Cryo-EM structures of the air-oxidized and dithionite-reduced photosynthetic alternative complex III from *Roseiflexus castenholzii*

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Alternative complex III (ACIII) is a multisubunit quinol:electron acceptor oxidoreductase that couples quinol oxidation with transmembrane proton translocation in both the respiratory and photosynthetic electron transport chains of bacteria. The coupling mechanism, however, is poorly understood. Here, we report the cryo-EM structures of air-oxidized and dithionite-reduced ACIII from the photosynthetic bacterium *Roseiflexus castenholzii* at 3.3- and 3.5-Å resolution, respectively. We identified a menaquinol binding pocket and an electron transfer wire comprising six hemes and four iron-sulfur clusters that is capable of transferring electrons to periplasmic acceptors. We detected a proton translocation passage in which three strictly conserved, mid-passage residues are likely essential for coupling the redox-driven proton translocation across the membrane. These results allow us to propose a previously unrecognized coupling mechanism that links the respiratory and photosynthetic functions of ACIII. This study provides a structural basis for further investigation of the energy transformation mechanisms in bacterial photosynthesis and respiration.

**INTRODUCTION**

Photosynthesis transforms solar energy to chemical energy and supports nearly all life on Earth. Sunlight is absorbed by pigments in the light-harvesting (LH) antenna system, and excitation energy is transferred to the reaction center (RC), where photochemistry occurs, initiating an electron transfer process. The electron transport chain (ETC) couples the redox reactions associated with electron donors and acceptors to proton translocation to build up a proton motive force across the membrane, which, in turn, drives the formation of adenosine triphosphate (ATP) and other energy-consuming processes. In photosynthetic and respiratory ETCs, complex III (mitochondrial and bacterial cytochrome bc1, chloroplast and cyanobacterial cytochrome bc) functions primarily to couple thermodynamically favorable electron transfer to proton translocation across the membrane (1–3). As quinol:electron acceptor oxidoreductases, these complexes create a transmembrane (TM) proton gradient through the Q-cycle mechanism: Four protons are translocated for every two electrons transferred to cytochrome c (cyt c) or plastocyanin upon quinol oxidation (3–5).

Notably, a functional counterpart for the cyt bc1 complex, alternative complex III (ACIII), has been identified in a wide range of bacterial taxa, and its presence usually coincides with the absence of the cyt bc1 complex (6–10). This complex is structurally and compositionally unrelated to the bacterial cyt bc1 complex, but it plays the same central role as a quinol:electron acceptor oxidoreductase in both the respiratory and photosynthetic ETCs (6, 8–13). In the respiratory chain, ACIII is usually associated with different cyt c oxidases and functions in aerobic electron transfer (14, 15). In the photosynthetic ETC of *Chloroflexus aurantiacus*, in which ACIII was originally discovered (16), the photosynthetic ACIII catalyzes the oxidation of menaquinol and mediates transfer of the released electrons to a periplasmic blue copper protein auracyanin, which, in turn, completes a cyclic electron transfer back to the RC (9, 12, 13).

Recent studies of the respiratory ACIII from *Rhodothermus marinus* (17) and *Flavobacterium johnsoniae* (18) have elucidated the structural features of this complex that are related to quinol coordination, cyt c oxidase association, and putative proton translocation. Regarding the association with different cyt c oxidases and the linear electron transfer mode of respiratory ACIII, the photosynthetic ACIII has a distinct composition and functions in a simple and efficient cyclic ETC using the electron donor menaquinol (8, 12, 13, 19, 20). However, the structure of the photosynthetic ACIII remains unknown. In particular, the fundamental coupling mechanisms underlying the menaquinol oxidation and proton translocation of the respiratory and photosynthetic ACIII complexes have received little research attention. Therefore, a structural investigation of the photosynthetic ACIII is necessary for a deeper understanding of the common coupling mechanism used by the ACIII from diverse bacterial taxa.

*Roseiflexus castenholzii* is a chlorosome-less filamentous anoxygenic phototroph. It contains a mosaic LH antenna, the type II phycobilin–quinone RC, and a cyclic electron transport system. The LH antenna of *R. castenholzii* is structurally similar to the LH1, but spectroscopically it resembles the LH2 of purple bacteria (19, 21, 22). Our previous structure of the *R. castenholzii* core complex (rcRC-LH) revealed a previously unknown architecture and a new type of menaquinone shuttle channel in the bacterial RC-LHs and illustrated the molecular basis underlying the LH and energy transfer mechanisms of early prokaryotes (23). We then extracted and purified *R. castenholzii* ACIII and its periplasmic electron acceptor auracyanin and revealed that ACIII oxidizes menaquinol-4 or menaquinol-7.

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and transfers the electrons to the copper ion coordinated in auracyanin (24). Here, we report the structures of the six-subunit *R. castenholzii* ACIII in air-oxidized and dithionite-reduced states, determined by single-particle cryo–electron microscopy (cryo-EM) at 3.3- and 3.5-Å resolution, respectively. We elucidated its structural features and here propose a previously unrecognized redox-coupled electron transfer and proton translocation mechanism that apparently links the respiratory and photosynthetic functions of the ACIII.

