Cryo-EM studies of the rotary H\textsuperscript{+}-ATPase/synthase from *Thermus thermophilus*

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Proton-translocating rotary ATPases couple proton influx across the membrane domain and ATP hydrolysis/synthesis in the soluble domain through rotation of the central rotor axis against the surrounding peripheral stator apparatus. It is a significant challenge to determine the structure of rotary ATPases due to their intrinsic conformational heterogeneity and instability. Recent progress of single particle analysis of protein complexes using cryogenic electron microscopy (cryo-EM) has enabled the determination of whole rotary ATPase structures and made it possible to classify different rotational states of the enzymes at a near atomic resolution. Three cryo-EM maps corresponding to different rotational states of the V/A type H\textsuperscript{+}-rotary ATPase from a bacterium *Thermus thermophilus* provide insights into the rotation of the whole complex, which allow us to determine the movement of each subunit during rotation. In addition, this review describes methodological developments to determine higher resolution cryo-EM structures, such as specimen preparation, to improve the image contrast of membrane proteins.

**Key words:** V-ATPase, rotary motor, ATP synthase

ATP produced by ATP synthases is a key molecule for the metabolism of every living organism. ATP synthases belong to a family of enzymes known as rotary ATP synthases and are categorized as F- and V-type ATPases (Fig. 1A, B). They share many structural and mechanistic features, such as mechanochemical coupling of ion translocation and ATP hydrolysis/synthesis through rotation [1–6]. Reactions mediated by rotary ATPases are reversible, and consequently can function as molecular motors to synthesize or hydrolyze ATP depending on the physiological conditions [7–9].

Some archaea and eubacteria contain an ATPase acting as a proton motive force-dependent ATP synthase [10,11]. Functional features and their general structures resemble F-type ATPases, whereas primary sequences of archaeal ATPases are closely related to eukaryotic V-type ATPases, which work as proton pumps depending on ATP hydrolysis in subcellular vesicles (Fig. 1C) [2,12]. The archaeal type ATPases are sometimes referred to as A-ATPases or V/A type ATPases [5,6,13], and these are regarded as an evolutionary origin of eukaryotic V-ATPases [14]. The V/A-ATPase from a thermophilic eubacterium *Thermus thermophilus* (Tth) is one of the best characterized rotary ATPases. The subunit composition of the Tth V/A-ATPase is similar to that of the eukaryotic enzyme; however, it has a simpler subunit structure and its physiological role is ATP synthesis \textit{in vivo}, using energy from an electrochemical

ATP hydrolysis/synthesis in the soluble domain of proton-translocating rotary ATPases is coupled with proton flux across the membrane domain via a rotation of the common central rotor complex against the surrounding peripheral stator apparatus. Structures of the complete V/A type H\textsuperscript{+}-rotary ATPase have been revealed at a near atomic resolution level using cryogenic electron microscopy (cryo-EM). In this review article, we will describe the information about specimen preparation to improve the image quality and cryo-EM single particle analysis to overcome the intrinsic conformational heterogeneity of the Tth V/A-ATPases.
On the other hand, V-type ATPases were first purified from vacuoles, and work as proton pumps dependent on ATP hydrolysis. Colored rotor complexes of B. PS3 (γε10), T. thermophilus (D1F1d1c12) and the yeast (D1F1d1c7c1) rotate relative to the surrounding stator apparatus α3β3γεδa1, A3B3E2G2a1, and A3B3C1E3G3H1a1, respectively.

Figure 1 Subunit arrangement in rotary ATPases. Schematic representations of bacterial F-type ATP synthase from Bacillus PS3 (A), V/A-type ATP synthase from Thermus thermophilus (B), and the yeast V-ATPase (C). F-type ATPases are found in the inner mitochondria membrane, in the thylakoid membrane of chloroplasts and in the bacterial plasma membrane, where they use proton gradient across the membrane to synthesize ATP. On the other hand, V-type ATPases were first purified from vacuoles, and work as proton pumps dependent on ATP hydrolysis. Colored rotor complexes of B. PS3 (γε10), T. thermophilus (D1F1d1c12) and the yeast (D1F1d1c7c1) rotate relative to the surrounding stator apparatus α3β3γεδa1, A3B3E2G2a1, and A3B3C1E3G3H1a1, respectively.

