Kinetic studies of the reactions of O$_2$ and NO with reduced *Thermus thermophilus* ba$_3$ and bovine aa$_3$ using photolabile carriers ☆

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**Abstract**

The reactions of molecular oxygen (O$_2$) and nitric oxide (NO) with reduced *Thermus thermophilus* (Tt) ba$_3$ and bovine heart aa$_3$ were investigated by time-resolved optical absorption spectroscopy to establish possible relationships between the structural diversity of these enzymes and their reaction dynamics. To determine whether the photodissociated carbon monoxide (CO) in the CO flow-flash experiment affects the ligand binding dynamics, we monitored the reactions in the absence and presence of CO using photolabile O$_2$ and NO complexes. The binding of O$_2$/NO to reduced ba$_3$ in the absence of CO occurs with a second-order rate constant of $1 \times 10^9$ M$^{-1}$ s$^{-1}$. This rate is 10-times faster than for the mammalian enzyme, and which is attributed to structural differences in the ligand channels of the two enzymes. Moreover, the O$_2$/NO binding in ba$_3$ is 10-times slower in the presence of the photodissociated CO while the rates are the same for the bovine enzyme. This indicates that the photodissociated CO directly or indirectly impedes O$_2$ and NO access to the active site in Tt ba$_3$, and that traditional CO flow-flash experiments do not accurately reflect the O$_2$ and NO binding kinetics in ba$_3$. We suggest that in ba$_3$ the binding of O$_2$ (NO) to heme aa$_3^{2+}$ causes rapid dissociation of CO from Cu$_n^+$ through steric or electronic effects or, alternatively, that the photodissociated CO does not bind to Cu$_n^+$. These findings indicate that structural differences between Tt ba$_3$ and the bovine aa$_3$ enzyme are tightly linked to mechanistic differences in the functions of these enzymes. This article is part of a Special Issue entitled: Respiratory Oxidases.

**Keywords**

*Thermus thermophilus* ba$_3$; O$_2$ and NO photolabile carriers; Double-laser transient absorption spectroscopy; CO photodissociation

**1. Introduction**

Activation of molecular oxygen and its four-electron reduction to water represent key processes of biological energy production in eukaryotes and many prokaryotes. The O$_2$
reduction is typically catalyzed by cytochrome and ubiquinol oxidases using electrons from respiratory electron transport [1–4]. This reaction is coupled to proton translocation across the inner mitochondrial or bacterial plasma membrane to generate an electrochemical proton gradient required for ATP synthesis [5].

Aside from the well known role of the heme–copper oxidases in energy conservation, cytochrome c oxidase is inhibited by nitric oxide (NO), and this inhibition has been implicated in the regulation of cellular respiration in eukaryotes [6,7]. Moreover, several bacterial heme–copper oxidases, including Thermus thermophilus (Tt) ba3, Escherichia coli bo3 ubiquinol oxidase and the cbb3 oxidases, can catalyze the reduction of NO to nitrous oxide (N2O), albeit with low efficiency [8–11]. NO denitrification by NO reductases (NORs) occurs in many prokaryotes and has been shown to endow various pathogenic bacteria with the ability to resist the mammalian immune response [12,13].

The mechanisms underlying the fast reactions of the heme–copper oxidases, O2 activation, electron and proton transfer, and coupling of electron transfer to proton translocation, remain a major challenge in the field of bioenergetics. Crystallographic studies of the bovine enzyme and several bacterial oxidases have provided a detailed picture of the static structures of the enzymes [14–19]. While sequence homology of the catalytic subunit containing the active site binuclear center is high between the bovine enzyme and the bacterial Rhodobacter sphaeroides and Paracoccus denitrificans aa3 oxidases (54 and 55%, respectively), it is much lower among other oxidases, including Tt ba3 (23%). Considering the very different functional environments of Tt ba3 and the bovine heart cytochrome oxidase, one might expect not only structural but also kinetic differences between the two enzymes. For instance, the significantly lower O2 solubility at 70 °C, the optimum growth temperature of the T. thermophilus bacterium, compared to that at room temperature, argues for more efficient O2 transport to the active site in Tt ba3. However, applying the traditional CO flow-flash method to study the O2 (and NO) binding dynamics in Tt ba3 may be compromised by the fate of the photodissociated CO. Whether this is the case can only be addressed by directly comparing the binding of ligands such as O2 and NO to Tt ba3 and the aa3 oxidases in the absence and presence of CO.

This paper summarizes recent investigations of NO binding and O2 binding and reduction in Tt ba3 and bovine aa3 using a novel time-resolved spectroscopic approach based on photolabile O2 and NO carriers. A comparative study of the two enzymes allows us to relate kinetic differences to their different structures, which evolved during adaptation to different physiological environments.

