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

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Abstract: V-ATPase is an energy converting enzyme, coupling ATP hydrolysis/synthesis in the hydrophilic $V_1$ moiety, with proton flow through the $V_o$ membrane moiety, via rotation of the central rotor complex relative to the surrounding stator apparatus. Upon dissociation from the $V_1$ domain, the $V_o$ of eukaryotic V-ATPase can adopt a physiologically relevant auto-inhibited form in which proton conductance through the $V_o$ is prevented, however the molecular mechanism of this inhibition is not fully understood. Using cryo-electron microscopy, we determined the structure of both the holo V/A-ATPase and the isolated $V_o$ at near-atomic resolution, respectively. These structures clarify how the isolated $V_o$ adopts the auto-inhibited form and how the holo complex prevents the formation of this inhibited $V_o$ form.

Short Title: The switching mechanism of rotary V-ATPase

One Sentence Summary: Cryo-EM structures of rotary V-ATPase reveal the ON-OFF switching mechanism of $H^+$ translocation in the $V_o$ membrane domain.
Main Text:

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

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

V type ATPase (V-ATPase, or $V_0V_1$) resides mainly in the membranes of acidic vesicles in eukaryote cells, functioning as a proton pump using a rotary catalytic mechanism (3, 9, 10). Eukaryotic V-ATPases probably evolved from the prokaryotic
enzymes (11, 12), which are termed Archaeal ATPase or V/A-ATPase (3, 13). V/A-
ATPase from a thermophilic bacterium, *Thermus thermophilus* (*Tth* V/A-ATPase) is a
rotary ATPase that has been well characterized using both structure and single molecular
observation studies (1, 9, 10, 14-18). The overall structure of *Tth* V/A-ATPase closely
resembles that of eukaryotic V-ATPase although it lacks some of the accessory subunits
of the eukaryotic enzyme (Figure 1B, C). The *Tth* V\textsubscript{1} moiety is composed of four
subunits with a stoichiometry of A\textsubscript{3}B\textsubscript{3}D\textsubscript{1}F\textsubscript{1} and is responsible for ATP synthesis or
hydrolysis (19, 20). Upon dissociation from V\textsubscript{o}, the isolated V\textsubscript{1} shows only ATP
hydrolysis activity accompanied by rotation of the DF shaft. The *Tth* V\textsubscript{o} moiety,
responsible for proton translocation across the membrane, contains a central rotor
complex (d\textsubscript{1}c\textsubscript{12}) and stator apparatus made up of the a subunit and two EG peripheral
stalks (a\textsubscript{1}E\textsubscript{2}G\textsubscript{2}). In *holo* *Tth* V/A-ATPase, proton motive force drives rotation of the
d\textsubscript{1}c\textsubscript{12} rotor complex relative to the surrounding stator, resulting in rotation of the entire
central rotor complex (D\textsubscript{1}F\textsubscript{1}d\textsubscript{1}c\textsubscript{12}) and inducing sequential conformation changes in the
A\textsubscript{3}B\textsubscript{3} catalytic hexamer to produce three ATP molecules from ADP and inorganic
phosphates per one rotation (Figure 1D).
Eukaryotic V-ATPase is regulated by a unique mechanism involving dissociation/association of V₁, likely to be key in controlling the pH of acidic vesicles (21-23). In yeast, glucose depletion condition in the culture medium induces dissociation of V₁ domain from V₀ domain resulting in reduced proton pumping activity of V-ATPase (Figure S1A). It is likely that the dissociated V₀ loses the ability to translocate protons as a result of auto-inhibition. In the structure of the dissociated V₀ of yeast, the hydrophilic region of the a subunit (aₚ) changes its conformation to prevent rotation of the rotor complex (24, 25). The yeast aₚ lies in close proximity to the d subunit, the rotor region of the isolated yeast V₀ structure. Both the aₚ domain and the d subunit are hallmarks of the V-ATPase family and are lacking in F-ATPases (see Figure 1A-C) (14). Thus, the aₚ and the d subunit are probably to be key in auto-inhibition of the dissociated V₀. However, the precise mechanism of V₀ auto-inhibition, and thus prevention of proton leakage, is currently unknown.