Sample preparation of Tth V/A-ATPase for single particle analysis

A key for Tth V/A-ATPases purification processes is the optimization of the solubilized conditions to generate mono-dispersed particles in solution. Membrane proteins are handled in solutions containing detergent above the critical micelle concentration (CMC) for their solubilization. However, the sample solution of detergent-solubilized membrane proteins contains detergent micelles and monomers, which increases the thickness of the amorphous ice by contributing a layered detergent at air-water interface of the specimen, resulting in decrease of the image contrast (Fig. 2A) [34,35]. To overcome this problem, lauryl maltose-neopentyl glycol (LMNG), a member of the maltose-neopentyl glycol class of amphipols, was used as a detergent to solubilize the ATPase [36]. LMNG and membrane proteins form a stable detergent-protein complex at a concentration even lower than CMC due to an extremely low off rate of LMNG from the membrane protein [37]. The removal of free detergent monomers and micelles from LMNG-solubilized samples improves the image contrast dramatically and enables acquisition of high-resolution data [27].
Another point for the cryo-grid preparation is that detergent-solubilized proteins tend to be adsorbed onto the surface of the carbon grid due to extensive glow discharged carbon surface. As a result, membrane protein particles are rarely found on the unsupported ice hole (Fig. 2B) [34,35]. This impels us to utilize membrane protein solutions at much higher concentrations than soluble proteins for the preparation of holey carbon cryo-grid. However, Tth V/A-ATPase particles were aggregated at high protein concentration on the unsupported ice hole. Conversely, a supporting carbon film has been used for cryo-grid preparation of ribosomes or membrane proteins [38,39]. The supporting carbon is capable of reducing charge-induced sample movement and provide an extra physical support for particle adsorption (Fig. 2C). When using the supported carbon cryo-grid, membrane protein particles appear to be concentrated on the surface of the carbon film due to extensive glow discharged carbon surface. As a result, membrane protein particles are rarely found on the unsupported ice hole (Fig. 2B) [34,35].

The supporting carbon film has been used for cryo-grid preparation of ribosomes or membrane proteins [38,39]. The supporting carbon is capable of reducing charge-induced sample movement and provide an extra physical support for particle adsorption (Fig. 2C). When using the supported carbon cryo-grid, membrane protein particles appear to be concentrated on the surface of the carbon film, which enables to obtain many particle images at even low membrane protein concentration. For the Tth V/A-ATPase, the monodispersed particles were clearly observed on the supporting carbon film [27]. Nonetheless, this system contributes substantially to increasing background signal [40]. Recently, other materials have been attested as supporting films. For instance, graphene is an excellent support material because it is a one-atom thick (0.34 nm) hexagonal lattice of carbon atoms with high electronic conductivity properties [41,42]. We suppose that it may be possible to improve the resolution of the map of the Tth V/A-ATPase by using graphene oxide instead of carbon film.

As mentioned above, a number of methods have been developed to overcome obstacles to determine high-resolution membrane protein structures. Specimen preparation of membrane protein complexes for cryo-EM still is a key process to determine their high resolution cryo-EM structures.

**Single particle analysis of V/A-ATPases**

Recent breakthroughs in structural studies using cryo-EM by improvements in the detector and image processing methods allowed for determination of the whole structure of rotary ATPases [13,27–33]. In 2015, the different rotational states of intact rotary ATPases were first observed in both the yeast V-ATPase and F-type ATP synthase, at 6–10 Å resolution, which has enabled a more detailed understanding of the molecular mechanism of these rotary motor proteins [28,29,33]. The resulting high-quality projection images of the ATPases made it possible to distinguish multiple conformational states during 3D classification [28,29]. However, resolving a high-resolution structure of the rotary ATPase has been extremely challenging because of their intrinsic conformational heterogeneity and instability.

