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Review Article

Operating principles of rotary molecular motors: differences between F_1 and V_1 motors

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Among the many types of bioenergy-transducing machineries, F- and V-ATPases are unique bio- and nanomolecular rotary motors. The rotational catalysis of F_1-ATPase has been investigated in detail, and molecular mechanisms have been proposed based on the crystal structures of the complex and on extensive single-molecule rotational observations. Recently, we obtained crystal structures of bacterial V_1-ATPase (A_3B_3 and A_3B_3DF complexes) in the presence and absence of nucleotides. Based on these new structures, we present a novel model for the rotational catalysis mechanism of V_1-ATPase, which is different from that of F_1-ATPases.

Key words: V-ATPase, F-ATPase, ATP, Crystal structure, molecular mechanism

Several mechanochemical energy transducers or molecular motors are present in living cells. These include linear motors such as myosins that function in muscle contraction and kinesins/dyneins that utilize the energy from ATP hydrolysis for vesicle transport. Molecular rotary motors, which include F-ATPases and V-ATPases, are another class of motors. This class utilizes the energy from ATP hydrolysis and the ionic current for axis rotation; flagellar motors also utilize ionic currents for rotation. F- and V-ATPases resemble one another and consist of a hydrophilic rotary motor region (F_1 and V_1) and a hydrophobic membrane embedded region (F_o and V_o). Rotation of the motor axis is coupled to ATP hydrolysis, whereas rotation of the embedded region drives proton transport across membranes. In this review, we focus on the rotary motor regions (F_1 and V_1). Although numerous structural and biophysical studies have been conducted, the molecular rotational mechanism of the F_1 motor is not fully understood. We also introduce our recent achievements in the three-dimensional structural analysis of the V_1 motor and discuss the unresolved questions related to the molecular mechanism of F_1 motors. This review is an extension of a previous Japanese review by Murata (2014) [1], which discusses recent progress in determining the rotation mechanism of mammalian F_1 motors [2,3] in addition.

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Studies on F_1 motors

F-ATPases (or ATP synthases) are found in the inner membranes of mitochondria, thylakoid membranes of chloroplasts, and cytoplasmic membranes of bacteria. ATP is synthesized by the F_1 part of F-ATPase, which utilizes rotational energy generated from the F_o part, which itself is driven by electro-chemical proton gradients across membranes (Fig. 1a). In the absence of an electro-chemical proton gradient and by utilizing the chemical energy derived from ATP hydrolysis, F-ATPases also function reversibly as proton-pumping motors. The F_1 part is also called F_1-ATPase, as the purified motor alone works as a rotary motor when it exhibits high ATP hydrolysis activity. It has been

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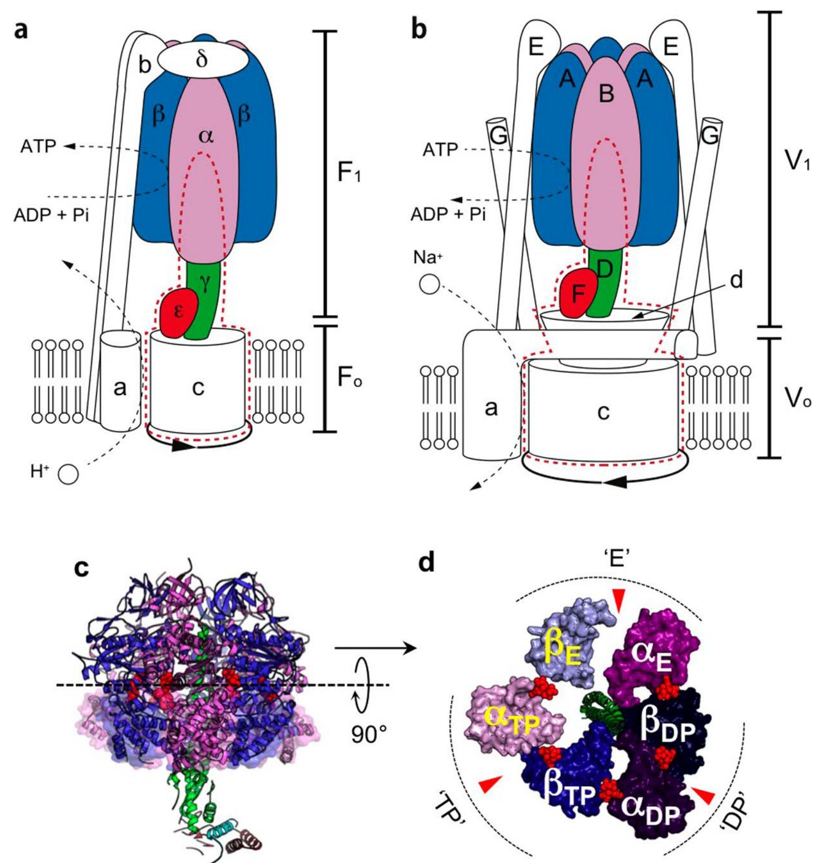

