

1 **Title: The mechanical inhibition of the isolated  $V_o$  from V-ATPase for**  
2 **proton conductance**

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2 **Abstract:** V-ATPase is an energy converting enzyme, coupling ATP hydrolysis/synthesis  
3 in the hydrophilic V<sub>1</sub> moiety, with proton flow through the V<sub>o</sub> membrane moiety, via  
4 rotation of the central rotor complex relative to the surrounding stator apparatus. Upon  
5 dissociation from the V<sub>1</sub> domain, the V<sub>o</sub> of eukaryotic V-ATPase can adopt a  
6 physiologically relevant auto-inhibited form in which proton conductance through the V<sub>o</sub>  
7 is prevented, however the molecular mechanism of this inhibition is not fully understood.  
8 Using cryo-electron microscopy, we determined the structure of both the *holo* V/A-  
9 ATPase and the isolated V<sub>o</sub> at near-atomic resolution, respectively. These structures  
10 clarify how the isolated V<sub>o</sub> adopts the auto-inhibited form and how the *holo* complex  
11 prevents the formation of this inhibited V<sub>o</sub> form.

12

13 **Short Title:** The switching mechanism of rotary V-ATPase

14 **One Sentence Summary:** Cryo-EM structures of rotary V-ATPase reveal the ON-OFF  
15 switching mechanism of H<sup>+</sup> translocation in the V<sub>o</sub> membrane domain.

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1 **Main Text:**

2 Rotary ATPase/ATP synthases, roughly classified into F type and V type  
3 ATPase, are marvelous, tiny rotary machines (1-5). These rotary motor proteins share a  
4 basic molecular architecture composed of a central rotor complex and the surrounding  
5 stator apparatus. These proteins function to couple ATP hydrolysis/synthesis in the  
6 hydrophilic  $F_1/V_1$  moiety with proton translocation through the membrane embedded  
7 hydrophobic  $F_0/V_0$  moiety by rotation of the central rotor complex relative to surrounding  
8 stator apparatus, via a rotary catalytic mechanism (Figure 1) (2-6).

9 Thus, both F and V type ATPases are capable of either ATP synthase coupled  
10 with proton motive force driven by membrane potential or proton pumping powered by  
11 ATP hydrolysis. F type ATPase (F-ATPase, or  $F_0F_1$ ) in mitochondria functions as an  
12 ATP synthase coupled to respiration, whilst in some bacteria F-ATPase can function as  
13 an ATP dependent proton pump (7, 8).

14 V type ATPase (V-ATPase, or  $V_0V_1$ ) resides mainly in the membranes of acidic  
15 vesicles in eukaryote cells, functioning as a proton pump using a rotary catalytic  
16 mechanism (3, 9, 10). Eukaryotic V-ATPases probably evolved from the prokaryotic

1 enzymes (11, 12), which are termed Archaeal ATPase or V/A-ATPase (3, 13). V/A-  
2 ATPase from a thermophilic bacterium, *Thermus thermophilus* (*Tth* V/A-ATPase) is a  
3 rotary ATPase that has been well characterized using both structure and single molecular  
4 observation studies (1, 9, 10, 14-18). The overall structure of *Tth* V/A-ATPase closely  
5 resembles that of eukaryotic V-ATPase although it lacks some of the accessory subunits  
6 of the eukaryotic enzyme (Figure 1B,C). The *Tth* V<sub>1</sub> moiety is composed of four  
7 subunits with a stoichiometry of A<sub>3</sub>B<sub>3</sub>D<sub>1</sub>F<sub>1</sub> and is responsible for ATP synthesis or  
8 hydrolysis (19, 20). Upon dissociation from V<sub>o</sub>, the isolated V<sub>1</sub> shows only ATP  
9 hydrolysis activity accompanied by rotation of the DF shaft. The *Tth* V<sub>o</sub> moiety,  
10 responsible for proton translocation across the membrane, contains a central rotor  
11 complex (*d*<sub>1</sub>*c*<sub>12</sub>) and stator apparatus made up of the *a* subunit and two EG peripheral  
12 stalks (*a*<sub>1</sub>E<sub>2</sub>G<sub>2</sub>). In *holo Tth* V/A-ATPase, proton motive force drives rotation of the  
13 *d*<sub>1</sub>*c*<sub>12</sub> rotor complex relative to the surrounding stator, resulting in rotation of the entire  
14 central rotor complex (D<sub>1</sub>F<sub>1</sub>*d*<sub>1</sub>*c*<sub>12</sub>) and inducing sequential conformation changes in the  
15 A<sub>3</sub>B<sub>3</sub> catalytic hexamer to produce three ATP molecules from ADP and inorganic  
16 phosphates per one rotation (Figure 1D).