Similar regulatory dissociation/association mechanism of V/A-ATPase in bacteria cells has not been reported, however, reconstitution experiments suggest an assembly pathway for the holo complex, in which the cytosolic V₁ associates with the V₀
in the membrane (Figure S1B)(26). Thus, proton leak through the \( V_o \) in the \( Tth \) membranes might be somehow also blocked by a similar autoinhibition mechanism to the eukaryotic enzyme. Indeed, the \( Tth \) V/A-ATPase and eukaryotic V-ATPase share very similar structures with both \( V_o \) moieties made up of the \( a \) and \( d \) subunits in addition to the \( c \) ring.

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

Here, we report a cryoEM structure of isolated \( Tth \) \( V_o \) at 3.9 Å resolution. Our results clarify the molecular mechanism of proton leak inhibition from \( Tth \) cells through an assembly intermediate \( V_o \) of \textit{holo} V/A-ATPase under physiological conditions.
CryoEM structures of the isolated V₀ and holo Tth V/A-ATPase

We purified both Tth V/A-ATPase and V₀ via a His₃-tagged c subunit from membranes of *T. thermophilus* cells using Ni-NTA resin. For Tth V/A-ATPase, acquisition of micrographs was carried out using a Titan Krios equipped with a Falcon II direct electron detector. Cryo-EM micrographs of the complexes reconstituted into nanodiscs resulted in higher resolution EM maps compared with the LMNG solubilized preparation previous reported (17). The strategy of single particle analysis for Tth V/A-ATPase is summarized in Figure S2A. The final structure of state 1 has an overall resolution of 3.6 Å (Figure 2A). After subtraction of the EM density of the membrane embedded domain from the density of the whole complex, we obtained a focused density map of A₃B₃D₁F₁d₁ with two EG peripheral stalks and the soluble arm domain of the a subunit (*a*ₘₐₙ) at 3.5 Å resolution. This map allowed us to build an atomic model of A₃B₃D₁F₁ (V₁). In our map, the obvious density of ADP-Mg was observed in the closed catalytic site, but not clearly observed in semi-closed site, in contrast to our previous structure of state 1 (5Y5Y). The secondary ADP in the semi-closed site shows lower occupancy, it is due to the low affinity of the semi-closed site for nucleotide and partial
flexibility in the complex (Figure S3A). In the recent cryoEM map of *Tth* V/A-ATPase (6QUM), clear densities likely to correspond to ADP were observed in the cavities of the crown-like structure formed by the six β barrel domains of A3B3 (27). In contrast, these densities were not clearly visible in our structure (Figure S3B). These differences are presumably due to the purification procedures; we purified the His-tagged *Tth* V/A-ATPase using a nickel column, while the authors of the other study isolated their *Tth* V/A-ATPase without affinity purification.

