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Rotational mechanism of *Enterococcus hirae* V_1-ATPase by crystal-structure and single-molecule analyses

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In ion-transporting rotary ATPases, the mechanical rotation of inner rotor subunits against other stator subunits in the complex mediates conversion of chemical free energy from ATP hydrolysis into electrochemical potential by pumping ions across the cell membrane. To fully understand the rotational mechanism of energy conversion, it is essential to analyze a target sample by multiple advanced methods that differ in spatiotemporal resolutions and sample environments. Here, we describe such a strategy applied to the water-soluble V_1 moiety of *Enterococcus hirae* V-ATPase; this strategy involves integration of crystal structure studies and single-molecule analysis of rotary dynamics and torque generation. In addition, we describe our current model of the chemo-mechanical coupling scheme obtained by this approach, as well as future prospects.

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Introduction

F- and V-ATPases use a unique 'rotary catalysis' mechanism [1,2•] in which highly coordinated sequential ATP hydrolysis reactions at three catalytic sites in the water-soluble F_1 or V_1 moiety lead to the unidirectional mechanical rotation of the inner rotor subunits against the stator subunits. The torque generated inside the single molecule is transmitted to the membrane-embedded F_o or V_o moiety, which actively transports ions and generates electrochemical potential across the cell membrane. In this sense, F- and V-ATPases are molecular machines that realize conversion among chemical, mechanical, and potential energies.

Multiple experimental approaches have contributed to our current understanding of the molecular mechanism by which rotary ATPases achieve energy conversion. In particular, structural information at the atomic level and the molecular dynamics at the single-molecule level are both important. The rotary catalysis of F_1 has been extensively studied by both approaches. However, the atomic level structural studies have primarily used samples from bovine or yeast mitochondria [3], while the single-molecule dynamics studies have used bacterial proteins [4]. The results obtained by different methods and from different species have raised some controversies on the model of chemo-mechanical coupling scheme of F_1 [5–7].

It is clear that using multiple, hybrid methods to study the same sample will provide us with better information for complete understanding of the rotary ATPase operation mechanism. One recent remarkable example is a single-molecule study of the human mitochondrial F_1 [8••], which has high amino-acid sequence homology to that of the bovine mitochondrial F_1. This study revealed a chemo-mechanical coupling scheme that differs from that obtained by single-molecule analysis of the bacterial F_1 [5,6], and provided a consistent model which can explain both the structural and single-molecule data.

We are now taking similar strategy to the study of V_1. Our target is the V_1 moiety of the *Enterococcus hirae* V-ATPase (EhV_1, note that *E. hirae* was previously called as *Streptococcus faecali* or *Streptococcus faecium*). As a rotary ATPase, the *E. hirae* V-ATPase has a unique feature: its V_o moiety primarily transports sodium ions rather than protons [9,10], similar to the F-ATPase from *Ilyobacter tartaricus* [11]. We have recently revealed the molecular architecture of EhV_1 at the atomic level by X-ray crystallography [12••] and analyzed its rotary dynamics by high-speed single-molecule rotation assays [13•]. Our results have revealed important features different from those of F_1 [14]. Furthermore, very recently, we have also established

a recombinant expression system for the complete *E. hirae* V-ATPase complex (EhV_oV_1) in *Escherichia coli*, and successfully compared rotary dynamics and generated torque between EhV_1, EhV_oV_1, and other rotary ATPases [15••]. In this short review, we will introduce these results with our current model of the chemo-mechanical coupling scheme of EhV_1.

Molecular architecture of EhV_1

Figure 1 shows the crystal structures of the A_3B_3 and A_3B_3DF (EhV_1) complexes, and the conformations of the individual A and B subunits [12••]. The A and B subunits form an alternately arranged stator A_3B_3 ring, and the D and F subunits form a rotor complex that penetrates into the central hole of the A_3B_3 ring (Figure 1, left and center). The nucleotide-binding catalytic sites are located at the interface of the A and B subunits (Figure 1, red arrowheads), although most of the amino acid residues involved in nucleotide binding are located in the A subunit. The overall architecture of EhV_1 is similar to that of the bovine and yeast mitochondrial F_1 [16,17], but the structures display certain distinct features as described below.

