V-ATPases are rotary motor proteins that convert the chemical energy of ATP into the electrochemical potential of ions across cell membranes. V-ATPases consist of two rotary motors, Vo and V1, and Enterococcus hirae V-ATPase (EhV0V1) actively transports Na⁺ in V₀ (EhV₀) by using torque generated by ATP hydrolysis in V₁ (EhV₁). Here, we observed ATP-driven stepping rotation of detergent-solubilized EhV₀V₁ wild-type, aE634A, and BR350K mutants under various Na⁺ and ATP concentrations ([Na⁺] and [ATP], respectively) by using a 40-nm gold nanoparticle as a low-load probe. When [Na⁺] was low and [ATP] was high, under the condition that only Na⁺ binding to EhV₀ is rate limiting, wild-type and aE634A exhibited 10 pausing positions reflecting 10-fold symmetry of the EhV₀ rotor and almost no backward steps. Duration time before the forward steps was inversely proportional to [Na⁺], confirming that Na⁺ binding triggers the steps. When both [ATP] and [Na⁺] were low, under the condition that both Na⁺ and ATP bindings are rate limiting, aE634A exhibited 13 pausing positions reflecting 10- and 3-fold symmetries of EhV₀ and EhV₁, respectively. The distribution of duration time before the forward step was fitted well by the sum of two exponential decay functions with distinct time constants. Furthermore, occasional backward steps smaller than 36° were observed. Small backward steps were also observed during three long ATP cleavage pauses of BR350K. These results indicate that EhV₀ and EhV₁ do not share pausing positions, Na⁺ and ATP bindings occur at different angles, and the coupling between EhV₀ and EhV₁ has a rigid component.

V-ATPase | single-molecule analysis | molecular motors

Rotary ATPases are ubiquitously expressed in living organisms and play important roles in biological energy conversions (1–6). These rotary ATPases are classified into F-, V-, and A-ATPases based on their amino acid sequences and physiological functions (6). Eukaryotic and bacterial F-ATPases (Fₒ/F₁) and archaeal A-ATPases (Aₐ/Aₘ) mainly function as ATP synthases driven by the electrochemical potential of ions across the cell membrane, although they can also act as active ion pumps driven by ATP hydrolysis depending on the cellular environment. In contrast, V-ATPases (Vₒ/V₁) in eukaryotes primarily function as active ion pumps. V-ATPases are also found in bacteria, and some of them are termed V/A-ATPases based on their origin and physiological function in ATP synthesis (6–8).

To date, numerous studies have been conducted to understand how the two motor proteins (i.e., Fₒ/A₁/V₁ and Fₓ/Aₓ/Vₓ) of the rotary ATPases couple their rotational motions and functions. Single-molecule studies using fluorescent probes (9–12), gold nanoparticle (AuNP) or nanorod probes (13–21), and Förster resonance energy transfer (16, 22, 23) have revealed the rotational dynamics of rotary ATPases for both ATP hydrolysis/synthesis directions. Furthermore, recent cryo–electron microscopic (cryo-EM) single-particle analyses have revealed entire architectures of the rotary ATPases with different structural states at atomic resolutions (24–35). In particular, several studies have demonstrated elastic coupling of Fₓ/F₁ due to large deformations of the peripheral stalk connecting Fₓ and F₁ (25, 29, 35). However, few studies on other types of rotary ATPases with different functions and subunit compositions have been performed, and a comprehensive understanding of the energy transduction mechanism remains elusive.

Enterococcus hirae V-ATPase (EhV₀/V₁) works as an ATP-driven sodium ion (Na⁺) pump to maintain Na⁺ concentrations ([Na⁺]) inside the cell (Fig. 1A) (37–41). Note that we use the term V-ATPase or Vₒ/V₁ because its physiological function is not ATP synthesis but active ion transport. EhV₁ is a multisubunit complex composed of nine different subunits, namely, a₁,b₁,d₁,E₁,G₁₂, and A₃,B₁,D₁ complexes in EhV₀ and EhV₁, respectively. In the EhV₁ A₃,B₁,D₁ complex, three pairs of the A and B subunits form a heterohexameric A₃B₃ stator ring, and the central rotor DF subcomplex is inserted into the A₃B₃ ring (Fig. 1B, Bottom) (42, 43). The EhV₁ ac₁₀E₂G₂ complex...
transports Na\(^+\) across the cell membrane. The membrane-embedded rotor ring is formed by a decamer of the tetrahedric transmembrane c subunit (c\(_{10}\) ring; Fig. 1B, Top) connected with the central DF stalk via the d subunit (26, 44). The stator a subunit works as an ion channel, and two EG peripheral stalks interact with the a and AB3 rings to assure rotary coupling between EhV\(_o\) and EhV\(_i\).

In EhV\(_i\), the ATP hydrolysis reaction is catalyzed at the interfaces of three A and B subunits. It drives a counterclockwise rotation of the DF rotor subunits as viewed from the EhV\(_o\) side (Fig. 1B, Bottom). Like other F\(_i\)/A\(_i\)/V\(_i\) (11, 45), EhV\(_i\) is a stepping motor that rotates 120° per one ATP hydrolysis (46). We previously revealed that the 120° step of isolated EhV\(_i\) is further divided into 40 and 80° substeps by using high-speed and high-precision single-molecule imaging analysis with AuNP as a low-load probe (47). A main pause before the 40° substep involves ATP cleavage, phosphate release, and ATP binding events. The ATP binding triggers the 40° substep because the duration time is inversely proportional to [ATP]. The 80° substep is triggered by ADP release after a subpause with [ATP]-independent duration time. While the chemomechanical coupling scheme in EhV\(_i\) has been revealed, because our previous single-molecule observation of EhV\(_i\) did not reveal the pauses and steps (17), the elementary steps in the rotation of EhV\(_i\) have not been revealed.

