Torque Generation of *Enterococcus hirae* V-ATPase*

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**Background:** Torque generation is important for the energy conversion of rotary ATPases.

**Results:** *Enterococcus hirae* V-ATPase (EhVoV1) generated larger torque than isolated EhV1.

**Conclusion:** Rotor-stator interactions in EhVoV1 are stabilized by the two peripheral stalks to generate larger torque compared with EhV1.

**Significance:** Torques generated by intact V-ATPase and isolated V1 moiety have been compared quantitatively for the first time.

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V-ATPase (V_o V_1) converts the chemical free energy of ATP into an ion-motive force across the cell membrane via mechanical rotation. This energy conversion requires proper interactions between the rotor and stator in V_o V_1 for tight coupling among chemical reaction, torque generation, and ion transport. We developed an *Escherichia coli* expression system for *Enterococcus hirae* V_1 V_o (EhV_o V_1) and established a single-molecule rotation assay to measure the torque generated. Recombinant and native EhV_o V_1 exhibited almost identical dependence of ATP hydrolysis activity on sodium ion and ATP concentrations, indicating their functional equivalence. In a single-molecule rotation assay with a low load probe at high ATP concentration, EhV_o V_1 only showed the “clear” state without apparent backward steps, whereas EhV_1 showed two states, “clear” and “unclear.” Furthermore, EhV_o V_1 showed slower rotation than EhV_1 without the three distinct pauses separated by 120° that were observed in EhV_1. When using a large probe, EhV_o V_1 showed faster rotation than EhV_1, and the torque of EhV_o V_1 estimated from the continuous rotation was nearly double that of EhV_1. On the other hand, stepping torque of EhV_1 in the clear state was comparable with that of EhV_o V_1. These results indicate that rotor-stator interactions of the V_o moiety and/or sodium ion transport limit the rotation driven by the V_1 moiety, and the rotor-stator interactions in EhV_o V_1 are stabilized by two peripheral stalks to generate a larger torque than that of isolated EhV_1. However, the torque value was substantially lower than that of other rotary ATPases, implying the low energy conversion efficiency of EhV_o V_1.

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V-ATPase (V_o V_1) is an ATP-dependent ion pump. Acidification of vesicles by intracellular V_1 V_o is important for various cellular processes, including receptor-mediated endocytosis, membrane trafficking, and protein processing and degradation (1). V_o V_1 can also function on the surface of certain cells, and because of its involvement in bone resorption and tumor metastasis (2), it is a drug target for osteoporosis and cancer treatments (2, 3). V_o V_1 is a large, multisubunit complex composed of a hydrophilic V_1 moiety for hydrolyzing ATP and a membrane-embedded V_o moiety for transporting ions (1, 2, 4). The V_1 and V_o moieties are connected by a central stalk and two or three peripheral stalks (1, 2, 4–6). In the simplest bacterial V_1 V_o, the catalytic core of V_1 moiety consists of A, B, D, and F subunits, in which three alternately arranged A and B subunits form a hexameric A3B3 ring, and the central shaft, composed of D and F subunits, penetrates the central cavity of the A3B3 ring (7–10). 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 (7). The membrane-embedded V_o consists of two different subunits, a and c subunits. The c subunits of the membrane-spanning ring are connected with the central DF axis via the d subunit, and the two peripheral stalks comprising the E and G subunits connect the channel-forming a subunit to the...
Aβ3 ring (5, 11–13). Enterococcus hirae V_oV_1 (EhV_oV_1), the target of this study, functions as a sodium ion (Na⁺) pump, similar in nature to eukaryotic V_oV_1 (13–16). In EhV_oV_1, the membrane rotor ring consists of 10 c subunits. Each c subunit has four transmembrane α-helices, in which a Na⁺ can be bound to the specific binding pocket (17–19).

V_oV_1 is structurally similar to F_1F_0 ATP synthase (F_1F_0) (2, 20, 21). They also have a common rotary catalytic mechanism. The V_1 (F_1) moiety generates a torque at the interface between the rotor and stator by using the chemical free energy change resulting from ATP hydrolysis, which causes rotation of the rotor subunits relative to the stator subunits. This mechanical rotation accompanies active ion transport in the V_o (F_o) moiety through the aqueous access channels formed at the α-c interface. Inversely, when the magnitude of the electrochemical potential of an ion is large enough, the downhill flow of ions through V_o (F_o) causes rotation of the rotor subunits in the reverse direction, which forces V_1 (F_1) to synthesize ATP (22).

Indeed, Thermus thermophilus V_oV_1 (TtV_oV_1) is known to function as an ATP synthase under physiological conditions (23–25); however, it remains unknown whether EhV_oV_1 can catalyze ATP synthesis.

The rotation of V_1 and F_1 driven by ATP hydrolysis has been directly visualized under an optical microscope by attachment of a probe to the rotary shaft (26–30). When viewed from the membrane side, T. thermophilus V_oV_1 (TtV_oV_1) is observed to rotate stepwise in a counterclockwise direction, consuming a single ATP molecule at each step (27). The step size is always 120° under conditions where ATP binding and/or hydrolysis (ATP cleavage and product release) are rate-limiting steps. This indicates that ATP binding and hydrolysis occur at the same angle in TtV_1 (26, 28), whereas these elementary reaction steps occur at different angles in thermophilic Bacillus PS3 F_1 (TF_1) and E. coli F_1 (EF_1) (Table 1). Recently, we characterized the rotary dynamics of E. hirae V_oATPase (EhV_o) by single-molecule analysis (29). EhV_o rotated in basically the same manner as TtV_1 and also showed no substeps (26, 28), despite their difference in physiological function, suggesting that the 120° stepping rotation without substeps is a common property of V_1. On the other hand, EhV_o also exhibited two characteristic rotational states, namely clear and unclear states, suggesting unstable interactions between the rotor and stator.