2. Ligand binding in the heme-copper oxidases

The ligand binding dynamics of O2 and NO in the heme-copper oxidases are key elements of the physiological O2 reduction, the NO inhibition of the mitochondrial enzyme, and the NO reductase activity of several of the bacterial oxidases. Carbon monoxide (CO), a competitive inhibitor of O2 binding, has frequently been used to model O2/NO binding in the heme-copper oxidases. Photodissociation and recombination studies of the CO-bound cytochrome oxidase dynamics have provided insights into the pathways of this ligand to and from heme a3 at the active site [4,20]. Moreover, CO forms a thermo-dynamically and kinetically stable complex with the reduced high-spin heme, and photodissociation of CO from the heme in the presence of either O2 or NO is generally used to initiate the reaction of these ligands with the reduced enzyme [21]. This “flow-flash” approach circumvents the rate limitation of conventional mixing techniques and has been used extensively to investigate O2 binding and reduction on time scales from microseconds to milliseconds [1–4].
The success of the CO flow-flash technique is contingent on the photodissociated CO not interfering with O$_2$ or NO binding at the binuclear center. Low-temperature Fourier transform infrared (FTIR) and time-resolved infrared (TRIR) studies have shown that the photodissociated CO binds to Cu$^{+}_n$ in several heme–copper oxidases, including the mitochondrial $aa_3$ [22–24] and $Tt$ ba$_3$ [25,26]. The pathway of CO from the solution to its binding site on heme $a_3^{2+}$ has likewise been proposed to involve a Cu$^{+}_n$–CO intermediate [23,24], with Cu$^{+}_n$ acting as a gateway for transporting CO and, by inference, other ligands such as NO and O$_2$ to and from the reduced high-spin heme $a_3$ [24,27]. In the mitochondrial enzyme, the Cu$^{+}_n$–CO photoproduct decays with a lifetime of $\sim$1.5 μs [23,24], while in $Tt$ ba$_3$, the reported lifetime is $\sim$30 ms [26]. The long lifetime of the Cu$^{+}_n$–CO complex in ba$_3$ raises questions whether the O$_2$/NO binding to heme $a_3^{2+}$ is impeded by the photodissociated CO in CO flow-flash experiments on this enzyme, and, secondly, whether the binding of these ligands to heme $a_3^{2+}$ in ba$_3$, and possibly other heme–copper oxidases, is in fact preceded by binding to Cu$^{+}_n$. The fate of the photodissociated CO is also significant with respect to establishing whether two ligands can be accommodated simultaneously at the active site. This is of particular relevance for the bacterial heme–copper oxidases with NO reductase activity, which requires at least the transient presence of two NO molecules at the active site [28]. Ultrafast transient absorption measurements of the photolyzed NO-bound bovine $aa_3$, $P$. denitrificans $aa_3$ and $Tt$ ba$_3$ have provided important insight into the NO recombination dynamics of these enzymes on a fast time scale [27–29]. However, kinetic studies of the direct binding of NO from solution to reduced ba$_3$ and the effect of CO on the NO binding dynamics have not been undertaken.

To address these issues, we investigated the reactions of O$_2$/NO with reduced $Tt$ ba$_3$ and the bovine cytochrome oxidase at room temperature using photolabile O$_2$ and NO carriers (see Supporting Information for experimental details). This method eliminates the interference from the photodissociated CO in the CO flow-flash method and circumvents the low NO quantum yield in NO flash-photolysis studies and the rate-limitation of traditional NO stopped-flow methods. Both the photolabile O$_2$ complex, [(μ-O$_2$)(μ-OH)(Co(bpy)$_2$)$_2$]$^{3+}$, and the NO- complex, K$_2$RuNOCl$_5$, are stable under anaerobic conditions at pH 7 and release O$_2$ or NO within the 7 ns 355 nm laser pulse (this study and [30,31]). Moreover, neither the complexes nor their respective photoproducts react with the enzymes during the transient absorption measurements. By exploring the binding of both O$_2$ and NO to the reduced enzymes, we can probe the microsecond time scale ligand binding dynamics during the physiological O$_2$ reduction as well as in the absence of redox chemistry (NO). We also monitored the O$_2$ reduction in ba$_3$ and extracted the absorption spectra of the respective intermediates based upon proposed mechanisms (these studies will be discussed in Section 3 on the O$_2$ reduction mechanism). For comparison, we investigated the reactions of O$_2$ and NO with both $Tt$ ba$_3$ and bovine $aa_3$ using a double-laser approach in which the fully reduced CO-bound enzymes were photolyzed with a 532 nm laser pulse and O$_2$ or NO was generated simultaneously by photolyzing the respective photolabile carrier with a 355 nm laser pulse [32]. This approach, unlike the CO flow-flash method, is not limited to enzymes with strong binding of CO to heme $a_3^{2+}$.

2.1. NO binding to reduced Tt ba$_3$ and bovine aa$_3$

Fig. 1 (a and b) shows the time-resolved optical absorption difference spectra (post-minus pre-photolysis) recorded during the reaction of reduced ba$_3$ with photoproduced NO in the absence and presence of CO, respectively. In the presence of CO, the reduced CO-bound enzyme was photolyzed with a 532 nm laser pulse and NO was generated simultaneously
with a 355 nm laser flash. The corresponding difference spectra for the bovine enzyme are shown in Fig. 2a and b, respectively.