Purified *V*o reconstituted into nanodiscs was subjected to single particle analysis using a cryoEM (CRYOARM200, JEOL) equipped with a K2 summit electron direct detector in electron counting mode. The 2D class averages showed the isolated *V*o with clearly visible transmembrane helices and a hydrophilic domain extending above the integral membrane region (Figure S2C). The density for the scaffold proteins and lipids of the nanodiscs is clearly visible surrounding the membrane domain of the isolated *V*o. Following 3D classification of the *V*o, only one major class was identified indicating that the isolated *V*o is very structurally homogenous, in contrast to the *Tth* V/A-ATPase which is clearly visible in three different rotational states (17). Our 3D reconstruction
map of the isolated $V_o$ was obtained with an overall resolution of 3.9 Å. The final map shows clear density for protein components of $V_o$, including subunit $a$, $d$, $c_{12}$ ring, but the EM density for both EG stalks, which attach to the $a_{sol}$, is weak indicating disorder in these regions, suggesting their flexibility (Figure 2B). In this structure, the C-terminal region of the EG stalk on the distal side is visible. With the exception of these two EG stalks, side-chain densities were visible for most of the proteins in the complex, allowing construction of a de novo atomic model using phenix and coot (Figure 3A,B). The map contains an apparent density inside the $c_{12}$ rotor ring, likely corresponding to the phospholipids capping the hole of the ring (Figure S4A). A further apparent density was identified in the cavity between the $a$ subunit and the $c_{12}$ ring on the upper periplasmic side (Figure S4B). This also might be corresponded to phospholipid and we postulate that this functions to plug the cavity between the $a$ subunit and the $c_{12}$ ring preventing proton leak from the periplasmic proton pathway. The densities corresponding to these phospholipids in our $V_o$ structure are also observed in recently published cryoEM density map of the holo complex (27). Notably, the diameter of the $c_{12}$ rotor ring in the isolated $V_o$ is slightly smaller than that in the $Tth$ V/A-ATPase (Figure S5A). It is likely that
penetration of the short helix of the subunit D into the cavity of subunit d enlarges the
diameter of the c_{12} rotor ring in the Tth V/A-ATPase.

Structure comparison of the isolated V_o with the holo complex

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

In contrast to the structure of V_o moiety in the holo complex, the a_{sol} in V_o only
is in close proximity to the d subunit as a result of kinking and twisting of the coiled coil
at residues a/L119 and a/A246 (Figure 3C, indicated by the arrows). In this structure,
there are several interactions between the residues in the a_{sol} and the d subunit (Figure
At the proximal site, three amino acid residues, $\alpha$/E57, $\alpha$/H65, and $\alpha$/Q106, form salt bridges or hydrogen bonds with residues $\delta$/R38, $\delta$/S41, and $\delta$/R64 in the $\delta$ subunit, respectively. The side chain of $\delta$/R59 likely forms $\pi$-$\pi$ stacking with $\alpha$/R103. Our structure also revealed clear connected densities between the distal subdomain of the $\alpha$$_{sol}$ and the $\delta$ subunit (Figure 3E). Four side chains, $\delta$/Q138, $\delta$/R152, $\delta$/R156, and $\alpha$/R196 probably form hydrogen bonds with the oxygen atoms in the main chain of $\alpha$/E201, $\alpha$/L144, $\alpha$/A197, and $\delta$/R156, respectively. With the exception of the interaction between $\alpha$/E57 and $\delta$/R38 in the proximal site, these interactions are broken by the dynamic movement of the $\alpha$$_{sol}$ and conformational change of $\delta$ subunit in the V$_o$ moiety of holo Tth V/A-ATPase. These conformational changes of the isolated V$_o$ induced by binding of V$_1$ (A$_3$B$_3$DF) to the V$_o$ are described in a separate section below.

**Voltage threshold for proton conductance activity of the isolated V$_o$**

Our structure of the isolated V$_o$ suggests that the rotation of $c_{12}$ rotor ring relative to the stator is mechanically hindered by a defined interaction between the $\alpha$$_{sol}$ and $\delta$ subunit. To investigate this mechanical hindrance of proton conductance through the V$_o$, we reconstituted the isolated V$_o$ into liposomes energized with a $\Delta\psi$ generated...
through a potassium ion (K⁺)/valinomycine diffusion potential. The pH change in the liposomes was monitored with 9-Amino-6-Chloro-2-Methoxyacridine (ACMA); the emission traces at 510 nm excited at 460 nm were recorded (Figure 4). The size of the membrane potential was modulated by varying the external K⁺ concentration. As shown in Figure 4B, a voltage threshold was observed in that the isolated Vₒ shows no proton conductance at less than 120 mV of membrane potential. When the membrane potential is 130 mV or more, the proton conductance through the Vₒ increases in proportion to the membrane potential (Figure 4B). The reported membrane potential in bacteria cells is -75 ~ -140 mV (28). Thus, the observed inhibitory mechanism of the isolated Vₒ can function to prevent proton leak through the Vₒ under physiological conditions. In contrast to the Vₒ, several experiments have indicated that proton conductance through Fₒ of bacteria does not show the threshold of membrane potential (29). Together, the observed results strongly suggest that the aₛₚₒ of the a subunit and the d subunit, absent in Fₒ and hallmarks structure of the V type ATPases, are key for mechanical inhibition of proton conductance through Vₒ.

