_b$$_{1+}$-CO complex, not present in the FR state, detected at 2046 and 2040 cm$^{-1}$, respectively (47, 48). The observed frequencies were 21 and 17 cm$^{-1}$ lower than the main Cu$_b$$_{1+}$-CO conformer, and the presence of those bands was attributed to the reverse electron transfer from heme a$_{3+}$/o$_{3+}$ to heme a$_{3+}$/b$^{1+}$. All the vibrational modes associated with the photolysis of the fully reduced and mixed-valence forms of various Co enzymes are summarized in Table 2.

### DISCUSSION

This study is mainly focused on the thermodynamic and kinetic investigation of two distinct forms of cytochrome ba$_3$-CO complex; that is, the fully reduced and the mixed valence. Through them we are also able to monitor possible heme a$_3$ $\rightarrow$ heme b and Cu$_b$ $\rightarrow$ Cu$_a$ ET processes of the enzyme. FTIR spectroscopy has been proven a structure- and redox-sensitive tool capable of monitoring the redox state of the active sites and their response to an external perturbation such as the photolysis of CO from heme a$_3$. This way we are able to reach physiologically relevant conclusions about the mechanism of action during catalysis.

### Binding, Photodissociation, and Recombination Kinetics of CO in ba$_3$-Cytochrome c Oxidase—The general aspect of the CO binding, photodissociation, and rebinding kinetics observed by the static FTIR and the time-resolved step-scan FTIR data presented in Figs. 1–7 can be understood through the model depicted in Scheme 3. It should be noted that in cytochrome ba$_3$-CO there is a 25–75% thermal distribution between states B and C not found in any other cytochrome c oxidase. However, the equilibrium Cu$_b$$_{1+}$-CO complex is not photolabile and remains a spectator through the photodissociation rebinding processes of heme a$_3$$^{3+}$-CO complex and formation decay processes of transient Cu$_b$$_{1+}$-CO complex.

In Scheme 3 $k_1$ represents the binding of CO to Cu$_b$ and $k_2$ represents its transfer to heme a$_3$. On the other hand, $k_{-1}$ and $k_{-2}$ reflect the reverse processes, and $k_3$ reflects the formation of the transient Cu$_b$$_{1+}$-CO complex from the geminate photoproduction (state D), which is expected to be formed in less than 100 fs as found in aa$_3$ oxidase (40, 55).

The five equations that describe kinetically our system are:

$$\frac{d[Fe-CO, Cu]}{dt} = k_2[Fe, Cu-CO] - k_{2}[Fe-CO, Cu] = k_{2}[Fe, Cu-CO]$$

(Eq. 1)
As \([\text{CO}]\) becomes equal to the term \(1/K_1\), our solution exhibits saturation behavior, so Equations 5 and 7 can be rearranged to

\[\text{[Fe, Cu]}_T \approx \text{[Fe, Cu-CO]} \quad (\text{Eq. 10})\]

\[k_2 = k_{2\text{obs}} \quad (\text{Eq. 11})\]

Equation 6 can now be rearranged as

\[k_2\text{[Fe, Cu]}_T = k_{2\text{obs}}\text{[Fe, Cu]}_T \quad (\text{Eq. 12})\]

In the case of cytochrome \(ba_3\)-CO, we can assume saturation conditions, as \([\text{CO}] = 10^{-3} \text{ M} \); therefore, \([\text{CO}] \gg 1/K_1 = 10^{-4} \text{ M}\). So, from Equation 11 and our experimental data, we are in position to calculate rate constant \(k_2\).

As shown in Figs. 5 and 6, the decay of the transient \(Cu_b^{1+}\)-CO complex is accompanied by the concomitant rebinding of CO to heme \(a_3\), suggesting the absence or decay of any thermodynamic and/or kinetic barrier. For example at pH 7.50 we measure a \(k_{1\text{obs}}\) of 29.4 s\(^{-1}\) and a \(k_{2\text{obs}}\) of 24.8 s\(^{-1}\), which is larger than \(k_{2}\) (0.8 s\(^{-1}\)) by a factor of 31, so the previous assumption \(k_2 \gg k_{-2}\) is valid. In addition we do not need to assume that the rate-determining step is \(k_{2}\) and, therefore, \(k_{-1} \gg k_2\).

The fast equilibration between states A and B (\(K_1 = 10^4 \text{ M}^{-1}\)) is responsible for the kinetic identification of the two processes leading to the formation of the \([\text{Fe-CO, Cu}]_T\) complex, the one that follows photodissociation \(k_{2\text{obs}}\) and the thermal one \(k_{-2}\). The final result of \(k_3 = k_{3\text{obs}}\) is valid only for cytochrome \(ba_3\), as it is the only oxidase that exhibits saturation kinetics behavior under normal CO pressure (1 atm) and temperature (293 K) conditions. The reason for this behavior is the exceptionally high affinity of \(Cu_b\) for \([\text{CO}]\) in contrast to what has been found to all the other heme-copper oxidases.