The effect of CO on the rate of NO binding to reduced \( b_{a_3} \) can be clearly visualized when the time dependence of the absorbance is plotted at a specific wavelength. Fig. 3a shows a comparison between the NO binding kinetics for \( b_{a_3} \) at 444 nm, the absorption maximum of the reduced heme \( a_3 \), in the absence (open circles) and presence of CO (closed circles). Fig. 3b demonstrates analogous traces for the bovine enzyme in the absence and presence of CO (open and closed triangles, respectively). The data are extracted from Figs. 1 and 2, and the solid lines represent absorbance changes calculated on the basis of global exponential fits to the data (see below). It is clear that the binding of NO to reduced \( b_{a_3} \) is significantly faster in the absence of CO (Fig. 3a), while the rates are the same for the bovine enzyme (Fig. 3b).

The time-resolved difference spectra were subjected to singular value decomposition (SVD) and global exponential fitting [33–36]. A single apparent lifetime of 16 \( \mu \)s (60 \( \mu \)M NO) was observed for \( b_{a_3} \) in the absence of CO. This process is attributed to NO binding to heme \( a_3^{2+} \) based on the corresponding spectral change, represented by the \( b \)-spectrum (Fig. 4a, solid curve). In the case of a single-step mechanism, the \( b \)-spectrum is the difference between the spectra of the first and second intermediates, i.e. the reduced and NO-bound enzymes, respectively. The time-independent \( b_0 \)-spectrum (dashed curve) is the difference spectrum at infinite time. The 16 \( \mu \)s lifetime at 60 \( \mu \)M NO concentration corresponds to a second-order rate constant of \( 1 \times 10^9 \) M\(^{-1}\) s\(^{-1}\) for NO binding to reduced \( b_{a_3} \) in the absence of CO. The value of the second-order rate constant and the linearity of the binding rate with NO concentration were confirmed using different NO concentrations (Fig. S1).

SVD-based global exponential fitting of the time-resolved difference spectra recorded during the reaction of reduced \( b_{a_3} \) with NO in the presence of CO revealed an apparent lifetime of 166 \( \mu \)s at 70 \( \mu \)M NO concentration. This corresponds to a second-order rate constant for NO binding of \( 9 \times 10^7 \) M\(^{-1}\) s\(^{-1}\), a rate \( \sim \)10-times slower than in the absence of CO. The respective spectral change is shown in Fig. 4b (solid curve); the dashed line represents the time-independent \( b_0 \)-spectrum.

For the bovine enzyme, apparent lifetimes of 127 \( \mu \)s (87 \( \mu \)M NO) and 105 \( \mu \)s (105 \( \mu \)M NO) were observed in the absence and presence of CO, respectively, with \( b \)-spectra analogous to those observed for the \( Tt b_{a_3} \). These lifetimes correspond to the same second-order rate constant, \( 9 \times 10^7 \) M\(^{-1}\) s\(^{-1}\), in the absence and presence of CO.

### 2.2. \( O_2 \) binding to \( Tt b_{a_3} \) and bovine \( a_{a_3} \)

Our laboratory recently investigated the \( O_2 \) binding and reduction in \( Tt b_{a_3} \) in the absence and presence of CO using a photolabile \( O_2 \) carrier [32]. SVD-based global exponential fitting and kinetic analysis of the time-resolved data recorded during the \( O_2 \) reduction by \( b_{a_3} \) showed that the \( O_2 \) binding at 90 \( \mu \)M \( O_2 \) concentration occurs with an apparent lifetime of 9.3 \( \mu \)s in the absence of CO and 110 \( \mu \)s in the presence of CO. This corresponds to second-order rate constants of \( 1 \times 10^9 \) and \( 1 \times 10^8 \) M\(^{-1}\) s\(^{-1}\), respectively; the details of the kinetic analysis are discussed in Ref. [32] and in Section 3 on the \( O_2 \) reduction mechanism. The rate of \( O_2 \) binding in the bovine enzyme was the same in the absence and presence of CO, \( \sim 1 \times 10^8 \) M\(^{-1}\) s\(^{-1}\).

Our results demonstrate that in the absence of CO, the rate of \( O_2 \) and NO binding to heme \( a_3^{2+} \) in \( b_{a_3} \), \( 1 \times 10^9 \) M\(^{-1}\) s\(^{-1}\), approaches the diffusion-controlled limit and is 10-fold faster than in the presence of photodissociated CO. In contrast, the rate of either \( O_2 \) or NO binding...
to heme $a_3^{2+}$ in the bovine enzyme is the same in the presence and absence of CO. These results indicate different ligand accessibility in $Tt$ ba$_3$ and the bovine enzyme, and suggest that in ba$_3$ the presence of CO on Cu$_h^+$ or at a nearby docking site impedes the access of O$_2$/NO to the heme $a_3$ site; the different possibilities will be discussed below. Our studies also show that for $Tt$ ba$_3$, the CO flow-flash method does not give accurate results for the “physiological” O$_2$ and NO binding kinetics, namely, that observed in the absence of CO.

