Structure and mechanism of a eukaryotic transmembrane ascorbate-dependent oxidoreductase

Peilong Lu[a,1], Dan Ma[a,1], Chuangye Yan[b], Xinqi Gong[b], Mingjian Du[a], and Yigong Shi[a,2]

[a]Ministry of Education Protein Science Laboratory and [b]State Key Laboratory of Biomembrane and Membrane Biotechnology, Joint Center for Life Sciences, Center for Structural Biology, School of Life Sciences and School of Medicine, Tsinghua University, Beijing 100084, China

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Vitamin C, also known as ascorbate, is required in numerous essential metabolic reactions in eukaryotes. The eukaryotic ascorbate-dependent oxidoreductase cytochrome b_{561} (Cyt b_{561}), a family of highly conserved transmembrane enzymes, plays an important role in ascorbate recycling and iron absorption. Although Cyt b_{561} was identified four decades ago, its atomic structure and functional mechanism remain largely unknown. Here, we report the high-resolution crystal structures of cytochrome b_{561} from *Arabidopsis thaliana* in both substrate-free and substrate-bound states. Cyt b_{561} forms a homodimer, with each protomer consisting of six transmembrane helices and two heme groups. The negatively charged substrate ascorbate, or monodehydroascorbate, is enclosed in a positively charged pocket on either side of the membrane. Two highly conserved amino acids, Lys^{81} and His^{106}, play an essential role in substrate recognition and catalysis. Our structural and biochemical analyses allow the proposition of a general electron transfer mechanism for members of the Cyt b_{561} family.

Vitamin C, an essential nutrient for humans (1), is important for the synthesis of collagen (2), carnitine (3), and the neurotransmitter norepinephrine (4). Vitamin C also plays an important role in protection against oxidative stress (5). Oxidation of ascorbate results in sequential production of monodehydroascorbate and dehydroascorbate through loss of one and two electrons, respectively (6). Ascorbate serves as an electron donor for various enzymes, such as prolyl and lysyl hydroxylase, dopamine β-hydroxylase, ascorbate peroxidase, and cytochrome b_{561} (Cyt b_{561}). Cyt b_{561}, initially identified in the chromaffin granules of bovine adrenal medullae about 40 y ago (7, 8), is a transmembrane ascorbate-dependent oxidoreductase (9–13) that plays a key role in ascorbate recycling and other physiological processes, such as iron absorption (14). To our knowledge, Cyt b_{561} is the only membrane-embedded oxidoreductase that relies on ascorbate as the electron donor.

Homologs of Cyt b_{561} are found only in eukaryotes (15). Mammalian chromaffin granule Cyt b_{561} (CGCyt b_{561}), mammalian duodenal Cyt b_{561} (DCyt b_{561}), and *Zea mays* Cyt b_{561} (ZmCyt b_{561}) have been extensively investigated. CGCyt b_{561} resides in the chromaffin vesicle membrane and transfers electrons from cytoplasmic ascorbate to the intravesicular monodehydroascorbate radical for the regeneration of ascorbate (9, 13, 16), which is a substrate of dopamine β-hydroxylase for the synthesis of neurotransmitter norepinephrine. DCyt b_{561} is present in the duodenal mucosa cell membrane, where it relays electrons from cytoplasmic ascorbate to ferric-chelate in the intestinal lumen, yielding soluble ferrous ion for absorption via a Fe^{2+}-transporter (17–20). Expression levels of DCyt b_{561} in the duodenal mucosa cell membrane are closely associated with iron metabolism disorders, such as chronic anemia and iron-deficiency anemia (19). Similar to CGCyt b_{561}, ZmCyt b_{561} uses ascorbate and the monodehydroascorbate radical as the physiological electron donor and acceptor, respectively (11, 21, 22).

Despite rigorous investigation, there is no detailed structural information for any member of the Cyt b_{561} family. Consequently, the electron transfer path is yet to be identified and the molecular mechanisms of substrate recognition and catalysis remain largely mysterious. These aspects are crucial for understanding the functional mechanism of the Cyt b_{561} protein family. In this study, we answer these questions by elucidating the crystal structures of Cyt b_{561} in both ascorbate-free and -bound forms and by performing systematic, structure-guided biochemical analyses.

