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Ligand Access to the Active Site in *Thermus thermophilus* *ba*₃ and Bovine Heart *aa*₃ Cytochrome Oxidases

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Abstract

Knowledge of the structure and dynamics of the ligand channel(s) in heme-copper oxidases is critical for understanding how the protein environment modulates their functions. Using photolabile NO and O₂ carriers, we recently found that NO and O₂ binding in *Thermus thermophilus* *ba*₃ (*Tt ba*₃) is ~10-times faster than in the bovine enzyme, indicating inherent structural differences that affect ligand access in these enzymes. Using x-ray crystallography, time-resolved optical absorption measurements, and theoretical calculations, we investigated ligand access in the *Tt ba*₃ mutants Y133W, T231F, and Y133W&T231F, in which tyrosine and/or threonine in the O₂-channel of *Tt ba*₃ are replaced by the corresponding bulkier tryptophan and phenylalanine present in the *aa*₃ enzymes. NO binding in Y133W and Y133W&T231F is 5-times slower than in wild-type *ba*₃ and the T231F mutant, and the results show that the *Tt ba*₃ Y133W mutation and the bovine W126 residue physically impede NO access to the binuclear center. In the bovine enzyme there is a hydrophobic “way-station,” which may further slow ligand access to the active site. Classical simulations of Xe diffusion to the active sites in *ba*₃ and bovine *aa*₃ show conformational freedom of the bovine F238 and the F231 side chain of the *Tt ba*₃ Y133W&T231F mutant, with both residues rotating out of the ligand channel, resulting in no effect on ligand access in either enzyme.

During the evolution of metallo-enzymes and their catalytic activity, the protein environment was likely to have been modified in such a way that it effectively tuned ligand pathways and metal-based prosthetic group(s) for optimal function in different conditions. The heme-copper oxidases play a key role in energy production of aerobic organisms (1-3), and their structural and functional similarities and differences make them an ideal system for exploring the effect of the protein environment on enzymatic function. The heme-copper oxidases have been classified into three sub-families, A, B, and C, based on their phylogenetic and structural analysis (4, 5). The A-family bacterial *Rhodobacter sphaeroides* (*Rs*) and *Paracoccus denitrificans* (*Pd*) *aa*₃ oxidases have high sequence homology to their

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Supplementary Information Available

Additional details regarding the simulation protocol, and system preparation and equilibration for the classical molecular dynamic simulation can be found in the Supplementary Information. Figure S1. The *Tt ba*₃ Y133W&T231F mutant in the context of the overall structure of the enzyme. Table S1 summarizes the data collection parameters and refinement statistics for the Y133W and Y133W&T231F mutants. Table S2 shows the distances defining the regions of the ligand channels in the wild-type *Tt ba*₃, Y133W *Tt ba*₃ and bovine *aa*₃. Table S3 shows the volumes of the cavities in the ligand channels of the wild-type *Tt ba*₃, Y133W *Tt ba*₃ and bovine *aa*₃. This material is available free of charge via the Internet at <http://pubs.acs.org>.

mitochondrial counterpart (5), while other heme-copper oxidases, including the B-family *Thermus thermophilus* (*Tt*) *ba₃* (6) and the C-family *cbb₃* oxidases (7) are phylogenetically more distant, with low sequence homology to the *aa₃* oxidases (5). Phylogenetic analysis has shown that the A-family is the most ancient, with both the B- and C-families evolving later (8).

The heme-copper oxidases catalyze the reduction of O₂ to water, which is coupled to the generation of a transmembrane electrochemical proton gradient required for ATP synthesis (1-3). Previous heme-copper oxidase research focused on the mechanisms of electron and proton transfer (1-3), but less emphasis has been given to the pathway(s) through which O₂ and other small ligands, such as NO and CO, move to the binuclear active site. A primary question concerns the structural and dynamic differences responsible for the adaptation of the heme-copper oxidases to different functional environments. For example, the gram-negative bacterium *T. thermophilus* grows optimally at 70°C (9) and it is found under microaerobic conditions, such as those existing at high temperatures, oxic/anoxic interfaces or in hypersaline microbial mats (8). The *Tt* HB8 strain has adapted to these environments as a facultative anaerobe (10), and it has been shown to express two types of oxidases depending on the oxygen tension (10, 11). The B-type *ba₃* oxidase has higher oxygen affinity and is typically expressed under microaerobic conditions (~10 μM), while the lower-oxygen affinity A-type *caa₃* oxidase is expressed at higher oxygen concentration (12-14)

On the basis of their crystal structures, putative O₂ channels have been suggested for the bovine heart *aa₃* enzyme (15), *Rs aa₃* (16), *Pd aa₃* (17), *Tt ba₃* (6), *Escherichia coli bo₃* (18), and *Pseudomonas stutzeri cbb₃* (7). Mutagenesis and biochemical experiments on *Rs aa₃*, *Pd aa₃* and *E. coli bo₃* suggest that these channels may indeed serve as paths for ligand diffusion into the binuclear site (19, 20). The residues and helices lining the channels demonstrate significant sequence and structural similarities among the different heme-copper oxidases (5). However, the global structure of the catalytic subunit shows structural variations that may be related to the different physiological and biochemical roles. A recent crystallographic study of xenon (Xe) binding to *Tt ba₃* identified a 'Y-shaped' hydrophobic channel, 18-20 Å in length, leading from the protein exterior of subunit I to the binuclear center; the channel is defined by five major and two minor discrete Xe atom binding sites (Xe1 through Xe7) (21, 22). Moreover, there is a narrowing of the channel confined by conserved tryptophan and phenylalanine residues in the ligand channel of the bovine, *Rs*, and *Pd aa₃* oxidases (16, 20, 23), while smaller residues, tyrosine (Y133) and threonine (T231), respectively, occupy these sites in *Tt ba₃* (6, 21) (Figure 1). Considering the 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. At low oxygen concentration (< 5-10 μM), the rate of O₂ binding would be rate limiting for the O₂ reduction assuming the bimolecular rate constant of $1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ reported for the *aa₃* enzymes. Although O₂ diffusion may be more efficient at higher temperature due to increased protein dynamics, it may not be able to compensate for this rate-limited O₂ binding. Using photolabile O₂ and NO carriers in conjunction with time-resolved optical absorption (TROA) spectroscopy, we recently demonstrated that the binding of O₂ and NO in *Tt ba₃* is 10-fold faster than in the bovine enzyme ($1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ vs. $1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$) (24, 25). These results suggest inherent differences between ligand access in the two enzymes, possibly related to the constriction point that is present in the *aa₃* oxidases but absent in *Tt ba₃*. Hence, the more open O₂ channel in *Tt ba₃* may reflect evolutionary adaptation in *Tt ba₃* to increase the rate of O₂ diffusion to the binuclear center, allowing the enzyme to maintain physiologically relevant reaction rates under low or microaerobic O₂ concentrations.

In this study, we used time-resolved optical absorption spectroscopy to investigate the NO binding in the *Tt ba₃* single mutants Y133W and T231F, and the Y133W&T231F double mutant, in which the conserved tryptophan and phenylalanine residues in the ligand channel of the bovine, *Rs*, and *Pd aa₃* enzymes are inserted into *Tt ba₃*. The results, together with the crystal structures of the wild-type and mutant *Tt ba₃*, and classical simulations of Xe diffusion into the active site of the bovine *aa₃* and *Tt ba₃*, provide strong support for the constriction point and a hydrophobic pocket in the ligand channel of the bovine enzyme impeding NO access to the active site in this enzyme but not in *Tt ba₃*.

Materials and Methods

Protein expression, purification and crystallization

Recombinant wild-type and mutant (Y133W, Y133F, T231F and Y133W&T231F) cytochrome *ba₃* oxidase genes were expressed in *Tt* HB8 and the enzymes purified as previously described (26). The proteins were concentrated to 20 mg/ml in 20 mM Tris buffer, pH 7.6, 0.05% n-dodecyl- β -D-maltoside buffer, and stored at 4 °C.

