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Rotation, Structure, and Classification of Prokaryotic V-ATPase

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The prokaryotic V-type ATPase/synthases (prokaryotic V-ATPases) have simpler subunit compositions than eukaryotic V-ATPases, and thus are useful subjects for studying chemical, physical and structural properties of V-ATPase. In this review, we focus on the results of recent studies on the structure/function relationships in the V-ATPase from the eubacterium *Thermus thermophilus*. First, we describe single-molecule analyses of *T. thermophilus* V-ATPase. Using the single-molecule technique, it was established that the V-ATPase is a rotary motor. Second, we discuss arrangement of subunits in V-ATPase. Third, the crystal structure of the C-subunit (homolog of eukaryotic d-subunit) is described. This funnel-shape subunit appears to cap the proteolipid ring in the V_0 domain in order to accommodate the V_1 central stalk. This structure seems essential for the regulatory reversible association/dissociation of the V_1 and the V_0 domains. Last, we discuss classification of the V-ATPase family. We propose that the term prokaryotic V-ATPases should be used rather than the term archaeal-type ATPase (A-ATPase).

KEY WORDS: V-ATPase; ATP synthase; single-molecule; X-ray crystallography; *Thermus thermophilus*.

INTRODUCTION

The vacuole-type ATPases (V-ATPases) are commonly found in many organisms involved in a variety of physiological processes. V-ATPases in eukaryotic cells (eukaryotic V-ATPases) pump protons across the membrane consuming ATP. They have complex architecture and consist of at least 13 kinds of different subunits (Graham *et al.*, 2003; Nishi and Forgac, 2002). The homologs of eukaryotic V-ATPases are also found in archaea and some eubacteria (prokaryotic V-ATPases) (Lolkema *et al.*, 2003; Murata *et al.*, 1997; Yokoyama *et al.*, 1994). The prokaryotic V-ATPases have a less complex architecture and consist of nine kinds of subunits. They function as either ATP synthases or sodium pumps. They can be considered as two classes of an ion translocating ATPase superfamily (rotary ATPase/synthase) consisting of enzymes that are hetero-oligomeric complexes that utilize a similar rotary mechanism for catalysis. Both the F-type and V-type enzymes are composed of two functional domains, the hydrophilic V_1 or F_1 domain and a membrane-embedded ion-translocating domain called V_0 or F_0. These distinct domains are connected by the central and peripheral stalks.

Detailed comparison of the structure/function relationships of V-ATPases with those of F-ATPases is important for establishing the basic features of subunit rotation necessary for coupling proton translocation across a membrane with ATP formation. It is difficult to obtain large amounts of pure V-ATPase from vacuolar membranes. This has limited detailed investigations of the structure/function relationships of the eukaryotic enzymes. In contrast, stable V-ATPases can be obtained in large amounts from some prokaryotes.

In this review, we summarize rotation, and structural information and classification of the prokaryotic

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V-ATPase, especially from the thermophilic eubacterium *Thermus thermophilus*.

PROKARYOTIC V-ATPase FROM *Thermus thermophilus*

We have previously identified a V-type H^+-ATPase in plasma membranes of *T. thermophilus* (Yokoyama *et al.*, 1990, 1994). This V-ATPase catalyzes both ATP-driven proton translocation and proton-driven ATP synthesis, and functions as ATP synthase in vivo. The *T. thermophilus* V-ATPase operon contains nine genes in the order of atpG-I-L-E-C-F-A-B-D, which encoded proteins with molecular masses of 13, 72, 10, 20, 35, 12, 64, 53, and 25 kDa, respectively (Yokoyama *et al.*, 2000). The product of atpL, the proteolipid subunit, lacks a 19 amino acid presequence and, unlike eukaryotic V-type ATPases, contains two membrane-spanning domains rather than four (Yokoyama *et al.*, 2000). The common operon encoding the *T. thermophilus* V-ATPase and other prokaryotic V-ATPases is well conserved (Lolkema *et al.*, 2003). Each subunit of *T. thermophilus* V-ATPase has significant sequence homology with its eukaryotic counterpart. However, the molecular masses of some subunits are less than those of the corresponding subunits in the eukaryotic enzymes (Yokoyama *et al.*, 2003a). For instance, the 72-kDa I-subunit has an overall sequence similarity to that of the eukaryotic 100-kDa a-subunit. Although the molecular mass of subunit L is ~50% of eukaryotic c-subunit (16k-Da proteolipid subunit), subunit L has an obvious sequence homology with the V_0 c-subunit.

