Basic properties of rotary dynamics of the molecular motor *Enterococcus hirae* V₁-ATPase

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Background: Chemo-mechanical coupling scheme of rotary motor V₁-ATPase is incompletely understood.

Results: *Enterococcus hirae* V₁-ATPase (EhV₁) showed 120° steps of rotation without sub-steps commonly seen with F₁-ATPase.

Conclusion: Basic properties of rotary dynamics of EhV₁ are similar to those of *Thermus thermophilus* V₁-ATPase.

Significance: This study revealed common properties of V₁-ATPases as rotary molecular motors, distinct from those of F₁-ATPases.

ABSTRACT

V-ATPases are rotary molecular motors that generally function as proton pumps. We have recently solved the crystal structures of the V₁ moiety of *Enterococcus hirae* V-ATPase (EhV₁) and proposed a model for its rotation mechanism. Here, we characterized the rotary dynamics of EhV₁ by using single-molecule analysis employing a load-free probe. EhV₁ rotated in a counterclockwise direction, exhibiting 2 distinct rotational states, namely, clear and unclear, suggesting unstable interactions between the rotor and stator. The clear state was analyzed in detail to obtain kinetic parameters. The rotation rates obeyed Michaelis-Menten kinetics with a maximal rotation rate ($V_{max}$) of 107 revolutions per second and a Michaelis constant ($K_m$) of 154 μM at 26°C. At all the ATP concentrations tested, EhV₁ showed only 3 pauses separated by 120° per turn, and no sub-steps were resolved, as was the case with...
**Thermus thermophilus** V$_1$-ATPase (TtV$_1$). At 10 µM ATP (<<$K_m$), the distribution of the durations of the ATP-waiting pause fit well with a single-exponential decay function. The second-order binding rate constant for ATP was $2.3 \times 10^6$ M$^{-1}$s$^{-1}$. At 40 mM ATP (>>$K_m$), the distribution of the durations of the catalytic pause was reproduced by a consecutive reaction with 2 time constants of 2.6 ms and 0.5 ms. These kinetic parameters were similar to those of TtV$_1$. Our results identify common properties of rotary catalysis of V$_1$-ATPases that are distinct from those of F$_1$-ATPases and will further our understanding of the general mechanisms of rotary molecular motors.

V-ATPase is a rotary molecular motor that couples ion transport to ATP hydrolysis and synthesis. The main function of V-ATPase in eukaryotes is to transport protons across a membrane by using the energy derived from ATP hydrolysis (1-3). V-ATPase also catalyzes ATP synthesis, harnessing the energy of proton flow in certain eubacteria, such as **Thermus thermophilus**. V-ATPases are composed of V$_1$-ATPase (V$_1$), a water-soluble moiety that hydrolyzes and synthesizes ATP, and a membrane-embedded moiety, V$_o$, that translocates ions. The V$_1$ and V$_o$ domains are connected by a rotary shaft and peripheral stalks (1-3). The V$_1$ complex is composed of A, B, D, and F subunits, in which the 3 A and 3 B subunits are alternately arranged, forming a hexameric stator A$_3$B$_3$ ring (4-7). ATP hydrolysis and synthesis occur on the catalytic sites that are located at the interfaces of the A and B subunits, with the majority of the catalytic residues residing in the A subunits. The rotary shaft is composed of D and F subunits penetrating into the central cavity of the A$_3$B$_3$ ring (6,7).

The rotation of V$_1$ has been visualized using optical microscopy by attachment of a probe to the rotary shaft (8-11). V$_1$ of **Thermus thermophilus** (TtV$_1$), which functions as an ATP synthase, rotates stepwise in a counterclockwise direction (8). The basic step size is 120° and, similar to F$_1$-ATPase (F$_1$), the water-soluble moiety of F$_o$F$_1$-ATP synthase (12), each step is coupled to the consumption of a single ATP molecule (10). While no sub-steps have yet been resolved in the rotation of TtV$_1$ (10,11), the 120° steps of F$_1$ from the thermophilic **Bacillus** PS3 (TF$_1$) and **Escherichia coli** (EF$_1$) have been shown to be further divided into 80° and 40° sub-steps, and 85° and 35° sub-steps, respectively (13-15). The 80° and 85° sub-steps are triggered by ATP binding and ADP release, whereas the 40° and 35° sub-steps are known to occur after ATP cleavage and release of inorganic phosphate (Pi). Accordingly, the pauses before the 80° and 85° sub-steps are referred to as ATP-binding (ATP-waiting) pauses, and those prior to the 40° and 35° sub-steps are known as catalytic pauses. As described above, the chemo-mechanical coupling scheme of TtV$_1$ appears to be distinct from that of F$_1$. However, to date, the stepping rotations of V$_1$ complexes other than TtV$_1$ have not been described, and the chemo-mechanical coupling scheme of V$_1$ remains unclear (9).

**Enterococcus hirae** V-ATPase functions as a primary ion pump, similar in nature to eukaryotic V-ATPases (16,17). We have recently solved the crystal structures of the V$_1$ component of **Enterococcus hirae** V-ATPase (EhV$_1$), and have proposed a model of its rotation mechanism (6). In this study, to characterize the stepping rotation of EhV$_1$, we analyzed and compared the basic properties of EhV$_1$ rotary dynamics with those of TtV$_1$, TF$_1$ and EF$_1$. As was the case with TtV$_1$, no sub-steps were resolved in the rotation of EhV$_1$, suggesting that 120° stepping rotation without sub-steps is a common property of V$_1$ complexes.