Steady-state activity measurements

The steady-state activity of the *Tt ba₃* mutants was measured using an oxygen electrode (26). Purified *Tt* cytochrome *c₅₅₂* in 10 mM Tris buffer (pH 7.6) was first added to the reaction chamber to generate a flat baseline. This was followed by addition of 6 μ l of 1.2 M ascorbate to obtain a background slope reading. After about 1 minute, 5 μ l of the *ba₃* sample (1 mg/ml) was added and the slope recorded until all O₂ in the chamber was consumed. The slope of the background was subtracted from the slope obtained during O₂ consumption; the resulting slope was used to calculate the steady-state activity.

Crystallization

The protein samples were spun at 15,000 g for 15 minutes prior to starting the crystallization trials. Following spinning, the protein was mixed with monoolein (Sigma) in a 2:3 v/v ratio and homogenized with a syringe mixer (27, 28). Crystallization trials were performed in a 24-well hanging drop plate using 200 nL protein-laden LCP (lipidic cubic phase) overlaid with 3 μ L reservoir solution (29). The crystallization plates were stored at 20 °C. Crystals were obtained after incubation for several days in 50 mM sodium cacodylate, pH 6.5, 1.6 M NaCl, 40-42% PEG 400. Crystals were harvested and immediately flash-frozen in liquid nitrogen.

X-ray data collection and structure determination

Crystallographic data were collected at the Stanford Synchrotron Radiation Lightsource. Data sets were integrated, scaled and merged using iMosflm (30). The wild-type *Tt ba₃* structure (PDB code 3S8F) was used as the model for molecular replacement with Phaser (31). The resulting models were adjusted manually in Coot and refined with Refmac5 (32). Crystallographic statistics for the refined structures of the Y133W and Y133W&T231F mutants are given in Table S1. The access codes in the Protein Data Bank for Y133W and Y133W&T231F are 4EV3 and 4ESL, respectively. The structure of the Y133F mutant was also determined at 2.80 Å resolution (PDB 4F05).

Time-resolved optical absorption measurements

The time-resolved optical absorption measurements were carried out using a CCD detector in combination with a photolabile NO carrier as previously described (24). The data were subjected to singular value decomposition (SVD) and global exponential fitting, which provided the spectral changes (*b*-spectra) and the apparent lifetimes (33-36). The

intermediate spectra were extracted based upon a proposed mechanism and compared to model spectra of the proposed intermediates

Classical molecular dynamics simulations of Xe diffusion to the binuclear active site in *Tt* ba_3 and the bovine aa_3

Classical molecular dynamics simulations of xenon (Xe) diffusion through subunit I of the wild-type *Tt* ba_3 , the Y133W and Y133W&T231F ba_3 mutants, and the bovine aa_3 were performed using the following protocol. Initial coordinates for the bovine enzyme were taken from the 1.90 Å resolution reduced enzyme crystal structure (PDB entry 2EIJ) (37), and those for *Tt* ba_3 were obtained from the 2.30 Å resolution oxidized enzyme resolution crystal structure (PDB entry 1XME) (38); no significant side-chain movements or backbone displacements are observed when comparing ba_3 crystal structures in different redox states (39). The initial conformations of the Y133W tryptophan and T231F phenylalanine side chains were obtained from the crystal structure published in this work after rmsd fitting the mutants to the simulated structure of an equilibrated wild-type model. The enzyme models contain subunit I from each structure, which includes the low-spin heme *a/b* (bovine/*Tt* ba_3 , respectively), the high-spin heme a_3 , and Cu_B . The bovine enzyme includes a Mg^{2+} ion. All lysine and arginine residues were positively charged, and all histidine residues were neutral. Aspartate residues and heme propionates were negatively charged, and—except for the bovine Glu242—glutamate residues were negatively charged. The proteins were inserted into a lipid bilayer consisting of two leaflets of 64 dimyristoyl phosphatidylcholine lipid molecules (DMPC) each, and then capped with TIP3P water molecules. The bovine enzyme model was then made neutral by the addition of 7 Na^+ ions, while the wild-type *Tt* ba_3 , the Y133W and the Y133W&T231F models were neutralized with 5 Cl^- ions. The final wild-type ba_3 , the Y133W and Y133W&T231F ba_3 mutants and bovine aa_3 simulations included 42457, 42460, 42466, and 35817 atoms, respectively.

The first three production simulations of 1-ns duration were started from the equilibrated system by placing a Xe atom in the Xe1, Xe2, and Xe5 binding sites of the *Tt* ba_3 subunit I identified in reference 21. Subsequent simulations were continuations of these first three. Equivalent Xe binding sites were identified in the bovine enzyme by structure alignment to the wild-type *Tt* ba_3 using the MatchMaker function in the UCSF Chimera software (40). Cavities were determined by binning all trajectories according to the average distance from Xe to Cu_B (see Table S2). Within each bin, the maximum and minimum distances from Xe to Cu_B and from Xe to two additional residues were calculated (W128 and F67 for bovine, Y133 and I78 for wild-type ba_3 , and W133 and I78 for the Y133W mutant). The ranges of distances were then adjusted to remove double counting of time points while maximizing the time in which Xe was in a respective cavity. Additional details regarding the simulation protocol, and system preparation and equilibration can be found in the Supplementary Information.

Results

Structures and steady-state activities of the O_2 channel mutants

The O_2 diffusion channel in wild-type *Tt* ba_3 , as defined by crystallographic occupancy of Xe atoms (21), is shown in Figure 1 in relation to Y133 and T231, which are superimposed onto the corresponding tryptophan and phenylalanine residues in bovine aa_3 (cyan), *Rs* aa_3 (yellow) and *Pd* aa_3 (magenta). In Figure 2, Y133 and T231 of the wild-type *Tt* ba_3 are superimposed onto W133 and F231 in the structure of the Y133W&T231F double mutant, showing how each of the larger side chains intrudes into the channel and the binding sites of three Xe atoms (see also Figure S1). Overall, the structures of the wild-type ba_3 and the Y133W and Y133W&T231F mutants are very similar (rms deviations of subunit I Ca atoms

0.18 – 0.25 Å). The principal difference at residue 133 is a change of the chi2 torsion angle by ~30° such that the tryptophan side chain rotates away from a propionate of heme a_3 , normally engaged in a hydrogen bond with Y133, and in so doing provides a hydrogen bond from the indole to the propionate. Consequently, the tryptophan extends into the channel while maintaining a key interaction with heme a_3 . It also displaces the H₂O molecule present in the native structure that is bridged to the active site via another bound H₂O. The tryptophan side chain positions in the single and double mutant structures are virtually identical. The shortest atom-to-atom distance between W133 and Cu_B is 8.44 and 8.43 Å for the single and double mutant, respectively.

The principal difference at residue 231 due to substitution of phenylalanine for threonine is 0.67 Å displacement of C β . A hydrogen bond from T231 to the amide of L200 in an adjacent α -helix is lost while the phenylalanine side chain gains hydrophobic contacts with W193, L200, and I235 at the interface of helices V and VI. Furthermore, the replacement of threonine by phenylalanine in the double mutant results in a shortest atom-to-atom distance between W133 and F231 of 5.1 Å, compared to the 7.3 Å distance between W133 and T231. The steady-state activities of Y133W, T231F and Y133W&T231F were found to be 57%, 82%, and 70%, respectively, of that of the recombinant wild-type ba_3 . While the rate of O₂ binding is unlikely to limit the steady-state kinetics under the room temperature experimental conditions, this may not be the case at the low O₂ concentration under which the enzyme is expressed.

