Structural snapshots of V/A-ATPase reveal the rotary catalytic mechanism of rotary ATPases

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V/A-ATPase is a motor protein that shares a common rotary catalytic mechanism with F0F1 ATP synthase. When powered by ATP hydrolysis, the V1 domain rotates the central rotor against the A3B3 hexamer, composed of three catalytic AB dimers adopting different conformations (AB_open, AB_semi, and AB_closed). Here, we report the atomic models of 18 catalytic intermediates of the V1 domain of V/A-ATPase under different reaction conditions, determined by single particle cryo-EM. The models reveal that the rotor does not rotate immediately after binding of ATP to the V1. Instead, three events proceed simultaneously with the 120° rotation of the shaft: hydrolysis of ATP in AB_semi, zipper movement in AB_open by the binding ATP, and unzipper movement in AB_closed with release of both ADP and Pi. This indicates the unidirectional rotation of V/A-ATPase by a ratchet-like mechanism owing to ATP hydrolysis in AB_semi, rather than the power stroke model proposed previously for F1-ATPase.
The proton translocation ATPase/synthase family includes F-type enzymes found in eubacteria, mitochondria, and chloroplasts, and the V/A type enzymes found in archaea and some eubacteria\(^1\)–\(^5\) (Fig. 1A). These ATPases produce the majority of cytosolic ATP from ADP and Pi using energy derived from the transmembrane proton motive force generated by cellular respiration\(^6\). These ATPases share a common molecular architecture, consisting of a hydrophilic V\(_1/F_1\) domain responsible for ATP hydrolysis or synthesis, and a hydrophobic V\(_0/F_0\) domain housing a proton translocation channel\(^7\)–\(^9\). The chemical reaction (ATP hydrolysis/synthesis) in V\(_1/F_1\) is tightly associated with proton movement through V\(_0/F_0\) using a rotary catalytic mechanism, where both reactions are coupled by rotation of the central rotor complex relative to the surrounding stator apparatus, which includes the ATPase active hexamer\(^6,10,11\) (Fig. 1B).

The V/A-ATPase from the thermophilic bacterium, \textit{Thermus thermophilus} (\textit{Tth}) is one of the best-characterized ATP synthases\(^3,12\). The overall architecture and subunit composition of V/A-ATPase is more similar to that of the eukaryotic V-ATPase, rather than F-type ATPase. However, the \textit{Tth} V/A-ATPase has a simpler subunit structure than the eukaryotic V-ATPase and shares the ATP synthase function of F-type ATPase\(^13\) (Fig. 1A). The V\(_1\) domain of \textit{Tth} V/A-ATPase (A\(_3\)B\(_3\)D\(_1\)F\(_1\)) is an ATP-driven rotary motor where the central DF shaft rotates inside the hexameric A\(_3\)B\(_3\) containing three catalytic sites, each composed of an AB dimer. The V\(_0\) domain (E\(_2\)G\(_2\)d\(_1\)c\(_{12}\)) is composed of stator parts including the \(a\) subunit and two EG peripheral stalks and the d\(_{1}\)c\(_{12}\) rotor complex, which consists of a central rotor complex with the DF subunits of V\(_1\)–\(_5\). When ATP hydrolysis by A\(_3\)B\(_3\) powers the DF shaft, the reverse rotation of the central rotor complex drives proton translocation in the membrane-embedded V\(_0\) domain (Fig. 1B).

According to the binding change mechanism of ATP synthesis\(^6\), the three catalytic sites in ATP synthases are in different conformations but interconvert sequentially between three different conformations as catalysis proceeds. Indeed, our previous structure demonstrated that the A\(_3\)B\(_3\) hexamer in the V/A-ATPase adopts an asymmetrical structure composed of three different AB dimers, termed open (AB\(_{\text{open}}\)), semi-closed (AB\(_{\text{semi}}\)), and closed (AB\(_{\text{closed}}\))\(^16,17\).

Experimental studies using specific rotational probes attached to DF revealed that ATP-driven rotation of the central shaft was unidirectionally clockwise when viewed from the V\(_1\) side\(^10\). At low ATP concentrations where ATP binding is rate-limiting, rotation proceeds in steps of 120°, commensurate with the three catalytic sites of AB dimers\(^18\). When using 40 nm gold beads with almost negligible viscous resistance, V\(_1\) also pauses every 120° even at an ATP concentration around \(K_{m}\) without a sign of substeps\(^19\). These single-molecule experiments on V\(_1\) suggest that both catalytic events, ATP hydrolysis and product (ADP and Pi) release occur at an individual ATP binding position, and imply the presence of chemo-mechanically stable catalytic intermediates (Fig. 1C and Supplementary Fig. 1).