**EXPERIMENTAL PROCEDURES**

Preparation of Recombinant EhV$_1$ and Avi-Tagged EhV$_1$ Expressed in Escherichia coli. – The EhV$_1$ holocomplex (A$_3$B$_3$DF) was expressed in **Escherichia coli** by using the expression plasmid pTR19-FABD. We synthesized a DNA fragment containing the ntpF, ntpA, ntpB and ntpD genes (in this order) and optimized its codon usage for **Escherichia coli** expression. This fragment was then cloned into plasmid pTR19, the expression vector for the F$_o$F$_1$-ATP synthase of thermophilic **Bacillus** PS3 (18), after which a His$_6$-tag was introduced at the N terminus of the A subunit by PCR to obtain the plasmid pTR19-FABD. For the rotation assay, we used the plasmid pTR19-FABD-Avi, in which the AviTag biotinylation sequence (GLNDIFEAQKIEWHE) (19) was inserted between Gly-121 and Tyr-122 of the D...
subunit by PCR-based mutagenesis. *Escherichia coli* BL21 (DE3) was transformed with pTR19-FABD or pTR19-FABD-Avi and cultured in Super Broth (32 g/l tryptone, 20 g/l yeast extract, and 5 g/l sodium chloride) containing 100 µg/ml ampicillin and 2 mM isopropyl-β-D-thiogalactopyranoside at 37°C for 20 h. Cells were suspended in buffer W (20 mM KPi, pH 7.0, 230 mM NaCl, and 20 mM imidazole) and disrupted by sonication. After removal of the cell debris by centrifugation (81,000 × g, 20 min, 4°C), the solution was applied to a Ni-nitrilotriacetic acid column (Ni-NTA Superflow, Qiagen). After washing with 10 column volumes of buffer W, recombinant EhV1 or Avi-tagged EhV1 was eluted with buffer E (20 mM KPi, pH 7.0, 50 mM NaCl, and 200 mM imidazole). The eluted fractions were concentrated with an Amicon Ultra 10 K unit (Merck Millipore), and then passed through a gel-filtration column (Superdex 200, GE Healthcare) equilibrated with buffer G (20 mM MES-NaOH, pH 6.5, 100 mM NaCl, 10% glycerol). The purified proteins were flash-frozen in liquid nitrogen and stored at –80°C until use.

**Preparation of the A3B3 Subcomplex** – The A3B3 subcomplex (A3B3) was expressed in *Escherichia coli* harboring the expression plasmid pTR19-AB. Plasmid pTR19-AB was constructed by removing the *ntpF* and *ntpD* genes from pTR19-FABD by PCR. Expression and purification of the A3B3 subcomplex were performed using the same procedure as for the EhV1 holocomplex, and purified proteins were stored at –80°C.

**Preparation of the DF Subcomplex** – An *Escherichia coli* cell-free protein-expression system was employed to synthesize the DF subcomplex (DF) by using a mixture of plasmids containing the corresponding genes. The expressed DF was purified as previously described (6). The homogeneity of each purified subcomplex was judged by SDS-PAGE analysis. After purification, cysteine residues in DF were biotinylated with a 3-molar excess of biotinylation reagent (biotin-PEAC5-maleimide, Dojindo) in 20 mM MOPS-KOH, pH 6.5, and 150 mM NaCl at room temperature for 20 min. The reaction was quenched using 10 mM DTT. We used a mutant DF containing 2 engineered cysteine residues in its D subunit (T60C/R131C), substituted using a QuickChange site-directed mutagenesis kit (Agilent Technologies), and a single endogenous cysteine residue in both D (C153) and F (C54) subunits. A maximum of 3 of these residues can be expected to react with the biotinylation reagent. Specific biotinylation of the D subunit was confirmed by western blotting with streptavidin-alkaline phosphatase conjugate (Fig. 1).

**Preparation of Reconstituted EhV1** – Reconstituted EhV1 was prepared according to the following procedure: Purified A3B3 and biotinylated-DF were mixed at a 1:2 molar ratio and incubated at room temperature for 2 h. Reconstituted EhV1 was purified using a gel-filtration column (Superdex 200, GE Healthcare) equilibrated with buffer G and flash-frozen in liquid nitrogen and stored at –80°C until use.

**Biochemical Assay** – The protein concentration of EhV1 was determined based on UV absorbance using a molar extinction coefficient of 310,910 M⁻¹ cm⁻¹ calculated from its amino acid sequence (ProtParam tool, ExPASy). The ATP hydrolysis rate of EhV1 was measured using an ATP-regenerating system. The reaction mixture contained 50 mM MES-KOH, pH 6.5, 50 mM KCl, 5 mM MgCl₂, 2.5 mM phosphoenol pyruvate, 0.2 mg/ml NADH, 0.1 mg/ml pyruvate kinase, and 0.1 mg/ml lactate dehydrogenase, in addition to various concentrations of ATP. The rate of ATP hydrolysis was monitored as the rate of NADH oxidation at times ranging from 0 to 10 s after addition of V₁, which was measured as a decrease in the absorbance at 340 nm. All measurements were carried out at 25°C ± 1°C.

