Crystal structures of the ATP-binding and ADP-release dwells of the $V_1$ rotary motor

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$V_1$-ATPases are highly conserved ATP-driven rotary molecular motors found in various membrane systems. We recently reported the crystal structures for the *Enterococcus hirae* A$_3$B$_2$DF ($V_1$) complex, corresponding to the catalytic dwell state waiting for ATP hydrolysis. Here we present the crystal structures for two other dwell states obtained by soaking nucleotide-free $V_1$ crystals in ADP. In the presence of 20 μM ADP, two ADP molecules bind to two of three binding sites and cooperatively induce conformational changes of the third site to an ATP-binding mode, corresponding to the ATP-binding dwell. In the presence of 2 mM ADP, all nucleotide-binding sites are occupied by ADP to induce conformational changes corresponding to the ADP-release dwell. Based on these and previous findings, we propose a $V_1$-ATPase rotational mechanism model.
on-transporting rotary ATPases are divided into three types based on their function and taxonomic origin: F-, V- and A-type ATPases. F-ATPases function as ATP synthases in mitochondria, chloroplasts and oxidative bacteria. V-ATPases function as proton pumps in acidic organelles and plasma membranes of eukaryotic cells. A-ATPases function as ATP syntheses similar to the F-ATPases in Archaea (the ‘A’ designation refers to Archaea), but the structure and subunit composition of A-ATPases are more similar to those of V-ATPases. These ATPases possess similar overall structures consisting of a globular catalytic domain (F1, V1 or A1) and a membrane-embedded ion-transporting domain (Fo, Vo or Ao). These catalytic domains are similar rotary molecular motors, in which the central axis complexes rotate within pseudo-hexagonally arranged catalytic complexes powered by energy from ATP hydrolysis.

The rotational catalysis of F1-ATPase has been investigated using structural analyses of bovine1–11, yeast12,13 and bacterial14–15 samples, and by single-molecule dynamics studies of bacterial samples16–21. However, contradictory findings have been obtained depending on the methods, conditions and species, leading to controversy regarding the general rotational model of F1 (refs 10,18). Recently, a model was proposed that could consistently explain both the structural and single-molecule data obtained for mammalian F1-ATPase22,23. In this model, the central axis rotates at 120° per ATP molecule with three dwell states: waiting for ATP binding (ATP-binding dwell) at 0° (and 120°), waiting for Pi release (Pi-release dwell) at 65°, and waiting for ATP hydrolysis (catalytic dwell) at 90° (see Fig. 1).

Similar V1-ATPase experiments have been conducted using bacterial enzymes from Thermus thermophilus24–28 and Enterococcus hirae29–33. These enzymes are sometimes called A-ATPases. However, they are derived from Eubacteria, rather than Archaea. Furthermore, E. hirae V-ATPase physiologically functions as an ion pump, similar to eukaryotic V-ATPases34–37, and is composed of nine subunits with amino acid sequences that are homologous to those of the corresponding subunits of eukaryotic V-ATPases38–40. Therefore, we believe the enzyme is a homologue of eukaryotic V-ATPases. We previously established the in vitro expression, purification and crystallization of E. hirae V1-ATPase (EhV1) from the A3B3 and DF complexes41,42. The crystal structures of the nucleotide-free and nucleotide-bound A3B3 (eA3B3 and bA3B3) and V1 (eV1 and bV1) complexes revealed conformational changes of the A3B3 complex induced by the binding of nucleotides and the DF axis (Supplementary Fig. 1), suggesting that the EhV1 structure corresponds to the catalytic dwell waiting for ATP hydrolysis in the rotary cycle43. We have also directly confirmed the unidirectional rotation of EhV1 with single-molecule observations44,45. EhV1 shows only three pausing positions separated by 120° at all ATP concentrations without distinct substeps, in contrast to that of F1-ATPase46,47. This suggests that the ATP hydrolysis step(s), for example, ATP binding, phosphate bond cleavage, ADP release or Pi release, is/are the rate-limiting step(s) in the three-pause rotation48,49. In this study, we performed experiments in which nucleotide-free V1 crystals were soaked with AMP-PNP (non-hydrolysable ATP analog adenosine 5′-(β,γ-imino)triphosphate), ADP or phosphate, and obtained two previously unidentified crystal structures corresponding to the ATP-binding dwell and ADP-release dwell states in the rotary cycle of EhV1. Our proposed rotational mechanism of EhV1 based on these crystal structures is apparently different from those previously reported for F1-ATPases48,49 (see Fig. 1).