As we will see below, cytochrome \(ba_3\) is the only oxidase where this assumption is solid.

With the assumption \(k_2 \gg k_{-2}\), Equation 1 can be rewritten,

\[k_2\text{[Fe, Cu-CO]} = k_{2\text{obs}}\text{[Fe, Cu]}_T \quad (\text{Eq. 6})\]

Combining Equations 3, 5, and 6, we reach

\[k_jk_1\text{[Fe, Cu]}_T + k_{2\text{obs}}\text{[Fe, Cu]}_T = k_{2\text{obs}}\text{[Fe, Cu]}_T \quad (\text{Eq. 7})\]

To reach to the above result, we assumed that states A and B are in equilibrium, so the rate-determining step is \(k_2 \gg k_{-2}\).

When \([\text{CO}] \ll 1/K_1\) (nonsaturation conditions) \([\text{Fe, Cu]}_T \approx [\text{Fe, Cu}]\), and accordingly Equation 7 leads to:

\[k_{2\text{obs}} = k_2k_1[\text{CO}] \quad (\text{Eq. 8})\]

and Equation 6 can then be transformed to

\[k_2k_1[\text{CO}][\text{Fe, Cu]}_T = k_{2\text{obs}}[\text{Fe, Cu]}_T \quad (\text{Eq. 9})\]
Spectroscopic and Kinetic Properties of \(\text{ba}_3\) Oxidase

Electron Transfer in Cytochrome \(c\) Oxidase—Cytochrome \(c\) oxidase utilizes a complex pattern of ET through strongly coupled substrate and redox-dependent sites that involve intermediate cofactors and nonadiabatic, long distance, single-electron tunneling between weakly coupled redox centers (5, 11, 45, 46, 66). In the aerobic electron transfer chain of \(T.\) thermophila, electrons are donated by cytochrome \(c_{552}\) and enter \(\text{ba}_3\)-cytochrome \(c\) oxidase through the dinuclear Cu\(A\) site (39, 58, 59, 67). The interactions between this pair were found to be mainly hydrophobic (68), in contrast to other oxidases where an electrostatic character was described. Furthermore, the physiological direction of the reaction is also thermodynamically favorable, as the midpoint \(E_m\) of cytochrome \(c_{552}\) and Cu\(A\) complex are 200–230 and 240–260 mV (57, 68), respectively. Similar values for the Cu\(A\) complex have been obtained for the bovine heart and \(P.\) denitrificans \(aa_3\) oxidases (69). The next step in cytochrome \(ba_3\) involves the Cu\(A\)-heme \(b\) electron transport, a reaction thermodynamically unfavorable as the low-spin heme \(b\) exhibits a redox potential of 210 mV (58, 59). Calculations with the method of tunneling currents in bovine heart \(aa_3\) oxidase suggested that this transfer is mediated through pathways involving the His-157, Gln-151, Cys-149, and Cys-153 Cu\(A\) ligands and the pair of Arg-449 and Arg-450. The current enters the heme mainly through the ring D propionate group (70).

Electron Transfer between Hemes in Cytochrome \(ba_3\) Oxidase—The final step in the ET chain is the transfer to the binuclear center through the heme \(b\)-heme \(a_3\) pathway. This step is characterized by a high driving force as the midpoint redox potentials of heme \(a_3\) and heme \(b\) were found to be 430 (58)/285 (59) and 210 mV, respectively. Although these values are highly model-dependent and no cooperative or anticooperative effects were taken into account, the downhill flow of the electrons is secured because of the above-mentioned inverted midpoint potentials. To study this ET step we performed photolysis of the mixed-valence state of cytochrome \(ba_3\)-CO to follow the possible reverse electron transfer between the hemes as previously shown on \(aa_3\) and \(ba_3\) oxidases. The data presented in Fig. 7 revealed that no such process takes place in cytochrome \(ba_3\), a unique property among the various cytochromes \(c\) oxidases examined (39–43). It should be noted that Silesky et al. (38) also studied the electron backflow in \(ba_3\)-oxidase using optical spectroscopy at the reduced heme \(a_3\) band (445 nm) and electrophoretic measurements. Although they observed a 4.2% electron redistribution between hemes \(a_3\) and \(b\), after CO photolysis those data are in relative agreement with ours in terms that in both studies cytochrome \(ba_3\) exhibited a significant reduced reverse electron transfer ratio relative to other heme-copper oxidases.

