Rotary catalysis of bovine mitochondrial F$_1$-ATPase studied by single-molecule experiments

Ryoei Kobayashi, Hiroshi Ueno, Chun-Biu Li, and Hiroyuki Noji

$^*$Department of Applied Chemistry, Graduate School of Engineering, The University of Tokyo, 113-8565 Tokyo, Japan; and $^*$Department of Mathematics, Stockholm University, 106 91 Stockholm, Sweden

Edited by John E. Walker, University of Cambridge, Cambridge, United Kingdom, and approved December 4, 2019 (received for review June 17, 2019)

The reaction scheme of rotary catalysis and the torque generation mechanism of bovine mitochondrial F$_1$ (bMF1) were studied in single-molecule experiments. Under ATP-saturated concentrations, high-speed imaging of a single 40-nm gold bead attached to the $\gamma$ subunit of bMF1 showed 2 types of intervening pauses during the rotation that were discriminated by short dwell and long dwell. Using ATP$\gamma$S as a slowly hydrolyzing ATP derivative as well as using a functional mutant E188D with slowed ATP hydrolysis, the 2 pauses were distinctively identified. Buffer-exchange experiments with a nonhydrolyzable analog (AMP-PNP) revealed that the long dwell corresponds to the catalytic dwell, that is, the waiting state for hydrolysis, while it remains elusive which catalytic state short pause represents. The angular position of catalytic dwell was determined to be at $+80^\circ$ from the ATP-binding angle, mostly consistent with other F$_1$s. The position of short dwell was found at 50 to 60° from catalytic dwell, that is, $+10$ to $20^\circ$ from the ATP-binding angle. This is a distinct difference from human mitochondrial F$_1$, which also shows intervening dwell that probably corresponds to the short dwell of bMF1 at $+65^\circ$ from the binding pause. Furthermore, we conducted “stall-and-release” experiments with a magnetic tweezers to reveal how the binding affinity and hydrolysis equilibrium are modulated by the $\gamma$ rotation. Similar to thermophilic F$_1$, bMF1 showed a strong exponential increase in ATP affinity, while the hydrolysis equilibrium did not change significantly. This indicates that the ATP binding process generates larger torque than the hydrolysis process.

F$_1$-ATPase | bovine mitochondrial F$_1$ | single-molecule analysis | molecular motor

The atomic structures of F$_1$ have been intensively studied by X-ray crystallography since the first report on bovine mitochondrial F$_1$, bMF1, in 1994 (3). The first crystal structure revealed most of the basic structural features of F$_1$, which were repeatedly confirmed in later structural studies on bMF1 and other F$_1$s (4–6). F$_1$ is composed of the $\alpha$$\beta_3$$\gamma$ stator ring and the central rotor complex of the $\gamma e$ in bacterial types and the $\gamma e\delta$ in mammalian types. In the $\alpha$$\beta_3$$\gamma$ stator ring, the $\alpha$ and the $\beta$ subunits are arranged alternately. The catalytic sites reside on one side of the $\alpha$ interface, while the other side of the $\alpha$ interface binds to ATP; however, it is catalytically impotent and thereby termed the non-catalytic site. The catalytic residues are mostly located on the $\beta$ subunit, except for the catalytically critical arginine residue termed the “arginine finger” on the $\alpha$ subunit (3, 7–9). Among the 3 $\beta$ subunits, 2 $\beta$ subunits bind to nucleotides: one $\beta$ binds to the ATP analog AMP-PNP and the other binds to ADP (later revealed to also bind to azide (5)). These $\beta$ subunits, termed $\beta_T$ and $\beta_D$, respectively, adopt so-called closed conformation, in which the C-terminal helical domain rotates inwardly to the rotor $\gamma$ subunit. The third subunit, $P_{\text{empty}}$, has no bound nucleotide and adopts an open conformation, swinging the C-terminal domain outwardly. From the structural features, it has been proposed that ATP binding triggers the open-to-closed conformational transition of the $\beta$ subunit, which is a major power-stroking motion. The conformational transition of the $\beta$ subunit was later visualized using the single-fluorescence polarization technique (10), Förster resonance energy transfer (11), and high-speed atomic force microscopy (12).