Recently, our group determined the three rotational state structures of Tth V/A-ATPase, based on the orientation of the central rotor subunit (Fig. 3A) [27]. The position of the central rotor subunits was different for each state, that was closely matched 120° steps which occurred during the ATP hydrolysis of V1. The EM structure corresponding to state 1 composed of major class gave a 5 Å resolution map, which is higher than previous reports for the V/A-ATPase structure at that time. This cryo-EM density map provided evidence for integrity of the V/A-ATPase against internal rotation of rotor part relative to stator. Close interactions between EG-subunits and B-subunits in V1 are identified, which appear to form rigid β sheet structures (Fig. 3B, C). This interaction is consistent with our results of previous reconstitution assay, showing that the A3B3 of the V/A-ATPase is tightly associated with the two peripheral stalks [43]. Additionally, interface structures of the central rotor subunits indicated the specific interactions among the rotor subunits in the complex (Fig. 3D). The interaction surface was identified between the short helix region of the D-subunit and the d-subunit cavity, suggesting an important interaction for appropriate fitting in our previous studies [44]. Tight electrostatic interactions between the two inner helices of the c12 ring and the N-terminal helix of the d-subunit are also clearly observed (Fig. 3E). We suppose that the robustness of the rotor complex during rotation relative to the stator is maintained by the interaction between the d- and the c12 ring in the Tth V/A-ATPase.
The three different structures provided insights into the rotation of the whole complex, which allowed to estimate the movement of each subunit during the rotation. The remarkably different conformation of the EG stalk region was observed in the three states, though superposition of the β barrel domain of the B-subunit attached to the peripheral stalk revealed no apparent difference in the globular region. A circular motion of the peripheral stalks is indicated among the three rotational states on the pivot point of the coiled-coil domain of the stalk regions in each state (Fig. 3F). With the circular movement of the EG stalk region, the N-terminal region of the α-subunit moves up-and-down at the linkage to the C-terminal region (Fig. 3G). The conformational changes in the stator subunits in the three rotational states is visible in Supplementary Movie 1 of our article [27]. This movement of the stator subunits in *Tth* V/A-ATPase is modestly com-