Exploring the reasons for this behavior, the basis of electron transfer in proteins is two or more single- or multi-electron redox centers usually connected through a chain of protein residues, with the three parameters affecting the intra-protein ET rates being the edge-to-edge distance, driving force \(\Delta G\), and reorganization energy \(\lambda\). Heme \(b\) and heme \(a_3\) are connected through His-386, Phe-385, and His-384 (Fig. 8A) a motif conserved in all the structurally known oxidases. Previous studies on \(aa_3\) oxidases have suggested that the dominant ET pathways are through space jumps between the two hemes and through the covalent bonds between His-386, Phe-385, and His-384 (cytochrome \(ba_3\) numbering). A comparison among the available crystal structures suggests that these pathways are also operative in cytochrome \(ba_3\). Therefore, assuming similar edge-to-edge distances and reorganization energies \(\lambda\), we attribute the observed difference in the heme \(b\)-heme \(a_3\) ET process to the high driving force \(\Delta G\) revealed by the electrochemical titrations of hemes \(b\) and \(a_3\) (58, 59).

Previous studies on \(aa_3\) oxidases revealed either undistinguishable potentials for the two hemes or higher potential for the low-spin heme. In contrast, cytochrome \(ba_3\) exhibits inverted potentials of 210 and 285/430 mV for the heme \(b\) and \(a_3\), respectively. Furthermore, both values are shifted to more positive potentials than other heme \(b\) proteins (sperm whale myoglobin 40 mV) and heme \(a_3\), containing oxidases (350 mV in \(P.\) denitrificans \(aa_3\) oxidase) (69). The difference between heme \(b\) and heme \(a_3\) potentials is due to the presence of a C-8 formyl substituent in heme \(a_3\), which is an electron-withdrawing group. The observed shifts to more positive potentials is a result of increasing positive charge on the hemes. This can be achieved by protonation of the heme(s) propionate groups, high hydrophobicity, and alterations of the heme(s) axial ligands. Indeed, subunit I of cytochrome \(ba_3\) is buried to an extreme hydrophobic region, having a pH of 10.4 and a net charge of +9 (21). This nonpolar, low-dielectric microenvironmental conditions favor the positive shift observed.
ment does not allow the heme to receive electron density from water molecules. In addition, spectroscopic studies have shown that the heme b and a₃ propionate groups are partially protonated in cytochrome ba₃ (34, 58) relative to other aa₃ oxidases and also that the heme a₃ axial ligand His-384 appears to have a larger Fe-N bond of 3.3 Å (20), preventing a high degree of π back-bonding electron donation to iron. All these three parameters deviate from what is known in other oxidases and have been tuned in such way to raise the positive charge feeling by the hemes. This leads to an increase of the midpoint potentials and stabilization of the reduced state of hemes. During catalysis, heme a₃ reduction is further favored due to the high driving force of the heme b-heme a₃ ET step, as discussed above.

Direct Electron Transfer from Cuₐ Complex to Heme a₃-Cuₐ Binuclear Center—From the first available crystal structure of bovine heart aa₃-cytochrome c oxidase, it was suggested that electrons can also be supplied to the binuclear center through a direct transfer from the Cuₐ complex. In addition, Soulimane et al. (20) reporting the x-ray structure of cytochrome ba₃ suggested a direct ET from Cuₐ to the binuclear center through a Cuₐ–Cu₈ electron pathway. This pathway, shown in Fig. 8B, involves the Cu₈ ligand Gln-151, Tyr-136, Trp-229, and H283 is-, one of the Cu₈ ligands at a distance of 21.6 Å between the two metals. In cytochrome ba₃, the Cu₈ complex exhibits a midpoint reduction potential of 250 mV similar to that observed in P. denitrificans aa₃ oxidase. The reduction potential of Cu₈ is not known yet; however, according to that determined in aa₃ oxidase (412 mV) (69), we expect similar or even higher potential. Therefore, this step if active is characterized by a high driving force. On the other hand, several spectroscopic studies and pathway analysis data suggested that the Cu₈-Cu₈ ET pathway is not active, presumably due to a long tunneling route between Cu₈ and Tyr-136 and a high reorganization energy. Although cytochrome ba₃ is an ideal system to study these events through the equilibrium heme a₃ Fe²⁺-CO and Cu₈⁻¹-CO modes, the data presented in Fig. 3 rule out the possibility of reverse electron transfer from Cu₈ or heme a₃ to Cu₈. However, the proposal of the forward ET from Cu₈ to Cu₈ or heme a₃ still cannot be excluded.

These results show that ba₃-cytochrome c oxidase uses a clear different structural pattern of energy conversion for the coupling of the electron transfer/oxygen chemistry and proton pumping processes. This pattern has taken into account all the extreme environmental factors that affect the function of the enzyme and is manufactured in such a way that ensures its effective operation.

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