Docking Site Dynamics of \textit{ba}_3-\textit{Cytochrome c Oxidase} from \textit{Thermus thermophilus}

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Running title: Trapping of CO into the docking site of cytochrome \textit{ba}_3

ABBREVIATIONS: FTIR= Fourier Transform Infrared; TRS$^2$-FTIR= Time-Resolved Step-Scan Fourier Transform Infrared; MCT=mercury cadmium telluride; Mb=myoglobin

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Abstract

Ligand trajectories trapped within a docking site or within an internal cavity near the active site of proteins are important issues towards the elucidation of the mechanism of reaction of such complex systems, whose activity requires the shuttling of oriented ligands to and from their active site. The ligand motion within ba₃-cytochrome c oxidase from *Thermus thermophilus* has been investigated by measuring time-resolved step-scan Fourier transform infrared difference spectra of photodissociated-CO from heme *a*₃ at ambient temperature. Upon photodissociation, 15-20% of the CO is not covalently attached to Cu₃b, but is trapped within a docking site near the ring A of heme *a*₃ propionate. Two trajectories of CO that are distinguished spectroscopically and kinetically (νₗ=2131 cm⁻¹/τₗ=10-35 µs and νₗ=2146 cm⁻¹/τₗ=85 µs) are observed. At later times (τₗ=110 µs) the docking site reorganizes about the CO and quickly establishes an energetic barrier that facilitates equilibration of the ligand with the protein solvent. The time-dependent shift of the CO trajectories we observe is attributed to a conformational motion of the docking site surrounding the ligand. The implications of these results with respect to the ability of the docking site to constrain ligand orientation, and the reaction dynamics of the docking site are discussed.
Introduction

The docking sites and internal cavities of proteins and enzymes play a significant role in controlling the pathway(s) for diffusion of substrates/ligands to the active site. Time-resolved x-ray and time-resolved IR experiments of photolyzed CO-bound myoglobin (Mb) have demonstrated the existence of docking sites and their relevance in physiological ligand binding (1-8). However, the different trajectories of the funneled photodissociated ligand in the docking site can be distinguished only through time-resolved IR spectroscopy (2-5). There is now a relatively good understanding in the ligand dynamics of photolyzed Mb-CO. Up to date Mb has played the role of a model system for determining docking sites and channels through which, ligands enter and escape proteins, establishing a foundation for their chemical dynamics (9-11). Similar measurements in enzymes, whose activity may require the shuttling of oriented ligands to and from their active site, however, have never been reported either under physiological conditions or at cryogenic temperatures. In addition to activating O\textsubscript{2} and conserving the energy of the O\textsubscript{2} reduction for subsequent ATP synthesis, \textit{ba}\textsubscript{3}-cytochrome \textit{c} oxidase is able to catalyze the reduction of nitric oxide (NO) to nitrous oxide (N\textsubscript{2}O) under reducing anaerobic conditions (12). To understand the mechanism of reaction in such complex systems, it is crucial to probe in detail the intermediates along the reaction pathway. The time evolution and the trajectories of ligand binding intermediates can be monitored by time-resolved FTIR spectroscopy providing profound information on whether ligands gain access to the binding sites through specific channels and docking sites, or by random diffusion through the protein matrix. Such measurements would enable us to address some new issues regarding the properties of the docking sites that may function to discriminate between ligands such as O\textsubscript{2}, NO and CO.

The crystal structure of cytochrome \textit{ba}\textsubscript{3} indicates that subunit I consists of a low-spin heme \textit{b} and a high-spin heme \textit{a}/Cu\textsubscript{b} binuclear center, where the dioxygen and nitric oxide...
reactions take place (13). Subunit II contains a homodinuclear copper complex. The CO- 
ligation/release mechanism in cytochrome \( ba_3 \) follows that found in other heme-copper 
oxidases (14-16) and proceeds according to the following scheme:

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

Cytochrome \( ba_3 \) has a relative high affinity for CO (\( K_1 > 10^4 \)); the transfer of CO to heme 
\( a_3^{2+} \) is characterized by a small \( k_2 = 8 \text{ s}^{-1} \), and by a \( k_2 = 0.8 \text{ s}^{-1} \) (15), that is 30-fold greater than 
that of the bovine \( aa_3 \). The Cu\(^{1+}\)-CO complex (Complex A) is not photolabile (17), and thus 
remains a bystander in the photodynamic events occurring to complex B. In the latter 
event, 80-85% of the photolyzed CO binds to Cu\(_B\) whereas the other 15-20% migrates to a 
specific docking site, and subsequently diffuses to protein channels prior to its rebinding to 
heme \( a_3 \); the rate of decay of the transient Cu\(^{1+}\)-CO complex is 34.3 \text{ s}^{-1} and rebinding to 
heme \( a_3 \) occurs with \( k_2 = 29.5 \text{ s}^{-1} \) (17,18).