## RESULTS

### Composition and overall structure

We purified the ACIII from phototrophically grown *R. castenholzii* using a modification of previous methods (7, 8, 16). We next used SDS–polyacrylamide gel electrophoresis (PAGE) and blue native PAGE to evaluate the purified ACIII (fig. S1A). Consistent with the molecular size expected from the corresponding gene sequences, we observed that the overall 300-kDa complex was composed of six subunits (ActA, ActB, ActC, ActD, ActE, and ActF), with molecular masses ranging from ∼10 to 110 kDa (fig. S1A). Each subunit was confirmed by peptide mass fingerprinting (PMF) (tables S1 and S2). Further, gel filtration analysis (fig. S1B) indicated that the purified ACIII was a monomer containing one copy of each subunit. Spectral analysis indicated that the purified ACIII was air-oxidized. It was reduced via addition of sodium dithionite (fig. S1C). The reduced-minus-oxidized difference spectrum showed two absorbance peaks at 524 and 554 nm, indicating the increase of the c-type heme absorbance after reduction (fig. S1D).

To elucidate the proposed conformational changes that were involved in the redox-driven proton translocation of respiratory ACIII (17), the vitrified air-oxidized and dithionite-reduced ACIII were individually subjected to cryo-EM single-particle analysis. A dataset of 257,815 particles of the air-oxidized ACIII was used to reconstruct an electron potential map with an average resolution of 3.3 Å and a local resolution extending to 2.5 Å (fig. S2 and movie S1). The final reconstructed cryo-EM map was resolved and enabled us to build an accurate model of the TM helices with side chains in the air-oxidized ACIII (fig. S3) and all the cofactors and lipid molecules (Table 1 and fig. S4). The cryo-EM map of the dithionite-reduced ACIII was reconstructed from 488,581 particles to 3.5-Å resolution, the composition and overall structure of which resembles that of the air-oxidized ACIII (Table 1, fig. S5, and movie S2).

Different from the respiratory ACIII from *R. marinus* that contains seven subunits (ActABCDEFH) and one additional unidentified subunit (17), the photosynthetic ACIII only contains six core subunits (ActA, ActB, ActC, ActD, ActE, and ActF) as in *F. johnsoniae* (18). Superimposition of *R. castenholzii* ACIII structure with that of *R. marinus* and *F. johnsoniae* gives a main-chain root mean square deviation (RMSD) of 1.5 and 3.2 Å, respectively. Like the two respiratory ACIII structures, *R. castenholzii* ACIII is assembled into an “L”-shaped architecture with dimensions of 141 Å; a TM arm (42 Å) containing 23 TM helices from subunits ActA, ActC, ActD, and ActF; and a peripheral arm comprising the periplasmic subunits ActA, ActB, and ActE. On the periplasmic side, subunit ActB forms extensive contacts with ActE, the penta-heme binding domain of ActA, and the periplasmic region of ActC, ActD, and ActF. The loop between the two TMs of ActD inserts into the interface of ActA, ActB, ActC, and ActF. The globular domain of ActD is located in the cytoplasm (Fig. 1A and B).

Table 1. Data collection, processing, and refinement statistics.

|                      | Air-oxidized ACIII (EMDB-0937) (PDB 6LOE) | Dithionite-reduced ACIII (EMDB-0936) (PDB 6LOD) |
|----------------------|------------------------------------------|-----------------------------------------------|
| Data collection and processing | Magnification | 22,500 | 22,500 |
|                      | Voltage (kV) | 300 | 300 |
|                      | Pixel size (Å) | 1.04 | 1.04 |
|                      | Defocus range (µm) | 1.2–3.3 | 1.0–3.0 |
|                      | Symmetry imposed | C1 | C1 |
|                      | Initial particle images (no.) | 257,815 | 488,581 |
|                      | Map resolution (Å) | 3.2 | 3.5 |
|                      | FSC threshold | 0.143 | 0.143 |
| Refinement | Model resolution (Å) | 3.3 | 3.5 |
|                      | FSC threshold | 0.5 | 0.5 |
|                      | Map sharpening B factor (Å²) | −125 | −135 |
|                      | Model composition | Nonhydrogen atoms | 19,024 | 19,024 |
|                      | Protein residues | 2,329 | 2,329 |
|                      | Ligands | 17 | 17 |
| RMSDs | Bond lengths (Å) | 0.025 | 0.025 |
|                      | Bond angles (°) | 1.548 | 1.420 |
| Validation | MolProbity score | 1.95 | 2.20 |
|                      | Clashscore | 8.57 | 14.09 |
|                      | Poor rotamers (%) | 0.42 | 0.21 |
|                      | Ramachandran plot | Favored (%) | 91.84 | 90.03 |
|                      | Allowed (%) | 8.16 | 9.97 |
|                      | Disallowed (%) | 0.00 | 0.00 |

Similar to *R. marinus* ACIII, given its known electron transport function, one [3Fe-4S] and three [4Fe-4S] clusters in ActB, and six c-type hemes (five in ActA and one in ActE) were modeled in the density map of *R. castenholzii* ACIII (Fig. 1C and fig. S4), apparently forming wires of the six hemes and the iron-sulfur clusters (Fig. 1D). The six c-type hemes exemplify identical positions and orientations as that in *R. marinus* and *F. johnsoniae* ACIII, but there are only one [3Fe-4S] cluster and one [4Fe-4S] cluster identified at deviated positions in *F. johnsoniae* ACIII (fig. S6A). The six hemes and four iron-sulfur clusters are all located within reasonable edge-to-edge
distances (less than 14 Å) to permit direct electron transfer along the wire.