Although the mechanism of ion transport in F\(_i\)/A\(_i\)/V\(_o\) is not fully understood, the so-called “two-channel” model has been widely accepted (48–54). In this model, the a-subunit has two half-channels for ion entry/exit into/from the ion-binding sites of the rotor c ring. In the case of EhV\(_o\), Na\(^+\) enters the half-channel from the cytoplasmic side and binds to the negatively charged Na\(^+\)-binding sites of the c subunit (44, 55). Then, the charge-neutralized c subunit can move into the hydrophobic lipid membrane (53, 56). The rotational torque generated by ATP hydrolysis in EhV\(_i\) is transmitted to EhV\(_o\) via the d subunit, allowing the c\(_{10}\) ring to rotate unidirectionally in the lipid membrane. Na\(^+\) translocated by a nearly single turn of the c\(_{10}\) ring reaches another half-channel of the a subunit, which connects the Na\(^+\)-binding site of a subunit to the extracellular side. Then, Na\(^+\) is pumped out of the cell by a hydrated microenvironment (57) and/or electrostatic repulsion with the positively charged residues in the a subunit, aGlu573 and aArg629, located at the interface between the two half-channels (Fig. 1C and SI Appendix, Fig. S1) (26). Because EhV\(_o\)V\(_i\) has the c\(_{10}\) ring, 10 Na\(^+\) are transported per single turn. Therefore, the step size of EhV\(_o\) is expected to be 36° (360°/10), similar to Escherichia coli and yeast F\(_i\)F\(_1\), which also have c\(_{10}\) rings (13, 14, 22, 35).

The ion-to-ATP ratio is a central issue in the coupling mechanism of rotary ATPases. All known F\(_i\)/A\(_i\)/V\(_i\) have three catalytic sites and threefold structural symmetry and hydrolyze or synthesize three ATP molecules per single turn. In contrast, the number of protomers forming the rotor c ring of F\(_i\)/A\(_i\)/V\(_o\) varies from 8 to 17 depending on the species, suggesting wide variations in the ion-to-ATP ratio of rotary ATPases (58, 59). In EhV\(_o\), because the rotor c ring of EhV\(_o\) has a 10-fold structural symmetry (Fig. 1B, Top), this enzyme has a structural symmetry mismatch and a noninteger ratio between transported Na\(^+\) and hydrolyzed ATP (10/3 = 3.3). If the rotational coupling between EhV\(_o\) and EhV\(_i\) is elastic, as reported for E. coli and yeast F\(_i\)F\(_1\), the symmetry mismatch is relieved by large deformations of the peripheral stalk and/or the central rotor (25, 29, 35, 60). On the other hand, if the coupling is rather rigid due to the multiple peripheral stalks of EhV\(_o\)V\(_i\), the pausing positions of both EhV\(_o\) and EhV\(_i\) would be observed independently in a single-molecule observation. To address this issue, it is required to directly visualize the rotational pauses and steps of EhV\(_o\)V\(_i\) under conditions where the elementary steps of the rotation such as the bindings of Na\(^+\) and ATP to EhV\(_o\) and EhV\(_i\), respectively, are both rate-limiting.

Here we carried out high-speed and high-precision single-molecule imaging of the rotation of detergent-solubilized EhV\(_o\)V\(_i\) by using 40-nm AuNP as a low-load probe. To resolve the rotational pauses and steps of EhV\(_o\), a glutamate residue in the stator a subunit (aGlu634) was replaced with alanine. Since the mutated aGlu634 is located on the surface of the Na\(^+\)-entry half-channel (Fig. 1C and SI Appendix, Fig. S2), Na\(^+\) binding to the c subunit in the EhV\(_o\)V\(_i\)(aE634A) mutant (hereinafter referred to as aE634A) is expected to become slower than in the wild type. The rotation rate of aE634A decreased about 10 times compared with that of the wild type, allowing us to clearly resolve the rotational pauses and steps in EhV\(_o\). Under the condition that only Na\(^+\) binding is rate-limiting, aE634A showed 10 pausing positions per single turn and a step size of about 36°, consistent with 10 protomers in the c\(_{10}\) ring of EhV\(_o\). The duration time before the forward step was inversely proportional to [Na\(^+\)], indicating that the dwell corresponds to the waiting time for Na\(^+\) binding. On the other hand, under the condition that both Na\(^+\) and ATP bindings are rate-limiting, 13 pausing positions per single turn were observed. Furthermore, backward steps smaller than 36° were occasionally observed only when ATP binding is also rate-limiting.
indicating that EhVoV1 undergoes Brownian motion between adjacent pausing positions of EhVo and EhV1 when no torque is applied from EhV1. Backward steps of 36° or larger than 36° were rarely observed, suggesting the suppression of reverse Na⁺ transport. Small backward steps were also frequently observed during three long ATP cleavage pauses of another mutant, EhVoV1(BR350K), in which ATP hydrolysis is rate-limiting for the rotation (47). From these results, we conclude that EhVo and EhV1 do not share their pausing positions, Na⁺ and ATP bindings occur at different angles, and their coupling has a rigid component.

Results

[[ATP] and [Na⁺]] Dependence of Rotation Rate of Wild Type and aE634A. To clearly visualize the ATP-driven rotation rate–limited by Na⁺ transport, site-directed mutagenesis was conducted on the a subunit of EhVo (aE634A). The purified aE634A solubilized with n-dodecyl-β-D-maltoside (DDM) exhibited subunit stoichiometry similar to the wild type in SDS-PAGE (SI Appendix, Fig. S3), indicating intactness of the complex.

Fig. 2A draws a schematic illustration of single-molecule observation. In this system, the ATP-driven rotation of V₉V₁, immobilized on a glass surface, was probed by AuNPs attached to the A subunit via biotin–streptavidin conjugation (SI Appendix). The localization precision in this system was 0.6 nm at 3,000 frames per second (fps) (0.33 ms temporal resolution) as determined by centroid analysis of the scattering images of single AuNPs non-specifically attached to the glass surface (SI Appendix, Fig. S4).