**Structure of the membrane embedded region of the isolated Vₒ**
Our atomic model of $V_0$ presented here reveals details of both proton paths formed by the membrane embedded C-terminal region of the $a$ subunit ($a_{CT}$) and its interface with the $c_{12}$ ring. The $a_{CT}$ contains eight membrane embedded helices, MH1 to MH8. MH7 and MH8 are highly tilted membrane embedded helices characteristic of rotary ATPases.

The cytoplasmic hydrophilic cavity is formed by the cytoplasmic side of MH4, MH5, MH7, and MH8, and the $c$ subunit /chainZ. The cavity is lined by polar residues, $a/R482$, $a/H491$, $a/H494$, $a/H497$, $a/E497$, $a/Y501$, $a/E550$, $a/Q554$, $a/T553$, $a/H557$, and $c(Z)/Thr54$ (Figure 5A), which seem to make up the cytoplasmic proton path. The periplasmic sides of MH1, MH2, MH7 and MH8 form the periplasmic hydrophilic cavity, lined with $a/D365$, $a/Y368$, $a/E426$, $a/H452$, $a/R453$, $a/D455$, and $c(Y)/E63$. The two hydrophilic channels are separated by a salt bridge formed between $c(Z)/63Glu$, a residue critical for proton translocation, and $a/Arg563$, $a/Arg622$ and $a/Gln619$ of MH7 (Figure 5B). This salt bridge is conserved in both eukaryotic and prokaryotic $V_0$ (25,26). In contrast the salt bridge forms between a single arginine residue and a single glutamic (or aspartic) acid residue in $F_o$(5, 30, 31). Similar to the two channel model described for other rotary ATPases (32, 33), the two arginine residues on the MH7 and 8 play an important role in
protonation and deprotonation of the carboxy groups on the $c_{12}$ ring, with the resulting rotation of $dc_{12}$ driven by proton translocation from periplasmic to cytoplasmic sides.

Notably, in addition to the rigid salt bridge formed between the two $a$/Arg residues, $a$/Gln and $c$/Glu, interactions between the $a_{ct}$ and $c_{12}$ ring are observed; $a$/Asp392 and Leu393 - $c$(Y)/Arg49 in the loop region of the $c$ subunit (Figure S6A), and the periplasmic sides of MH5 and MH6 are in close proximity to the C-terminal end of the $c$ subunit (Figure S6B).

Overall, our $V_o$ structure is largely identical to the $V_o$ moiety in holo complex with the exception of key alterations in hydrophilic domain (27).

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

The inhibition mechanism of $V_o$ depends upon conformational changes in two subunits. In the isolated $V_o$, the $d$ subunit adopts the closed form in which three side chains of the $d$ subunit are able to interact with the distal subdomain of $a_{sol}$. Once the short helix of the D subunit inserts into the cavity of the $d$ subunit, the interaction between H6 and H11 via $d$/R90 and $d$/E195 is broken (Figure 6A and Movie S1), resulting in the $d$ subunit adopting an open form where the orientation of three side chains move away from the distal subdomain of $a_{sol}$. 
Another contributing factor is dynamic motion of the $a_{sol}$ induced by binding of the distal EG stalk to the top of the A$_3$B$_3$. In the isolated V$_o$, the C-terminal region of the EG stalk binding onto the distal subdomain of $a_{sol}$ is at a much steeper angle relative to the horizontal coiled coil structure of $a_{sol}$ than that in the holo enzyme (Figure 6B, C and S7). Once the N-terminal globular domain of the distal EG stalk binds onto the top of A$_3$B$_3$, the angled distal EG adopts a vertical standing form, resulting in both a twisting and kinking of the coiled coil of the hydrophilic arm and the distal globular subdomain (Figure 6C, Movie S2). These dynamic motions of the $a_{sol}$ of a subunit induces disruption of the specific interactions of $a_{sol}$ with d subunit.

