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Review

# The pathway of $O_2$ to the active site in heme–copper oxidases ☆

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ABSTRACT

The route of $O_2$ to and from the high-spin heme in heme–copper oxidases has generally been believed to emulate that of carbon monoxide (CO). Time-resolved and stationary infrared experiments in our laboratories of the fully reduced CO-bound enzymes, as well as transient optical absorption saturation kinetics studies as a function of CO pressure, have provided strong support for CO binding to $Cu_B^+$ on the pathway to and from the high-spin heme. The presence of CO on $Cu_B^+$ suggests that $O_2$ binding may be compromised in CO flow-flash experiments. Time-resolved optical absorption studies show that the rate of $O_2$ and NO binding in the bovine enzyme ($1 \times 10^8\ M^{-1}\ s^{-1}$) is unaffected by the presence of CO, which is consistent with the rapid dissociation ($t_{1/2} = 1.5\ \mu s$) of CO from $Cu_B^+$. In contrast, in *Thermus thermophilus* (*Tt*) cytochrome $ba_3$ the $O_2$ and NO binding to heme $a_3$ slows by an order of magnitude in the presence of CO (from $1 \times 10^9$ to $1 \times 10^8\ M^{-1}\ s^{-1}$), but is still considerably faster (~10 μs at 1 atm $O_2$) than the CO off-rate from $Cu_B$ in the absence of $O_2$ (milliseconds). These results show that traditional CO flow-flash experiments do not give accurate results for the physiological binding of $O_2$ and NO in *Tt* $ba_3$, namely, in the absence of CO. They also raise the question whether in CO flow-flash experiments on *Tt* $ba_3$ the presence of CO on $Cu_B^+$ impedes the binding of $O_2$ to $Cu_B^+$ or, if $O_2$ does not bind to $Cu_B^+$ prior to heme $a_3$, whether the $Cu_B^+$–CO complex sterically restricts access of $O_2$ to the heme. Both possibilities are discussed, and we argue that $O_2$ binds directly to heme $a_3$ in *Tt* $ba_3$, causing CO to dissociate from $Cu_B^+$ in a concerted manner through steric and/or electronic effects. This would allow $Cu_B^+$ to function as an electron donor during the fast (5 μs) breaking of the O – O bond. These results suggest that the binding of CO to $Cu_B^+$ on the path to and from heme $a_3$ may not be applicable to $O_2$ and NO in all heme-copper oxidases. This article is part of a Special Issue entitled: Vibrational spectroscopies and bioenergetic systems.



## 1. Introduction

Heme–copper oxidases, which include the cytochrome and ubiquinol oxidases, play a crucial role in energy production by aerobic organisms [1–3]. Their primary function is to catalyze the reduction of dioxygen to water using electrons from respiratory electron transport. The energy made available by the reaction generates a transmembrane electrochemical proton gradient that drives ATP synthesis [4]. These enzymes are responsible for over 90% of biological dioxygen reduction and for nearly half of the redox energy of cellular respiration [5,6]. Significantly, cytochrome *c* oxidase is inhibited by nitric oxide (NO) [7], a signaling molecule involved in diverse biochemical and physiological processes [8]. This inhibition of cytochrome oxidase may play an important role in regulating cellular respiration [7,9]. Several bacterial heme–copper oxidases are also able to catalyze the reduction of nitric oxide (NO) to nitrous oxide ($N_2O$) [10–13]; however, there are conflicting reports whether the bovine cytochrome oxidase has NO reductase activity [14,15].

The heme–copper oxidases are subdivided into three families, denoted A, B, and C [16,17], and all three families contain a high-spin heme ($a_3$, $o_3$ or $b_3$) which together with a copper center, $Cu_B$, forms the binuclear heme–copper site of $O_2$ binding and reduction. While sequence homology of the catalytic subunit containing the binuclear center is high between the bovine enzyme and the bacterial *Rhodobacter sphaeroides* (*Rs*) and *Paracoccus denitrificans (Pd)* $aa_3$ oxidases (54 and 55%, respectively), it is much lower in *Thermus thermophilus (Tt)* $ba_3$ and *Pseudomonas stutzeri* $cbb_3$ oxidases (23 and 15%, respectively). Despite this diversity, $Cu_B$ in its oxidized form in all three oxidase families is trigonally ligated to the imidazole side chains of three conserved histidines, and the high-spin iron is ligated to an invariant histidine on the "proximal" side (opposite to the $O_2$ binding site) of the heme; in the $cbb_3$ oxidases, the proximal histidine is hydrogen bonded to the carboxylate of a glutamate residue [18]. All heme–copper oxidases also contain a post-translational modification, a cross-link between C6 of a tyrosine residue (Tyr 244 in the bovine enzyme) and the ε-

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nitrogen of one of the histidine ligands to $Cu_B$ [19–21]. In the $cbb_3$ oxidases, the tyrosine originates from a different helix than in the A- and B-type oxidases [18].

Knowledge of the structural and dynamic features of the binuclear active site and the intraprotein channel(s) for ligation and proton transfer is critical for understanding the mechanisms of $O_2$ reduction, inhibition by NO, and NO reduction. The first step in $O_2$ reduction and NO inhibition and reduction relies on access of $O_2$ and NO, respectively, to the active site and how the protein environment modulates this access. Based on crystal structures, ligand pathways have been postulated for many heme–copper oxidases [18,20,22–25]. While there are significant sequence and structural similarities among these ligand pathways, there are variations in the global structure of the catalytic subunit, which may reflect the different functional environments of these enzymes. For instance, recent crystallographic studies of xenon (Xe) binding in *Tt* $ba_3$ [26] indicated that a constriction point in the oxygen channel of the $aa_3$ oxidases [24,27,28] is not present in *Tt* $ba_3$. This observation suggests easier access of ligands to the binuclear site in $ba_3$, which may be related to the different physiological requirements of the $aa_3$ and $ba_3$ oxidases [29].

Migration of $O_2$ through a ligand channel to the active site is followed by $O_2$ binding to the binuclear center. The role of $Cu_B$ in transporting ligands such as $O_2$ and NO to and from the high-spin heme is of particular interest. Carbon monoxide (CO), a competitive inhibitor of $O_2$ reduction in cytochrome oxidase, has frequently been used as a model for $O_2$ binding [30–34], and the reactions following photodissociation of CO have been thought to exemplify the pathways of $O_2$ to and from the active site. This knowledge, however, is only relevant to the physiological $O_2$ reduction as long as the coordination chemistry of CO mimics that of $O_2$. The photodissociation of CO from the high-spin heme in the presence of $O_2$ has also been used extensively to initiate the $O_2$ reduction reaction [2,3,35–39]. However, the fate of the photodissociated CO may compromise the $O_2$ (or NO) binding and electron transfer dynamics [40]. This issue can only be addressed by exploring the photodissociation and recombination dynamics of the CO ligand, and by comparing the binding of $O_2$ and NO in the heme–copper oxidases in the presence of CO to the binding of these ligands under more realistic physiological conditions, namely, in the absence of CO.

In this review, we summarize vibrational and UV–vis spectroscopic studies of 1) the flash-induced photodissociation and rebinding of CO in the heme–copper oxidases, and 2) the reactions of $O_2$ and NO with these enzymes in the presence and absence of CO. These results provide insight into the ligand binding dynamics of the heme-copper oxidases and how the protein environment modulates the ligand pathways and metal centers for different physiological environments.

