The Electron Transfer Complex between Cytochrome $c_552$ and the Cu$_A$ Domain of the \textit{Thermus thermophilus} ba$_3$ Oxidase

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The structural analysis of the redox complex between the soluble cytochrome $c_552$ and the membrane-integral cytochrome ba$_3$ oxidase of \textit{Thermus thermophilus} is complicated by the transient nature of this protein-protein interaction. Using NMR-based chemical shift perturbation mapping, however, we identified the contact regions between cytochrome $c_552$ and the Cu$_A$ domain, the fully functional water-soluble fragment of subunit II of the ba$_3$ oxidase. First we determined the complete backbone resonance assignments of both proteins for each redox state. Subsequently, two-dimensional $[^{1}^{15}N,^{1}H]$TROSY spectra were recorded for both redox partner both in free and complexed state indicated those surface residues affected by complex formation between the two proteins. This chemical shift analysis performed for both redox states provided a topological description of the contact surface on each partner molecule. Remarkably, very pronounced indirect effects, which were observed on the back side of the heme cleft only in the reduced state, suggested that alterations of the electron distribution in the porphyrin ring due to formation of the protein-protein complex are apparently sensed even beyond the heme propionate groups. The contact residues of each redox partner, as derived from the chemical shift perturbation mapping, were employed for a protein-protein docking calculation that provided a structure ensemble of 10 closely related conformers representing the complex between cytochrome $c_552$ and the Cu$_A$ domain. Based on these structures, the electron transfer pathway from the heme of cytochrome $c_552$ to the Cu$_A$ center of the ba$_3$ oxidase has been predicted.

\textit{Thermus thermophilus}, a Gram-negative eubacterium originally isolated from hot springs, produces highly thermostable proteins under obligately aerobic conditions (1, 2). Its respiratory chain contains several major redox complexes, such as an NADH-oxidizing complex I (3) and a recently identified bc$_1$ complex (4). For this terminal part of the electron transport chain, \textit{T. thermophilus} possesses two alternative pathways for oxygen reduction to water, depending on the oxygen partial pressure: (i) the cytochrome ba$_3$ oxidase branch with cytochrome $c_552$ as water-soluble electron donor or (ii) the c$_{aa}$ oxidase, where the c-type cytochrome domain is covalently bound to subunit II of this terminal oxidase (5).

Under low aeration conditions, the preferred product in \textit{T. thermophilus} is the ba$_3$ branch (6, 7). Electron transport from the bc$_1$ complex to the ba$_3$ oxidase is mediated by the 14.4-kDa periplasmic cytochrome $c_552$ (8). This c-type cytochrome contains a covalently bound heme moiety, with thioether linkages to two cysteine residues in a conserved Cys$_{11}$-Xaa$_{12}$-Yaa$_{13}$-Cys$_{14}$-His$_{15}$ motif. The heme iron is octahedrally coordinated by the four porphyrin nitrogens and two axial ligands, His$_1^{15}$ and Met$_{69}$, thus representing a “low spin” heme complex (9, 10). In the redox cycle, the iron atom alternates between the reduced, diamagnetic Fe$^{2+}$ and the oxidized, paramagnetic Fe$^{3+}$ state. Thereby, the reduced cytochrome $c_552$ transfers one electron to the oxidized Cu$_A$ domain, the first electron acceptor of the terminal ba$_3$ oxidase. From this solvent-exposed part of subunit II (11), the electron subsequently reaches the redox centers in the membrane-embedded subunit I, i.e. heme $b$ and the binuclear heme $a_3$/Cu$_A$ center where oxygen reduction takes place.

The entire ba$_3$ oxidase of \textit{T. thermophilus} consists of subunits I, II, and IIA (874 residues, 84.9 kDa) and represents one of the smallest known terminal oxidases in both prokaryotic and eukaryotic organisms (12, 13). Moreover, contrary to most other members of the oxidase superfamily, this oxidase lacks most of the canonical amino acid “signatures” and was reported to pump only 0.5 H$^+$/e$^-$ (13, 14). In the present NMR study, we use the Cu$_A$ domain, the fully functional water-soluble fragment of subunit II. Its binuclear Cu$_A$ center is asymmetric, with one copper atom (Cu2) coordinated by His$_{154}$, Cys$_{149}$, Cys$_{153}$, and Met$_{160}$ and the other (Cu1) by the same two cysteine residues, His$_{157}$ and presumably Gln$_{151}$ (11, 13, 15). The charge of each copper atom alternates in the redox cycle between +1.5 (“mixed valence”) and +1 (16, 17).

It had been shown previously (18, 19), that the complex formation between cytochrome $c_552$ and the $a_3$ oxidase of \textit{Paracoccus denitrificans} is based on electrostatic interactions between the hydrophilic residues surrounding Trp$_{121}$ in the Cu$_A$ domain and the positively charged lysine residues encircling the heme cleft in cytochrome $c_552$ in \textit{T. thermophilus}, on the other hand, the interaction between the corresponding proteins involves mainly hydrophobic and non-ionic contacts (18, 20),
possibly because the electrostatic attractions would rather be weakened at the high temperatures these bacteria are exposed to. This different specificity between the reaction partners is also supported by the fact that cytochrome c552 from P. denitrificans does not interact with the CuA domain of T. thermophilus (18).