NO binding to reduced wild-type ba_3 and its constriction mutants

The reaction of NO with fully reduced recombinant wild-type *Tt* ba_3 , and the Y133W, T231F and Y133W&T231F ba_3 mutants (hereafter referred to as the constriction mutants) was investigated in the absence of CO using a photolabile NO carrier (24). Recent studies in our laboratory have shown that NO binds to wild-type *Tt* ba_3 with the same rate as O₂, suggesting that the two ligands follow the same ligand path and that NO serves as a good mimic for O₂ binding but without the complexities of the subsequent microsecond timescale redox reactions (24). Using the NO photolabile carrier also circumvents the low NO quantum yield in NO flash-photolysis studies, the rate-limitation of conventional NO stopped-flow methods and, importantly, the recently reported interference from the photodissociated CO when using the CO flow-flash method (24, 25). Figure 3 (a-c) shows the time-resolved difference spectra recorded during the reaction of the photoproduct NO with the reduced *Tt* ba_3 mutants, Y133W (panel a), T231F (panel b), and Y133W&T231F (panel c). The absorbance difference spectra are those obtained following subtraction of the spectral contribution of the photolabile NO-complex, determined in a separate experiment.

Figure 4 shows the NO binding kinetics at 444 nm, the absorbance maximum of the reduced heme a_3 , for the recombinant wild-type ba_3 (filled squares), Y133W (open circles), T231F (open squares), Y133W&T231F (filled circles) and the bovine aa_3 (filled triangles); the absorption has been normalized to 1. The data are from the multi-wavelength data presented above and are plotted on a logarithmic time scale. Clearly, mutating Y133 in ba_3 to the corresponding tryptophan constriction residue in the aa_3 oxidases (W126 in the bovine enzyme) significantly reduces the rate of NO binding, although not to the level observed in the bovine enzyme; however, this is not the case for the T231F mutation, an issue addressed in more detail below. The solid lines represent the calculated traces obtained based on the global exponential fits discussed below.

SVD-based global exponential fitting of the time-resolved absorption spectra recorded during the reaction of NO with the Y133W, Y133W&T231F and T231F *Tt* ba_3 mutants revealed a single apparent lifetime of 54 μ s (85 μ M NO), 46 μ s (110 μ M NO), and 7.1 μ s (120 μ M NO), respectively. These lifetimes represent NO binding to the reduced heme a_3 ,

an assignment based on the spectral changes, and show linear dependence on NO concentration. The lifetimes correspond to second-order rate constants of $2.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, $2.0 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, and $\sim 1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ for the Y133W, Y133W&T231F and T231F *Tt ba₃* mutants, respectively. The second-order rate constant for NO binding to the recombinant wild-type *ba₃* was found to be $1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, which is equal to our previously reported second-order rate constant for the non-recombinant wild-type enzyme (24). In contrast, the analogous rate for the bovine enzyme is $\sim 1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ (24, 41). Thus the rate of NO binding in the Y133W and Y133W&T231F mutants is approximately ~5 times slower than observed for the wild-type *Tt ba₃*, while it is unaffected by the T231F mutation.

Classical simulations of Xe diffusion to the active site in *Tt ba₃* and bovine *aa₃*

To explore the ligand access to the binuclear site in *Tt ba₃* and the bovine *aa₃*, we performed multiple 1-ns classical molecular dynamics simulations of a xenon (Xe) atom diffusing through subunit I of the enzymes immersed in a dimyristoyl phosphatidyl choline (DMPC) bilayer and capped with TIP3P water molecules (see Methods and Supporting Information for details). The Xe atom was chosen to be consistent with the crystallographic experiments of references 21 and 22, and because it is non-polar and its van der Waals radius is similar to that of O₂; however, Xe can be described entirely by a non-bonded potential in the classical simulations. The interface of subunits I and III of the bovine enzyme has recently been suggested to provide an entry point for dioxygen (42). While subunit III (and subunit II) is absent from the simulations, this does not alter the global structure of subunit I as evidenced by root mean squared fluctuations (rmsf) in simulated C α positions of $2.64 \pm 0.08 \text{ \AA}$ relative to the crystal structure. Consequently, the absence of subunits II and III is not expected to alter the size or location of cavities within the catalytic subunit.

Ligand diffusion through the ligand channel in wild-type *Tt ba₃* and the Y133W and Y133W&T231F mutants

The Xe diffusion within the ligand channel of *Tt ba₃* and its mutants was monitored using the following three distances: (1) the distance from Xe to Cu_B; (2) the distance from Xe to Y(W)133; and (3) the distance from Xe to I78. The time series for the Xe—Cu_B (red trace), Xe—Y(W)133 (blue trace), and Xe—I78 (green trace) distances for four representative trajectories each for the wild-type *Tt ba₃* (A-D) and the mutant (E-H) are presented in Figure 5. The time series of the three distances for both the wild-type enzyme and the mutants suggest that the ligand channel may be divided into four regions, presented graphically in Figure 6A and B for the wild-type enzyme and Figure 6C and D for the Y133W mutant; panels 6A/6C and 6B/6D represent a view from the P-side of the membrane and from within the membrane, respectively. These regions include an inner cavity (blue spheres/ellipsoids), two outer cavities, hereafter referred to as outer-1 (red spheres) and outer-2 (orange spheres), and a docking site (magenta spheres) between the outer-1 and outer-2 cavities; comparative studies on the bovine enzyme will be discussed separately below. The distances defining these cavities are presented in Tables S2A and S2B for the wild-type *ba₃* and Y133W mutant, respectively. As is clear from Figure 6, the Y133W mutation does alter the ligand channel in significant ways to be discussed in detail below.

The Y133W mutation has little effect on the dimensions and position of the outer-1 cavity (Figure 6, red spheres), and this is confirmed by the simulations (see Supporting Information, Table S2). However, the increased steric bulk of the tryptophan compared to the native tyrosine decreases the volume of the inner cavity (blue spheres) as reflected by a comparison of the distances in Table S2A to those in Table S2B. Furthermore, in the Y133W mutant, the inner cavity moves “up” in the protein, *i.e.* closer to the P-side of the membrane, compared to the wild-type enzyme. While the inner cavity in the Y133W mutant is still bounded by F385 on helix IX as in the wild-type *ba₃*, a Xe atom does not approach

A77 as in the wild-type enzyme. Rather, it stops within van der Waal's contact of W133 (the site of the point mutation), and stays somewhat "above" V236, nearer to W229 on helix VI. The outer-2 cavity (Figure 6, orange spheres) in the Y133W mutant is elongated and increased in volume compared to that of the wild-type enzyme (compare Table S2A to Table S2B), and while still bounded by T231, the outer-2 cavity stretches from W133 to F135 in the mutant. The docking site between the outer-1 and outer-2 cavities is decreased in volume and is pushed "lower" (*i.e.* closer to the N-side of the membrane) in the Y133W mutant compared to the native enzyme (Figure 6, magenta spheres).

The occupation times of the inner, outer-1 and outer-2 regions, and the docking site in the wild-type *ba₃* and the Y133W mutant, expressed as a percentage of simulation time, are presented in Tables 1 and 2, respectively. For the trajectories in which the Xe was placed in the outer-2 cavity, the occupation time of the inner cavity in the mutant simulations (Table 2) is half that of the wild-type enzyme (Table 1) while retention in the outer-2 cavity increases from 54 to 87% in the presence of W133. These results suggest that the movement of the Xe atom between the inner and outer-2 cavities is more restricted in the Y133W mutant. The same behavior is observed in simulations of the Y133W&T231F mutant (not shown).