## 2. CO photolysis and recombination dynamics

### 2.1. Fourier transform infrared (FTIR) experiments: CO binding to $Cu_B^+$

Characterization of the $O_2$ binding site is critical for understanding the mechanism of $O_2$ reduction to water and for elucidation of the protein structures that facilitate this reaction. Infrared spectroscopy provides a direct approach for studying the binding of ligands to heme proteins, including the heme–copper oxidases [30–34,41]. Carbon monoxide (CO) is commonly used as an infrared probe for $O_2$ binding because it generally binds to the same sites as $O_2$ and because of its strong infrared absorption and non-reducible nature. The frequencies and bandwidths of the CO infrared stretching bands can give valuable information not only about the identity of the metal center to which CO binds but also about the environment surrounding the CO ligand [31,34,41]. For example, CO binding to the high-spin heme in the bovine enzyme gives rise to a major peak at 1963 $cm^{-1}$ (the Fe-bound C–O stretching frequency) in the infrared spectrum [31,34]. Infrared spectroscopy has also allowed us to follow the binding of CO to $Cu_B^+$, a metal center that is inaccessible to other spectroscopic techniques. Pioneering FTIR studies by Alben and coworkers showed that the photodissociated CO binds to $Cu_B^+$ in mitochondrial preparations under cryogenic conditions based on the infrared frequency at 2062 $cm^{-1}$ [30,42].

Our laboratories have carried out extensive infrared studies of the photodissociation and recombination of the fully reduced CO-bound heme–copper oxidases [31,32,33,43–48]. FTIR difference spectra (dark minus light) were recorded over a wide temperature range (21–298 K) for the bovine enzyme, and *Tt* $caa_3$ and $ba_3$ [33]. Fig. 1 shows the spectra recorded between ~22 and 300 K for the bovine enzyme (left panels) and *Tt* $ba_3$ (right panels). In addition to the major heme $a_3$-CO band at 1963 $cm^{-1}$, the bovine enzyme has two minor bands at 1949 and 1944 $cm^{-1}$. At 21 K, *Tt* $caa_3$ shows two positive peaks at 1953 and 1947 $cm^{-1}$ representing the heme $a_3$-CO [33], while CO binding to heme $a_3$ in *Tt* $ba_3$ gives rise to two major positive bands at 1974 and 1983 $cm^{-1}$ (Fig. 1, right panels). The FTIR difference spectra show negative peaks at 2066, 2054 and 2039 $cm^{-1}$ for the bovine enzyme, at 2060 and 2036 $cm^{-1}$ for *Tt* $caa_3$ [33] and at 2054 $cm^{-1}$ for *Tt* $ba_3$, all of which are attributed to CO binding to $Cu_B^+$.

The $Cu_B^+$–CO complex in the bovine and *Tt* $caa_3$ enzymes is kinetically stable below 140 K and 170 K, respectively, but dissociates at higher temperature. However, for *Tt* $ba_3$ we were able to observe binding of CO to $Cu_B^+$ upon continuous photolysis at room temperature in the FTIR dark-minus-light difference spectrum (Fig. 1, right panel, top spectrum). The differences in the CO stretching frequency for the Fe–CO (and $Cu_B^+$–CO) infrared absorption among these three oxidases indicate significant variations in the details of the CO binding and the stability of the $Cu_B^+$–CO intermediate. Nonetheless, the bandwidths of the IR peaks remain narrow over a large temperature range for all the oxidases, indicating a very homogeneous environment around the CO ligand. A recent combined crystallographic and infrared spectral study supports CO binding to $Cu_B^+$ in *Tt* $ba_3$ following photolysis of CO from heme $a_3$ [49].

We were also able to follow CO transfer from $Cu_B^+$ to $Fe_{a3}^{2+}$ between 158 and 179 K in the bovine enzyme, 175–195 K in *Tt* $caa_3$

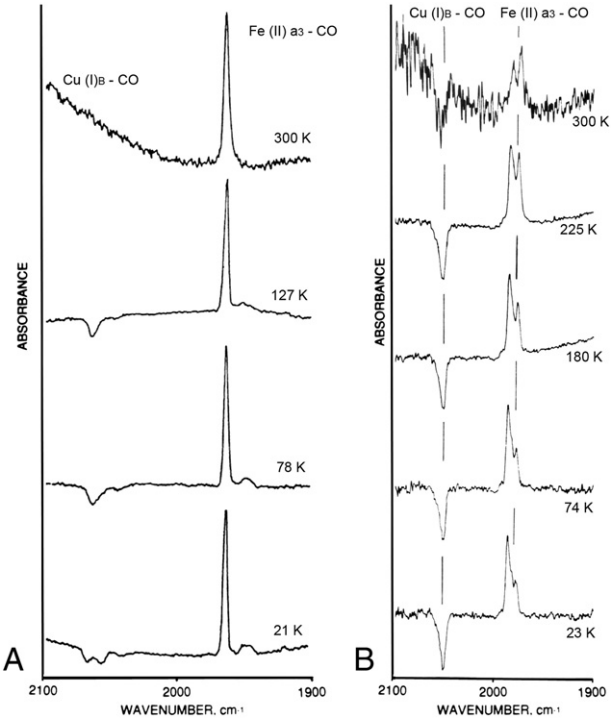



**Fig. 1.** FTIR difference spectra (dark minus light) of carbonmonoxy fully reduced bovine cytochrome $aa_3$ (left) and *Thermus thermophilus* cytochrome $ba_3$ (right) at various temperatures. The 300 K light spectrum of the $ba_3$ enzyme was recorded under continuous photolysis. Conditions are those described in [32] and [33].

and 205–230 K in *Tt* $ba_3$ [33]. Taking into account the relative absorptivities of heme and copper CO-complexes, the relative integrated areas of the Fe–CO and $Cu_B^+$–CO infrared peaks represent quantitative transfer of CO from the heme to $Cu_B$ following CO photodissociation, supporting a closed pocket isolated from the surrounding medium [32]. The activation parameters derived from an Eyring plot of the CO recombination in the three enzymes (Fig. 2) are listed in Table 1.

The multiple infrared heme – CO stretching bands represent discrete CO conformers of different structures that interconvert rapidly (on the FTIR time scale, i.e. minutes) [31,33]. For *Tt* $ba_3$, the Fe–CO conformers interconvert down to ~150 K, and they are energetically close as reflected by their relative populations between 180 K and room temperature. The thermodynamic parameters, $\Delta H^0 = 0.84 +/- 0.17$ kcal/mol and $\Delta S^0$ of 3.5 +/− 0.9 cal/mol-K, were obtained for the interconversion of the 1983 and 1974 $cm^{-1}$ conformers based on the relative areas of the IR peaks of the two conformers as a function of temperature [33]. These conformers were also observed in the CO-FTIR spectra of intact plasma membranes.

### 2.2. Resonance Raman experiments: multiple Fe–N(Im) conformers in Tt $ba_3$

We explored the different heme–copper oxidases by resonance Raman spectroscopy [48,50,51]. The resonance Raman spectra of the unliganded reduced *Tt* $ba_3$ in the low-frequency region, which contains the out-of-plane iron-imidazole nitrogen, Fe–N(Im), stretching vibration, show two bands around 192 and 208 $cm^{-1}$ (Fig. 3). The use of isotopically enriched $^{57}Fe$ (95%) confirmed the assignments of these bands as the Fe–N(Im) stretching frequencies [51]. In contrast, the bovine enzyme shows a single Fe–N(Im) frequency at 214 $cm^{-1}$. The relative intensities of the two conformers in $ba_3$ are temperature dependent over a large range and track those of the Fe-CO peaks in the infrared spectra. This is reflected in almost identical thermodynamic parameters based on the Fe–CO (see above) and Fe–N(Im) conformers ($\Delta H^0 = 0.75 +/-$ 1.2 kcal/mol and $\Delta S^0$ of 2.1 +/− 1.2 cal/mol-K) thermodynamics plot (Fig. 4). This suggests that the Fe–CO infrared conformers and the Fe–N(Im) conformers arise from the same conformational changes, although the small thermodynamics values do not indicate major global structural changes. The different conformers may represent rotamers of the imidazole plane about the Fe–N axis, giving rise to different steric interactions between the imidazole and the heme of possible relevance to the coordination at the heme.