Structural superimposition of the air-oxidized and dithionite-reduced ACIII showed a main-chain RMSD of 0.4 Å (Fig. 2A), indicating that dithionite reduction does not induce obvious conformational changes at the current resolution. However, the difference map of ACIII (the air-oxidized map minus dithionite-reduced map) showed major electron potential differences at the periplasmic subunits (ActA, ActE, and ActB) and the cytoplasmic side of the TM region of ActA, ActC, and ActD (Fig. 2A). The increased electron potentials were observed at the six heme groups as well as the four iron-sulfur clusters (Fig. 2A and movie S3), indicating that these electron carriers are essentially reduced after dithionite treatment, which is consistent with the increased heme spectral differences upon dithionite reduction (fig. S1D).

The heme electron transfer wire in ActA and ActE subunits
ActA (Gln\textsuperscript{9} - Arg\textsuperscript{226}) and ActE (Cys\textsuperscript{33} - Asn\textsuperscript{193}) were found to be pentaheme and mono-heme subunits, respectively, which form the main electron transfer wire of the photosynthetic ACIII. ActA is membrane-anchored, with a N-terminal TM helix (α1, Gln\textsuperscript{8} - Trp\textsuperscript{38}). Five c-type hemes were bound in the loop regions between its six α helices on the periplasmic side (Fig. 2B). The C-terminal mono-heme binding domain of ActE is composed of three α helices and two turns (Fig. 2B), and our model showed a lipid anchor that is present at the N terminus of ActE (fig. S4D). This observation suggested the possibility that the consensus lipobox sequence L/V-A/T-G/A-C (M\textsuperscript{30}TAC\textsuperscript{33}) (fig. S6B) in the actE gene sequence may be excised from the transcript or degraded following translation in cells or at some point before the final complex assembly. This phenomenon was also observed in the respiratory ACIII (17).

The six heme groups bound by ActA and ActE are each covalently attached via thioester linkages to cysteine residues of highly conserved heme binding motifs (C-X-X-C-H), and their iron ions are axially coordinated through bi-His or His-Met residue couplets (Fig. 2C and fig. S6B). The five hemes of the ActA subunit are arranged in alternating parallel (heme\textsubscript{2,5} and heme\textsubscript{3,4}) and perpendicular pairs (heme\textsubscript{2,3} and heme\textsubscript{4,5}) (Fig. 2C). In particular, the heme\textsubscript{3,4} pair adopts typical stacked motif in van der Waals contact (edge-to-edge distance, 4.8 Å), whereas heme\textsubscript{2,3} (5.1 Å) and heme\textsubscript{4,5} (4.5 Å) exemplify the T-shaped heme pairs (Figs. 1D and 2C). The spatial organization of heme\textsubscript{2} to heme\textsubscript{5} resembles that of the tetraheme in Shewanella oneidensis STC, in which the electron transfer between stacked heme pairs is approximately an order of magnitude greater than for the T-shaped heme pairs (25). But the electronic coupling of T-shaped heme pairs would be strongly enhanced by cysteine linkages inserted in the space between these pairs (26). The heme\textsubscript{1} is closest in terms of edge-to-edge distance to [3Fe-4S] (8.3 Å), and it is buried in a hydrophobic pocket formed by residues from ActB, ActC, ActD, and ActE (fig. S6C). The porphyrin ring of the mono-heme in ActE is inclined about 60° compared to that of heme\textsubscript{5} in ActA, with an edge-to-edge distance of 9.0 Å and a center-to-center distance of 16.7 Å (Figs. 1D and 2C).
The iron-sulfur clusters bound in the ActB subunit

The largest subunit, ActB (Gly^{77}-Glu^{1006}), was found to be composed of 26 α helices and 17 β strands that can be divided into two subdomains: the B1 domain (Gly^{77}-Phe^{714}) and B2 iron-sulfur binding domain (Leu^{715}-Glu^{1006}) (Fig. 2D). The N terminus of ActB was resolved from Gly^{77}, just behind the signal peptidase cleavage site A^{71}LA^{73}. The twin-arginine translocate signal peptide assists with the translocation of ActB to periplasm (27). Superimposition analysis of ActB with PsrA and PsrB subunits of polysulfide reductase (PsrABC), an integral membrane-bound enzyme that performs quinone-coupled reduction of polysulfide substrates (28), revealed that the B2 iron-sulfur binding domain is similar to PsrB and that both the folding and positions of the four iron-sulfur clusters match well between the two subunits (Fig. S7A). The analysis also revealed that the B1 domain of ActB forms a fold similar to the known substrate binding pocket of PsrA (fig. S9B), yet the absence of any cofactors in our model suggests that the function of ActB does not mirror the reduction activity of PsrA.