**Figure 1 | Coupling scheme for the 120° rotation and ATP hydrolysis of mammalian F1 and Enterococcus hirae V1-ATPases.** Each circle represents the chemical state of the nucleotide-binding site, viewed from the cytoplasmic side (that is, the N-terminal β-barrel side of V1). The central arrows in the ellipses represent the orientation of the central axis beginning from the twelve o’clock position, which corresponds to the ATP-binding dwell (a waiting state for ATP binding). PDB ID numbers of the corresponding crystal structures are shown under the schemes. ATP* represents an ATP molecule that is committed to hydrolysis. (a–d) A model for mammalian F1 (refs 22,23). ATP binding to the ATP-binding dwell (a) induces a 65° rotation concomitant with ADP release from another binding site and resulting conformational changes to the P1-release dwell5,9,23 (b). P1 release induces a 25° rotation and consequent conformational changes to the catalytic dwell11,23 (c), which is waiting for ATP hydrolysis. ATP* hydrolysis to produce ADP and Pi induces a 30° rotation and consequent conformational changes to the ATP-binding dwell (d). (e–h) A model for E. hirae V1 (this study). ATP binding to the ATP-binding dwell (e) induces conformational changes to the ADP-release dwell (f) without an apparent rotational substep of the central axis. ADP release induces a 120° rotation and consequent conformational changes to the catalytic dwell (g). ATP* is hydrolysed to produce ADP and Pi, and the Pi release induces conformational changes to the ATP-binding dwell state (h) without a rotational substep.
molecules bound in the 'bound' and 'tight' forms and was almost identical to that of bV1 (r.m.s.d. = 0.51 Å) (ref. 31) (Fig. 2e,f). No electron density peak for AMP-PNP was found in the 'empty' form (Supplementary Fig. 2), indicating that it has a very low affinity for AMP-PNP.

Structure of the 2ADP-bound V1 complex. Next, we soaked the crystals of eV1 in 20 μM ADP, and the crystal structure (denoted 2ADPV1) was solved at a resolution of 3.3 Å (Table 1). Two ADP·Mg$^{2+}$ molecules were bound to the 'bound' and 'tight' forms of eV1, as in the case of 2ATPV1 (Supplementary Fig. 3), and induced conformational changes with the crystal packing rearrangements (Supplementary Fig. 4). The structure of 2ADPV1 was validated for possible model bias by generating omit maps of conformationally changed regions (Supplementary Fig. 5). ADP binding changed the structure of eV1, but the crystal packing force might have the potential to distort the actual conformational changes for 2ADPV1.

The structural differences between eV1 and 2ADPV1 that should have been induced by ADP binding are compared in Fig. 3 (see also Supplementary Movie 1). The eV1 'bound' form did not show a conformational change upon ADP binding (r.m.s.d. = 0.48 Å; Fig. 3c,d). However, the eV1 'tight' form changed to a more open conformation (A_C from A_CCR from B_CR) upon ADP binding (Fig. 3e,f). We designated the new ADP-bound A_C:B_C pair of 2ADPV1 as the 'ADP-bound' form. The γ-phosphate contained in AMP-PNP of the bV1 'tight' form interacted with the Lys238 residue of the P-loop (P-loop binding) and the Arg262 residue of the 'arm' region (fixed α-helix during the conformational changes: residues 261–275) in Eh-A, and the Arg350 residue (the so-called 'Arg-finger' in ATPases) in Eh-B to stabilize the 'tight' conformation (Fig. 4b), thus preventing any further conformational change. In contrast, ADP, which does not contain γ-phosphate, interacted with these side chains by binding to β-phosphate (Fig. 4a and Supplementary Figs 6 and 7). These different binding contacts induced an apparent conformational change to the 'ADP-bound' form (Fig. 4a–c), which seems to be a more stable conformation for the ADP-binding mode than the 'tight' form. According to the observed conformational changes, the DF axis became tilted towards the 'ADP-bound' form to maintain the extensive protein–protein interactions between DF and the 'ADP-bound' form (Fig. 3b–h).

The last conformation of the AB pair (eV1; 'empty'), which did not bind to ADP, also showed a cooperative conformational change. Specifically, Eh-A (A_O) and Eh-B (B_C) of the 'empty' form were attracted to the DF axis and the 'ADP-bound' form, respectively (Fig. 3b,g,h and Supplementary Movie 1). The wider conformation of the resultant AB (A_O:B_C) pair was most similar to that of the eA3B3-'bindable' form (A_O:B_C; ATP-accessible state) among all AB pairs (r.m.s.d. = 0.94 Å) (Supplementary Table 2), and was thus denoted a 'bindable-like' form. The structure at the nucleotide-binding site was also more similar to that of eA3B3-'bindable' (ATP-accessible state) than to that of eV1-'empty' (ATP-unbound state). Similar to the 'bindable' form, the topology between the Arg-finger (Eh-B-Arg350) and Eh-A-Arg262 of the 'bindable-like' form was more open than that of the 'empty' form (Fig. 4d–f; green boxes). Therefore, the 'bindable-like' conformation seemed to be able to bind a nucleotide and probably changes to the 'bound' form, as observed for eA3B3-'bindable' (31). Based on these findings, we inferred that the structure of 2ADPV1 corresponds to the state of waiting for ATP binding (that is, the ATP-binding dwell) in the rotation.