1 Eukaryotic V-ATPase is regulated by a unique mechanism involving  
2 dissociation/association of  $V_1$ , likely to be key in controlling the pH of acidic vesicles  
3 (21-23). In yeast, glucose depletion condition in the culture medium induces  
4 dissociation of  $V_1$  domain from  $V_o$  domain resulting in reduced proton pumping activity  
5 of V-ATPase (Figure S1A). It is likely that the dissociated  $V_o$  loses the ability to  
6 translocate protons as a result of auto-inhibition. In the structure of the dissociated  $V_o$   
7 of yeast, the hydrophilic region of the  $a$  subunit ( $a_{sol}$ ) changes its conformation to prevent  
8 rotation of the rotor complex (24, 25). The yeast  $a_{sol}$  lies in close proximity to the  $d$   
9 subunit, the rotor region of the isolated yeast  $V_o$  structure. Both the  $a_{sol}$  domain and the  
10  $d$  subunit are hallmarks of the V-ATPase family and are lacking in F-ATPases (see Figure  
11 1A-C) (14). Thus, the  $a_{sol}$  and the  $d$  subunit are probably to be key in auto-inhibition of  
12 the dissociated  $V_o$ . However, the precise mechanism of  $V_o$  auto-inhibition, and thus  
13 prevention of proton leakage, is currently unknown.

14 Similar regulatory dissociation/association mechanism of V/A-ATPase in  
15 bacteria cells has not been reported, however, reconstitution experiments suggest an  
16 assembly pathway for the *holo* complex, in which the cytosolic  $V_1$  associates with the  $V_o$

1 in the membrane (Figure S1B)(26). Thus, proton leak through the  $V_o$  in the *Tth*  
2 membranes might be somehow also blocked by a similar autoinhibition mechanism to the  
3 eukaryotic enzyme. Indeed, the *Tth* V/A-ATPase and eukaryotic V-ATPase share very  
4 similar structures with both  $V_o$  moieties made up of the *a* and *d* subunits in addition to  
5 the *c* ring.

6 Structural analysis using cryogenic microscopy (cryoEM) of the *holo* V/A-  
7 ATPase, including our recent study, revealed several rotational states of the entire *holo*  
8 complex (17, 27). However, understanding of the inhibition mechanism of the isolated  
9 *Tth*  $V_o$  is currently limited due to a lack of a high resolution structure.

10 Here, we report a cryoEM structure of isolated *Tth*  $V_o$  at 3.9 Å resolution. Our  
11 results clarify the molecular mechanism of proton leak inhibition from *Tth* cells through  
12 an assembly intermediate  $V_o$  of *holo* V/A-ATPase under physiological conditions.

## 1 **CryoEM structures of the isolated $V_o$ and *holo Tth* V/A-ATPase**

2 We purified both *Tth* V/A-ATPase and  $V_o$  via a His<sub>3</sub>-tagged *c* subunit from  
3 membranes of *T. thermophilus* cells using Ni-NTA resin. For *Tth* V/A-ATPase,  
4 acquisition of micrographs was carried out using a Titan Krios equipped with a Falcon II  
5 direct electron detector. Cryo-EM micrographs of the complexes reconstituted into  
6 nanodiscs resulted in higher resolution EM maps compared with the LMNG solubilized  
7 preparation previous reported (17). The strategy of single particle analysis for *Tth* V/A-  
8 ATPase is summarized in Figure S2A. The final structure of state 1 has an overall  
9 resolution of 3.6 Å (Figure 2A). After subtraction of the EM density of the membrane  
10 embedded domain from the density of the whole complex, we obtained a focused density  
11 map of A<sub>3</sub>B<sub>3</sub>D<sub>1</sub>F<sub>1</sub>d<sub>1</sub> with two EG peripheral stalks and the soluble arm domain of the *a*  
12 subunit (*a*<sub>sol</sub>) at 3.5 Å resolution. This map allowed us to build an atomic model of  
13 A<sub>3</sub>B<sub>3</sub>D<sub>1</sub>F<sub>1</sub> (*V*<sub>1</sub>). In our map, the obvious density of ADP-Mg was observed in the closed  
14 catalytic site, but not clearly observed in semi-closed site, in contrast to our previous  
15 structure of state 1 (5Y5Y). The secondary ADP in the semi-closed site shows lower  
16 occupancy, it is due to the low affinity of the semi-closed site for nucleotide and partial

1 flexibility in the complex (Figure S3A). In the recent cryoEM map of *Tth* V/A-ATPase  
2 (6QUM), clear densities likely to correspond to ADP were observed in the cavities of the  
3 crown-like structure formed by the six  $\beta$  barrel domains of A<sub>3</sub>B<sub>3</sub> (27). In contrast, these  
4 densities were not clearly visible in our structure (Figure S3B). These differences are  
5 presumably due to the purification procedures; we purified the His-tagged *Tth* V/A-  
6 ATPase using a nickel column, while the authors of the other study isolated their *Tth* V/A-  
7 ATPase without affinity purification.