**Rotation Assay** – To observe ATP hydrolysis-driven rotation of EhV1, the A3B3 stator ring was immobilized on an Ni²⁺-NTA-coated glass surface via a His₆-tag introduced at the N-terminus of the A subunit. Streptavidin-coated 40-nm gold colloid was then attached to the biotinylated cysteine or Avi-tag in the rotor DF as a probe. The gold colloids were prepared as described previously (20). The rotations of the gold colloid were observed at 26°C ± 1°C by an objective-type total internal reflection dark-field microscope constructed on an inverted microscope (IX-71, Olympus) (20). The images were recorded with a high-speed CMOS camera (FASTCAM-1024PCI, Photron) at 1,000–10,000 frames per second (fps). The flow cell was assembled
from a Ni\textsuperscript{2+}-NTA-coated glass (24 × 36 mm\textsuperscript{2}) and an uncoated coverglass (18 × 18 mm\textsuperscript{2}) separated by 2 spacers of ~50 μm thickness. First, buffer R (5 mg/ml BSA, 20 mM KPi, pH 7.0, 230 mM NaCl, 20 mM imidazole) was infused into the flow cell to prevent nonspecific binding of the EhV\textsubscript{1} and gold colloid. After incubation for 10 min, EhV\textsubscript{1} (0.5−2 nM in buffer R) was infused into the flow cell. After incubation for 5 min, unbound EhV\textsubscript{1} was washed out with buffer R, after which gold colloid suspended in buffer R was infused. After 10 min, unbound gold colloid was washed out. Observation of rotation was initiated after infusion of buffer S (50 mM MES-KOH, pH 6.5, 50 mM KCl, 5 mM MgCl\textsubscript{2}) containing ATP (10 μM−3 mM) or MgATP (4 mM, 40 mM) and an ATP-regenerating system.

RESULTS

Recombinant EhV\textsubscript{1} – We first tried to carry out a rotation assay using recombinant EhV\textsubscript{1} (A\textsubscript{3}B\textsubscript{3}DF complex) expressed in Escherichia coli. To observe the rotation, the rotation probe must be attached to the rotor DF subunits through biotin-streptavidin linkage. Since 3 endogenous cysteine residues in the stator A subunit (C28, C174, and C259) may react with the biotinylation reagent, we substituted all these residues with serine or alanine residues (C28A/C174S/C259S) and measured the activity of this mutant. The ATP hydrolysis rate of mutant EhV\textsubscript{1}, measured by a biochemical assay, was less than 10% of that of wild-type EhV\textsubscript{1} (data not shown), indicating that the effect of substitution was significant.

Reconstituted EhV\textsubscript{1} – Since the rotor DF subunits can be biotinylated separately from the stator A\textsubscript{3}B\textsubscript{3} ring prior to reconstitution, we next used reconstituted EhV\textsubscript{1} in the rotation assay. Reconstituted EhV\textsubscript{1} has also been used recently for crystal structural analysis requiring a pure homogeneous sample (6). No differences were observed in the subunit composition for reconstituted EhV\textsubscript{1} and recombinant EhV\textsubscript{1} (Fig. 1), indicating high reconstitution efficiency. Furthermore, reconstituted EhV\textsubscript{1} had an ATP hydrolysis rate comparable to that of recombinant EhV\textsubscript{1} at all the ATP concentrations used, ranging from 10 μM to 4 mM (Fig. 2). The expected rotation rates of reconstituted EhV\textsubscript{1} and recombinant EhV\textsubscript{1}, calculated as one-third of the ATP hydrolysis rates, followed Michaelis-Menten kinetics. The maximal rotation rate ($V_{max}$) and Michaelis constant ($K_m$) were 73 ± 2 revolutions per second (rps) and 221 ± 17 μM, respectively, for reconstituted EhV\textsubscript{1} and 72 ± 1 rps and 246 ± 16 μM (mean ± S.E. of fitting), respectively, for recombinant EhV\textsubscript{1} (Fig. 2B, red and blue dashed lines in top panel). These results indicated that the kinetic parameters of reconstituted EhV\textsubscript{1} are almost identical to those of recombinant EhV\textsubscript{1}, suggesting that the catalytic properties of EhV\textsubscript{1} are not affected by the reconstitution process. Therefore, we decided to use reconstituted EhV\textsubscript{1} for the rotation assay.

EhV\textsubscript{1} Has Two Distinct Rotational States – The rotary motion of reconstituted EhV\textsubscript{1} was observed in a single-molecule assay using a 40-nm streptavidin-coated gold colloid as a load-free probe at a rate of 1,000–10,000 fps (Fig. 3). We found that the reconstituted EhV\textsubscript{1} complexes exhibited 2 distinct reversible states of rotation, namely, clear and unclear (Fig. 4A-C). In the clear rotational state, the majority of the centroids of gold colloid in each frame were distributed in the 3 positions separated by 120° and were remote from the rotation center. Moreover, the time course showed clear unidirectional rotation in a counterclockwise direction (Fig. 4A). On the other hand, in the unclear state, the centroids showed wide fluctuations toward the rotational center (Fig. 4B). Since the rotation rate in the unclear state seemed to be similar to that in the clear state, the complex appears to rotate unidirectionally, although the measured rotation rate in the unclear state may not be accurate.

To assess whether the 2 rotational states were observed only in the reconstituted EhV\textsubscript{1}, the D subunit of recombinant EhV\textsubscript{1} was fused to the Avi-tag (Avi-tagged EhV\textsubscript{1}), a 15-amin acid sequence that is subjected to biotinylation by biotin ligase in Escherichia coli (19). No differences were observed in the subunit compositions of Avi-tagged EhV\textsubscript{1} and reconstituted or recombinant EhV\textsubscript{1} (Fig. 1). Moreover, the ATP hydrolysis rate of Avi-tagged EhV\textsubscript{1} was comparable to that of reconstituted and recombinant EhV\textsubscript{1} in the biochemical assay (Fig. 2). Importantly, as was the case in the rotation assay with reconstituted EhV\textsubscript{1}, the Avi-tagged EhV\textsubscript{1} also exhibited 2 reversible rotational states (Fig. 4D-F). This result clearly shows that the 2 rotational states were not an artifact of
damage or inactivity caused by the reconstitution procedure, but rather represent an intrinsic property of EhV1. Given that the efficiency of biotinylation (Fig. 1) and the frequency of rotating probes for Avi-tagged EhV1 were significantly lower than those of the reconstituted EhV1, we used reconstituted EhV1 in the subsequent experiments.