The hydrophilic V_1 domain of *T. thermophilus* is made up of four kinds of subunits with a stoichiometry of $A_3B_3D_1F_1$. Although the G-, E-, and C-subunits are also hydrophilic, they are identified as V_0 subunits together with hydrophobic subunits I and L.

ROTATION OF V-ATPase

In order to transport protons across the membrane during ATP hydrolysis, structural changes in the proton channel must occur. However, the catalytic sites for reversible ATP hydrolysis and synthesis in V_1 are at least 100 Å apart from the proton channel in V_0. V-ATPases as well as F-ATPases utilize rotary mechanisms to overcome this distance barrier. A rotary catalytic mechanism was first proposed for the F_1-ATPase by Boyer in early 1980s (Boyer and Kohlbrenner, 1981). Noji *et al.* (1997) directly demonstrated rotation of F_1-ATPase during single-molecule catalysis in 1997. In the case of F_1, ATP hydrolysis drives rotation of the central γ-subunit within the $\alpha_3\beta_3$ core. In the case of ATP hydrolysis by single molecules of the F_0F_1 complex, the γ-subunit rotates together with the ring of c-subunits, which contains the proton-binding sites (Tsunoda *et al.*, 2001). Although structural homologies indicated that the V-ATPase is also a rotary motor, direct demonstration of rotation during ATP hydrolysis by the V-ATPase was not demonstrated until we reported rotational catalysis by single molecules of the *T. thermophilus* enzyme in 2003 (Imamura *et al.*, 2003; Yokoyama *et al.*, 2003b). Rotation was probed with a large ($\approx$0.6 μm) bead attached to rotor subunits which was monitored under a microscope. First, we demonstrated rotational catalysis by isolated V_1 of *T. thermophilus*, which is capable of hydrolyzing MgATP. In contrast, the eukaryotic V_1 is inactivated when detached from V_0, presumably by the inhibitory action of the regulatory H-subunit, which is not present in prokaryotic V-ATPases (Parra *et al.*, 2000). ATP-dependent rotation was observed with a bead attached to the D- or F-subunit (Imamura *et al.*, 2003). Rotation is counterclockwise when viewed from the membrane side, which is the same direction of γ-subunit rotation during ATP hydrolysis by F_1. Next, we demonstrated rotational catalysis during ATP hydrolysis by the complete V_0V_1 complex of *T. thermophilus* with a bead attached to the proteolipid subunits. This rotation was significantly inhibited by treatment of the V_0V_1 complex with *N,N′*-dicyclohexyl-carbodiimide (DCCD) (Yokoyama *et al.*, 2003b). Thus, mechanical rotation couples proton translocation to ATP hydrolysis by both the V_0V_1-ATPase and the F_0F_1-ATPase. Soon after our demonstration of the rotation of *T. thermophilus* V-ATPase, Hirata *et al.* (2003) reported the rotation of the yeast enzyme. They showed that an actin-filament attached to the G-subunit rotated relative to the proteolipid ring (c-subunit) in the presence of ATP. However, it is not clear whether the rotation they observed is coupled to V-ATPase activity, because they did not show DCCD sensitivity of the rotation. Other experiments have also supported the rotary movement of V-ATPase (Aviezer-Hagai *et al.*, 2003). When anti-HA antibody was bound to the HA-tagged rotor F- or c″-subunits of yeast V-ATPase, which are rotor subunits, both ATPase activity and ATP-dependent proton translocation were inhibited. In contrast, binding of the antibody to the HA-tagged G-subunit did not.