Structure of the 3ADP-bound V1 complex. Next, we soaked the eV1 crystals in high concentration (2 mM) of ADP to verify
nucleotide binding to the third ‘bindable-like’ form of 2ADPV1, and obtained the crystal structure (denoted 3ADPV1) at a resolution of 3.0 Å (Table 1). Three ADP:Mg2+ molecules were bound at all three nucleotide-binding sites (Supplementary Fig. 8) and induced conformational changes with the crystal packing rearrangements (Fig. 5a, Supplementary Fig. 4, and Supplementary Movie 2). The structure was verified by generating omit maps of crystals with 20 and 200 mM Pi, and solved the crystal structures (Supplementary Fig. 9), and ATP hydrolysis activity of purified EhV1 was not inhibited, even in the presence of 20 mM Pi (Supplementary Table 1). These findings suggest that the binding affinity for ADP of the ‘tight-like’ form is lower than that of the ‘ADP-bound’ form. Consequently, an ADP molecule will be easily released from the binding site. Therefore, we inferred that the structure of 3ADPV1 corresponds to the state of waiting for ADP release (that is, ADP-release dwell) in the rotation. ATP hydrolysis activity of purified EhV1 was inhibited at a high (2 mM) concentration of ADP (Supplementary Table 1), which is significantly higher than the natural concentration in E. hirae cells. Therefore, the ADP-release dwell state might be a minor intermediate state, which might exist in the catalytic cycle of EhV1.

Furthermore, the nucleotide-binding site was also more similar to that of the ‘tight’ form than to that of the ‘ADP-bound’ form (Fig. 6b–d, and Supplementary Fig. 7). We, therefore, designated this shifted ‘ADP-bound’ form of 3ADPV1 as the ‘tight-like’ form. The distances between the β-phosphate of ADP and the interacting residues in the ‘tight-like’ form were slightly longer than those in the ‘ADP-bound’ form (Supplementary Fig. 6), suggesting that the binding affinity for ADP of the ‘tight-like’ form is lower than that of the ‘ADP-bound’ form. Consequently, an ADP molecule will be easily released from the binding site. Therefore, we inferred that the structure of 3ADPV1 corresponds to the state of waiting for ADP release (that is, ADP-release dwell) in the rotation. ATP hydrolysis activity of purified EhV1 was inhibited at a high (2 mM) concentration of ADP (Supplementary Table 1), which is significantly higher than the natural concentration in E. hirae cells. Therefore, the ADP-release dwell state might be a minor intermediate state, which might exist in the catalytic cycle with high [ADP] and low [ATP].

Table 1 | Data collection and refinement statistics of the V1-ATPase.

| Denoted as | 2ATPV1 | 2ADPV1 | 3ADPV1 | 0.5V1:20 μM | 0.5V1:200 μM | 1V1 |
|------------|--------|--------|--------|-------------|-------------|-----|
| **Crystallization condition** | | | | | | |
| Soaking with | 2 mM AMP-PNP | 20 mM ADP | 2 mM ADP | 20 μM Pi | 200 μM Pi | 2 mM Pi |
| Soaking time | 6.5 h | 4.5 h | 4.5 h | 5.5 h | 5.0 h | 5.0 h |
| **Data collection** | | | | | | |
| Beamline | PF BL1A | PF BL1A | PF BL1A | PF BL1A | PF BL1A | PF BL1A |
| Wavelength (Å) | 1.1000 | 1.1000 | 1.1000 | 0.9800 | 1.0000 | 1.0000 |
| Space group | P212121 | P212121 | P212121 | P212121 | P212121 | P212121 |
| Cell dimensions | | | | | | |
| a, b, c (Å) | 128.3, 128.4, 226.9 | 127.4, 129.6, 237.2 | 121.7, 126.5, 225.3 | 128.5, 128.5, 225.3 | 127.9, 128.4, 226.7 | 128.2, 128.4, 228.0 |
| α,β,γ (°) | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 |
| Resolution (Å) | 50.2–2.73 | 50.3–2.35 | 50.3–2.02 | 50.3–2.04 | 50.2–2.84 | 49.04–2.89 |
| | (2.89–2.73)* | (3.45–3.25) | (3.21–3.02) | (3.23–3.04) | (3.01–2.84) | (2.99–2.89) |
| Rmerge | 0.170 (0.916) | 0.199 (1.064) | 0.221 (1.064) | 0.251 (0.873) | 0.222 (0.961) | 0.157 (0.824) |
| l/σl | 10.14 (1.87) | 10.33 (1.88) | 10.08 (1.92) | 8.79 (2.08) | 9.34 (1.99) | 12.5 (2.2) |
| Completeness (%) | 98.9 (94.4) | 99.4 (97.2) | 99.2 (95.4) | 99.7 (99.2) | 99.7 (98.7) | 99.9 (100) |
| Redundancy | 6.5 (6.3) | 6.7 (6.6) | 6.6 (6.5) | 6.3 (6.5) | 6.7 (6.8) | 6.6 (5.3) |
| **Refinement** | | | | | | |
| Resolution (Å) | 50.2–2.73 | 50.3–2.35 | 50.3–2.02 | 48.93–3.04 | 50.2–2.84 | 49.04–2.89 |
| No. of reflections | 99,064 | 62,128 | 67,952 | 72,486 | 88,898 | 84,446 |
| Rwork/ Rfree (%) | 20.5/23.2 | 20.9/24.5 | 21.4/25.3 | 23.0/27.3 | 18.5/20.1 | 20.7/25.1 |
| No. of atoms | 26,653 | 25,976 | 26,554 | 26,389 | 26,414 | 26,309 |
| Protein | 26,653 | 25,976 | 26,554 | 26,389 | 26,414 | 26,309 |
| Ligand/ion | 137 | 68 | 173 | 44 | 80 | 43 |
| Water | 299 | 33 | 72 | 29 | 268 | 64 |
| B-factors | | | | | | |
| Protein | 55.37 | 83.02 | 55.82 | 49.78 | 44.90 | 64.84 |
| Ligand/ion | 47.81 | 57.03 | 57.91 | 56.10 | 50.85 | 70.55 |
| Water | 38.22 | 52.84 | 39.93 | 17.61 | 29.66 | 46.61 |
| r.m.s. deviations | | | | | | |
| Bond lengths (Å) | 0.004 | 0.003 | 0.003 | 0.002 | 0.002 | 0.003 |
| Bond angles (°) | 0.872 | 0.673 | 0.660 | 0.548 | 0.757 | 0.575 |
| PDB ID | - | 5KNB | 5KNC | - | - | 5KND |