The rotation rates of the wild type and aE634A at various substrate concentrations were examined from the slope of the time course of rotation (Fig. 2 B and C and SI Appendix, Fig. S5). [ATP] dependences of the rotation rate for the wild type and aE634A in the presence of 300 mM Na⁺ are shown in Fig. 2B. Under this high-[Na⁺] condition, Na⁺ binding is fast and not rate-limiting, and consequently, [ATP] dependence obeyed Michaelis–Menten kinetics. Obtained kinetic parameters, the Michaelis constant (Kₘ[ATP]) and the maximum velocity (Vₘax[ATP]), are summarized in Table 1. The value of Kₘ[ATP] for the wild type was 60.4 ± 2.1 μM (fitted value ± SE of the fit). This value was comparable to that for the isolated EhV₁ (43 ± 6 μM), indicating that the apparent affinity of ATP was not significantly affected by EhVo. On the other hand, the value of Vₘax[ATP] (40.0 ± 0.3 rps, fitted value ± SE of the fit) was significantly smaller than that of the isolated EhV₁ (117 ± 3 rps) (47). As a result, the binding rate constant of ATP ([Kₘ[ATP]] by estimated as 3 × Vₘax[ATP]/Kₘ[ATP] was smaller than that of the isolated EhV₁ (Table 1). We attributed this difference to the intact interaction between the rotor c₁₀ ring and stator a subunit of EhVo and concluded that Na⁺ transport in EhVo limits the rotation (47). From these results, we conclude that EhVo limits the rotation (47). From these results, we conclude that EhVo limits the rotation (47). From these results, we conclude that EhVo limits the rotation (47).
fitted value ± SE of the fit) and the wild type (0.29 ± 0.09 and 160.2 ± 88.8 mM). Then, the binding rate constant of Na\(^+\) (\(k_\text{m,Na}\)) as estimated by 10 × \(V_{\text{max,Na}}/K_{\text{m,Na}}\) largely decreased in aE634A, notably more than 10 times for the high-affinity site (\(k_{\text{on,Na}}\); Table 2). The significant decrease in the \(V_{\text{max,Na}}\) for aE634A compared with that of the wild type also suggests that the rate of elementary reaction steps other than Na\(^+\) binding also decreased in EhVo. Because Na\(^+\) binding to EhVo is certainly rate-limiting at low [Na\(^+\)] for both the wild type and aE634A, we then conducted a detailed analysis of the rotational pauses and steps at low [Na\(^+\)].

**Table 1. Kinetic parameters for [ATP] dependence of rotation rate of EhVoV\(_1\) and EhV\(_1\)**

| Protein          | NaCl (mM) | \(K_m\text{ATP}\) (\(\mu\)M) | \(V_{\text{max,ATP}}\) (rps) | \(k_{\text{on,ATP}}^\text{Na}\) (M\(^{-1}\) s\(^{-1}\)) | Reference                      |
|------------------|-----------|-----------------------------|-----------------------------|---------------------------------|--------------------------------|
| EhVoV\(_1\) wild type | 300       | 60.4 ± 2.1                  | 40.0 ± 0.3                  | 2.0 × 10\(^6\)                 | This study (single molecule)   |
| EhVoV\(_1\) aE634A | 300       | 6.6 ± 0.3                   | 4.58 ± 0.04                 | 2.1 × 10\(^6\)                 | This study (single molecule)   |
| EhV\(_1\) wild type | 300       | 134 ± 12                    | 59 ± 1                      | 1.3 × 10\(^6\)                 | Ref. 17 (biochemical)          |
| EhV\(_1\) wild type | 0 (50 mM KCl) | 43 ± 6                      | 117 ± 3                     | 8.2 × 10\(^6\)                 | Ref. 47 (single molecule)      |

\(\text{Na}^+\) (mM) was 5 mM. *The binding rate constant of ATP estimated by \(k_{\text{on,ATP}} = 3 \times V_{\text{max,ATP}}/K_m\text{ATP}.\)

**Table 2. Kinetic parameters for [Na\(^+\)] dependence of rotation rate of wild type and aE634A**

| Protein          | \(k_{\text{m,Na}}\) (\(\mu\)M) | \(V_{\text{max,Na}}\) (rps) | \(k_{\text{on,Na}}\) (M\(^{-1}\) s\(^{-1}\)) | \(K_m\text{Na}\) (mM) | \(V_{\text{max,Na}}\) (rps) | \(k_{\text{on,Na}}\) (M\(^{-1}\) s\(^{-1}\)) |
|------------------|-------------------------------|-------------------------------|---------------------------------|-----------------|-------------------------------|---------------------------------|
| EhVoV\(_1\) wild type | 0.29 ± 0.09                    | 15.7 ± 1.6                   | 5.4 × 10\(^5\)                 | 160.2 ± 88.8    | 35.0 ± 7.1                   | 2.2 × 10\(^4\)                 |
| EhVoV\(_1\) aE634A | 0.32 ± 0.28                    | 1.1 ± 0.3                    | 3.4 × 10\(^4\)                 | 58.3 ± 26.5     | 4.4 ± 0.5                    | 7.5 × 10\(^3\)                 |

[ATP] was 5 mM. *The binding rate constant of Na\(^+\) estimated by \(k_{\text{on,Na}} = 10 \times V_{\text{max,Na}}/K_m\text{Na}.\)
of the step size is shown in Fig. 7B. The average value for the forward step was 29.9°, which is distinctly smaller than 36°. When we superimposed the two histograms in Fig. 5B (0.3 mM Na⁺ and 5 mM ATP) and Fig. 7B (0.3 mM Na⁺ and 1 μM ATP) after normalizing the maximum values, the difference was obvious (SI Appendix, Fig. S11). Furthermore, interestingly, in contrast to the condition in which Na⁺ binding was the sole rate-limiting factor, the ratio of the backward step increased to 6.1%. Therefore, we fitted the distribution with the sum of three Gaussians: one peak in the backward (minus) direction and two peaks in the forward (plus) direction. Note that one of the forward peaks was fixed at 36°, which is assumed as the step of EhV₀. Then, we obtained three peaks at −14.2 ± 6.8, 23.1 ± 10.4, and 36.0 ± 12.8° (peak ± SD; Fig. 7B). For comparison, the fitting with two Gaussians with peaks in minus and plus directions is shown in SI Appendix, Fig. S12A. The distribution of duration time before the forward step fitted better with the sum of two exponential decay functions (coefficient of determination \( R^2 = 0.98 \); Fig. 7C) than with a single exponential decay function (\( R^2 = 0.94 \); SI Appendix, Fig. S12B), and time constants of 135 ± 24 and 642 ± 322 ms (fitted value ± SE of the fit) were obtained. These values presumably correspond to the time constants for Na⁺ and ATP bindings, respectively, because the time constant for Na⁺ binding was 137 ms at 0.3 mM Na⁺ and 5 mM ATP (Fig. 5C), and that for ATP binding was 476 ms at 1 μM ATP as estimated from \( k_{\text{on,ATP}} \) (2.1 × 10⁵ M⁻¹ s⁻¹; Table 1). These results are consistent with the notion that both Na⁺ and ATP bindings are rate-limiting for rotation.