**Fig. 1 Schematic representation of rotary ATPases and the conventional rotary mechanism.** A Illustration of subunit composition of different types of rotary ATPases; prokaryotic V/A-ATPase (left), eukaryotic V-ATPase (middle), prokaryotic F-ATPase (right). The stators are represented in various colors and the rotors are represented in gray. B A schematic model of the rotary catalytic mechanism of the V/A-ATPase. When powered by ATP, the central rotor composed of D\(_1\)F\(_1\)c\(_{10}\) (gray) rotates against a surrounding stator composed of A\(_3\)B\(_3\)E\(_2\)G\(_2\)a\(_1\) (white), coupled with proton translocation across the membrane. C The conventional catalytic cycle of V/A-ATPase. At low ATP concentration, the ATP binding dwell time is increased. ATP\(_{\text{S}}\) also prolongs the ATP hydrolysis dwell.
However, single-molecule observation experiments only allow us to see the motion of the shaft to which the observation probe is bound, and do not tell us what events are occurring at each catalytic site. To elucidate the entire rotational mechanism of the V/A-ATPase, we must determine the structures of catalytic intermediates of the rotary ATPase during rotation. There are many reaction intermediates of the enzyme during turnover, and this structural heterogeneity makes successful crystallization of a specific state very challenging.

Technological breakthroughs in single-particle Cryo-EM, such as the development of direct electron detectors, and advances in image processing and automation, have triggered a revolution in structural biology, making this the technique of choice for large and dynamic complexes unsuitable for crystallization. In addition, by freezing Cryo-EM grids at different time points or under different reaction conditions, it is possible to trap intermediate states and thus build up a picture of the chemomechanical cycle of biological macromolecular complexes step by step. To date, there are few examples of studies that have successfully captured such details of a catalytic cycle at atomic resolution using Cryo-EM.

Here, we report several keys, and thus far uncharacterized, intermediate states of V/A-ATPase, obtained under different reaction conditions. Comparison of these structures provides insight into the cooperativity between the three catalytic sites and demonstrates a rotary catalytic mechanism powered by ATP hydrolysis.

Results
Sample preparation for Cryo-EM structural analysis. We previously determined the Cryo-EM structures of the wild-type V/A-ATPase containing an ADP in the catalytic site of ABclosed, ABsemi, and ABopen (Fig. 2). The V/A-ATPase bound to the inhibitory ADP exhibits no ATPase activity until the ADP is removed. Partial ADP removal from ABclosed is possible by dialysis against an EDTA-containing buffer, but it is difficult to obtain a homogenous nucleotide-free V/A-ATPase after such a treatment, due to the high binding affinity of the ADP to ABclosed (Supplementary Table 1). To obtain a homogeneous ATPase active enzyme, mutant V/A-ATPase (A/S232A, T235S) with reduced nucleotide-binding affinity was purified from T. thermophilus membranes.

The mutated V/A-ATPase exhibits higher $V_{max}$ values for nucleotide in both the ATP hydrolysis and synthesis reactions than the wild-type enzyme, but the enzymatic and rotational properties are almost the same as those of the wild-type enzyme. The mutated V/A-ATPase is fully activated for ATPase activity after EDTA/phosphate buffer washing. We used the nucleotide-free V/A-ATPase (Nucfree) for cryo-grid preparation under different ATPase reaction conditions (Supplementary Fig. 1c). Results of the structural analysis of the protein under each set of reaction conditions are summarized in Supplementary Fig. 3a–d.

The structures of V/A-ATPase without nucleotide (Vnucfree). The flow charts showing image acquisition and reconstitution of the 3D structure of V/A-ATPase without nucleotide are summarized in Supplementary Fig. 3a. We obtained structures of three rotational states of V/A-ATPase without nucleotide; state1 at 3.1 Å, state 2 at 4.7 Å, and state 3 at 6.3 Å resolution, with the DF shaft positions differing by 120° in each case (Fig. 2). Using signal subtraction of the V3 domain, we achieved resolution of 3.0 and 4.1 Å for the V1 domain including half of the EG stalk in state1 and 2, allowing us to build atomic models of the V3 domain of these states.

The three AB dimers in the V3 domain adopted open (ABopen), semi-closed (ABsemi), and closed (ABclosed) states, respectively (Fig. 2B, C). The tip of the C-terminal helix bundle (CHB) of Aopen is in contact with the C-terminal helix of the D subunit, and the wide part of the CHB of Bopen is in contact with the N-terminal helix of the D subunit, respectively (Supplementary Fig. 4a–c). The ABsemi and ABclosed also interact with the coiled-coil of subunit D in specific regions of the CHB, respectively (Supplementary Fig. 4d–g).