Figure 1 (a) Structural model of the F-ATPase; (b) structural model of the V-ATPase; (c, d) crystal structures of the F_1 motor. (c) Side view of the F_1 motor. The C-terminal domain is shown as a transparent surface representation. The AMP-PNP molecules, bound in two catalytic and three non-catalytic sites, are shown as red spheres. d) Top view of the F_1 motor generated by 90° rotation around the axis (dotted line) of (c). Red arrows show the locations of the catalytic sites.

recognized that the three catalytic sites of this ATPase work cooperatively to hydrolyze ATP; however, the rotational movement of its rotary motor was the subject of long-term intensive study. Based on careful experimentation using [^{18}O]phosphate isotopes, Boyer [4] proposed a new model in 1980, suggesting that each of the three catalytic sites of the ATPase alternates its hydrolyzing activities and drives rotational movements. At the time, it seemed implausible that such a small protein could rotate and function as a rotary motor. However, in 1994, Abrahams *et al.* [5] determined the crystal structure of the bovine mitochondrial F_1-ATPase, demonstrating the plausibility of the rotational catalysis model. The structure of the F_1 motor showed an $\alpha_3\beta_3$ ring structure containing three non-catalytic subunits (α) and three catalytic subunits (β), alternately arranged around a central axis γ subunit. Furthermore, the three catalytic sites showed asymmetrical structures with ATP bound (TP form), ADP bound (DP form), and empty (E form) (Fig. 1c, d). Three years later, using a fluorescently labeled actin filament attached to the γ-axis of a thermophilic bacterial F_1 motor, Noji *et al*. [6] directly visualized the ATP-driven rotation of the γ-axis by fluorescence microscopy. For their work, Boyer and Walker became Nobel Laureates of Chemistry in 1997.

During this time, the leading research on the molecular mechanism of the F_1 motor was conducted by crystallography research groups in England and single-molecule observation research groups in Japan. Crystallographic studies revealed various F_1-ATPase structures in complex with various nucleotides and inhibitors [7–11]. The use of various newly developed single molecule techniques revealed detailed molecular and rotational characteristics of ATP synthesis and hydrolysis reactions of the F_1 motor [12–15]. However, the rotation mechanism models, generated based on two research approaches, differed in several aspects [11,14,16]. Recently, a model was proposed that may explain both the structural and single-molecule data obtained from mammalian F_1-ATPase [2,3]. In this model, the central axis rotates by 120° per ATP molecule and has three intermediate states: a state "waiting for ATP binding" at 0° (and 120°), a state "waiting for Pi release" at 65°, and a state "waiting for ATP hydrolysis" at 90° [2]. Crystal structures corresponding to the states "waiting for Pi release" and "waiting for ATP hydrolysis" were obtained, which suggested how Pi is released in the rotary substeps [3]. How-

ever, the crystal structure corresponding to the state "waiting for ATP binding" of this model of F_1-ATPases has not yet been obtained.