Under steady-state turnover conditions at 25 °C, molar redox activities with k_{max} = 250 s^-1 have been reported between cytochrome c552 and the ba3 oxidase of T. thermophilus (5). The complex therefore has to be short lived to ensure efficient electron transport (ET). This transient nature of the redox interaction precludes the detection of any intermolecular NOE connectivities to define the contact region between the proteins. However, the highly sensitive amide resonances allow the observation of chemical shift changes as a result of transient alterations in the local environment due to the presence of the redox partner, as previously demonstrated with other systems such as plastocyanin/cytochrome c, plastocyanin/cytochrome f, cytochrome c peroxidase/iso-1-cytochrome c, and CuA domain/cytochrome c552 from P. denitrificans for example (19, 21–23).

We therefore employed two isolated, soluble components, i.e. cytochrome c552 and the CuA domain, to determine the biologically relevant ET complex of the T. thermophilus system. Contrary to the CuA domain from P. denitrificans, which was not sufficiently stable for prolonged NMR data collection at room temperature, the CuA domain from T. thermophilus proved highly stable. Both proteins were complexed under uniform redox conditions that precluded ET; but the transient complex interaction apparently still took place, as in the P. denitrificans system (19). Interestingly, analogous to the previous P. denitrificans study, in the case of reduced cytochrome c552 from T. thermophilus we again detected the most pronounced shifts at residues located in the protein interior behind the heme ring. These indirect effects are an indication for redox state-dependent alterations of the electron delocalization in the porphyrin system. Based on chemical shift perturbation mapping, protein-protein docking calculations subsequently yielded the first structural characterization of the ET complex between cytochrome c552 and the CuA domain that is founded on experimental data. Using this information, the shortest ET pathway from the heme iron to the CuA center was calculated based on the pathway model, revealing an involvement of Phe88, which however does not play such a crucial role as the corresponding Trp121 residue in the P. denitrificans system (24). Two alternative ET scenarios, matching our experimental mutagenesis data, will be discussed.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—Cytochrome c552 (133 amino acid residues; 14,405 Da including the heme cofactor) and the CuA domain (136 residues; 15,062 Da including the two copper atoms) of T. thermophilus were both expressed heterologously in *Escherichia coli* and subsequently purified as described previously (18, 25). For 15N enrichment, both proteins were expressed in M9 medium. In the case of cytochrome c552, heme maturation was achieved by co-transformation of the E. coli cells with the ccmABCDEFGH gene cluster (26) present on the pEC86 plasmid. Copper atoms were introduced into the apo-CuA domain by addition of Cu(His)2 after cell lysis. The NMR resonance assignments revealed that the soluble cytochrome c552 protein carried an alanine-to-threonine point mutation in position 123; subsequent activity tests, however, showed the same functionality as the wild-type protein. The CuA domain, the water-soluble fragment of the ba3 oxidase, also was fully functional as revealed by redox spectroscopy (18).

Mutations were introduced by the “altered sites” protocol (Promega, Heidelberg, Germany) as described previously (24). All stopped-flow experiments were carried out in 20 mM BisTris buffer (pH 7.0; 10 mM KCl) at 20 °C, as described elsewhere (18).

For the resonance assignments, NMR samples of 2 mM protein concentration were prepared for each redox partner, containing 20 mM potassium phosphate buffer (pH 6.0), 0.15 mM 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as internal chemical shift reference (Cambridge Isotope Laboratories, Andover, MA) and 5% D2O. Depending on the redox state to be investigated, 5 mM sodium ascorbate or 5 mM potassium hexacyanoferrate(III) were added to obtain a fully reduced or oxidized sample, respectively.

For the chemical shift perturbation study, the protein samples were prepared according to the following procedures: 1) two samples both containing 0.5 mM 15N-labeled cytochrome c552 in 20 mM potassium phosphate buffer (pH 6.0), 0.15 mM DSS, and 5% D2O were treated in parallel. Non-labeled CuA was added in 4-fold molar excess to one of the two samples. The pH values of both samples were adjusted precisely. To obtain cytochrome c552 Sample pairs under reduced or oxidized conditions, the solutions included 4 mM sodium ascorbate or 5 mM potassium hexacyanoferrate(III), respectively. 2) Two samples both containing 0.5 mM 15N-labeled CuA fragment in 20 mM potassium phosphate buffer (pH 6.0), 0.15 mM DSS, and 5% D2O were treated likewise. Non-labeled cytochrome c552 was added in 4-fold excess to only one of the two samples and pH values checked for both solutions. To obtain CuA domain sample pairs under reduced or oxidized conditions, the solutions included 4 mM sodium ascorbate or 5 mM potassium hexacyanoferrate(III), respectively.