Interactions between the rotor and stator will affect the torque generated by rotary ATPase. Because rotary ATPase interconverts the chemical free energy and the electrochemical potential via mechanical rotation, torque is an important factor affecting the energy conversion efficiency. Torque against viscous drag has been estimated for TtV_1 (26, 37), TF_1 (37, 38), and EF_1 (33, 39, 40) (Table 1), all of which act as ATP synthases, and high energy conversion efficiency of TF_1 under external torque was demonstrated (41). Therefore, it is intriguing to consider whether EhV_o, which functions as an ion pump, might also generate a comparable torque.

The ATP-driven rotation of V_oV_1 and F_oF_1 has also been observed directly (28, 42–46). High spatiotemporal imaging employing a low load probe revealed the presence of short pauses and small steps that seem to reflect the interactions between the rotor (c subunit) and stator (a subunit) in V_o (28) or F_o (44). However, no coupling between the pauses and proton transport was shown (28), and the observed short pauses were also found to be independent of proton transport (44). The torque has also been estimated for Saccharomyces cerevisiae V_oV_1 (SV_oV_1) (42) and E. coli F_1F_0 (EF_1F_1) (Table 1). However, the torque of V_1 and V_oV_1 from the same species has not been compared quantitatively, and the effect of the peripheral stalks on torque generation has not been assessed.

In this study, we developed an expression system of recombinant EhV_oV_1 in E. coli and established a single-molecule rotation assay for EhV_oV_1. Recombinant EhV_oV_1 exhibited equivalent dependence of ATP hydrolysis activity on Na⁺ and ATP concentrations to that observed in native EhV_oV_1, indicating that recombinant EhV_oV_1 is as functional as native EhV_oV_1. We further used the single-molecule rotation assay to compare the rotational state and torque of EhV_oV_1 and EhV_1 in the rotation driven by ATP hydrolysis. EhV_oV_1 showed only clear rotation and generated a larger torque than EhV_1, indicating that the rotor-stator interactions in EhV_oV_1 are stabilized by two peripheral stalks to generate larger torque compared with that of isolated EhV_1. However, the torque generated by EhV_oV_1 was substantially lower than those of other rotary ATPases, implying low energy conversion efficiency.

### Table 1: Torque Generation of E. hirae V-ATPases

| Protein | Continuous rotation | Stepping rotation | Reference |
|---------|---------------------|-------------------|-----------|
| EhV_oV_1 | 23 ± 10^a | 27 ± 5^b | This study |
| EhV_1 | 13 ± 3^a | 35° | This study |
| TtV_1 | 33 ± 2°^a | 30° | Ref. 26 |
| SV_oV_1 | 36° | 35 ± 3°^a | Ref. 37 |
| TF_1 | 39 ± 4°^a | 42° | Ref. 38 |
| EF_1F_1 | 35 ± 3°^a | 38 ± 3°^a | Ref. 37 |
| EF_1 | 50 ± 6° | Ref. 47 |
| TtV_1 | 39° | Ref. 39 |
| EF_1 | 30° | Ref. 40 |
| EhV_1 | 40° | Ref. 40 |

* The values were determined by the fluctuation theorem.
* The values are the means ± S.D.
* The values were determined from the angular velocity and frictional drag coefficient of the probe, gold nanoparticle, or polystyrene beads.
* The values were determined from the angular velocity and frictional drag coefficient of the probe, actin filament.
* The values were determined from the bending of the probe, actin filament.

The values were the means ± S.E.

**Experimental Procedures**

Preparation of EhV_oV_1 and EhV_1—Expression Assays—Recombinant EhV_oV_1 (ac_{69}DE_{G_2-A_9}B_6DF) was expressed in E. coli by using the expression plasmid pTR19-EhV_oV_1. We synthesized a DNA fragment containing nine genes of the ntp operon, ntp-FIKEGABD, and optimized its codon usage and ribosome-binding site for E. coli expression. This fragment was then cloned into the plasmid pTR19 (48). For the rotation assay, a His_{63} tag and AviTag biotinylation sequence (GLNDIFEAQKIEWHE) (49) were introduced at the
membranes were suspended in buffer B (50 mM potassium phosphate, pH 7.5, 5 mM MgCl₂, 10% glycerol) and disrupted by sonication. After removal of the cell debris by centrifugation (21,000 × g, 20 min, 4 °C), the membrane fraction was precipitated by centrifugation (>100,000 × g, 1 h, 4 °C). After washing the membranes with buffer A, the membranes were suspended in buffer B (50 mM potassium phosphate, pH 7.5, 100 mM KCl, 5 mM MgCl₂, 20 mMimidazole, 10% glycerol), and then EhVₒV₁ was solubilized from the membranes by incubation with 2% n-dodecyl β-D-maltoside (DDM) for 30 min on ice. The insoluble fraction was removed by centrifugation (>100,000 × g, 30 min, 4 °C), and the supernatant was diluted 5-fold with buffer B. This suspension was applied to a nickel-nitrilotriacetic acid column (Ni²⁺-NTA Superflow; Qiagen) equilibrated with buffer B containing 0.05% DDM. After washing with 10 column volumes of buffer B containing 0.05% DDM, recombinant EhVₒV₁ was eluted with buffer C (50 mM potassium phosphate, pH 7.5, 50 mM KCl, 5 mM MgCl₂, 300 mMimidazole, 10% glycerol) containing 0.05% DDM. The eluted fractions were concentrated with an Amicon Ultra 100 K flow cell. After incubation for 5 min, unbound EhVoV₁ was washed out with buffer E, and then the gold colloid in buffer E was visualized on an objective type total internal reflection flow assay. The flow cell was assembled from a Ni²⁺-NTA-coated glass surface via a His₃ tag introduced at the N terminus of the A subunit. A streptavidin-coated 40-nm gold colloid was then attached to the biotinylated cysteines in the rotor DF as a probe. The rotation of EhVₒV₁ using the low load probe was observed as follows. EhVₒV₁ was immobilized on a Ni²⁺-NTA glass surface through His₃ tags in the subunit, and a 40-nm gold colloid (British BioCell International) coated with streptavidin was attached to the AviTag in the A subunit as a low load probe. The flow cell was assembled from a Ni²⁺-NTA-coated glass and an uncoated coverglass. First, buffer E (buffer B containing 0.05% DDM, 5 mg/ml bovine serum albumin, and 250 mM NaCl) was infused into the flow cell to prevent nonspecific binding of the EhVₒV₁ and gold colloid. After incubation for 10 min, EhVₒV₁ (5–10 nM in buffer E) was infused into the flow cell. After incubation for 5 min, unbound EhVₒV₁ was washed out with buffer E, and then the gold colloid in buffer E was infused. After 10 min, any unbound gold colloid was washed out. Observations of the rotation of EhVₒV₁ and EhV₁ were initiated after infusion of buffer F (50 mM MES-KOH, pH 6.5, 5 mM MgCl₂, indicated concentrations of ATP, ATP-regenerating system) containing 300 mM NaCl and 0.05% DDM for EhVₒV₁ or 50 mM KCl for EhV₁. The rotations of the gold colloid were visualized on an objective type total internal reflection dark field microscope constructed on an inverted microscope (IX-71; Olympus) (51). The images were recorded with a high speed CMOS camera (FASTCAM-1024PCI; Photron) at 5,000–10,000 frames per second (fps).