All data sets were obtained from single crystal each. *Highest resolution shell is shown in parentheses.
affinity for Pi is lower than that of either AMP-PNP or ADP. We further soaked the crystals in a higher concentration (2 mM) of Pi, and the crystal structure (denoted 1PV1) was determined at a resolution of 2.9 Å (Fig. 7 and Table 1). A Pi molecule with Mg2⁺ was found in the 'tight' form, which was fixed by the Arg-finger as observed for the binding of the γ-phosphate of AMP-PNP in bV1 (Fig. 7 and Supplementary Fig. 7). Importantly, no conformational change was observed upon Pi:Mg2⁺ soaking (Fig. 7 and Table 1). The soaking of eV1 crystals in Pi did not induce conformational changes, as in the case of 2ATPV1 after soaking with AMP-PNP. We also soaked the crystals of eV1 in the mixture of various concentrations of AMP-PNP, ADP and/or Pi to obtain other intermediate states. However, diffraction of these soaked crystals was not sufficient to solve the structure. Careful optimization of the ligand concentrations and crystal soaking times are necessary to improve the resolutions.

**Binding affinities of nucleotide to V1 complex.** We performed isothermal titration calorimetry (ITC) experiments to estimate the binding affinities of AMP-PNP to nucleotide-free EhV1. Exothermic reactions were observed upon the addition of AMP-PNP (Fig. 8a). The binding isotherm was saturated for titrations to an AMP-PNP/EhV1 molar ratio of 2.2 (~14 μM AMP-PNP); no additional binding was observed for titrations up to the molar ratio of 1,400 (2.4 mM AMP-PNP) (Supplementary Fig. 10). The curve was fit by the two sets of sites model with the following parameters. The numbers (n1 and n2) of binding sites per EhV1 were 0.68 and 0.77, respectively. The Kd1 and Kd2 values were 9.4 and 40 nM, respectively. The ΔH1 and ΔH2 values were 9.3 and 9.7 kcal mol⁻¹, respectively. The ΔS1 and ΔS2 values were 5.5 and 1.4 cal mol⁻¹ per degree, respectively. These ITC data suggested that the binding affinities of AMP-PNP to the 'bound' and 'tight' forms were both high, and that of the third 'empty' form was very low (<2 mM), corresponding to the structural findings described above.

Next, we quantified the binding affinities of ADP to nucleotide-free EhV1 using ITC. The binding isotherm for ADP titration was remarkably different from that for AMP-PNP titration, and showed three distinct zones (Fig. 8b). The first zone, below an ADP/EhV1 molar ratio of 2, was characterized by a continuous decrease in the exothermic signal. The second zone, between ADP/EhV1 molar ratios of 2 and 2.8, exhibited the opposite trend, with an increase in the exothermic signal throughout the titration. Finally, in the third zone, the exothermic signal decreased as the ADP/EhV1 molar ratios increased from 2.8 to 5.6 (a ratio at which saturation was reached), and no additional exothermic signal was observed for titrations up to an ADP/EhV1 molar ratio of 1,400 (2.4 mM ADP) (Supplementary Fig. 10).

Thus, the triphasic curve, which was likely to contain three different binding reactions, was analysed using the three sets of sites model with the following parameters. The numbers (n1, n2 and n3) of binding sites per EhV1 were 1.4, 0.82 and 0.65, respectively. The Kd1, Kd2 and Kd3 values were 6.7 nM, 13 nM and 3.6 μM, respectively. The ΔH1, ΔH2 and ΔH3 values were −4.3, 1.3 and −10 kcal mol⁻¹, respectively. The ΔS1, ΔS2 and ΔS3 values were 23, 41 and -10 cal mol⁻¹ per degree, respectively. Interestingly, ADP binding to site-2 showed an endothermic reaction, whereas those to site-1 and site-3 involved exothermic reactions. This implies a dynamic structural change with ADP.
binding to site-2, corresponding to the structural findings that ADP binding to the ‘tight’ form induces conformational changes to the ‘ADP-bound’ form and the adjacent ‘empty’ form then changes to a ‘bindable-like’ form in a cooperative manner. According to this interpretation, site-1, -2 and -3 correspond to the ‘bound’, ‘tight’ and ‘bindable-like’ forms of EhV1, respectively.