2.3. Does Cu$_h^+$ act as an obligatory way-station for ligand binding to heme $a_3$?

Our observations have important implications and raise several questions regarding the possible mechanisms of ligand binding at the binuclear center in $Tt$ ba$_3$. One question concerns whether the binding of O$_2$/NO to Cu$_h^+$ on route to and from heme $a_3^{2+}$ is indeed a common mechanistic feature among the different oxidases as proposed for CO [20]. In ba$_3$, the rate of O$_2$/NO binding to heme $a_3^{2+}$ in the absence of CO is close to the diffusion-controlled limit. Therefore, if O$_2$/NO binds to Cu$_h^+$ on its route to heme $a_3$, this binding does not appear to limit the approach of the ligands to heme $a_3^{2+}$ to any significant extent. Carbon monoxide has been reported to bind to Cu$_h^+$ in the bovine enzyme with an upper limit of 1 ps following photolysis of CO from heme $a_3^{2+}$ [37]. Thus, by inference, the photodissociated CO would be expected to bind to Cu$_h^+$ in ba$_3$ much faster than the photoproduced O$_2$/NO. The reported affinity of Cu$_h^+$ for NO is also much lower than that for CO [9], and thus NO is unlikely to replace CO at the Cu$_h^+$ site. The affinity of the reduced Cu$_B$ for CO would also be expected to be significantly higher than that of O$_2$. Therefore, if the photodissociated CO stays bound to Cu$_h^+$ in ba$_3$ for hundreds of microseconds, Cu$_B$ could not be an obligatory way-station for O$_2$/NO binding to heme $a_3^{2+}$.

2.4. Does the reduced binuclear center in $Tt$ ba$_3$ bind one or two ligands simultaneously?

The above discussion raises a fundamental question whether the active site of reduced $Tt$ ba$_3$ can accommodate the simultaneous binding of two ligand molecules, one on heme $a_3$ and the other on Cu$_B$. Low-temperature EPR and room temperature optical studies of the NO binding dynamics of reduced ba$_3$ and $P$. denitrificans aa$_3$ concluded that only one NO was bound with high affinity to heme $a_3^{2+}$ in ba$_3$ whereas two NO molecules could bind at the active site of the Paracoccus enzyme, one at heme $a_3^{2+}$ and the other presumably at Cu$_h^+$ the binding of a second molecule in ba$_3$ with low affinity was not excluded [28]. Low-temperature (30 K) FTIR experiments indicated that the reduced E. coli ba$_3$, which like $Tt$ ba$_3$, reduces NO to N$_2$O [8], simultaneously binds NO and CO to the reduced high-spin heme and Cu$_B$, respectively, while Thermus ba$_3$ does not [38]. This difference was attributed to the greater distance between Cu$_B$ and the heme $a_3$ iron in the ba$_3$ oxidase (5.30 Å) compared to that in ba$_3$ (4.4–4.7 Å) [14,17,39]; these distances refer X-ray data of the oxidized enzymes. The shorter iron-copper distance in ba$_3$ suggests that the simultaneous binding of CO to Cu$_h^+$ and either O$_2$ or NO to heme $a_3^{2+}$ would likely require significant conformational changes at the active site. Recent crystallographic studies have indicated that the reduction of ba$_3$ increases the distance between heme $a_3^{2+}$ and Cu$_h^+$ by 0.3 Å, with the concomitant loss of a H$_2$O molecule between the two metal centers [40]; these redox-induced conformational changes at the active site are likely to be of mechanistic importance for O$_2$/NO binding and reduction. It should also be noted that in ba$_3$, the high-spin heme $a_3$ is tilted away from Cu$_B$, making the surface area of the binuclear center that is presented to an approaching ligand larger than in the aa$_3$ oxidases. With regards to the NO reductase activity of ba$_3$, this might allow a “second” NO molecule to react with NO already bound to

Biochim Biophys Acta.
the high-spin heme. Based on theoretical studies of the NO reductase activity in ba$_3$, Blomberg and coworkers proposed a reaction mechanism in which the binding of the first NO molecule to heme a$_3^{2+}$ activates the attack of a second NO molecule, generating a cyclic hyponitrous acid anhydride intermediate with the two oxygens coordinating to Cu$_n^+$ [41].

2.5. How does CO impede ligand access to the active site in Tt ba$_3$?

If CO remains on Cu$_n^+$ for milliseconds and the binuclear center of ba$_3$ is only able to accommodate one ligand, then the rapid binding of O$_2$/NO would not be expected to occur. This is clearly not the case. This raises questions whether the photodissociated CO does indeed bind to Cu$_n^+$ and whether the infrared frequency observed at 2053 cm$^{-1}$ following photolysis of the CO-bound ba$_3$ arises from CO bound to Cu$_n^+$ [25,26] or from CO being trapped at “docking” site [42]. Interestingly, FTIR studies of the signal transducer O$_2$-sensing protein HemAT, a heme protein lacking copper, revealed a well-defined peak at 2065 cm$^{-1}$ in the spectrum of the equilibrium CO-bound sensor and in its light-minus-dark FTIR difference spectrum [43]. A recent crystal structure of the bovine aa$_3$-CO shows that CO binds to Cu$_n^+$ in a side-on fashion after photolysis of CO from heme a$_3^{2+}$. The Cu$_n^+$-to-carbon and Cu$_n^+$-to-oxygen atom distances of 2.4 and 2.7 Å, respectively, indicate a very weak Cu$_n^+$–CO bond [44,45]. Significantly, no infrared linear dichroism was observed for the transient photoproduct of the CO-bound bovine aa$_3$, which was interpreted in terms of CO being bound to Cu$_n^+$ at the “magic angle” to the heme normal [46]; another explanation is that the photodissociated CO does not bind to Cu$_n^+$. If the latter is the case for ba$_3$, the presence of CO at a “docking site” [42] may impede the access of O$_2$/NO to heme a$_3^{2+}$, resulting in the 10-fold difference in the ligand binding rate between the reduced ba$_3$ and the CO-photolyzed reduced ba$_3$.