High-speed atomic force microscopy was developed by Ando *et al.*, enabling real-time single-molecular imaging. This technique was used to reveal that the thermophilic F_1 motor without the γ-axis ($\alpha_3\beta_3$) rotates and changes its conformation in one decisive orientation and follows the correct order of ADP and Pi release after ATP hydrolysis [17]. Based on these observations, the unidirectional rotation of the F_1 motor is driven by conformational changes originating from the $\alpha_3\beta_3$ unit in an ATP hydrolysis-dependent manner. However, since the crystal structure of $\alpha_3\beta_3$ without bound nucleotide showed symmetrical arrangement of its subunits [18], the mechanism of the unidirectional movement of $\alpha_3\beta_3$ remains unknown. This is the first question that will be addressed in this review (Question I). Furthermore, it is not clear whether the γ-axis induces conformational changes in $\alpha_3\beta_3$, which will be addressed in our second question (Question II). These questions must be answered to fully understand the rotational mechanism of these motor proteins.

Studies on V_1 motors

The energy produced by the ATP-hydrolyzing activity of F-ATPase drives many cellular metabolic processes. V-ATPase (vacuolar type ATPase) also utilizes this energy to transport protons across organelle membranes in eukaryotes, acidifying their membranous compartments. Thus, as a proton pump, the function of the V-ATPase is opposite to that of F-ATPase. V-ATPases are found in the plasma membranes of osteoblasts and cancer cells, which function to acidify the intercellular matrix for bone adsorption and to aid in the metastasis of cancer cells [19]. Therefore, V-ATPases are an important target for drug design and prevention of osteoporosis and cancer. V-ATPase is thought to have evolved from a common ancestor with F-ATPase [20] and thus shares common structural features. These include a hydrophilic ATP-hydrolyzing part (V_1) and a hydrophobic membrane-embedded ion pump (V_o), which are connected by a central axis and peripheral stalks. However, their subunit compositions exhibit several differences (Fig. 1b).

Studies on V_1-ATPase were conducted using a thermophilic bacterial enzyme, which has subunits resembling that of V-ATPase but functions as an ATP synthase similar to F-ATPase. The crystal structures of the A_3B_3 complex [21] (2.8 Å resolution) and V_1-ATPase (A_3B_3DF complex) [22] (4.5–4.8 Å resolution) have been obtained, and single-molecule observation [23] revealed similarities and differences with the F_1-ATPase. However, because the high-resolution structure of V_1-ATPase has not been obtained, numerous questions remain unsolved regarding the details of the rotational mechanism.

Using molecular, biochemical, and structural techniques, we showed that a V-ATPase from *Enterococcus hirae* is an ATP-hydrolyzing enzyme homologous to the eukaryotic V-ATPase. We studied the structure and function of the V_o part of this enzyme, and based on its Na^+-translocating activity, we proposed a model for its ion-transporting mechanism [24–26]. Beginning in 1996, we attempted crystallization trials of the V_1 motor part in order to obtain X-ray crystal structures. We initially attempted to purify and isolate the V_1 part from the whole V-ATPase complex. Our crystal did not diffract to high resolution, and our preparation of V_1 may have been contaminated with the motor without the axis (DF complex). Recently, using an *E. coli* cell-free protein synthesis system [27], we established expression and purification procedures for the motor part (A_3B_3 complex) without the DF complex, and subsequently solved its X-ray crystal structures [28].

Asymmetrical crystal structures of A_3B_3 complex

A crystal structure of the apo A_3B_3 complex obtained without the nucleotides ATP or ADP was solved to 2.8 Å resolution. The overall structure resembles that of the F_1 motor $\alpha_3\beta_3$ complex, revealing a hetero-hexameric ring composed of three catalytic subunits A and three non-catalytic subunits B arranged in an alternating configuration (Fig. 2a). Each subunit consists of an N-terminal β-barrel, middle α/β domain, and C-terminal helical domain. Since the hexameric ring is joined at the N-terminal β-barrel part, this region was fixed during structural comparison of the three A subunits. Superimposition revealed that all subunits adopt different conformations from one another. One of the A subunits is in the closed form (A_C) and is located closer to the ring center of the A_3B_3 complex, while the other two A subunits showed similar open forms (A_O and $A_{O'}$) (Fig. 2b). Similarly, the three B subunits showed different conformations from one another; one exhibited a closed form (B_C), while the other two exhibited open forms (B_O and $B_{O'}$) (Fig. 2b). Three nucleotide binding (catalytic) sites are located at the boundaries between the A/B pairs A_OB_C, $A_{O'}B_O$, and $A_CB_{O'}$ (red arrowheads in Fig. 2b). Surprisingly, even in the absence of nucleotide, the three catalytic sites formed by the same AB pair types show different conformations from one another. Previous reports of the apo structures of the thermophilic $\alpha_3\beta_3$ F_1 motor [18] and the A_3B_3 unit of the V_1 motor [21] both showed 3-fold rotational symmetry. Therefore, our structure is the first report of a motor protein structure with asymmetrical arrangement at the catalytic head.