**NMR Experiments and Analysis**—For the resonance assignments of both proteins, NMR spectra were acquired at 25 °C using Bruker DMX 500 and DMX 600 spectrometers operating at 500.13 and 600.13 MHz proton resonance frequencies, respectively, and both equipped with 5-mm triple-resonance [1H,1H] NOESY, two-dimensional [15N,1H] TROSY, NOESY-[15N,1H] TROSY, and three-dimensional TOCSY-[15N,1H] TROSY, and three-dimensional NOESY-[15N,1H] TROSY.

In the homonuclear one- and two-dimensional 1H experiments, the water signal was suppressed by selective presaturation during the relaxation delay, with the carrier placed in the center of the spectrum on the water resonance. All heteronuclear experiments made use of pulsed field gradients for coherence selection and artifact suppression and utilized gradient sensitivity enhancement schemes (29). Quadrature detection in the indirectly detected dimensions was obtained either by the States-TPPI (time proportional phase incrementation) or by the echo/antiche method. All NMR spectra were acquired and processed on Silicon Graphics computers using the program XWINNMR 3.5 (Bruker Bio-Spin, Rheinstetten, Germany). A 90° phase-shifted squared sine bell function was used for apodization in all dimensions. Polynomial base-line correction was applied to the processed spectra in the directly detected 1H dimension. Peak picking and data analysis of the transformed spectra were performed using the AURELIA 2.5.9 (Bruker Bio-Spin) software package. The chemical shifts were referenced to internal DSS to ensure consistency among all spectra (30).
For the chemical shift perturbation mapping, two-dimensional [15N,1H]TROSY spectra were recorded, as described previously (19), at 25 °C and pH 6 on a Bruker Avance 900 spectrometer, operating at 900.13 MHz proton resonance frequency and equipped with a 5 mm cryogenic z-gradient 1H-13C/15N triple-resonance probe. Data acquisition and processing were performed as described above. The backbone amide peaks were picked with the program FELIX 2000 (Accelrys Inc., San Diego, CA). Chemical shift differences in the amide proton (Δδ(H,15N)) and nitrogen (Δδ(N,15N)) resonances of the free and complexed protein forms were combined for each residue by using the expression 
\[
(Δδ(H,15N)^2 + (Δδ(N,15N)/6.5)^2)^{1/2}
\]
(31). These combined chemical shift differences were illustrated with the program MOLMOL (32) by color-coding each respective surface residue.

Docking Calculations—The structure of the cytochrome c552-CuA domain complex was determined with the program HADDOCK (high ambiguity driven protein-protein docking) (33) that has been implemented in the program CNS (34), making use of python scripts derived from ARIA (35) for automation. HADDOCK employs bio-physical interaction data, such as chemical shift perturbation resulting from NMR titration experiments, that are introduced into the structure calculation as ambiguous interaction restraints (AIRs) to drive the docking process. In our application, four independent sets of amide proton chemical shift perturbation data were available: 15N-labeled cytochrome c552 titrated with non-labeled CuA domain, both in the reduced and oxidized state, and 15N-labeled CuA domain titrated with non-labeled cytochrome c552, again both in the reduced and oxidized state. From each independent data set, the residues with the strongest chemical shift perturbations were selected as “active AIRs” for the HADDOCK calculations; residues with less than 20% accessible surface area, however, were rejected. In addition, surface residues located next to the selected active AIRs were chosen as “passive AIRs”, if their relative surface accessibility was also above 20%. The x-ray coordinates of cytochrome c552 (PDB ID code 1DT1) and the hao oxidase (PDB ID code 1EHK) were used both to calculate the surface accessibility with the program NACCESS (36) and for the subsequent docking calculations.

First, 100 structures of the cytochrome c552-CuA domain complex were calculated using the rigid docking protocol of HADDOCK. Next, the 20 structures showing the lowest AIR violations were further energy minimized with the side chains of the active and passive residues left flexible. Finally, the 10 best structures were minimized once more in a 8 Å shell of explicit TIP3P (37) water molecules (for details, see Ref. 33). AIR violations, interaction energies, and buried surface areas of the final structures were compared. In addition, the complexes were characterized in terms of electron transfer by the evaluation of electron-tunneling coupling factors using the program GREENPATH (38). In the latter program, the highly interconnected network of bonded and non-bonded contacts within the protein matrix is searched to specify the pathways that maximize the electron-tunneling coupling between the electron donor and acceptor (i.e. the iron atom of the cytochrome c552 heme moiety and the copper center of the CuA domain, respectively). This is achieved using the theories and methods developed by Onuchic, Netz, and Betts (39), which quantify the ET (without interference) using the pathway model. A pathway is defined as a combination of interacting atoms that link the donor with the acceptor via covalent bonds, hydrogen bonds, and through-space jumps. Rates of non-adiabatic ET reactions can be defined by the expression
\[
\frac{k_{ET}}{T_{DA}^{\theta}(FC)} = \frac{2\pi h}{T_{DA}^{\theta}(FC)}
\]
Thereby, the terms TDA describes the donor-acceptor interaction associated with electron tunneling, while the FC (Franck-Condon) term contains the free energy dependence (reorganization and reaction free energy) related to nuclear motion.