Alternatively, the binding of the photodissociated CO to Cu$_n^+$ may directly impede O$_2$/NO access to heme in ba$_3$. If the simultaneous binding of CO to Cu$_n^+$ and O$_2$/NO to heme a$_3^{2+}$ does occur, and if CO remains bound to Cu$_n^+$ for longer than a few microseconds, Cu$_n^+$ would be unable to act as an electron donor for the rapid O–O bond cleavage (4–5 μs) in ba$_3$ (see below and Ref. [32]). However, the binding of O$_2$ to heme a$_3^{2+}$ and the driving force for the subsequent O–O bond cleavage may cause a change in the tetrahedral geometry of Cu$_n^+$ toward more square planar, which would facilitate the oxidation of Cu$_n^+$ and the dissociation of CO from Cu$_n^+$. The observation that in ba$_3$ the 5 μs rate of O–O bond cleavage is the same in the absence (see below and Ref. [32]) and presence of CO [47], suggests that the CO dissociation from Cu$_n^+$ is not the rate-limiting step for the electron transfer to the bound dioxygen. In the bovine enzyme, the CO dissociates from Cu$_n^+$ on a time scale of 1.5 μs [23,24], rapidly enough not to interfere with the binding of O$_2$/NO to heme a$_3^{2+}$.

The slower O$_2$/NO binding to heme a$_3^{2+}$ in ba$_3$ in the presence of CO may also arise because the reduced enzyme generated after CO photo-dissociation has a conformation less favorable for ligand access to heme a$_3^{2+}$ than the normal reduced enzyme. A “global” structural change at the active site of ba$_3$ as a result of ligand binding to heme a$_3^{2+}$ is supported by time-resolved magnetic circular dichroism and circular dichroism photolysis experiments of the fully reduced CO-bound ba$_3$ [48]. These measurements showed that the high-spin photoproduced heme a$_3^{2+}$ is spectrally distinct from that observed for the steady-state reduced ba$_3$. These spectral differences were interpreted in terms of a small but global
conformational change in the protein upon CO binding to heme $a_3^{3+}$, persisting after the Fe-CO photodissociation [48].

2.6. Ligand binding in Tt $ba_3$ versus bovine $aa_3$

The binding of $O_2$ and NO to heme $a_3^{3+}$ in $ba_3$ in the absence of CO is 10-times faster than the binding of these ligands to the bovine enzyme under similar conditions. We attribute the order of magnitude difference in the ligand binding rates to structural variations in the dioxygen channel of the two enzymes, with a more open ligand access in $ba_3$. In the bovine enzyme, as well as $R. sphæroides$ and $P. denitrificans$ $aa_3$, a constriction point reduces the diameter of the $O_2$ channel, which likely impedes the access of ligands to heme $a_3$ [49,50]. These structural differences in the $O_2$ channel may be related to the functional requirements of the two enzymes, with the $Thermus$ enzyme operating optimally at 70 °C, at which temperature the $O_2$ solubility is about half of that in water at 25 °C [32,49]. Further understanding of the structural features controlling ligand ($O_2$, NO and CO) binding dynamics and reactivities of the different oxidase families will provide insight into the function of these enzymes and how the protein scaffold tunes ligand pathways for different physiological environments.

3. The $O_2$ reduction mechanism in the heme–copper oxidases

The reduction of $O_2$ to water in the heme–copper oxidases takes place at the Cu$_B$ and the high-spin heme ($a_3$, $b_3$, $o_3$); a low-spin heme serves as the electron donor to the binuclear center. Kinetic studies of the $O_2$ reduction have generally relied on the CO flow-flash technique, with the primary focus on the mitochondrial and the A-type bacterial oxidases [1–4]. Less emphasis has, until recently, been placed on the B-type oxidases, including the $Thermus$ $ba_3$ enzyme [32,47,51,52]. Most of these transient optical measurements are based on single-wavelength detection in which the $O_2$ kinetics and corresponding spectral changes are recorded at a few selected wavelengths. Because of the limited spectral information, it is difficult to derive a detailed kinetic picture using this technique. The $O_2$ reduction kinetics has generally been interpreted in terms of a unidirectional sequential mechanism (Scheme 1), with decreasing values of the apparent rate constants assigned to consecutive steps. However, recent studies in our laboratory using multi-wavelength detection and advanced data analysis have raised questions about these conventional rate assignments in $Tt$ $ba_3$. These studies are summarized below.

