Vacuole-type ATPases ($V_0V_1$) and $F_oF_1$ ATP synthases couple ATP hydrolysis/synthesis in the soluble $V_1$ or $F_1$ portion with proton (or Na$^+$) flow in the membrane-embedded $V_o$ or $F_o$ portion through rotation of one common shaft. Here we show at submillisecond resolutions the ATP-driven rotation of isolated $V_1$ and the whole $V_oV_1$ from *Thermus thermophilus*, by attaching a 40-nm gold bead for which viscous drag is almost negligible. $V_1$ made 120° steps, commensurate with the presence of three catalytic sites. Dwells between the steps involved at least two events other than ATP binding, one likely to be ATP hydrolysis. $V_oV_1$ exhibited 12 dwell positions per revolution, consistent with the 12-fold symmetry of the $V_o$ rotor in *T. thermophilus*. Unlike $F_1$ that undergoes 80°–40° substepping, chemo-mechanical checkpoints in isolated $V_1$ are all at the ATP-waiting position, and $V_o$ adds further bumps through stator-rotor interactions outside and remote from $V_1$.  

Shou Furuike$^{1,2}$, Masahiro Nakano$^{3,4}$, Kengo Adachi$^{1,1}$, Hiroyuki Noji$^4$, Kazuhiko Kinosita Jr$^1$ & Ken Yokoyama$^{3,5,6}$  

1 Department of Physics, Faculty of Science and Engineering, Waseda University, Shinjuku-ku, Tokyo 169-8555, Japan. 2 Department of Physics, Osaka Medical College, Takatsuki, Osaka 569-8686, Japan. 3 Chemical Resources Laboratory, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8503, Japan. 4 Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka 567-0047, Japan. 5 ICORP, ATP Synthesis Regulation Project, Japan Science and Technology Agency (JST), National Museum of Emerging Science and Innovation, 2-41 Aomi, Koto-ku, Tokyo 135-0064, Japan. 6 Department of Molecular Biosciences, Kyoto Sangyo University, Motoyama Kamigamo, Kita-ku, Kyoto 603-8555, Japan. †Present address: Department of Physics, Faculty of Science, Gakushuin University, Toshima-ku, Tokyo 171-8588, Japan. Correspondence and requests for materials should be addressed to K.Y. (email: yokoken@cc.kyoto-su.ac.jp) or to K.K. (email: kazuhiko@waseda.jp).
The F_1,F_0- and V-type ATPase/ATP synthase superfamily utilizes a rotary mechanism to perform their specific functions.

The basic structures of these ATPases/synthases are conserved among species. The soluble, cytoplasmic portion of F_1,F_0- and V-type ATPases (called F_1 and V_1, respectively), responsible for ATP hydrolysis/synthesis, is connected via the central rotor stalk and the peripheral stator stalk to the transmembrane portion (F_0 and V_0) that houses the ion-transporting pathway. In the bacterial V-type ATPase of Therbus thermophilus (V_0V_1), the V_1 portion is composed of a hexameric A_3B_3 cylinder and a central shaft composed of D and F subunits (see Fig. 1a). The V_0 portion of T. thermophilus is composed of two distinct domains: a hydrophobic rotor ring made of V_0-c subunits supplemented with a funnel shape V_0-d subunit and a stator apparatus composed of a transmembrane V_0-a subunit and EG subunits forming the peripheral stalk (see Fig. 1b).

Cryo-electron micrographs of two-dimensional crystals of the V_0 ring at 7.0 Å resolution showed the presence of 12 V_0-c subunits, each composed of two transmembrane helices. The bacterial V-ATPase that we describe here works as an ATP synthase, whereas its eukaryotic counterpart is vacuolar proton pump and thus some mechanistic differences may exist. A number of researchers refer to the bacterial V-ATPase as archaeal-ATPase or A_oA_1-ATP synthase, but here we adopt the broader terminology.

It is believed that V_0 (and F_0) is a rotary motor driven by the transmembrane flow of protons (or Na^+) and V_1 (and F_1) is another rotary motor driven by ATP hydrolysis, and that the two motors have a common rotary shaft yet their genuine rotary directions are opposite to each other. Thus, when V_0 (F_0) takes control, V_1 (F_1) is in turn deprived of ATP. Therefore, the F_0F_1 and V_0V_1 motors operate in opposite directions, and their genuine rotary directions are opposite to each other. Thus, when V_0 (F_0) takes control, V_1 (F_1) is in turn deprived of ATP.