Time-resolved step scan FTIR (TRS\(^2\)) spectra can now be unambiguously interpreted to 
yield specific information concerning the docking sites and their respective dynamic 
behavior in heme-copper oxidases. These observations may be made at room temperature 
under physiologically relevant conditions. In the work presented here, we have continued 
our TRS\(^2\)-FTIR approach to heme-copper oxidases (18-21) and report the dynamics of the 
docking site that shelters the photodissociated CO. In analogy to Mb studies, the results 
demonstrate the formation of the \( B_0 \) and \( B_0 \) states that are associated with the photolyzed 
CO within the docking site of \( ba_3 \)-cytochrome \( c \) oxidase.

**Materials and Methods**

Cytochrome \( ba_3 \) was isolated from *Th. thermophilus* HB8 cells according to previously 
published procedures (15). The samples were stored in liquid nitrogen until further use. The
samples used for the FTIR measurements had an enzyme concentration of ~ 1 mM and were placed in MES buffer (pH 5.25) or in HEPES buffer (pD 7.50). The fully reduced enzyme was prepared by addition of dithionite after cycling the samples five times between vacuum and Ar. Dithionite reduced samples were exposed to 1 atm CO (1 mM) in an anaerobic cell to prepare the carbonmonoxy adduct and transferred to a tightly sealed FTIR cell composed of two 3 mm-thick CaF$_2$ windows, under anaerobic conditions. The pathlength was sufficiently small (15 µm) to avoid the strong absorbance of the water around 1650 cm$^{-1}$ and keep the response of the MCT detector linear. The TRS$^2$ experiments were performed on a system described elsewhere (17). $^{12}$CO gas (99.9 %) and isotopic CO (91.6 % $^{13}$C$^{16}$O and 8.4 % $^{13}$C$^{18}$O) were obtained from Messer (Germany) and Isotec (Miamisburg, OH), respectively. 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. No sample degradation occurred during the time course of the experiments.

**Results and Discussion**

For the sake of comparison with earlier work on Mb, we ascribe the 2131 and 2146 cm$^{-1}$ modes we have detected in the TRS$^2$-FTIR difference spectra of fully reduced $ba_3$-CO to the states referred to in the literature as $B_1$ and $B_0$, respectively.