3.1. The reaction of reduced Tt $ba_3$ with $O_2$ in the absence of CO

To investigate the applicability of the conventional sequential $O_2$ reduction mechanism (Scheme 1) to the different heme-copper oxidases, we investigated the reactions of the reduced $Tt$ $ba_3$ and the bovine heart enzyme with photoproduced $O_2$ in the absence of CO (see Supporting Information and Ref. [32] for experimental details). The time-resolved difference spectra are shown in Fig. 5a and b, respectively. The difference spectra were analyzed using SVD and global exponential fitting. The time dependence of the spectra recorded during the reaction of reduced $ba_3$ with $O_2$ (90 μM $O_2$) in the absence of CO was best fitted with four apparent lifetimes, 4.8 μs, 9.3 μs, 55 μs and 1.0 ms. Fig. 6a (solid lines) shows the intermediate spectra (referenced versus the reduced $ba_3$) extracted based on a fast-slow sequential mechanism in which decreasing values of the apparent rate constants (increasing lifetimes) are assigned to consecutive steps (Scheme 2). Fig. 6b shows the corresponding model spectra generated from the linear combination of the ground state model spectra; the model difference spectrum of compound A is that of the bovine enzyme.

When the data were analyzed in terms of this traditional “fast–slow” mechanism, the difference spectrum of compound A (Fig. 6a, green solid curve) had the same shape as the
Einarsdóttir et al.  Page 8

corresponding difference spectrum of the bovine enzyme (Fig. 6b, green curve) but with half the amplitude. This discrepancy was also noted in previous fast-mixing CO flow-flash studies on bT2 [47]. Using a kinetic analysis based on the apparent rates and b-spectra, we were able exclude the reversibility of dioxygen binding as being the source of the reduced amplitude of the compound A spectrum [32]. A correspondence between the experimental and model spectra of compound A was attained only when the data were analyzed using a mechanism in which the 9.3 μs lifetime for O₂ binding was followed by the shorter 4.8 μs lifetime of O–O bond cleavage (Scheme 3). The green dashed curve in Fig. 6a shows the increased amplitude of the spectrum of compound A extracted using this slow-fast mechanism. The increased amplitude is in excellent agreement with the expected amplitude (Fig. 6b, green curve). The 9.3 μs lifetime at 90 μM O₂ concentration corresponds to a second-order rate constant of 1×10⁹ M⁻¹ s⁻¹, which is 10-times faster than observed for the bovine enzyme. Repeating the experiment at half the O₂ concentration (45 μM) increased the 9.3 μs lifetime to 18 μs while the 4.8 μs life-time was unchanged. This observation supports the assignment of the 9.3 μs process to O₂ binding [32]. The rate constant of 1×10⁹ M⁻¹ s⁻¹ is the same as we observed for NO binding to heme a⁵⁺ in bT2 in the absence of CO, further supporting the assignment of the 9.3 μs lifetime to the O₂-binding step.

There is an interesting parallel between a slow-fast mechanism and a mechanism containing a reversible step followed by an irreversible one; this can be demonstrated using the algebraic kinetic analysis method [32]. Both of these physical mechanisms produce very similar experimental data. When these data are analyzed by the traditional fast–slow scheme, the spectrum of the second inter-mediate in the scheme appears to be a mixture of the spectra of the first and second intermediates of the true mechanism [32]. However, a combination of multi-wavelength detection and quantitative kinetic analysis can distinguish between the two choices and identify the most probable underlying mechanism [32]. It should be noted that the 9.3 μs lifetime at 90 μM O₂ concentration corresponds to an apparent lifetime of ∼1 μs at 1 mM O₂, at which concentration the reaction would indeed follow a mechanism with increasing lifetimes assigned to consecutive steps. However, at lower O₂ concentrations (∼100 μM O₂) in the absence of CO [32] or when using the CO flow-flash method at 1 mM O₂ the slower O₂ binding, ∼10 μs in both cases, would be followed by the faster 5 μs O–O bond cleavage.

The third and fourth intermediates (Fig. 6a, red and cyan, respectively) generated during O₂ reduction in bT2, both correspond to P but with heme b oxidized in the former (P₁) and rereduced in the latter (P₄) (Scheme 3); no 580-nm oxyferryl F form is observed, in agreement with recent CO flow-flash experiments on bT2 [47,52]. The P₁ intermediate formed upon the decay of compound A (Fig. 6a, red curve) is well modeled by the P₅ spectrum of bT2 (heme b oxidized) (Fig. 6b, red curve). In contrast, the spectrum of P₅ of the bovine enzyme, extracted from the time-resolved data obtained in a traditional CO flow-flash experiment, is considerably broader in the visible region than that of P₁ for bT2 [53]. These observations support that P₅ in the bovine enzyme and P₁ in bT2 are indeed spectrally different. Our previous studies show that P₅ is best modeled by a mixture of the spectra of compound A, P and F, which we interpreted in terms of a branched mechanism [36, 54].