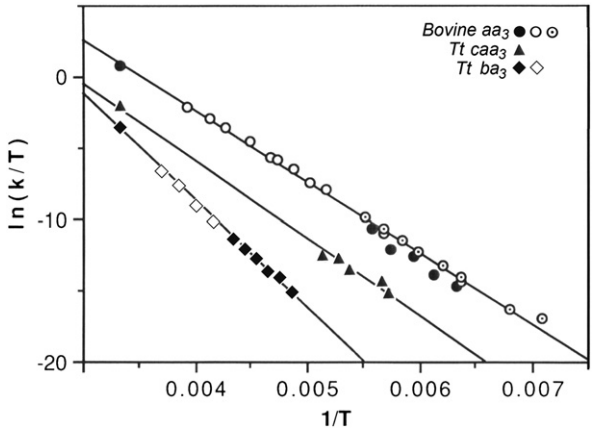



**Fig. 2.** The Eyring plot for *T. thermophilus* cytochrome $ba_3$–CO, *T. thermophilus* $caa_3$–CO and bovine $aa_3$–CO recombination, measured from the Fe–CO infrared peaks (low temperature) and by kinetic UV–vis spectrophotometry at room temperature. Data are from current work and [32,33]. For the bovine enzyme, open circles are from Sharrock and Yonetani [77] and the circles with concentric dots are from Fiamingo et al. [42].

**Table 1**
Activation parameters for CO recombination in *Tt* $ba_3$, *Tt* $caa_3$ and bovine $aa_3$.

| | $\Delta H^‡$ (kcal/mol) | $\Delta S^‡$ (cal/mol-K) |
|---|---|---|
| *T. thermophilus* $ba_3$ | 14.9 | −5 |
| *T. thermophilus* $caa_3$ | 10.8 | −16 |
| Bovine $aa_3$ | 10.0 | −12 |

### 2.3. Dynamic time-resolved infrared (TRIR) experiments: Transient binding of CO to $Cu_B^+$ at ambient temperature

While the low-temperature FTIR measurements provided important information about CO binding to $Cu_B^+$ in the various heme–copper oxidases, it was imperative to demonstrate whether the photodissociated CO could also bind to $Cu_B^+$ at room temperature, particularly with respect to CO flow-flash experiments that rely on the photolability of the CO complex to initiate the reaction with $O_2$. Time-resolved infrared (TRIR) spectroscopy of the photodissociated CO-bound bovine enzyme in our laboratories provided the first evidence for CO binding to $Cu_B^+$ at room temperature following photodissociation of CO from heme $a_3$ [43]. Fig. 5 (top) displays the infrared transient at 2061 $cm^{-1}$ due to the $Cu_B^+$–CO complex, and a single exponential fit shows that the $Cu_B^+$–CO transient decays with a half-life of 1.5 μs (Fig. 5, bottom). The time resolution of these early experiments was 200 ns but later experiments showed that CO binds to $Cu_B^+$ within 3 ps [45]. More recent studies by others have shown photoinitiated CO ligand transfer to $Cu_B$ of 60 fs [52]. The subsequent recombination of CO with $Fe_{a3}$ occurs with an observed rate constant of ~90 $s^{-1}$ at 1 atm of CO [32].

The photodissociated CO also binds to $Cu_B^+$ in *Tt* $caa_3$ and $ba_3$ at room temperature following photodissociation of CO from heme $a_3$ as reflected by the infrared transients at 2036 $cm^{-1}$ and 2054 $cm^{-1}$, respectively. In *Tt* $caa_3$, CO equilibrates with the surroundings on a

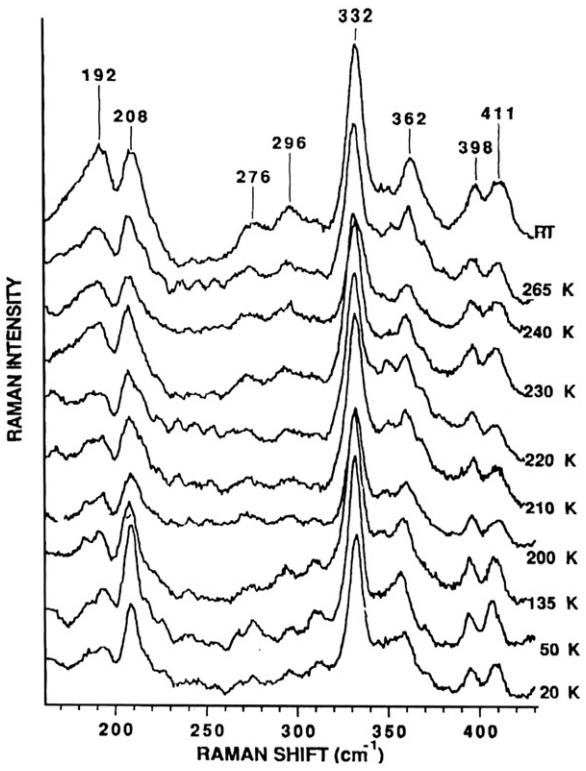



**Fig. 3.** Resonance Raman spectra showing the Fe–N(Im) stretching peaks of *T. thermophilus* cytochrome $ba_3$ as a function of temperature.

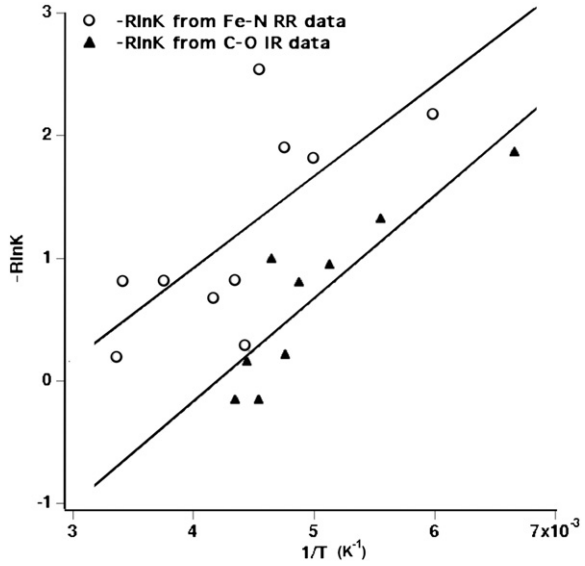



**Fig. 4.** Conformer thermodynamics. The temperature dependent intensities of the 192 and 208 $cm^{-1}$ Fe-N(Im) bands in the resonance Raman spectra of *Tt* $ba_3$ and those of the two major Fe-CO stretching peaks at 1973 and 1984 $cm^{-1}$.

microsecond time scale, $2 \times 10^4$ $s^{-1}$, and rebinds to heme $a_3$ with an apparent rate constant of 40 $s^{-1}$ [47]. The room temperature infrared transients for *Tt* $ba_3$-CO before photolysis (1974 $cm^{-1}$; heme $a_3$-CO bound) and after photolysis (2053 $cm^{-1}$; $Cu_B^+$–CO) are shown in Fig. 6. The $Cu_B^+$–CO transient is much more stable in *Tt* $ba_3$ than in the bovine enzyme, and based on a single exponential fit decays with an apparent lifetime of ~46 ms, which is the same within experimental error as the value of 50 ms obtained for CO rebinding to heme $a_3$ (Fig. 6). These values are similar to those obtained previously (~30 $s^{-1}$) by time-resolved step-scan FTIR difference spectroscopy [53]. However, our UV–vis time-resolved optical absorption measurements show that the photodissociated CO rebinds to heme $a_3$ in $ba_3$ with an apparent lifetime of 260 ms, which is significantly slower than observed in our TRIR experiment and previous time-resolved step-scan FTIR measurements [53]. The cause of the discrepancy between the two approaches is unknown and is currently under investigation. Regardless, the slow CO dissociation from $Cu_B^+$ in *Tt* $ba_3$ raises the question whether the photodissociated CO interferes with the reaction of *Tt* $ba_3$ with $O_2$. TRIR spectroscopy has also demonstrated the binding of the photodissociated CO to $Cu_B^+$ in other heme–copper oxidases. For example, in *Escherichia coli* $bo_3$, CO dissociation from $Cu_B^+$ was reported with a rate constant of ~500 $s^{-1}$ [47], although later studies reported a multiphasic dissociation of CO from $Cu_B^+$ on both microsecond and millisecond time scales [54]. The CO recombines with heme $o_3$ with an apparent rate constant of 40 $s^{-1}$ (25 ms) based on UV–vis time-resolved optical absorption measurements in our laboratory.