Next, we analyzed the duration of the clear and unclear states for 10 μM to 40 mM ATP. Since we did not find a clear dependence of the duration on ATP concentration, we analyzed the data at various ATP concentrations collectively. The distributions of the duration times fit well to a single-exponential decay function, suggesting a single rate-limiting step in the transition between the clear and unclear states. The time constants were 0.13 ± 0.003 s (mean ± S.E. of fitting, 238 events from 58 molecules) for the clear state and 0.33 ± 0.009 s (mean ± S.E. of fitting, 199 events from 58 molecules) for the unclear state (Fig. 4G, H). The ratio of the clear state to the total observation time was ~0.3 (= 0.13/[0.13+0.33]).

To date, it has been unclear why transitions occur between the clear and unclear states. A study reported that while unusual fluctuations have been reported in the rotation of TtV1 (11), the behaviors are not entirely identical; in that study (11), the authors attributed the fluctuating state to the probe adopting 2 orientations relative to the D subunit and excluded these data from the analysis. Alternatively, the unclear state may be due to less stable interactions between the rotor and stator in V1 compared to those for F1. Since Vn and V1 are connected not only by the rotor but also by the 2 peripheral stalks composed of the E and G subunits, this unstable interaction would be anticipated to occur only in the isolated V1 complex and not in the physiological VnV1 complex. We nevertheless concluded that the tight chemo-mechanical coupling of EhV1 is achieved at least in the clear state and, accordingly, restricted our analysis in the remainder of the study to that state.

**ATP Dependence of Rotation** – The rotation rates of reconstituted EhV1 were measured at various concentrations of ATP ranging from 10 μM to 40 mM (Fig. 2B, red dots and open circles in top panel). Below 100 μM ATP, the rotation rates were almost proportional to the ATP concentration, indicating that ATP binding was rate-limiting in this range. Above 1 mM ATP, the rotation rate was essentially constant. The rotation rates followed Michaelis-Menten kinetics with a $V_{\text{max}}$ of 107 ± 5 rps and a $K_m$ of 154 ± 33 μM (mean ± S.E. of fitting) (Fig. 2B, red solid line in top panel). The second-order binding rate constant for ATP ($k_{\text{on}}^{\text{ATP}}$) determined from $3 \times V_{\text{max}}/K_m$ was (2.2 ± 0.4) × 10^6 M^-1s^-1 under the assumption that 3 ATP molecules were hydrolyzed per turn.

The value of $K_m$ determined by the single-molecule rotation assay was slightly lower than that determined by the biochemical assay. Furthermore, the value of $V_{\text{max}}$ estimated by the single-molecule rotation assay was approximately 50% greater than that deduced from the biochemical assay. This ratio was essentially constant at each ATP concentration (Fig. 2B, bottom). This result may arise from inaccuracy in protein concentration determination based on the molar absorbance coefficient calculated from the amino-acid sequence and/or imply that the ATP hydrolysis rate during the unclear state is slightly lower than that during the clear state. Furthermore, in the biochemical assay, the ATP hydrolysis rate gradually decreased during measurement (Fig. 2A). It is well known that the ATP hydrolysis of TtV1 which functions as ATP synthase is strongly regulated by Mg-ADP inhibition to prevent wasteful ATP consumption (21). ATP hydrolysis activity of TtV1 is inhibited rapidly and irreversibly in the presence of ATP. Since the decrease of ATP hydrolysis rate of EhV1 was much slower than that of TtV1 (Fig. 2A) and some EhV1 molecules showed reversible and irreversible long pauses in the rotation assay, the unknown inhibited-states of EhV1 other than Mg-ADP inhibition may exist.

Typical examples of rotation at 40 mM, 100 μM, and 10 μM ATP are shown in Fig. 5. At all ATP concentrations, EhV1 exhibited stepwise rotation with 3 intervening pauses separated by 120°, as shown in the X-Y trajectories and the distribution of the rotary angle (insets of Fig. 5). Since the 120° steps were completed within 0.2 ms (1–2 frames captured at 10,000 fps), the rotation rate was determined primarily by the duration of the intervening pause. At 40 mM ATP, a concentration considerably higher than $K_m$, the intervening pauses would represent the catalytic pauses (Fig. 5A), since the expected binding time constant for ATP (0.011 ms, estimated from 1/[40 × 10^3 M × 2.2 × 10^6 M^-1s^-1]) was 100-fold smaller than...
the duration times (3.1 ms) of pauses expected from $V_{\text{max}}$ (1/(107 × 3)). At 10 μM ATP, a concentration substantially lower than $K_m$, the intervening pauses would correspond to the ATP-waiting pauses, since ATP binding is rate-limiting under these conditions (Fig. 5C).

Even at 100 μM ATP, a concentration near $K_m$ and where the duration of the ATP-waiting pause approaches that of the catalytic pause, EhV1 exhibited only 3 pauses separated by 120° (Fig. 5B). This finding suggests that there are no sub-steps in the rotation of EhV1, as is the case for TtV1 (10,11).