The ATP-driven rotation of the DF shaft in V_0 has been observed directly: a bead (nominal diameter 0.56 μm) attached to the D subunit rotated unidirectionally anticlockwise when viewed from the membrane side. At low ATP concentrations where ATP binding is rate limiting, the rotation proceeded in steps of 120°, commensurate with the presence of three catalytic sites on A-B interfaces.

Rotation of the V_0-c ring in V_0V_1 has also been observed, with 120° steps at low ATP concentrations.

For F_0, which also undergoes anticlockwise 120° stepping at low ATP, high-speed imaging with 40-nm gold particles, with little drag, has revealed that a 120° step consists of 80–90° and 40–30° substeps. F_1 cycles through an ATP-waiting dwell, ~80° substep rotation driven by ATP binding and subsequent ADP release, a catalytic dwell where ATP is hydrolyzed and the phosphate is released, and ~40° substep rotation driven by the phosphate release.

ATP-driven rotation of F_0F_1 has also been demonstrated for Escherichia coli and thermophilic Bacillus PS3 enzymes, with features basically similar to those of F_1. So far, ATP-driven rotation either in V_0V_1 or in F_0F_1 has failed to reveal a sign of specific interactions between a rotor and a stator subunit in the V_0/F_0 portion, even in the high-resolution study.

Here, we have analysed ATP-driven rotation of both V_1 and V_0V_1 (holo V-ATPase) derived from T. thermophilus, using a 40-nm bead and a submillisecond fast camera. V_1 molecules rotated with 120° steps without adopting the 80°–40° substep scheme of F_0F_1, in contrast, showed ~30° steps that likely reflect stator–rotor interactions in the V_0 domain. All rate-limiting reactions in the V_1 chemico-mechanical cycle occur in one angle, whereas stator–rotor interactions in V_0 pose additional bumps that might check rotation depending on protonation/deprotonation.

Figure 1 | Rotation of V_1 and V_0V_1 carrying a 40-nm bead. Schematic observation systems for rotation of V_1 (a) and V_0V_1 (b). (a) V_1 was fixed to the Ni^{2+}-NTA-coated glass surface with His_{10} tags at A subunits. A 40-nm bead (or duplex) was attached to the biotinylated cysteine residues (E48BC/Q5SC) of the D subunit via streptavidin. In this system, the central shaft composed of D and F subunits rotates relative to A subcomplex containing catalytic sites. (b) V_0V_1 was fixed to the Ni^{2+}-NTA-coated glass surface with His tags at V_0-c subunits. In this system, the stator apparatus composed of A_3B_3, G, and V_0-a subunit rotates relative to the fixed central rotor shaft composed of V_0-c ring, V_0-d, D and F subunits. A 40 nm bead was attached to the AviTag at A subunit(s) by biotin–streptavidin linkage. Rotation was observed under an optical microscope with dark-field illumination, and recorded with a high-speed camera at 250–8000 frames per s (fps).

(c) Rotation rates of beads attached onto V_1 (circles) and V_0V_1 (triangles) at the indicated ATP concentrations. Red and black circles indicate the presence and absence of 0.05% (w/v) DDM, respectively. Squares indicate the averages of V_1 rotation rates (n=28; s.d. greater than the symbol size shown with bars).

Line indicates the fit with Michaelis–Menten kinetics: $V = V_{\text{max}} \cdot [\text{ATP}] / (K_m + [\text{ATP}])$, where $V_{\text{max}}$ and $K_m$ are 64 r.p.s. and 229 μM, respectively, giving the apparent ATP-binding rate $k_{\text{on}}$ of 0.84 × 10^4 M^{-1} s^{-1} (3×$V_{\text{max}}$/K_m). For V_0V_1, the rotation buffer contained 0.05% DDM. Time-averaged rotation rates of V_1 or V_0V_1 were estimated over tens of consecutive revolutions as listed in Supplementary Table S1. The molecules of V_0V_1, which showed relatively clean 120° steps, are shown as closed blue triangles.

Results

Stepwise rotation of V_1. V_1 was immobilized on a nickel-nitritrioltriacetic acid (Ni^{2+}-NTA)–coated glass surface through His (histidine)_{10}-tags introduced at the amino terminus of the A subunits, and a 40-nm streptavidin-coated gold colloidal (40 nm) bead was attached to the biotin-labelled D subunit (Fig. 1a).