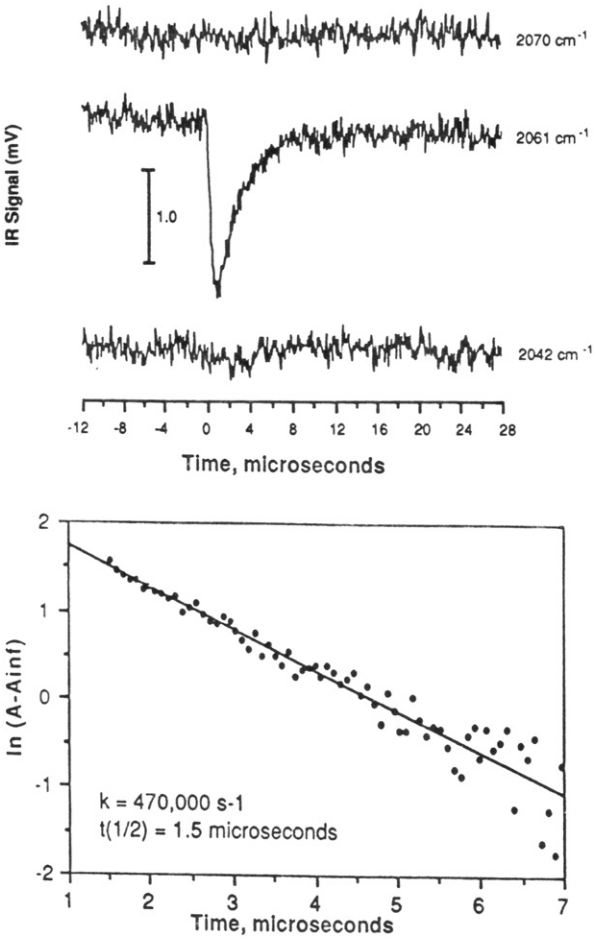



**Fig. 5.** (Top) The room temperature TRIR transients of bovine heart cytochrome $aa_3$–CO following photolysis of CO from the heme. (Bottom) A single exponential fit to the $Cu_B^+$–CO transient decay (see ref. [43]) for details.

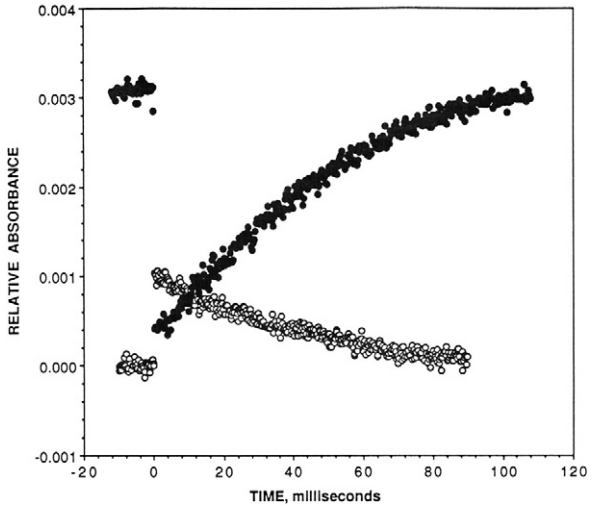



**Fig. 6.** The post-photodissociation TRIR transient absorbance trace for *Tt* $ba_3$-CO, recorded at the maximum of the $Fe_{a3}$–CO absorbance peak at 1974 $cm^{-1}$ (filled circles) and the $Cu_B^+$–CO absorbance peak at 2053 $cm^{-1}$ (open circles).

### *2.4. Time-resolved infrared linear dichroism: the orientation of CO in heme $a_3$–CO and $Cu_B$–CO complexes of bovine $aa_3$*

TRIR linear dichroism (TRIRLID) measurements allowed us to determine the orientation of the C–O bond axis with respect to the heme normal in the $Fe_{a3}^{2+}$–CO complex of the bovine enzyme as well as the $Cu_B^+$–CO photoproduct [44]. The TRIRLID is based on the differential absorption (photoselection) of parallel versus perpendicular polarized infrared light with respect to that of the photodissociation pulse and is measured as a function of the transmittance of the infrared probe beam. The results of such an experiment for both the $Fe_{a3}$–CO complex and the $Cu_B$–CO photoproduct of the bovine enzyme are shown in Fig. 7. TRIRLID experiments gave an angle between the heme normal and the C–O bond vector of 21° (+/− 2°) for the $Fe_{a3}^{2+}$–CO in the bovine enzyme, which was supported by subsequent picosecond TRIR measurements [46]. For the $Fe_{a3}^{2+}$–CO, a discrete angle (α) rather than a distribution of angles was assumed because the IR peak is very narrow, indicating the absence of inhomogeneous broadening [46]. The angle

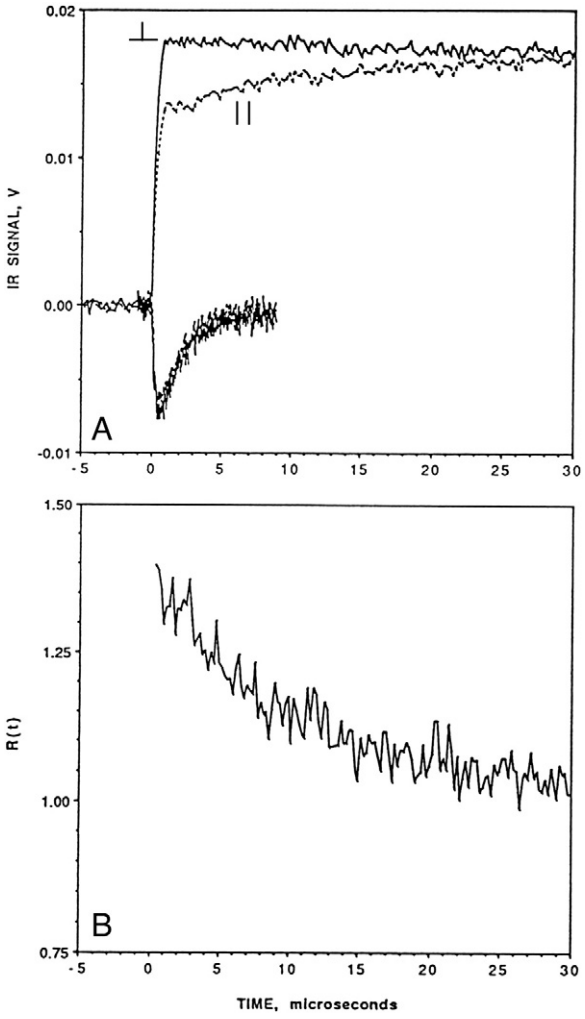



**Fig. 7.** (A) The TRIR linear dichroism signals of CO-bound bovine heart cytochrome oxidase between −5 and 30 μs with respect to the time of the photodissociation pulse. The upper traces are for the Fe–CO complex and the lower traces for the $Cu_B$–CO photoproduct. The polarization of the infrared probe beam relative to the photodissociation pulse is indicated. The solid traces represent perpendicular polarization and the dashed traces represent the parallel polarization. (B) The time-dependence of the polarization ratio, $R(t) = (\Delta A_{perpendicular} / \Delta A_{parallel})$ (see ref. [44] for further details).
Modified from [44].

of 21° is in good agreement with an analogous angle of 16.6° derived based on the heme–CO bound crystal structure [55].