The four iron-sulfur clusters are covalently coordinated by conserved Cys residues (Fig. 2E and fig. S6D), with the largest edge-to-edge distance of 9.7 Å (Fig. 1D). The [3Fe-4S] is located at the interface with ActC and in the most proximity to the periplasmic side of the four-helix bundle that hosts the menaquinol binding pocket (Fig. 3A). This iron-sulfur cluster is the most probable primary electron acceptor from the menaquinol bound in the ActC subunit. The midpoint redox potential of [3Fe-4S] in R. marinus ACIII was determined to be +140 mV (11, 17), which is sufficient for an uphill electron transfer from menaquinol (−70 mV at pH 7) (29). The role of the three [4Fe-4S] clusters in both respiratory and photosynthetic ACIIIs is still unknown. The air-oxidized minus dithionite-reduced electron potential differences at the [3Fe-4S] and three [4Fe-4S] clusters indicate that these iron-sulfur clusters can be reduced upon dithionite treatment (Fig. 2A and movie S3). An edge-to-edge distance of 8.3 Å was observed between the [3Fe-4S] and heme_1 in the ActC subunit (Fig. 1D), which suggests that the electrons accepted by the [3Fe-4S] are most probably transferred along the heme wire to reduce a periplasmic electron carrier.

A menaquinol binding pocket located at the periplasmic side of ActC subunit

The ActC (Lys^{8}-Ala^{464}) and ActF (Gln^{4}-Ser^{399}) subunits each contain 10 TM helices. The middle eight helices are arranged into two four-helix bundles (TM2-5 and TM6-9 of ActC, and TM2’-5’ and TM6’-9’ of ActF), which were sandwiched by the intersection of TM1 (TM1’) and TM10 (TM10’) (Fig. 3A). The helix bundles of ActC and ActF resemble the structure of PsrC dimer (fig. S7C). Superimposition of the structures of ActC and PsrC gives a main-chain RMSD of 1.1 Å. The quinone binding pocket of PsrC, which is formed by the N-terminal four-helix bundle and located at the periplasmic side, was identified according to the structures complexed with MK-7, pentachlorophenol, and ubiquinone-1 (28).

No midpoint redox potential data are available for the six hemes and iron-sulfur clusters in R. castenholzii ACIII. The heme redox potentials of R. marinus ACIII was shown to range from −45 mV to +230 mV at neutral pH (11). Potentiometric titration of the c hemes in F. johnsoniae ACIII gives redox potentials at +331 mV and +439 mV (18). For C. aurantiacus ACIII, which shares 59% sequence identities with R. castenholzii ACIII, the heme midpot redox potentials were determined to be −228 mV, −110 mV, +94 mV, and +391 mV (8). With the highest redox potential at +391 mV (8), the monoheme of ActE is believed to be the final electron prosthetic group to accept the electrons transferred from the five hemes in ActA. Regarding the high sequence homology and functional similarity (9) of ActA and ActE with that of C. aurantiacus and respiratory ACIIIs from R. marinus and F. johnsoniae (fig. S6, A to C), as well as the spatial distribution of the six hemes (Fig. 2, A to C), electrons can be sequentially transferred along a wire that begins with the heme_1 in ActA and ends with the monoheme in the ActE subunit, and then eventually to the acceptor auracyanin (13, 24).
Although no menaquinol was found in the current structures, we observed an open cavity between the TM helices of ActA, ActD, and TM3/4 of ActC subunits, which is equivalent to the quinol binding pocket of PsrC (Fig. 3B and movie S4).

On the basis of structural analysis and comparison as well as sequence alignment (fig. S8), we identified a menaquinol binding pocket of ACIII at the periplasmic side of the first four-helix bundle in ActC, about 12 Å away from the [3Fe-4S] cluster (Fig. 3C). Adjacent to [3Fe-4S], a strictly conserved His141 residue replaces the Glu67 of PsrC quinol binding pocket (Fig. 3C and figs. S7D and S8), which is involved in proton transfer from the menaquinol (30). The side chains of Trp84, Ile88, Phe91, Pro138, and Leu168 further form a hydrophobic pocket that is capable of immobilizing the menaquinol head group (Fig. 3D). The two carbonyl oxygen atoms of the modeled menaquinol head are capable of forming hydrogen bonds with the imidazole group of His141 (2.8 Å) and the hydroxyl group of Asp171 (2.8 Å), which further forms hydrogen bonds with Asp252 (2.9 Å) (Fig. 3D). At the bottom of the pocket, Ile249 takes the position of Tyr130 in PsrC, which forms a hydrogen bond (2.6 Å) with the O1 carbonyl group of MK-7 (fig. S7D) (28). The menaquinol binding pocket of R. castenholzii ACIII shares high sequence homology and conformational similarity with that of R. marinus and F. johnsoniae ACIII (fig. S7, E and F), indicating that ACIIIIs play essentially similar enzymatic function in the photosynthesis and respiration.

**Putative proton translocation passages in the ActC and ActF subunits**

On the basis of the structural comparison with the respiratory ACIII, we further identified a putative proton translocation passage in the ActC subunit. The passage begins at the cytoplasmic residues Arg198 and Asp199 and proceeds to the TM region located primarily at the first four-helix bundle of the periplasmic region (Fig. 4, A and C). This passage is composed of 22 proton-carrying residues that provide side chains for hydrogen bonding with protons (Fig. 4A). The air-oxidized minus dithionite-reduced electron potential differences were mainly distributed at the cytoplasmic side of TM1, TM3, TM4, TM5, and TM10 of ActC (Figs. 2A and 4C), where the menaquinol binding pocket and proton translocation passage are absent. Furthermore, we did not observe obvious structural differences at the proton translocation passage between the air-oxidized and dithionite-reduced structures (Fig. 2A).