8 Purified V<sub>o</sub> reconstituted into nanodiscs was subjected to single particle  
9 analysis using a cryoEM (CRYOARM200, JEOL) equipped with a K2 summit electron  
10 direct detector in electron counting mode. The 2D class averages showed the isolated  
11 V<sub>o</sub> with clearly visible transmembrane helices and a hydrophilic domain extending above  
12 the integral membrane region (Figure S2C). The density for the scaffold proteins and  
13 lipids of the nanodiscs is clearly visible surrounding the membrane domain of the isolated  
14 V<sub>o</sub>. Following 3D classification of the V<sub>o</sub>, only one major class was identified indicating  
15 that the isolated V<sub>o</sub> is very structurally homogenous, in contrast to the *Tth* V/A-ATPase  
16 which is clearly visible in three different rotational states (17). Our 3D reconstruction



1 map of the isolated  $V_o$  was obtained with an overall resolution of 3.9 Å. The final map  
2 shows clear density for protein components of  $V_o$ , including subunit  $a$ ,  $d$ ,  $c_{12}$  ring, but the  
3 EM density for both EG stalks, which attach to the  $a_{sol}$ , is weak indicating disorder in  
4 these regions, suggesting their flexibility (Figure 2B). In this structure, the C-terminal  
5 region of the EG stalk on the distal side is visible. With the exception of these two EG  
6 stalks, side-chain densities were visible for most of the proteins in the complex, allowing  
7 construction of a *de novo* atomic model using phenix and coot (Figure 3A,B). The map  
8 contains an apparent density inside the  $c_{12}$  rotor ring, likely corresponding to the  
9 phospholipids capping the hole of the ring (Figure S4A). A further apparent density was  
10 identified in the cavity between the  $a$  subunit and the  $c_{12}$  ring on the upper periplasmic  
11 side (Figure S4B). This also might be corresponded to phospholipid and we postulate  
12 that this functions to plug the cavity between the  $a$  subunit and the  $c_{12}$  ring preventing  
13 proton leak from the periplasmic proton pathway. The densities corresponding to these  
14 phospholipids in our  $V_o$  structure are also observed in recently published cryoEM density  
15 map of the *holo* complex (27). Notably, the diameter of the  $c_{12}$  rotor ring in the isolated  
16  $V_o$  is slightly smaller than that in the *Tth* V/A-ATPase (Figure S5A). It is likely that

1 penetration of the short helix of the subunit D into the cavity of subunit *d* enlarges the  
2 diameter of the  $c_{12}$  rotor ring in the *Tth* V/A-ATPase.

### 3 **Structure comparison of the isolated $V_o$ with the *holo* complex**

4 A comparison of our structure of the isolated  $V_o$  with that of  $V_o$  moiety in *holo*  
5 complex revealed a high degree of similarity in the membrane embedded region.  
6 However, there were significant differences in the *a* subunit. The basic structure of the  
7 *a* subunit of *Tth*  $V_o$  is almost identical to the eukaryotic counterpart, with both composed  
8 of a soluble arm domain ( $a_{sol}$ ) and a C-terminal hydrophobic domain responsible for  
9 proton translocation via rotation of the  $c_{12}$  ring. The  $a_{sol}$  contains two globular  $\alpha/\beta$   
10 folding subdomains responsible for binding of both the proximal and distal EG stalks  
11 (Figure 3A and B). Both globular subdomains are connected by a hydrophilic coiled  
12 coil with a bent conformation.

13 In contrast to the structure of  $V_o$  moiety in the *holo* complex, the  $a_{sol}$  in  $V_o$  only  
14 is in close proximity to the *d* subunit as a result of kinking and twisting of the coiled coil  
15 at residues *a*/L119 and *a*/A246 (Figure 3C, indicated by the arrows). In this structure,  
16 there are several interactions between the residues in the  $a_{sol}$  and the *d* subunit (Figure

1 3D). At the proximal site, three amino acid residues, *a*/E57, *a* /H65, and *a*/Q106, form  
2 salt bridges or hydrogen bonds with residues *d*/R38, *d*/S41, and *d*/R64 in the *d* subunit,  
3 respectively. The side chain of *d*/R59 likely forms  $\pi$ - $\pi$  stacking with *a*/R103. Our  
4 structure also revealed clear connected densities between the distal subdomain of the *a*<sub>sol</sub>  
5 and the *d* subunit (Figure 3E). Four side chains, *d*/Q138, *d*/R152, *d*/R156, and *a*/R196  
6 probably form hydrogen bonds with the oxygen atoms in the main chain of *a*/E201,  
7 *a*/L144, *a*/A197, and *d*/R156, respectively. With the exception of the interaction  
8 between *a*/E57 and *d*/R38 in the proximal site, these interactions are broken by the  
9 dynamic movement of the *a*<sub>sol</sub> and conformational change of *d* subunit in the V<sub>o</sub> moiety  
10 of *holo* *Tth* V/A-ATPase. These conformational changes of the isolated V<sub>o</sub> induced by  
11 binding of V<sub>1</sub> (A<sub>3</sub>B<sub>3</sub>DF) to the V<sub>o</sub> are described in a separate section below.