Next, we obtained a crystal structure of the A_3B_3 complex in the presence of AMP-PNP, a non-hydrolysable analogue of ATP, at 3.4 Å resolution. In this structure, two of the three catalytic sites are occupied with electron density corresponding to AMP-PNP (Fig. 2c, d). The AB pair without bound AMP-PNP resembles the structure of the A_OB_C pair in the apo A_3B_3 complex. We named this the empty form, as it appears to have low affinity for the nucleotide. The two other AMP-PNP-binding AB subunits show similar conforma-

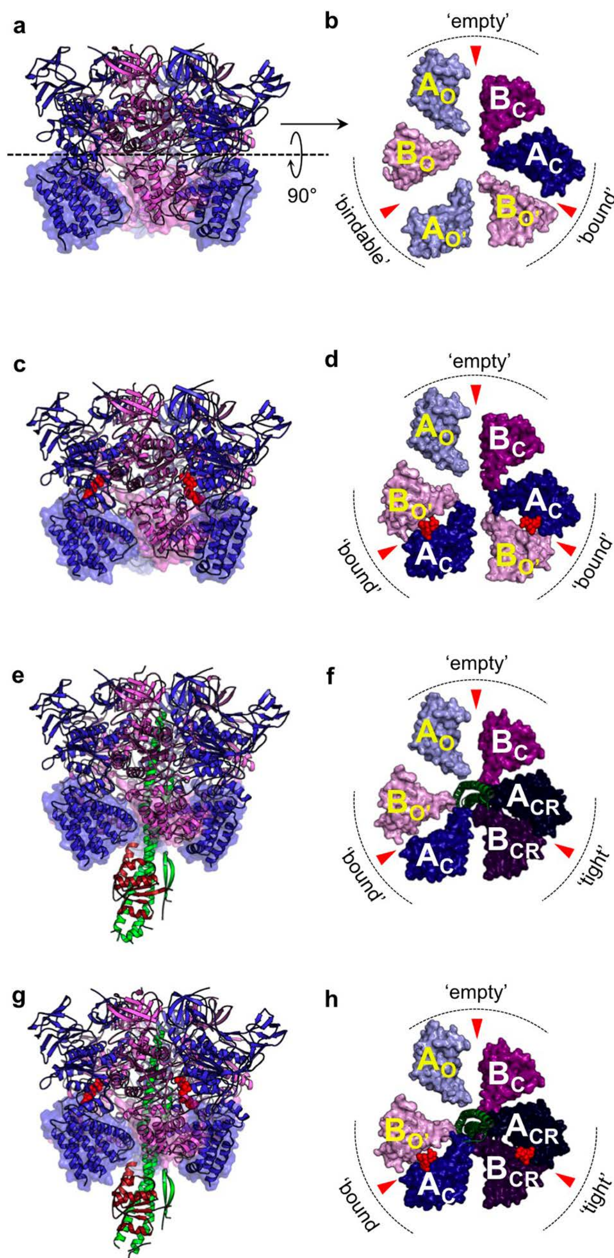

Figure 2 Crystal structures of the *E. hirae* V_1 motor. The figures are drawn as described in Fig. 1c, d. (a, b) apo A_3B_3; (c, d) A_3B_3 with bound AMP-PNP; (e, f) apo A_3B_3DF; (g, h) A_3B_3DF with bound nucleotide.

tions to one another and resemble the $A_CB_{O'}$ pair in the apo A_3B_3. This $A_CB_{O'}$ pair was thought to be the conformation that binds nucleotide and thus was named as the bound form. For the third $A_OB_{O'}$ pair in the apo A_3B_3 complex, it has been proposed that its conformation changes to the bound form upon AMP-PNP binding. Thus, this $A_OB_{O'}$ was named as the bindable form. This new bindable form has not yet been observed in the F_1 motor structure and this structure may be the state "waiting for ATP binding". This state will be described in a later section.