In the middle of the passage, three strictly conserved residues—Arg198, His246, and His99—form a hydrogen bonding network that links the menaquinol binding pocket and proton translocation passage (Fig. 4B). The imidazole group of His246 forms hydrogen bonds with the guanidine group of Arg198 (3.1 Å) and imidazole nitrogen of His99 (3.3 Å), which forms a weak hydrogen bond with the main chain of Ile95 (3.3 Å). The main-chain nitrogen of Ile95 is further hydrogen-bonded with the main-chain oxygen of Phe91 (3.1 Å), one of the key residues involved in menaquinol coordination. In close
proximity to His$^{246}$, Ile$^{248}$ forms a hydrogen bond with Asp$^{171}$ (3.0 Å), which is hydrogen-bonded with Asp$^{252}$ (2.9 Å) at the top of the menaquinol binding pocket (Fig. 4B).

Arg$^{394}$, His$^{346}$, and His$^{99}$ are strictly conserved in both the respiratory and photosynthetic ACIII (fig. S8). Superimposition analyses showed that the “triplet” residues adopt the same side-chain orientations and hydrogen bonding network as that from R. marinus and F. johnsoniae (Fig. 5, A and B), suggesting that these residues share a similar function in the respiratory and photosynthetic ACIII. Asp$^{394}$ is also conserved in other polysulfide, tetrathionate, nitrate, and dimethyl sulfoxide reductases (30). Mutation of Arg$^{394}$ in Wolinella succinogenes PsrC resulted in an inactive enzyme, which was suggested that it stabilizes the deprotonated quinol (30). Regarding the sequence conservation, location, and extensive hydrogen bonding interactions with the menaquinol binding pocket, the triplet residues are likely essential for coupling the menaquinol oxidation and proton translocation.

We observed a similar proton translocation passage in the ActF subunit formed by 20 less conserved amino acids (about 20% identities) from the cytoplasmic to periplasmic side (Fig. 5C and fig. S9). In the middle of the ActF passage, side chains of Glu$^{335}$, Ser$^{177}$, and Tyr$^{339}$ are capable of forming hydrogen bonding interactions, but no menaquinol-binding pocket and similar hydrogen bonding networks as that in ActC were found (Fig. 5C). In addition, Ser$^{177}$ and Tyr$^{339}$ are less conserved in both the photosynthetic and respiratory ActF, and Glu$^{335}$ is replaced by Arg in C. aurantiacus and His residue in the respiratory ActF (Fig. 5D and fig. S9). Minor electron potential differences were only observed at His$^{346}$ of ActC and Ser$^{244}$ of ActC (2.1 Å), which was close to the His$^{246}$ of ActC that would be essential for coupling the menaquinol oxidation and proton translocation (Fig. 4B). Thus, ActD might play a primary role in stabilizing the TM region of ACIII, which thereby contributes to a stable menaquinol binding pocket and proton translocation pathway.

DISCUSSION
As a functional counterpart of the bc$_1$ complex, ACIII plays a central role in both the photosynthetic and respiratory ETC of a wide range of prokaryotes. The discovery of these novel proton translocation pathways in ACIII opens up new possibilities for understanding the mechanism of proton translocation in these important biological systems.
range of bacterial taxa (6–10). It couples quinol oxidation with TM proton translocation to build up a TM proton gradient, which drives the formation of ATP required for bacterial growth. However, the nature of the coupling mechanism(s) for the respiratory and photosynthetic functions of ACIII has not been well discussed.

The photosynthetic bacterium *R. castenholzii* has evolved a simple but efficient cyclic ETC to transform solar energy into chemical energy that is different from the linear respiratory chain (31–33). Our study has revealed the structure of the first photosynthetic ACIII comprising six conserved subunits, in both the air-oxidized and dithionite-reduced states, as well as the nature and position of the cofactors, including six hemes and four iron-sulfur clusters. We also detected a menaquinol binding pocket positioned at the periplasmic side of the TM subunit ActC. This pocket is capable of immobilizing the menaquinol head group via strictly conserved residues (Fig. 3D), which is linked by extensive hydrogen bonding interactions with three proton-carrying residues in the middle of an apparent proton translocation passage. In addition, the ActD subunit is shown to coordinate extensive interactions with subunits ActA, ActB, ActC, and ActF.

Previous enzymatic analyses confirmed the activity of photosynthetic ACIII as a menaquinol:auracyanin or cyt c oxidoreductase (9). Recently, we revealed that *R. castenholzii* ACIII oxidizes menaquinol-4 or menaquinol-7 and transfers electrons to its periplasmic electron acceptor auracyanin (24). It has been revealed that there is a single quinol binding site in *R. marinus* ACIII by isothermal titration calorimetry experiments (17). The high sequence and structural similarity among photosynthetic and respiratory ACIIIs would also suggest a single menaquinol binding pocket of *R. castenholzii* ACIII. Within this pocket, menaquinol binds and is oxidized by the terminal electron acceptor auracyanin, releasing two protons into periplasm. Considering that menaquinone is reduced at the binding site of RC-LH complex (23), accepting two protons from cytoplasm, an apparent efficient quinone shuttling cycle is formed among RC-LH, the membrane quinone pool, and ACIII in the *R. castenholzii* simple cyclic photosynthetic ETC. As a result, with the reduction of one molecule menaquinone at RC-LH and the oxidation of one shuttled menaquinol at ACIII, two transferred electrons are accompanied with two protons transferred from cytoplasm to periplasm, yielding a $\text{H}^+ /e^-$ ratio of 2:2.