## 12 **Voltage threshold for proton conductance activity of the isolated V<sub>o</sub>**

13 Our structure of the isolated V<sub>o</sub> suggests that the rotation of *c*<sub>12</sub> rotor ring  
14 relative to the stator is mechanically hindered by a defined interaction between the *a*<sub>sol</sub>  
15 and *d* subunit. To investigate this mechanical hindrance of proton conductance through  
16 the V<sub>o</sub>, we reconstituted the isolated V<sub>o</sub> into liposomes energized with a  $\Delta\psi$  generated

1 through a potassium ion ( $K^+$ )/valinomycin diffusion potential. The pH change in the  
2 liposomes was monitored with 9-Amino-6-Chloro-2-Methoxyacridine (ACMA); the  
3 emission traces at 510 nm excited at 460 nm were recorded (Figure 4). The size of the  
4 membrane potential was modulated by varying the external  $K^+$  concentration. As shown  
5 in Figure 4B, a voltage threshold was observed in that the isolated  $V_o$  shows no proton  
6 conductance at less than 120 mV of membrane potential. When the membrane potential  
7 is 130 mV or more, the proton conductance through the  $V_o$  increases in proportion to the  
8 membrane potential (Figure 4B). The reported membrane potential in bacteria cells is -  
9 75 ~ -140 mV (28). Thus, the observed inhibitory mechanism of the isolated  $V_o$  can  
10 function to prevent proton leak through the  $V_o$  under physiological conditions. In  
11 contrast to the  $V_o$ , several experiments have indicated that proton conductance through  
12  $F_o$  of bacteria does not show the threshold of membrane potential (29). Together, the  
13 observed results strongly suggest that the  $a_{sol}$  of the  $a$  subunit and the  $d$  subunit, absent in  
14  $F_o$  and hallmarks structure of the V type ATPases, are key for mechanical inhibition of  
15 proton conductance through  $V_o$ .

16 **Structure of the membrane embedded region of the isolated  $V_o$**

1 Our atomic model of  $V_o$  presented here reveals details of both proton paths formed by the  
2 membrane embedded C-terminal region of the  $a$  subunit ( $a_{CT}$ ) and its interface with the  
3  $c_{12}$  ring. The  $a_{CT}$  contains eight membrane embedded helices, MH1 to MH8. MH7  
4 and MH8 are highly tilted membrane embedded helices characteristic of rotary ATPases.  
5 The cytoplasmic hydrophilic cavity is formed by the cytoplasmic side of MH4, MH5,  
6 MH7, and MH8, and the  $c$  subunit /chainZ. The cavity is lined by polar residues,  $a/R482$ ,  
7  $a/H491$ ,  $a/H494$ ,  $a/E497$ ,  $a/Y501$ ,  $a/E550$ ,  $a/Q554$ ,  $a/T553$ ,  $a/H557$ , and  $c(Z)/Thr54$   
8 (Figure 5A), which seem to make up the cytoplasmic proton path. The periplasmic sides  
9 of MH1, MH2, MH7 and MH8 form the periplasmic hydrophilic cavity, lined with  
10  $a/D365$ ,  $a/Y368$ ,  $a/E426$ ,  $a/H452$ ,  $a/R453$ ,  $a/D455$ , and  $c(Y)/E63$ . The two hydrophilic  
11 channels are separated by a salt bridge formed between  $c(Z)/63Glu$ , a residue critical for  
12 proton translocation, and  $a/Arg563$ ,  $a/Arg622$  and  $a/Gln619$  of MH7 (Figure 5B). This  
13 salt bridge is conserved in both eukaryotic and prokaryotic  $V_o$  (25,26). In contrast the  
14 salt bridge forms between a single arginine residue and a single glutamic (or aspartic)  
15 acid residue in  $F_o(5, 30, 31)$ . Similar to the two channel model described for other rotary  
16 ATPases (32, 33), the two arginine residues on the MH7 and 8 play an important role in

1 protonation and deprotonation of the carboxy groups on the  $c_{12}$  ring, with the resulting  
2 rotation of  $dc_{12}$  driven by proton translocation from periplasmic to cytoplasmic sides.  
3 Notably, in addition to the rigid salt bridge formed between the two  $a$ /Arg residues,  $a$ /Gln  
4 and  $c$ /Glu, interactions between the  $a_{ct}$  and  $c_{12}$  ring are observed;  $a$ /Asp392 and Leu393 -  
5  $c$ (Y)/Arg49 in the loop region of the  $c$  subunit (Figure S6A), and the periplasmic sides of  
6 MH5 and MH6 are in close proximity to the C-terminal end of the  $c$  subunit (Figure S6B).  
7 Overall, our  $V_o$  structure is largely identical to the  $V_o$  moiety in *holo* complex with the  
8 exception of key alterations in hydrophilic domain (27).

### 9 **Molecular basis of the auto-inhibition of proton conductance in the isolated $V_o$**

10           The inhibition mechanism of  $V_o$  depends upon conformational changes in two  
11 subunits. In the isolated  $V_o$ , the  $d$  subunit adopts the closed form in which three side  
12 chains of the  $d$  subunit are able to interact with the distal subdomain of  $a_{sol}$ . Once the  
13 short helix of the D subunit inserts into the cavity of the  $d$  subunit, the interaction between  
14 H6 and H11 via  $d$ /R90 and  $d$ /E195 is broken (Figure 6A and Movie S1), resulting in the  
15  $d$  subunit adopting an open form where the orientation of three side chains move away  
16 from the distal subdomain of  $a_{sol}$ .