**Backward and Recovery Steps between Adjacent Pauses of EhV₀ and EhV₁.** As described above, occasional backward steps (6.1% of total steps) were observed under conditions where both Na⁺ and ATP bindings were rate-limiting for rotation (Fig. 7B). Typical examples of backward steps are shown in Fig. 8A. Green, cyan, and purple lines indicate the forward step just before the backward step, the backward step, and the forward step after the backward step (recovery step), respectively. Fig. 8 B–D shows the step size distributions for these events. The average value of a backward step size was −18.8° (Fig. 8C), which is smaller than the expected step size (36°) of EhV₀. Because backward steps were observed when not only Na⁺ binding but also ATP binding was rate-limiting for rotation, it is reasonable to assume that backward steps occur during the pauses waiting for ATP binding. A backward step size smaller than 36° is also consistent with the notion that the pausing positions waiting for Na⁺ and ATP bindings are different in the EhV₀/V₁ complex, and backward steps occur at the intermediate pausing position of EhV₁ between pausing positions of EhV₀. The forward steps just before the backward and recovery steps seemed to show two kinds of step sizes, smaller than 36° and close to 36° (Fig. 8 B and D). The backward and forward steps smaller than 36° suggest Brownian motion between the adjacent pausing positions waiting for ATP and Na⁺ bindings. Furthermore, the 36° forward steps just before the backward steps suggest a standard forward step in EhV₀, followed by the Brownian backward step to the adjacent pausing position of EhV₁, and the 36° recovery steps after the backward steps suggest a resumption of the rotation after ATP binding to EhV₁.

Then, to test the hypothesis that the backward steps occur equally at three pausing positions of EhV₁, we prepared other mutants, isolated EhV₁(BR350K) and EhV₀/V₁(BR350K). Because an arginine residue in the B subunit (BArg350) of EhV₁ plays a crucial role in ATP hydrolysis (SI Appendix, Fig. S13) (42), the rotation rate of the EhV₁(BR350K) mutant
In the present study, we directly visualized ATP-driven rotation of EhV1, which is rate-limited by ion transport. EhV1, used in this study transports Na+, while most rotary ATPases transport H+. This property of EhV1 has advantages for resolving the stepping rotation, which is rate-limited by ion transport in V1. First, H+ transport in rotary ATPases is thought to be achieved by a Grothuss mechanism (15, 65) in which H+ transfer is not a rate-limiting process. Indeed, the duration of transient dwells of F3 in E. coli FoF1 does not exhibit pH dependence ranging from pH 5 to 9, although the frequency of pause occurrence is highly affected by pH (15). In addition, changes in pH can largely affect protein stabilities and enzymatic reactions, whereas changes in [Na+] would mildly affect them. Here we resolved the pauses and steps of EhV1 driven by using a mutant in which the aGlucose residue in the stator a subunit of EhV1 was substituted with alanine (aE634A) and found that the duration time before the forward steps is inversely proportional to [Na+] (Fig. 6B). We previously confirmed that the coupling in EhV1 is retained by using DCCD (N, N'-dicyclohexylcarbodiimide) modification assays (17). [Na+] dependency of the rotation rate in both the wild type and aE634A (Fig. 2C) is also strong evidence for the intact coupling. The present study demonstrates ATP-driven rotation of a rotary ATPase rate-limited by ion transport and tight coupling between ion binding and the rotational step, although single-molecule studies of ATP-driven rotation of H+-transporting rotary ATPases have been reported (13–15, 66–69).

We found that both the wild type and aE634A mainly have 10 pauses per single turn and 36° steps (Figs. 3 and 5), which reflect the structural symmetry of the c10 ring under conditions in which only Na+ binding is rate-limiting for rotation (0.3 mM Na+ and 5 mM ATP). In addition, numbers of pauses larger or smaller than 10 per single turn were also observed (Fig. 4). From the time constants for the elementary steps of the ATP hydrolysis did not show clear backward steps during the long ATP cleavage pauses under this condition (SI Appendix, Fig. S14A) (47). On the other hand, EhV1(Vr350K) exhibited frequent backward and recovery steps during the long ATP cleavage pauses (SI Appendix, Fig. S14B). The distribution of the step size showed two peaks at −11 and 11°, the sizes were smaller than 36° (SI Appendix, Fig. S14C), and these small backward and recovery steps occurred equally in all three ATP cleavage pauses of EhV1.

**Discussion**

**ATP-Driven Rotation of EhV1 Is Tightly Coupled with Na+ transport.** In the present study, we directly visualized ATP-driven rotation of EhV1, which is rate-limited by ion transport. EhV1(Vr350K) exhibited frequent backward and recovery steps during the long ATP cleavage pauses (SI Appendix, Fig. S14B). The distribution of the step size showed two peaks at −11 and 11°, the sizes were smaller than 36° (SI Appendix, Fig. S14C), and these small backward and recovery steps occurred equally in all three ATP cleavage pauses of EhV1.

**Fig. 6.** Single-molecule analysis of aE634A at saturated [ATP] (5 mM). Experimental conditions are described on the left. Examples of trajectories are shown in SI Appendix, Fig. S10. (A and B) Distribution of the step size at 0.9 and 1.3 mM Na+. Black lines represent fitting with single Gaussians. The values at the top right are the fitted parameters (peak ± SD) and averages. (C and D) Distribution of the duration time before the forward step. Black lines represent fitting with single exponential decay functions. The value at the top right is the obtained time constant (fitted value ± SE of the fit). (E) Plot between [Na+] (0.09, 0.3, and 1.3 mM) and time constant obtained by the fitting at 5 mM ATP. The solid red line represents a straight line connecting two data points at 0.09 and 0.3 mM Na+, and its slope is −1.1. The solid blue line is the result of linear fitting among all three data points. The obtained slope is −1.0.
assumed that steps are triggered by Na\textsuperscript{+}bindings. Whether or not EhV\textsubscript{0}V\textsubscript{1}really transports H\textsuperscript{+}under low-[Na\textsuperscript{+}]conditions will be addressed elsewhere. Furthermore, we need to mention that the step-finding algorithm we used cannot perfectly eliminate overfittings and underfittings (62, 64). Due to these experimental and analytical limitations, we cannot confidently identify which dwells correspond to EhV\textsubscript{1}or EhV\textsubscript{0}.