The isolated yeast V$_o$ also adopts a similar inhibited conformation where the $a_{sol}$ is in close proximity to the d subunit, resulting in interaction between the stator and the rotor and inhibition of proton conductance (24, 25). Although an atomic model of yeast holo V-ATPase has yet to be determined, the $a_{sol}$ is some distance from the d subunit in the V$_o$ moiety of the poly alanine model of yeast V-ATPase (34). These structures hint at a similar conformational change in V$_o$ induced by binding of the V$_1$ domain as predicted in the *Tth* V/A-ATPase. Notably, the d subunit in the yeast holo complex adopts the
open form, in contrast to the *Tih* V<sub>o</sub> where the *d* subunit is in the closed form (Figure S8).

With this single exception, the eukaryotic and prokaryotic V-ATPases seem to share a similar auto-inhibited mechanism of V<sub>o</sub> preventing proton leakage from cells or acidic vesicles. This suggests that the auto-inhibition mechanism of V<sub>o</sub> is conserved during the evolution of V type ATPases.

The interaction between the *a<sub>sol</sub>* and *d* subunit stabilizes the isolated V<sub>o</sub> structure and protects against loss of *d*-subunit in the absence of the rotor-stator interactions mediated by V<sub>1</sub> as a result of the dissociation of the two domains (35). This stabilization of V<sub>o</sub> is most likely to be key for both assembly of holo V-type ATPase complexes and regulation of eukaryotic V-ATPase via dissociation of V<sub>1</sub> from V<sub>o</sub>. 
References and Notes

1. K. Yokoyama, H. Imamura, Rotation, structure, and classification of prokaryotic V-ATPase. *J. Bioenerg. Biomembr.* **37**, 405-410 (2005)

2. K. Kinosita, Jr., F(1)-ATPase: a prototypical rotary molecular motor. *Adv. Exp. Med. Biol.* **726**, 5-16 (2012)

3. M. Forgac, Vacuolar ATPases: rotary proton pumps in physiology and pathophysiology. *Nat. Rev. Mol. Cell. Biol.* **8**, 917-929 (2007)

4. M. Yoshida, E. Muneyuki, T. Hisabori, ATP synthase - A marvellous rotary engine of the cell. *Nat. Rev. Mol. Cell. Bio.* **2**, 669-677 (2001)

5. W. Kuhlbrandt, Structure and Mechanisms of F-Type ATP Synthases. *Annu. Rev. Biochem.* **88**, 515-549 (2019)

6. H. Guo, J. L. Rubinstein, Cryo-EM of ATP synthases. *Curr. Opin. Struct. Biol.* **52**, 71-79 (2018)

7. C. Shibata, T. Ehara, K. Tomura, K. Igarashi, H. Kobayashi, Gene structure of *Enterococcus hirae* (*Streptococcus faecalis*) F1F0-ATPase, which functions as a regulator of cytoplasmic pH. *J. Bacteriol.* **174**, 6117-6124 (1992)
1. M. J. Kullen, T. R. Klaenhammer, Identification of the pH-inducible, proton-translocating F1F0-ATPase (atpBEFHAGDC) operon of Lactobacillus acidophilus by differential display: gene structure, cloning and characterization. *Mol. Microbiol.* **33**, 1152-1161 (1999)

2. K. Yokoyama, M. Nakano, H. Imamura, M. Yoshida, M. Tamakoshi, Rotation of the proteolipid ring in the V-ATPase. *J. Biol. Chem.* **278**, 24255-24258 (2003)

3. H. Imamura, M. Nakano, H. Noji, E. Muneyuki, S. Ohkuma, M. Yoshida, K. Yokoyama, Evidence for rotation of V1-ATPase. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 2312-2315 (2003)