1           Another contributing factor is dynamic motion of the  $a_{\text{sol}}$  induced by binding  
2 of the distal EG stalk to the top of the  $A_3B_3$ . In the isolated  $V_o$ , the C-terminal region of  
3 the EG stalk binding onto the distal subdomain of  $a_{\text{sol}}$  is at a much steeper angle relative  
4 to the horizontal coiled coil structure of  $a_{\text{sol}}$  than that in the *holo* enzyme (Figure 6B, C  
5 and S7). Once the N-terminal globular domain of the distal EG stalk binds onto the top  
6 of  $A_3B_3$ , the angled distal EG adopts a vertical standing form, resulting in both a twisting  
7 and kinking of the coiled coil of the hydrophilic arm and the distal globular subdomain  
8 (Figure 6C, Movie S2). These dynamic motions of the  $a_{\text{sol}}$  of  $a$  subunit induces  
9 disruption of the specific interactions of  $a_{\text{sol}}$  with  $d$  subunit.

10           The isolated yeast  $V_o$  also adopts a similar inhibited conformation where the  
11  $a_{\text{sol}}$  is in close proximity to the  $d$  subunit, resulting in interaction between the stator and  
12 the rotor and inhibition of proton conductance (24, 25). Although an atomic model of  
13 yeast *holo* V-ATPase has yet to be determined, the  $a_{\text{sol}}$  is some distance from the  $d$  subunit  
14 in the  $V_o$  moiety of the poly alanine model of yeast V-ATPase (34). These structures hint  
15 at a similar conformational change in  $V_o$  induced by binding of the  $V_1$  domain as predicted  
16 in the *Tth* V/A-ATPase. Notably, the  $d$  subunit in the yeast *holo* complex adopts the

1 open form, in contrast to the *Tth*  $V_o$  where the *d* subunit is in the closed form (Figure S8).  
2 With this single exception, the eukaryotic and prokaryotic V-ATPases seem to share a  
3 similar auto-inhibited mechanism of  $V_o$  preventing proton leakage from cells or acidic  
4 vesicles. This suggests that the auto-inhibition mechanism of  $V_o$  is conserved during  
5 the evolution of V type ATPases.

6 The interaction between the  $a_{sol}$  and *d* subunit stabilizes the isolated  $V_o$  structure  
7 and protects against loss of *d*-subunit in the absence of the rotor-stator interactions  
8 mediated by  $V_1$  as a result of the dissociation of the two domains (35). This stabilization  
9 of  $V_o$  is most likely to be key for both assembly of *holo* V-type ATPase complexes and  
10 regulation of eukaryotic V-ATPase via dissociation of  $V_1$  from  $V_o$ .



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5

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11    figures. MT constructed vectors for expression of mutant proteins. TK, and KM  
12    provided technical support and conceptual advice. KY designed and supervised the  
13    experiments and wrote the manuscript. All authors discussed the results and commented  
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15    associated with this manuscript. **Data and materials availability:** The density maps  
16    and the built models for *Tth* V<sub>0</sub>V<sub>1</sub>, *Tth* V<sub>1</sub> (focused refined), and *Tth* V<sub>0</sub> were deposited



1 in EMDB (EMDB DI; 30013, 30014, and 30015) and PDB (PDB ID; 6LY8 for V<sub>1</sub> and  
2 6LY9 for isolated V<sub>o</sub>), respectively. All data is available in the main text or the  
3 supplementary materials.