Results

Members of the Cyt b_{561} family share strong sequence homology and are functionally conserved, and structural information on any Cyt b_{561} enzyme is expected to reveal conserved structural features and functional insights for the entire family. To obtain Cyt b_{561} with a high expression level, we cloned and screened a number of orthologs from humans and plants. These proteins were also examined for solution behavior and crystallization. Based on preliminary analyses, we focused on the recombinant full-length WT Cyt b_{561}-B protein (residues 1–230) from *Arabidopsis thaliana*, which gave rise to weak-diffracting crystals. Treatment with ferricyanide during protein purification, which presumably led to oxidation of the heme groups in Cyt b_{561}, and hence more homogeneous protein, led to markedly improved diffraction for these crystals. The structure was determined by ferric-based, single-wavelength anomalous dispersion, and the atomic model was refined at a resolution of 1.7 Å (Table S1). The sulfur anomalous signals of internal methionine and cysteine residues were clearly detected (Fig. S1*A*).

Each asymmetrical unit contains two molecules of Cyt b_{561}, arranged as a pseudosymmetrical dimer through a twofold axis perpendicular to the plane of the lipid membrane (Fig. 1*A*). The

Significance

Vitamin C (also known as ascorbate), an essential nutrient for humans, plays an important role in protection against oxidative stress. The ascorbate-dependent oxidoreductase cytochrome b_{561} (Cyt b_{561}) is a family of highly conserved, multipass transmembrane enzymes found only in eukaryotes. Cyt b_{561} plays a key role in ascorbate recycling and many other important physiological processes, such as iron absorption. The atomic structure and functional mechanism of Cyt b_{561} remain unknown. In this study, we report the high-resolution crystal structures of Cyt b_{561} in both ascorbate-free and ascorbate-bound states. Our structural and biochemical analyses identify a general functional mechanism for the Cyt b_{561} family.

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Data deposition: The coordinates and structure factors for the ascorbate-free and ascorbate-bound structures have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 4O6Y, 4O79, and 4O7G).

[1]P.L. and D.M. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: shi-lab@tsinghua.edu.cn.

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two Cyt b_{561} protomers, named Mol A and Mol B, exhibit nearly identical structural features, with a pairwise rmsd of 0.49 Å over 210 aligned Cα atoms. Each Cyt b_{561} protomer comprises six transmembrane segments (TMs) (Fig. 1*A*), with both the N and C termini located in the cytoplasm based on prior knowledge (23, 24). Each protomer contains two heme groups, which are partially exposed to solvent (Fig. 1*B*). Notably, two cavities are present on each side of the membrane; each cavity is surrounded by positive charges, with a heme group located underneath. These analyses strongly suggest that these positively charged cavities may be the substrate-binding sites. The potential substrate-binding site on the cytoplasmic side of Mol A is occupied by a sulfate ion, which mediates crystal packing interactions (Fig. S1 *B* and *C*).

The two Cyt b_{561} protomers associate with each other through a hydrophobic interface, resulting in a buried surface area of ~1,410 $Å^2$ (Fig. S2*A*). To examine the notion that Cyt b_{561} may exist as a homodimer, we sought to engineer a dimerization-dependent disulfide bond in Cyt b_{561}. The Cα-Cα distance between Tyr^{115} in Mol A and Arg^{191} on Mol B is ~5.8 Å (Fig. S2*B*, *Left*), which is ideally suited for the formation of a disulfide bond if these two residues are mutated to cysteine. The WT Cyt b_{561} protein appears mostly as a monomer on the denaturing SDS/PAGE; by contrast, a major proportion of the mutant Y115C/R191C protein formed a cross-linked homodimer even in the absence of the oxidation catalyst *o*-phenanthroline copper complex (25, 26) (Fig. S2*B*, *Right*, lanes 1 and 5). Incubation with the oxidation catalyst led to more complete formation of the cross-linked homodimer for Y115C/R191C but had little impact on WT Cyt b_{561} (Fig. S2*B*, *Right*, lanes 3 and 7). The presence of the reducing agent DTT reduced the proportion of cross-linked homodimer for Y115C/R191C (Fig. S2*B*, *Right*, lanes 6 and 8) but had no impact on WT Cyt b_{561} (Fig. S2*B*, *Right*, lanes 2 and 4). Further supporting this analysis, the disulfide-bonded Y115C/R191C was eluted from gel filtration with approximately the same elution volume as that for WT Cyt b_{561} (Fig. S2*C*). Taken together, our biochemical characterization demonstrates that the Cyt b_{561} protein exists as a homodimer.