To date, no experimental data on the $\text{H}^+ /e^-$ stoichiometry for any ACIII were reported. Previous studies proposed that ACIII could also actively pump additional protons from cytoplasm into periplasm (10, 12, 14, 15), which would yield a different $\text{H}^+ /e^-$ stoichiometry deduced from above quinone shuttling cycle. However, the detailed mechanism of its active proton translocation has not been elucidated. The lack of any redox-active cofactors in the TM and cytoplasmic regions of ACIII argues against a Q-cycle type...
H⁺ pumping mechanism, such as is used in the cyt bc₁ and cyt b₅f complexes.

A hypothesis of the redox-coupled proton translocation mechanism

Structural comparison and analyses revealed two putative proton translocation passages in ActC and ActF, respectively, for both photosynthetic and respiratory ACIII (Fig. 5, A and C). The side chains of the middle-passage triplet residues Arg₃⁹⁴, His₂⁶⁴, and His₉⁹ of ActC adopt exactly the same conformation for all three ACIIIs (Fig. 5B). However, the proton-carrying residues in the passage of ActF are less conserved than that of ActC (Fig. 5, C and D). Notably, the respiratory ACIIIs from R. marinus (17) and F. johnsoniae (18) contain two conserved His and Asp residues in the middle passage of ActF, but these two residues are replaced by Glu and Tyr in the R. castenholzii ACIII (Fig. 5D and fig. S9). In addition, neither menaquinol binding pocket nor hydrogen bonding network was found in ActF. Less differences of electron potential around ActF between the air-oxidized and dithionite-reduced states (Fig. 2A) suggest that ActF is insensitive to the changes of redox potential. Therefore, most probably, ActF passage lacks a driving force for efficient TM proton translocation. If there exists a redox-coupled active proton translocation in ACIII, it would be mostly located in the ActC subunit and driven by the coupling between menaquinol oxidation and putative proton passage, without the necessary conformational change.

On the basis of the above structural analysis and discussion, we propose a redox-coupled proton translocation mechanism for the photosynthetic ACIII, which occurs within the subunit of ActC (Fig. 6). In the menaquinol binding pocket, at the close-to-neutral pH environment (pH ~6.5) of periplasmic space, both Asp¹⁷¹ and His¹⁴¹ are deprotonated and coordinate the bound menaquinol (MQH₂) by hydrogen bonds. The hydroxyl hydrogens of menaquinol can be bound by the hydroxyl oxygen of Asp¹⁷¹ and imidazole nitrogen of His¹⁴¹, respectively. Upon oxidation, the hydroxyl group of menaquinol that faces the side chain of Asp¹⁷¹ is first oxidized to form an intermediate semi-menadione. The released hydrogen protonates Asp¹⁷¹. Lacking the coordination by Asp¹⁷¹, the semi-menadione would be relocalized in the binding pocket and thus enable extraction of one proton from the proximal proton passage of ActC, resulting in one proton translocated from the cytoplasm. The binding of the extracted proton will induce a reorganized electronic structure of semi-menadione, releasing another hydroxyl hydrogen to protonate His¹⁴¹. The reorganized semi-menadione can be further coordinated by the hydroxyl group of Asp¹⁷¹. Then, the semi-menadione is further oxidized to form menadione (MQ) and release the exacted proton. After the release of menadione and the extracted proton from the menadione binding pocket, the two protons from oxidation of menadione are released to periplasmic space with the deprotonation of Asp¹⁷¹ and His¹⁴¹. During this proposed process, one instance of menadione oxidation is coupled to one proton pumped from the cytoplasm. As a result, three protons are released into the periplasm per two electrons transferred (Fig. 6).
The potential role of the [4Fe-4S] clusters
In both the respiratory and photosynthetic ACIII structures, a [3Fe-4S] cluster in the ActB subunit functions as the primary electron acceptor from menaquinol (17, 18), donating the electrons along the six-heme wire and finally onto the periplasmic electron acceptor. Both the photosynthetic ACIII from R. castenholzii and the respiratory ACIII from R. marinus contain additional three [4Fe-4S] clusters, while only one [4Fe-4S] cluster was identified in the F. johnsoniae ACIII (18). The function of [4Fe-4S] clusters remains largely unknown.

Our observation of the electron potential differences of these [4Fe-4S] clusters between air-oxidized and dithionite-reduced states indicates that these clusters are either accessible to dithionite or connected to the electron transfer wire. In Psr with the absence of heme groups, two electrons released from MK-7 are transferred via five [4Fe-4S] clusters to the bis-MGD (bis-molybdopterin guanine dinucleotide) cofactor and then reduce polysulfide (28). Unfortunately, no cofactors were observed in the B1 domain of ActB subunit (fig. S7B), indicating an electron transfer dead end in these [4Fe-4S] clusters. How they contribute to the electron transfer of ACIII needs to be further considered.