4. J. P. Gogarten, H. Kibak, P. Dittrich, L. Taiz, E. J. Bowman, B. J. Bowman, M. F. Manolson, R. J. Poole, T. Date, T. Oshima, J. Konishi, K. Denda, M. Yoshida, Evolution of the vacuolar H+-ATPase: implications for the origin of eukaryotes. *Proc. Natl. Acad. Sci. U. S. A.* **86**, 6661-6665 (1989)

5. S. Tsutsumi, K. Denda, K. Yokoyama, T. Oshima, T. Date, M. Yoshida, Molecular cloning of genes encoding major two subunits of a eubacterial V-type ATPase from *Thermus thermophilus*. *Biochim. Biophys. Acta.* **1098**, 13-20 (1991)
13. W. Kuhlbrandt, K. M. Davies, Rotary ATPases: A New Twist to an Ancient Machine. *Trends Biochem. Sci.* **41**, 106-116 (2016)

14. M. Iwata, H. Imamura, E. Stambouli, C. Ikeda, M. Tamakoshi, K. Nagata, H. Makyio, B. Hankamer, J. Barber, M. Yoshida, K. Yokoyama, S. Iwata, Crystal structure of a central stalk subunit C and reversible association/dissociation of vacuole-type ATPase. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 59-64 (2004)

15. H. Makyio, R. Iino, C. Ikeda, H. Imamura, M. Tamakoshi, M. Iwata, D. Stock, R. A. Bernal, E. P. Carpenter, M. Yoshida, K. Yokoyama, S. Iwata, Structure of a central stalk subunit F of prokaryotic V-type ATPase/synthase from *Thermus thermophilus*. *EMBO J.* **24**, 3974-3983 (2005)

16. M. Toei, C. Gerle, M. Nakano, K. Tani, N. Gyobu, M. Tamakoshi, N. Sone, M. Yoshida, Y. Fujiyoshi, K. Mitsuoka, K. Yokoyama, Dodecamer rotor ring defines H⁺/ATP ratio for ATP synthesis of prokaryotic V-ATPase from *Thermus thermophilus*. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 20256-20261 (2007)

17. A. Nakanishi, J. Kishikawa, M. Tamakoshi, K. Mitsuoka, K. Yokoyama, Cryo EM structure of intact rotary H(+)−ATPase/synthase from *Thermus thermophilus*. *Nat.*
18. D. G. Schep, J. Zhao, J. L. Rubinstein, Models for the a subunits of the *Thermus thermophilus* V/A-ATPase and *Saccharomyces cerevisiae* V-ATPase enzymes by cryo-EM and evolutionary covariance. *Proc. Natl. Acad. Sci. U. S. A.* **113**, 3245-3250 (2016)

19. K. Yokoyama, E. Muneyuki, T. Amano, S. Mizutani, M. Yoshida, M. Ishida, S. Ohkuma, V-ATPase of *Thermus thermophilus* is inactivated during ATP hydrolysis but can synthesize ATP. *J. Biol. Chem.* **273**, 20504-20510 (1998)

20. K. Yokoyama, T. Oshima, M. Yoshida, *Thermus thermophilus* membrane-associated ATPase. Indication of a eubacterial V-type ATPase. *J. Biol. Chem.* **265**, 21946-21950 (1990)

21. P. M. Kane, K. J. Parra, Assembly and regulation of the yeast vacuolar H(+) ATPase. *J. Exp. Biol.* **203**, 81-87 (2000)

22. S. Sharma, R. A. Oot, M. M. Khan, S. Wilkens, Functional reconstitution of vacuolar H(+)-ATPase from Vo proton channel and mutant V¹-ATPase provides insight into the mechanism of reversible disassembly. *J. Biol. Chem.* **294**, 6439-
23. M. Toei, R. Saum, M. Forgac, Regulation and isoform function of the V-ATPases. *Biochemistry* **49**, 4715-4723 (2010)