Torque Measurement—The torque (N) was estimated from the rotation trajectories of the duplex beads (287 nm in diameter; Seradyn) attached to EhV₁ or EhVₒV₁ by using the fluctuation theorem (FT) (37). Observation of rotation of EhV₁ and EhVₒV₁ was performed basically the same procedure as for quantification, potassium phosphate monobasic (0.2, 0.5, 1.0, and 1.5 mM) was dispensed into the 96-well plate. Color development was initiated by adding 75 µl of color development reagent consisting of 5% (w/v) ferrous sulfate, 1.6% (w/v) ammonium molybdate, and 1 M sulfuric acid. The plate was read immediately for end point absorbance at 750 nm. The concentration of inorganic phosphate was calculated from the standard curve.

Dependence of the ATPase Activity of EhVₒV₁ on ATP Concentration—-The effect of ATP concentration on the ATPase activities of EhVₒV₁ and EhV₁ was determined using ATP-regenerating systems. For EhV₁, this assay was performed as described previously (29). The reaction mixture for EhVₒV₁ contained 100 mM Tris-HCl, pH 8.5, 300 mM NaCl, 5 mM MgCl₂, and 10% glycerol or 50 mM MES-KOH, pH 6.5, 300 mM NaCl, and 5 mM MgCl₂, each containing 0.05% DDM, 2.5 mM phosphoenolpyruvate, 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. ATP hydrolysis was initiated by the addition of EhVₒV₁ (final concentration, 5 nM). The rate of hydrolysis was monitored as the rate of NADH oxidation, which was measured by the absorbance decrease at 340 nm and determined as the highest rate for 10 s.

Rotation Assay Using a Low Load Probe—The rotation of EhV₁ using a low load probe was observed as described previously (29). Briefly, the A₃B₃ stator ring was immobilized on a Ni²⁺-NTA-coated glass surface via a His₃ tag introduced at the N terminus of the A subunit. A streptavidin-coated 40-nm gold colloid was then attached to the biotinylated cysteines in the rotor DF as a probe. The rotation of EhVₒV₁ using the low load probe was observed as follows. EhVₒV₁ was immobilized on a Ni²⁺-NTA glass surface through His₃ tags in the subunit, and a 40-nm gold colloid (British BioCell International) coated with streptavidin was attached to the AviTag in the A subunit as a low load probe. The flow cell was assembled from a Ni²⁺-NTA-coated glass and an uncoated coverglass. First, buffer E (buffer B containing 0.05% DDM, 5 mg/ml bovine serum albumin, and 250 mM NaCl) was infused into the flow cell to prevent nonspecific binding of the EhVₒV₁ and gold colloid. After incubation for 10 min, EhVₒV₁ (5–10 nM in buffer E) was infused into the flow cell. After incubation for 5 min, unbound EhVₒV₁ was washed out with buffer E, and then the gold colloid in buffer E was infused. After 10 min, any unbound gold colloid was washed out. Observations of the rotation of EhVₒV₁ and EhV₁ were initiated after infusion of buffer F (50 mM MES-KOH, pH 6.5, 5 mM MgCl₂, indicated concentrations of ATP, ATP-regenerating system) containing 300 mM NaCl and 0.05% DDM for EhVₒV₁ or 50 mM KCl for EhV₁. The rotations of the gold colloid were visualized on an objective type total internal reflection dark field microscope constructed on an inverted microscope (IX-71; Olympus) (51). The images were recorded with a high speed CMOS camera (FASTCAM-1024PCI; Photron) at 5,000–10,000 frames per second (fps).
described above. In the case of the rotary motor proteins that rotate in one direction, the following expression is derived from the FT,

$$\ln[P(\Delta \theta)/P(-\Delta \theta)] = N\Delta \theta/k_\theta T$$  \hspace{1cm} \text{(Eq. 1)}$$

where $\theta(t)$ is the rotary angle of the bead, $\Delta \theta = \theta(t + \Delta t) - \theta(t)$, and $P(\Delta \theta)$ is the probability distribution of $\Delta \theta$. $k_\theta$ is the Boltzmann constant, and $T$ is the room temperature (298 K). For molecules continuous rotating for at least 5 s, $P(\Delta \theta)$ was calculated for the case $\Delta t = 10$ ms, and then $\ln[P(\Delta \theta)/P(-\Delta \theta)]$ versus $\Delta \theta/k_\theta T$ was plotted. The slope of the graph corresponds to the torque. The torque of each molecule was defined as the maximum value obtained from the FT analysis when employing a 5-s moving window, with windows starting at 1-ms intervals.