The $V_{\text{max}}^\text{ATP}$ of the wild-type EhV\textsubscript{0}V\textsubscript{1} (40.0 ± 0.3 rps) at 300 mM Na\textsuperscript{+}was smaller than that of the isolated EhV\textsubscript{1} (117 ± 3 rps) (Fig. 2B and Table 1) (47). The difference is presumably caused by the elementary steps of Na\textsuperscript{+}transport in EhV\textsubscript{0}, especially by Na\textsuperscript{+}release, and/or the interaction (molecular friction) between the rotor c\textsubscript{10}ring and stator a subunit. If we assume that Na\textsuperscript{+}release is rate-limiting, we can estimate the time constant for Na\textsuperscript{+}release to be 1.6 ms from the difference of the $V_{\text{max}}^\text{ATP}$ between EhV\textsubscript{0}V\textsubscript{1}and EhV\textsubscript{1}. Note that in the present study, both entry and exit half-channels of the a subunit were exposed to the solution with 300 mM Na\textsuperscript{+}because we used detergent-solubilized EhV\textsubscript{0}V\textsubscript{1}. The effect of Na\textsuperscript{+}rebinding from the exit half-channel on the rotation rate of EhV\textsubscript{0}V\textsubscript{1}is an interesting question that needs to be clarified. On the other hand, the $V_{\text{max}}^\text{ATP}$ of aE634A (4.58 ± 0.04 rps) at 300 mM Na\textsuperscript{+}was approximately one-tenth that of the wild type (40.0 ± 0.3 rps) (Fig. 2B and Table 1). Michaelis–Menten kinetics analysis showed that the $k_{\text{on}}^\text{ATP}$ of aE634A was similar to that of the wild type, although the $k_{\text{on}}^\text{Na}$ of aE634A was reaction in EhV\textsubscript{1}and the time resolution of measurement (0.33 and 1 ms for the wild type and aE634A, respectively), we can consider the case where more than 10 pauses are detected (Fig. 4). From the $k_{\text{on}}^\text{ATP}$ of the wild type and aE634A (2.0 × 10\textsuperscript{6} and 2.1 × 10\textsuperscript{6} M\textsuperscript{-1} s\textsuperscript{-1}, respectively; Table 1), the time constants for ATP binding are estimated to be about 0.1 ms at 5 mM ATP. In addition, by using isolated EhV\textsubscript{1}, we previously reported that the time constants for ATP cleavage and phosphate release are smaller than 1 ms, and that for ADP release is 2.5 ms. Furthermore, ADP release occurs at a different angle from that of ATP binding, while ATP cleavage and phosphate release occur at the same angle as ATP binding (47). Considering the stochastic nature of the pause duration and current time resolution, these elementary events, especially ADP release, can be also detected. If the time resolution and the localization precision are further improved, detection of 16 pauses (10, 3, and 3 pauses for Na\textsuperscript{+}binding, ATP binding, and ADP release, respectively) would become possible and give us further insight into the energy transduction mechanism of the EhV\textsubscript{0}V\textsubscript{1}complex. Similarly, a number of pauses fewer than 10 can also occur stochastically due to Na\textsuperscript{+}binding events with shorter duration times than the time resolution. Another possible explanation is stochastic H\textsuperscript{+}transport instead of Na\textsuperscript{+}transport in EhV\textsubscript{0}V\textsubscript{1}at low [Na\textsuperscript{+}]. It has been reported that H\textsuperscript{+}can bind to the ion-binding site of the EhV\textsubscript{0}c subunit when [Na\textsuperscript{+}] is sufficiently low (44). Because H\textsuperscript{+}binding will not be the rate-limiting factor as described above, the pauses could not be detected at the current time resolution (14). In the present study, we could not distinguish Na\textsuperscript{+} and H\textsuperscript{+}bindings and

![Figure 7](image-url)

**Fig. 7.** (A) Typical trajectory of ATP-driven rotation of aE634A at 1 μM ATP and 0.3 mM Na\textsuperscript{+}recorded at 1,000 fps (1 ms time resolution). (Right) Enlarged view of one revolution (360°). Pink, red, and black traces represent raw, median-filtered (current ± 7 frames), and fitted trajectories, respectively. (Inset) The corresponding x-y trajectory. Pink lines and red dots represent the raw and median-filtered (current ± 7 frames) coordinates, respectively. We collected 920 steps from 21 molecules. Other examples of trajectories are shown in SI Appendix, Fig. S11. (B) Distribution of the step size fitted with the sum of three Gaussians: one peak in backward (minus) direction and two peaks in forward (plus) direction, one of which was fixed at 36°, assuming that it was the step of EhV\textsubscript{0}. The ratio of backward steps was 61%. (C) Distribution of the duration time before the forward step fitted with the sum of two exponential decay functions. The values at the top right are obtained time constants (fitted value ± SE of the fit) and the coefficient of determination ($R^2$) of fitting.

![Figure 8](image-url)

**Fig. 8.** Backward steps of aE634A observed at 1 μM ATP and 0.3 mM Na\textsuperscript{+}with 1,000 fps (1 ms time resolution). (A) Examples of trajectories showing the backward steps. The pink, red, and black traces represent the raw, median-filtered (current ± 7 frames), and fitted trajectories of the median-filtered data identified by the algorithm, respectively. The green, cyan, and purple lines indicate forward steps just before backward steps, backward steps, and forward steps just after backward steps (recovery steps), respectively. In B and D, the distributions seemed to show two peaks at <36° and 36°. In C, the peak position was larger than ~36°, and the average value was ~18.8°.

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significantly decreased compared with that of the wild type (Tables 1 and 2). These results indicate that the aE634A mutation has little effect on ATP binding to EhV1. The mutated glutamate residue is located on the surface of entry half-channel of the a subunit (Fig. 1C), and the decrease in $k_{\text{on}}^{\text{Na}^+}$ is likely caused by the loss of a negative charge. Interestingly, this glutamate residue is completely conserved among Na$^+$- and H$^+$-transporting V-ATPases (*SI Appendix*, Figs. S1 and S2) (24, 26, 70, 71). Therefore, it may have a common role in efficient ion uptake for both Na$^+$ and H$^+$. Consistent with this notion, ion selectivity seems to be determined by the ion-binding site of the c ring rather than the half-channels in the a subunit (72, 73).