Ligand diffusion through the ligand channel in the bovine *aa₃* enzyme

While the Y133W mutation in *Tt ba₃* slows down the rate of NO binding 5-fold, it alone is not solely responsible for the observed 10-fold difference in the ligand binding between the wild-type *Tt ba₃* and the bovine enzyme. To explore this issue further, we carried out comparative simulations of Xe atom diffusion to the active site in the bovine enzyme. The diffusion of Xe within the internal cavity of the bovine enzyme was followed using the analogous three distances to that of the *ba₃* enzyme: (1) the distance from Xe to Cu_B; (2) the distance from Xe to W126 (one of the two constriction residues); and (3) the distance from Xe to F67 (the bottom residue of the hydrophobic pocket identified in our simulations; see below). The bovine W126 and F67 residues correspond to the wild-type *Tt ba₃* Y133 and I78 residues, respectively. The time evolution of the Xe—Cu_B (red trace), Xe—W126 (blue trace), and Xe—F67 (green trace) distances for four representative trajectories of the bovine enzyme are presented in Figure 7A-D. From the time series of these three distances, we suggest that the ligand channel may be divided into four regions, presented graphically in Figure 7E and 7F, an inner cavity (blue spheres), an outer-1 cavity (red spheres), an outer-2 cavity (orange spheres), and a hydrophobic pocket (green spheres) that separates the inner cavity from the outer-1 cavity.

The time of occupation of each region, expressed as a percentage of the simulation time, is presented in Table 3. A Xe atom placed in the hydrophobic pocket tends to remain there (~76% of the time). Similarly, a Xe atom placed in the outer-2 cavity remains in the outer-2 cavity (88% of the time). Furthermore, the standard deviations in retention times for the simulations where the Xe was initially placed in the hydrophobic pocket and outer-2 cavity are small (14 and 12%, respectively, Table 3), reflecting a consistent level of high occupancy across the simulations. These observations, combined with the narrow range of distances sampled by Xe, suggest that the hydrophobic pocket and, secondarily, the outer-2 cavity are well-defined "docking" sites in the ligand channel of the bovine enzyme.

The ~15 Å Xe—Cu_B distance of the last 900 and 400 ps of the wild-type *ba₃* trajectories in Figure 5B and 5C, respectively, and the initial Xe placement in Figure 5D within the docking site are similar to the Xe—Cu_B distances to the hydrophobic pocket of the bovine enzyme (Figure 7D, red trajectory). However, the bovine hydrophobic pocket is in van der Waal's contact with W126 and "above" F67, in contrast to the docking site of the *ba₃* simulations, which is in contact with I78, and somewhat "lower" in the cavity than Y133

(compare Figures 6 and 7). Furthermore, the volume swept out by the Xe in the docking site of the wild-type *ba₃* enzyme is larger than the volume sampled by Xe in the hydrophobic pocket of the bovine enzyme (Table S3). Moreover, the standard deviation in the occupation time of the wild-type *ba₃* docking site is large (32%, see Table 1) compared to the standard deviation in the occupation time of the bovine hydrophobic pocket (14%, Table 3), suggesting that the *ba₃* docking site is less stable than the hydrophobic pocket of the bovine enzyme. It should be noted that for simulations where the Xe was placed in the outer-2 cavity, the occupation times of the *ba₃* Y133W mutant (Table 2) are similar to those of the bovine enzyme (Table 3), suggesting that the Y133W mutation and the native bovine W126 restrict ligand passage between the inner and outer-2 cavities. The volume of the inner cavity in the Y133W mutant is also decreased relative to the wild-type enzyme and is closer to that of the bovine enzyme (Table S3). Furthermore, while the ligand channel is passive and the Xe simulations are dynamic with all the restraints removed (see the Methods section), both the crystal structures and the simulations show that ligand access in the Y133W and Y133W&T231F mutants and the bovine enzyme is impeded because the tryptophan residue extends into the middle of the cavity; we suggest that the presence of a hydrophobic pocket in the bovine enzyme may further reduce the rate of ligand binding in this enzyme.

Discussion

The effect of the Y133W mutation on ligand access in *Tt ba₃*

Our recent time-resolved optical absorption studies show ~10-times faster rates of O₂/NO binding in the wild-type *Tt ba₃* compared to the bovine enzyme (24, 25), a result attributed to the narrower ligand channel in the bovine enzyme compared to the *Tt ba₃*. The closest distance across the O₂/NO channel in the crystal structures of the bovine enzyme, *Rs aa₃* and *Pd aa₃* is that between the tryptophan (W126) and phenylalanine (F238) residues, 4.6 Å (PDB 2EIJ (37), PDB 3FYE (43) and PDB 3HB3 (44), respectively), while in the wild-type *ba₃* the closest distance (atom-to-atom) between the corresponding but less bulky Y133 and T231 residues is 10.4 Å (3S8F). Neither Y133 nor T231 extend into the ligand channel cavity in the wild-type *ba₃* based on its crystal structure, and in the molecular dynamics Xe simulations, the side chains of the Y133 and T231 have approximately the same orientations as in the crystal structure (side chain fluctuations of ~1 Å). In the bovine enzyme, the corresponding tryptophan (W126) and phenylalanine (F238) both extend into the ligand channel (Figure 1), suggesting that the slower ligand access in this enzyme arises from the constriction point present in the oxygen channel of the *aa₃* oxidases but absent in *Tt ba₃*.

The time-resolved optical absorption results reported in this study demonstrate that mutating the *Tt ba₃* Y133 to the corresponding tryptophan constriction residue present in the ligand channel of the *aa₃* oxidases, significantly (5 times) slows access of NO to the active site in the mutant and, by inference, in the *aa₃* enzymes. This is consistent with the crystal structure of the *Tt ba₃* Y133W, which shows that replacing the Y133 in *ba₃* with the corresponding tryptophan residue physically constricts the ligand channel (Figure 2). The molecular dynamics simulation of Xe diffusion to the active site in the Y133W mutant shows that the tryptophan residue maintains the same orientation during the simulation as in the crystal structure, with small side chain fluctuations of 0.8-1.1 Å. The Y133 in the wild-type *ba₃*, the W133 in the Y133W mutant and the W126 in the bovine enzyme are all oriented as to maintain the H-bond to the propionate. In our simulations, the H-bond is more transient in the Y133W mutant, i.e., the time-averaged H-bond distance is larger compared to the wild-type enzyme, and the W133 residue is twice as likely to be separated by distances outside the H-bonding range compared to Y133. While the differences are small, they do demonstrate the transient and dynamic nature of the hydrogen bonding observed in crystal structures at 100 K. As discussed below, the Y133W mutation alters the ligand channel

compared to the wild-type enzyme by decreasing the volume of some cavities while increasing the volume of others.

The role of the F231 side chain dynamics on ligand access in the Y133W&T231F mutant

In contrast to the Y133W mutant, the mutation of the *Tt ba₃* T231 to the corresponding phenylalanine “constriction” residue present in the bovine enzyme had no effect on the rate of NO binding to heme *a₃*. However, the crystal structure of the Y133W&T231F mutant shows that F231 partially blocks the ligand channel (Figure 2). This suggests that the dynamics of residue 231 must play an important role in ligand access to the active site in the T231F mutant. This proposal is supported by our molecular dynamics simulations of the Y133W&T231F mutant (see below). Furthermore, the b-factor for the wild-type T231 side chain is smaller than the average b-factor for the respective protein, while the b-factors for the F231 side chain are larger than the average b-factor for the double mutant. This suggests increased positional variability in the phenylalanine side chain of the W133Y&T231F mutant; this is also true for the T231F single mutant.