Fig. 1 shows the TRS$^2$-FTIR difference spectra ($t_d$= 10-110 µs, 8 cm$^{-1}$ spectral resolution) of fully reduced $ba_3$-CO subsequent to CO photolysis by a 7-nanosecond laser pulse (532 nm). The negative peak at 1976 cm$^{-1}$ arises from the photolyzed heme $a_3$-CO complex. The positive peak that appears at 2053 cm$^{-1}$ is the C-O stretch ($\nu_{CO}$) of Cu$_B$, as previously reported (17). Concurrently with the formation of the Cu$_B^{1+}$-CO complex, a positive peak appears at 2131 cm$^{-1}$ that persists for 35 µs subsequent to CO photolysis, and is displaced by a peak at 2146 cm$^{-1}$ at later times ($t_d$= 85 µs). The TRS$^2$-FTIR difference spectra presented here in
conjunction with the reported extinction coefficients for heme $a_3$-CO and Cu$_B$-CO (16), demonstrate that 80-85% of the photodissociated CO binds to Cu$_B$. The remaining 15-20% is attributed to the population of the 2131 cm$^{-1}$ mode (see below). The frequency of the 2146 cm$^{-1}$ mode that we observe is close to the free-gas value of CO ($\nu_{CO} = 2143.3$ cm$^{-1}$), and exactly the same to that found in Mb characterizing the $B_0$ state at cryogenic temperatures (22). The 2131 cm$^{-1}$ mode corresponds to the $B_1$ state in which the CO is funneled into a docking site near the ring A of heme $a_3$ propionate of cytochrome $ba_3$ (18). The time evolution of the $B_1$ conversion to $B_0$ is depicted in Fig. 1B. At 10 and 35 $\mu$s subsequent to CO photolysis only the 2131 cm$^{-1}$ ($B_1$ state) mode is present. A close inspection of the data indicates that this mode has lost most of its intensity at $t_d$=85 $\mu$s. As the 2131 cm$^{-1}$ mode decays with time, another mode located at 2146 cm$^{-1}$ gains intensity. At $t_d$ =10-50 $\mu$s, the 2146 cm$^{-1}$ mode is below noise level, but at 85 $\mu$s, it is clearly observed in the spectrum. This feature decays at $t_d$=110 $\mu$s, becoming “buried” to the background as free “solvated” CO. Importantly, the decay of the 2146 cm$^{-1}$ mode occurs prior to rebinding of CO to heme $a_3$ Fe. Fig. 2A presents the TRS$^2$-FTIR difference spectra of fully reduced $ba_3^{13}$CO subsequent to $^{13}$CO photolysis at pH=7.5. The 2099 and 2084 cm$^{-1}$ modes correspond to those observed in the $^{12}$CO photolysis experiment (Fig. 1), and remain unchanged when the experiment was repeated in H$_2$O (data not shown). This indicates that the 2146 and 2131 cm$^{-1}$ modes are not sensitive to H/D exchange. A close inspection of the data presented in Fig. 2B indicates that the decay of the 2084 and 2099 cm$^{-1}$ modes occurs faster in D$_2$O than in H$_2$O, and with reduced intensities. The unligated CO and its time-evolution are clearly identified by the intensity of the 2146 and 2131 cm$^{-1}$ modes relative to that of the bound CO to Cu$_B$ ($\nu_{CO} =2053$ cm$^{-1}$), and the $^{12}$CO to $^{13}$CO isotope shift. The intensities of the 1976 (photolyzed heme $a_3$-CO) and 2053 cm$^{-1}$ (Cu$_B$-CO) modes remain unchanged during the entire measurement associated with the 2131 and 2146
cm\(^{-1}\) modes, indicating that the binuclear center (heme \(a_3\) Fe/Cu\(_{II}\)-CO) remains a spectator to the CO events occurring within the docking site.

We have recently demonstrated that the ligand docking site in cytochrome \(ba_3\) is located near the ring A propionate of heme \(a_3\), and that the release of CO from the docking site is not followed by recombination to heme \(a_3\) Fe (18). This behavior was interpreted as reflecting a mechanism in which the protein near the ring A propionate of heme \(a_3\) reorganizes about the released CO from the docking site, and subsequently establishes a transient barrier that inhibits the recombination process to the heme \(a_3\) Fe for a few ms; rebinding to heme \(a_3\) occurs at later times with \(k_2 = 29.5\) s\(^{-1}\). The 2131 cm\(^{-1}\) mode we observe is 12 cm\(^{-1}\) lower than the gas frequency of CO (2143.3 cm\(^{-1}\)) suggesting a weak electrostatic interaction of the ligand with the protein. Furthermore, the sharpness of this feature denotes a well-defined environment that accommodates the photolyzed CO. The 2146 cm\(^{-1}\) mode is slightly higher than the gas frequency of CO and retains the same bandwidth as that of the 2131 cm\(^{-1}\) mode indicating that the docking site controls not only the electrostatic interaction(s) of the “docked” CO during the \(B_1\) to \(B_0\) transition, but also the degree of its rotation. Furthermore, the formation and decay of the \(B_0\) state signals the onset of CO release from the docking site to the protein environment.