The rotation rate of both the wild type and aE634A showed a biphasic response to [Na$^+$] (Fig. 2C and Table 2). This biphasic response was also reported previously for the ATPase activity of detergent-solubilized EhV$_{3/4}$V$_1$ (17, 61). The Michaelis–Menten parameters obtained in this study are slightly different from those in previous studies, presumably due to differences in experimental conditions, especially pH and [Na$^+$] contaminating the observation buffer. Regarding the Na$^+$ contamination in the experimental system, it should be noted that it is difficult to determine affinity at the submicromolar range since the observation buffer contains at least 90 $\mu$M Na$^+$ ([SI Appendix, Fig. S6]). The biphasic response to [Na$^+$] strongly suggests two different Na$^+$-binding sites of EhV$_{3/4}$V$_1$ with largely different affinities. One possible explanation is that these sites are related to the entry and exit half-channels of the a subunit. The high-affinity binding site may correspond to Na$^+$ binding to the c ring from the entry site because the dissociation constant of Na$^+$ to the c subunit in the EhV$_{3/4}$V$_1$ complex has been reported as 12 $\mu$M in the absence of ATP (44). On the other hand, the low-affinity Na$^+$ binding site corresponding to $k_{\text{on}}^{\text{Na}^+}$ has not been identified yet. We assume that the low-affinity site would be physiologically important because the intracellular [Na$^+$] in *E. hirae* has been reported to be several tens of millimolar (38, 41, 74). To clarify the mechanism of the biphasic response of EhV$_{3/4}$V$_1$ to [Na$^+$], it will be required to embed EhV$_{3/4}$V$_1$ in the lipid membrane and change the [Na$^+$] in the entry and exit sides independently (75). Alternatively, mutagenesis in the Na$^+$ exit half-channel of the a subunit will provide insights into the biphasic response. Recently, Yanagisawa and Frasch (15) investigated *E. coli* F$_1$F$_{0}$ mutants by replacing charged or polar residues in the entry and exit half-channels with nonpolar leucine residues and successfully revealed that these residues possess optimal p$K_a$ values for unidirectional H$^+$ transfer. A similar approach would also be helpful for EhV$_{3/4}$V$_1$.

**Rigid Component in Coupling between EhV$_o$ and EhV$_1$ in the EhV$_{3/4}$V$_1$ Complex.** By using single-molecule imaging of aE634A under conditions where both Na$^+$ and ATP bindings are rate-limiting for rotation, we visualized the pauses of EhV$_o$ and EhV$_1$ simultaneously. We found that aE634A mainly shows 13 pauses per single turn under this condition (Figs. 4C and 7A and *SI Appendix*, Fig. S10). Furthermore, occasional backward and recovery steps smaller than 36° were observed (Fig. 8), not only in aE634A but also during the three long ATP cleavage pauses of EhV$_{3/4}$V$_1$(BR350K) ([SI Appendix, Fig. S14]). These results indicate that the pausing positions waiting for Na$^+$ and ATP bindings are different, and transitions occur between adjacent pausing positions of EhV$_o$ and EhV$_1$. These results also indicate that the ATP hydrolysis reaction in EhV$_1$ is tightly coupled with Na$^+$ transport in EhV$_o$, and this coupling has a rigid component. Considering our observation system where the c ring of EhV$_o$ is immobilized on the glass surface and the AuNP probe is attached to the A subunit of EhV$_1$ (Fig. 2A), if the complex shows a fully elastic coupling, the pausing positions of EhV$_o$ and EhV$_1$ would be superimposed by deformation of the peripheral E$_2$G$_2$ stalks or the central DF shaft. In this case, a single turn (360° revolution) would be divided into two 108° (36° × 3) and one 144° (36° × 4) rotations with extensive deformation. However, our results clearly showed 10 pauses per single turn (36° step) at high [ATP] and 13 pauses at low [ATP], suggesting rigid coupling in which the 120° steps in EhV$_1$ are retained in the presence of a structural symmetry mismatch between EhV$_o$ and EhV$_1$. Therefore, we assume that our observation system, even if elastic deformation of the peripheral stalks causes the motion of the a subunit of EhV$_o$ to move to forward or backward Na$^+$ binding angle during the pause of EhV$_1$ waiting for ATP binding, it cannot be detected as a step because we attached the AuNP probe to the A subunit of EhV$_1$. Therefore, the present results do not completely rule out the possibility of elastic deformation in EhV$_{3/4}$V$_1$ ([SI Appendix, Fig. S15]).

Different pausing positions between V$_o$ and V$_1$ have also been reported by a single-molecule study of detergent-solubilized H$^+$-transporting *Thermus thermophilus* V1/ATPase, which acts as an ATP synthase (20). Here we denote this enzyme as TtV$_{10}$V$_1$ following previous studies (20, 24), although it is believed to have been introduced by horizontal gene transfer from a habitat sharing archaea (8). During rotation in the ATP hydrolysis direction, TtV$_{10}$V$_1$ showed 30° steps derived from the c$_{12}$ ring of TtV$_o$ and extra pauses between two adjacent pauses of TtV$_o$. These extra pauses were attributed to catalytic dwells of TtV$_1$. Furthermore, corresponding structures were also recently revealed by a cryo-EM study of nanodisc-embedded TtV$_{10}$V$_1$ (24). Zhou and Sanazan (24) resolved the structures of two substates in addition to three main states, which reflect the threefold structural symmetry of TtV$_1$. Those substates, 1L and 1R, which differ in the relative positions of TtV$_o$ and TtV$_1$, exhibited a slight twist of the central shaft and deformation of the peripheral stalks against state 1. It remains uncertain which pauses found in the single-molecule study correspond to those substates. It is intriguing that the angles of TtV$_o$ and TtV$_1$ do not coincide although TtV$_1$ has a c$_{12}$ ring and no symmetry mismatch with TtV$_1$.

The rigid component in EhV$_{3/4}$V$_1$ coupling is distinct from *E. coli*, *Bacillus* PS3, and yeast mitochondrial F-ATPases, in which cryo-EM studies have indicated an elastic coupling (25, 29, 35). These F-ATPases have c$_{10}$ rings (34, 76) and the same structural symmetry mismatches as EhV$_{3/4}$V$_1$. Structural analysis of *E. coli* or *Bacillus* PS3 F$_0$F$_{1}$ has revealed three rotational steps that are classified into two 108° steps and one 144° step. This significant mismatch is tolerated by the flexible peripheral stalk rather than the central shaft (25, 29, 77). Actually, a very recent cryo-EM study of yeast mitochondrial F$_0$F$_{1}$ revealed extensive deformation of the peripheral stalk during catalysis (35). In rotary ATPases, the number of peripheral stalks varies from one to three, depending on the enzymes (6, 78, 79). *E. coli*, *Bacillus* PS3, and yeast F$_0$F$_{1}$ possess only one peripheral stalk (25, 29), whereas EhV$_{3/4}$V$_1$ has two peripheral stalks that can strongly connect two motor domains (Fig. 1A) (26). As a result, EhV$_{3/4}$V$_1$ would rotate rigidly without large, elastic deformations. Consistent with this notion, a cryo-EM study of yeast V$_o$V$_1$, which has three peripheral stalks, showed three almost uniform 120° steps despite having a c$_{10}$ ring (27), although the possibility of uniform steps due to asymmetry in the c ring of yeast V$_o$V$_1$ should be considered as proposed by the author.
A detailed model is shown in Fig. 9. The orange circles and dark green squares indicate the pausing positions waiting for Na\(^{+}\) binding to EhV\(_{o}\) and ATP binding to EhV\(_{1}\), respectively. The red arrows indicate the 36° steps between adjacent pausing positions for the EhV\(_{o}\). The blue arrows indicate the backward and forward steps smaller than 36° between adjacent pausing positions for EhV\(_{o}\) and EhV\(_{1}\).

