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 bc6f) 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 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 pheophytin-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 shuttling 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 at 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 [594.0x756.0] and the periplasmic region of ActC, ActD, and ActB forms extensive contacts with ActE, the penta-heme binding subunits ActA, ActB, and ActE.

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

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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^{9}-Arg^{226}) and ActE (Cys^{33}-Asn^{193}) were found to be penta-heme and mono-heme subunits, respectively, which form the main electron transfer wire of the photosynthetic ACIII. ActA is membrane-anchored, with an N-terminal TM helix (α1, Gln^{8}-Trp^{13}). 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^{30}TAC^{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_2,5 and heme_3,4) and perpendicular pairs (heme_2,3 and heme_4,5) (Fig. 2C). In particular, the heme_3,4 pair adopts typical stacked motif in van der Waals contact (edge-to-edge distance, 4.8 Å), whereas heme_2,3 (5.1 Å) and heme_4,5 (4.5 Å) exemplify the T-shaped heme pairs (Figs. 1D and 2C). The spatial organization of heme_2 to heme_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_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_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).
Fig. 2. Heme and iron-sulfur clusters in ActA and ActE subunits. (A) The air-oxidized minus dithionite-reduced electron potential difference map (orange) of the ActCIII is shown from the front (left) and back (right) view. The structures of the air-oxidized and dithionite-reduced ActCIII (white) are superimposed, with the iron-sulfur clusters and heme groups shown in sphere and stick models. The color code for each subunit and cofactors of the air-oxidized ActCIII is the same as that in Fig. 1. (B) Ribbon representation of the ActA and ActE subunits bound with pentaheme and monoheme groups (red sticks). The N and C termini of the protein are highlighted with a black dot and labeled. (C) Spatial organization and immobilization of the pentaheme and monoheme groups in ActA and ActE subunits. The residues that axially coordinate the heme iron ions are shown as sticks and labeled; the center-to-center distances of the hemes are shown and labeled. (D) Overall structure of ActB subunit. The B1 and B2 domains are colored in blue and magenta, respectively. The iron-sulfur clusters are shown as spheres. (E) Coordination of the iron-sulfur clusters in the ActB subunit. The conserved cysteine residues that coordinate the iron-sulfur clusters are shown as sticks and labeled, and the B2 domain is shown as a ribbon with 80% transparency.

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 midpoint 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 ACIII 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).

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−L_73. The twin-arginine translocase 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 subunit 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] clusters 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_64) 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).
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 ACIIIs 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—Arg294, 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 Arg294 (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\textsuperscript{246}, Ile\textsuperscript{248} forms a hydrogen bond with Asp\textsuperscript{171}, which is hydrogen-bonded with Asp\textsuperscript{252} at the top of the menaquinol binding pocket (Fig. 4B).

Arg\textsuperscript{394}, His\textsuperscript{171}, and His\textsuperscript{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 \textit{R. marinus} and \textit{F. johnsoniae} (Fig. 5, A and B), suggesting that these residues share a similar function in the respiratory and photosynthetic ACIII. Asp\textsuperscript{394} is also conserved in other polysulfide, tetrathionate, nitrate, and dimethyl sulfoxide reductases (30). Mutation of Arg\textsuperscript{394} in \textit{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\textsuperscript{335}, Ser\textsuperscript{217}, and Tyr\textsuperscript{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\textsuperscript{217} and Tyr\textsuperscript{339} are less conserved in both the photosynthetic and respiratory ActF, and Glu\textsuperscript{335} is replaced by Arg in \textit{C. aurantiacus} and His residue in the respiratory ActF (Fig. 5D and fig. S9). Minor electron potential differences were only observed at His\textsuperscript{246}, Ala\textsuperscript{188}, and Met\textsuperscript{285} of the ActF subunit, suggesting that this subunit is not sensitive to the air-oxidized and dithionite-reduced state of ACIII. To be noted, a conserved residue Tyr\textsuperscript{264F} forms a hydrogen bond with the main-chain oxygen of Pro\textsuperscript{267C}, which is close to the periplasmic portion of the proton translocation passage in ActC (Fig. 4B). The distinct conservation of these proton translocation passages indicates that the ActC subunit plays a consensus important role in both the respiratory and photosynthetic ACIII.

**Extensive interactions between ActD and other subunits**

The function of the ActD subunit in the ACIII remains obscure. We observed hydrogen bonding interactions between Asn\textsuperscript{100} of ActD and Tyr\textsuperscript{755} of ActB, as well as between Leu\textsuperscript{106} of ActD and Tyr\textsuperscript{753} of ActB (fig. S10A). We also observed extensive hydrophobic interactions between residues located in the ActD loop and subunits ActB, ActF, and ActC. These interactions can stabilize the conformation of TM5, which contributes to the menaquinol binding pocket (fig. S10A). Near the menaquinol binding pocket, a hydrogen bond is formed between the hydroxyl groups of Glu\textsuperscript{118} of ActD and Ser\textsuperscript{244} of ActC (2.1 Å), which was close to the His\textsuperscript{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 passage.

**DISCUSSION**

As a functional counterpart of the bc\textsubscript{1} complex, ACIII plays a central role in both the photosynthetic and respiratory ETC of a wide
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 $H^+/e^-$ ratio of 2:2.

To date, no experimental data on the $H^+/e^-$ 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 $H^+/e^-$ 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 \(bc\)_\(_1\) and cyt \(b\)_\(_6\)f 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 Arg\(^{394}\), His\(^{264}\), and His\(^{99}\) 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 Asp\(^{171}\) and His\(^{141}\) are deprotonated and coordinate the bound menaquinol (MQH\(_2\)) by hydrogen bonds. The hydroxyl hydrogens of menaquinol can be bound by the hydroxyl oxygen of Asp\(^{171}\) and imidazole nitrogen of His\(^{141}\), respectively. Upon oxidation, the hydroxy group of menaquinol that faces the side chain of Asp\(^{171}\) is first oxidized to form an intermediate semi-menaquinol. The released hydrogen protonates Asp\(^{171}\). Lacking the coordination by Asp\(^{171}\), the semi-menaquinol would be relocated in the binding pocket and thus enable extraction of one proton from the proximal proton passage of ActC, resulting in the two protons translocated from the cytoplasm. During this proposed process, one instance of menaquinol 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 semi-menaquinol, 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 anaerobi-cally 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 OD680 (optical density at 880 nm) = 20 cm−1] in 20 mM tris-HCl 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 ressuspended 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 MilliQ 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 A270/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 spectroscopy

Stained bands from the SDS-PAGE were excised and destained and washed 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 GIG R1.2/1.3 300-mesh gold holey carbon grid (Jiangsu Lantuo Biotechnology, China) and blotted for

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

**REFERENCES AND NOTES**


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