3.2. The reaction of reduced Tt bT2 with O₂ in the presence of CO

The reaction of reduced bT2 with O₂ in the presence of CO was initiated by simultaneously photolyzing the fully reduced CO-bound enzyme and the photolabile O₂ complex with 532 and 355 nm laser pulses, respectively [32]. In the presence of CO, three apparent life-times, 59 μs, 110 μs and 0.82 ms, were resolved for the reaction of O₂ with bT2 at 90 μM O₂ concentration. Kinetic analysis based on the apparent rates and the corresponding spectral changes (b-spectra), together with a comparison of the extracted intermediate spectra with
model spectra, showed that the 110 μs lifetime is due to O₂ binding, followed by the O–O bond cleavage on 5 μs time scale (A to P₁) [32]; the latter process is not resolved in our experiment due to the rate-limiting nature of the O₂-binding step. The generation of P₁ is followed by the formation of P₁I (59 μs) and the subsequent generation of the oxidized enzyme (0.82 ms).

3.3. The reaction of O₂ with the reduced bovine heart oxidase in the absence of CO

The reaction of the reduced bovine heart enzyme with dioxygen was investigated in the absence of CO using the photolabile O₂ carrier. SVD-based global exponential fitting of the time-resolved difference spectra resolved four lifetimes, 42 μs, 160 μs, 1.1 ms and 4.3 ms at 70 μM O₂ concentration. Kinetic analysis similar to that applied to the ba₃ showed that the 160 μs lifetime represents the binding of O₂ to heme a₃²⁺. This lifetime corresponds to a second-order rate constant of O₂ binding of ~1×10⁸ M⁻¹ s⁻¹, which is equivalent to that observed in CO flow-flash experiments [55–57]. The 42 μs lifetime is the result of heme a oxidation, a dominant spectral change following O₂ binding. However, the individual formation of Pᵣ and P₁F₁I was not resolved because both processes are faster than the O₂-binding step. Both millisecond processes show spectral changes characteristic of the final oxidation step [36].

3.4. Comparison of the early kinetic steps in Tt ba₃ and bovine aa₃

In addition to the 10-times faster O₂/NO binding in ba₃ compared to the bovine enzyme, the conversion of compound A to P₁ is ~8 times faster in ba₃ (4.8 μs compared to ~40 μs in the bovine enzyme). This may be due to different redox potentials of the high- and low-spin hemes in the two enzymes. The absence of electron back-flow following photolysis of the mixed-valence CO-bound ba₃ is in line with this suggestion (unpublished results). A relatively short heme a₃ iron-CuB distance in ba₃ may also facilitate electron transfer from Cu⁺ to the oxygen-bound heme a₃.

Our results show that following the decay of compound A in ba₃ to P₁, the subsequent reduction of the oxidized heme b is not associated with the formation of the 580-nm oxyferryl F intermediate, as in the bovine enzyme, but instead the generation of an intermediate which maintains the spectral signature of P (P₁I). This observation is in agreement with recent CO flow-flash results on ba₃ [47,52]. Thus despite similarities between the bovine enzyme and Thermus ba₃ at the active site, there are sufficient structural and dynamic differences to produce different intermediates in the two enzymes. As previously suggested, the proton acceptor postulated to cause the spectral differences between P and F, i.e. the deprotonated cross-linked tyrosine, Y244⁻, or Cu⁺-OH⁻, may be different in the two enzymes [47,52].

4. Conclusions

Several important conclusions can be drawn from our studies. First, the results show superfast O₂ and NO binding to reduced ba₃ in the absence of CO, with the rates of O₂ and NO binding approaching the diffusion-controlled limit. These rates are 10-times greater than found for ba₃ in the presence of CO, indicating that the photodissociated CO directly or indirectly impedes O₂ and NO access to the active site in this enzyme. Therefore, the CO flow-flash approach does not accurately reflect the O₂ and NO binding kinetics in ba₃. Second, our kinetic analysis demonstrates that the breaking of the O–O bond occurs on ~5 μs time scale, 8-times faster than in the bovine enzyme. Third, our results show that the binding of O₂ and NO to heme a₃²⁺ in ba₃ in the absence of the photodissociated CO is 10-times faster than in the bovine enzyme under equivalent conditions or when using the CO
flow-flash method. Together these results indicate important structural differences between the accessibility of O$_2$/NO to the active site in ba$_3$ and the bovine heart enzyme. We suggest that the different ligand access is related to a constriction point in the oxygen channel of the aa$_3$ oxidases, which is not present in ba$_3$ [49].

The fast rates of O$_2$ and NO binding to heme aa$_3^2+$ in ba$_3$ in both the absence and presence of CO and the reported long lifetime of the Cu$_n^+$–CO complex suggest that Cu$_n^+$ may not act as a gateway for O$_2$/NO binding to heme aa$_3^2+$ in ba$_3$. Our findings also raise a fundamental question about whether the photodissociated CO impedes the access of O$_2$/NO to heme aa$_3^2+$, either by binding to Cu$_n^+$ or being present at ligand docking site. If the former is true, we propose that during the CO flow-flash method, or when using the double-laser approach, the O$_2$ binding to heme aa$_3^2+$ causes CO to dissociate from Cu$_n^+$ through steric and electronic effects, thereby allowing Cu$_n^+$ to act as an electron donor during O–O bond cleavage.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Biochim Biophys Acta.