The TRIR linear dichroism technique also allowed us to determine the orientation of the C–O bond axis with respect to the heme normal in the $Cu_B^+$–CO transient photoproduct in the bovine enzyme. The TRIRLID signal for the transient photoproduct was practically the same for the parallel and perpendicular polarization of the infrared probe beam with respect to the photodissociation pulse (Fig. 7A, lower traces). The near absence of linear dichroism was interpreted in terms of angle, α, of 51 +/− 3° between the heme normal and the C–O bond vector of the $Cu_B^+$–CO complex. Subsequent picosecond infrared measurements gave α of 55 +/− 3° [46]. It should be noted that the α values for the heme iron and $Cu_B$ are cone half-angles because the TRIRLID does not provide angular orientation of the $Fe_{a3}$C–O and $Cu_B$–CO vectors. Recent x-ray structure of the CO-bound bovine enzyme at 100 K indicates that CO is bound to $Cu_B$ in a side-on fashion, with metal-to-carbon and metal-to-oxygen atom distances of 2.4 and 2.7 Å, respectively, indicating a weak $Cu_B$–CO bond [55]. Based on the crystal structure of the proposed $Cu_B^+$–CO complex (the crystal structure of the CO derivative determined at 100 K), the angle between the heme normal and the C–O bond vector for the $Cu_B^+$–CO complex is 65.5°, somewhat higher than observed in solution in the TRIRLID measurements. Based on a recent x-ray crystal structure of the CO-bound *Tt* $ba_3$ and the photodissociated product [49], the angle between the heme normal and the C–O bond vector is 66° for $Fe_{a3}^{2+}$–CO and 64° for $Cu_B^+$–CO. Although the C–O bond vector for the $Cu_B^+$-CO complex makes a similar angle with respect to the heme normal in both the *Tt* $ba_3$ (64°) and bovine enzymes (65.5°), the CO is oriented within the binuclear center quite differently in these two enzymes as illustrated in Fig. 8. In the *Tt* $ba_3$ $Cu_B^+$–CO complex, the carbon atom is bonded to $Cu_B^+$ at a distance of 1.9 Å (top panel), while the oxygen atom is bonded to $Fe_{a3}$ at a distance of 2.3 Å [49]. In addition, in the *Tt* $ba_3$ $Cu_B^+$–CO photoproduct, the ligand is directly above the $Fe_{a3}$ atom along the heme normal vector. By contrast, the CO ligand is bound quite weakly to $Cu_B$ in the bovine $Cu_B$–CO photoproduct, and the CO ligand is not above the $Fe_{a3}$ atom, but is displaced away from $Cu_B$ and the K-proton channel and toward the ligand entrance channel (Fig. 8, lower panel).

### *2.5. Transient UV–vis spectroscopy: the photodissociation and recombination dynamics of CO-cytochrome oxidase*

While infrared spectroscopy has structural specificity and allows us to follow species that are inaccessible by other spectroscopic approaches, such as $Cu_B^+$-CO in cytochrome oxidase, transient UV–visible kinetic studies of the CO photodissociation and rebinding in a variety of heme proteins have provided important information about the heme environment and the dynamics of CO in the active site cavity

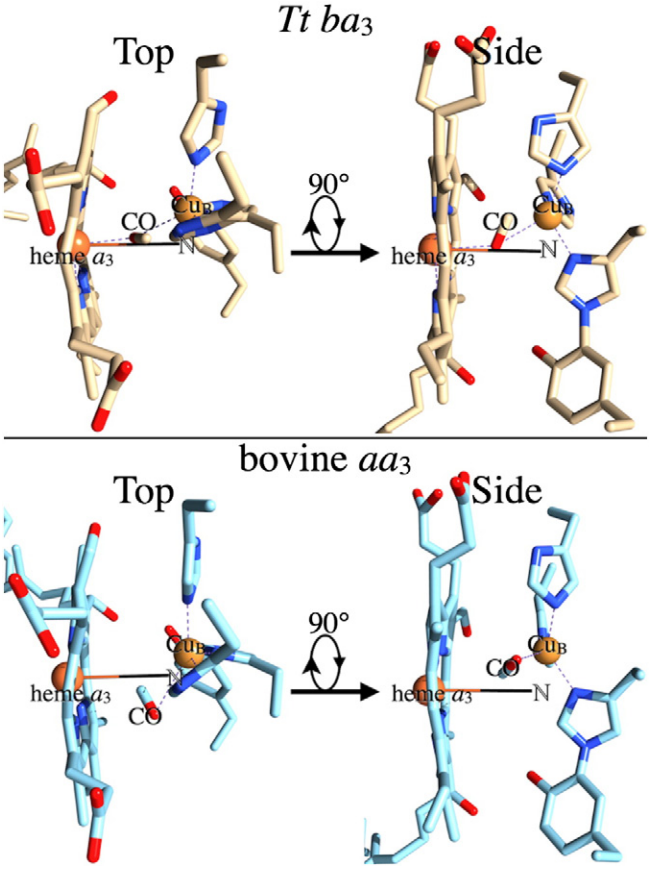



**Fig. 8.** Top panel: The *Tt* $ba_3$ $Cu_B$–CO transient photoproduct (PDB 3QJR, *49*). Lower panel: The bovine $Cu_B$–CO transient photoproduct (PDB 3AG2, *55*). In both panels, the left figure (Top) is viewed from the positive side of membrane, while the right figure (Side) is viewed from the ligand entrance channel. The "Side" view is generated from the "Top" by a left-handed 90° rotation about the horizontal axis.

and its migration pathways through the proteins [56,57]. In bovine cytochrome oxidase, an increase in the intensity of the α-band (615 nm) on picosecond time scale was observed following the initial femtosecond events accompanying the photodissociation of CO from heme $a_3$ [32]. A subsequent decrease in the α-band was observed on ~1 μs times scale, simultaneously with the loss of CO from $Cu_B^+$. These picosecond and microsecond changes were associated with structural effects at heme $a_3$ following the formation and dissociation of the $Cu_B^+$–CO complex [32]. The observed pseudo-first-order rate of rebinding of the flash-dissociated CO to cytochrome $a_3$ of the bovine heart enzyme showed the onset of saturation at [CO] >1 mM ($P_{CO}$ ~1–22 atm) when measured at room temperature on micro- and millisecond time scales. This was interpreted in terms of CO, and by extension other ligands such as $O_2$, first binding to a non-heme site, i.e. $Cu_B^+$, as it migrated to heme $a_3$. Saturation kinetics was also reported for the rebinding of the photodissociated CO to *E. coli* $bo_3$ ubiquinol oxidase as a function of CO concentration [58]. Interestingly, two mutant oxidases (His333Leu and His334Leu), in which the $Cu_B$ site was significantly altered, or $Cu_B$ was lacking, showed no evidence of CO binding saturation up to 21 mM CO. This was interpreted as $Cu_B$ acting as a way-station for CO moving to the heme. In contrast, in *Tt* $ba_3$, the observed rate constant for CO rebinding to the heme $a_3$ appears to be independent of CO over a large concentration range, 25 μM–3 mM [47]. This indicates that the pre-equilibrium of CO with a non-heme site, i.e. $Cu_B$, in $ba_3$ is saturated at the lowest CO concentration used.

The TRIR and transient UV–visible results of the kinetics of CO rebinding following its photodissociation from the high-spin heme have been explained by the kinetics model shown in Scheme 1, which includes an obligatory binding of CO to $Cu_B^+$ to and from the high-spin heme. In Scheme 1, $k_1$ represents CO binding to $Cu_B$ from solution prior to the rate-limiting CO transfer to $Fe_{a3}^{2+}$, represented by $k_2$. The thermal dissociation of CO from $Fe_{a3}^{2+}$ is represented by $k_{-2}$. The equilibrium constants are represented by $K_1 = k_1/k_{-1}$ and $K_2 = k_2/k_{-2}$. Based on our CO recombination kinetics of the bovine enzyme as a function of CO concentration, we calculated a value for $k_{-2}$ of 0.027 $s^{-1}$, which is in excellent agreement with the value of 0.023 $s^{-1}$, reported previously by Gibson and Greenwood [36]. In *Tt* $ba_3$, this rate is much faster or 0.8 $s^{-1}$ [59].