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Bead rotation was imaged by laser dark-field microscope and recorded on a fast-framing CMOS camera at speeds up to 8,000 frames per s.

ATP dependence of the time-averaged rotation rate of \( V_o \) is shown in Figure 1c. Below 100 \( \mu \)M ATP, ATP binding was rate limiting, the rotation speed being practically proportional to the ATP concentration ([ATP]). The rate constant for apparent, or effective, ATP binding was 8.0 \( \times 10^4 \) M\(^{-1}\) s\(^{-1}\), assuming three ATP molecules consumed per revolution. Above 1 mM ATP, the rotary speed saturated, reaching \( V_{\text{max}} \) of 64 revolutions per s (r.p.s.). This is the full speed of \( V_o \) rotation at 23°C, not limited by the viscous drag on the bead (see below). The Michaelis–Menten constant, \( K_m \), of 229 \( \mu \)M (Fig. 1c) agrees with that for the bulk ATP hydrolysis assay without beads resolved in the previous study with a 340-nm bead duplex 12, where rotation takes place. The 120° steps at saturating [ATP] were not by the ~5 ms dwells where a reaction(s) that does not accompany this was also at, or close to, ATP-waiting angles. This was also confirmed by the average rotation speed of 64 r.p.s. at saturating [ATP] is limited and thus mechanical stepping does not limit the overall rotation rate. The events that underlie the \( V_o \) dwell. \( V_o \) dwells basically (see below) at every 120°, or once per catalytic cycle, irrespective of [ATP]. We now enquire what causes these dwells. At least four events occur in a catalytic cycle of \( V_o \): ATP binding, ATP hydrolysis, phosphate release and ADP release. Of these, ATP binding must trigger, and likely drives at least partially, the 120° step. Our previous study 12 with a slowly hydrolyzed ATP analogue ATP-\( \gamma \)-S indicated that ATP hydrolysis occurs at an ATP-waiting angle, and thus the time required for hydrolysis is a determinant of the dwell.

To see whether hydrolysis alone is responsible for the dwell, we have analyzed the distribution of dwell times, measured as the time between the midpoints of two successive 120° steps (Fig. 2f–i). At all four [ATP] examined, the dwell-time histogram was not exponen-
tial and rose from the origin (not well resolved at 4 \( \mu \)M), indicating the involvement of two or more rate-limiting reactions. Sequential two-reaction scheme could reasonably fit the histograms (orange lines in Fig. 2f–i). At 4 mM ATP, the two rates seemed indistinguishable and were 0.36 ms\(^{-1}\). One rate should correspond to that of ATP hydrolysis, unless a third reaction is also involved. The nature of the other reaction is unknown, but it cannot be ATP binding, which must be rapid at 4 mM ATP (binding rate for ATP is calculated as 3.2 ms\(^{-1}\) by multiplying 4 mM by 0.8 \( \times 10^6 \) M\(^{-1}\) s\(^{-1}\)). Likely candidates are phosphate or ADP release (or both combined).

At and below 200\( \mu \)M ATP, the dwells must also involve the time for ATP binding in addition to the two (or more) reactions at 4 mM. We therefore attempted a global fit to the three histograms (Fig. 2f–h, blue lines) around \( K_m \), where the rise from the origin was well resolved, with a sequential scheme for three reactions, of which one is ATP binding with the apparent rate constant \( k_m \). Although the fit was not perfect, the recovered \( k_m \) of 1.2 \( \times 10^6 \) M\(^{-1}\) s\(^{-1}\) is consistent with that for 4 \( \mu \)M ATP, and with the estimate from Figure 1c above and a previous value of \( \sim 1.3 \times 10^6 \) M\(^{-1}\) s\(^{-1}\) obtained with 220-nm duplex beads 14. The other two rates were 0.49 ms\(^{-1}\) and 0.34 ms\(^{-1}\), roughly consistent with the two-rate fit of the 4 mM beads above.

In addition to the relatively clean 120° steps as in Figure 2, some beads (52 out of 169; see Supplementary Table S1) exhibited peculiar fluctuations such as jumping to and fro between two angles separated by ~40° (see Supplementary Fig. S1). Because the basic 120° stepping feature was preserved, we ignore these minor fluctuating beads in the analyses above.