4

## 5 **Supplementary Materials**

6 Materials Methods

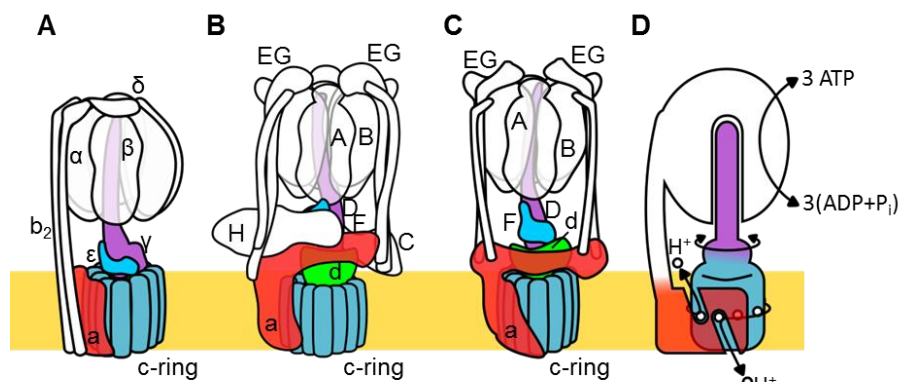
7 Figure S1-S10

8 Tables S1

9 Movies S1 and S2

10 References (36-47)

11



1

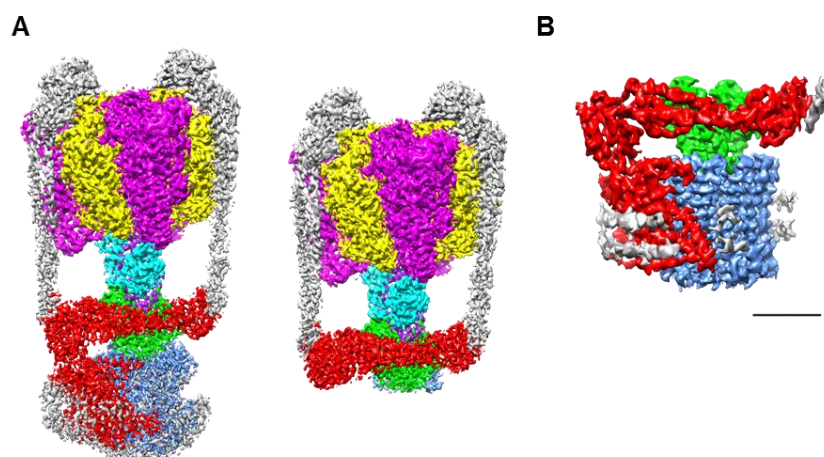
2 **Figure 1. Schematic of rotary ATPase/synthases and the rotary catalytic mechanism.**

3 A. bacterial F<sub>0</sub>F<sub>1</sub>, B. yeast V-ATPase, C. *Tth* V/A-ATPase, D. schematic model of rotary

4 catalytic mechanism. The subunits of the central rotor complex are colored: c-ring; dark

5 blue, a-subunit; red, central axis; purple and cyan, and d-subunit; green.

6



1

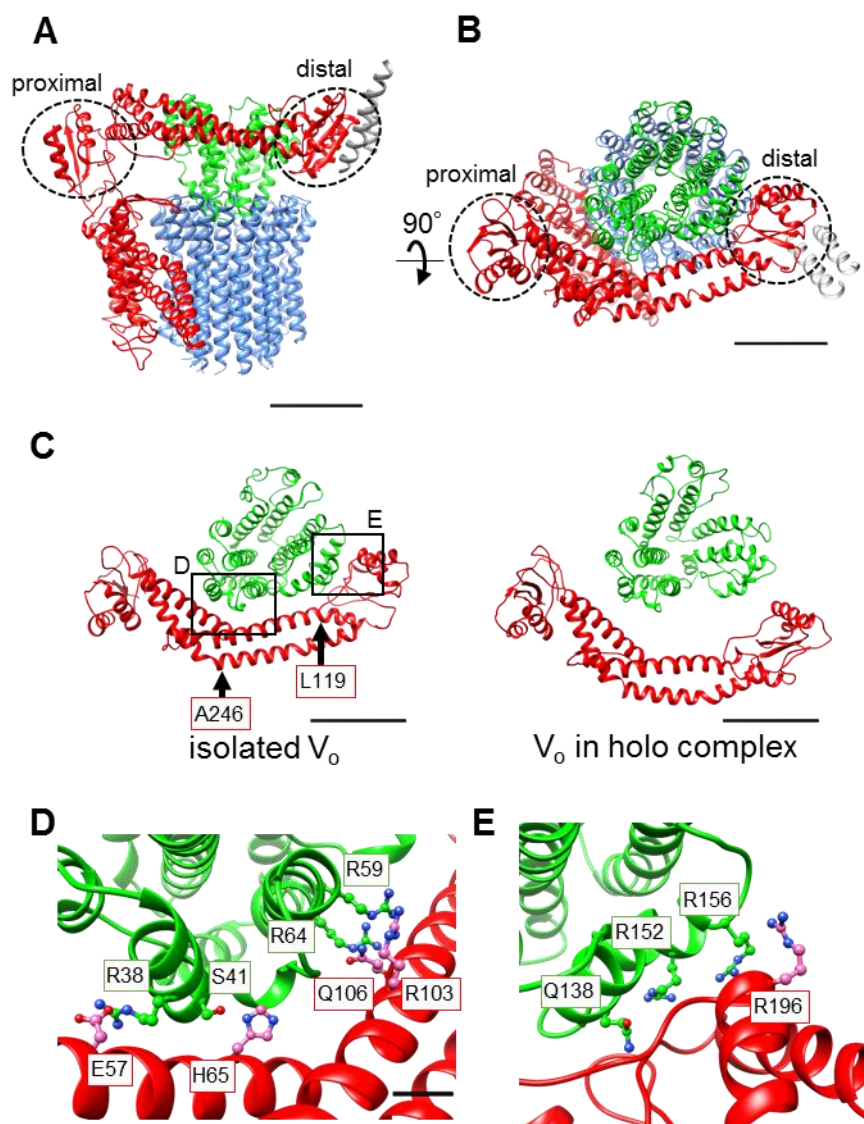
2 **Figure 2. EM density map of complex.** A. *holo Tth V/A-ATPase* (left) and focused

3 refined map of  $A_3B_3DFd(EG)_2a_{sol}$  (right) B. the isolated  $V_o$  (B). The density