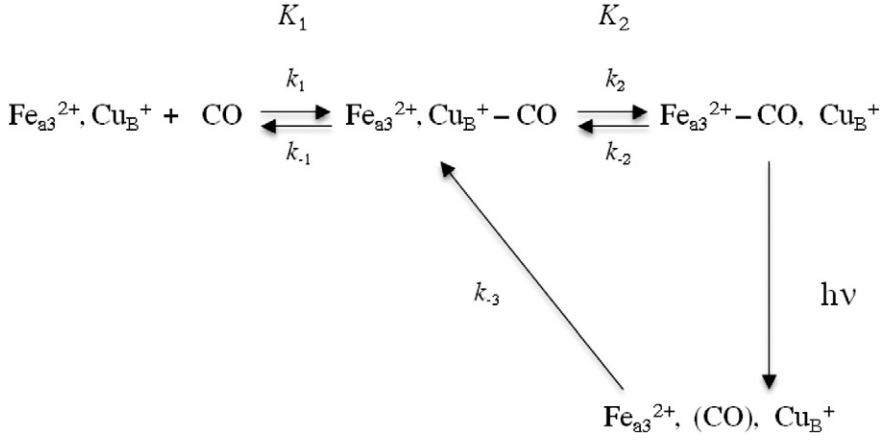
## 3. $O_2$ and NO binding in heme–copper oxidases in the absence and presence of CO

If the binding of CO to $Cu_B$ in the heme-copper oxidases precedes the binding of CO to the high-spin heme, does this binding affect the access of other ligands, such as $O_2$ and NO, to the active site in CO/NO and CO/$O_2$ flow-flash experiments? As shown above, the $Cu_B^+$–CO complex decays with a half-life of ~1.5 μs in the bovine enzyme [32,43] while in *Tt* $ba_3$ the $Cu_B^+$–CO complex decays on a millisecond time scale (Fig. 6 and [53]), much slower than that of $O_2$ binding to heme $a_3^{2+}$ in the $aa_3$ oxidases (~10–20 μs at ~0.5–1 mM $O_2$). This long lifetime of the $Cu_B^+$–CO complex in $ba_3$ raises questions whether $O_2$ binding to heme $a_3$ may be impeded by the binding of the photodissociated CO to $Cu_B^+$ in CO/$O_2$ flow-flash experiments on this enzyme, and secondly, whether the binding of $O_2$ and NO to $Cu_B^+$ on route to heme $a_3$ is a general feature of the ligand binding dynamics of the heme–copper oxidases as observed for CO. Results from studies aimed at answering these questions are summarized below.

### 3.1. Does CO impede $O_2$ and NO access to the active site in heme-copper oxidases?

To investigate whether CO impedes access of $O_2$ and NO to the active site in the heme-copper oxidases, we monitored the reactions of $O_2$ and NO with bovine $aa_3$ and *Tt* $ba_3$ under more physiological conditions, namely, in the absence of CO using time-resolved optical absorption spectroscopy in combination with photolabile $O_2$ and NO carriers [40,60]. This technique eliminates the possible interferences from the photodissociated CO in typical CO flow-flash experiments as well as circumvents the low NO quantum yield in NO flash-photolysis studies. The results from these studies were compared to those obtained in the presence of CO using a double-laser approach in which the $O_2$ and NO were generated by photolyzing the respective $O_2$ and NO photolabile carrier with a 355 nm laser pulse and the CO-bound enzyme was photolyzed simultaneously using a second 532 nm laser pulse [40,60]. Time-resolved optical absorption spectra were recorded and the data were analyzed with a combination of singular value decomposition (SVD), global exponential fitting and an algebraic kinetic approach and the spectra of the intermediates were determined. The data show that $O_2$ and NO bind to cytochrome $a_3$ in *Tt* $ba_3$ in the absence of CO with a second order rate constant of $1 \times 10^9$ $M^{-1}$ $s^{-1}$, which is 10-times faster than observed in the bovine enzyme ($1 \times 10^8$ $M^{-1}$ $s^{-1}$) under the same conditions [40,60]. Moreover, the $O_2$ and NO binding in *Tt* $ba_3$ is 10-times slower in the presence of CO ($1 \times 10^8$ $M^{-1}$ $s^{-1}$) while the presence of CO does not affect the rate of $O_2$ and NO binding in the bovine enzyme (Fig. 9). These results show that the reactions of $O_2$ and NO with reduced *Tt* $ba_3$ are indeed compromised by the transient binding of the photodissociated CO to $Cu_B^+$ and that the CO flow-flash method does not give accurate results for "physiological" $O_2$ and NO binding in $ba_3$, i.e. that observed in the absence of CO.

The post-photolysis structure of the bovine enzyme shows that CO is quite weakly bound to $Cu_B^+$ [55], while CO is bound much more tightly to $Cu_B$ in *Tt* $ba_3$ [49]. For example, the $Cu_B$–C distance is 2.4 Å and 1.9 Å in the bovine and $ba_3$ structures, respectively [49,55]. Likewise, the Fe–O distance is 3.8 Å (not bound) and 2.3 Å in the bovine and $ba_3$




**Scheme 1.** Proposed mechanism for CO photodissociation and rebinding in heme-copper oxidases.

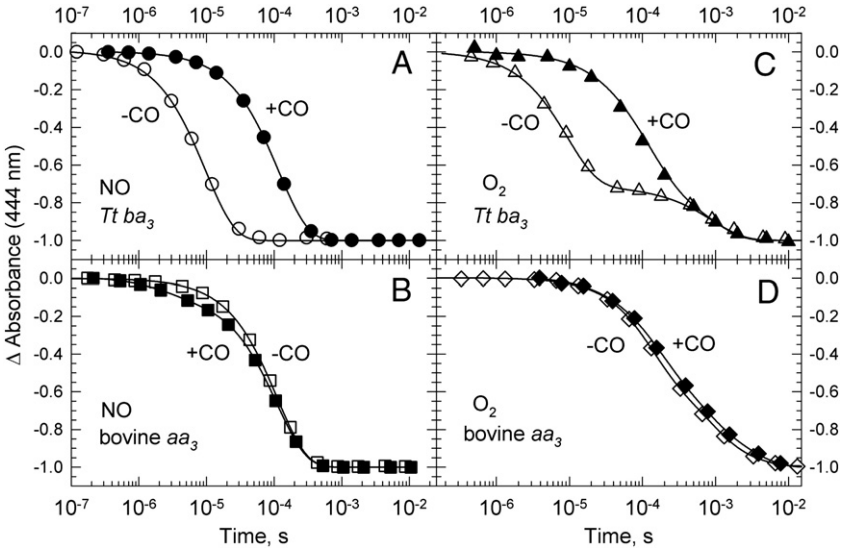



**Fig. 9.** Comparison of the transient absorbance changes at 444 nm during the reaction of the fully reduced $ba_3$ (panels A and C) and bovine $aa_3$ (panels B and D) with photoproduced NO (A and B) and photoproduced $O_2$ (C and D) in the presence of CO (filled symbols) and absence of CO (open symbols). The kinetics traces are from time-resolved optical absorption data recorded at multiple wavelengths and are normalized to the total absorbance change. The solid lines represent the absorbance traces at 444 nm calculated on the basis of a single exponential fit. The conditions are those reported in [40,60].

post-photolysis structures, respectively. It is noteworthy that the CO in the post-photolysis bovine enzyme moves "out" of the binuclear center, i.e. away from the $H_2O$ exit channel and back toward the ligand entrance channel. This suggests that in the post-photolysis fully reduced bovine enzyme, CO is already "escaping" the binuclear center; this is in contrast to the $ba_3$ enzymes, in which the photodissociated CO rotates within the binuclear cavity, but remains bound to the metals. This may explain why CO impedes $O_2$ binding in $ba_3$ but not in the bovine enzyme.