24. S. H. Roh, N. J. Stam, C. F. Hryc, S. Couoh-Cardel, G. Pintilie, W. Chiu, S. Wilkens, The 3.5-A CryoEM Structure of Nanodisc-Reconstituted Yeast Vacuolar ATPase Vo Proton Channel. *Mol. Cell.* **69**, 993-1004 e1003 (2018)

25. M. T. Mazhab-Jafari, A. Rohou, C. Schmidt, S. A. Bueler, S. Benlekbir, C. V. Robinson, J. L. Rubinstein, Atomic model for the membrane-embedded VO motor of a eukaryotic V-ATPase. *Nature* **539**, 118-122 (2016)

26. J. Kishikawa, K. Yokoyama, Reconstitution of vacuolar-type rotary H⁺-ATPase/synthase from *Thermus thermophilus*. *J. Biol. Chem.* **287**, 24597-24603 (2012)

27. L. Zhou, L. A. Sazanov, Structure and conformational plasticity of the intact *Thermus thermophilus* V/A-type ATPase. *Science* **365**, eaaw9144 (2019)

28. C. J. Lo, M. C. Leake, T. Pilizota, R. M. Berry, Nonequivalence of membrane voltage and ion-gradient as driving forces for the bacterial flagellar motor at low
29. A. Wiedenmann, P. Dimroth, C. von Ballmoos, Deltapsi and DeltapH are equivalent driving forces for proton transport through isolated F(0) complexes of ATP synthases. *Biochim. Biophys. Acta.* **1777**, 1301-1310 (2008)

30. B. J. Murphy, N. Klusch, J. Langer, D. J. Mills, Ö. Yildiz, W. Kühlbrandt W, Rotary substates of mitochondrial ATP synthase reveal the basis of flexible F1-Fo coupling. *Science* **364**, eaaw9128 (2019)

31. H. Guo, T. Suzuki, J. L. Rubinstein, Structure of a bacterial ATP synthase. *Elife* **8**, e43128 (2019)

32. A. P. Srivastava, M. Luo, W. Zhou, J. Symersky, D. Bai, M. G. Chambers, J. D. Faraldo-Gómez, M. Liao, D. M. Mueller, High-resolution cryo-EM analysis of the yeast ATP synthase in a lipid membrane. *Science* **360**, eaas9699 (2018)

33. A. Hahn, J. Vonck, D. J. Mills, T. Meier, W. Kuhlbrandt, Structure, mechanism, and regulation of the chloroplast ATP synthase. *Science* **360**, eaat4318 (2018)

34. J. Zhao, S. Benlekbir, J. L. Rubinstein, Electron cryomicroscopy observation of rotational states in a eukaryotic V-ATPase. *Nature* **521**, 241-245 (2015)
B. Ediger, S. D. Melman, D. L. Pappas Jr., M. Finch, J. Applen, K. J. Parra, The tether connecting cytosolic (N terminus) and membrane (C terminus) domains of yeast V-ATPase subunit a (Vph1) is required for assembly of V0 subunit d. *J. Biol. Chem.* **284**, 19522-19532 (2009). doi: 10.1074/jbc.M109.013375
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Author contributions: JK, and AN designed, performed and analyzed the experiments. JK, AN, AF, TK, and KM analyzed the data and contributed to the preparation of the figures. MT constructed vectors for expression of mutant proteins. TK, and KM provided technical support and conceptual advice. KY designed and supervised the experiments and wrote the manuscript. All authors discussed the results and commented on the manuscript. Competing interests: The authors declare no conflicts of interest associated with this manuscript. Data and materials availability: The density maps and the built models for _Tth VoV1_, _Tth V1_ (focused refined), and _Tth Vo_ were deposited.
in EMDB (EMDB DI; 30013, 30014, and 30015) and PDB (PDB ID; 6LY8 for V₁ and 6LY9 for isolated V₀), respectively. All data is available in the main text or the supplementary materials.