As described above, the apo A_3B_3 complex appears to be composed of three different AB pairs adopting three conformations: an empty form that cannot bind ATP, a bindable form that can bind ATP, and a bound form that has the same conformation as the bound form. In the presence of ATP, the complex is thought to adopt two bound forms derived from binding ATP in the bindable and bound forms. When ATP in the bound form, originally the bound form in the apo structure, is hydrolyzed, the ADP and Pi products are likely released. The A_3B_3 complex may then resemble the structure of the initial stable apo-structure, as the ATP on the bound form derived from the original bindable form may remain without being hydrolyzed and maintain the bound form, which resembles the original bound form in the apo-structure. Thus, since the apo-structure of the A_3B_3 complex is stable when AB pairs adopt the empty, bindable, and bound forms, it is conceivable that when one AB pair adopts a bound form, its neighbor to the right takes the empty form, while the next to right neighbor takes the bindable form. This description suggests that after ATP binding, hydrolysis, and release of ADP at the bound form, the complex rotates by 120° and each form takes on the next form in the turn. This is considered the principle of the unidirectional (or clockwise) rotational mechanism of the motor, driven by ATP hydrolysis. This may provide answers to Question I.

Crystal structures of V_1-ATPase

Given the proposed mechanism for the unidirectional movement (Question I) described in the above section, we next address the role of the DF complex in the conformational change and rotation of the A_3B_3 complex (Question II). To answer this question, it is necessary to compare the structures in the presence and absence of the DF complex. Therefore, we reconstituted the V_1-ATPase (A_3B_3DF complex) from A_3B_3 and DF and obtained high-resolution diffracting crystals [28].

We determined the crystal structure of apo V_1-ATPase to 2.2 Å resolution. As expected, the structure showed the DF complex penetrating through the center of the A_3B_3 ring (Fig. 2e, f). We compared the structures of the apo A_3B_3 complex with A_3B_3DF to examine the structural changes induced by DF binding. Similarly to the A_3B_3 complex, the A_3B_3DF complex also showed an empty form (A_OB_C pair) and bound form ($A_CB_{O'}$ pair) (Fig. 2f). However, when the structures were superposed at the empty form positions, the bindable form in the A_3B_3 complex aligned with the bound form in the A_3B_3DF complex (Fig. 2b, f). This finding indicates that the conformational change from the bindable to the bound form, induced by nucleotide binding, can also be induced by DF binding. The third AB pair in V_1-ATPase exhibited a closer conformation not found in the A_3B_3 complex. This pair ($A_{CR}B_{CR}$), consisting of the closed A_{CR} and B_{CR} structures, was subsequently named the tight form (Figs. 2f, 3e, 3f). Thus, upon binding of DF, the bound form ob-