**Rotation of \( V_o V_1 \).** To examine the effect(s) of the \( V_o \) domain on the ATP-driven rotation of \( V_1 \) in intact \( V_o V_1 \), we constructed the experimental system in Figure 1b. \( V_o V_1 \) was fixed, in the presence of 0.05% (w/v) N-dodecyl β-D-maltoside (DDM) upside down on a Ni\(^2+\)-NTA-coated glass surface via His, tags on the \( V_1 \)-c subunits. A 40-nm gold bead was attached to \( V_1 \)-A subunit(s) through the Avitag–bistin–streptavidin linkage. Immediately after infusion of millimolar ATP, we found a few rotating beads per field of view (7.1 \( \times 1.7 \) μm\(^2\)). The number decreased with time, particularly at high [ATP] where finding the rotation became difficult after 1 h. Both \( V_o \) and \( V_1 \) are highly susceptible to ADP inhibition even in the presence of an ATP-regeneration system 14,32. Part of the dormant molecules was somehow reactivated by re-infusion of the observation buffer, allowing further observations.

All molecules that rotated for many revolutions (as listed in Supplementary Table S1) without an obvious sign of obstruction at a particular angle were subjected to analysis. Rotation speed of \( V_o V_1 \) was variable and was distributed around 1–10 r.p.s. at 4 mM ATP (Fig. 1c). Typical rotation time courses are shown in Figure 3a–e. Unlike \( V_1 \), which basically paused every 120°, \( V_o V_1 \) made short pauses at many angles at all [ATP] examined. A relatively fast rotation (~10 r.p.s.) at 4 mM ATP is shown in Figure 3e, which still contains many pauses. At this [ATP], most \( V_1 \) molecules rotated much faster, at ~60 r.p.s. (Fig. 1c). The \( V_o \) domain seems to introduce bumps that lead to the small steps and the reduced average speed of \( V_o V_1 \) rotation. In this observation system, the whole stator apparatus (A\(_3\)B\(_3\)EGV\(_o\)-a) rotates against the central rotor spanning the \( V_o V_1 \) (DFV\(_o\)-dDFV\(_c\)-c ring). The bumps likely represent the interaction between \( V_o \)-c ring and \( V_1 \)-a in the \( V_o \) domain. In 15 analysed molecules, we found three beads that showed clean 120° steps (Fig. 3f), and these beads (Fig. 1c, blue triangles) rotated fast (~30 r.p.s.). Detailed analyses of the short pauses in the presence of Triton below suggest that these 120° stepping beads are attached to defective \( V_o V_1 \), in which the \( V_o \) interaction is somehow impaired, although the opposite possibility of short pauses being an artefact cannot be ruled out.

**Approximately 30° stepping.** The detergent Triton X-100 (Triton) has been reported to be deleterious to the integrity of F\(_{10}\), presumably affecting stator–rotor interaction in F\(_{10}\). Unexpectedly, however, the substep behaviour of \( V_o V_1 \) above, indicative of rotor–stator interaction in the \( V_o \) domain, was enhanced when DDM was replaced with Triton. The small substeps could be more clearly discerned in the presence of Triton. When Triton-solubilized \( V_o V_1 \) was reconstituted into liposomes, it actively pumped protons, indicating that Triton treatment leaves \( V_o V_1 \) intact 14. The same lot of \( V_o V_1 \) has also been shown to be inactivated by N,N′-dicyclohexylcarbodiimide 14, another sign of integrity particularly in the \( V_1 \) portion. Below, we analyze the clearer substeps observed in the presence of Triton.