There are four heme groups in the Cyt b_{561} homodimer (Fig. 2*A*). Each heme is sandwiched by the same set of four transmembrane helices, TMs 2/3/4/5, and bound by two invariant His residues. The heme group on the cytoplasmic side is recognized by His^{84} from TM3 and His^{157} from TM5, whereas the heme on the noncytoplasmic side is coordinated by His^{51} from TM2 and His^{118} from TM4 (Fig. 2*B* and Fig. S3). The closest edge-to-edge distance between the two heme groups within the same Cyt b_{561} protomer is about 15.3 Å (Fig. 2*B*), out of the range (14 Å or less) for robust through-space electron transfer (27, 28). Intriguingly, a water molecule is located between the two heme centers, 4.5 Å from the cytoplasmic heme and 12 Å from the noncytoplasmic heme (Fig. 2*C*). This water molecule is hydrogen-bonded to the side chain of Asn^{63} from TM2 and the carbonyl oxygen atom of Gly^{161} from TM5 (Fig. 2*D*). In addition, the side chain of the conserved Phe^{129} is located between the two heme centers, 5.5 Å from the cytoplasmic heme and 10.5 Å from the noncytoplasmic heme (Fig. 2 *C* and *D*). All these distances are suitable for efficient electron transfer, suggesting potential roles for the water molecule and/or Phe^{129} in bridging a tunneling electron.

On the noncytoplasmic side, the two propanoate groups of the heme center mediate a number of hydrogen bonds (H-bonds) with surrounding amino acids (Fig. 2*E*). The A-propanoate interacts with the side chains of Ser^{116} and Glu^{178}, whereas the D-propanoate is hydrogen-bonded to the amide nitrogen of Phe^{114} and the side chains of His^{106} and Asn^{113}. On the cytoplasmic side, the A-propanoate of the heme center forms two H-bonds, one with the side chain of Lys^{71} in TM2 and the other through a water molecule to the amide nitrogen of Lys^{71}, whereas the D-propanoate of the heme center is exposed to the solvent (Fig. 2*F*). The amino acids that use their side chains to form H-bonds with the heme groups are highly conserved among Cyt b_{561} family members (Fig. S3).