We also performed displacement ITC experiments of ADP-bound and AMP-PNP-bound EhV1 by addition of AMP-PNP and ADP, respectively. The titration experiment of AMP-PNP into 3ADP-bound EhV1 with 35 μM ADP (saturated concentration) showed that the exothermicity was remarkably lower than that into nucleotide-free EhV1 (Fig. 8a,c). No noticeable exothermic signal was observed for titrations up to an ADP/EhV1 molar ratio of 600 (2.4 mM ADP) (Supplementary Fig. 10). This suggests that AMP-PNP binding sites are already occupied by ADP in 3ADP-bound EhV1, and these nucleotides competitively bind to EhV1.

In the competitive displacement experiment, the apparent $K_m$ values for AMP-PNP to 3ADP-bound EhV1 were expected to be very high$^{42}$, and were actually estimated to be very weak. The binding of AMP-PNP yields a nearly horizontal trace in binding isotherm whether or not the real displacement of nucleotides takes place$^{43}$. Therefore, it is difficult to investigate the exchange reaction precisely from the ITC data. Similarly, the titration experiment of ADP into 2AMP-PNP-bound EhV1 with 21 μM AMP-PNP (saturated concentration) also showed that the exothermicity was remarkably lower than that into nucleotide-free EhV1 (Fig. 8b,d), and no noticeable exothermic signals were observed for titrations up to an ADP/EhV1 molar ratio of 600 (2.4 mM ADP) (Supplementary Fig. 10). It is also predicted the exothermic signal should be very small because the apparent $K_d$ values for ADP to 2AMP-PNP-bound EhV1 were estimated very high$^{42,43}$.

This finding suggests that ADP is not able to bind to the ‘empty’ form of $2_{ATP}V_1$, owing to low affinity, as in the case of AMP-PNP. Therefore, the ‘half-closed’ form of $3_{ADP}V_1$ seems to be obtained by ADP binding to the ‘bindable-like’ form of $2_{ADP}V_1$, but not to the ‘empty’ form of $eV_1$ in the soaking experiment of ADP to $eV_1$ crystals, consistent with the structural findings in this study.

**Tryptophan fluorescence change of $V_1$ complex.** Tryptophan fluorescence is very sensitive to conformational changes in proteins$^{44}$. In order to verify the conformational change induced by ADP binding, which was observed by X-ray crystallography of ADP-soaked crystals, we measured the tryptophan fluorescence of EhV1 in the presence of AMP-PNP and/or ADP. Eh-A and Eh-B subunits of EhV1 have 8 and 1 tryptophan residues, respectively. Emission spectra of the intrinsic tryptophan fluorescence of EhV1 without added nucleotides showed a peak at around 335 nm (Supplementary Fig. 11). The fluorescence intensity around 335 nm had distinct increase (2.3 ± 0.2 a.u.) by the addition of 500 nM AMP-PNP (a higher concentration than estimated $K_d$ values for AMP-PNP by ITC) (Fig. 8e, lane 1). However, the overall structure of $2_{ATP}V_1$ was very similar to that of $eV_1$ (Fig. 2). Therefore, we attributed this change in intensity to a side-chain shift of the Trp248 residue near the P-loop from AMP-PNP binding, rather than overall conformational changes of EhV1 (Supplementary Fig. 12). The fluorescence intensity was not affected by the re-addition of AMP-PNP (21 and 100 μM) (Fig. 8e, lane 2 and 3), consistent with the ITC data. On the other hand, the addition of 500 nM ADP (a higher concentration than the two $K_d$ values for ADP and lower than the third $K_d$ value for AMP-PNP) induced fluorescence change (3.1 ± 0.2 a.u.), which was higher than that of AMP-PNP (Fig. 8e, lane 4). This fluorescence increase is consistent with the conformational changes to a ‘bindable-like’ form (as in a), corresponding to the green box of Fig. 3g. (e,f) The viewing position, colours and representations of the binding sites correspond to those described in the right panel of e. eV1-‘empty’. f $eA_B$-‘bindable’. Green boxes (d-f) show the topological locations of Eh-A-Arg262 and Eh-B-Arg350.

**Figure 4** | Nucleotide-binding sites of the 2ADP-bound V1 complex (2ADPV1). (a) Magnified view of the nucleotide-binding sites with conserved residues of the ‘ADP-bound’ form in 2ADPV1, corresponding to the red box of Fig. 3e. Right panels, A-B interfaces rotated 90° around a vertical axis from the left columns. The |Fo|-|Fc| maps calculated without ADP:Mg$^{2+}$ at the binding pockets contoured at 4.0 sigma are shown in red (negative) and green (positive). (b,c) The viewing position, colours and representations of the binding sites correspond to those described in the right panel of a. bV1-‘tight’. Dotted lines indicate the distances (Å) between atoms. (c) a (right panel) is superimposed at the adenosine part onto that of b (shown in transparent grey). (d) Magnified nucleotide-binding site of the ‘bindable-like’ form (as in a), corresponding to the red box of Fig. 3e. (e,f) The viewing position, colours and representations of the binding sites correspond to those described in the right panel of d. eV1-‘empty’. f $eA_B$-‘bindable’. Green boxes (d-f) show the topological locations of Eh-A-Arg262 and Eh-B-Arg350.
changes observed for the 2ADP-bound crystal structure (2ADPV1) (Supplementary Fig. 12). Addition of 35 μM ADP (a higher concentration than the third K_d value for ADP) induced further changes in fluorescence intensity (Fig. 8e, lane 5), and the intensity was not affected by the addition of ADP at a higher concentration (that is, 100 μM ADP) (Fig. 8e, lane 6). This intensity change might correspond to the conformational changes to 3ADPV1 by ADP binding to the third 'bindable-like' form of 2ADPV1 (Supplementary Fig. 12).