Buffer Exchange Experiment – To further confirm that the angles of the ATP-waiting pauses observed at low ATP concentrations correspond to those of the catalytic pauses observed at saturating ATP concentrations, we next conducted a buffer-exchange experiment. After recording the stepwise rotation of EhV1 pausing every 120° at 10 μM ATP, we increased the ATP concentration to 3 mM by infusing buffer containing 3 mM ATP into the flow cell. After buffer exchange, while the rotation rate increased significantly, EhV1 continued to rotate with discrete 120° steps, pausing at almost the same angles (Fig. 6A, B). The distributions of the rotary angles indicated 3 peaks corresponding to 3 pauses in the rotation (Fig. 6B). To assess the difference in the angular position ($\Delta \theta$) between 10 μM and 3 mM ATP, the positions of the peaks were determined by fitting the histogram with Gaussian functions, and then comparing them with the nearest ones (Fig. 6B). The distribution of $\Delta \theta$ showed a single peak around 0°, with a mean value of 3.2 ± 12° (mean ± S.D., 21 events from 7 molecules, Fig. 6C). Based on these data, we confirmed again that the rotation of EhV1 contains no sub-steps.

Dwell Time Analysis – To obtain the time constants and kinetic parameters for elementary reaction steps such as ATP binding, ATP cleavage, and product release, we analyzed the duration of the pauses. On analyzing 4 molecules at saturating ATP concentrations (40 mM), all distributions of the durations of the catalytic pauses showed a convex shape (Fig. 7A-D). At 40 mM ATP, the expected time constant for ATP binding (0.011 ms) was too short to be resolved. Therefore, 3 elementary reaction steps could occur during the catalytic pauses, namely, ATP cleavage, ADP release, and phosphate release. We first attempted to fit the distributions with a model of 3 consecutive reactions. This failed to improve the fits compared to a model of 2 consecutive reactions with 2 time constants. The average time constants from 4 molecules were 2.4 ± 1.1 ms and 0.6 ± 0.2 ms (mean ± S.D.) (Fig. 7F, red open circle and square). These values are consistent with the time constants of 2.6 ± 0.1 ms and 0.5 ± 0.02 ms (mean ± S.E. of fitting, 2097 events), determined by reproducing the accumulated distribution of the pause duration time from all 4 molecules (Fig. 7E). In addition, at 4 mM ATP (expected time constant for ATP binding is 0.11 ms), similar accumulated distribution and time constants of 2.4 ± 0.05 ms and 0.5 ± 0.02 ms (mean ± S.E. of fitting, 4303 events from 9 molecules) were obtained (Fig. 7G and H), consistent with the saturation of the rotation rate at this concentration (Fig. 2B).

These time constants would correspond to (i) ATP cleavage and (ii) ADP and/or phosphate release, although it is currently unclear which time constant corresponds to which elementary reaction step. At a low ATP concentration (10 μM), as discussed above, the duration of the pauses corresponded to that of ATP waiting. Analysis of the 4 molecules showed that the distributions of the ATP-waiting duration time followed single-exponential decay (Fig. 7I-L), indicating that EhV1 consumed 1 ATP molecule per 120° step. The average time constant from 4 molecules was 45 ± 9 ms (mean ± S.D.) (Fig. 7N, red open circle), which is consistent with a time constant of 43 ± 1 ms (mean ± S.E. of fitting, 2058 events) determined by reproducing the accumulated distribution of duration time from all 4 molecules (Fig. 7M). This value corresponds to the $k_{\text{on}}^{\text{ATP}}$ of $2.3 \pm 0.03 \times 10^6$ M$^{-1}$s$^{-1}$, which is consistent with that determined by $3 \times V_{\text{max}}/K_m$ ((2.2 ± 0.4) × 10$^6$ M$^{-1}$s$^{-1}$), shown in Fig. 2B.

DISCUSSION

In this study, using a single-molecule assay, we have shown that EhV1 is a rotary molecular motor. To our knowledge, this is the first report to show that a eubacterial V1 functions as an ATP-driven ion pump under physiological conditions. EhV1 exhibited 2 rotation states, namely, clear and unclear (Fig. 4). Assuming that the clear rotation state represents the tight chemo-mechanical coupling of EhV1, we
analyzed this state to elucidate the basic rotational properties of EhV1. Our hypothesis that the unclear state is caused by unstable interactions between the rotor and stator of EhV1 must be examined by rotation assay of the entire Enterococcus hirae V-ATPase complex, in which the interactions between the rotor and stator are stabilized by 2 peripheral stalks. In order to perform this study, we are currently designing an Escherichia coli expression system in which an appropriately-tagged recombinant V-ATPase complex can be produced for a rotation assay.

In the clear rotational state, at all ATP concentrations ranging from below to above the $K_m$, EhV1 rotated unidirectionally in a counterclockwise direction, exhibiting 3 pauses separated by 120° (Fig. 5). No sub-steps were resolved, as has been reported for TtV1 (10,11). In contrast, in the region of their respective $K_m$ values, TF1 and EF1 have been reported to rotate with 6 pauses per turn (13-15). Recently, the overall crystal structures of TtV1 and EhV1 have been shown to be similar (4-7), especially with respect to the interaction sites between the rotor and stator. These structures are distinct from the structure of F1 (22), although many amino acid residues associated with catalysis in the binding pocket are conserved between V1 and F1. These results imply that the degree of similarity in the interactions between the rotor and stator determines the presence or absence of sub-steps in the rotation.

Table 1 contains a comparison of the kinetic parameters determined by the single-molecule assay for EhV1, showing values for TtV1, TF1, and EF1. Despite the difference in physiological function between EhV1 and TtV1 and notwithstanding the large difference (>30°C) in the optimal growth temperatures between Enterococcus hirae and Thermus thermophilus, the values for EhV1 are closer to those for TtV1 than to TF1 and EF1. This result implies that the basic properties of rotary dynamics are determined by their overall structures and that the difference in the physiological function derives from regulatory mechanisms such as Mg-ADP inhibition.