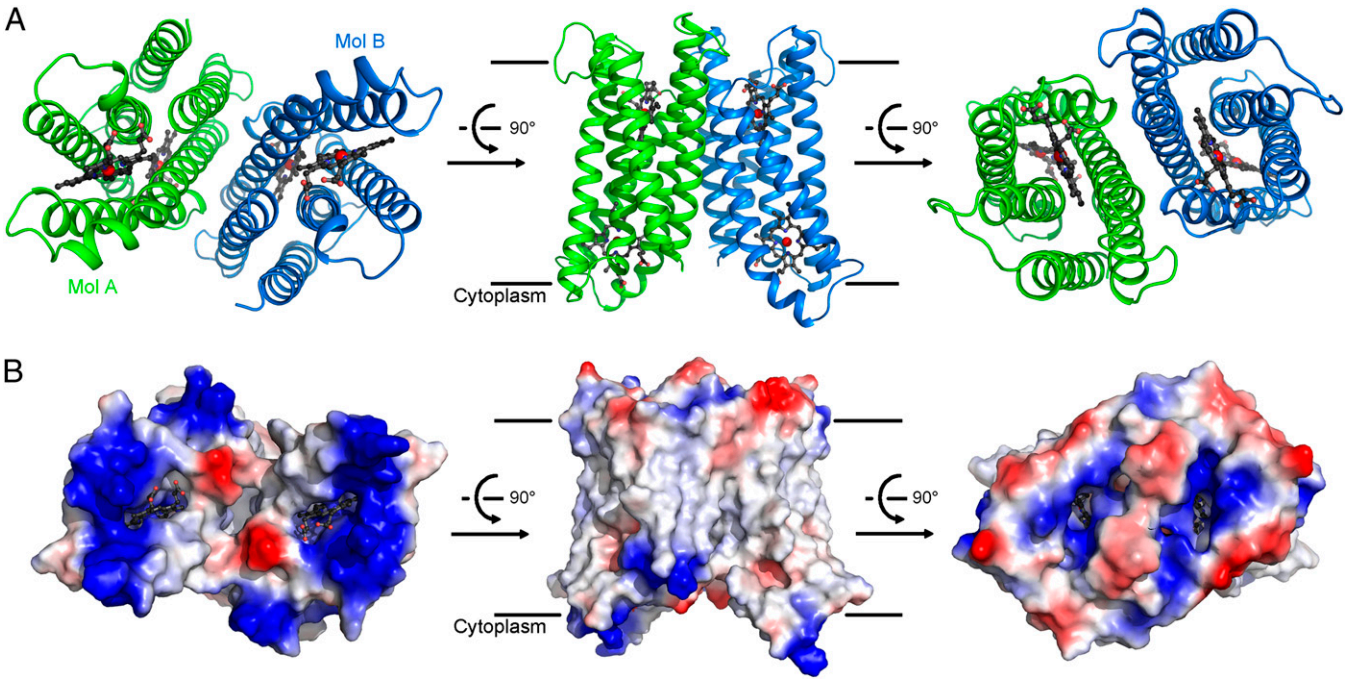

Fig. 1. Overall structure of Cyt b_{561}. (*A*) Overall structure of WT, full-length Cyt b_{561}. The structure of Cyt b_{561} is shown in three successive views. There are two molecules of Cyt b_{561} in each asymmetrical unit, named Mol A (green) and Mol B (blue). (*B*) Surface features of the Cyt b_{561} homodimer by electrostatic potential. The three views shown correspond to those in *A*. Two cavities on either side are surrounded by positively charged amino acids. All structural figures were prepared using PyMOL Molecular Graphics System, Version 1.5 (Schrödinger, LLC).