The rotor shaft of V_1 is composed of the D- and F-subunits. Even when the F-subunit is removed from V_1, rotation of the D-subunit was also observed (Imamura *et al.*, 2004). The direction of the rotation was the same as that observed with V_1 (A_3B_3DF). This result indicates that the F-subunit is not an essential for rotary catalysis of V_1, and that A_3B_3D is the minimum ATP-driven rotary unit of

V-ATPase rather than A_3B_3DF. Thus, the F-subunit should bind peripherally to the D-subunit. Interestingly, ATPase activity of A_3B_3D was substantially lower than that of A_3B_3DF. When the F-subunit was mixed with A_3B_3D, ATPase activity was recovered. This indicates that the action of the F-subunit is markedly different from the inhibitory function of the ε-subunit in F-ATPase (Kato *et al*., 1997; Nelson *et al*., 1972; Smith and Sternweis, 1977).

It is known that the central γ-subunit of F_1 rotates by repeating a sequence of four processes: (i) binding of ATP to a catalytic site, (ii) 80° rotation of the γ-subunit, (iii) cleavage of ATP and/or release of hydrolysis product(s), (iv) 40° rotation of γ (Shimabukuro *et al*., 2003; Yasuda *et al*., 1998, 2001). One of the fundamental questions concerning V_1 rotation is whether or not the rotational mechanism of V_1 is completely the same as that of F_1, because the detailed rotation scheme of V_1 has been established. Recently, we found from analyses of rotational steps that V_1, like F_1, consumes one ATP during each 120° rotational step. However, the rotational scheme of V_1 has marked differences from that of F_1. We will describe this result elsewhere in detail.

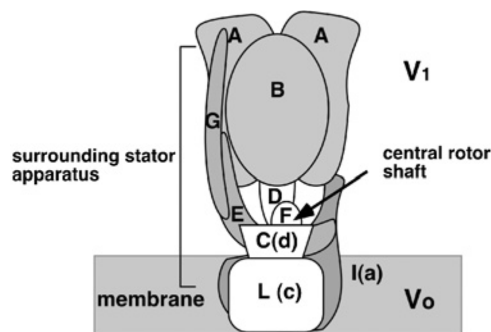

Fig. 1. Structural model of the prokaryotic V-ATPase. The subunits D, F, C (d), and L (shown in white) form a central rotor shaft, contrary subunits A, B, E, G, I (a) (shown in grey) form a surrounding stator apparatus. The subunits A and B form the A_3B_3 hexameric ring responsible for ATP synthesis or hydrolysis. Subunit D fills the cavity of the A_3B_3 ring and constitutes V_1 shaft with subunit F. ATP hydrolysis is proposed to drive rotation of the V_1 shaft, which in turn drives rotation of the L ring in V_0 via subunit C. There are two membranes proteins in the V_0 domain, one is proteolipid subunit L and the other is subunit I, a homolog of eukaryotic V_0 a-subunit. Subunit I, together with L ring, plays a critical role for ion translocation across the membrane. The membrane-embedded domain of subunit I is connected to the A_3B_3 ring by the peripheral stalks composed of subunit E, G, and the hydrophilic domain of subunit I. The precise structure of the peripheral stalk remains uncertain. Rotation of the L ring relative to subunit I drives unidirectional proton transport. On the other hand, the L ring also rotates when the proton motive force drives rotation of the V_1 shaft. As a result, ATP is synthesized from ADP and Pi in the V_1 domain.