The differences in the structures of the three AB dimers, when superimposed on the β barrel domains of both A and B subunits, are the result of the movement of the N-terminal bulge domain, the nucleotide-binding domain (NB) of the A subunit and the CHBs of both the A and B subunits (Fig. 2D). When comparing the structure of ABopen and ABsemi, both the NB and CHB of the ABsemi are in closer proximity to Bsemi than Bopen, resulting in a closed structure of ABclosed (Fig. 2C, D). The structure of Bopen is very similar to Bsemi as shown in Fig. 2D. In the ABclosed both the CHB and NB domains of Aclosed are in closer proximity to Bclosed and the CHB of Bclosed moves to Aclosed resulting in the more closed structure of ABclosed compared to ABsemi (Fig. 2C, D).

In ABclosed and ABsemi, densities for the catalytic side chains are well resolved, but no density corresponding to nucleotide was observed (Fig. 3A). Hereafter we refer to the structure as the Vnucfree. The structure of Vnucfree is very similar to the previously reported ADP inhibited structure. For state1, the rmsd value for the Cα chains of A3B3DF of the Vnucfree and ADP inhibited structures is 1.98 Å (Supplementary Fig. 5). In addition, the Vnucfree is also similar to the structures under the saturated-ATP condition determined in this study, with the positions of the catalytic side chains almost identical in both cases (Supplementary Fig. 6). This indicates that the V3 domain adopts the same conformation, including the arrangement of the DF shaft in the A3B3 and the geometry of the catalytic side chains, irrespective of the presence or absence of bound ATP.

In the density maps obtained for state1 of Vnucfree, the CHB of the AB dimers was slightly blurred, likely due to structural heterogeneity. To classify the probable substates of state1, we performed focused 3D classification using a mask covering ABopen and Bsemi (Supplementary Fig. 3a). We identified a cryoEM structure of the original state1 from 39,902 particles at 3.1 Å resolution and another substate from 24,101 particles at 3.1 Å resolution. We termed the substates reconstructed from these major particle classes as state1-1 and state1-2, respectively. The atomic model initially constructed as state1 is identical to the atomic model of state1–1. The structures of the sub-states are very similar, with most differences due to the movement of the CHB of the A and B subunits. Therefore, we quantified the difference in the structure of the CHB observed when the structures were superimposed on the N-barrel domain (Supplementary Tables 2 and 3). Substates were also obtained under other reaction conditions (see below) and the rmsd values shown in Supplementary Tables 2 and 3 are used to discuss which subunits are responsible for the differences in the structure of the substates obtained under different reaction conditions.

Structures obtained at a saturating ATP concentration. Cryo-girds were prepared using a reaction mixture of nucleotide-free V/A-ATPase, containing the regenerating system and ATP at a saturating concentration of 6 mM. The reaction mixture was
incubated for 120 s at 25 °C and then loaded onto a holey grid, followed by flash freezing.

We determined three rotational states followed by focused refinement using a V1EG mask for each state (Supplementary Fig. 3b). In the density maps obtained for each state, the amino acid residues of the nucleotide-binding sites in both ABclosed and ABsemi were well resolved, but the CHB domains of the AB dimers were blurred due to structural heterogeneity, as with the Vnucfree. For state1, we identified an atomic resolution structure of state1–1 from 40,831 particles at 3.1 Å resolution and state1–2 from 28,801 particles at 3.2 Å resolution by further 3D classification without alignment (Supplementary Fig. 3b). The same classification analysis was performed for state2 and state3, yielding state 2–1 (3.0 Å resolution) and state 2–2 (3.4 Å resolution).
resolution), and state 3–1 (3.0 Å resolution) and state 3–2 (3.4 Å resolution) respectively. In these structures, nucleotide densities have been identified in the three catalytic sites. Hereafter, we refer to the structures obtained at ATP saturating conditions as \( V_{3\text{nuc}} \).

The structure of \( AB_{\text{open}} \) of \( V_{3\text{nuc}} \) state 1–1 is almost identical to that of \( V_{\text{nucfree}} \) state 1–1 (Supplementary Fig. 6). This is confirmed by the fact that the \( \text{rmsd} \) values in the CHB of \( A_{\text{open}} \) and \( B_{\text{open}} \) for \( V_{3\text{nuc}} \) state 1–1 and \( V_{\text{nucfree}} \) are less than 1 Å (Supplementary Tables 2 and 3). The \( AB_{\text{open}} \) of state 1–2 adopts a slightly more closed conformation compared to that of state 1–1, which results from a movement of CHB of \( B_{\text{open}} \) toward the \( \beta \)-barrel domain (Fig. 4C). Nevertheless, the \( AB_{\text{open}} \) of \( V_{3\text{nuc}} \) with bound ATP retains the interaction with the DF shaft, indicating that ATP binding to the \( AB_{\text{open}} \) does not move the DF shaft.