Estimation of the Stepping Torque of EhV$_1$—The stepping torque was calculated from the frictional drag coefficient ($\Gamma$) and angular velocity ($\omega$) of a 120° step between intervening pauses by using the equation $n = \Gamma \omega$ (52). The streptavidin-coated 50-nm gold colloid (British BioCell International) was used as a probe. The 50-nm gold colloid has two times larger $\Gamma$ than that of 40-nm gold colloid. The $\Gamma$ for a single bead was estimated using the equation $\Gamma = 8\pi \eta a^2 + 6\pi \eta a^2$, where $a$, $x$, and $\eta$ are the radius of the gold colloid ($a = 25$ nm), the rotation radius, and the viscosity of the medium ($\eta = 0.89 \times 10^{-3}$ pN s/nm$^2$ at 25 °C), respectively. The rotation radius, $x$, was determined from the long axis of the ellipse obtained by ellipse fitting to the $x$-$y$ trajectories of the centroid of a rotating gold colloid (53). The ellipse shape resulting from tilted EhV$_1$ on the glass was transformed to a circle to obtain the angular velocity from the original 120° step. The angular velocity was estimated from the time course of stepping rotation obtained by the rotation assay as described previously (31). The rotation assay was conducted as described above with slight modifications. The rotation of the gold colloid was recorded with a high speed CMOS camera (FASTCAM SA5; Photron) at 300,000–372,000 fps. To obtain the angular velocity, we first identified the main 120° steps observed within a continuous run by eye. Then the individual steps were aligned on the time and the angle axes by positioning a point closest to the midpoint of each 120° step. Then the average of the time courses of the individual steps was calculated. Finally, the angular velocity was determined by fitting the line to the linearly increasing region.

Other Biochemical Assays—The protein concentrations of EhV$_1$ and EhV$_o$V$_1$ were determined based on absorbance at 280 nm using a molar extinction coefficient of $3.1 \times 10^5$ M$^{-1}$ cm$^{-1}$ calculated according to its amino acid sequence (ProtParam tool; ExPaSy) and a BCA protein assay kit (Pierce) with bovine serum albumin as a standard, respectively. Inhibitory effect of N,N’-dicetylhexylcarbodiimide (DCCD) on ATPase activity of detergent-solubilized recombinant EhV$_o$V$_1$ was examined by incubating the enzyme ($\sim 0.8 \mu$M) in buffer D with 200 $\mu$M DCCD or solvent methanol only for indicated times at 25 °C and subsequent ATPase measurement using ATP-regenerating systems under the same buffer condition as rotation assay for EhV$_o$V$_1$ (50 mM MES-KOH, pH 6.5, 300 mM NaCl, 5 mM MgCl$_2$, 4 mM ATP, 0.05% DDM) with or without 0.15% (w/v) lauryldimethylamine oxide (LDAO). 100% ATPase activity was obtained from the methanol only control. Residual activity was estimated as relative activity (%) against the 100% ATPase activity of the control. All measurements were carried out at 25 ± 1 °C.

RESULTS

Recombinant EhV$_o$V$_1$ for Use in a Rotation Assay—We developed an E. coli expression system for recombinant EhV$_o$V$_1$ to obtain mutant EhV$_o$V$_1$ for a rotation assay. The synthetic ntp genes coding for EhVoV1 were designed to optimize their codon usage for E. coli expression and were inserted into the plasmid pTR19 (48). This recombinant EhV$_o$V$_1$ has His$_6$ tags at the C terminus of the $c$ subunits, one His$_6$ tag on each of the 10 $c$ subunits, to achieve immobilization onto the Ni$^{2+}$-NTA glass surface, and an AviTag biotinylation sequence (49), a 15-amino acid sequence that is biotinylated by biotin ligase in E. coli, at the N terminus of the A subunit to attach the rotation probe through a biotin-streptavidin interaction. The enzyme was expressed in E. coli strain C43(DE3) and purified by Ni$^{2+}$-NTA chromatography and subsequent gel filtration. The yield of the recombinant protein was ~2–3 mg/liter of culture, which is sufficient for functional and structural analyses. Homogeneity of the enzyme and specific biotinylation of the A subunit was confirmed by SDS-PAGE and immunoblotting, respectively (Fig. 1). The recombinant EhV$_o$V$_1$ had the same subunit composition as the native EhV$_o$V$_1$, purified from E. hirae, although the His$_6$ tag caused a band shift of the $c$ subunit to a similar molecular weight of the G subunit (Fig. 1).