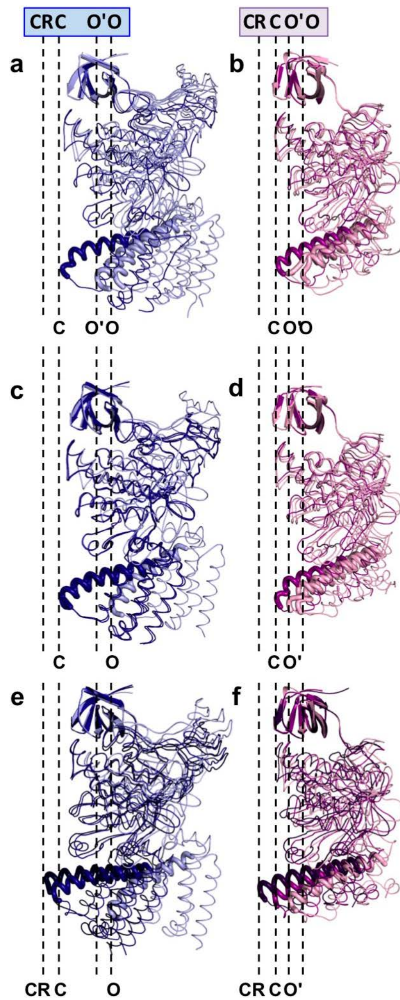

Figure 3 Structural comparison between subunits. Subunits are superimposed at the N-terminal β-barrel domain of subunit A (left) and subunit B (right). To aid in the orientation and for marking structural differences, a protruding helix in the C-terminal domain is highlighted with thick lines. (a, b) apo A_3B_3; (c, d) A_3B_3 with bound AMP-PNP; (e, f) apo A_3B_3DF.

served in the A_3B_3 complex may change conformation to the tight form. These observations provide answers to Question II.

Comparison of the tight form and bound forms shows that at the catalytic site, the R-finger (R^{350}) in B_{CR} is closer to the R^{262} residue in the A_{CR} of the tight form than those in $B_{O'}$ and A_C of the bound form (Fig. 4a). To examine the effect of this structural difference in R^{350} on nucleotide binding, we determined the crystal structure of the V_1-ATPase bound with AMP-PNP to 2.7 Å resolution. Electron density corresponding to AMP-PNP was found at two sites, the bound and tight forms, and no electron density was observed in the empty form (Fig. 2g, h). As suggested from the crystal structure of the A_3B_3-nucleotide complex, the empty form had low affinity for the nucleotide. Similarly to the interactions found between A_3B_3 and the bound nucleotide, the γ-phosphate in AMP-PNP and Mg^{2+} interacts with K^{238}, T^{239}, and R^{262} in

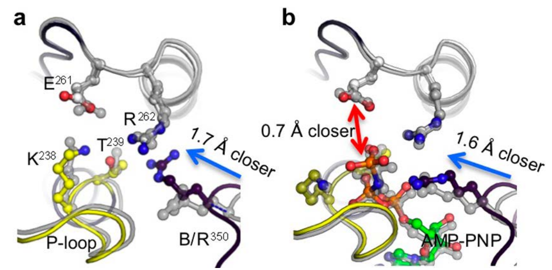

Figure 4 Catalytic site architecture. (a) Superposition of the bound form of apo A_3B_3 (grey) and tight form of apo A_3B_3DF (colored). (b) Superposition of bound form of (grey) and its tight form (colored) of the A_3B_3DF AMP-PNP complex.

subunit A and the R-finger (R^{350}) in subunit B (Fig. 4b). Comparison of the catalytic sites of the tight and bound forms in the V_1-ATPase-nucleotide complex showed that the R-finger in the former structure is 1.6 Å closer to the γ-phosphate. This phosphate group is in turn 0.7 Å closer to E^{261} in subunit A (Fig. 4b). E^{261} was shown to be essential for ATPase activity in *Saccharomyces cerevisiae* V_1-ATPase [29]. Additionally, the corresponding E^{188} residue in bovine F_1-ATPase is an important residue for ATP hydrolysis and is known to interact with the oxygen atom of the γ-phosphate and with an intermittent water molecule [30]. The closer proximity of the R-finger to the γ-phosphate, brought about by DF binding, may enhance the ATP hydrolysis reaction. Thus, we propose that this crystal structure corresponds to the state "waiting for ATP hydrolysis".

Further experimental results; demonstration of V_1-ATPase rotation by single-molecule observation

ATP hydrolysis generates torque for the rotation of the DF axis. Single-molecule observation of the rotation is useful for obtaining direct evidence of rotational movement. In collaboration with Iino *et al.*, we demonstrated 120° rotation of the DF axis in our V_1 complex [31,32]. At 4 mM ATP, the axis rotated continuously at a rotational speed of approximately 100 rps. The K_M was approximately 100 μM. The angle distribution was analyzed, showing a 3-step rotation for each cycle or 120° rotation per step, at all ATP concentrations. Torque generation in the continuous ATP hydrolysis state was estimated to be approximately 20 pNnm [32], which is lower than values reported for F-ATPases.