The \( AB_{\text{semi}} \) in state 1–2 has a more closed structure than that in state 1–1 mainly due to the movement of CHB in \( B_{\text{semi}} \) (Fig. 4B, Supplementary Table 3). In summary, \( V_{3\text{nuc}} \) state 1–2 has a more closed structure than state 1–1 due to the movement of the CHB of both \( A_{\text{semi}} \) and \( B_{\text{semi}} \), but the slightly closed conformation of state 1–2 is independent of ATP binding to the \( AB_{\text{open}} \).

**Structures of the catalytic sites at AB dimers of \( V_{3\text{nuc}} \)** In both state 1–1 and state 1–2 structures obtained under ATP saturating conditions, a bound ATP molecule is clearly observed in the catalytic site of \( AB_{\text{open}} \) (Fig. 3B and Supplementary Fig. 7c). The catalytic sites in the \( AB_{\text{closed}} \) and \( AB_{\text{semi}} \) in both the state 1–1 and state 1–2 also contained density corresponding to an ATP molecule, and in these cases, the associated magnesium ions were visible (Fig. 3B and Supplementary Fig. 7a, b). In the \( V_{3\text{nuc}} \) structure, we did not find density corresponding to nucleotides between the D and A subunits as reported in a previous paper\(^{25}\) (Supplementary Fig. 8).

In the catalytic site of \( AB_{\text{semi}} \) of \( V_{3\text{nuc}} \), the density of each nucleotide phosphate atom was easily identifiable (Supplementary Fig. 7b), indicating that the ATP molecule occupies the catalytic site in \( AB_{\text{semi}} \). The protein structure is sufficiently close to also provide a detailed picture of the configuration of the catalytic side chains (Fig. 5A–C). The \( \gamma \)-phosphate of ATP and the magnesium ion are coordinated by the A/K234 and A/S235 residues on the P-loop, which contains the conserved nucleotide-binding motif\(^{26}\). The aromatic ring of A/F230, not conserved in F type ATPase, is oriented away from the triphosphate moiety, allowing access of the guanidium group to the arginine finger (Supplementary Fig. 9). Considering clear EM density for the \( \gamma \)-phosphate of the ATP bound in \( AB_{\text{semi}} \), hydrolysis of ATP is unlikely to proceed in \( AB_{\text{semi}} \).

The nucleotide-binding site of the \( AB_{\text{closed}} \) is shown in Fig. 5B. The geometry of ATP binding in \( AB_{\text{closed}} \) is very similar to that found in the \( AB_{\text{semi}} \); however, the carbonyl group of A/E257 is closer to the \( \gamma \)-phosphate by about 1 Å than \( AB_{\text{semi}} \). In state 1–2 of \( V_{3\text{nuc}} \), the \( \gamma \)-phosphate of ATP bound to the \( AB_{\text{closed}} \) appears to be separated from \( \beta \)-phosphate when a relatively high density threshold is used (Supplementary Fig. 10). These findings strongly suggest that ATP bound to \( AB_{\text{closed}} \) is either already hydrolyzed or in the process of being hydrolyzed. The state of ATP in \( AB_{\text{closed}} \) is discussed further in Supplementary Note.

In the nucleotide-binding site of the \( AB_{\text{open}} \) of \( V_{3\text{nuc}} \), the adenosine moiety of ATP is occluded as in the \( AB_{\text{semi}} \) and \( AB_{\text{closed}} \), with A/F415, A/Y500, and A/V236 forming the adenine binding pocket, however, the hydrogen bonding of the ribose moiety to the side chain of B/N363 is lost due to movement of the CHB of \( A_{\text{open}} \) (Fig. 5C). Unlike in the \( AB_{\text{closed}} \) and \( AB_{\text{semi}} \), the phenyl group of A/230F in the \( AB_{\text{open}} \) is closer to the triphosphate group of ATP due to the torsion of the main chain, resulting in the formation of a hydrophobic barrier between the
In both $\text{AB}_{\text{semi}}$ and $\text{AB}_{\text{closed}}$ of state 1–1, the apparent density of ATP-magnesium was observed, but the density of the γ-phosphate at the $\text{AB}_{\text{closed}}$ is weaker than that at the $\text{AB}_{\text{semi}}$ (Fig. 3C and Supplementary Fig. 7a, b). In contrast, density was not observed in the nucleotide-binding site of $\text{AB}_{\text{open}}$. For state 1–2, as in state 1–1, nucleotides are present in both $\text{AB}_{\text{semi}}$ and $\text{AB}_{\text{closed}}$, while $\text{AB}_{\text{open}}$ is empty. Hereafter we refer to the structure as $\text{V}_{\text{2nuc}}$.