During the unclear rotation state, the centroids of the gold colloid showed wide fluctuations toward the rotation center. It should be noted that EhV1 nevertheless rotates unidirectionally, implying that even if the interactions between the rotor and stator are not perfect, EhV1 maintains unidirectional and cooperative rotary catalysis. Recently, rotary catalysis of the rotor-less stator $\alpha_3\beta_3$ ring of TF1 has been demonstrated by high-speed atomic force microscopy (23), and we speculate that the stator $\alpha_3\beta_3$ ring also likely exhibits rotary catalysis in the absence of the rotor DF subunits.

The chemo-mechanical coupling scheme of TF1 has been extensively studied by advanced single-molecule techniques such as a rotation assay of hybrid molecules and single-molecule manipulation with magnetic tweezers (24-26). For a single catalytic site of TF1, after ATP binding at 0°, ATP cleavage, ADP release, and phosphate release occur at 200°, 240° and 320°, respectively (26). Further studies on EhV1 by using advanced single-molecule techniques and high-resolution structural analysis will provide details on its chemo-mechanical coupling scheme. Moreover, comparison of the schemes of V1 and F1 from various species will shed light on the general mechanism of rotary molecular motors.

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FOOTNOTES

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2The abbreviations used are as follows: VoV1, V-ATPase; V1, V1-ATPase; F1, F1-ATPase; EhV1, V1-ATPase from *Enterococcus hirae*; TrV1, V1-ATPase from *Thermus thermophilus*; TF1, F1-ATPase from thermophilic *Bacillus* PS3; EF1, F1-ATPase from *Escherichia coli*.

FIGURE LEGENDS

**FIGURE 1. Gel electrophoresis**

Lanes 1–3, SDS-PAGE of reconstituted EhV1, recombinant EhV1, and Avi-tagged EhV1. A 16% gel was used; 12 pmol protein was loaded in each lane. The molecular weights of the A, B, D, F subunits are 65, 51, 24, and 11 kDa, respectively. Proteins were stained with Coomassie Brilliant Blue. Lanes 4–6, immunoblots stained by alkaline phosphatase-streptavidin conjugates, showing biotin labeling of the D subunit. Lane 4, reconstituted EhV1 containing biotinylated D subunit; lane 5, non-biotinylated recombinant EhV1; lane 6, Avi-tagged EhV1 containing biotinylated D subunit.

**FIGURE 2. ATP dependence of ATP hydrolysis rate and rotation rate**

A, Time course of ATP hydrolysis by reconstituted EhV1 (solid line), recombinant EhV1 (dashed line), and Avi-tagged EhV1 (dotted line) at 25°C ± 1°C at 10 μM, 50 μM, 100 μM, 300 μM, 1 mM, and 4 mM ATP. ATP hydrolysis was monitored as NADH oxidation in the ATP-regeneration system. The reaction was initiated by addition of EhV1 at 0 s (final, 10 nM). The ATP hydrolysis rate of EhV1 was estimated from the slope of 0–10 s.

B, (Top panel) Red dots indicate rotation rates determined by single-molecule rotation assay of reconstituted EhV1. Red open circles indicate average rotation rates (n ≥ 3). Standard deviations (S.D.) are shown as error bars. Red open squares, blue open triangles, and green crosses indicate the average of one-third of ATP hydrolysis rates determined by the biochemical assay of reconstituted EhV1, recombinant EhV1, and Avi-tagged EhV1, respectively (n ≥ 3). In biochemical assay, S.D. is smaller than the size of the symbols. The solid and dashed lines indicate fits with the Michaelis-Menten equation, \( V = V_{max} \times [ATP]/(K_m + [ATP]) \). \( V_{max} = 107 ± 5 \) rps and \( K_m = 154 ± 33 \) μM (mean ± S.E. of fitting) were obtained for the single-molecule rotation assay of reconstituted EhV1, \( V_{max} = 73 ± 2 \) rps and \( K_m = 221 ± 17 \) μM were obtained for the biochemical assay of reconstituted EhV1, \( V_{max} = 72 ± 1 \) rps and \( K_m = 246 ± 16 \) μM for recombinant EhV1, and \( V_{max} = 76 ± 1 \) rps and \( K_m = 300 ± 19 \) μM.

![Image](https://example.com/image.png)
The apparent binding constants for ATP ($3 \times V_{\text{max}}/K_m$) were estimated as $(2.2 \pm 0.4) \times 10^6$ M$^{-1}$s$^{-1}$ from the rotation assay. (Bottom panel) Ratio of the rotation rate determined by the rotation assay to one-third of the ATP hydrolysis rate determined by the biochemical assay of reconstituted EhV$_1$.

**FIGURE 3. Schematic image of the rotation assay.**
Experimental setup for single-molecule rotation assay of reconstituted EhV$_1$. The stator A$_2$B$_3$ ring of EhV$_1$ was fixed on the glass surface with His$_6$-tags at the N-terminus of the A subunits. A 40-nm streptavidin-coated gold colloid was attached to biotinylated cysteine residues in the rotor DF.