We also performed competitive displacement experiments of ADP-bound and AMP-PNP-bound EhV1 by addition of AMP-PNP and ADP, respectively. When 2 mM AMP-PNP was
added into 3ADP-bound EhV1 pre-incubated with 35 μM ADP (saturated concentration), the fluorescence intensity decreased rapidly (Supplementary Fig. 11), and reached an equilibrium within 5 min (2.3 ± 0.1 a.u.; change from nucleotide-free EhV1) (Fig. 8f, lane 1), which was very similar to that of AMP-PNP bound EhV1 (Fig. 8e, lanes 1–3). Similarly, when 2 mM ADP was added into 2AMP-PNP-bound EhV1 pre-incubated with 21 μM AMP-PNP (saturated concentration), the fluorescence intensity increased slowly to 3.6 ± 0.1 a.u. (change from nucleotide-free EhV1) (Fig. 8f, lane 2 and Supplementary Fig. 11), which was very similar to that of 3ADP-bound EhV1 (Fig. 8e, lanes 5 and 6). These findings suggest that EhV1 is able to bind AMP-PNP and ADP at two or three binding sites competitively and to reversibly change the conformations.

Discussion
We previously reported that the structure of bV1 represents the catalytic dwell state (that is, a state of waiting for ATP hydrolysis) in which two ATP analogs (AMP-PNP) are bound, one in the ‘bound’ and the other in the ‘tight’ form. ATP hydrolysis is thought to occur in the ‘tight’ form due to induction caused by

Figure 7 | Structure of the P1-bound V1 complex (1PiV1). (a) Side view. (b) Top views of the C-terminal domain (transparent surface in a) from the cytoplasmic side in which the ‘bound’ form is superimposed onto that of eV1 (grey). (c) Magnified nucleotide-binding site of the ‘tight’ form in 1PiV1, in which the Eh-A residues (67–593) are superimposed onto the same residues of the ‘tight’ form (shown in transparent grey) in eV1, as in Fig. 4c. The bound Pi molecules are depicted in stick format and coloured orange.

Figure 8 | Biochemical properties of the Enterococcus hirae V1 complex. (a–d) Isothermal titration calorimetry (ITC) analysis. Nucleotides (200 μM) were injected into 7 mM EhV1 at 25 °C. The integrated heat values from raw heats (inset) were plotted against the molar ratio of nucleotides to EhV1 after subtraction of the nucleotide dilution heat values from the corresponding heat values of the EhV1-nucleotide titration. (a, b) show the binding isotherm titrated to nucleotide-free EhV1 with AMP-PNP (a) and ADP (b). The solid line represents the best fit to a binding model including the two sets of sites model for AMP-PNP (a) and the three sets of sites model for ADP (b). (c) shows the binding isotherm titrated to ADP-bound EhV1 with AMP-PNP. (d) shows the binding isotherm titrated to AMP-PNP-bound EhV1 with ADP. (e) Tryptophan fluorescence changes of nucleotide-free EhV1 by addition of 500 nM (lane 1), 21 μM (lane 2) and 100 μM (lane 3) AMP-PNP and 500 nM (lane 4), 35 μM (lane 5) and 100 μM (lane 6) ADP. (f) Tryptophan fluorescence changes of 35 μM AMP-PNP-bound and 21 μM AMP-PNP-bound EhV1 from nucleotide-free EhV1 by addition of 2 mM AMP-PNP (lane 1) and 2 mM ADP (lane 2), respectively. The intensity was averaged between 330 and 340 nm. All data represent means ± standard estimated errors (s.e.m.) of three independent experiments.
the approach of the Arg-finger. However, the details of the reaction after hydrolysis remain unresolved. A new ATP molecule is unable to bind to the 'empty' form owing to its low affinity. In order for the reaction to continue, certain structural changes in the 'tight' form need to be induced via conversion to ADP and Pi. In this study, we solved the crystal structures of the 2ADP-bound V1 complex (2ADPV1) by soaking eV1 crystals in 20 μM ADP. The 'tight' form changed to the 'ADP-bound' form, and cooperatively induced conformational changes from the 'empty' to 'bindable-like' forms. In contrast, soaking with 20 and 200 μM Pi did not produce an electron density peak for P_i or any conformational change. Thus, ADP has a much higher binding affinity to the 'tight' form than does P_i. In the presence of 2 mM P_i, P_iMg^{2+} was bound to the 'tight' form, but this binding did not induce conformational changes. Therefore, we concluded that P_i is released first after ATP hydrolysis, which changes the conformation to the ATP-binding dwell state (2ADPV1). If ADP was released first, the conformational change required to continue the rotational reaction would not be induced, as observed for the 1P_iV1 structure.