RESULTS AND DISCUSSION

To allow chemical shift perturbation mapping, the full set of backbone amide resonance values had to be determined for both proteins, i.e. cytochrome c552 and the CuA domain from T. thermophilus. The resonance assignments were performed according to the classical strategy based on NOE connectivities between adjacent residues (40), with 15N labeling used to achieve a better signal dispersion. The resulting 1H and 15N assignments of cytochrome c552 and the CuA domain in both redox states have been deposited at the BioMagResBank data base.

Both redox partners investigated in this study had been shown earlier to be fully functional when expressed in a heterologous host organism (18). Moreover, the T. thermophilus proteins, in particular the CuA domain, displayed a higher stability compared to the homologous proteins from P. denitrificans, which had been employed in an earlier NMR study (19).

NMR Resonance Assignments—The reduced (i.e. diamagnetic) cytochrome c552 was fully assigned (BMRB-6966); heme proton resonances were determined based on NOE data that agreed with interproton distances in the x-ray structure (PDB ID code 1DT1). Several residues showed highly unusual chemical shift resonances because of ring-current effects; the backbone amide proton resonance of Leu116, for example, is located upfield of the water signal at 4.35 ppm (Fig. 1). The oxidized (i.e. paramagnetic) cytochrome c552 was assigned nearly completely (BMRB-6967), except for the heme ring and three protein residues: His15 and Met149, the two axial ligands of the iron atom, and Cys145, which is bound covalently to the heme ring. Only a few of the heme proton resonances could be identified by using NOE information from the two-dimensional and three-dimensional NOESY data. Certain resonances showed strong line broadening due to the proximity of the paramagnetic center.

The reduced (i.e. diamagnetic) CuA domain had been previously assigned using triple-resonance experiments (25); however, several aromatic ring proton resonances have been additionally identified in the present work based on homonuclear two-dimensional TOCSY and NOESY spectra (update of BMRB-5819). Interestingly, the 1H resonances of the Phe88 ring, which is situated close to the CuA center and has been postulated to play a role in the ET (13), were the only aromatic ring resonances that could not be identified. In the case of the oxidized (i.e. paramagnetic) CuA domain, the assignment of the 1H and 15N resonances was again not complete (BMRB-6965), due to the paramagnetic CuA center. Moreover, as reported elsewhere (41), several resonances are extremely shifted, such as an amide group at 300 ppm and certain CB protons at 30 ppm. Nevertheless, except for 9 residues (i.e. the three N-terminal amino acids Met137–Tyr135, Gly115, and Cys145–Cys153), all other backbone amide groups could be identified for the oxidized CuA domain.

Chemical Shift Perturbation Mapping—To obtain structural data on the transient complex between cytochrome c552 and the CuA domain, two-dimensional [15N,1H]TROSY spectra comparing the free and the complexed state of each protein were collected. Employing 15N-labeled protein samples, the chemical shift changes upon addition of 4-fold molar excess of the non-labeled reaction partner provided crucial information about the residues that are affected by the formation of the complex. The non-labeled redox partner was always added in excess, to shift the equilibrium toward the associated complex state. Nevertheless, the observed effects were rather small, presumably due to the very short-lived nature of the cytochrome c552-CuA domain complex. The
chemical shift changes in the $^1$H and $^{15}$N dimensions between the free and complexed protein form were subsequently combined for each residue (31), as indicated in Fig. 2.