**FIGURE 4. Two distinct states in the rotation of EhV$_1$.**
A and D, Time courses of rotation, including 2 reversible states of a single-molecule-reconstituted EhV$_1$ (3mM ATP) and Avi-tagged EhV$_1$ (2 mM ATP). The rotations in the clear and unclear states are highlighted in red and blue, respectively. B and E, The X-Y trajectories of the centroid of a rotating gold colloid shown in A and D. C and F, Distributions of rotary angles shown in A and D. The numbers in A-C and D-F indicate the corresponding parts. G and H, Distributions of duration times of clear and unclear states of reconstituted EhV$_1$. The data at various ATP concentrations (from 10 μM to 40 mM) were analyzed collectively. Bin width: 0.1 s. The solid curves show the fit with single-exponential decay: constant $\times (\exp(-t/\tau))$, where $\tau = 0.13 \pm 0.003$ s (mean $\pm$ S.E. of fitting, 238 events from 58 molecules) and $0.33 \pm 0.009$ s (mean $\pm$ S.E., 199 events from 58 molecules) for the clear (G) and unclear (H) states, respectively.

**FIGURE 5. Steps and pauses in the rotation of reconstituted EhV$_1$.**
Typical time courses of rotation of reconstituted EhV$_1$ at various ATP concentrations. A, Rotation at 40 mM ATP, captured at 10,000 fps. B, Rotation at 100 μM ATP, captured at 10,000 fps. C, Rotation at 10 μM ATP, captured at 5,000 fps. The top-left insets show the X-Y trajectories of the centroid of a rotating gold colloid. The bottom-right inset shows the distributions of the rotary angle.

**FIGURE 6. Buffer-exchange experiments.**
A, Time courses of rotation of the same molecule at 10 μM ATP (red) and 3 mM ATP (blue). The ATP concentration was increased from 10 μM to 3 mM. B, Distributions of the rotary angle at 10 μM ATP (top) and 3 mM ATP (bottom) shown in A. The $\Delta \theta$ represents the angular differences between the pause angles (peaks) before and after buffer exchange. C, Distribution of $\Delta \theta$. The mean value was $3.2 \pm 12^\circ$ (mean $\pm$ S.D., 21 events from 7 molecules).

**FIGURE 7. Distributions of duration time of the pauses.**
A-D, Distributions of duration times of 4 single molecules at 40 mM ATP with 0.2 ms bin width captured at 10,000 fps. The solid curves show fits with a model of 2 consecutive reactions: constant $\times (\exp(t/\tau_1)-\exp(t/\tau_2))$, where $\tau_1 = 3.0 \pm 0.2$ ms and $\tau_2 = 0.5 \pm 0.1$ ms (mean $\pm$ S.E. of fitting, 398 events). B, $3.5 \pm 0.1$ ms and $0.6 \pm 0.04$ ms (863 events). C, $1.2 \pm 0.2$ ms and $0.4 \pm 0.1$ ms (228 events). D, $1.8 \pm 0.3$ ms and $0.8 \pm 0.2$ ms (608 events). E, Accumulated distribution of duration times of all 4 molecules at 40 mM. The solid curve shows a fit with a model of 2 consecutive reactions with time constants of $2.6 \pm 0.1$ ms and $0.5 \pm 0.02$ ms (2097 events). F, Red circle and square dots indicate $\tau_1$ and $\tau_2$, respectively, shown in A-D. Red open circle and square indicate average $\tau_1$ ($2.4 \pm 1.1$ ms) and $\tau_2$ ($0.6 \pm 0.2$ ms) (mean $\pm$ S.D.) from 4 molecules. S.D. values are shown with error bars. Blue open circle and square indicate $\tau_1$ and $\tau_2$, respectively, shown in E. G, Accumulated distribution of duration times (9 molecules) at 4 mM ATP with 0.2 ms bin width captured at 10,000 fps. The solid curve shows a fit with a model of 2 consecutive reactions: constant $\times (\exp(t/\tau_1)-\exp(t/\tau_2))$, where $\tau_1 = 2.4 \pm 0.05$ ms and $\tau_2 = 0.5 \pm 0.02$ ms (mean $\pm$ S.E. of fitting, 4303 events). H, Red circles and square dots indicate $\tau_1$ and $\tau_2$ determined by fitting the individual distributions of duration times of 9 single molecules with a model of 2 consecutive
reactions, respectively (each distribution are not shown). Red open circle and square indicate average $\tau_1$ (2.3 ± 0.5 ms) and $\tau_2$ (0.4 ± 0.2 ms) (mean ± S.D.) from 9 molecules. S.D. values are shown with error bars. Blue open circle and square indicate $\tau_1$ and $\tau_2$, respectively, shown in G. I-L, Distributions of duration times of 4 single-molecules at 10 µM ATP with 10 ms bin width captured at 5000 or 2000 fps. The solid curves show fits with single-exponential decay: constant × (exp(-t/\tau)), where I, $\tau = 33 ± 1$ ms (mean ± S.E. of fitting, 581 events). J, 47 ± 6 ms (67 events). K, 54 ± 1 ms (432 events). L, 46 ± 1 ms (978 events). M, Accumulated distribution of duration times of all 4 molecules at 10 µM. The solid curve shows a fit with single-exponential decay with time constant of 43 ± 1 ms (2058 events). N, Red circle dots indicate $\tau$ shown in I-L. Red open circle indicates average $\tau$ (45 ± 9 ms) (mean ± S.D.). S.D. is shown with error bar. Blue open circle indicates $\tau$ shown in M.
Table 1. Kinetic parameters of V1-ATPases and F1-ATPases from different sources.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Measurement Temperatures (°C)</th>
<th>$k_{on}^{ATP}$ (M⁻¹s⁻¹)¹</th>
<th>$k_{on}^{ATP}$ (3 $V_{max}/K_m$) (M⁻¹s⁻¹)²</th>
<th>$\tau_1, \tau_2$ (ms)³</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>EhV1</td>
<td>26 ± 1</td>
<td>(2.3 ± 0.03) × 10⁶</td>
<td>(2.2 ± 0.4) × 10⁶</td>
<td>2.6 ± 0.1, 0.5 ± 0.02⁵</td>
<td>This work</td>
</tr>
<tr>
<td>TtV1</td>
<td>23</td>
<td>1.5 × 10⁶</td>
<td>0.84 × 10⁶</td>
<td>2.8, 2.8</td>
<td>(11)</td>
</tr>
<tr>
<td></td>
<td>24 ± 1</td>
<td>3.0 × 10⁷</td>
<td>2.6 × 10⁷</td>
<td>1.6, 0.71</td>
<td>(13)</td>
</tr>
<tr>
<td>TF1</td>
<td>25 ± 1</td>
<td>4.7 × 10⁷</td>
<td>6.4 × 10⁷</td>
<td>0.41, 0.29</td>
<td>(15)</td>
</tr>
<tr>
<td>EF1</td>
<td>23</td>
<td>1.39 × 10⁶</td>
<td>2.2 × 10⁷</td>
<td>1.34, 0.29</td>
<td>(27)</td>
</tr>
</tbody>
</table>