Model of rotation mechanism of V_1-ATPase [difference from F_1 model [2,3]]

The following is a model for the rotation mechanism for V_1-ATPase based on crystal structures. This model resembles that of the F_1 motor proposed according to single-molecule observation studies, but several points are different and are highlighted in brackets [ ].

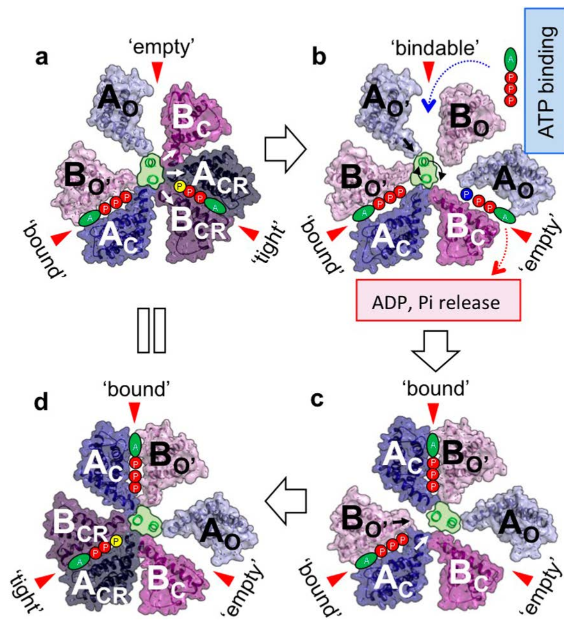

Figure 5 Model for the rotational mechanism of V_1 motor. Upper view (from the N-terminal end) of the C-terminal domains of the respective crystal structures. (a, d) AMP-PNP bound A_3B_3DF, (b) apo A_3B_3, and (c) AMP-PNP bound A_3B_3. ATP colored with a yellow Pi is in the hydrolyzing state and ATP colored with a blue Pi is in the released state, after hydrolysis. Refer to details in the text.

The rotation mechanism model starts with the structure of V_1-ATPase with ATP at the bound and tight forms. Since the R-finger in the tight form is in close proximity to the ATP γ-phosphate, this ATP is "waiting for hydrolysis". The process starts with hydrolysis of this ATP (Fig. 5a) [similar in the F_1 motor]. Next, as the empty form shows low affinity for nucleotide, it is unable to bind ATP [similar to F_1 [33]]. After ATP is hydrolyzed to ADP and Pi, the tight form may take on another conformation, and the whole A_3B_3 complex may undergo a large conformational change. Based on single-molecule analysis of F_1-ATPase, ATP binding and ADP release appear to occur after ATP hydrolysis, causing a large conformational change that induces axis rotation; therefore, the ATP binding conformation is expected to appear at this step. If the effects of the DF axis are ignored, the ATP hydrolysis reaction may induce the conformational change into the apo A_3B_3 structure, and the empty and tight forms appear to transform into bindable and empty forms, respectively (Fig. 5b) [no such step is predicted in the F_1 motor model]. However, strong interactions between the DF and the tight form prevent the conformational change of the tight form into the empty form, and another intermediate state is likely to exist in place of Fig. 5b [this conformational change is not present in the model of the F_1 motor]. This unidentified structure may be the "waiting for ATP binding" state and resemble the structure of the A_3B_3 apo-structure, which has a bindable form that can bind ATP [In the F_1 motor, a structure corresponding to the bindable form has not been obtained]. In the next step, ATP binding to the bindable form induces a conformational change of this AB pair to the bound form and 120° rotation of the DF axis. Upon ATP binding and DF rotation, the bindable form changes into the bound form and a nucleotide-bound A_3B_3-like structure transiently appears (Fig. 5c). Finally, the original bound form is induced to change its conformation to the tight form by interacting with the DF complex [this is not thought to be the case in the F_1 motor]. The R-finger approaches the γ-phosphate of ATP and resumes the state of "waiting for ATP hydrolysis" of V_1-ATPase (Fig. 5d = a). This cycle is repeated. Because of the asymmetrical structure of the A_3B_3 ring, it is easy to conceive that the order of ATP hydrolysis and direction of rotation are intrinsically determined by the structure.