Somewhat, rotation trajectory of \( V_o V_1 \) was unstable in the presence of a detergent, whether Triton or DDM, and gradually drifted both rotationally and translationally up to a few nanometers. Nevertheless, we could identify pauses clearly in trajectories of
Figure 2 | Rotation of Vₕ. (a–e) Typical time courses of rotation with a 40-nm bead (or duplex). (a) Rotation at 4 mM ATP captured at 8,000 fps; (b) 200 μM ATP at 2,000 fps; (c) 40 μM ATP at 250 fps; (d) 4 μM ATP at 4,000 fps and (e) 2 mM ATP at 4,000 fps, obtained from the same molecule as in d after medium exchange. Trajectories of the bead centroid (axis divisions: 11.1 nm) and histograms of angular positions, both for the indicated portion of the records, are shown in the upper and lower insets, respectively. (f–i) Histograms of dwell times between 120° steps. (f) Dwell times at 4 mM ATP with 125 μs bin size obtained from 6 molecules observed at 8,000 fps; (g) 200 μM ATP, 250 μs bin size, 6 molecules at 8,000 fps; (h) 40 μM ATP, 1 ms bin size, 6 molecules at 4,000 fps; (i) 4 μM ATP, 4 ms bin, 15 molecules at 2,000 fps. Orange curves show fit with the sequential two-reaction scheme with rates $k_1$ and $k_2$: constant $\times \exp(-k_1 t)$ + $(k_1 - k_{\text{on}}(\text{ATP})) \times \exp(-k_{\text{on}}(\text{ATP}) t)$ + $(k_{\text{on}}(\text{ATP}) - k_2) \times \exp(-k_2 t)$ + $(k_1 - k_{\text{on}}(\text{ATP})) \exp(-k_2 t)$. The estimated rates and associated s.e. are: $k_{\text{4M}} = 0.36 \pm 0.01 \text{ ms}^{-1}$, $k_{\text{200μM}} = 0.17 \pm 0.02 \text{ ms}^{-1}$, $k_{\text{40μM}} = 0.28 \pm 0.03 \text{ ms}^{-1}$, $k_{\text{4μM}} = 31 \pm 1 \text{ s}^{-1}$, $k_{\text{40μM}} = 0.40 \pm 0.03 \text{ ms}^{-1}$, and $k_{\text{4μM}} = 6.1 \pm 0.1 \text{ s}^{-1}$, $k_{\text{4μM}} = 2.6 \pm 0.02 \text{ ms}^{-1}$. If we assume that $k_{\text{on}}$ represents the rate of ATP binding ($k_{\text{on}} = k_{\text{on}}(\text{ATP})$), $k_{\text{on}}$ is given as $0.85 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ at 200 μM ATP, $0.78 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ at 40 μM and $1.5 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ at 4 μM. At 4 μM, $k_{\text{on}}$ should dominate the histogram, and the green fit with constant $\times \exp(-k_{\text{on}}(\text{ATP}) t)$ gave $k_{\text{on}}$ of $1.5 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$. Blue curves show a global fit to f–h (equal weight for each count), with sequential reactions starting with ATP binding at the rate $k_{\text{on}}(\text{ATP})$ and two ATP-independent reactions with rates $k_1$ and $k_2$: constant $\times \exp(-k_{\text{on}}(\text{ATP}) t)$ + $(k_1 - k_{\text{on}}(\text{ATP})) \exp(-k_{\text{on}}(\text{ATP}) t) + (k_1 - k_{\text{on}}(\text{ATP})) \exp(-k_2 t) + (k_1 - k_{\text{on}}(\text{ATP})) \exp(-k_2 t)$ with $k_{\text{on}} = (1.2 \pm 0.1) \times 10^6 \text{ M}^{-1} \text{s}^{-1}$, $k_1 = 0.49 \pm 0.05 \text{ ms}^{-1}$, $k_2 = 0.34 \pm 0.04 \text{ ms}^{-1}$.
successive segments for one to two revolutions (Fig. 4a, square insets, with frames coloured as in the segmented time course). We could also estimate pausing angles by fitting an ellipse to each segmented trajectory and assuming that the ellipse represents the projection of a circular orbit oblique to the glass surface (Fig. 4b). The angular histogram of the time course is shown on the left axis of Figure 4a. In most parts, the histogram as well as the trajectories show dwells that occur every ~30°, missing positions ascribed to rapid passage. An autocorrelation of the histogram, equivalent with the pairwise angular distribution function\(^{23,24}\), is shown in Figure 4c together with its power spectrum (Fig. 4d). The latter shows a peak at (27°)\(^{-1}\), indicated by the arrowhead at the resolution of ~4°. In Figure 4e,f, the average of all autocorrelations of individual angular histograms and its power spectrum, including other examples of ~30° step rotation shown in Figure 5 and Supplementary Figures S2a,b is shown. The power spectrum in Figure 4f shows a peak at (32°)\(^{-1}\).