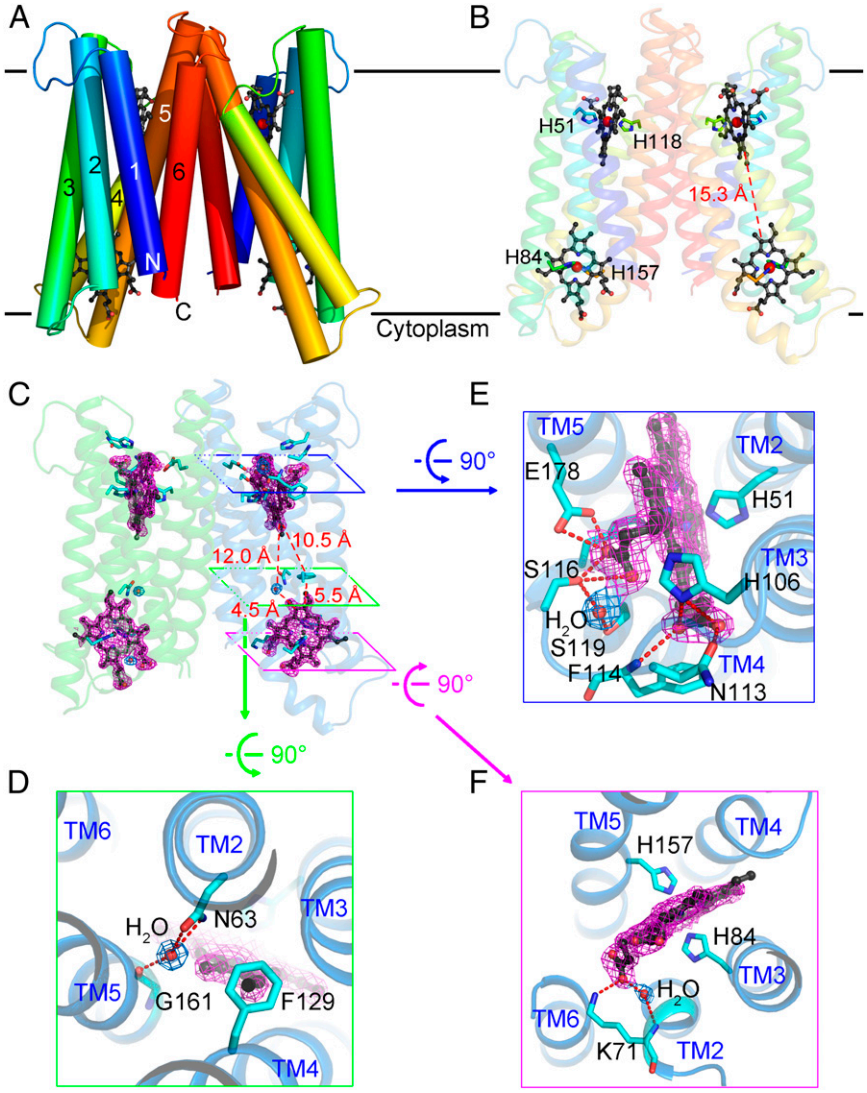

Fig. 2. Heme-binding sites of Cyt b_{561}. (*A*) Ribbon representation of the Cyt b_{561} homodimer. Cyt b_{561} is rainbow-colored, with its N terminus in blue and its C terminus in red. (*B*) Heme arrangement in Cyt b_{561}. The heme groups are in a ball-and-stick configuration, with the iron atoms as red spheres. The four histidines that coordinate heme iron are shown. (*C*) Location of a water molecule and Phe^{129} between two heme groups in each protomer. The electron density maps, contoured at 1.0σ, are colored blue for waters and magenta for the heme groups. (*D*) Close-up view of the intervening water molecule and Phe^{129} between the two heme groups in each protomer of Cyt b_{561}. H-bonds are indicated by red dashed lines. (*E*) Close-up view of the heme group on the noncytoplasmic side. (*F*) Close-up view of the heme group on the cytoplasmic side.

The crystal structure of Cyt b_{561} is related to that of the heme-containing protein cytochrome b_6, a subunit of the cytochrome b_6f complex (29, 30). In both cases, the central four TMs have similar conformations (Fig. S4). The four TMs of cytochrome b_6 can be superimposed to those in Cyt b_{561}, with an rmsd of 2.77 Å over 118 aligned Cα atoms. Despite this similarity, coordination of the heme group is different between these two proteins. In the case of Cyt b_{561}, heme groups are bound by residues from all four TMs; in cytochrome b_6, however, only residues from TM helices B and D participate in heme binding. In addition, Cyt b_{561} has unique defining features. Most importantly, the electron donor/acceptor pair for Cyt b_{561} is ascorbate/monodehydroascorbate or ascorbate/ferric-chelate, whereas the electron donor/acceptor pair for cytochrome b_6f is hydroquinone/plastocyanin. The closest edge-to-edge distance between the two heme centers of Cyt b_{561} is considerably longer than that in cytochrome b_6. Moreover, Cyt b_{561} contains two additional TMs and has a dimer arrangement different from that of cytochrome b_6.

The primary sequences of *A. thaliana* $Cytb_{561}$-B share 34% identity and 54% similarity with those of human $CGCytb_{561}$ and 36% identity and 52% similarity with those of human $DCytb_{561}$ (Fig. S3). This suggests that $Cytb_{561}$-B may function similarly as human $CGCytb_{561}$ or $DCytb_{561}$ to participate in ascorbate recycling or ascorbate-dependent ferric-chelate reduction. To elucidate the mechanism of substrate recognition, we sought to determine the crystal structure of Cyt b_{561} bound to L-ascorbate by soaking Cyt b_{561} crystals in 1 M L-ascorbate for 10 min. The resulting crystals were used for X-ray data collection and structure determination at a resolution of 2.0 Å (Table S1).

The overall structure of Cyt b_{561} is unaffected by ascorbate binding (Fig. S5*A*). After all protein atoms were modeled, the electron density allowed assignment for L-ascorbate in Mol B on the cytoplasmic side (Fig. 3*A*), but not on the noncytoplasmic side, under this condition. The same position in Mol A is occupied by a sulfate anion, which facilitated crystal packing. Ascorbate is caged in a positively charged pocket located in close proximity to the heme group on the cytoplasmic side (Fig. 3*A* and Fig. S5*B*). This