### 3.2. Differences in ligand access to the active site in bovine $aa_3$ and Tt $ba_3$

The 10-fold faster rate of $O_2$ and NO binding in *Tt* $ba_3$ compared to bovine $aa_3$ in the absence of CO indicates inherent structural differences between ligand access in the two enzymes, which may reflect their different physiological requirements. In the $aa_3$ oxidases, a constriction point defined by conserved tryptophan and phenylalanine residues was identified [24,27,28], while in *Tt* $ba_3$ these sites are occupied by smaller residues, tyrosine (Y133) and threonine (T231), respectively [26]. Recent experiments in our laboratory have shown that $O_2$ and NO binding to heme $a_3$ in the absence of CO in the Y133W and Y133W/T231F mutants of *Tt* $ba_3$ is ~5 times slower than in the wild type enzyme [29]. This suggests that the significantly slower ligand binding in the bovine enzyme ($1 \times 10^8$ $M^{-1}$ $s^{-1}$) compared to that in *Tt* $ba_3$ ($1 \times 10^9$ $M^{-1}$ $s^{-1}$) in the absence of CO is in part due to the tryptophan constriction residue in the ligand channel of the bovine $aa_3$ (W126) impeding $O_2$ and NO access to the active site [29]. Interestingly, mutation of the T231 residue in *Tt* $ba_3$ to the corresponding phenylalanine in the $aa_3$ oxidases did not have any effect on the ligand binding rate [29]. Classical molecular dynamics simulations of Xe and $O_2$ diffusion to the active sites in $ba_3$ and bovine $aa_3$ showed that the native bovine F238 residue and the F231 side chain of the Y133W/T231F mutant, which in the crystal structures extend into the ligand channels, rotate out of the channels, resulting in no effect on ligand access in the T231F mutant and, by extension, in the bovine enzyme [29]. The rate of $O_2$ and NO binding in the Y133W and Y133W/T231F mutants of *Tt* $ba_3$ in the presence of CO was also 10-times slower than in the corresponding mutants in the absence of CO and 50 times slower than in the wild-type enzyme in the absence of CO. This demonstrates that the photodissociated CO directly or indirectly slows down ligand binding in the mutants to the same extent as in the wild type *Tt* $ba_3$.

### 3.3. How does CO impede access of $O_2$ and NO to the active site in Tt $ba_3$?

In the mitochondrial enzyme, the $Cu_B^+$–CO photoproduct decays with a half-life of ~1.5 μs based on the TRIR transient at 2062 $cm^{-1}$ [32,43], significantly faster than the ~10 μs binding of $O_2$ to heme $a_3$ (at 625 μM [$O_2$]) observed in either the absence or presence of CO. Thus the dissociation of CO from $Cu_B$ does not limit $O_2$ or NO binding at the active site ($Cu_B$ or heme $a_3$) in the bovine enzyme at this $O_2$ concentration.

In the absence of CO, the rate of $O_2$ and NO binding to heme $a_3$ in *Tt* $ba_3$ is close to the diffusion-controlled limit. Therefore, if $O_2$ and NO were to bind to $Cu_B^+$ prior to heme $a_3$ in *Tt* $ba_3$, this obligatory binding would not appear to limit access of the ligands to heme $a_3$. Even in the presence of CO, the $O_2$ and NO binding in *Tt* $ba_3$ is $1 \times 10^8$ $M^{-1}$ $s^{-1}$ (~ 10 μs at 625 μM $O_2$), significantly faster than the millisecond CO dissociation from $Cu_B$ observed in the absence of $O_2$.

If CO remains on $Cu_B^+$ in *Tt* $ba_3$ on millisecond time scale in the presence of $O_2$ and the active site is only able to accommodate one ligand at the active site, $O_2$ or NO would not be able to bind to the heme, which is clearly not the case. On the other hand, if the active site is able to accommodate two ligands, albeit transiently, and if the photodissociated CO stays bound to $Cu_B^+$ in *Tt* $ba_3$ for tens of milliseconds in the presence of $O_2$, then $Cu_B$ would not be an obligatory way-station for $O_2$ (or NO) binding to heme $a_3$. In this case, $O_2$ (NO) would presumably bind to heme $a_3$ with CO remaining on $Cu_B^+$. However, a prolonged presence of CO on $Cu_B$ would not allow $Cu_B$ to act as an electron donor for the rapid O – O bond cleavage (4–5 μs) observed during $O_2$ reduction in $ba_3$ both in the absence [40,60] and presence of CO [61]. This leads us to the conclusion that the photodissociated CO in $ba_3$ does not remain on $Cu_B$ for a prolonged period of time (hundred of microseconds) when $O_2$ is present but still long enough to decrease the $O_2$ binding rate to the high-spin heme by an order of magnitude. The binding of CO to $Cu_B$ in *Tt* $ba_3$ could either impede the binding of $O_2$ to $Cu_B^+$ or if $O_2$ does not bind to $Cu_B^+$ prior to heme $a_3$, the $Cu_B^+$–CO complex could sterically restrict access of $O_2$ to the heme. The two scenarios will be discussed in more detail below.

### 3.4. Is $Cu_B^+$ an obligatory gate for $O_2$ binding to the high-spin heme in bovine $aa_3$ and Tt $ba_3$?

The binding of $O_2$ in the heme–copper oxidases has been proposed to follow Scheme 1, namely, that the obligatory path of $O_2$ to and from

the high-spin heme involves the transient binding of $O_2$ to $Cu_B^+$, as observed for CO. In early flow-flash studies of the reaction of the reduced bovine enzyme with $O_2$, the fast phase was found to increase proportionally with $O_2$ concentration at low concentrations but saturate at higher concentration (~1 mM) [37,62]. Later studies used two lasers to study the kinetics of $O_2$ binding in the bovine enzyme as a function of $O_2$ concentration, in which the first pulse photolyzed the heme $a_3$–CO complex and the second pulse photolyzed the early transient species in the $O_2$ reaction, presumably the heme $a_3^{2+}$–$O_2$ complex [63]. The authors found that the fast component of the reaction displayed a rate limit at higher $O_2$ concentration (up to 700 μM). However, there was no evidence of rate limitation in the flow-flash experiment with NO, and the authors suggested that if the escape of CO from the active site in the bovine enzyme was the cause of the $O_2$ saturation kinetics, such a mechanism would not be in place for NO; these types of measurements have not been carried out on *Tt* $ba_3$. It should be noted that the limited $O_2$ concentration in these experiments of 1 mM or less makes it difficult to ascertain unequivocally saturation kinetics behavior at high $O_2$ concentrations as well as separate the redox processes following $O_2$ binding. Subsequent high $O_2$ pressure flow-flash studies of the reaction of dioxygen with the bovine enzyme (up to 16 mM $O_2$) reported that the rate of $O_2$ binding showed saturation kinetics at high $O_2$ concentration with a limiting rate constant of $1 \times 10^6$ $s^{-1}$ [64]. It was suggested that this rate (~1 μs) was set by the dissociation of CO from $Cu_B^+$, which occurs at approximately the same rate. The authors concluded that either the $Cu_B^+$–$O_2$ complex forms prior to transfer of $O_2$ to the high-spin heme or that the $Cu_B^+$–CO directly blocks access of the incoming $O_2$ to the heme. However, as discussed above, at ~625 μM $O_2$ concentration the lifetime of the $Cu_B^+$–CO photoproduct of the bovine enzyme is too short (1.5 μs) to interfere with the ~10 μs $O_2$ binding. High $O_2$ pressure flow-flash measurements have not been carried out on the reaction of $O_2$ with *Tt* $ba_3$. In an FTIR study of the steady-state *Tt* $ba_3$ CO complex in the presence of limited amount of $O_2$ (70 μM), the C–O stretching frequency of the $Cu_B$–CO complex was found to shift from the usual 2053 $cm^{-1}$ to 2045 $cm^{-1}$ [65]. The latter peak belonged to CO as demonstrated by the appropriate shift in the $^{13}CO$ spectrum. These results were interpreted as being the result of structural changes at $Cu_B$ caused by $O_2$ being close to but not bound to $Cu_B$.