SUBUNIT ARRANGEMENT IN V-ATPase

The precise arrangement of subunits in V-ATPase remains an important, unclarified issue. Particularly, the structure and subunit compositions of both the central and peripheral stalks have not been established. The D-subunit is the most probable candidate for a rotor subunit in V_1 portion. Cross-linking studies on yeast V-ATPase have suggested that the D-subunit is adjacent to the B-subunit in the central cavity region of the A_3B_3 hexamer and the F-subunit is associated with the D-subunit (Arata *et al*., 2002; Xu *et al*., 1999). On the contrary, some studies suggested that the E-subunit, rather than the D-subunit, is the rotor subunit (Grüber *et al*., 2002; Rizzo *et al*., 2003). As described earlier, single molecule analysis for *T. thermophilus* V-ATPase showed that the D- and F-subunits rotate relative to A_3B_3 (Imamura *et al*., 2003). This result clearly indicates that these two subunits are involved in the central stalk.

The V_0 domain of *T. thermophilus* V-ATPase is composed of two hydrophobic subunits I and L, and three hydrophilic subunits, C, E, and G. The V_0 domain is resolved into two subcomplexes, when exposed to low pH or 8 M urea. One is composed of the E-, G-, and I-subunits, and the other is composed of the L- and C-subunits (Yokoyama *et al*., 2003a). The secondary structural prediction for the G-subunit shows the presence of a long hydrophilic α-helix at the C-terminal region as observed in the F_1 b-subunit. Tomashek *et al*. (1997) and Xu *et al*. (1999) showed that the yeast E-subunit (Vma4p) interacts with the G-subunit (Vma10p). A cross-linking study also suggested interaction between E- and a-subunits in the yeast V-ATPase (Xu *et al*., 1999). Taken together, hydrophilic subunits E and G are associated with hydrophobic subunit I (homolog of the eukaryotic a-subunit) to form the peripheral stalk (Fig. 1). Unlike the *T. thermophilus* V-ATPase, both the E- and G-subunits of the eukaryotic V-ATPase are components of the V_1 moiety (Graham *et al*., 2003; Nishi and Forgac, 2002). The different localizations of both subunits in the *T. thermophilus* and eukaryotic enzymes might be due to difference in the affinity of EG complex for A_3B_3DF and the 100-kDa subunit.

The C-subunit, a homolog of Vma6p (or d-subunit) assigned to be the V_0 domain in yeast V-ATPase, is also part of the V_0 part in *T. thermophilus* V-ATPase. The CL subcomplex was stable against the treatment with 8 M Urea, suggesting that the C-subunit tightly binds to the L-subunits ring. Based on the electron microscopic study of subcomplexes with different subunit composition, Chaban *et al*. suggested that the C-subunit of *C. fervidus* V-ATPase