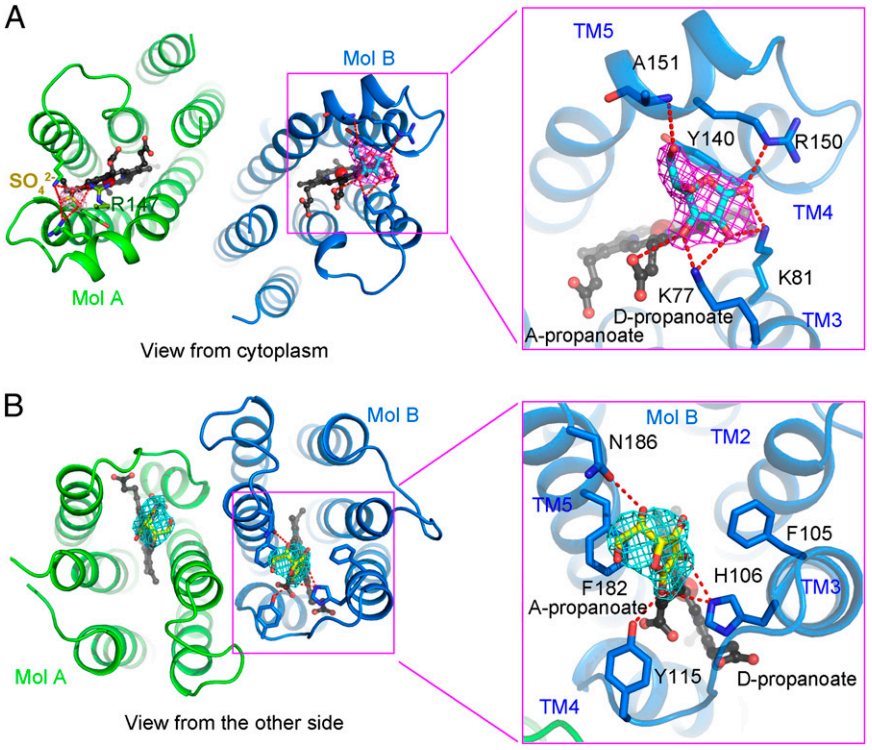

Fig. 3. Recognition of ascorbate by conserved amino acids. (*A*) Recognition of ascorbate on the cytoplasmic side. Ascorbate is bound on the cytoplasmic side of Mol B, coordinated by the heme group and residues from TM3, TM4, and TM5. The same position of Mol A is occupied by a sulfate ion and the side chain of Arg^{147} from a neighboring molecule, Mol A′. The $F_{obs} - F_{calc}$ electron density for ascorbate (magenta) is contoured at 2.0σ. (*B*) Recognition of monodehydroascorbate on the noncytoplasmic side. Monodehydroascorbate is bound on the noncytoplasmic side of the Cyt b_{561} dimer, coordinated by residues from TM3, Loop3, and TM5. The $F_{obs} - F_{calc}$ electron density for ascorbate (cyan) is contoured at 2.5σ.

binding mode is similar to that of the ascorbate–ascorbate peroxidase complex structure (31). The ketone group (position 1) (Fig. S5*C*) of ascorbate accepts two H-bonds from the side chains of Lys^{81} and Arg^{150}, whereas the hydroxyl group at position 2 of ascorbate makes two H-bonds to the side chains of Lys^{77} and Lys^{81}. The hydroxyl group at position 3 interacts with the side chain of Lys^{77} and the D-propanoate of the heme group. In addition, the 6-OH group of ascorbate mediates one H-bond with the main chain amide group of Ala^{151}. The five-membered ring of ascorbate interacts with the heme group and Tyr^{140} from TM4 via van der Waals contacts. All residues that interact with ascorbate are highly conserved in Cyt b_{561} from plants to humans (Fig. S3).

To examine substrate binding on the noncytoplasmic side, we screened different substrate soaking times. Fortunately, upon soaking crystals in 1 M ascorbate for 40 min, we were able to determine the structure at a resolution of 2.2 Å (Table S1). Again, prolonged soaking had little impact on the overall structure (Fig. S5*D*) but produced two lobes of electron density in the positively charged pockets of the Cyt b_{561} dimer on the noncytoplasmic side (Fig. 3*B* and Fig. S5*E*). Because the monodehydroascorbate radical, the physiological substrate for human CGCyt b_{561} on the noncytoplasmic side (9, 10), is structurally indistinguishable from ascorbate at this resolution, we modeled two molecules of monodehydroascorbate, instead of ascorbate, to fit the density. Monodehydroascorbates are positioned above the heme groups on the noncytoplasmic side, each surrounded by two polar residues and three aromatic amino acids (Fig. 3*B*). The side chains of His^{106}, Tyr^{115}, and Asn^{186} mediate four potential H-bonds to monodehydroascorbate. In addition, monodehydroascorbate is stacked by the benzene rings of Phe^{105} and Phe^{182}. Except for Asn^{186}, all residues that interact with monodehydroascorbate are highly conserved among members of the Cyt b_{561} family. Notably, these substrate-binding residues observed in our crystal structures are different from the predicted substrate-binding motifs ALLVYR (residues 66–71) and SLHSW (residues 116–120) (14, 19, 32) (Fig. S3).