### 3.5. Does the $Cu_B^+$–CO complex directly block access of $O_2$ to heme $a_3$ in Tt $ba_3$?

Alternatively, $O_2$ may not bind to $Cu_B^+$ in the heme–copper oxidases, in particular in *Tt* $ba_3$, prior to being transferred to the high-spin heme. In the case of $ba_3$, the direct binding of $O_2$ to heme $a_3$ may give rise to changes in the geometry around $Cu_B$ from tetrahedrally-distorted square planar to more square planar, which in a concerted manner could give rise to the dissociation of CO from $Cu_B^+$, thus allowing $Cu_B$ to act as an electron donor to the bound dioxygen. This would require the active site in *Tt* $ba_3$ to be able to transiently accommodate two ligand molecules, CO on $Cu_B$ and $O_2$ on heme $a_3$. The relatively short distance between heme and $Cu_B^+$ in *Tt* $ba_3$ (4.4 Å) [66] compared to that in bovine $aa_3$ (5.1 Å) [67] would likely preclude the simultaneous binding of CO to $Cu_B^+$ and either $O_2$ (or NO) to heme $a_3^{2+}$ without some conformational changes [68]. Such structural changes as a result of ligand binding to heme $a_3$ are supported by time-resolved magnetic circular dichroism and circular dichroism measurements of the unligated *Tt* $ba_3$ formed after photodissociation of its CO complex [69]. These measurements showed spectral differences between the photolyzed enzyme and the steady-state unliganded enzyme, which were explained in terms of CO binding to heme $a_3$ inducing a global conformational change at the active site, which persisted on a time scale comparable to that of CO rebinding. Both the "dark" heme $a_3$–CO and "light" $Cu_B^+$–CO structures [49] show a structural distortion in heme $a_3$, with somewhat greater distortion (the porphyrin being not planar) in the dark structure. The $Fe_{a3}$–$Cu_B$ distance increases from 4.7 Å in the "dark" structure to 5.1 Å in the "light" structure, which may be due to tighter binding of CO to $Cu_B$ than to $Fe_{a3}$. It should also be noted that the crystal structure of *Tt* $ba_3$ shows that the high spin heme $a_3$ is tilted away from $Cu_B$, thus providing a larger surface area to the incoming ligand than in the $aa_3$ oxidases. This might allow a second ligand to be accommodated at the active site, an essential requirement of the NO reductase activity of this enzyme [12]. Based on theoretical studies, Blomberg and coworkers have proposed that two NO molecules bind at the active site of *Tt* $ba_3$, one to heme $a_3$ and one to $Cu_B$ (through the two oxygen atoms) during the conversion of NO to $N_2O$ [70]. Low-temperature FTIR photolysis experiments of the $ba_3$–NO complex led to the proposal of a $Fe_{a3}$–NO NO–$Cu_B$ intermediate during the NO reductase reaction, suggesting that the $ba_3$ binuclear center is able to transiently bind two ligands [71]. Previous spectroscopic measurements also show that two $CN^-$ molecules can be accommodated simultaneously at the active site of *Tt* $ba_3$, one bound to $Fe_{a3}^{2+}$ and the other one to $Cu_B^{2+}$ [72]. Several studies have reported that the A-type oxidases can simultaneously accommodate two ligands at the active site [14,15,73–76].

## 4. Conclusions

Several important conclusions can be drawn from the results described here. 1) Our infrared experiments, as well as UV–vis saturation CO rebinding studies as a function of CO pressure provide strong evidence that the obligatory path of CO to and from the high-spin heme in the heme–copper oxidases involves the transient binding of CO to $Cu_B^+$. 2) Our TRIRLID measurements allowed us to determine the orientation of the C–O bond axis with respect to the heme normal in both the heme $a_3$–CO complex and the $Cu_B$–CO product of the bovine enzyme, and the results are in good agreement with later crystallographic results. 3) Our time-resolved optical absorption measurements show superfast $O_2$ and NO binding to $Fe_{a3}$ in *Tt* $ba_3$ in the absence of CO, $1 \times 10^9$ $M^{-1}$ $s^{-1}$, which approaches the diffusion-controlled limit and is 10-times faster than in the bovine enzyme under the same conditions. The slower $O_2$ and NO binding in the bovine enzyme compared to $ba_3$ is partially due to the tryptophan constriction point residue in the $O_2$ channel of the bovine enzyme impeding access of $O_2$ and NO to the active site. The more open channel in *Tt* $ba_3$, which allows easier access of $O_2$ to the heme, likely reflects the functional requirements of the thermophilic bacterium, which is found under microaerobic conditions and grows optimally at 70 °C (at which temperature $O_2$ solubility is half of that in water at 25 °C). 4) The rate of $O_2$ and NO binding is 10-times slower in the presence of CO while in the bovine enzyme the $O_2$ and NO binding rate is the same in the presence and absence of CO ($1 \times 10^8$ $M^{-1}$ $s^{-1}$). These results indicate that the CO flow-flash method does not accurately reflect the $O_2$ and NO binding in *Tt* $ba_3$ under physiological conditions, namely, in the absence of CO.

Despite considerable progress, there are still some outstanding issues regarding ligand binding in the heme–copper oxidases, most importantly whether the route of $O_2$ (NO) to and from the high-spin heme involves an obligatory binding to $Cu_B$, as has been proposed for CO, and whether this path is the same for all heme–copper oxidases. While the CO flow-flash UV–visible measurements carried out at high $O_2$ pressure on the bovine enzyme reported a nonlinear dependence of the observed rate of $O_2$ binding as a function of $O_2$ concentration [64], no saturation limit was observed in flow-flash experiments on the binding of NO to the bovine enzyme [63], a ligand that is expected to model $O_2$ binding. In the bovine enzyme, our TRIR experiments show that CO dissociates from $Cu_B$ with a half-life of 1.5 μs, rapidly enough not to interfere with ~10 μs $O_2$ (NO) binding to heme $a_3$ at 1 mM $O_2$. Moreover, the rate of $O_2$ binding to heme $a_3$ is the same in the presence and absence of CO, indicating that the $Cu_B^+$–CO complex is not sterically restricting access of $O_2$ to the heme. Thus the evidence is inconclusive whether $Cu_B^+$ acts a way-station for $O_2$ (NO) in the bovine enzyme.

In *Tt* $ba_3$, our UV–visible results show that the binding of the photodissociated CO to $Cu_B^+$ slows the access of $O_2$ (NO) to heme $a_3$ by

an order of magnitude compared to that observed in the absence of CO. The $Cu_B$ in $ba_3$ has significantly higher affinity for CO compared to the bovine enzyme [47], and the crystal structure of the $Cu_B$–CO complex shows that the $Cu_B$–C bond is significantly shorter (1.88 Å) [49] and stronger than the corresponding bond in the bovine enzyme (2.43 Å) [55]. Thus it seems unlikely that $O_2$ would replace CO on $Cu_B^+$. If CO remains bound to $Cu_B$ for longer than a few microseconds, $Cu_B^+$ would not be able to provide one of the electrons required for the rapid (5 μs) breaking of the O–O bond. This is clearly not the case. We propose that the direct binding of $O_2$ to heme $a_3$ in *Tt* $ba_3$ and the driving force for the breaking of the O–O bond cause CO to dissociate from $Cu_B^+$ in a concerted manner through steric and/or electronic effects, thereby allowing $Cu_B^+$ to act as an electron donor during the breaking of the O–O bond. For this to happen would require the transient presence of two ligands, one on heme $a_3$ and the other on $Cu_B$. This proposal is supported by the NO reductase activity of *Tt* $ba_3$, which would require two NO molecules to be transiently bound at the active site. FTIR studies aimed at resolving whether the active site in *Tt* $ba_3$ is indeed able to accommodate two ligands are in progress.

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