In Figure 5, in particular, \(V_{V,0}\) was fixed upside up on a Ni\(^{2+}\)-NTA-coated glass surface via His\(_{10}\) tags in the A subunits and

Figure 3 | Rotation of \(V_{V,0}\). Typical time courses of the rotation of a 40-nm gold bead attached on \(V_{V,0}\) in the presence of 0.05% DDM. Horizontal lines are 30° apart, except in f. (a) Rotation at 4 mM ATP captured at 2,000 fps; (b) 40 μM ATP at 2,000 fps; (c) 4 μM ATP at 1,000 fps and (d) 400 nM ATP at 250 fps. (e) A relatively fast rotation (~10 r.p.s.) with small substeps at 4 mM ATP captured at 1,000 fps. (f) A minor case of rotation with 120° steps at 4 mM ATP captured at 2,000 fps. Trajectories of the bead centroid (axis divisions: 11.1 nm) and histograms of angular positions for the indicated portion of the records are shown in the upper and lower insets, respectively.
Figure 4 | Well-resolved substeps in V_{o}V_{1}. (a) An expanded time course of the rotation of a 40-nm gold bead attached on a V_{o}V_{1} at 40 μM ATP, in the presence of 0.1% (w/v) Triton captured at 2,000 fps. Horizontal lines are 30° apart. The time course is split into three and horizontally shifted (magenta and orange curves partially overlap). To minimize the effect of small, gradual drift on the angle analysis, the record was divided into six coloured portions (black, magenta, orange, green, blue and purple) covering ~1 revolution and analysed as follows. First, the bead trajectory in each portion (coloured square insets; grey points show raw data and black after 21-point median filtering of x and y time courses) was fitted with an ellipsoid (orange). Rotary angle was calculated by assuming the ellipsoid to be a projection of a circular orbit (b). The angle 0, a start of a revolution on the vertical axis of the figure, was assigned to the red dot in each inset, chosen from the 12 orange spokes that fitted the dwells. The green line on the time courses shows 41-point (20 ms) median. The histograms on the left axis represent logarithm of the number of data points per 2°. Red arrowheads, dwells that are clearly out of the 30° periodicity. Black arrowheads, excursions to a neighbouring (closed, forward; open, backward) dwell position for >20° and >20 ms. Boxes enclosing trajectories show a fixed 89 × 89 nm² area, such that drifts manifest as differences between insets. (b) Circular orbit (cyan) of a bead projected on the image plane (pink). Direction of observation is indicated by a green arrow. For the data in a, the angle θ ranged between 43° and 55°. (c) The autocorrelation of the angular histogram derived from a; the continuous time course over 2,500° was 21-point median filtered and then binned at 0.25° intervals. For this analysis, we calculated the angular histogram without adjusting the angular origins of the six portions, that is, without correction for the rotational drift, to eliminate possible subjectivity. (d) The power spectrum of c, the arrowhead showing a peak at (27°)^{-1}. (e) The average of autocorrelations of individual angular histograms for Figures 4c, 5c and Supplementary Figures S2a,b. (f) The power spectrum of e, the arrowhead showing a peak at (32°)^{-1}. 
beads were attached with biotinylated \(V_o\)-c subunit (see Fig. 5a). The \(\sim 30^\circ\) steps are not the consequences of the upside down configuration (Fig. 5b–d).

Taking into account the variations in the peak position in the individual power spectra, we conclude that substeps in \(V_oV_1\) rotation are characterized by an amplitude between \(27^\circ – 32^\circ\).

We noticed that some dwells were observed between two \(\sim 30^\circ\) dwell positions (Fig. 4, orange arrow heads). These may represent ATP-waiting dwells, because they were roughly \(120^\circ\) apart, taking the drift into account. If so, the \(\sim 30^\circ\) steps are not synchronous with ATP binding. This is not entirely unexpected, if the \(\sim 30^\circ\) steps arise from the stator–rotor interaction in the \(V_o\) domain, whereas ATP binding takes place in \(V_1\). As mentioned above, ATP-waiting dwells in \(V_oV_1\) do not stand out even at low [ATP]. This suggests that the driving torque produced in the \(V_1\) portion, the torque that can drive the DF rotor of \(V_1\) over \(120^\circ\) in a matter of 0.25 ms or less, is sustained for many seconds while the \(V_o\) rotor slowly proceeds over the bumps presented by the \(V_o\) stator every \(\sim 30^\circ\). An alternative, less likely scenario is that every \(\sim 30^\circ\) step is driven by ATP binding: because of friction in \(V_o\), \(V_oV_1\) works in a half-engaged clutch mode where \(120^\circ\) rotation in \(V_1\) results in \(\sim 30^\circ\) rotation in \(V_o\).