In the case of cytochrome $c_552$, for both redox states the most pronounced shift perturbations upon addition of the CuA domain were seen in residues located around the heme cleft (Fig. 3), indicating that the “front side” of the protein is the contact surface during the interaction with the redox partner, similar to the corresponding $P$. denitrificans system (19). Moreover, also analogous to the effects noted for $P$. denitrificans, the largest shifts in the reduced state of cytochrome $c_552$ from $T$. thermophilus were observed on the back side of the heme cleft, at residues Ala$^{34}$ and His$^{32}$ (Fig. 4); since both residues are not exposed at the protein surface, these dominant shifts must be indirect effects that are apparently relayed from the contact surface through the heme pocket. Interestingly, these indirect effects (at Ala$^{34}$ and His$^{32}$ in $T$. thermophilus and at Gly$^{54}$, Gly$^{55}$, and Asp$^{56}$ in $P$. denitrificans) stand out only in the reduced but not in the oxidized state of cytochrome $c_552$. This finding suggests that these chemical shift perturbations in the back of the heme moiety are a result of the electronic differences between the two redox states. More precisely, His$^{32}$ and Arg$^{125}$ form a hydrogen bonding network with the propionate A chain at the back of the heme ring (Fig. 5, bottom), resembling the arrangement of Trp$^{57}$, Arg$^{36}$, and propionate A in the $P$. denitrificans system (Fig. 5, top). We therefore propose that the electronic state of the heme is propagated through the propionate A substituent and across the hydrogen bond to the aromatic ring, i.e. His$^{32}$ in $T$. thermophilus or Trp$^{57}$ in $P$. denitrificans. In the case of $P$. denitrificans, for example, fluorescence spectra of reduced and oxidized cytochrome $c_552$ (see Fig. S1 in the supplemental data) had shown a 20-nm shift of the tryptophan band (42). This is apparently due to an alteration in the electronic structure of Trp$^{57}$, since the protein conformation is identical in both redox states as confirmed by both x-ray and NMR structure analysis (42, 43), thus excluding an explanation that is based on conformational changes in the protein structure. Hence, the only distinction that could explain this redox state-dependent effect in the fluorescence spectrum of $P$. denitrificans cytochrome $c_552$ is the additional electron delocalized across the porphyrin system. This is a clear indication that the electronic state of the heme system is also sensed in the protein region located beyond the propionate groups. Presumably, the electrons of the heme iron show an effective delocalization toward the periphery of the porphyrin ring including its substituents, as previ-
ously suggested by Johansson et al. (44, 45): the actual change of the central iron charge in the redox reaction is only about 0.1–0.2 electrons, despite the unit difference in the formal oxidation state. This relatively small difference in electron probability at the heme iron implies a considerable electron delocalization into the periphery of the porphyrin system, which seems to be very important for both the ET rates and the accommodation of the charged heme moiety in a low dielectric environment such as the interior of a protein (44, 45). In our chemical shift perturbation study, the reduced cytochrome c_{552} is additionally complexed with the reduced Cu_{A} domain. Hence, the electron delocalization in the heme porphyrin ring of the protein complex may be distributed even further into the back of the heme pocket, to minimize unfavorable Coulomb interactions that arise because of increased electron repulsion in the combined heme-Cu_{A} system, as both redox centers are fully occupied with electrons. This shift in the electron density upon complex formation apparently is sensed by the ring systems of Trp^{97} in P. denitrifcans or His^{32} in T. thermophilus via the hydrogen bond connection to propionate A, thereby in turn presumably affecting their respective local environments (see Fig. 5).

In case of the Cu_{A} domain, the most pronounced shift perturbations upon addition of cytochrome c_{552} occurred in different regions (Fig. 6). For the docking calculations, however, several of the affected residues could be excluded because of either low surface accessibility or location at the interface to subunit I of the ba oxidase, as described below. The contact region relevant for the ET is located near the Cu_{A} center, at the surface residues Ala^{87}, Phe^{89}, Gln^{158}, and Asn^{159}.

Theoretically, in the fully oxidized state pseudocontact shifts (see Ref. 46 and references therein) could occur in residues of cytochrome c_{552} that are closest to the paramagnetic copper center of the Cu_{A} domain, and vice versa; such shifts, however, would hardly be distinguishable from those due to “true” intermolecular contacts. They would arise at the interface between the copper and iron metal centers where most of the intermolecular contacts occur, and thus both effects on the chemical shift would superpose. The impact on the structure calculation using AIRs would therefore be negligible, as indicated also by the consistency of our calculations (see below).

Docking Calculations—To perform docking calculations between cytochrome c_{552} and the Cu_{A} domain, it was necessary to make a reasonable selection among the residues affected in the chemical shift per-
In the case of reduced cytochrome c_{552}, residues Ala^{34}, His^{32}, Gly^{24}, Ser^{70}, Gln^{16}, His^{15}, Leu^{116}, Leu^{29}, Cys^{14}, and Lys^{98} (in this order) showed the largest combined chemical shift perturbations (Δδ ≅ 0.008 ppm). Some of these residues were excluded as possible contact partners for the following reasons: Ala^{34}, His^{32}, His^{15}, Leu^{116}, Leu^{29}, and Cys^{14} were rejected because of a too low surface accessibility (<20%); Lys^{98} could be neglected due to its location on the back side of the molecule. Thus, residues Gly^{24} and Ser^{70} (both with over 40% relative surface accessibility) were chosen as active AIRs, whereas Gln^{16} with only 28.7% relative surface accessibility was classified as passive AIR.

In the oxidized cytochrome c_{552}, residues Lys^{115}, Gln^{57}, Ala^{113}, Gly^{99}, Ala^{105}, Gln^{119}, Asn^{18}, Gln^{120}, Gly^{13}, Gly^{24}, and Val^{168} (in this order) showed the largest combined chemical shift perturbations (Δδ ≅ 0.0124 ppm). Ala^{105} was excluded because of its position on the back side of the molecule. Gly^{99} (with 23.4% relative surface accessibility) was classified as passive AIR. All the other affected residues show over 40% relative surface accessibility and were therefore accepted as active AIRs.

In the case of the reduced Cu_{A} domain, residues Gly^{120}, Arg^{141}, Ile^{45}, Gln^{31}, Arg^{25}, Gln^{126}, Leu^{50}, Phe^{19}, Asn^{19}, Gln^{158}, Arg^{146}, Lys^{140}, and His^{57} (in this order) showed the largest combined chemical shift perturbations (Δδ ≅ 0.010 ppm). Gly^{120}, Ile^{45}, and Gln^{126} could be excluded because of their position at the interface to subunit I of the b_{a3} oxidase.
Arg141, Arg52, Leu50, and Lys140 were also neglected, since these residues are only accessible in the soluble CuA fragment, while in the full ba₃ oxidase their side chains should be immersed into the lipid membrane. Glu51 was not taken into account, since it is located at the opposite side of the Cu₄ domain relative to the copper center. His117 and Gin158 were rejected because of too low surface accessibilities (<20%). The remaining residues Phe98, Arg146, and Asn159 were chosen as active AIRs.