4 corresponding each subunit is colored: A; magenta, B; yellow, D; purple, F; cyan, E and

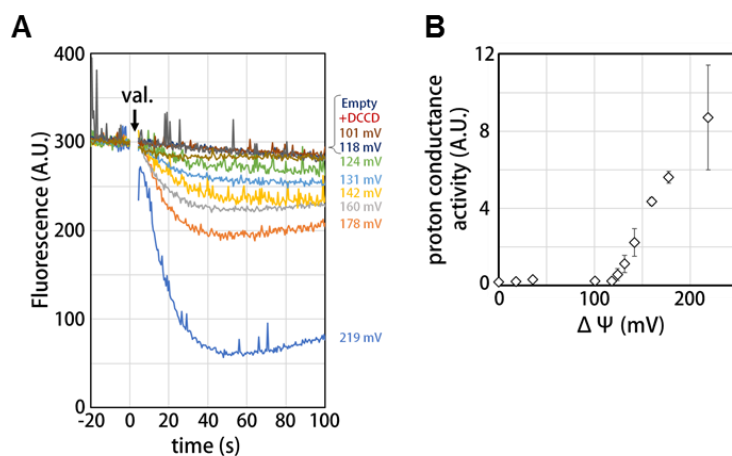
5 G; gray, *a*; red, *d*; green, and *c*; dark blue. Scale bar; 30 Å.

6



1  
 2 **Figure 3. Atomic model of the isolated  $V_0$ .** A. Side view and B. Upper view of  $a$ -,  $d$ -,  
 3  $c$ -, and EG subunits colored as in Figure 2, respectively. Scale bar represents 30 Å.  
 4 The proximal and distal subdomains of  $a$ -subunit are circled by the dotted lines. C.  
 5 Comparison of the relative positions of  $a_{sol}$  (red) and the d subunit (green) in the isolated  
 6  $V_0$  (left) and the  $V_0$  moiety in the *holo* complex (right). Arrows indicate the kinking and

1 twisting points in the  $a_{\text{sol}}$  in isolated  $V_o$ . Scale bar represents 30 Å. D, E. Specific  
2 interactions between the  $a_{\text{sol}}$  and  $d$  subunit at proximal (D) and distal (E) regions. The  
3 regions are specified in black squares in C. The residues are represented as balls and  
4 sticks. Scale bar; 5 Å.  
5



1

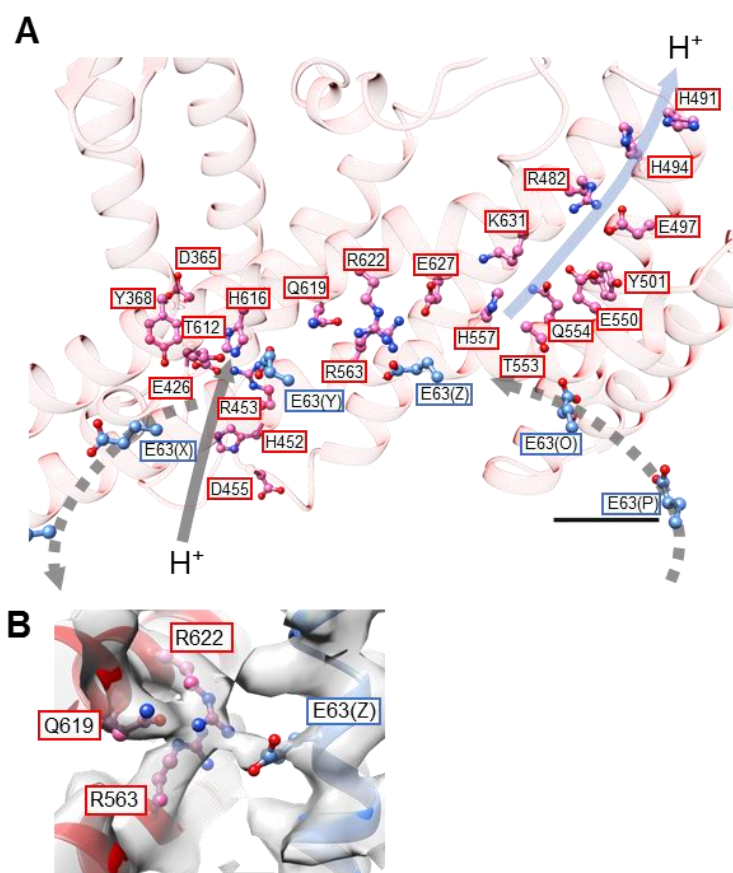
2 **Figure 4. Proton conductance through the isolated  $V_o$ .** A. Changes of fluorescence

3 of ACMA due to pH changes inside the  $V_o$  proteo-liposomes. Membrane potential ( $\Delta\Psi$ )

4 values were estimated by the Nernst equation;  $\Delta\Psi = \frac{RT}{zF} \ln \frac{[KCl]_o}{[KCl]_i}$ , described in

5 the Methods section. B. Voltage threshold of the proton conductance through the  $V_o$ .