During the simulation of the wild-type *ba₃*, the T231 residue is positioned near the bottom edge of the outer-2 cavity (Figure 6A and 6B). The root mean squared fluctuations in atomic positions for the T231 side chain are small, ranging from 0.7-1.1 Å, with the largest motions belonging to CH₃ rotation. This indicates that T231 is rigid, in accordance with a hydrogen bond between the side chain and the amide of L200, and does not extend into the cavity. However, this is not the case for F231 in the respective mutants. In the simulation of the Y133W&T231F mutant, the F231 residue changes its conformation from that in the crystal structure, with the side chain rotating “up” (pointing towards the P-side of the membrane) and a chi1 torsion angle of -70° (this torsion angle is 11.3° in the crystal structure presented here) (Figure 8, right panel). As a result of this repacking, F231 no longer extends into the cavity but increases contacts with L200 and F228 on helices V and VI, respectively. These hydrophobic contacts may compensate for loss of the T231-L200 hydrogen bond and explain the limited range of conformers of F231. In none of the simulations does the Xe atom interact with F231. Rotations of aromatic side chains are observed in molecular dynamics simulations. For example, rapid equilibration of aromatic side chains is observed in the “relaxed-complex” method for ligand docking simulations (45). Furthermore, the E92Q/N155H and G140S/Q148H HIV-I integrase double mutants significantly alter the distribution of H67 conformations (46), a residue that is crucial to virulence (47).

The dynamics of the F238 constriction residue in the bovine enzyme

In the bovine crystal structure, the F238 extends into the ligand channel (Figure 1). During the molecular dynamics simulation of the bovine enzyme, the F238 constriction point residue lies “higher” in the ligand channel, *i.e.* closer to the P-side of the membrane, in relation to the outer-2 cavity (Fig. 7C), while the *ba₃* T231 is located at the lower edge of the *ba₃* outer cavity (Fig. 6A). In the bovine enzyme, the F238 side chain adopts two conformations with chi1 torsion angles of 55±9° (“down”; Figure 8A, colored cyan) and -70±8° (“up”; Figure 8A, colored magenta), with occupation times of 63 and 37%, respectively; this angle is 27° in the published bovine crystal structure (37). The conformer with the higher occupation time (55°) is that pictured in Figure 7 (E and F). The rotation of F238 side chain “down” (*i.e.* towards the N-side of the membrane) relative to the crystal structure, places the F238 side chain between helices V and VI in contact with L202. The alternative conformation rotates the F238 side chain “up” (*i.e.* towards the P-side of the membrane) relative to the crystal structure in contact with L234 (Figure 8A); the F231 in the Y133WT231F *ba₃* mutant adopts a conformation analogous to the “up” conformation of the bovine enzyme (Figure 8B). In neither conformation does the bovine F238 interact with the Xe atom, which suggests that this residue does not interact with a diatomic ligand. Hence,

the simulations show conformational freedom of phenylalanine at this position in the O₂ channel in both bovine *aa₃* and *Tt ba₃*, which is not observed in the crystal structures.

Molecular simulations of Xe diffusion versus Xe sites in the crystal structure of *Tt ba₃*

In a recent freeze-trap, kinetic crystallography experiments of Xe-laden *Tt ba₃* crystals, the Xe migration out of the internal channel was modeled by following the Xe-crystallographic occupancy of five major crystallographic Xe binding sites (Xe1-Xe5) (22). While the volumes swept out by a Xe atom in our simulations are larger than the crystallographic binding sites and encompass more than one site (see Table S3 for the volumes of the cavities), in general, the locations of the principal sites in the crystal structures and simulations are in accord, with the Xe1, Xe2 and Xe5 binding sites being within the inner cavity, outer-1 cavity and outer-2 cavity, respectively. The position of the Xe4 site overlaps our docking site but the Xe4 and Xe3 sites lie closer to the N- and P-side of the membrane, respectively, and both are nearer to the protein exterior than our docking site.

The hydrophobic sink in the bovine enzyme

In agreement with previous simulations, we find that the internal ligand channel of the bovine oxidase is divided into several cavities (23). As observed by Hofacker and Schulten, the outer-2 cavity near H151 on helix IV provides access to the inner cavity and an escape from the protein interior (23). We find that in the bovine enzyme, a ligand is able to exit—and presumably enter—the protein through the outer-1 cavity. This is in agreement with Xe-pressurized *Rs aa₃* crystallographic studies suggesting that two cavities, the first near L157, I104, A153, and L105 (S116, F63, L112, and V64 in the bovine numbering) and connecting to our outer-1 cavity between helices II and III, and the second near W172, F108, L246, and I250 (W126, F67, L202, and I206 in the bovine numbering, respectively), in rough correspondence to our outer-1 cavity, provide possible oxygen channels (16). Significantly, based on our classical simulations we have identified a “sink” in the bovine enzyme that traps a Xe atom in a hydrophobic pocket between the inner and outer cavities at Cu_B distances of ~15 Å. We propose that this trapping of the ligand, together with the constriction created by W126, give rise to the 10-fold decrease in the rate of ligand binding in the bovine enzyme compared to that in *Tt ba₃*.

This previously unobserved hydrophobic pocket consists of residues F63, I66, F67, and W126 (Figure 7A/B). It should be noted that these residues define the edges of the volume that the Xe explores. The corresponding residues in *Tt ba₃* are V74, A77, I78, and Y133, respectively, obtained by comparing the published crystal structure to that of the bovine enzyme (Table S2). The bovine F63 and I66 are replaced by the much smaller V74 and A77 in *ba₃*. In *Tt ba₃*, the smaller V74 and A77 side chains (and G73) create a larger cavity, but it is occupied by a structural water molecule that is hydrogen bonded to two main chain atoms and a heme *b* propionate; presumably this more polar environment excludes Xe.

Docking sites in *Tt ba₃* and its mutants

In contrast to the bovine enzyme, the *Tt ba₃* enzyme does not seem to contain a hydrophobic pocket. Rather, we identify a well-defined docking site between the outer-1 and outer-2 cavities in *ba₃* (Figure 6, magenta sphere). In *ba₃*, the top and the bottom residues of the bovine enzyme hydrophobic pocket, W126 and F67, are reduced in steric bulk to Y133 and I78, respectively, and the ligand is free to diffuse within the inner cavity and into the binuclear center. According to the classical simulations, the Xe atom moves from the outer-1 cavity in *Tt ba₃* to the relatively stable docking site between the outer-1 and outer-2 cavities. From this site, the ligand in the wild-type enzyme has straight unhindered access to the binuclear center and can move fairly easily between the docking site and the outer-1 and outer-2 cavities, and the inner cavity as reflected by the large standard deviations in

occupation times (Table 1) and the many passes Xe makes between the docking site and the other cavities.

As discussed above, our simulations indicate a relatively stable docking site in *Tt ba₃* at the intersection of the outer-1 and outer-2 cavities (Figure 6, magenta spheres). The classical simulations of Varotsis and coworkers identified a docking site in the inner cavity of the *Tt ba₃*, close to the ring propionate of heme *a₃* (48). This site corresponds to the initial placement of Xe at the Xe1 site in the inner cavity (Figures 2 and 5A). As the ligand stays in this site for 900 ps before moving to the outer-2 cavity, we cannot eliminate this site as a docking site. However, the inner cavity identified in our simulations encompasses twice the volume (100 Å³) of the docking site at the intersection of the outer-1 and outer-2 cavities (49 Å³) (see Tables S2A and S3), as reflected by the larger range of distances sampled by the Xe atom (Table S2A). However, the two results are not necessarily inconsistent as the simulations of Varotsis and coworkers (48) are an order of magnitude shorter than those represented here, which would preclude the observation of the ligand escaping the inner cavity to the outer-2 cavity or the nearby docking site.