The overall structure and geometry of the catalytic residues of state 1–1 of $\text{V}_{\text{2nuc}}$ are largely identical to state 1–1 of $\text{V}_{\text{nucfree}}$ and $\text{V}_{\text{3nuc}}$ (Supplementary Fig. 11). This structural similarity between $\text{V}_{\text{2nuc}}$, $\text{V}_{\text{nucfree}}$, and $\text{V}_{\text{3nuc}}$ is confirmed by the low RMSD values when comparing the CHB of the A and B subunits of these structures (Supplementary Tables 2 and 3). The similarity of the structures of these substrates indicates that the structural polymorphism of the $\text{V}_1$ domain is independent of the binding of ATP to AB dimers.

**Structures obtained at a saturating concentration of ATP·γ·S ($\text{V}_{\text{prehyd}}$).** The $\text{V}_1$-ATPase from *T. thermophilus* is capable of hydrolyzing ATP·γ·S, however, the turnover rate of ATP·γ·S is much lower than that of ATP due to the decrease in hydrolysis rate (Supplementary Fig. 2d). Thus, pre-hydrolysis structures of $\text{V}_1$-ATPase can be obtained at 4 mM ATP·γ·S. The cryo-grid was prepared by blotting the reaction mixture comprising Nucleotide-free $\text{V}_1$-ATPase and 4 mM of ATP·γ·S in the absence of the regenerating system in order to exclude any effect of regenerated ATP produced from hydrolyzed ATP·γ·S. We reconstructed three rotational states from the acquired EM images using the CRYOARM300 (JEOL). After the focused masked refinement of the $\text{V}_1$-EG domain, we obtained atomic resolution structures of each state (state 1, 2.7 Å, state 2, 3.4 Å, and state 3, 3.6 Å), respectively (Supplementary Fig. 3d). We refer to these structures as $\text{V}_{\text{prehyd}}$. For state 1, two sub-states, state 1–1 and state 1–2, were obtained at 2.7 and 2.9 Å resolution respectively, by focused classification using a mask with $\text{AB}_{\text{open}}$ and $\text{AB}_{\text{semi}}$ (Supplementary Fig. 3d). For the $\text{AB}_{\text{open}}$ of $\text{V}_{\text{prehyd}}$ bound ATP·γ·S is clearly observed in the catalytic site, which has an almost identical structure to that of the ATP bound state of $\text{V}_{\text{3nuc}}$ (Fig. 3B).

The nucleotide-binding sites of the $\text{AB}_{\text{closed}}$ and $\text{AB}_{\text{semi}}$ of $\text{V}_{\text{prehyd}}$ are almost identical to those of $\text{V}_{\text{3nuc}}$ respectively, as shown in Fig. 5. The γ-phosphate group of the bound ATP·γ·S molecule in the $\text{AB}_{\text{semi}}$ is well resolved as seen in for ATP in $\text{V}_{\text{nucfree}}$ and $\text{V}_{\text{3nuc}}$ (Fig. 3d and Supplementary Fig. 7b). In contrast, the density of γ-phosphate of ATP·γ·S at the $\text{AB}_{\text{closed}}$ is faint (Supplementary Fig. 7a), suggesting that the ATP·γ·S in the $\text{AB}_{\text{closed}}$ has already been hydrolyzed and the bound nucleotide in the $\text{AB}_{\text{closed}}$ is ADP. This indicates that the $\text{AB}_{\text{semi}}$ is in the pre-hydrolysis conformation, waiting for ATP hydrolysis.