(4) Condition in which only Na\(^{+}\) binding to EhV\(_{o}\) is rate-limiting. In this condition, the pauses waiting for ATP binding to EhV\(_{1}\) are too short to be detected, and EhV\(_{o}\) rotates unidirectionally without backward steps.

(5) Condition in which both Na\(^{+}\) and ATP bindings are rate-limiting. The pausing positions waiting for ATP binding are visualized, and then 1.6 pausing positions are detected per single turn. Because no torque is generated during the pauses waiting for ATP binding to EhV\(_{1}\), EhV\(_{o}\)V\(_{1}\) rotates to the backward and forward pausing positions of EhV\(_{o}\) driven by Brownian motion. As a result, backward and forward steps smaller than 36° are observed. A detailed model is shown in SI Appendix, Fig. S15.

In addition to the number of peripheral stalks, the number of c subunits in the rotor ring also varies widely from 8 to 17 among species (58, 59). This makes the issue more complicated because a different number of c subunits will change the step size and the ion-to-ATP ratio. Furthermore, the number of transmembrane helices in a c subunit monomer also differs among species. For example, although both EhV\(_{o}\)V\(_{1}\) and E. coli F\(_{o}\)F\(_{1}\) have c\(_{10}\) rings, each c subunit consists of tetratransmembrane and double-transmembrane helices, respectively (25, 55). This results in largely different diameters of the c\(_{10}\) ring, 8 and 5 nm for EhV\(_{o}\)V\(_{1}\) and E. coli F\(_{o}\)F\(_{1}\), respectively (26). Obviously, larger rings would require larger deformations to adjust the relative angles between \(V_{o}/F_{o}\) and \(V_{1}/F_{1}\) if elastic coupling is assumed. Another interesting feature of EhV\(_{o}\)V\(_{1}\) with the large c ring is its off-axis rotation, which may affect the coupling between EhV\(_{o}\) and EhV\(_{1}\) (26). However, the off-axis rotation was not resolved in the present single-molecule experiments. To understand the common and diverse mechanisms of energy transduction in rotary ATPases, a comprehensive study of various ATPases with rotor c rings of different c subunit numbers and sizes will be required.

Brownian Ratchet Rotation of EhV\(_{o}\). Fig. 9 shows a schematic model of the stepping rotation of EhV\(_{o}\)V\(_{1}\) at high [ATP] and low [Na\(^{+}\)] (Fig. 9A) and at low [ATP] and low [Na\(^{+}\)] (Fig. 9B). In the present study, backward steps were rarely observed when [ATP] is high (5 mM) in both the wild type and aE634A (Figs. 3B, 5B, and 6 A and B). In EhV\(_{o}\)V\(_{1}\), the torque for ATP-driven rotation is generated by ATP binding and ADP release in EhV\(_{1}\) (47), and the value has been estimated to be 20 pNnm (17). When [ATP] is high, time constants for ATP binding and ADP release are both small, and the torque from EhV\(_{1}\) will be applied to EhV\(_{o}\) almost constantly. Therefore, EhV\(_{o}\)V\(_{1}\) will show unidirectional rotation without backward steps (Fig. 9A).

On the other hand, when [ATP] is low (1 nM) and where the time constant for ATP binding is large (476 or 642 ms; Table 1 and Fig. 7C), backward steps were observed occasionally (Fig. 8A). The size of the backward steps was much smaller than 36° (14.2 or 18.8°; Figs. 7B and 8O). Because no torque is applied when EhV\(_{o}\)V\(_{1}\) is waiting for ATP binding at the pausing position of EhV\(_{1}\) (Fig. 9B, dark green), it can move to the adjacent backward (and also forward) pausing position of EhV\(_{o}\) by Brownian motion (Fig. 9B, blue arrows). Backward and recovery steps smaller than 36° were more frequently observed in EhV\(_{o}\)V\(_{1}\) (BR350K), in which ATP cleavage pauses are prominently longer than in the wild type (SI Appendix, Fig. S14). Importantly, backward steps occurred equally during the three ATP cleavage pauses of EhV\(_{1}\), consistent with the notion that backward steps are driven by thermal fluctuation, not by elastic strain accumulation. These results support a Brownian ratchet model of EhV\(_{o}\) rotation in addition to the rigid coupling component of EhV\(_{o}\)V\(_{1}\).

In our previous study using isolated EhV\(_{1}\), no backward steps were observed in ATP-driven rotation except under extreme experimental conditions (47). Therefore, backward and forward steps smaller than 36° observed in EhV\(_{o}\)V\(_{1}\) are caused by EhV\(_{o}\) and are presumably coupled with Na\(^{+}\) binding/release to/from the c subunit through the two half-channels of the a subunit.

A recent single-molecule study observed similar small backward steps of 11° in E. coli F\(_{o}\)F\(_{1}\) during ATP-driven rotation and successfully revealed that backward steps are related to H\(^{+}\) translocation between the c ring and half-channels of the a subunit (15). In a study of E. coli F\(_{o}\)F\(_{1}\), the small backward step was attributed to the fact that E. coli F\(_{o}\)F\(_{1}\) functions as an ATP synthase. In the case of EhV\(_{o}\)V\(_{1}\), because backward steps of 36° or larger than 36° rarely occur, the backward flow of Na\(^{+}\) seems to be suppressed. The detailed mechanisms of the backward step may be different by ATP synthesis capacity, size of the c ring, and ion species being transported. In the present study, however, the electrochemical potential of Na\(^{+}\) could not be applied because detergent-solubilized EhV\(_{o}\)V\(_{1}\) was used. To address the question of whether the electrochemical potential of Na\(^{+}\) can drive the rotation of EhV\(_{o}\)V\(_{1}\) in the opposite direction and synthesize ATP, our next projects will be single-molecule imaging and biochemical assays of EhV\(_{o}\)V\(_{1}\) embedded in a lipid membrane.