Both heme and iron-sulfur cluster are single electron carriers that are unable to transfer two electrons simultaneously. Thus, a sequential transfer of electrons upon menaquinol oxidation is necessary. In addition, the latency time between the formation of semimenaquinol and its further oxidation needs long enough to allow extraction of proton from the translocation passage, but it should not be too long to avoid the formation of reactive oxygen species. On the other side, the final periplasmic electron acceptor auracyanin can only accept one electron each time. Therefore, the speed of electron transfer in ACIII should be well controlled. The alternating T-shaped spatial organization of the six hemes in ACIII would limit it in one order the electron transfer efficiency of the heme wire, which would increase the steady time of semi-menaquinol. This limitation could be further compensated by the [4Fe-4S] clusters playing as an electron sink. Overall, the possible electron transfer during menaquinol oxidation would look like that, the first electron would quickly sink into the [4Fe-4S] clusters via [3Fe-4S] with the formation of semimenaquinol, and the second electron could then be transferred to the final periplasmic acceptor auracyanin via the heme wire; with a second auracyanin binding, the sinking electron in the [4Fe-4S] clusters could be further transferred to the final acceptor via the heme wire. As a result, the existence of the [4Fe-4S] clusters would be very important in assisting sequential and efficient transfer of two electrons with an intrinsic time interval.

In summary, our work provides a structural basis and conceptual insight into the coupling mechanism underlying menaquinol oxidation, electron transfer, and proton translocation for the photosynthetic ACIII, which seems likely to play the same role as a menaquinol: electron acceptor oxidoreductase in respiratory ACIIIs. Direct experimental will be required for definitive characterization the proton pumping mechanism of these ACIIIs.

MATERIALS AND METHODS
Extraction and purification of the photosynthetic ACIII from R. castenholzii
R. castenholzii DSM 13941 was grown in a batch culture anaerobically in modified PE medium at 50°C under high-light conditions for 10 days (19). Cells were harvested by centrifugation at 10,000g for 20 min, and the pellet was washed twice with 20 mM tris buffer (pH 7.4) and then stored at −40°C.

A suspension of whole membranes [with OD 880 (optical density at 880 nm) = 20 cm −1 ] in 20 mM tris-HCl (pH 8.0; buffer A) was treated with 1% β-octyl glucoside and stirred for 1 hour at room temperature in the dark. The extraction was centrifuged at 200,000g for 2 hours (Ti 70 rotor, 45,000 rpm) at 4°C. The pellets were resuspended in 50 mM sodium acetate (pH 5.0; buffer B) and treated with 0.5% β-dodecyl maltoside as above with 1% β-octyl glucoside. The supernatant from the second ultracentrifugation was collected and filtered through a 0.22-μm Millipore filter and subsequently loaded on a prepacked cation exchange chromatography column (SPHP5, GE Healthcare), which had been equilibrated with buffer B containing 0.04% β-dodecyl maltoside (which makes up buffer C). The column was extensively washed with 50 mM NaCl in buffer C until the eluent was colorless. Last, the crude ACIII was eluted from the column by a sodium gradient from 0.1 M NaCl to 0.4 M NaCl with 50 ml of buffer C at 2 ml min −1 . The collected fractions were concentrated and further purified by Superdex-200 gel filtration in buffer D [100 mM NaCl, 0.02% β-dodecyl maltoside, and 20 mM tris-HCl (pH 8.0)]. The fractions with an absorption ratio of A413/A280 higher than 1.38 were pooled and used for cryo-EM analysis.

The polypeptide composition of the purified complex was determined by SDS-PAGE and blue-native PAGE. The sample solubility was optimized by dissolving samples in buffer containing 5% 2-mercaptoethanol for 30 min at 65°C; these conditions yielded the sharpest protein bands. The identity of SDS-PAGE and blue-native PAGE bands was confirmed by PMF using matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry.

PMF analysis of ACIII through MALDI-TOF mass spectrometry
Stained bands from the SDS-PAGE were excised and destained with 50% acetonitrile in 50 mM aqueous NH4HCO3. Proteins were then reduced with 10 mM dithiothreitol in 100 mM NH4HCO3 for 30 min. Cysteine residues in the proteins were further alkylated by 55 mM iodoacetamide in 100 mM NH4HCO3 for an additional 30 min. Trypsin (Promega Trypsin Gold, TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone)–treated) in 50 mM NH4HCO3 was added to the gel pieces, and the enzymatic reaction proceeded overnight at 37°C. Afterward, peptides were extracted twice with 1% trifluoroacetic acid in 60% acetonitrile for 30 min. Extracted solutions were collected, dried completely in a speed-vac, and then redissolved in 50% acetonitrile containing 0.1% trifluoroacetic acid for mass spectrometry analysis.

The identities of proteins were determined by PMF using an ABI 4700 MALDI-TOF mass spectrometer. A mixture of the peptide sample and freshly prepared matrix solution (10 mg ml −1 α-cyano-4-hydroxycinnamic acid in 50% acetonitrile) was spotted on a stainless-steel target plate. Peptide mass value searches were performed against the National Center for Biotechnology Information (NCBI) database using Mascot software (www.matrixscience.com). The alkylation of cysteine was included as a possible modification. The mass tolerance for the monoisotopic peptide mass was set to ±0.6 Da.