¹Second-order binding rate constant for ATP ($k_{on}^{ATP}$) determined from the distribution of duration of the ATP-waiting pause.
²Second-order binding rate constant for ATP ($k_{on}^{ATP}$) determined from $3 \times V_{max}/K_m$.
³Time constants determined from distribution of duration of the catalytic pause, which corresponds to ATP cleavage and either ADP or phosphate release (or both).
⁴The values are mean ± S.E. of fitting.
⁵The values obtained at 40 mM ATP.
Figure 1

Figure 1
**Figure 2**

A

![Absorbance at 340 nm vs Time (s)](chart)

Reconstituted EhV1 (solid line)
Recombinant EhV1 (dashed line)
Avi-tagged EhV1 (dotted line)

- **10 μM**
- **50 μM**
- **100 μM**
- **300 μM**
- **1 mM**
- **4 mM**

**ATP (μM)**

100 101 102 103 105

**Rotation rate**

1/3 ATPase (s⁻¹)

2.0 1.0 0.0

Ratio

B

![Rotation rate (rps) vs ATP (μM)](chart)

Rotation Assay
- **Reconstituted EhV1**
  - \( K_m = 154 \, \mu M \)
  - \( V_{max} = 107 \, \text{rps} \)
- **Recombinant EhV1**
  - \( K_m = 246 \, \mu M \)
  - \( V_{max} = 72 \, \text{rps} \)
- **Avi-tagged EhV1**
  - \( K_m = 300 \, \mu M \)
  - \( V_{max} = 76 \, \text{rps} \)

Biochemical Assay
- **Reconstituted EhV1**
  - \( K_m = 221 \, \mu M \)
  - \( V_{max} = 73 \, \text{rps} \)
Figure 5

(A) [ATP] = 40 mM, v = 93 rps

(B) [ATP] = 100 μM, v = 42 rps

(C) [ATP] = 10 μM, v = 10 rps
Figure 6

A

\[ v = 98.9 \text{ rps} \] (ATP 3 mM)

\[ v = 7.9 \text{ rps} \] (ATP 10 μM)

ATP 10 μM

ATP 3 mM

B

\( \Delta \theta \)

\( \Delta \theta \)

\( \Delta \theta \)

Number of events

0

10

20

30

40

50

60

70

80

90

100

110

120

130

140

0

120

240

360

Angle

Number of events

C

N = 21 (7 molecules)
Figure 7

- **A**: [ATP] = 40 mM, Molecule 1
  - $\tau_1 = 3.0 \text{ ms}$
  - $\tau_2 = 0.5 \text{ ms}$

- **B**: Molecule 2
  - $\tau_1 = 3.5 \text{ ms}$
  - $\tau_2 = 0.6 \text{ ms}$

- **C**: Molecule 3
  - $\tau_1 = 1.2 \text{ ms}$
  - $\tau_2 = 0.4 \text{ ms}$

- **D**: Molecule 4
  - $\tau_1 = 1.8 \text{ ms}$
  - $\tau_2 = 0.8 \text{ ms}$

- **E**: [ATP] = 40 mM, Accumulated
  - $\tau_1 = 2.6 \text{ ms}$
  - $\tau_2 = 0.5 \text{ ms}$

- **F**: [ATP] = 40 mM
  - Time constant (ms)
  - [ATP] = 40 mM

- **G**: [ATP] = 4 mM, Accumulated
  - $\tau_1 = 2.4 \text{ ms}$
  - $\tau_2 = 0.5 \text{ ms}$

- **H**: [ATP] = 4 mM
  - Time Constant (ms)

- **I**: [ATP] = 10 μM, Molecule 1
  - $\tau = 33 \text{ ms}$

- **J**: Molecule 2
  - $\tau = 47 \text{ ms}$

- **K**: Molecule 3
  - $\tau = 54 \text{ ms}$

- **L**: Molecule 4
  - $\tau = 46 \text{ ms}$

- **M**: [ATP] = 10 μM, Accumulated
  - $\tau = 43 \text{ ms}$

- **N**: [ATP] = 10 μM
  - Time constant (ms)
Basic properties of rotary dynamics of the molecular motor Enterococcus hirae V$_1^{-}$ATPase

Yoshihiro Minagawa, Hiroshi Ueno, Mayu Hara, Yoshiko Ishizuka-Katsura, Noboru Ohsawa, Takahiro Terada, Mikako Shirouzu, Shigeyuki Yokoyama, Ichiro Yamato, Eiro Muneyuki, Hiroyuki Noji, Takeshi Murata and Ryota Iino

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