6



1

2 **Figure 5. Structure of the hydrophobic domain of the isolated  $V_o$ .** A. Proton paths

3 on both the cytoplasmic and periplasmic sides of the isolated  $V_o$ . Residues lining the

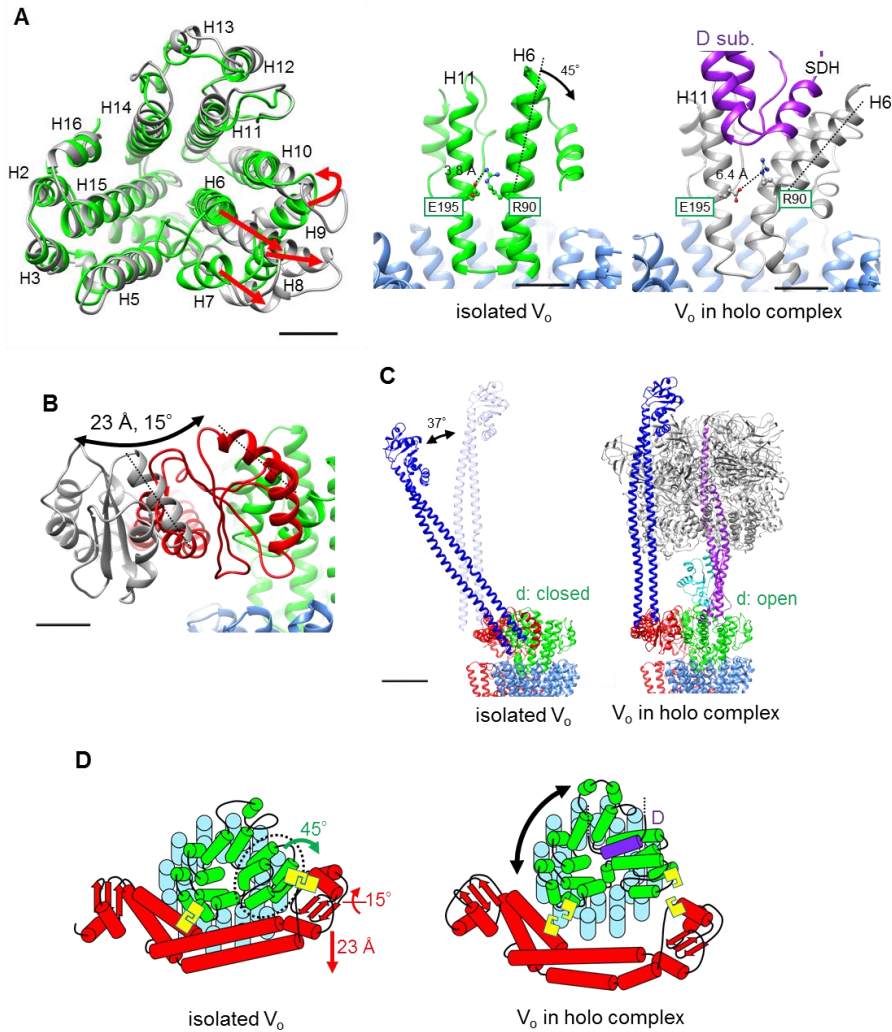
4 paths are represented as balls and sticks. Residues from the *a*-subunit and *c*-subunit are

5 indicated in the red and blue boxes, respectively. Proton flow from the periplasmic side

6 is represented by the grey arrow as it would occur in the case of ATP synthesis. Scale

7 bar; 10 Å. B. Salt bridge between *a*/Arg563, Arg622, Gln619 and *c*/Glu63. Scale

8 bar; 3 Å.



1  
 2 **Figure 6. Conformational changes occurring in both the *d*- and *a*<sub>sol</sub> subunits as a**  
 3 **result of binding of  $V_1$  to  $V_0$ .** A. Structural changes in the *d* subunit caused by insertion  
 4 of the screw driver helix (SDH). (left) Top view of *d*-subunit. The *d*-subunit from the  
 5 isolated  $V_0$  and the *holo* enzyme are colored in green and grey, respectively. Red arrows  
 6 indicate the movements of helices 6-9 (H6-9). (center, right) Key helices (H6 and 11)  
 7 of *d*-subunit in the isolated  $V_0$  and the *holo* complex. The H6 bends 45° as a result of



- 1 binding of SDH of D-subunit. B. Structural change of the distal subdomain of  $a_{\text{sol}}$ .
- 2 Upon the pivoting movement of  $a_{\text{sol}}$  on the proximal subdomain, the distal subdomain
- 3 swings 25 Å and twist 15° between the isolated  $V_o$  (red) and the *holo* complex (gray).
- 4 C. EG structure in the distal subdomain of  $a_{\text{sol}}$  (EG<sub>d</sub>) in the isolated  $V_o$  (left) and in the
- 5 *holo* complex (right). D. Schematic representation of the mechanical inhibition of the
- 6  $V_o$  induced by dissociation of  $V_1$ . In isolated  $V_o$ , the rotation of central rotor is inhibited
- 7 by interactions between *d*- and  $a_{\text{sol}}$  (yellow box, Figure 3D, E).