Materials and Methods

Sample Preparation. The construction of the expression plasmid for the wild-type EhV\(_{o}\)V\(_{1}\) (pTR19-EhV\(_{o}\)V\(_{1}\)) was reported previously (17). For the plasmid construction of aE634A, EhV\(_{o}\)V\(_{1}\) (BR350K), and EhV\(_{o}\) (BR350K), PCR-based site-directed mutagenesis was performed by KOD one PCR Master Mix (Toyobo) with pTR19-EhV\(_{o}\) as a template. For expression and purification of the wild type, aE634A, EhV\(_{o}\)V\(_{1}\) (BR350K), and EhV\(_{o}\) (BR350K), the procedures described in our previous study were used with some modifications (17, 47). Details are described in SI Appendix.

Single-Molecule Imaging. AuNPs with a diameter of 40 nm (EMGC40, BBI) were biotinylated with biotin-alkane-thiol (HS-C11-EG3, Surfmods) and coated by streptavidin (PRO791, PROSPEC) as described in previous reports (47, 80). We followed the setting of a total internal reflection dark-field microscope system based on a high-speed complementary metal oxide semiconductor (CMOS) camera and an inverted microscope as described in our previous studies with some modifications (47). Details are described in SI Appendix.
ICP-OES Measurement. To estimate the concentrations of contaminating Na⁺ in the observation buffers, we measured emission spectra using an ICP-OES (5100 ICP-OES, Agilent Technologies). The emission signal at λ = 589.592 nm was used for analysis. The flow rates of the plasma and assist gas (Ar) were 14 and 1.2 L/min, respectively. The standard addition method was used to avoid physical and ionization interferences. Namely, a sodium standard solution (FUJIFILM Wako) was added to the observation buffers in a range of 0.1 to 10 mg/L (4.3 to 430 μM) as a final concentration of Na⁺. The calibration curve was extrapolated, and the absolute value of the x intercept was taken as the concentration of contaminating Na⁺.

Data, Materials, and Software Availability. All study data are included in the article and/or SI Appendix.

ACKNOWLEDGMENTS. We thank Monique Honsa for advice about data analysis; Kazuyoshi Murata, Chihong Song, and Raymond N. Burton-Smith for providing us with the structural data and for their fruitful discussions; Yayoi Kori for her technical support; and all laboratory members for helpful discussions and technical advice. This work was supported by Grants-in-Aid for Scientific Research on Innovative Areas “Molecular Engine” (JP19H05380 to H.U., JP19H05425 to T.M., and JP18H05424 to R.I.); Grants-in-Aid for Scientific Research (JP21H02454 to R.I. and JP21K15600 and JP20J01316 to A.O.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; and the National Institutes of Natural Sciences (NINS) program for cross-disciplinary study (Grant O11312001 to A.O.). A part of this work was performed with the aid of the Instrument Center of the Institute for Molecular Science.
65. T. E. Spikes, M. G. Montgomery, J. E. Walker. Structure of the dimeric ATP synthase from bovine mitochondria. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 23519–23526 (2020).

66. K. Nishio, A. Iwamoto-Kihara, A. Yamamoto, Y. Wada, M. Futai. Subunit rotation of ATP synthase embedded in membranes: a γβ Subunit rotation relative to the c subunit ring. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 1333–1338 (2002).

67. H. Ueno, T. Suzuki, K. Kinosita Jr., M. Yoshida. ATP-driven stepwise rotation of FoF1-ATP synthase. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 1333–1338 (2005).

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

69. T. Hirata et al. Subunit rotation of vascular-type proton pumping ATPase: Relative rotation of the G and C subunits. *J. Biol. Chem.* **278**, 23714–23719 (2003).

70. D. G. Schep, J. Zhao, J. L. Rubinstein. Models for the a subunits of the Thermus thermophilus V1A-ATPase and Saccharomyces cerevisiae V1ATPase enzymes by cryo-EM and evolutionary covariance. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 3245–3250 (2016).

71. T. Vasanthakumar et al. Structural comparison of the vacuolar and Golgi V-ATPases from Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U.S.A.* **116**, 7272–7277 (2019).

72. V. Leone, D. Pogoryelov, T. Meier, J. D. Faraldo-Gomez. On the principle of ion selectivity in Na⁺/H⁺-coupled membrane proteins: Experimental and theoretical studies of an ATP synthase rotor. *Proc. Natl. Acad. Sci. U.S.A.* **112**, E1057–E1066 (2015).

73. K. Schlegel, V. Leone, J. D. Faraldo-Gomez, V. Muller. Promiscuous archaeal ATP synthase concurrently coupled to Na⁺ and H⁺ translocation. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 947–952 (2012).

74. T. Murata, K. Takase, I. Yamato, K. Igarashi, Y. Kakinuma. The ntpJ gene in the Enterococcus hirae ntp operon encodes a component of Xtnl potassium transport system functionally independent of vascular Na⁺-ATPase. *J. Biol. Chem.* **271**, 10042–10047 (1996).

75. R. Watanabe et al. Biased Brownian stepping rotation of F₁F₀-ATP synthase driven by proton motive force. *Nat. Commun.* **4**, 1631 (2013).

76. W. Jiang, J. Hermolin, R. H. Fillingame. The preferred stoichiometry of c subunits in the rotary motor sector of Escherichia coli ATP synthase is 10. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 4966–4971 (2001).

77. A. Wächter et al. Two rotary motors in F-ATP synthase are elastically coupled by a flexible rotor and a stiff stator stalk. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 3924–3929 (2011).

78. L. Colina-Tenorio, A. Dautant, H. Miranda-Astudillo, M.-F. Giraud, D. Gonzalez-Halphen. The peripheral stalk of rotary ATPases. *Front. Physiol.* **9**, 1243 (2018).

79. J. E. Walker, V. K. Dickson. The peripheral stalk of the mitochondrial ATP synthase. *Biochim. Biophys. Acta* **1757**, 286–296 (2006).

80. J. Ardo et al. Single-nanoparticle tracking with angstrom localization precision and microsecond time resolution. *Biophys. J.* **115**, 2413–2427 (2018).