The conformations of each A and B subunit in the A_3B_3 ring are apparently different even in the absence of the rotor DF complex and bound nucleotides, and show two open (O or O') and one closed (c) conformations (Figure 1a, right). These distinct conformations result in the different structures of the three catalytic sites designated 'empty,' 'bound,' and 'bindable' sites, respectively. This asymmetric feature of the rotor-less stator ring is different from that of F_1, which displays a three-fold symmetric structure with three identical catalytic sites in the absence of bound nucleotides in the crystal structure [18] and high-speed atomic force microscopy [19,20]. In the presence of the non-hydrolyzable ATP analog AMPPNP, A_3B_3 binds two AMPPNP molecules at the 'bound' and 'bindable' sites, resulting in a conformational change of the A (O' to C) and B (O to O') subunits (Figure 1b) and a transformation of the 'bindable' site into the 'bound' site (Figure 1b).

A crucial feature is the effect of the central rotor (DF) on the structure of the three catalytic sites of the stator (A_3B_3). Insertion of the DF complex into the A_3B_3 ring induces conformational changes in the A and B subunits even in the absence of bound nucleotides, and consequently results in the appearance of the more closed 'closer' (CR) conformations of the A and B subunits (Figure 1a and c, right). This results in not only the formation of the 'bound' site from the 'bindable' site, but also the formation of the 'tight' site from the 'bound' site (Figure 1c, center). The 'tight' site presumably corresponds to the catalytic site in the pre-ATP-hydrolysis stage. Conversely, the binding of AMPPNP to the 'bound' and 'tight' sites of the A_3B_3DF complex does not cause further conformational changes of the A and B subunits (not shown, PDB ID: 3VR6), and nucleotide-free and nucleotide-bound A_3B_3DF complexes show almost identical structures [12••]. Almost identical nucleotide-free and nucleotide-bound structures were also reported previously in the studies of yeast mitochondrial F_1 [21], although the structure of the $\alpha_3\beta_3$ complex has not yet been obtained. These results imply that the interactions between the stator and rotor are as critical as nucleotide binding in determining the structure of the catalytic sites of rotary ATPases.

Rotary dynamics of EhV_1 and EhV_oV_1

The experimental system for high-speed single-molecule rotation assays of EhV_1 and EhV_oV_1 is depicted schematically in Figure 2a. In this system, a 40-nm gold colloid was used as a low-load probe for rotation. The rotary motion was observed by using total internal reflection dark-field microscopy [22] at an imaging rate of 5000–10 000 frames per second. In the presence of ATP, EhV_1 showed unidirectional successive rotation in the counterclockwise direction, exhibiting two distinct reversible states of rotation, namely clear and unclear (Figure 2b) [13•]. We assigned the rotation state based on the distribution of the centroid of the probe and the presence of apparent backward rotations. If the centroid of the probe was distributed near the rotation center (Figure 2b, right) and this distribution caused the apparent backward rotations (Figure 2b, left), the rotation was assigned as the unclear. If the majority of the centroids of the probe were distant from the rotation center and the time course showed clear unidirectional rotations, the rotation was assigned as the clear. This result suggests that the interactions between the rotor and stator subunits in isolated EhV_1 are less stable than these subunits in EhV_oV_1. Actually, the unclear state occurs only in the isolated EhV_1 and it is not observed in EhV_oV_1 [15••]. Thus, two peripheral stalks likely stabilize the interactions between the rotor and stator subunits in EhV_oV_1. Furthermore, interestingly, EhV_oV_1 showed slower rotation than EhV_1 without the three distinct pauses separated by 120° that were observed in EhV_1 (Figure 2b). This result indicates that rotor–stator interactions of the V_o moiety and/or sodium ion transport limit the rotation of EhV_oV_1 driven by the V_1 moiety.

From the results described above, we concluded that, in EhV_1, the tight chemo-mechanical coupling is achieved at least in the clear state. Then, rotary dynamics in the clear state were analyzed in detail. In the clear rotation state, EhV_1 showed only three pausing positions separated by 120° at all ATP concentrations ranging from below to above the Michaelis constant, at which distinct elementary reaction steps of the ATP hydrolysis, such as ATP binding, phosphate-bond cleavage, or product release, become the rate-limiting steps of the rotation (Figure 2c). In contrast to the rotation of thermophilic *Bacillus* PS3 F_1 [23], *E. coli* F_1 [24] and human mitochondrial F_1 [8••], the