**Discussion**

We have obtained catalytic intermediates of the $\text{V}_1$ domain, $\text{V}_{\text{nucfree}}$, $\text{V}_{\text{3nuc}}$, $\text{V}_{\text{2nuc}}$, and $\text{V}_{\text{prehyd}}$ with different nucleotide occupancy. Despite the different nucleotide occupancy of these structures, their overall conformations are very similar. For instance, the RMSD of the Ca chains of $\text{A}_3\text{B}_3$ in $\text{V}_{\text{nucfree}}$ and $\text{V}_{\text{3nuc}}$ state 1–1 is 1.98 Å. In addition, the relative position of the central DF shaft within the asymmetric $\text{A}_3\text{B}_3$ is almost the same in the $\text{V}_{\text{nucfree}}$ and $\text{V}_{\text{3nuc}}$ structures. These findings demonstrate that the configuration between the DF shaft and individual AB dimers is independent of the state of nucleotide occupancy of each AB dimer. In other words, the structure of the $\text{V}_1$ domain adopts three rotational states, 1–3, during continuous ATP hydrolysis, with the conformational changes in the $\text{A}_3\text{B}_3$ hexamer driven by ATP hydrolysis, being discrete rather than continuous.
The V$_{3\text{nuc}}$ structure, obtained under ATP saturation conditions, shows all three catalytic sites occupied by ATP or the products of hydrolysis (ADP + Pi). Since the hydrolyzed Pi is clearly visible in the AB$_{\text{closed}}$ of V$_{3\text{nuc}}$ (Supplementary Fig. 10), it is assumed that V$_{3\text{nuc}}$ is the structure before dissociation of Pi from the catalytic site in AB$_{\text{closed}}$. We also obtained the V$_{2\text{nuc}}$ structure in which ATP and product(s) are bound to AB$_{\text{semi}}$ and AB$_{\text{closed}}$, respectively, but AB$_{\text{open}}$ is empty. The V$_{2\text{nuc}}$ is therefore assumed to be the structure of the protein awaiting ATP binding to AB$_{\text{open}}$. When using ATPyS as a substrate, which has a very slow hydrolysis rate, the high-resolution atomic structure of the V$_{1}$ domain allowed visualization of ATPyS molecules bound to the catalytic sites of AB$_{\text{open}}$ and AB$_{\text{semi}}$, as well as identification of the hydrolyzed ATPyS at the AB$_{\text{closed}}$. The V$_{\text{prehyd}}$ reveals both that the AB$_{\text{closed}}$ adopts the post-hydrolysis state where the product of phosphate (Pi) is dissociated, and that the AB$_{\text{semi}}$ is awaiting ATP hydrolysis.

Fig. 5 Coordination of nucleotides in the binding sites of V$_{3\text{nuc}}$ and V$_{\text{prehyd}}$. Left panels: Comparison of the three nucleotide-binding sites (AB$_{\text{semi}}$ (A), AB$_{\text{semi}}$ (B), and AB$_{\text{closed}}$ (C)) of state 1 of V$_{3\text{nuc}}$, shown with colored (green, blue, and pink) atoms and bonds, and main chain, and state 1 of V$_{\text{prehyd}}$ shown with gray atoms, bonds and main chain. Right panels: schematic representations of the coordination of the ATP group in the three binding sites of V$_{3\text{nuc}}$ and V$_{\text{prehyd}}$ in parentheses. The distances between the atoms are shown in dotted lines. All distances are in Å.
The structures provide important insights into the chemo-mechanical cycle of V/A-ATPase. The V/A-ATPase undergoes a unidirectional conformational change from state 1 to state 2 when powered by ATP. Thus, V$_3$nucl of state 1, in which three catalytic sites are already occupied by nucleotides, should change to state 2 of V$_2$nucl following ATP hydrolysis at AB$_{semi}$ and the subsequent or simultaneously dissociation of ADP and Pi by the discrete structural transition of AB$_{closed}$ to AB$_{open}$ (Fig. 6). This demonstrates that rotation of the rotary ATPase proceeds via the tri-site model with the protein progressing through a two nucleotide bound state and a three-nucleotide bound state, setting the long-standing debate on whether the bi-site model or tri-site model is appropriate for rotary ATPases.

Based on previous single molecular observation experiments for both F$_1$ and V$_1$-ATPase, ATP binding onto the enzyme directly triggers the first 120° rotation step of the DF shaft (Supplementary Fig. 1a). In light of our findings presented here, this scheme needs to be redrawn; the rotor does not immediately travel 120° as a result of ATP binding to enzyme.

The next catalytic event after ATP binding is ATP hydrolysis in AB$_{semi}$. Each conformational change, from AB$_{open}$ to AB$_{semi}$, AB$_{semi}$ to AB$_{closed}$, and AB$_{closed}$ to AB$_{open}$ occurs simultaneously, with the rotation of the shaft, and with the hydrolysis of ATP in the AB$_{semi}$ and release of products (ADP and Pi) from the AB$_{closed}$ (Fig. 6 and Supplementary Movie 1). This is in contrast to the classical rotary model, where catalytic events occur in sequence at the three catalytic sites, until now the broadly accepted mechanism of action of the F$_1$-ATPase.

In the V$_2$nucl and V$_3$nucl two sub-states, state 1–1 and state 1–2 were identified (Supplementary Fig. 12). These substrates were also identified in V$_{nucfree}$ thus the conformational dynamics of the V$_1$ domain are independent of ATP binding. In other words, state 1–1 and state 1–2 are in a thermal equilibrium state, irrespective of nucleotide occupancy in each catalytic site. Both AB$_{semi}$ and AB$_{open}$ in state 1–2 adopt more closed structures than those in state 1–1, suggesting that state 1–2 of V$_3$nucl is likely an intermediate structure just prior to the 120° rotation step of the DF shaft. Compared to state 1–2 of V$_3$nucl state 1–2 of V$_{prehyd}$ exhibits slightly more closed structures of AB$_{open}$ and AB$_{semi}$ (Supplementary Fig. 13), likely to be associated with the progress of the catalytic reaction in AB$_{closed}$, i.e., the dissociation of the phosphate. In this respect, state 1–2 of V$_{prehyd}$ may be another reaction intermediate structure in which the Pi in the AB$_{closed}$ is released prior to the 120° step (Supplementary Fig. 14).