First, there were clear structural differences between the catalytic dwell (eV1 = 2ATPV1) and ATP-binding dwell (2ADPV1). The DF axis of 2ADPV1 did not rotate significantly, but was instead tilted toward the 'ADP-bound' form owing to the conformational changes induced by the binding of ADP to the 'tight' form of eV1 (see Supplementary Movie 1). Such a tilt of DF without apparent rotation would be difficult to detect using the single-molecule observations as an additional substep. Second, there were clear structural differences between the ATP-binding dwell (2ADPV1) and the ADP-release dwell (3ADPV1). The DF axis of 3ADPV1 was slightly bent towards the 'tight-like' form, but did not induce any rotational changes (see Supplementary Movie 3). This small shift in DF without apparent rotation would also be difficult to be detected using our single-molecule observations as additional substeps. These findings suggested that EhV1 exists in at least three dwell states in the 120° rotation without any rotational substeps. Thus, although the number of dwell states in EhV1 and mammalian F1 appears to be the same, these V1 and F1 motors show clear differences in the release order of cleavage products, rotational arrest points, dynamics and conformational changes.

Three-dimensional structures for three rotational states of the whole V-ATPase complex of *Saccharomyces cerevisiae* have been obtained by electron cryo-microscopy. The samples for this analysis were obtained in the absence of nucleotides during the purification procedures. Therefore, the V1 part of the three structures (PDB number: 3JT, 3JU and 3JV) seemed to correspond to the nucleotide-free form. These three structures of yeast V1 are comparable to those of EhV1, and are the most similar to that of eV1 (the nucleotide-free form of EhV1 corresponding to the catalytic dwell state), although the tilts of these DF complexes are different (Supplementary Fig. 13). Thus, nucleotide-free V1-ATPases seem to form the catalytic dwell state, rather than the ATP-binding dwell and ADP-release dwell states. Recently, the crystal structure of *S. cerevisiae* V1-ATPase has been obtained at a 6.2 Å resolution. This structure appears to be an inhibitory state wherein the subunit H inhibits the ATPase activity by stabilizing ADP binding to the catalytic site. We compared these structures of yeast V1 and EhV1, and found a lack of similarity between the yeast structure and EhV1.

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**Figure 9** Proposed model of the rotation mechanism of *Enterococcus hirae* V1-ATPase. (a–d) The structure models are based on the crystal structures of 2ATPV1 (catalytic dwell; a,d), 2ADPV1 (ATP-binding dwell; b), and 3ADPV1 (ADP-release dwell; c) determined in this study. ATP indicated as a yellow ‘P’ in (a) and (d) represents an ATP molecule that is committed to hydrolysis. (e) Correspondence table for all AB pairs observed in the crystal structures of the AβB3 and V1 complexes. See text for additional details.

| AB pair | State     | ATP-unbound | ATP-accessible | ATP-binding | ATP-hydrolysing | ADP-bound |
|---------|-----------|-------------|----------------|-------------|-----------------|-----------|
| Empty   | ATP-unbound | ATP-accessible | ATP-binding | ATP-hydrolysing | ADP-bound |
| ATP     | ATP-unbound | ATP-accessible | ATP-binding | ATP-hydrolysing | ADP-bound |
| ADP     | ATP-unbound | ATP-accessible | ATP-binding | ATP-hydrolysing | ADP-bound |
| V1      | ATP-unbound | ATP-accessible | ATP-binding | ATP-hydrolysing | ADP-bound |

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**Table** Correspondence table for all AB pairs observed in the crystal structures of the AβB3 and V1 complexes. See text for additional details.
structures \( \left( ^{2} \text{ATP} V_{1}, ^{2} \text{ADP} V_{1}, ^{3} \text{ADP} V_{1} \right) \), suggesting that the yeast \( V_{1} \) structure provides a unique view of an inhibitory state of a eukaryotic \( V \)-ATPase (Supplementary Fig. 13). Finally, we propose a potential model to describe the rotation mechanism of \( E. hirae \) \( V_{1} \)-ATPase based on the observed crystal structures and single-molecule observations\(^{32,33}\). Figure 9 shows the 120° rotation model starting from the catalytic dwell (Fig. 9a), in which the surface structure of the C-terminal domain of \( 2 \text{AMP-PNP-bound} V_{1} \left( ^{2} \text{ATP} V_{1} \right) \) is depicted, and the two ATP molecules are bound to the ‘bound’ and ‘tight’ forms (see Supplementary Fig. 14 and Supplementary Movie 4 for the 360° rotation model). The ATP that is tightly bound to the ‘tight’ form is hydrolysed to produce ADP and \( P_{i} \). The \( P_{i} \) molecule, which has a lower affinity than ADP, is released, inducing a change from the ‘tight’ to ‘ADP-bound’ form. Consequently, the DF axis tilts toward the ‘ADP-bound’ form, but this does not induce a rotational event of the DF axis. The adjacent ‘empty’ form (ATP-unbound state) then changes to a ‘bindable-like’ form (ATP-accessible state) in a cooperative manner (Fig. 9b: ATP-binding dwell). Next the ATP molecule binds to the ‘bindable-like’ form, which induces a conformational change to the ‘bound’ form, thereby releasing the bound ADP from the ‘ADP-bound’ form. If the ADP stays in the ‘ADP-bound’ form, the ‘bindable-like’ form will become ‘half-closed’ due to ATP binding (more specifically, due to ADP-\( M G^{2+} \) binding with \( S O_{4}^{2-} \), as shown in Fig. 6a), which is accompanied by a small shift of the DF axis, but no apparent rotational substep. Consequently, the adjacent ‘ADP-bound’ form cooperatively returns to the ‘tight-like’ conformation, and the binding affinity for ADP is reduced, as described above (Fig. 9c: ADP-release dwell). Then, ADP is released from the ‘tight-like’ form, and the ‘half-closed’ form is converted to the ‘bound’ conformation. Following these conformational changes, the DF axis rotates 120° with a torque of \( \sim 25 \text{pNnm} \) (ref. 33), and conformational changes from ‘tight-like’ to ‘empty’ and from ‘bound’ to ‘tight’ occur as a result of protein–protein interactions with the DF axis\(^{31}\). Finally, the enzyme resumes its initial catalytic dwell state, shown in Fig. 9a (Fig. 9d). Thus, the \( V_{1} \) motor achieves its final catalytic dwell state, shown in Fig. 9a (Fig. 9d). The ATPase activity and protein concentrations of the purified \( V_{1} \)-ATPase in the presence of various concentrations of sodium phosphate were measured using an ATP regenerating system\(^{31,59}\). ATP hydrolysis rates at 23 °C were determined in terms of the rate of NADH oxidation, which was measured as a decrease in absorbance of 340 nm, and the measurement was repeated three times. Protein concentrations were determined using Pierce \( BCA \) Protein Assay Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with bovine serum albumin as the standard.