As with the wild-type *ba₃*, the Y133W mutant does not seem to contain a hydrophobic pocket with the same relative position within the ligand channel as in the bovine enzyme (compare Figure 6C/D to Figure 7E/F). This highlights the importance of the bovine F67 in defining this structural motif. However, the Y133W mutation significantly alters the ligand channel relative to the wild-type enzyme, decreasing the volume of the inner cavity from 100 to 48 Å³, and increasing the volume of the outer-2 cavity from 24 to 80 Å³ (see Table S3). In addition, the position of the inner cavity in the Y133W mutant is shifted closer to the P-side of the membrane, while the docking site between the outer-2 and outer-1 cavities moves closer to the N-side of the membrane in the mutant compared to the wild-type enzyme. Furthermore, the increased occupation time and decreased standard deviation in the occupation time of the outer-2 cavity in all the Y133W *ba₃* simulations (Table 2) compared to the wild type (Table 1) suggests that the outer-2 cavity is a more stable docking site in the mutant.

Our simulations suggest that a ligand entering the protein interior *via* the outer-1 cavity of the Y133W mutant moves with high probability into the docking site located between the outer-1 and outer-2 cavities (Figure 6C/D). From this site, a ligand is equally likely to move forward into the outer-2 cavity or backward to the outer-1 cavity (Table 2). However, the outer-2 cavity presents a stable docking site to the non-polar Xe—and presumably O₂—and once a ligand occupies this site, there is reduced probability for the ligand to move to the inner cavity of the mutant compared to that of the wild-type enzyme. This reduced probability seems to result from the interaction of the Xe atom with W133 and F135, located in an extended loop above the ligand channel, and W229, a residue that π -stacks to H282, a ligand to Cu_B, providing a structural basis for the reduced ligand binding rate in the Y133W *ba₃* enzyme compared to the wild-type enzyme (Figure 6).

Conclusions

Several conclusions can be drawn from our studies. First, our results demonstrate that mutation of the Y133 residue in the ligand channel of *Tt ba₃* to the bulkier tryptophan residue present in the bovine enzyme impedes ligand access to the active site in the Y133W and Y133W&T231F mutants. It can be inferred that the tryptophan “constriction” residue in the bovine enzyme at least partially controls ligand accessibility to the active site in the bovine *aa₃* oxidase. Second, the molecular dynamics simulations show that the F231 side chain, which in the Y133W&T231F crystal structure extends into the ligand channel, rotates out of the ligand channel, resulting in no effect on the rate of NO binding in the T231F

mutant. This reflects the importance of protein dynamics in controlling ligand access to the active site. Third, the molecular dynamics simulations of Xe diffusion to the active site in the wild-type *Tt ba₃*, the Y133W and Y133W&T231F *ba₃* mutants and the bovine enzyme show that protein cavities or “docking sites” appear important in controlling ligand accessibility to the active site. In particular, we have identified a hydrophobic pocket in the bovine enzyme that is absent in the thermophilic enzyme. We propose that this hydrophobic sink in the bovine enzyme traps the ligand, further decreasing the rate of ligand binding. Moreover, the top and the bottom residues of the hydrophobic pocket of the bovine enzyme, W126 and F67, respectively, are conserved in *Rs aa₃*, *Pd aa₃* and *E. coli bo₃* (W170 and F112, respectively), suggesting these residues may define similar ligand cavities in these enzymes. In contrast, *ba₃* does not contain a hydrophobic pocket. Consequently, the inner cavity is effectively larger than that in the bovine enzyme, which is characterized by distances to Cu_B of 5–10 Å; in the *Tt ba₃* Y133W mutant, the volume of the inner cavity is decreased and is closer to that of the bovine enzyme. The absence of the constriction point and hydrophobic pocket in the wild-type *Tt ba₃* allows the ligand to diffuse freely into the binuclear center in this enzyme and provides a structural and dynamic basis for the differences in ligand binding between the *aa₃* oxidases and cytochrome *ba₃* from the *T. thermophilus*. These differences likely reflect evolutionary adaptation of the *Tt ba₃* to microaerobic conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SVD	singular value decomposition
<i>b</i>-spectrum	spectral changes associated with an apparent rate (lifetime)
<i>Tt ba₃</i>	<i>Thermus thermophilus ba₃</i> cytochrome oxidase
<i>Rs</i>	<i>Rhodobacter sphaeroides</i>
<i>Pd</i>	<i>Paracoccus denitrificans</i>

References

1. Brzezinski P, Larsson G. Redox-driven proton pumping by heme-copper oxidases. *Biochim. Biophys. Acta.* 2003; 1605:1–13. [PubMed: 12907296]
2. Ferguson-Miller S, Babcock GT. Heme/copper terminal oxidases. *Chem. Rev.* 1996; 96:2889–2907. [PubMed: 11848844]
3. Kaila VR, Verkhovsky MI, Wikström M. Proton-coupled electron transfer in cytochrome oxidase. *Chem. Rev.* 2010; 110:7062–7081. [PubMed: 21053971]

4. Hemp J, Gennis RB. Diversity of the heme-copper superfamily in *Archaea*: insights from genomics and structural modeling. *Results. Probl. Cell. Differ.* 2008; 45:1–31. [PubMed: 18183358]
5. Pereira MM, Santana M, Teixeira M. A novel scenario for the evolution of haem-copper oxygen reductases. *Biochim. Biophys. Acta.* 2001; 1505:185–208. [PubMed: 11334784]
6. Soulimane T, Buse G, Bourenkov GP, Bartunik HD, Huber R, Than ME. Structure and mechanism of the aberrant *ba*₃-cytochrome *c* oxidase from *Thermus thermophilus*. *EMBO J.* 2000; 19:1766–1776. [PubMed: 10775261]
7. Buschmann S, Warkentin E, Xie H, Langer JD, Ermler U, Michel H. The structure of *cbb*₃ cytochrome oxidase provides insights into proton pumping. *Science.* 2010; 329:327–330. [PubMed: 20576851]
8. Han H, Hemp J, Pace LA, Ouyang H, Ganesan K, Roh JH, Daldal F, Blanke SR, Gennis RB. Adaptation of aerobic respiration to low O₂ environments. *Proc. Natl. Acad. Sci. USA.* 2011; 108:14109–14114. [PubMed: 21844375]
9. Oshima T, Imahori K. Description of *Thermus thermophilus* comb. nov., a nonsporulating thermophilic bacterium from a Japanese thermal spa. *Int. J. Syst. Bacteriol.* 1974; 24:102–112.
10. Radzi Noor M, Soulimane T. Bioenergetics at extreme temperature: *Thermus thermophilus* *ba*₃- and *caa*₃-type cytochrome *c* oxidases. *Biochim. Biophys. Acta.* 2012; 1817:638–649. [PubMed: 22385645]
11. Zimmermann BH, Nitsche CI, Fee JA, Rusnak F, Münck E. Properties of a copper-containing cytochrome *ba*₃: A second terminal oxidase from the extreme thermophile *Thermus thermophilus*. *Proc. Nat. Acad. Sci. USA.* 1988; 85:5779–5783. [PubMed: 2842747]
12. Fee JA, Choc MG, Findling KL, Lorence R, Yoshida T. Properties of a copper-containing cytochrome *c*₁*aa*₃ complex: a terminal oxidase of the extreme thermophile *Thermus thermophilus* HB8. *Proc. Natl. Acad. Sci. USA.* 1980; 77:147–151. [PubMed: 6244539]
13. Keightley J, Zimmermann BH, Mather MW, Springer P, Pastuszyn A, Lawrence DM, Fee JA. Molecular genetic and protein chemical characterization of the cytochrome *ba*₃ from *Thermus thermophilus* HB8. *J. Biol. Chem.* 1995; 270:20345–20358. [PubMed: 7657607]
14. Soulimane T, von Walter M, Hof P, Than ME, Huber R, Buse G. Cytochrome-*c*₅₅₂ from *Thermus thermophilus*: a functional and crystallographic investigation. *Biochem. Biophys. Res. Commun.* 1997; 237:572–576. [PubMed: 9299406]
15. Tsukihara T, Aoyama H, Yamashita E, Tomizaki T, Yamaguchi H, Shinzawa-Itoh K, Nakashima R, Yaono R, Yoshikawa S. The whole structure of the 13-subunit oxidized cytochrome *c* oxidase at 2.8 Å. *Science.* 1996; 272:1136–1144. [PubMed: 8638158]
16. Svensson-Ek M, Abramson J, Larsson G, Törnroth S, Brzezinski P, Iwata S. The X-ray crystal structures of wild-type and EQ(I-286) mutant cytochrome *c* oxidases from *Rhodobacter sphaeroides*. *J. Mol. Biol.* 2002; 321:329–339. [PubMed: 12144789]
17. Iwata S, Ostermeier C, Ludwig B, Michel H. Structure at 2.8 Å resolution of cytochrome *c* oxidase from *Paracoccus denitrificans*. *Nature.* 1995; 376:660–669. [PubMed: 7651515]
18. Abramson J, Riistama S, Larsson G, Jasaitis A, Svensson-Ek M, Laakkonen L, Puustinen A, Iwata S, Wikström M. The structure of the ubiquinol oxidase from *Escherichia coli* and its ubiquinone binding site. *Nat. Struct. Biol.* 2000; 7:910–917. [PubMed: 11017202]
19. Riistama S, Puustinen A, Verkhovsky MI, Morgan JE, Wikström M. Binding of O₂ and its reduction are both retarded by replacement of valine 279 by isoleucine in cytochrome *c* oxidase from *Paracoccus denitrificans*. *Biochemistry.* 2000; 39:6365–6372. [PubMed: 10828950]
20. Salomonsson L, Lee A, Gennis RB, Brzezinski P. A single-amino-acid lid renders a gas-tight compartment within a membrane-bound transporter. *Proc. Natl. Acad. Sci. USA.* 2004; 101:11617–11621. [PubMed: 15289603]
21. Luna VM, Chen Y, Fee JA, Stout CD. Crystallographic studies of Xe and Kr binding within the large internal cavity of cytochrome *ba*₃ from *Thermus thermophilus*: structural analysis and role of oxygen transport channels in the heme-Cu oxidases. *Biochemistry.* 2008; 47:4657–4665. [PubMed: 18376849]
22. Luna WM, Fee JA, Deniz AA, Stout CD. Mobility of Xe atoms within the oxygen diffusion channel of cytochrome *ba*₃ oxidase. *Biochemistry.* 2012; 51:4669–4676. [PubMed: 22607023]