Cyt b_{561} is an oxidoreductase, transferring electrons from ascorbate to monodehydroascorbate or ferric-chelate. As previously reported (11, 33), ferricyanide-oxidized Cyt b_{561} shows a characteristic peak at 416 nm in its visible absorption spectra and, upon reduction by ascorbate, displays three unique peaks at 428, 531, and ~561 nm (Figs. S6, *Upper Left* and S7A, *Left*). The oxidized Cyt b_{561} can be reduced by ascorbate but not by other electron donors, such as vitamin E, reduced glutathione, NADH, or NADPH (Fig. S6). Next, using a stopped-flow apparatus, we investigated the kinetics of ascorbate-mediated reduction of Cyt b_{561} by monitoring the absorbance change at 430 nm (Fig. S7*A*, *Right*). The result shows rapid reduction of Cyt b_{561} within 15 s.

The route of electron transfer might begin with ascorbate on either side of the membrane due to the two substrate-binding sites. To corroborate our structural finding, we generated 11 Cyt b_{561} variants, each containing one or multiple mutations targeting ascorbate-binding residues and purified these variants to homogeneity (Fig. S7*B*). Mutations on the cytoplasmic side only, K81A/R150A or Y140W, or mutations on the noncytoplasmic side only, F105W/H106E or Y115W, still allowed the oxidized Cyt b_{561} to be reduced by ascorbate (Fig. S7*C*). In sharp contrast, the Cyt b_{561} variant K81A/R150A/F105W/H106E, which carries ascorbate-binding mutations on both cytoplasmic and noncytoplasmic sides, completely lost its ability to be reduced by ascorbate (Fig. S7*D*). This analysis also suggests that two key mutations from either side, K81A/R150A on the cytoplasmic side or F105W/H106E on the noncytoplasmic side, are sufficient for abrogating electron transfer from the corresponding side.

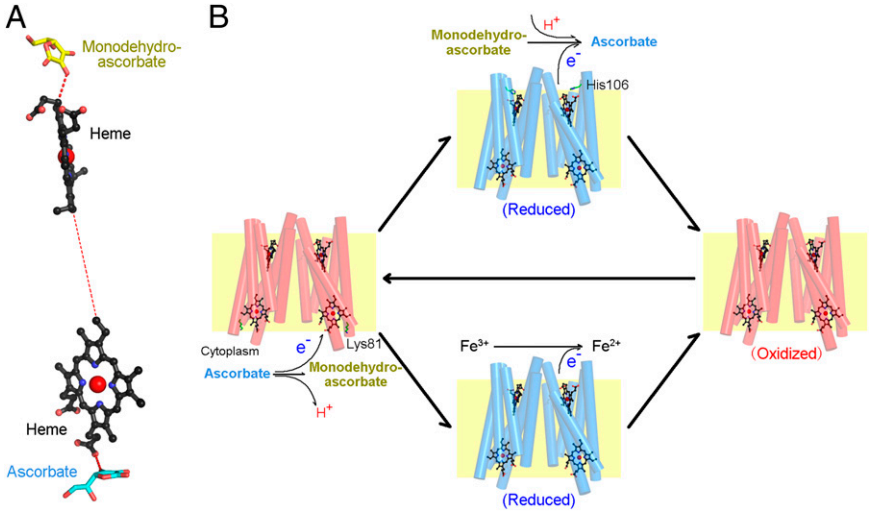

Fig. 4. Proposed model for electron transfer. (*A*) Proposed electron transfer path. This illustration shows the electron transfer path from ascorbate in the cytoplasm to monodehydroascorbate on the noncytoplasmic side. (*B*) Proposed mechanism of ascorbate recycling and ascorbate-dependent ferric reduction catalyzed by Cyt b_{561}. The reactions occur on both sides of the membrane, with Cyt b_{561} catalyzing the transfer of electrons.

Notably, an important role of the amino acid that corresponds to Lys81 of Cyt b_{561} has previously been reported for ZmCyt b_{561} and bovine CGCyt b_{561}, where mutation or chemical modification of the corresponding residue was shown to affect electron acceptance from ascorbate in a negative manner (11, 13, 22).