The ultrafast dynamics of CO motion subsequent to its dissociation from the heme of Mb at ambient temperatures have revealed the presence of the \(B_1\) and \(B_2\) states (2, 3, 8). The two CO trajectories were spectroscopically distinguishable due to the vibrational Stark effect that arises from the electrostatic field surrounding the ligand. The \(B_1\) and \(B_2\) states were resolved even at 0.2 ps with more than half of the vibrational Stark shift developed at that time (2). The center frequency of the \(B_2\) state (\(v_{CO} = 2119\) cm\(^{-1}\)) appeared static from 0.2 to 10 ps and thus, it was concluded that the spectral shifting appeared to come only from the trajectory leading to the \(B_1\) state. In addition, from the surprisingly narrow band shapes of \(B_1\) (\(\Delta v_{1/2} = 9.1\) cm\(^{-1}\))
cm\(^{-1}\) and B\(_2\) (\(\Delta \nu_{1/2}=6.0 \text{ cm}\(^{-1}\)\), it was suggested that the orientation of the docked CO is constrained by a static potential (7). It was finally concluded that the two B-states correspond to opposite orientations of CO within the same docking site and that interconversion between B\(_1\) and B\(_2\) requires end-to-end rotation of CO (2). The B-state spectrum of photolyzed Mb-CO at cryogenic temperatures (5.5 K), however, shows all three features that are denoted to B\(_0\), B\(_1\), and B\(_2\) states (22). The absence of the B\(_0\) state in the 283 K spectrum was attributed to a conformational relaxation that occurs only under ambient temperatures. Obviously, the detection of the B\(_0\) state in our experiments demonstrates that such conformational relaxation, that prevents the detection of the 2146 cm\(^{-1}\) mode, is absent in cytochrome \(ba_3\).

The present \(\mu s\)-TRS\(^2\)-FTIR results provide clear evidence that the B\(_1\) and B\(_0\) states are formed in cytochrome \(ba_3\), even though the corresponding states in Mb are formed in the ps-time scale (B\(_1\) and B\(_2\)) or under cryogenic conditions (B\(_0\)). Although we do not detect the B\(_2\) state under our limited 5 \(\mu s\) time resolution, we cannot exclude the possibility that both the B\(_1\) and B\(_2\) states are initially formed and conversion of B\(_2\) to B\(_1\) has already occurred in the \(\mu s\)-time scale. The close correlation for the B\(_1\) state between the picosecond reactions of Mb and Hb and those of cytochrome \(ba_3\) occurring in the \(\mu s\) time-scale does not exist for the B\(_0\) state. Both the B\(_0\) and B\(_1\) state in cytochrome \(ba_3\) have \(\Delta \nu_{1/2} \approx 12 \text{ cm}\(^{-1}\)\) indicating that the B\(_0\) state arises from CO that remains in the docking site. If the CO ligand corresponding to the B\(_0\) state was laid outside the docking site as solvated, its detection would have been difficult because the spectrum of CO in \(H_2O\) is too diffuse to be observed (5, 7). Despite the close resemblance in the energies of the B\(_1\) and B\(_0\) states of unligated CO between Mb and cytochrome \(ba_3\) there are profound differences in the dynamic properties of their docking sites.

An additional difference that is apparent between the properties of the docking site in Mb and cytochrome \(ba_3\) is the lifetime of their B states. Both the B\(_1\) and B\(_2\) states in Mb persist
into nanosecond time regime and decay simultaneously with half-time of 100-200 ns at room temperature (6). In cytochrome \( ba_3 \), however, there is a thermal conversion of \( B_1 \) to \( B_0 \) state indicating that the docking site which shelters CO, exhibits a conformational relaxation that facilitates the interconversion and subsequently the CO escape to the protein solvent. Consistent with this interpretation is the shorter lifetime of the \( B_1 \) and \( B_0 \) states in \( D_2O \) where conformational relaxation is most rapid. The long lifetime of the \( B_1 \) state in cytochrome \( ba_3 \) as compared to that of Mb, and the interconversion of the \( B_1 \) to \( B_0 \) state indicate that the docking site in cytochrome \( ba_3 \) consists of an environment quite different from that of Mb. In contrast to the Mb docking site, where the release of CO to the protein matrix occurs in a concerted manner by the simultaneously decay of both CO trajectories (\( B_1 \) and \( B_2 \) states), in cytochrome \( ba_3 \) the escape of CO from the docking site occurs in a sequential conversion of \( B_1 \) to \( B_0 \). A plausible explanation is that the rate of conformational relaxation near the docking site of Mb is much faster than that of cytochrome \( ba_3 \).