Catalytic Properties of Recombinant EhV$_o$V$_1$—To characterize the catalytic properties of recombinant EhV$_o$V$_1$, we measured the effect of Na$^+$ on ATPase activity of the purified enzymes. To avoid contamination of Na$^+$ in this experiment, ATPase activities were determined by measuring the concentrations of inorganic phosphate liberated from ATP rather than using an ATP-regenerating system. The recombinant EhV$_o$V$_1$ showed a ATP hydrolysis rate comparable with that of native EhV$_o$V$_1$ at all Na$^+$ concentrations tested (Fig. 2A). As the Na$^+$ concentration increased, the ATP hydrolysis rate of both enzymes increased, and partial and complete saturations occurred at about 1 and 200 mM Na$^+$ concentrations, respectively. Double reciprocal plots of both data sets suggested the presence of two $K_m$ values for Na$^+$ (Fig. 2B) as reported previously (15); therefore, the data were fitted with the sum of two Michaelis-Menten equations (Fig. 2A, solid and dashed lines) assuming two independent binding sites of Na$^+$. The Michaelis constants ($K_m^{\text{Na}}$, $K_m^{\text{Na}}$) for native and recombinant EhV$_o$V$_1$ were 20 ± 5 $\mu$M, 42 ± 8 mM and 13 ± 3 $\mu$M, 50 ± 9 mM (fitted value ± S.E. of the fit), respectively. The value of $K_m^{\text{Na}}$ (high affinity for Na$^+$) was similar between recombinant and native EhV$_o$V$_1$ and was also close to the previously reported $K_m$ values (20 $\mu$M) for Na$^+$-dependent ATPase activity of the purified native EhV$_o$V$_1$ in detergent (15) and in liposomes (16). At zero Na$^+$ concentration, recombinant and native enzymes showed 20% of the maximum ATPase activity. However, it is difficult to completely prevent contamination of Na$^+$ in the assay buffer (15, 16), which typically contains 5–10 $\mu$M Na$^+$; therefore, the ATP hydrolysis of both enzymes is likely to be tightly coupled to Na$^+$ transport. Although the values of $K_m^{\text{Na}}$ (low affinity for Na$^+$)
were larger than the reported value (4 mM) of the purified native EhVoV1 (15), there was only a slight difference in the values of the Na⁺-affinity binding site for Na⁺-inhibited recombinant and native enzymes, respectively, and were comparable with that reported for recombinant EhVoV1. The second order binding rate constants for ATP (∼10⁶ M⁻¹ s⁻¹) (fitted value) were obtained for EhV1 (Table 2) (29). These results indicated that most of the detergent-solubilized recombinant EhVoV1, resist to the labeling of the essential carboxyl group in the c subunit of Vᵦ mole, in the catalytic sites of Vᵦ mole. Therefore, we concluded that most of the detergent-solubilized recombinant EhVoV1, used in our study is intact and fully coupled with Na⁺ transport.

**Rotation Rate of EhVᵦVoV₁ and EhV₁ at Saturating ATP Concentration**—We observed the ATP-driven rotation of EhVᵦVoV₁ in detergent to investigate the effect of the Vᵦ mole on the rotation driven by the V₁ mole. EhVᵦVoV₁ was immobilized on a Ni²⁺-NTA glass surface through His₃ tags in the c subunit, and a 40-nm streptavidin-coated gold colloidal was attached to the A subunit as a low load probe (Fig. 3A). It is worth noting that in this “upside-down” configuration, the stator part (αΕ₂G₂-Å₂B₃) rotates against the rotor part (c₁₀-d-DF) with the probe because the c ring is immobilized on a glass surface, and the direction of ATP-driven rotation is counterclockwise when viewed from the top as shown in Fig. 3A. To compare the results with those obtained in our earlier study of rotation of EhV₁ (29), whose ATPase activity decreased at pH > 7 (50), the rotation assay of EhVᵦVoV₁ was performed using the same buffer condition (pH 6.5) as in our previous study but containing 0.05% DDM and 300 mM NaCl.

EhVᵦVoV₁ rotated unidirectionally counterclockwise, similar to other rotary ATPases reported previously. At saturated ATP (4 mM), which is much higher than the $K_{\text{m}}^{\text{ATP}}$ values for EhVᵦVoV₁ (134 μM) and EhV₁ (221 μM) (Fig. 3B and Table 2) (29), the rotation rate (45 ± 12 rps, mean ± S.D.) of EhVᵦVoV₁ was about half that (102 ± 13 rps) of EhV₁ (Fig. 3B, open red and blue triangles, respectively). Considering the averages and standard deviations of these rotation rates, almost all molecules of
We analyzed the in-depth rotary dynamics of EhV₁ at 4 mM ATP by using a low load probe. Typical time courses of rotation of EhV₁ (29) and EhV₀V₁ are shown in Fig. 4 (A and E). As reported previously, EhV₁ exhibited two reversible states of clear and unclear rotation (Fig. 4, A–D) (29). We assigned the rotation state based on the criteria if the centroid of the probe is distributed near the rotation center and that causes the apparent backward rotation (Fig. 4, panels 1a and 1c), the majority of the centroids of the probe were distant from the torque generation of E. hirae V-ATPase.

**FIGURE 2. Biochemical assay of the ATPase activity of EhV₁, V₀, and V₁.** A, dependence of ATPase activity on Na⁺ concentration at 5 mM ATP. Average ATPase rates of native (open circles) and recombinant (open triangles) EhV₀V₁ are shown (n ≥ 3). The error bars represent standard deviations. The inset shows the expanded plot for lower Na⁺ concentrations. Solid and dashed curves show the fit of the model with a sum of two Michaelis-Menten equations assuming two independent binding sites of Na⁺: V = Vmax[Na⁺]/(Km₁ + [Na⁺]) + Vmax2[Na⁺]/(Km₂ + [Na⁺]). The Km₁, Vmax₁, and Vmax₂ values were 20 ± 5 μM, 42 ± 8 mM, 38 ± 1 s⁻¹, and 55 ± 2 s⁻¹ for native EhV₀V₁ and 13 ± 3 μM, 50 ± 9 mM, 37 ± 1 s⁻¹, and 49 ± 2 s⁻¹ for recombinant EhV₀V₁ (fitted value ± S.E. of the fit), respectively. B, double-reciprocal plots of ATPase activity on ATP concentration at high Na⁺ concentration (300 mM). Averages of one-third of the ATPase rate (corresponding to the rotation rate) for native (open circles) and recombinant (open triangles) EhV₀V₁, determined by a biochemical assay at pH 8.5 (n ≥ 3) are shown. The solid and dashed lines indicate the fit with the Michaelis-Menten equation: V = VmaxATP/[ATP]/(VmaxATP + [ATP]). The values of VmaxATP and the second order binding rate constant for ATP (3 × VmaxATP/KmATP) are shown in Table 2. D, inhibitory effect of DCCD on ATPase activity of detergent-solubilized recombinant EhV₀V₁. After incubation of recombinant EhV₀V₁ with 200 μM DCCD for indicated times, residual ATPase activity was examined (n ≥ 3) under the same buffer condition as the rotation assay at 4 mM ATP without (open triangles) or with 0.15% LDAO (filled triangles). Residual ATPase activities decreased to 24 ± 3% and 21 ± 3% after 60 and 90 min of incubation with DCCD, respectively (mean ± S.D.). Residual ATPase activities were recovered to 90 ± 1% and 88 ± 3% by the addition of LDAO, even after 60 and 90 min of incubation with DCCD, respectively. The error bars represent standard deviations.