Next, we blocked the ascorbate-binding site on the noncytoplasmic side and examined the functional importance of the ascorbate-binding residues on the cytoplasmic side (Fig. S7*E*). Under the background of F105W/H106E, the variant K81A displayed a more severe loss of the ability to be reduced by ascorbate compared with the variant Y140W or R150A. Using a similar strategy, we examined the ascorbate-binding residues on the noncytoplasmic side (Fig. S7*F*). Under the background of K81A/R150A, the variant H106E, but not F105W or Y115W, nearly lost the ability to be reduced by ascorbate. Thus, consistent with our structural observations, Lys^{81} on the cytoplasmic side and His^{106} on the noncytoplasmic side play a particularly important functional role. This in vitro system allows efficient evaluation of the functional importance of select amino acids in Cyt b_{561}; our observations strongly suggest that Cyt b_{561} indeed has two binding sites for ascorbate/monodehydroascorbate.

Discussion

Our structural and biochemical analyses identify key elements of the electron transfer path for Cyt b_{561}, which comprises two heme groups and two associated substrate molecules (Fig. 4*A*). The donor electron from ascorbate on the cytoplasmic side is transferred first to the heme group on the cytoplasmic side; then to the heme group on the noncytoplasmic side; and finally to the electron acceptor, monodehydroascorbate. To pinpoint the electron transfer route between the two heme groups, we performed in silico electron transfer modeling. Intriguingly, the results show that the energetically favored electron propagation occurs exclusively through bonding, via a number of covalent bonds and H-bonds (Fig. S8). Nonetheless, we cannot rule out a potential role by the en route water molecule and Phe^{129} in through-space electron tunneling.

Cyt b_{561} family members are mainly involved in two physiological processes: ascorbate recycling and ferric-chelate reduction, as exemplified by CGCyt b_{561} and DCyt b_{561}, respectively. Notably, CGCyt b_{561} also has ferrireductase activity (12). In addition, other members of the Cyt b_{561} family, such as ZmCyt b_{561} (11), Cyt b_{561}-A (34), and Cyt b_{561}-B from *A. thaliana* and lysosomal Cyt b_{561} (12), could be oxidized by ferric-chelates. On the other hand, reduced CGCyt b_{561} and ZmCyt b_{561} could react with the monodehydroascorbate radical (11, 13), consistent with our observation that Cyt b_{561} has two ascorbate/monodehydroascorbate-binding sites. Importantly, the substrate-binding and heme-coordinating residues are highly conserved, suggesting a similar functional mechanism for the entire family. These lines of evidence suggest that Cyt b_{561} family members may have electron-donating activities to both monodehydroascorbate and ferric-chelates (Fig. 4*B*).

In our model, one molecule of ascorbate binds to the conserved substrate-binding site on the cytoplasmic side (involving Lys^{81} in *A. thaliana* $Cytb_{561}$-B) and donates an electron to Cyt b_{561}, releasing a proton and generating one molecule of monodehydroascorbate. Next, on the other side of membrane, monodehydroascorbate or ferric-chelate may react with the reduced Cyt b_{561}. Monodehydroascorbate likely binds to His^{106} of *A. thaliana* $Cytb_{561}$-B or the corresponding residues in other homologs, accepting an electron, incorporating a proton, and producing one molecule of ascorbate. This model is consistent with the observations that the concentration of ascorbate is higher in the cytoplasm than on the other side (32), whereas the concentration of proton is the opposite (35). During the electron transfer process, Lys^{81} on the cytoplasmic side and His^{106} on the noncytoplasmic side might be involved not only in substrate recognition but in catalysis, perhaps through cycles of protonation and deprotonation. In addition, ferric-chelate might bind to the pocket on the noncytoplasmic side of Cyt b_{561}, being reduced to a ferrous ion by accepting an electron from the heme center. The resulting oxidized Cyt b_{561} protein is now ready for another cycle of reduction and oxidation initiated by ascorbate in the cytoplasm.

Materials and Methods

All proteins in this study were expressed in *Escherichia coli* and purified by affinity chromatography and gel filtration. Crystals were obtained by the hanging drop, vapor diffusion method. Detailed methods describing protein preparation, crystallization, data collection, structure determination, and biochemical assays can be found in *SI Materials and Methods*.

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