**Measurement of \( V_{1} \)-ATPase activity and protein concentrations.** ATPase activity of the purified \( V_{1} \)-ATPase in the presence of AMP-PNP or ADP was measured by the colorimetric method using molybdic acid\(^{22,23}\). The reaction was initiated by the addition of \( 1 \text{mM} \) ATP, after a 10 min pre-incubation with various concentrations of AMP-PNP or ADP, and terminated by the addition of \( 10 \text{mM} \) sodium dodecyl sulphate. The initial rate of the ATPase reaction at 23 °C was determined within 4 min, and the measurement was repeated three times. ATPase activities of the purified \( V_{1} \)-ATPase in the presence of various concentrations of sodium phosphate were measured using an ATP regenerating system\(^{31,59}\). ATP hydrolysis rates at 23 °C were determined in terms of the rate of NADH oxidation, which was measured as a decrease in absorbance of 340 nm, and the measurement was repeated three times. Protein concentrations were determined using Pierce \( BCA \) Protein Assay Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with bovine serum albumin as the standard.

**Isotothermal titration calorimetry (ITC).** \( V_{1} \)-ATPase was purified by mixing 12 mM \( A_{2}B_{3} \) and 60 mM \( D \) in 900 \text{mM} of buffer \( D \), and the suspended sample buffer was replaced with buffer-\( F \) (100 mM Tris-HCl, 100 mM NaCl, and 5 mM \( M G S O_{4} \) \( \text{pH} 7.5 \)) using Spectra/Por 3 Dialysis Tubing (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA). ITC experiments were performed using the MicroCal iTC200 calorimeter (Malvern Instruments Ltd., Malvern, Worcestershire, UK), and the samples \( (7 \text{mM}) \) with \( 21 \text{mM} \) AMP-PNP or 35 mM ADP were loaded into the sample cell. Either 200 mM AMP-PNP or ADP in buffer-\( F \) was injected into the sample cell at 25 °C using one initial injection of 1.0 \text{mL} followed by 18
injections of 2.0 µL. Binding data were fitted to the two sets of site model using Origin 7.0 (MacroCal) or the three sets of site model using MATLAB.25

Tryptophan fluorescence. V1-ATPase was prepared by mixing 100 nM Aβ6 and 300 nM DF in buffer F in 1.2 ml. Fluorescence experiments were performed using the FP-6500 spectrofluorimeter (JASCO, Tokyo, Japan) at 25 °C. Fluorescence spectra were recorded with excitation at 300 nm (slit width 1 nm) and emission between 310 and 450 nm (slit width 20 nm). Time courses of exchange reactions of AMP-PNP and ADP were measured every 0.5 s at 335 nm with excitation at 300 nm. Time courses were averaged for 20 data points around each point.

Data availability. Coordinates and structure factors for the ADP- and Pi-bound V1-ATPase complexes have been deposited in the Protein Data Bank under the access codes 5KNB (doi: 10.2210/pdb5knb/pdb; 2ADPV1 at 3.3 Å) (ref. 60), NATURE COMMUNICATIONS| 7:13235 | DOI: 10.1038/ncomms13235 | www.nature.com/naturecommunications

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Author contributions

T.M. designed the study. Y.K., Y.I.-K, M.S. and S.Y. prepared the proteins. K. Suzuki crystallized the proteins. K. Suzuki and K.M. collected X-ray data. K. Suzuki, K.M. and F.I.I. processed and refined X-ray data. K. Suzuki, S.M, K. Shimono and E.M. performed functional analysis. K. Suzuki, K. Shimono, E.M., I.Y. and T.M. analysed the results. K. Suzuki prepared figures and movies. T.M. wrote the paper. All authors discussed the results and commented on the manuscript.

Additional information

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