In the oxidized Cu₄ domain, residues Asn122, Val112, His157, Asn159, Gly115, Gly156, Val127, Ala85, His117, and Ala87 (in this order) showed the largest chemical shift perturbations (Δδ ≥ 0.020 ppm). Val112, His157, Gly115, Val127, and Ala85 could be excluded because of too low surface accessibilities (<20%). Asn122 and His117 were also neglected because they are located at the interface to subunit I of the ba₃ oxidase. The remaining residues Ala87, Gly156, and Asn159 were accepted as active AIRs.

All residues that were thus chosen for the docking calculations as active AIRs (10 and 5 for cytochrome c₅₅₂ and the Cu₄ domain, respectively) are listed in Table 1. Consequently, neighboring residues with relative surface accessibility above 20% were selected as passive AIRs for the calculations. Based on these AIRs, 100 rigid structures of the cytochrome c₅₅₂/Cu₄ domain complex were calculated with the HADDOCK program (33). The 20 structures with the lowest interaction energies were further energy minimized by keeping the side chains of the active and passive residues flexible. Finally, the 10 lowest energy structures were minimized once more in a shell of explicit water molecules. Listed in Table 2 are the energy terms, buried surface areas, ET distances, ET pathway lengths, and ET efficiencies of the 10 final structures. The distance between the electron donor (i.e., the iron atom of cytochrome c₅₅₂) and acceptor (i.e., the copper atom CU2 of the Cu₄ domain) varies between 15.6 and 16.8 Å. The estimated electron-tunneling coupling factor (log |Tₑₑ|²) ranges from ~11.2 to ~12.8 and the electron pathway length from 19.6 to 24.5 Å. The total interaction energy varies in the ensemble between ~124 and ~79 kcal/mol.

As the contact surfaces of the protein molecules are rather flat, and since the AIRs allow different contact combinations between the active and passive residues of the two redox partners, no single preferred solu-
The Cytochrome c{sub}552{sup}Cu{sub}A Complex from T. thermophilus

TABLE 1
Residues of cytochrome c{sub}552 and the Cu{sub}A domain used as active and passive AIRs in the docking calculations

| Redox partner | Active AIRs | Passive AIRs |
|---------------|-------------|--------------|
| Cytochrome c{sub}552 | Gly{sup}7, Asn{sup}14, Gly{sup}27, Glu{sup}57, Val{sup}69, Ser{sup}76, Ala{sup}115, Lys{sup}117, Glu{sup}120, Gly{sup}128, Met{sup}129 | Cys{sup}1, Glu{sup}125, Glu{sup}127, Glu{sup}128, Ile{sup}129, Pro{sup}130, Ala{sup}132, Phe{sup}133, Glu{sup}135, Gly{sup}136, Asn{sup}139, Gly{sup}146, Ser{sup}1, Thr{sup}11, Lys{sup}113, Arg{sup}114, Lys{sup}115, Thr{sup}117, Pro{sup}118, Thr{sup}120, Glu{sup}124 |
| Cu{sub}A domain | Ala{sup}22, Phe{sup}23, Arg{sup}24, Gly{sup}35, Asn{sup}38 | Phe{sup}29, Glu{sup}31, Glu{sup}44, Leu{sup}55, Phe{sup}161 |

TABLE 2
Properties of the 10 final structures representing the cytochrome c{sub}552-Cu{sub}A domain complex, sorted according to the distance between the iron atom of cytochrome c{sub}552 and the closest copper atom of the Cu{sub}A center (CU2 in all cases)

| No. | Distance (FE-CU2) | Intermolecular energy<sup>a</sup> | Buried surface | Electron transfer<sup>b</sup> | r.m.s.d.<sup>d</sup> |
|-----|------------------|-------------------------------|---------------|---------------------------|-----------------|
|     | Å                | kcal/mol                      | Å<sup>c</sup> | Length [Å<sub>a</sub>] | Å               |
| 1   | 15.59            | -102.3                        | -64.4         | 19.60                     | -11.2           | NA<sup>d</sup> |
| 2   | 15.78            | -110.0                        | -67.5         | 19.55                     | -11.2           | 2.31 |
| 3   | 15.87            | -123.6                        | 17.0          | 1563                      | 4.54            |
| 4   | 16.03            | -91.9                         | 18.3          | 1602                      | 3.21            |
| 5   | 16.05            | -86.1                         | 17.9          | 1426                      | 2.34            |
| 6   | 16.18            | -98.9                         | 18.4          | 1488                      | 1.57            |
| 7   | 16.31            | -91.2                         | 14.6          | 1314                      | 2.72            |
| 8   | 16.32            | -78.3                         | 18.8          | 1495                      | 3.33            |
| 9   | 16.61            | -92.2                         | 16.7          | 1360                      | 3.82            |
| 10  | 16.77            | -90.2                         | 18.3          | 1354                      | 2.35            |

<sup>a</sup> The total, van der Waals (vdw), electrostatic (Elec), and AIR energy terms for the intermolecular interaction.