**TABLE 2**

Kinetic parameters for ATP hydrolysis determined by a biochemical assay of EhV₀V₁ and EhV₁

| Protein         | pH | NaCl  | KₐᵣP | VₐᵣP₁000 | kₐᵣP₁000 | kₐᵣPₐᵣP₁000 |
|-----------------|----|-------|-------|-----------|-----------|--------------|
| Native EhV₀V₁   | 8.5| 300   | 86 ± 7| 40 ± 0.8  | (1.4 ± 0.1)×10⁶| Reference    |
| Recombinant EhV₀V₁ | 8.5| 300   | 65 ± 2| 37 ± 0.2  | (1.7 ± 0.1)×10⁶| Reference    |
| Recombinant EhV₁ | 6.5| 300   | 134 ± 12| 59 ± 1.4  | (1.3 ± 0.1)×10⁶| Reference    |
| Recombinant EhV₁ | 6.5| (50 mM KCl) | 221 ± 17| 73 ± 1.5  | (1.0 ± 0.1)×10⁶| Reference    |

*The second order binding rate constant for ATP (KₐᵣP) determined from 3 × VₐᵣP/KₐᵣP.*
rotation center, and the time course showed clear unidirectional rotations. On the other hand, in the unclear state (Fig. 4, A–D, panels 1b), the centroids showed wide fluctuations toward the rotational center, and the time course resulted in some apparent backward rotations. In contrast to EhV₁, EhVoV₁ only showed the clear state exhibiting centroid distribution distant from the rotation center and clear unidirectional rotations (Fig. 4, E–H). In our previous report (29), we proposed that the unclear state is caused by the unstable interactions between the rotor (DF) and stator (A₃B₃) in EhV₁. We also expected that these unstable interactions would occur only in the isolated EhV₁ and not in EhVoV₁, because the two peripheral stalks composed of the E and G subunits should stabilize the rotor-stator interactions (29). The lack of an unclear state in EhVoV₁ is consistent with these contentions.

In contrast to the relatively large difference between the rotation rate of EhV₁ determined by a single-molecule assay (102 rps) and that determined with a biochemical assay (73 rps) at pH 6.5 in the presence of 4 mM ATP (Fig. 3B, open blue triangle and circles, respectively), the rotation rate of EhVoV₁ was similar when determined by a single-molecule assay (45 rps) and by a biochemical assay (59 rps) (Fig. 3B, open red triangle and circles, respectively). This result also supports the hypothesis that EhVoV₁ is more stable than isolated EhV₁.

Furthermore, interestingly, although EhVoV₁ showed only clear rotation without apparent backward rotation (Fig. 4, E–H), three pauses and steps in the V₁ moiety became obscure as compared with the clear rotation of EhV₁ (Fig. 4, A–D). Considering the elastic coupling of rotary ATPase (58), it is likely that the coupling with EhV₀ causes the multiple transient paus-
ing positions corresponding to the pausing angle of the EhVα moiety in the catalytic dwell. However, under the present condition (300 mM Na\textsuperscript{+}/H\textsubscript{2}O\textsubscript{1100}), no clear multiple pauses were resolved in the rotation of EhV\textsubscript{0}, whereas the ATP-driven rotation of TtV\textsubscript{c} showed pauses separated by \(30^\circ\) (28), consistent with the 12-fold symmetry of the \(c\) ring. Further studies at lower Na\textsuperscript{+} concentrations with high spatiotemporal resolution will be necessary to resolve the clear small pauses and steps in EhV\textsubscript{0}.

**Torque Generation in EhV\textsubscript{1} and EhV\textsubscript{0}**—The torque of EhV\textsubscript{1} and EhV\textsubscript{0} was measured and compared to examine the interactions between the rotor and stator in EhV\textsubscript{1} and EhV\textsubscript{0} (Fig. 5). We employed the FT to estimate the torque (37). This method estimates the torque only from the time courses of rotary angles without requiring an estimate of the frictional drag coefficient of the probe. In this experiment, large duplex beads (287 nm in diameter) were employed as the rotation probe instead of the low load probe shown in Fig. 3, and we observed continuous rotation at 4 mM ATP.

In contrast to the results of rotation rate using the low load probe, EhV\textsubscript{0} rotated faster than EhV\textsubscript{1} when using the duplex beads (Fig. 5, columns 1 and 3). Consistent with this difference in rotation rate, EhV\textsubscript{0} generated a torque that was nearly twice that of EhV\textsubscript{1} (23 ± 10 pNnm versus 13 ± 3 pNnm, mean ± S.D.) (Fig. 5, columns 1 and 3, and Table 1). We also examined the rotation rate and torque of EhV\textsubscript{1} with DDM, but there were no changes in the torque values (Fig. 5, columns 2 and 4). The rotation assay of EhV\textsubscript{1} using a slightly smaller probe (200 nm in diameter) resulted in an increase in the rotation rate (Fig. 5, column 5), but no change in the torque was observed (Fig. 5, column 5), indicating the reliability and reproducibility of torque estimation with FT.