23. Hofacker I, Schulten K. Oxygen and proton pathways in cytochrome *c* oxidase. *Proteins: Struct., Funct., Genet.* 1998; 30:100–107. [PubMed: 9443344]
24. Einarsson Ó, Funatogawa C, Soulimane T, Szundi I. Kinetic studies of the reactions of O₂ and NO with reduced *Thermus thermophilus* *ba*₃ and bovine *aa*₃ using photolabile carriers. *Biochim. Biophys. Acta.* 2012; 1817:672–679. [PubMed: 22201543]
25. Szundi I, Funatogawa C, Fee JA, Soulimane T, Einarsson Ó. CO impedes superfast O₂ binding in *ba*₃ cytochrome oxidase from *Thermus thermophilus*. *Proc. Natl. Acad. Sci. USA.* 2010; 107:21010–21015. [PubMed: 21097703]
26. Chen Y, Hunsicker-Wang L, Pacoma RL, Luna E, Fee JA. A homologous expression system for obtaining engineered cytochrome *ba*₃ from *Thermus thermophilus* HB8. *Protein. Expr. Purif.* 2005; 40:299–318. [PubMed: 15766872]
27. Caffrey M, Cherezov V. Crystallizing membrane proteins using lipidic mesophases. *Nat. Protoc.* 2009; 4:706–731. [PubMed: 19390528]
28. Cheng A, Hummel B, Qiu H, Caffrey M. A simple mechanical mixer for small viscous lipid-containing samples. *Chem. Phys. Lipids.* 1998; 95:11–21. [PubMed: 9807807]
29. Tiefenbrunn T, Liu W, Chen Y, Katritch V, Stout CD, Fee JA, Cherezov V. High resolution structure of the *ba*₃ cytochrome *c* oxidase from *Thermus thermophilus* in a lipidic environment. *PLoS One.* 2011; 6:e22348. [PubMed: 21814577]
30. Batty TG, Kontogiannis L, Johnson O, Powell HR, Leslie AG. iMOSFLM: a new graphical interface for diffraction-image processing with MOSFLM. *Acta Crystallogr. D Biol. Crystallogr.* 2011; 67:271–281. [PubMed: 21460445]
31. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. Phaser crystallographic software. *J. Appl. Crystallogr.* 2007; 40:658–674. [PubMed: 19461840]
32. Murshudov GN, Skubak P, Lebedev AA, Pannu NS, Steiner RA, Nicholls RA, Winn MD, Long F, Vagin AA. REFMAC5 for the refinement of macromolecular crystal structures. *Acta Crystallogr. D Biol. Crystallogr.* 2011; 67:355–367. [PubMed: 21460454]
33. Georgiadis KE, Jhon N-I, Einarsson Ó. Time-resolved optical absorption studies of intramolecular electron transfer in cytochrome *c* oxidase. *Biochemistry.* 1994; 33:9245–9256. [PubMed: 8049226]
34. Hug SJ, Lewis JW, Einterz CM, Thorgeirsson TE, Kliger DS. Nanosecond photolysis of rhodopsin: Evidence for a new, blue-shifted intermediate. *Biochemistry.* 1990; 29:1475–1485. [PubMed: 2334708]
35. Szundi I, Lewis JW, Kliger DS. Deriving reaction mechanisms from kinetic spectroscopy. Application to late rhodopsin intermediates. *Biophys. J.* 1997; 73:688–702. [PubMed: 9251787]
36. Szundi I, Van Eps N, Einarsson Ó. pH dependence of the reduction of dioxygen to water by cytochrome *c* oxidase. 2 Branched electron transfer pathways linked by proton transfer. *Biochemistry.* 2003; 42:5074–5090. [PubMed: 12718551]
37. Muramoto K, Hirata K, Shinzawa-Itoh K, Yoko-o S, Yamashita E, Aoyama H, Tsukihara T, Yoshikawa S. A histidine residue acting as a controlling site for dioxygen reduction and proton pumping by cytochrome *c* oxidase. *Proc. Natl. Acad. Sci. USA.* 2007; 104:7881–7886.
38. Hunsicker-Wang LM, Pacoma RL, Chen Y, Fee JA, Stout CD. A novel cryoprotection scheme for enhancing the diffraction of crystals of recombinant cytochrome *ba*₃ oxidase from *Thermus thermophilus*. *Acta Crystallogr.* 2005; D61:340–343.
39. Liu B, Chen Y, Doukov T, Soltis SM, Stout CD, Fee JA. Combined microspectrophotometric and crystallographic examination of chemically reduced and X-ray radiation-reduced forms of cytochrome *ba*₃ oxidase from *Thermus thermophilus*: structure of the reduced form of the enzyme. *Biochemistry.* 2009; 48:820–826. [PubMed: 19140675]
40. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. UCSF chimera - A visualization system for exploratory research and analysis. *J. Comput. Chem.* 2004; 25:1605–1612. [PubMed: 15264254]
41. Szundi I, Rose MJ, Sen I, Eroy-Reveles AA, Mascharak PK, Einarsson Ó. A new approach for studying fast biological reactions involving nitric oxide: generation of NO using photolabile ruthenium and manganese NO donors. *Photochem. Photobiol.* 2006; 82:1377–1384. [PubMed: 17421079]