The same docking site that shelters the photodissociated CO from heme \( a_3 \) is expected to trap the thermally dissociated CO from Cu\(_8\) (\( k_2 = 29.5 \text{ s}^{-1} \)), as well as the thermally dissociated CO from heme \( a_3 \) under non-photolytic conditions. The latter process occurs very slowly with a time constant of \( k_2 = 0.8 \text{ s}^{-1} \). However, we found no spectroscopic evidence that the thermally dissociated CO from Cu\(_8\) is trapped within the docking site prior to its rebinding to the Fe of heme \( a_3 \). We also expect the same docking site to trap any ligand having similar size to CO such as NO and O\(_2\), and the residues constituting the docking site to be responsible for the kinetic control of ligand binding and escape. The short lifetime of the \( B_0 \) and \( B_1 \) states in conjunction with the slow recombination of CO to heme \( a_3 \) is not a consequence of a diffusional barrier created by the docking site, but it is rather due to steric restrictions directly adjacent to the binuclear center.
In Fig. 3 we present a model for the ligand trajectory of the B$_1$ state that is consistent with the time-resolved vibrational spectra of photolyzed cytochrome $b_{a3}$. The CO trajectory is illustrated at three different times; after photodissociation from heme $a_3$; in its way to the docking site, in its transient binding to Cu$_B$, and finally, as trapped within the docking site. The detection of free CO in our experiments indicates that the CO transfer to Cu$_B$ is a true bond-breakage/formation process. Other studies have proposed, however, that in the absence of “free” CO the Fe-C bond starts to lengthen and thus, the CO feels the influence of the Cu$_B$ potential. This way, the formation of the Cu$_B$-C bond is rapid and concurrent with the Fe-C rupture (23-25).

The TRS$^2$-FTIR difference spectra reported here provide for the first time, an incisive view for ligand motion within cytochrome $b_{a3}$, and show unambiguously the formation of the B$_1$ and B$_0$ states within a docking site. Taken together, the results demonstrate a facile pathway connecting the catalytic binuclear center and the docking site. Since the docking sites and their potential fields determine the discrimination in favor or against ligand binding, the measured values of the unligated C-O can be used to predict the parameters of physiologically relevant ligand, such as O$_2$ and NO. Experiments are in progress with emphasis on these questions.

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**Figure legends**

**Figure 1.** (A) TRS²-FTIR difference spectra of the $^{12}$CO-bound form of fully reduced cytochrome $ba_3$ (pH 5.25) at 10, 35, 85 and 110 µs subsequent to CO photolysis. Enzyme concentration was $\sim$ 1 mM and the pathlength 15 µm. The spectral resolution was 8 cm$^{-1}$, the time resolution 5 µs and 10 co-additions were collected per retardation data point. The excitation wavelength was 532 nm (4 mJ/pulse). (B) Expanded view of the “free” $^{12}$CO spectral region (2080-2200 cm$^{-1}$), of the cytochrome $ba_3$-$^{12}$CO complex at 10, 35, 85 and 110 µs subsequent to CO photolysis.

**Figure 2.** (A) TRS²-FTIR difference spectra of the $^{13}$CO-bound form of fully reduced cytochrome $ba_3$ (pD 7.50) at 10, 35 and 60 µs subsequent to CO photolysis. Enzyme concentration was $\sim$ 1 mM and the pathlength 15 µm. The spectral resolution was 8 cm$^{-1}$, the time resolution 5 µs and 10 co-additions were collected per retardation data point. The excitation wavelength was 532 nm (4 mJ/pulse). The peak at 1883 cm$^{-1}$ represents the 8.4 % Fe-$^{13}$C$^{18}$O mode. (B) Expanded view of the “free” $^{13}$CO spectral region (2040-2160 cm$^{-1}$), of the cytochrome $ba_3$-$^{13}$CO complex at 10, 35 and 60 µs subsequent to CO photolysis.

**Figure 3.** Schematic view of the CO trajectory (B₁ state, 2131 cm$^{-1}$ mode), subsequent to photolysis from heme $a_3$. The CO trajectory is depicted at three different times; (i) immediately after photolysis, (ii) in its way to the docking site, and finally trapped within the docking site, where the C end of C-O points away from the heme $a_3$. The dimensions are not in scale.
Figure 1

**Figure 1**

**A**

STEP-SCAN FTIR

$T = 293 \text{ K}$

Wavenumbers (cm$^{-1}$)

ΔAbsorbance

λA = 0.00005

1976 -

cytochrome $b_{5}$ oxidase

pH 5.25

2053

2146

2131

B

Wavenumbers (cm$^{-1}$)

ΔAbsorbance

10 μg

35 μg

85 μg

110 μg

x2

2146

2131
Figure 2
Figure 3
Docking site dynamics of ba-cytochrome c oxidase from Thermus thermophilus
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