For comparison with other rotary ATPases under the same experimental conditions, we also measured the rotation rate and torque of TF\textsubscript{1}. The value of torque estimated for TF\textsubscript{1} (39 ± 4 pNnm) was comparable with those previously reported (Table 1) (37, 38), indicating the reliability of the estimated torque. TF\textsubscript{1} rotated faster than EhV\textsubscript{0} (Fig. 5, column 6) and showed nearly twice the torque as that of EhV\textsubscript{0} (Fig. 5, column 6). It should be noted that the S.D. for the torque of E. hirae V-ATPase

**FIGURE 4. Low load rotation of EhV\textsubscript{0} and EhV\textsubscript{1}.** A and E, typical time course of rotation of EhV\textsubscript{1} at 3 mM ATP recorded at 10,000 fps (29) and of EhV\textsubscript{0} at 4 mM ATP in the presence of 300 mM NaCl recorded at 5,000–10,000 fps by using a 40-nm gold colloid. The time course of EhV\textsubscript{1} includes two reversible distinct states: clear (black) and unclear (gray) (29). B and F, the \(x\)–\(y\) trajectories of the centroid of the rotating gold colloid shown in A and E. C and G, the distribution of the rotary angle shown in B and F. D and H, the distribution of the distance of the centroid of the gold colloid from the rotation center. Average distance in unclear state (gray, 16–18 nm) is distinctly shorter than those in the clear state (black, 18–20 nm), and the histogram in the unclear state is distributed closer to the rotation center (13% within 10 nm) than that in clear state (<5% within 10 nm). The numbers in A–H indicate corresponding parts and molecules.
EhV\texttextsuperscript{o}V\texttextsubscript{1} was relatively larger than those of EhV\textsubscript{1} and TF\textsubscript{1}. We speculate that relatively large uncertainty for the torque of EhV\texttextsuperscript{o}V\textsubscript{1} results from the upside-down configuration of rotation assay for EhV\texttextsuperscript{o}V\textsubscript{1} (Fig. 3A, right panel). In this configuration, the \textit{a} subunit rotates against the \textit{c} ring very close to the glass surface and is more likely to nonspecifically interact with the glass surface during rotation as compared with that of the DF subunits in the rotation assay for EhV\textsubscript{1} (Fig. 3A, left panel). The nonspecific interaction between the \textit{a} subunit and the glass surface will occasionally cause transient pauses and/or restrict fluctuation of the probe, which results in the lower estimate of the torque using the fluctuation theorem (37). This would result in the relatively large distribution (uncertainty) of the torque of EhV\texttextsuperscript{o}V\textsubscript{1}. Therefore, the torque of EhV\texttextsuperscript{o}V\textsubscript{1} could be underestimated slightly.

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**Stepping Torque of EhV\textsubscript{1}**—The lower torque of EhV\textsubscript{1} than EhV\texttextsuperscript{o}V\textsubscript{1} as estimated from continuous rotation can be attributed to the transition between the clear and unclear states in rotation (Fig. 4, A–D), assuming that EhV\textsubscript{1} can generate very low torque in the unclear state. To test this assumption, we determined the stepping torque of EhV\textsubscript{1} in the clear state. Because observation of clear rotational steps using a large probe was difficult, we used a 50-nm gold colloid as a frictional probe. Rotation was observed at 3 mM ATP and was recorded at 300,000–372,000 fps. The torque was estimated from the angular velocity and the frictional drag coefficient of the probe. The 120\degree steps of individual EhV\textsubscript{1} molecules are superimposed in Fig. 6. The angular velocity of the 120\degree step rotation was determined by a linear fit to the data (Fig. 6, dotted lines). The stepping torque of EhV\textsubscript{1} was estimated to be 27 ± 5 pNnm (mean ± S.D.) and was higher than that of EhV\textsubscript{1} (13 ± 3 pNnm) estimated from the continuous rotation (Table 1). On the other hand, the stepping torque of EhV\textsubscript{1} was comparable with that of EhV\texttextsuperscript{o}V\textsubscript{1} (23 ± 10 pNnm), which showed only the clear state (Table 1). These results support our assumption that EhV\textsubscript{1} in the unclear state generates lower torque than that in the clear state. Our results also indicate that even in the clear state, EhV\textsubscript{1}
and EhV_oV_1 generate lower torque than other rotary ATPases (Table 1).

DISCUSSION

We developed an E. coli expression system for EhV_oV_1 using nine synthetic ntp genes in an ntp operon (Fig. 1). Recombinant EhV_oV_1 exhibited identical catalytic properties to native EhV_oV_1 (Fig. 2 and Table 2), which indicates that recombinant EhV_oV_1 is as functional as native EhV_oV_1. The dependence of the ATPase activity of both native and recombinant EhV_oV_1 on Na^+ concentration showed biphasic Michaelis-Menten kinetics (Fig. 2, A and B). The values of $K_{m}^{\text{Na}}$ (20 $\mu$M for native and 13 $\mu$M for recombinant EhV_oV_1) for the high affinity Na^+-binding site were close to the dissociation constant (15 $\mu$M) for Na^+ ($K_{d}^{\text{Na}}$) of purified native EhV_oV_1 in detergent (14) and were comparable with the apparent Michaelis-Menten constant for Na^+ transport, $K_c$ (40 $\mu$M), measured as the half-maximal transport of Na^+ by native EhV_oV_1 liposomes (16). This suggests that ATP hydrolysis of detergent-solubilized EhV_oV_1 is tightly coupled to Na^+ transport and that Na^+ binding to the high affinity site in EhV_o limits the ATP hydrolysis rate (rotation) at concentrations below the $K_{m}^{\text{Na}}$ value. There has been no report about the relationship between the low affinity site with higher $K_{m}^{\text{Na}}$ values (42 mM for native and 50 mM for recombinant EhV_oV_1) and Na^+ transport. Therefore, it will be interesting to determine whether the Na^+ bound to this low affinity site is transported or not. The mutagenesis approach of the recombinant EhV_oV_1 developed in this study will help to identify the residues that are important for Na^+ binding and reveal the cause of the observed biphasic kinetics.