<sup>b</sup> Electron-tunneling coupling factor, log [T<sub>DA</sub>], and electron pathway length of the best pathway found in each structure with the program GREENPATH (38).

<sup>c</sup> r.m.s.d. of the cytochrome c{sub}552 backbone atoms relative to structure 1, when the Cu{sub}A domain is superposed in all 10 conformers.

<sup>d</sup> Not applicable.

FIGURE 7. Stereo view of the 10 final structures of the cytochrome c{sub}552-Cu{sub}A domain complex, with the backbone atoms of the Cu{sub}A domain superposed on the bottom. The ensemble shows a single structure cluster, whereby the less aligned cytochrome c{sub}552 conformers still produce very similar docking arrangements with the water-soluble part of subunit II.

Possess the most favorable total interaction energies (<~100 kcal/mol) and exhibit an identical ET pathway; they were therefore selected as most representative of the cytochrome c{sub}552-Cu{sub}A domain complex and their atom coordinates deposited at the Brookhaven Data Bank under PDB ID code 2FWL. (The cytochrome c{sub}552 backbone r.m.s.d. between the superposed complex structures 2 and 3 is 2.55 Å).

None of the calculated complexes was able to fully compensate the potential energy that is associated with the AIRs; its contribution, however, remains significantly smaller than the van der Waals or electrostatic terms. More importantly, intermolecular contacts with the partner molecule were shown either directly by the residues classified as active AIRs or at least by one of the respective neighboring residues representing passive AIRs. The intermolecular contacts in structure 1, as displayed in Fig. 8, are therefore in agreement with the experimental picture; this has been achieved with a set of high-quality AIRs, derived from four independent experiments. Moreover, in agreement with the postulated hydrophobic/non-ionic character of the cytochrome c{sub}552-Cu{sub}A domain interaction in the T. thermophilus system (18, 20), about 40% of the contact surfaces are composed of hydrophobic residues. This is due to a large number of nonpolar intermolecular interactions (see Fig. 8), for example by Ile{sup}22 and Val{sup}69 (both in cytochrome c{sub}552) as well as Phe{sup}188 and Leu{sup}155 (both in the Cu{sub}A domain), whereas only two charged residues (Lys{sup}117 in cytochrome c{sub}552 and Arg{sup}114 in the Cu{sub}A domain) are found within the protein-protein contact zone, in comparison to an inner ring of four positively charged lysine residues encircling the heme cleft in P. denitrificans cytochrome c{sub}552 (19).

An additional consideration regarding the quality of the complex structures involves the Cu{sub}A domain that was used in the chemical shift perturbation experiments. This Cu{sub}A domain represents merely the solvent-exposed part of the entire ba<sub>3</sub> oxidase. It is reasonable to assume, however, that the complete ba<sub>3</sub> oxidase forms the same type of complex with cytochrome c{sub}552 like the free Cu{sub}A domain. To test whether this is true for complex structures 1–3, the Cu{sub}A domain coordinates were reattached to subunit I of the ba<sub>3</sub> oxidase (Fig. 9). Subsequent analysis for steric overlap with the corresponding cytochrome c{sub}552 molecule displayed only few addi-
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The Electron Pathway—The program GREENPATH (38) was used to compare, based on the pathway model, the ET efficiencies within the complex structures by calculating the electron-tunneling coupling factors. The Franck-Condon term (see “Experimental Procedures”) contains the free energy dependence related to nuclear motion; this is difficult to quantify, since it requires the evaluation of reorganization energies, but should be approximately constant for different complex conformations of the same partners. The results obtained with this method have been quite successful in the prediction of ET properties of proteins (see Refs. 47 and 48 and references therein), although the semi-empirical formulation behind it is a simplification of more complete descriptions (49). The shortest electron pathway proposed for the cytochrome c<sub>552</sub>-Cu<sub>α</sub> domain complex was found in structure 1. The electron originates at the porphyrin system in cytochrome c<sub>552</sub>, formally traveling from the iron center along the heme NC, C4C, C3C, and CBC atoms, and crosses over to the Ala<sup>87</sup> backbone oxygen in the Cu<sub>α</sub> domain. The electron continues along the Ala<sup>87</sup>-Phe<sup>88</sup> peptide bond to the amide proton of Phe<sup>88</sup>, where another jump occurs to the imidazole ring of His<sup>114</sup>, a direct ligand of the Cu<sub>α</sub> center. Continuing along the His<sup>114</sup> ring atoms HE1, CE1, and ND1, the electron eventually reaches the copper atom CU2 (Fig. 10).