Based on the catalytic intermediates of the V$_1$ domain of V/A-ATPases obtained under different reaction conditions, we propose a model for the ATP-driven rotation mechanism of V/A-ATPases (Fig. 7, Supplementary Fig. 15).

When ATP binds to V$_{nucfree}$ which is in a stable initial state (ground state), the enzyme transits into the steady-state for ATP hydrolysis (Fig. 7, upper row). The V$_3$nucl structure is formed by binding of ATP to the AB$_{open}$ of V$_3$nucl but the binding of ATP itself does not cause structural transitions between AB dimers associated with the 120° step of the DF shaft.

In the V$_3$nucl structure, where nucleotides are bound to all three AB dimers, three distinct but associated catalytic events occur at
ABopen and ATP hydrolysis in ABsemi induce unzipper motion of ABclosed accompanying the release of ADP and ATP to the catalytic sites. In V2nuc awaiting ATP binding, binding of ATP to ABopen does not induce the 120° rotation step. In V3nuc, both zipper motion of ABclosed hampers the structural transition of ABopen to ABsemi by binding of ATP to ABopen. The Vnucfree in the ground state is activated by the binding of dimers occur simultaneously with the 120° step of the DF shaft, resulting in the three AB dimers simultaneously and these events are coupled to the first 120° rotation step of the DF shaft. One of the driving events for this transition is the conformational change from the ATP-bound ABopen to the more closed ABsemi, which can be explained by a zipper motion of ABopen upon ATP binding. From our structures, a comparison between ABopen and ABsemi implies that new hydrogen bonds form between the triphosphate moiety of ATP and the surrounding side chain groups of B/R360, A/258R, A/257E, and A/K234 (Fig. 5).

The Vprehyd structures indicate that the ATP bound to ABsemi is awaiting hydrolysis. The conformational change from ABsemi to ABclosed should occur spontaneously because it involves ATP hydrolysis, an exergonic reaction. In contrast, ADP bound to ABopen hampers the unzipper motion in ABclosed, thereby preventing the overall structural transition of the V1 domain. This is supported by the fact that V/A-ATPase adopts the ADP inhibited state in which ADP is entrapped in ABclosed (Fig. 7, upper line). The enzyme in the ADP-inhibited state does not show ATP hydrolysis activity even at saturated ATP concentration.

In summary, the ATP-driven unidirectional rotation of V/A-ATPase proceeds by a discrete structural transition between the three rotational states, i.e., the potential barrier to the structural transition of ABclosed to ABopen accompanied by the release of ADP and Pi, is overcome by both a zipper motion of ABopen by the bound ATP and ATP hydrolysis in ABsemi. Since the ATP hydrolysis reaction is a heat dissipation process, the structural transition of ABsemi to ABclosed associated with the ATP hydrolysis occurs spontaneously and irreversibly, resulting in a unidirectionality of the 120° steps of the rotor. In other words, our model explains the unidirectional rotation of V/A-ATPase by a ratchet-like mechanism driven by ATP hydrolysis, rather than the power stroke model proposed previously for F1-ATPase.

V/A-ATPase and F1,F0 are molecular machines based on the same construction principle, and thus are likely to share the same rotary mechanism. In fact, observation of rotation with high time-resolved rotation analysis using a tiny gold rod showed that bacterial F-ATPase and an A-ATPase share the same rotation mechanism. For both rotary ATPases, a 120° rotation step together with ATP hydrolysis occurs after the catalytic dwell under ATP-saturated conditions. Importantly, for the thermophilic F1, the first 120° rotation step also includes ~80° and ~40° substeps, suggesting the existence of at least one additional catalytic intermediate of F1. A recent structural study of thermophilic F1-ATPase indicated a possible intermediate structure responsible for the substeps. In the V/A-ATPase, any intermediate structure containing phosphate after ADP or Pi release is likely to be in an unstable state, and therefore studies on the ATP-driven rotation of V/A-ATPase have failed to reveal the presence of any substep.