We also noticed that, during a long dwell, momentary excursions to a neighbouring dwell position took place in either direction, mostly forward. In Figure 4a and Supplementary Figure S2, we indicate conspicuous excursions (amplitude \(> 20^\circ\) and duration \(> 20\) ms) with black arrowheads, counting 49 forward (closed arrowheads) and ten backward (open) ones in the total of 17 revolutions. The basically rectangular time courses seen in the expanded insets indicate metastable nature of the neighbouring dwell positions, consistent with bumps of structural origin as with the \(V_o\)-c and \(V_o\)-a interaction.

**Discussion**

We have characterized the ATP-driven rotation of both \(V_1\) and \(V_oV_1\) under the conditions where the viscous drag between the probe and medium is negligible. For \(V_1\), the major results are that it pauses every \(120^\circ\) at all [ATP] (Fig. 2), implying that the pauses occur at ATP-waiting angles, and that at least two reactions other than ATP-binding limit each dwell. No dwells at other positions are resolved, at the resolution of \(0.1^\circ\), in contrast to \(F_1\) that shows millisecond dwells at \(\sim 80^\circ\) past ATP-waiting angles.

The previous study using a mutated \(V_1\) and a slowly hydrolyzed ATP analogue suggested that hydrolysis in \(V_1\) occurs at \(0^\circ\).
(ATP-waiting angle), as opposed to the 80° hydrolysis in F₁, but absence of an 80° reaction(s) could not be demonstrated. In F₁, another reaction, Pi release, takes place at ~80°, contributing to the millisecond ~80° dwells that are resolved even at saturating [ATP] if the temporal resolution is sufficiently high. By contrast, the present results show that catalytic events in V₃, at least those that take longer than a submillisecond, all occur at the ATP-binding position. At least two events other than ATP binding occur at this position, one likely to be ATP hydrolysis and the other phosphate or ADP release (or both combined). Together, it is safe to conclude that the canonical “80° and 40° scheme” for F₁ does not apply to V₃. V₃V₅ shows significantly different rotation behaviours from that of V₁. V₃V₅ rotated an order of magnitude slower. V₃V₅ did not show clear 120° steps as observed in V₃, and instead exhibited short pauses separated by ~30°. We could not judge whether the [ATP] dependence of the rotation speed of V₃V₅ follows simple Michaelis–Menten kinetics because of the large scatters in the data (Fig. 1c). At all [ATP], the rotary speed of V₃V₅ was significantly lower than that of V₃. The bumps introduced by the V₃ addition are high, such that passage through any of the ~30° bumps is of structural origin. Presumably, the resulting ATP-waiting angle, which must pose an energy valley until the next ATP binds, the motor would wait for ATP on either side of a bump, resulting in more than three ATP-waiting angles.

The slow stepwise rotation of V₃V₅ observed here is at odds with our previous observation with a duplex of 220-nm beads on the same upside down system (the A subunits were mutated to render the enzyme less prone to MgADP inhibition): the average rate of rotation was ~10 r.p.s. at saturating [ATP], and the molecules basically showed 120° stepwise rotation at low [ATP]. Defective interaction in the V₃ domain could explain the discrepancy, although we are not sure if this was really the case.

The ~30° steps that we resolve relatively clearly in the presence of Triton are commensurate with the periodicity of the V₃ rotor ring in T. thermophila V-ATPase. It is highly likely that dwells result from specific interaction between a V₃-c subunit in the ring and the V₃-a subunit in the stator. When V₃V₅ works as an ATP-driven proton pump in a membrane, proton translocation occurs at the interface between V₃-c and V₃-a. It is possible that protons were also translocated in our experiment with detergent-solubilized V₃V₅ on a glass surface at one proton per ~30° step.

The momentary excursions to a neighbouring ~30° position reinforce the ~30° bumps are of structural origin. Presumably, ATP hydrolysis reaction in the V₃ domain sets up an energy slope that biases the thermal ride over bumps in the anticlockwise direction, and the elastic nature of the rotor helps go over the bumps. Note that this view alone does not account for the strong tendency to rotate back to the original dwelling position after an excursion: the original position is somehow more stable than that of its neighbours. An obvious explanation would be the stable positions being next to an ATP-waiting angle, which must pose an energy valley until the next ATP binds. Indeed, starting angles of the excursions are grossly clustered at ~120° intervals, supporting this interpretation. The 120° intervals, however, were not strictly observed and there were excursions from other angles. These are likely statistical exceptions, but might point to a remote possibility that the rotor–stator interaction is not static and each time it is reconfigured, possibly accompanying protonation/deprotonation, to make the new position stable; until that happens, the previous position remains more stable.