This pathway involves residues Ala<sup>87</sup> and Phe<sup>88</sup> of the Cu<sub>α</sub> domain, which both showed significant perturbations of the amide <sup>1</sup>H and <sup>15</sup>N chemical shifts upon titration with cytochrome c<sub>552</sub>. Moreover, the involvement of Phe<sup>88</sup> in the ET was confirmed by site-directed mutagenesis experiments: replacement of Phe<sup>88</sup> by a leucine residue did not completely abolish the ET rates in stopped-flow kinetics (see Fig. S3 in the supplemental data) but diminished its efficiency significantly to ~68% of the apparent bimolecular rate constant in the physiological direction compared with the wild-type (WT) protein (<i>k<sub>forward</sub></i>(WT): 5.0 × 10<sup>6</sup> M<sup>−1</sup> s<sup>−1</sup>, <i>k<sub>forward</sub></i>(F88L): 3.4 × 10<sup>6</sup> M<sup>−1</sup> s<sup>−1</sup>). This situation is different to the <i>P. denitrificans</i> system, where the corresponding Trp<sup>121</sup> residue in the Cu<sub>α</sub> domain has a key role in the ET to cytochrome c<sub>552</sub> (24, 50, 51): substitution of Trp<sup>121</sup>, e.g. by glutamine, rendered the enzyme inactive. The fact that the F88L mutant of <i>T. thermophilus</i> still shows 68% ET activity therefore suggests that one or more alternative pathways may exist. The neighboring Phe<sup>88</sup>, corresponding to Tyr<sup>122</sup> in <i>P. denitrificans</i>, could be excluded as possible ET component for several reasons: first, the F86L mutant was fully functional like the wild-type protein (<i>k<sub>forward</i></i>; <i>WT</i>): 5.0 × 10<sup>6</sup> M<sup>−1</sup> s<sup>−1</sup>, <i>k<sub>forward</i></i>(F86L): 3.4 × 10<sup>6</sup> M<sup>−1</sup> s<sup>−1</sup>). As a consequence, the influence of Phe<sup>88</sup> on the ET can be narrowed down to two possible scenarios. Either the ET pathway proposed by the GREENPATH program is the only biologically relevant route the electron can take, in which case the effect of the F88L mutation on the ET activity must be due to the resulting decrease of the hydrophobic portion in the contact surface and/or changes in the reorganization energy; or as we assume more likely, the electron can principally take two alternative pathways both involving position 88 of the Cu<sub>α</sub> domain (Fig. 10). The through-bond pathway of ~19–20 Å length, as proposed by GREENPATH, has only short through-space jumps of 1.83 Å (between heme HBC and Ala<sup>87</sup> O) and 2.95 Å (between Phe<sup>88</sup> HN and His<sup>114</sup> HE1). In this case, the closest edge-to-edge distance between the conjugated donor and acceptor systems (i.e. the heme ring and the His<sup>114</sup> imidazole ring, respectively) is 10.9 Å. This distance can be bridged easily and efficiently by a tunneling electron, as the majority of known ET reac-

The scheme was prepared with the program 

![FIGURE 8. Intermolecular interaction matrix of cytochrome c<sub>552</sub> (cytc<sub>552</sub>) and the Cu<sub>α</sub> domain (cua) in complex structure 1 (see Table 2), showing the number of atom-to-atom contacts closer than 2.8 Å for each residue involved in the complex formation. This scheme was prepared with the program mmp2fit (27).](image)
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inations between natural redox centers occur over distances of 14 Å or less (52). These ET reactions are remarkably rapid and specific with favor- timescales according to current ET theories (52, 53). Substitution of the phenyl ring by an aliphatic side chain in the F88L mutant would there- fore eliminate only one of the possible ET pathways. In fact, the through bond pathway along the backbone of Ala<sup>88</sup> and Phe<sup>88</sup> might even rep- resent a rational solution from an evolutionary point of view, since in this case the ET will not be significantly affected by spontaneous point mutations that could otherwise possibly render the system inactive by eliminating an essential side chain.

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FIGURE 10. The ET pathways proposed for the cytochrome c<sub>552</sub>-Cu<sub>A</sub> domain complex of T. thermophilus based on the energetically best complex structure 1 (see Table 2). In the through-bond pathway (white arrow), the electron travels from the heme to the Phe<sup>88</sup> ring (see dashed arrows in Fig. 10), thereby bridging a total distance of around 13.5 Å. However, in this case the transfer rates should be rather low, as electrons tunnel "through space" from one center to another with a rate that decreases exponen- tially with distance. Although this edge-to-edge distance is still within the productivity limit for ET through space, it should be less efficient compared with the "through-bond" path outlined above, as indicated by the fact that the F88L mutation reduced the ET activity by not more than 32%.

Hence, both pathways appear possible within biologically relevant time scales according to current ET theories (52, 53). Substitution of the phenyl ring by an aliphatic side chain in the F88L mutant would therefore eliminate only one of the possible ET pathways. In fact, the through bond pathway along the backbone of Ala<sup>88</sup> and Phe<sup>88</sup> might even represent a rational solution from an evolutionary point of view, since in this case the ET will not be significantly affected by spontaneous point mutations that could otherwise possibly render the system inactive by eliminating an essential side chain.
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