42. Shinzawa-Itoh K, Aoyama H, Muramoto K, Terada H, Kurauchi T, Tadehara Y, Yamasaki A, Sugimura T, Kurono S, Tsujimoto K, Mizushima T, Yamashita E, Tsukihara T, Yoshikawa S. Structures and physiological roles of 13 integral lipids of bovine heart cytochrome *c* oxidase. *EMBO J.* 2007; 26:1713–1725. [PubMed: 17332748]
43. Qin L, Liu J, Mills DA, Proshlyakov DA, Hiser C, Ferguson-Miller S. Redox-dependent conformational changes in cytochrome *c* oxidase suggest a gating mechanism for proton uptake. *Biochemistry.* 2009; 48:5121–5130. [PubMed: 19397279]
44. Koepke J, Olkhova E, Angerer H, Muller H, Peng G, Michel H. High resolution crystal structure of *Paracoccus denitrificans* cytochrome *c* oxidase: new insights into the active site and the proton transfer pathways. *Biochim. Biophys. Acta.* 2009; 1787:635–645. [PubMed: 19374884]
45. Lin JH, Perryman AL, Schames JR, McCammon JA. Computational drug design accommodating receptor flexibility: the relaxed complex scheme. *J. Am. Chem. Soc.* 2002; 124:5632–5633. [PubMed: 12010024]
46. Perryman AL, Forli S, Morris GM, Burt C, Cheng Y, Palmer MJ, Whitby K, McCammon JA, Phillips C, Olson AJ. A dynamic model of HIV integrase inhibition and drug resistance. *J. Mol. Biol.* 2010; 397:600–615. [PubMed: 20096702]
47. Lu R, Limon A, Ghory HZ, Engelman A. Genetic analyses of DNA-binding mutants in the catalytic core domain of human immunodeficiency virus type 1 integrase. *J. Virol.* 2005; 79:2493–2505. [PubMed: 15681450]
48. Koutsoupakis C, Soulimane T, Varotsis C. Docking site dynamics of *ba3*-cytochrome *c* oxidase from *Thermus thermophilus*. *J. Biol. Chem.* 2003; 278:36806–36809. [PubMed: 12851397]

Figure 1.

Superimposition of the constriction residues, tryptophan and phenylalanine of bovine *aa*₃ (cyan sticks) *R. sphaeroides aa*₃ (yellow sticks) and *P. denitrificans aa*₃ (magenta sticks) onto the corresponding residues, Y133 and T231, respectively, in the wild-type cytochrome *ba*₃ oxidase (orange sticks). The red mesh represents the oxygen channel. The volumes were calculated by the program Voidoo.

Figure 2.

Comparison of the wild-type *ba3* and the Y133W&T231F mutant showing constriction of the O₂ diffusion channel. The red mesh represents the computed oxygen channel as in Figure 1. Blue balls represent seven gas binding sites observed in Xe-pressurized *Tt ba3* crystals (21). Orange sticks represent residues Y133 and T231 of the wild-type *Tt ba3* and the green sticks the W133 and F231 residues of the Y133W&T231F double mutant, respectively.

Figure 3.

The time-resolved optical absorption difference spectra recorded during the reaction of the photoproduct NO with the reduced *Tt ba₃* mutants: Y133W (panel a), T231F (panel b), and Y133W&T231F (panel c). The experiments were carried out with 2 mM ascorbic acid and 1 μ M phenazine methosulfate (PMS). The absorbance difference spectra are those obtained following subtraction of the spectral contribution of the photolabile NO complex, determined in a separate experiment. The spectra were recorded at logarithmically space delay times between 1 μ s – 5 ms (panel a), 200 ns – 200 μ s (panel b) and 1 μ s–1 ms (panel c). Effective enzyme concentration: 2.5 μ M (panel a), 2.8 μ M (panel b) and 1.4 μ M (panel c). The NO concentration was 85 μ M (panel a), 120 μ M (panel b) and 110 μ M (panel c).

Figure 4.

The NO binding kinetics at 444 nm for the wild-type *Tt ba*₃ (filled squares), T231F (open squares), Y133W (open circles), Y133W&T231F (filled circles), and the bovine *aa*₃ (filled triangles). The traces are plotted on a logarithmic time scale, with the maximum absorbance values normalized to 1. The experimental time dependence has been converted to correspond to the same 100 μ M NO concentration for each sample. The solid lines represent the calculated traces obtained based on the global exponential fits.

Figure 5.

Representative time series of the Xe-Cu_B distance (red), the Xe-Y133 distance (blue), and Xe-I78 distance (green) for the wild-type *ba3* (panels A-D) and the Y133W mutant (panels E-H). The Xe atom was initially placed at: (A, E) the Xe1 site in the inner cavity; (B, F), the Xe2 site in the outer-1 cavity; (C, G) the Xe5 site in the outer-2 cavity; (D, H) and the docking site.

Figure 6.

The four regions of the wild-type *Tt ba₃* (A and B) and Y133W (C and D) ligand channel: the inner cavity is the blue ellipsoid, the outer-1 cavity is red, and the outer-2 cavity is the orange sphere. The magenta sphere is the docking site between the outer-1 and outer-2 cavities as viewed from: (A and C) the P-side of the membrane; and (B and D) within the membrane. Y(W)133 is colored blue, and I78 is colored green.

Figure 7.

(A-D) Representative time series of the Xe-Cu_B distance (red), the Xe-W126 distance (blue), and Xe-F67 (green) for the bovine enzyme. The Xe atom was initially placed at: (A) in the inner cavity; (B) in the outer-1 cavity; (C) in the outer-2 cavity; and (D) in the hydrophobic pocket (hp). (E and F) The four regions of the bovine ligand channel: the inner cavity is the blue ellipsoid, the hydrophobic pocket (hp) is green, the outer-1 cavity is red, and the outer-2 cavity is the orange sphere as viewed from: (E) the P-side of the membrane; (F) within the membrane. W126 is colored blue, and F67 is colored magenta.

Figure 8.

Comparison of the conformations of the bovine aa₃ F238/*Tt* ba₃ F231 side chains observed in the crystal structures (exp) to those in the molecular dynamics simulations. (A) the bovine crystal structure is colored red; (B) the Y133W&T231F mutant crystal structure is colored orange.

Table 1
 Time of Occupation (% simulation time) of the Four Regions of the Wild-Type *ba*₃ Ligand Channel

Number of Simulations	Initial Xe placement	time of occupation (%)			
		Inner cavity	Outer1	Docking Site	Outer2
4	Inner cavity	95 (7) ^a	0	0	5 (7)
4	Outer1	0	71 (44)	29 (44)	0
5	Docking Site	0	34 (35)	62 (32)	4 (6)
6	Outer2	11 (28)	21 (39)	14 (18)	54 (39)

^aValues in parentheses are standard deviations

Table 2
Time of Occupation (% simulation time) of the Four Regions of the Y133W *ba*₃ Ligand Channel

Number of Simulations	Initial Xe placement	time of occupation (%)			
		Inner cavity	Outer1	Docking Site	Outer2
4	Inner cavity	77 (46) ^a	0	1 (2)	22 (45)
5	Outer1	0	46 (20)	49 (19)	5 (6)
4	Docking Site	0	26 (31)	48 (41)	25 (50)
5	Outer2	5 (11)	0	8 (11)	87 (12)

^aValues in parentheses are standard deviations

Table 3
 Time of Occupation (% simulation time) of the Four Regions of the bovine *aa₃* Ligand Channel

Number of Simulations	Initial Xe placement	time of occupation (%)			
		Inner cavity	Pocket	Outer1	Outer2
7	Inner cavity	55 (21) ^a	34 (20)	9 (12)	0
4	Pocket	19 (15)	76 (14)	0	0
5	Outer1 ^b	7 (16)	2 (4)	33 (39)	2 (1)
6	Outer2	2 (6)	1 (2)	6 (9)	88 (12)

^aValues in parentheses are standard deviations

^bthis row does not sum to 100% because in 2 of 5 simulations the Xe atom escapes the cavity interior into the lipids and is not within the ligand channel of protein