**Supplemental Materials**

Materials Methods

Figure S1-S10

Tables S1

Movies S1 and S2

References (36-47)
Figure 1. Schematic of rotary ATPase/synthases and the rotary catalytic mechanism.

A. bacterial F$_0$F$_1$, B. yeast V-ATPase, C. Tth V/A-ATPase, D. schematic model of rotary catalytic mechanism. The subunits of the central rotor complex are colored: c-ring; dark blue, a-subunit; red, central axis; purple and cyan, and d-subunit; green.
Figure 2. EM density map of complex. A. *holo Tth* V/A-ATPase (left) and focused refined map of A$_3$B$_3$DF$_d$(EG)$_2$a$_{sol}$ (right) B. the isolated V$_o$ (B). The density corresponding each subunit is colored: A; magenta, B; yellow, D; purple, F; cyan, E and G; gray, a; red, d; green, and c; dark blue. Scale bar; 30 Å.
Figure 3. Atomic model of the isolated $V_0$.  A. Side view and B. Upper view of $a$, $d$, $c$, and EG subunits colored as in Figure 2, respectively.  Scale bar represents 30 Å.  The proximal and distal subdomains of $a$-subunit are circled by the dotted lines.  C. Comparison of the relative positions of $a_{sol}$ (red) and the $d$ subunit (green) in the isolated $V_0$ (left) and the $V_0$ moiety in the holo complex (right).  Arrows indicate the kinking and
twisting points in the $a_{sol}$ in isolated $V_0$. Scale bar represents 30 Å. D, E. Specific interactions between the $a_{sol}$ and $d$ subunit at proximal (D) and distal (E) regions. The regions are specified in black squares in C. The residues are represented as balls and sticks. Scale bar; 5 Å.
Figure 4. Proton conductance through the isolated $V_0$. A. Changes of fluorescence of ACMA due to pH changes inside the $V_0$ proteo-liposomes. Membrane potential ($\Delta \Psi$) values were estimated by the Nernst equation; $\Delta \Psi = RF/\epsilon F \ln[KCl]_o/[KCl]_i$, described in the Methods section. B. Voltage threshold of the proton conductance through the $V_0$. 
Figure 5. Structure of the hydrophobic domain of the isolated V$_\alpha$. A. Proton paths on both the cytoplasmic and periplasmic sides of the isolated V$_\alpha$. Residues lining the paths are represented as balls and sticks. Residues from the $a$-subunit and $c$-subunit are indicated in the red and blue boxes, respectively. Proton flow from the periplasmic side is represented by the grey arrow as it would occur in the case of ATP synthesis. Scale bar; 10 Å. B. Salt bridge between $a$/Arg563, Arg622, Gln619 and $c$/Glu63. Scale bar; 3 Å.
Figure 6. Conformational changes occurring in both the $d$- and $a_{\text{sol}}$ subunits as a result of binding of $V_1$ to $V_o$.  

A. Structural changes in the $d$ subunit caused by insertion of the screw driver helix (SDH).  (left) Top view of $d$-subunit. The $d$-subunit from the isolated $V_o$ and the holo enzyme are colored in green and grey, respectively. Red arrows indicate the movements of helices 6-9 (H6-9).  (center, right) Key helices (H6 and 11) of $d$-subunit in the isolated $V_o$ and the holo complex. The H6 bends 45° as a result of
binding of SDH of D-subunit. B. Structural change of the distal subdomain of $a_{sol}$.

Upon the pivoting movement of $a_{sol}$ on the proximal subdomain, the distal subdomain swings 25 Å and twist 15° between the isolated $V_o$ (red) and the holo complex (gray).

C. EG structure in the distal subdomain of $a_{sol}$ ($E_{Gd}$) in the isolated $V_o$ (left) and in the holo complex (right). D. Schematic representation of the mechanical inhibition of the $V_o$ induced by dissociation of $V_1$. In isolated $V_o$, the rotation of central rotor is inhibited by interactions between $d$- and $a_{sol}$ (yellow box, Figure 3D, E).