We characterized the ATP-driven rotation of EhV_oV_1 using a low load probe, and its rotation behaviors were compared with those of EhV_1 at the single-molecule level. Although EhV_1 rotated with clear and unclear states (Fig. 4, A–D), EhV_oV_1 showed only the clear state (Fig. 4, E–H), as predicted based on the results of our previous study (29). Interestingly, the rotation rate of EhV_oV_1 was almost twice as slow as that of EhV_1 at saturating ATP concentration (Fig. 3B) with no clear pauses separated by 120° observed for EhV_1 (Fig. 4). We speculate that the interactions between the rotor (c ring) and stator (a subunit) in EhV_o resist the rotation driven by EhV_1, which results in the slow rotation of EhV_oV_1, as previously proposed for TvV_oV_1 (28) and EF_F1 (44). It is also highly likely that the Na^+ transport through EhV_o limits the rotation; however, considering the $K_{m}^{\text{Na}}$ (13 $\mu$M), the reported $K_{d}^{\text{Na}}$ values (15 $\mu$M) (14), and the Na^+ concentration used in our rotation assay (300 mM), the slow rotation could be caused by the Na^+ transfer process between the c ring and a subunit and/or Na^+ release into the periplasm side rather than by Na^+ binding onto the a or c subunit. Similar slow rotation caused by the effect of V_o has also been reported in the rotation of TvV_oV_1 (28). The authors reported that the bumps caused by the V_o moiety obscured the ATP-waiting pauses (i.e., the catalytic pauses) separated by 120° observed in TvV_1. Furthermore, they found that the bumps introduced by the V_o moiety led to ~30° steps, which is consistent with the 12-fold symmetry of the c ring. In our rotation assay of EhV_oV_1, where catalytic events are expected to be rate-limiting, we also did not find the three clear pauses separated by 120° that were observed in EhV_1 (Fig. 4). On the other hand, we did not observe small steps reflecting the c ring of the V_o moiety (Fig. 4, E–G). Because the c ring in EhV_o consists of 10 c subunits that each have an essential glutamate for Na^+ transport (18), it is possible that EhV_oV_1 exhibits ~36° steps (360°/10). We are currently trying to observe the rotation of EhV_oV_1 at lower Na^+ concentrations and resolve this small step.

The values of torque for EhV_oV_1 and EhV_1 determined in this study are summarized in Table 1 along with the reported values for various rotary ATPases. EhV_oV_1 only showed the clear rotation state without apparent backward rotations unlike EhV_1, and when the torque was measured with the FT analysis of continuous rotation, EhV_oV_1 generated larger torque than EhV_1. These results are consistent with our notion that two peripheral stalks stabilize the interactions between the rotor and stator (29). This hypothesis is further supported by the result that the stepping torque of EhV_1 measured in the clear rotation state was much higher than that measured by the FT analysis of continuous rotation. The difference between the torque of EhV_1 estimated from continuous rotation and that from stepping rotation in the clear state strongly suggest that EhV_1 in the unclear state generates much lower torque than that in the clear state. Given the ratio of the clear state to the total observation time (~0.3) determined in a previous study (29), the torque of EhV_1 in the unclear state is estimated to be roughly 7 pNnm (i.e., [13 – 0.3 × 27]/0.7). A similar low torque property and rotation behavior as those observed for EhV_1 showing the unclear state have been reported in EF_F1 mutants in which the N- and C-terminal residues of the rotor y subunit were deleted (59).

We also found that the torque of EhV_oV_1 was substantially lower than that of other rotary ATPases, although there is a possibility of underestimating the torque of EhV_oV_1 slightly as described above (Table 1). Thermophilic Bacillus PS3 F_F1 (TF_F1) mutants generating half the torque observed in the wild type was previously reported (60). This half-torque TF_F1 mutant could carry out ATP-driven proton pumping and ATP synthesis, although these activities were low. Therefore, one important question arising from our results is the amount of Na^+-motive force (electrochemical potential of Na^+) that can be generated by EhV_oV_1. Resolving this issue will require the development of a quantitative method to accurately measure the Na^+-motive force. Another important question is the capability of EhV_oV_1 to synthesize ATP. It has been reported that SV_oV_1 in the vacuolar membrane, which acts as a proton pump under physiological conditions, synthesized ATP when a proton-motive force was applied by the exogenously expressed pyrophosphatase (61). However, a quantitative correlation between the amplitude of the proton-motive force and the ATP synthesis rate has not been determined. As previously described for EF_F1 (62) and TF_F1 (63), our recombinant EhV_oV_1 will enable quantitative measurements of the ATP synthesis rate under defined conditions and should provide detailed insight to help resolve this issue.

As described above, small steps have been observed in the rotation of TvV_oV_1 and EF_F1 driven by ATP hydrolysis, which is consistent with the structural symmetry of the rotor c ring (28, 44). However, in one study, no relationship between small steps and proton transport was shown (28), and the results of
another study indicated that the observed short dwells were independent of proton transport (44). The correlation between the small steps and proton transport could be established by using the single-molecule rotation assay with different proton concentrations at which proton binding/release becomes rate-limiting. However, large changes in proton concentration will also induce large changes in pH, which will greatly affect the stability of the sample. On the other hand, EhV0V1 has a significant advantage in that the concentration of the transport substrate, Na+, can be easily and widely changed without affecting the sample stability. This property will allow us to perform quantitative single-molecule analyses of EhV0V1 rotation coupled with ion binding/release events in the V0 moiety and help to further understand the mechano-electrochemical coupling mechanism in V0V1.

Acknowledgments—We thank Shou Furuike for valuable comments, as well as all the members of the laboratory for valuable discussions and comments.

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