Recently, Düser et al. have reported stepwise c-ring rotation relative to the stator a subunits, equivalent to V₃-a subunit of our V₃V₅, in E. coli F₅F₁, during ATP synthesis using single-molecule fluorescence resonance energy transfer. They estimate the step size as ~36°, which is consistent with the proposed c subunit stoichiometry of 10 in E. coli F₅F₁. In their experiment, protons, presumably each one of them, directly drive the rotation of the F₅ motor, whereas in our experiment the V₃ motor is passively driven by the V₃ motor and proton translocation would be the result and not the cause. The ~30° steps we have observed indicate that passive interactions in the V₃ domain, possibly coupled to proton translocation, check and set the pace of ATP-driven rotation.

**Methods**

Proteins. The his-tagged V₃ (V₃his-ΔC-ΔN-B62L12D-H62R-FQCT-SQ, F) was expressed in E. coli. After disruption of the cells by sonication, the his-tagged V₃ was purified by Ni⁺⁻ affinity column (Qiagen) and RESOURCE Q column (GE healthcare). The purified his-tagged V₃ was biotinylated at two cysteines using 6-[N-(N-maleimidemethyl)-N-piperazinyl]amide]hexyl-d-biotinamide (Dojindo). The V₃, for rotation assay was obtained by reconstitution of the V₃ containing a His₃ tag in each V₃-c subunit and the AviTag V₅. The bound ADP in V₃ or V₃V₅ was partially removed by successive EDTA–heat treatments.

**Observation of rotation of 40-nm gold beads.** Streptavidin-coated 40-nm gold beads and Ni⁺⁻-NTA-coated glass cover were prepared. A flow cell (5–10 μl) was made of two coverslips: a Ni⁺⁻-NTA-coated bottom one (24×36 mm) and an untreated top one (24×24 mm) separated by two spacers of 50 μm thickness. The biotinylated V₃ or Avitagged V₃V₅ (1–5 nM) in buffer A (50 mM Hepes-KOH, pH 8.0, 100 mM KCl, with 0.05% (v/v) DDM only for V₃V₅) was applied to the flow cell and incubated for a few minutes. Unbound V₃ or V₃V₅ was washed out with 20 μl of buffer A more than three times. Then, 20 μl of buffer A with 10 μg/ml BSA was infused to the flow cell and incubated for ~30 s to prevent nonspecific binding. The BSA solution in the chamber was washed out with 20 μl of buffer A more than five times. Then, buffer A containing streptavidin-coated 40-nm beads (10⁻⁴–10⁻⁵ particles per ml) were infused into the flow cell and incubated for a few min. Unbound gold beads were washed out with 20 μl of buffer A more than five times. After incubation of 80 μl of buffer A containing Mg-ATP at the indicated concentration, 2 mM MgCl₂, 2.5 mM phosphoenol pyruvate and 0.5 mg ml⁻¹ pyruvate kinase, bead rotation was observed at 23 °C by laser dark-field microscope on an inverted microscope (Olympus IX70) with a stable microscope stage (KS-O, Chukoshikaseisaku), with some modifications (S. Furukie, unpublished): in place of the oblique laser-illumination, the specimen was illuminated along the optical axis with parallel beam (diameter ~10 μm, power <10 μW), by collimating a laser beam (Millenium IIs, Spectra Physics) with an objective placed just before the specimen. After the specimen was illuminated, the transmitted light was let out through a pinhole at the centre of a mirror while the mirror deflected the scattered light to form a dark-field image of the beads. Images were captured with a high-speed CMOS camera (FASTCAM-DJV, Photron) at 250 to 8,000 frames per s as an 8-bit AVI file. Centroid of bead images was calculated.

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Author contributions
S.F. and K.Y. performed the experiments. M.N. and H.N. performed the sample preparation. S.F., K.K. and K.A. analysed the data. K.Y. designed the study. K.K., K.Y. and S.F. wrote the paper.

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