General discussion regarding bioenergy transduction

A more fundamental or basic question is about the energy transduction mechanism, referred to as the affinity change model by Yamato [34] or as the thermal ratchet model for muscle contraction by Vale and Oosawa [35]. Does the DF axis rotate by thermal fluctuation or by real force (torque) generated in the A_3B_3 motor complex coupled with ATP hydrolysis? In thermal fluctuation, the hydrolysis energy utilized to change the affinity of a protein or sub-complex for a substrate or other complex (i.e., DF complex or actin filament) is converted into directional information of movement. In real force model, actin filament movement or DF complex rotational movement is driven by force energy (also proposed as velocity change). In active transport systems, depending on the reaction direction, the energy utilized to alter substrate affinity may be converted into directional information. In the actomyosin system, the nucleotide-dependent change in affinity of myosin for the actin filament has been demonstrated [36]. Here, the energy of movement is likely derived from thermal fluctuation energy. We have not yet characterized the detailed affinity of the DF complex with the A_3B_3 motor, but the affinity appears to be quite high [37]. Therefore, without dissociating the axis, it would be difficult to generate torque through mechanical rotation. If the detaching/rotation/attaching movement of DF occurs to mechanically generate torque (force), the detaching/attaching steps should be seamless. However, in the crystal structures obtained so far, it is not conceivable for the motor to make large conformational changes to enable seamless detachment and reattachment. The DF binding sites between the tight form (or intermediary conformation) and the next bound form are too far apart (> 10 Å) to allow for seamless and efficient exchange of the binding residues between the motor and DF complex. Thus, the DF axis should be released, allowing for thermal fluctuation and searching for the correct landing site in both directions. Without information input into the direction of rotation, this will result in poor efficiency of energy utilization. Therefore, using an energy input, the affinity change model allows the motor

molecule to select its direction of rotation. The conversion of free chemical energy from ATP hydrolysis into selective information decreases informational entropy. In this case, the selective information is the directional movement of the DF axis, which is guided by changes in affinity. A model system of such information-to-energy conversion has been demonstrated [38]. In a few instances, such informational entropy may be quantitatively correlated with thermodynamic free energy. However, this should be evaluated in future studies.

Conclusions

As described above, the model of rotation mechanism of *E. hirae* V_1-ATPase resembles that proposed by the single-molecule observation of F_1. However, there are several differences between the two models because of their fundamental differences in function; one functions in ATP synthesis, while the other in ATP hydrolysis. Although our model describes structural differences on the atomic level, it cannot predict the dynamic nature of structural changes, as time-dependent and reaction scheme information has not been fully determined. Thus, we have obtained supporting information from single-molecule observation of our enzyme, which will be useful for elucidating the reaction mechanism [31,32]. Furthermore, we are attempting to obtain other crystal structures to reflect intermediate rotational structures. We are also collaborating with a research group to perform molecular dynamics simulations to model the structural changes between the snapshot structures obtained from crystallography. In this respect, the MD results of Nam *et al.* indicate a F_1 motor conformation corresponding to the state “waiting for ATP binding” [39]. Using techniques that differ in spatio-temporal resolution and sample environment, we hope to clarify the detailed rotational mechanism of these rotary motors.

Acknowledgments

This review is dedicated to Late Prof. Yoshimi Kakinuma (he died at the age of 64 in January 2016) as a memorial for his great contribution to the research of V-type ATPases. He studied Na^+-translocating ATPase in *Enterococcus hirae* and found it to be a bacterial V-type ATPase. Since then, we have collaborated to elucidate the bioenergy transduction mechanism of this V-type Na^+-ATPase. With his support, we succeeded in obtaining 3D structures of our V_1-ATPase as described in this review and are now close to understanding its bioenergy transduction mechanism.

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Conflict of Interest

The authors declare no conflict of interests.

Author Contributions

I. Y., Y. K., and T. M. reviewed the field of rotary motors. I. Y. and T. M. wrote the manuscript.

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