Fig. 1.
Time-resolved optical absorption difference spectra (post-minus pre-photolysis) of the reaction of the fully reduced *Tt ba*$_3$ with photoproduced NO in the absence of CO (panel a) and the presence of CO (panel b). The spectra were obtained by subtracting the spectral contribution of the photolyzed NO complex, determined in a separate experiment. The spectra (SVD-filtered) were recorded at 12–16 delay times, equally spaced on a logarithmic scale, between 200 ns–10 ms in the absence of CO and 500 ns–50 ms in the presence of CO. The arrows represent the direction of the absorbance change with time. Conditions: the effective enzyme concentration, 1.2 μM; 0.1 M HEPES (pH 7.5); 0.1% DM; effective NO concentration, 60 μM (panel a) and 70 μM (panel b); optical path, 0.5 cm. The CO concentration was 0.5 mM after mixing.
Fig. 2.
Time-resolved optical absorption difference spectra for the reaction of the fully reduced bovine $a_2B_3$ with photoproduced NO in the absence of CO (panel a; 100 ns–10 ms) and the presence of CO (panel b; 200 ns–10 ms) (see Fig. 1 for details). Conditions: the effective enzyme concentration, 5.2 μM; 50 mM Na-phosphate (pH 7.5); effective NO concentration: 87 μM (a) and 105 μM (b). The CO concentration was 0.5 mM after mixing.
Fig. 3.
Comparison of the transient absorbance changes taking place at 444 nm during the reaction of fully reduced \(Tt\) ba\(_3\) (panel a) and bovine aa\(_3\) (panel b) with photoproduced NO in the presence of CO (filled circles and triangles) and in the absence of CO (open circles and triangles). The kinetic traces in panels a and b are from the time-resolved data in Figs. 1 and 2, respectively, and are normalized to the total absorbance change. The solid lines represent the absorbance traces at 444 nm, calculated on the basis of the global exponential fits (see text for details).
Fig. 4.
The spectral amplitudes ($b$-spectra; solid curves) and lifetimes from a global-exponential fit to the time-resolved data recorded during the reaction of NO with reduced $Tt$ $ba_3$ in the absence (a) and presence (b) of CO. The time-independent $b_0$-spectra (dashed curves) represent the difference spectra extrapolated to infinite time, namely, the difference between the spectra of the NO-bound and reduced enzymes (a), and the NO-bound and CO-bound enzymes (b).
Time-resolved optical absorption difference spectra (post-minus pre-photolysis) recorded during the reaction of dioxygen with the fully reduced *Tt ba*$_3$ (panel a) and bovine enzyme (panel b) in the absence of CO. The spectra are those obtained after subtracting the spectral contribution of the photolyzed O$_2$ complex, determined in a separate experiment. The spectra (SVD-filtered) were recorded at 15–17 delay times, equally spaced on a logarithmic scale, between 500 ns–20 ms (panel a) and 200 ns–50 ms (panel b). The arrows represent the direction of the absorbance change with time. Conditions: *ba*$_3$: 0.1 M HEPES (pH 7.5); 0.1% DM; effective enzyme concentration, 2.6 μM; bovine enzyme: 50 m N NaPi (pH 7.5) effective enzyme concentration, 4.3 μM; optical path, 0.5 cm.
Fig. 6.
(Panel a) (Solid lines) The experimental intermediate spectra (referenced versus the reduced enzyme) for the reaction of reduced *Tt ba*$_3$ with O$_2$ in the absence of CO. The spectra were extracted on the basis of the fast–slow mechanism in Scheme 2: compound A (green), P$_I$ (red), P$_II$ (cyan) and O (magenta). The spectrum of Int 2 extracted using the fast–slow Scheme 2 has the shape of compound A of the bovine heart oxidase but significantly lower amplitude. (Dashed, green curve) The spectrum of compound A extracted using the slow–fast mechanism in Scheme 3, in which the 9.3 μs process is followed by the 4.8 μs step. (Panel b) The model spectra of the proposed intermediates, compoundA(green), P$_I$ (red), P$_II$ (cyan) and O (magenta), were generated based on the linear combinations of the ground-state spectra of ba$_3$; the difference spectrum of compound A is that of the bovine enzyme. The spectrum in panel a are normalized to the enzyme concentration in panel b.
Scheme 1.
The conventional sequential unidirectional mechanism for the reduction of dioxygen to water.
Scheme 2.
A unidirectional “fast-slow” sequential mechanism of O₂ reduction for ba₃ in the absence of CO.

\[ R \rightarrow A \rightarrow P_I \rightarrow P_{II} \rightarrow O \]
Scheme 3.
The “slow-fast” sequential mechanism for the reaction of $ba_5$ with photo-produced $O_2$ in the absence of CO.