Methods
Preparation of Tth V/A-ATPase for biochemical assay and cryo-EM imaging
The Tth V/A-ATPase containing His3 tags on the C-terminus of each c subunit and the TSSA mutation (M223A and T235S) on the A subunit was isolated from T. thermophilus membranes as previously described with the following modifications. The enzyme, solubilized from the membranes with 10% Triton X-100 was purified by Ni2+-NTA affinity with 0.03% dodecyl-β-D-maltoside (DDM). For bound nucleotide removal, the eluted fractions containing Tth V/A-ATPase were dialyzed against 200 mM Sodium phosphate, pH 8.0, 10 mM EDTA, and 0.03% DDM overnight at 25 °C with three buffer changes, followed by dialysis against 20 mM Tris–Cl, pH 8.0, 1 mM EDTA, and 0.03% DDM (TE buffer) prior to anion exchange chromatography using a 6 ml Resource Q column (GE healthcare). The Tth V/A-ATPase was eluted by a linear NaCl gradient using a TE buffer.
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(0–500 mM NaCl, 0.03% DDM). The eluted fractions containing holo-Tth V/A-ATPase were concentrated to ~10 mg/ml using Amicon 100k molecular weight cut-off filters. For the nuclear disintegration, 2'-Dimercaptoethanol (DMPE, Avanti) was used to form lipid bilayers in reconstruction as previously described. Purified Tth V/A-ATPase solubilized in 0.03% n-DDM was mixed with the lipid stock and membrane scaffold protein MSP1E3D1 (Sigma) at a specific molar ratio V1/V1:MSPI/MSPC lipid = 1:4.5 and incubated on ice for 0.5 h. Then, 200 μl of Bio Beads SM-2 equivalent buffer (20 mM Tris-HCL, pH8.0, 150 mM NaCl) was added to the 500 μl mixture. After 2 h incubation at 4 °C with gentle stirring, an additional 300 μl of Bio Beads was added and the mixture was incubated overnight at 4 °C to form the nanodiscs. The supernatant of the mixture containing nanodisc-Tth V/A-ATPase (nd-V/A-ATPase) was loaded onto the Superdex 200 Increase 10/300 column equilibrated with wash buffer. The peak fractions were collected, analyzed by SDS-PAGE, and concentrated to ~4 mg/ml. The prepared nd-V/A-ATPase was immediately used for biochemical assay or cryo-grid preparation since nd-V/A-ATPase aggregates within a few days.

Biochemical assay. The quantitative analysis of bound nucleotides of Tth V/A-ATPase was carried out using anion-exchange high-performance liquid chromatography with F200. The papules were released from the enzyme by the addition of 5 μl of 60% perchloric acid to 50 μl of the solution. Thereafter, the mixture was incubated on ice for 10 min. Then, 5 μl of 5 M K2CO3 solution was added and the mixture was incubated on ice for 10 min. The resulting pellet was removed by centrifugation at 4 °C. The supernatant was applied to a Cosmopak-200 column (Millipore). For nanodisc incorporation, the 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC, Avanti) was used to form lipid bilayers in

Model building and refinement. To generate the atomic model for the V1EG region of V/A-ATPase, the individual subunits of the V1EG model from the previous structure of V/A-ATPase (PDBID: 6QUM) were fitted into the density map as rigid bodies with specific focus on the N terminal region of EG stalk (E: 1–77 aa; G: 2–33 aa). The rough initial model was refined against the map with Phenix real space refine program. The initial model was extensively manually corrected residue by residue in COOT in terms of side-chain conformations. Peripheral stalks were removed due to low resolution in this region. The corrected model was again refined by the phenix.real_space_refine program with secondary structure and Ramachandran restraints, then the resulting model was manually checked by COOT. This iterative process was performed for several rounds to correct remaining errors until the model was in good agreement with geometry, as reflected by the MolProbity score of 1.08–1.74 and EMRinger score of 1.59–3.45. For model validation against over-fitting, the built models were used for calculation of FSC curves against both half maps, and compared with the FSC of the final model against the final density map used for model building by phenix.real_space program. The statistics of the obtained maps and the atomic model were summarized in Supplemental Tables 4–11. RMSD values between the atomic models were calculated using UCSF chimera. All the figures were rendered using UCSF chimera. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The cryo-EM maps have been deposited in the EMDB under accession codes 31841, 31842, 31843, 31844, 31845, 31846, 31847, 31848, 31849, 31850, 31851, 31852, 31853, 31854, 31855, 31856, 31857, 31858, 31859, 31860, 31861, 31862, 31863, 31864, 31865, 31866, 31867, 31868, 31869, 31870, 31871, 31872, and 31873. The atomic models have been deposited in the Protein Data Bank under accession codes 7VAI, 7VJA, 7VAK, 7VAO, 7VAQ, 7VAR, 7VAU, 7VAV, 7VAY, 7VAY, 7VAY, and 7VBO. The initial model for model building is accessible in PDB under accession number 6QUM. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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