**Figure 3** Three rotational states of *Tth* V/A-ATPase. Cryo-EM maps of three different conformational states fitting atomic models of the subunits from *Tth* V/A-ATPase, respectively (A). A schematic model of *Tth* V/A-ATPase is shown on the right. Colors of the subunits displayed in the schematic model correspond to the colors of the subunits in atomic models fitted into the maps of *Tth* V/A-ATPase. Scale bar, 50 Å. Magnified views of interface structures between B- and E-subunit (B, C), d- and D-subunit (D) and d- and c12 ring (E) in state 1 are indicated respectively. The positions of glutamate residue on the D-subunit (E74) and the histidine residue on the d-subunit (H94) are indicated by sphere format (D). The positions of the glutamate on the c-subunit (E47) and the arginine on the d-subunit (R11, R13) are shown in sphere format (E). The N-terminal helix of the d-subunit is indicated by an arrow in E. Scale bar, 5 Å. The viewing positions of B-E are indicated as squares in A. Circular motions of EG subunits during transition of three rotational states (F). Bottom views of the N-terminal region of the EG-subunits are indicated when three states structures are superimposed at the N-terminal β barrel domain of B-subunit. Colors corresponding to the state1, state2 and state3 are red, blue and green, respectively. Circular motion of EG subunits are indicated by arrows. Scale bar, 30 Å. Comparison of the N-terminal domain of the α-subunit in three states when subunits are superimposed at the C-terminal domain of the α-subunit (G). The color of each state corresponds to F. Up-and-down motion of the N-terminal domain of α-subunit in three states is indicated by an arrowhead. Scale bar, 20 Å. Model IDs corresponding to the rotational states of *Tth* V/A-ATPase are 5Y5X and EMDB-6810 for state 1, 5Y5Z and EMDB-6812 for state 2, and 5Y60 and EMDB-6813 for state 3.
ing to the model, the α-subunit provides entry and exit of protons that protonate glutamate residues on the c₁₂ ring in contact with aqueous half channels (Fig. 4A). The EM structure of the membrane-embedded region of the V/A-ATPase provided a higher resolution structure of α₁c₁₂ region than previous intact rotary ATPase structures at that time, and enabled the main chains of the α-subunit and the c₁₂ ring to fit well into the EM map [27]. The two highly tilted α helices of the α-subunit and the adjacent four outer α helices of c₁₂ ring were clearly identified in the model. The highly conserved arginine residue (R563) was positioned in the central region of the transmembrane helix 7 of the α-subunit (α-TM7), where the α-subunit was in close proximity to the outer helices of c-subunit (Fig. 4B). Homology mapping suggested that the conserved R563 on the α-subunit was positioned close to the conserved E63 on the c-subunit (Fig. 4B), which are necessary for proton pumping activity in yeast [47,48]. The cryo-EM map of state 1 with solvent density showed that the detergent shell was recessed at each side of the membrane to form two aqueous cavities (Fig. 4C). The cytoplasmic aqueous cavity provides direct access to the hydrophilic residues on the α-TM7, reaching the conserved Arg-Glu residue pair at the interface of the α-TM7 and the outer helix of c-subunit (Fig. 4B), indicating that the cavity corresponds to the cytoplasmic half channel. The peri-

**Figure 4** Structure of the membrane embedded domain of *Tth* V/A-ATPase. Two-channel model of proton translocation of *Tth* V/A-ATPase (A). Proton translocation occurs through the periplasmic and cytoplasmic pores shown by dotted lines. A part of the α-subunit colored in transparent magenta is indicated in (A), showing the important two transmembrane helices (α-TM7, α-TM8). Density map colored semi-transparent gray is fitted atomic model of state 1 (PDB ID: 5Y5X) with the detergent shell surrounding the Vₒ (B). A top view of contact surface between the c₁₂ ring and the α-subunit at cytoplasmic pore is shown in right. Spheres representing the essential residues of the arginine on the transmembrane helix 7 of the α-subunit and the glutamate residues on the c₁₂ ring are colored yellow and green, respectively. Scale bar, 20 Å. Close-up views from the cytoplasmic (above) and periplasmic (below) side (C). Density map colored semi-transparent gray shows large cavities outlined in black. Spheres showing the essential residues of the arginine on the transmembrane helix 7 of the α-subunit and the glutamate residues on the c₁₂ ring are colored yellow and green, respectively. Scale bar, 10 Å.
plasmic cavity faces the helix bundle composed of a-TM1, a-TM2, and a-TM3, including a line of hydrophilic residues, and connects to the aqueous hole giving access to the tilted helices.

**Perspective**

Future work will be focused on obtaining a higher resolution cryo-EM map of *Tth* V/A-ATPase to reveal the mechanism by which proton translocation across the V1 channel powers the rotation of the rotor ring. Therefore, clearer EM images of V/A-ATPase single particles are necessary to obtain higher resolution structures, which will enable us to determine the accurate orientation of water molecules in the V1 channels. As mentioned above, methodologies for both sample and cryo-grid preparation of membrane proteins have been developed by several groups. A nanodisc is a powerful tool for the removal of detergents which tend to decrease proton translocation [17].

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**Conflicts of interest**

A. N., J. K., K. M., and K. Y declare that they have no conflict of interest.

**Author Contribution**

A. N., J. K., K. M., and K. Y wrote the paper.

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