Cryo–electron microscopy
Three-microliter aliquots of air-oxidized ACIII (4 mg ml −1) was placed on the glow-discharged G1 R1.2/1.3 300-mesh gold holey carbon grid (Jiangsu Lantuo Biotechnology, China) and blotted for

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3.0 s under a blot force of 1 at 100% humidity and 16°C before being flash-frozen in liquid ethane with a Mark IV Vitrobot system (FEI). Micrographs were acquired on a Titan Krios microscope (FEI) operated at 300 kV with a K2 Summit direct electron detector (Gatan). SerialEM (34) was used for automatic data collection. A nominal magnification of ×22,500 was used for imaging, which yielded a pixel size of 1.04 Å. The defocus range was between 1.2 and 3.3 μm. Each micrograph was dose-fractionated to 32 frames under a dose rate of 9.2 e⁻/Å² per second and an exposure time of 6.4 s, which resulted in a total dose of about 59 e⁻/Å².

For the sodium dithionite–reduced ACIII, 3-μl aliquots of a sample (4.5 mg ml⁻¹) were placed on the glow-discharged CryoMatrix R1.2/1.3 300-mesh amorphous alloy film (product no. M024-Au300-R12/13, Zhenjiang Lehua Technology Co. Ltd., China) and blotted for 3 s under a blot force of 0 at 100% humidity and 16°C before being flash-frozen in liquid ethane with a Mark IV Vitrobot system (FEI). Micrographs were acquired on a Titan Krios microscope (FEI) operated at 300 kV with a K2 Summit direct electron detector (Gatan). SerialEM was used for automatic data collection. A nominal magnification of ×22,500 was used for imaging, which yielded a pixel size of 1.04 Å. The defocus range was between 1.5 and 2.5 μm. Each micrograph was dose-fractionated to 32 frames under a dose rate of 9.4 e⁻/Å² per second and an exposure time of 6.4 s, which resulted in a total dose of about 60 e⁻/Å².

**Image processing**
Motion correction and exposure weighting was performed by the MotionCorr2 program (35), and the CTF (contrast transfer function) parameter was estimated using the Gctf program (36). The automatic particle picking was performed by Gautomatch (developed by K. Zhang, MRC Laboratory of Molecular Biology, Cambridge, UK) and Auto-picking module in RELION; an initial model was made by e2initial model.py in EMAN2 software package (37), and all other steps were performed using RELION (38). For the air-oxidized ACIII dataset, 600 particles were manually picked and extracted for two-dimensional (2D) classification. The resulting 2D class averages were used as the templates for the automated particle picking, which yielded 257,815 particles from 1700 micrographs. The picked particles were extracted at 2 × 2 binning and subjected to three rounds of 2D classification. A total of 197,496 particles were finally selected for 3D classification.

Good 2D class averages in different orientations were selected to generate the initial model. A total of 177,489 particles were left after two rounds of 3D classification and re-extracted into the original pixel size of 1.04 Å. The following 3D refinement and postprocessing yielded an EM map with a resolution of 3.45 Å. At performing CTF refinement in RELION3, the resolution was increased to 3.24 Å. Reported resolutions were estimated with a soft-edge mask around the protein complex and micelle densities and based on the gold-standard FSC (Fourier Shell Correlation) = 0.143 criterion. Local resolution was estimated with Resmap (39).

For the reduced ACIII dataset, 1970 unscreened micrographs were subjected to 3D reference–based auto-picking in RELION3; reconstruction of the ACIII dataset was the 3D reference low-pass–filtered to 20 Å. The resulting 488,581 particles were used to extract particles at 2 × 2 binning. After two rounds of 2D classification, 297,122 particles were selected for a 3D refinement and alignment-free 3D classification, and 219,913 particles from the best 3D class were re-extracted without downsampling. The following 3D refinement and postprocessing yielded an EM map with a resolution of 3.68 Å. CTF refinement and another alignment-free 3D classification improved the resolution to 3.51 and 3.46 Å, respectively. The final subset had 207,633 particles.

**Model building, refinement, and validation**
De novo atomic model building was conducted in Coot (40). Sequence assignments were guided by residues with bulky side chains. The starting models of the cofactors were taken from the CCP4 ligand library. The model was real space–refined by PHENIX (41, 42) with intra-cofactor and protein-cofactor geometric constraints. The refinement and model statistics are listed in Table 1. All figures were prepared in PyMOL (www.pymol.org) or UCSF Chimera (43).

**Calculation of the electron potential difference map**
The difference map between air-oxidized and dithionite-reduced ACIII was calculated using EMAN2 (37). First, the cryo-EM map of dithionite-reduced ACIII was fitted to that of air-oxidized ACIII by Chimera and then was clipped into the same box size using “e2proc3d.py” in EMAN2. Then, the structural amplitudes of both maps were scaled using e2proc3d.py in EMAN2. Last, the difference map between the corrected maps was computed by the “e2.py” python tool in EMAN2 and further low-pass–filtered at a quarter of the Nyquist criterion.

**SUPPLEMENTARY MATERIALS**
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/31/eaba2739/DC1

View/request a protocol for this paper from Bio-protocol.

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Cryo-EM structures of the air-oxidized and dithionite-reduced photosynthetic alternative complex III from *Roseiflexus castenholzii*

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