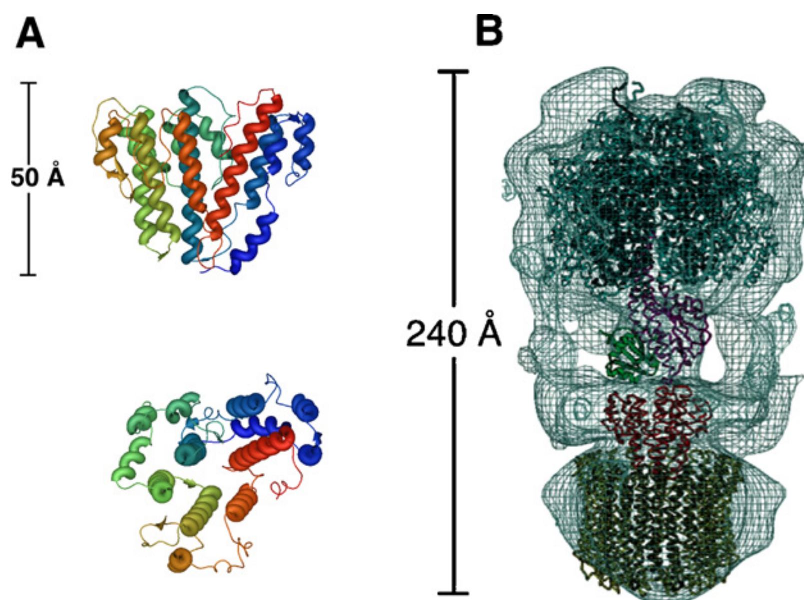

Fig. 2. The structure of *Thermus thermophilus* V-ATPase. (A) Crystal structure of subunit C; *upper*, side view; *lower*, top view from the cytoplasmic side. (B) Fitting of the known X-ray coordinates was done using the electron density map of the V-ATPase that was determined by single particle analysis (mention two different contour levels). Subunit F (green) is shown with the NtpK ring structure (PDB ID 2BL2; Murata *et al*., 2005) (yellow). Bovine mitochondrial F_1-ATPase $\alpha_3\beta_3\gamma$ complex (PDB ID 1E79; Gibbons *et al*., 2000) (cyan), a model of subunit D (magenta) and the subunit C (PDB ID 1R5Z; Iwata *et al*., 2004) (red) of V-ATPase are also fitted to the V_0 domain as a reference.

is a component of the central stalk (Chaban *et al*., 2002). These results, together with our rotational experiments, clearly indicate that the C-subunit is a constituent of the central rotor shaft of V-ATPase together with the D- and F-subunits, and transmits the torque generated in V_1 to the ring of L-subunits (Fig. 1).

STRUCTURE OF THE CENTRAL STALK SUBUNIT

The crystal structure of the C-subunit of *T. thermophilus*, which was determined at 1.95 Å resolution, suggested how this unique subunit functions in V-ATPase (Iwata *et al*., 2004). The molecule is highly α-helical. It is composed of 6 central helices and 12 peripheral helices arranged in a novel funnel-shaped structure (Fig. 2A). There is a polar cavity inside (8 Å diameter, 25 Å depth), open to only one side. The surface of the subunit is mainly polar. No possible membrane anchor region was observed. The peripheral helices form a rim, $\sim$25 Å high around the central helices. Based on the results of cross-linking experiments, the C-subunit is located in the center of the "flat mass" in the electron density of the holo-enzyme complex obtained by single particle analysis (Fig. 2B). The thickness of the "flat mass" is equal to the height of the peripheral helix rim of the C-subunit (25 Å). In this model, the C-subunit caps one end of the L-subunit ring, and the internal cavity of the C-subunit is open towards the upper V_1 side. As described earlier, the C-subunit is likely to interact with the "DF central shaft." The C-subunit should recognize this complex, like a "socket."

In the F-ATPase, both the ε- and γ-subunits directly bind to the proteolipid ring. In contrast, the C-subunit forms a socket-like structure to accommodate the DF central shaft at the V_1 and V_0 interface of the V-ATPase. Thus, the V_0–V_1 interface is significantly different from the F_0–F_1 interface, and this could be relevant to the unique reversible association/dissociation of V_1 and V_0, which is found in eukaryotic V-ATPase (Kane and Smardon, 2003).

The crystal structure of the F-subunit of *T. thermophilus* V-ATPase was also determined to 2.2 Å resolution very recently. Although the F-subunit had been proposed to have a function and structure similar to that of the F_1 ε-subunit, the structure of the F-subunit of the V-ATPase shows no structural similarity to the ε-subunit. This unique structure of the F-subunit will be described elsewhere.

CLASSIFICATION OF ROTARY ATPase/SYNTHASE

The prokaryotic V-ATPases are sometimes called archaeal-type ATPases (A-ATPase). Müller and coworkers have proposed that prokaryotic (Archaeal) H^+-ATPases and eukaryotic V-ATPases are distinct ATPase families, and that the rotary ATPase/synthase should be classified into three categories, A-, F-, and V-ATPase families (Grüber *et al.*, 2001). In their review, the prokaryotic V-ATPase (A-ATPase) was proposed to differ considerably from eukaryotic V-ATPase. However, several lines of evidences indicate the prokaryotic V/A-ATPase and eukaryotic V-ATPase are very similar, but are distinct from the F-ATPase family. Each subunit of *T. thermophilus* V-ATPase shows a significant sequence similarity to its eukaryotic counterpart. For example, amino acid homology between *T. thermophilus* V_1 subunits and yeast subunits are; the A-subunit (51% identity/69% similarity), the B-subunit (54/72%), the F-subunit (28/48%), and the F-subunit (22/38%). In contrast, the subunits of *T. thermophilus* show no apparent sequence similarity to subunits of F-ATPases except the A-, B-, and L-subunits, which are similar to β-, α-, and c-subunit of F-ATPases, respectively. The phylogenic tree analysis for catalytic subunits of both F- and V-ATPases clearly indicated that prokaryotic V/A-ATPases are much closer to eukaryotic V-ATPases than to F-ATPases (Gogarten *et al.*, 1989). Electron micrographs (EMs) also indicate a closer relationship between prokaryotic V/A-ATPase and eukaryotic V-ATPase. The F-ATPases contain only one peripheral stalk (Rubinstein *et al.*, 2003), whereas EM images of the yeast V-ATPase clearly indicate the presence of two peripheral stalks (Wilkens *et al.*, 2004). The central stalk of V-ATPase also differs from that of F-ATPase. The V-ATPase has an apparently longer central stalk than that of F-ATPase due to the large mass observed at the center of central stalk (Bernal and Stock, 2004; Chaban *et al.*, 2002; Iwata *et al.*, 2004; Wilkens *et al.*, 2004). The features in the peripheral and central stalk regions of eukaryotic V-ATPases, which are well conserved in *T. thermophilus* V/A-ATPase, are also distinct from those found in F-ATPases. Based on subunit similarity and overall structures described earlier, it is clear that prokaryotic V/A-ATPase is a member of a common V-ATPase family rather than a distinct A-ATPase family. We also think that classification of ATPase families should not be based on in vivo function alone, because these ATPases are reversible motors whether they function as ion-pumps or ATP synthases. Although *T. thermophilus* V/A-ATPase functions as an ATP synthase in vivo, it can pump protons driven by ATP hydrolysis in vitro (Yokoyama *et al.*, 2003b). Also, yeast V-ATPase, which is a proton pump in vivo, can synthesize ATP driven by an artificial proton motive force in vitro (Hirata *et al.*, 2000). Furthermore, it does not seem appropriate to link the evolution of eukaryote, archaea, and eubacteria to evolution of rotary ATPase/synthase.

Taken together, it is appropriate to classify prokaryotic V/A-ATPases into a subclass of the V-ATPase family. It is clear that the term "*A-ATPase*" is inappropriate for the reasons stated earlier. We strongly propose to use the term "prokaryotic V-ATPase" instead of "A-ATPase."

CONCLUSION

The homologs of V-type ATPase in eukaryotic cells are found in plasma membranes of archaea and some eubacteria. These prokaryotic V-ATPases are composed of nine kinds of essential subunits and function as ATP synthases or sodium pumps. Single-molecule analyses of the V-ATPase from *T. thermophilus* have established that the V-ATPase is an ATP-driven rotary motor resembling the F-ATPase. Although the detailed arrangement of subunits in V-ATPase has not been established with certainty, our recent studies have confirmed that the C-, D-, F-, and L-subunits constitute the central rotor shaft and the A-, B-, E-, G-, and I-subunits comprise the surrounding stator apparatus in the V-ATPase. The crystal structure of subunits C revealed that the V-ATPase has a quite different central stalk structure from F-ATPase. It is likely that this unique central structure plays an important role for a regulatory system of eukaryotic V-ATPase, dissociation/association of V_1 domain from V_0 domain.

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