Significance

The gold-standard model for structural analysis of F$_1$-ATPase has been bovine mitochondrial F$_1$ (bMF1), but its rotational dynamics remain elusive. This study analyzes rotational characteristics of bMF1. bMF1 showed 3 distinct dwells in rotation, “binding dwell,” “catalytic dwell,” and “short dwell,” in each 120° step of rotation. While the positions of binding and catalytic dwell are similar to those of human mitochondrial F$_1$ (hMF1), bMF1 shows short dwell at a distinctively different position from the corresponding dwell of hMF1, implying variety in the timing of the putative reaction at short dwell, phosphate release or ADP release. Single-molecule manipulation experiments revealed that the affinity change of ATP is a major torque-generating step.

Author contributions: R.K., H.U., and H.N. designed research; R.K. and H.U. performed research; R.K. and C.-B.L. analyzed data; and R.K., H.U., C.-B.L., and H.N. wrote the paper. The authors declare no competing interest.

This article is a PNAS Direct Submission.

This open access article is distributed under the Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

Data deposition: Data reported in this paper have been deposited in the Dryad Digital Repository (https://doi.org/10.5061/dryad.pg44pkj).

*To whom correspondence may be addressed. Email: hnoji@appchem.t.u-tokyo.ac.jp.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1909407117[1/-/DCSupplemental].

First Published January 2, 2020.
Later crystallographic studies showed that βDP can bind to AMP-PNP (4) or a transition-state analog (13), while βTP predominantly binds to AMP-PNP unless AMP-PNP is omitted from the crystallization medium. Therefore, βDP is thought to represent the catalytically active conformational state for cleavage of bound ATP. This contention is supported by several studies (7, 14, 15). In a typical rotation assay, the αβγ ring is immobilized on the glass surface, and a probe for rotational imaging is attached onto the protruding component of the γ subunit. For high-speed imaging of rotation practically not affected with viscous friction against a rotating probe, nanoprobes are used such as 40-nm gold colloid (19, 20) or nanorods (21). For the torque measurement or rotation manipulation, 100- to 500-nm-diameter polystyrene beads or magnetic beads are used. Single-molecule rotation analysis has elucidated the basic features of F1 rotation and the chemomechanical coupling scheme of rotation.

Among F1’s characterized by the rotation assay thus far, thermophilic Bacillus PS3 (TF1) is the best characterized due to its high conformational stability and clear stepping behaviors. The rotation analysis of TF1 established the reference reaction scheme, although some variations for different F1’s have been observed as described later. The unitary step size of the rotation is 120°, each coupled with a single turnover of ATP hydrolysis, reflecting the pseudo 3-fold symmetry of the structure. The 120° step rotation is divided into 2 substeps of 80° and 40° (19), each intervened by ATP-waiting dwell (binding dwell) or catalysis-waiting dwell (catalytic dwell), respectively (22). During binding dwell, another β releases ADP (23, 24), and P release is suggested to occur during catalytic dwell (24, 25). Considering that each β exerts a single turnover of ATP hydrolysis upon a single turn of the rotor, and the reaction phase is different by 120° among 3 βs, the reaction scheme is proposed as shown in Fig. 1D (25), although another scheme has also been proposed (26).

Recent statistical analysis (27) revealed that TF1 makes a small rotation upon catalysis during catalytic dwell that is too small to be detected in conventional image analysis, suggesting that the catalytic dwell is split into hydrolysis and P release dwells. The split of the catalytic dwell was also proposed in studies on the rotation of yeast mitochondrial F1 (28) and human mitochondrial F1 (hMF1) (29). The work on hMF1 showed that the 120° rotation was resolved into 3 substeps: 65°, 25°, and 30°. Each step was initiated by ATP binding, presumably P release and hydrolysis. Therefore, the dwells before the 65°, 25°, and 30° substeps are referred to as the binding dwell, P release dwell, and catalytic dwell, respectively. The reaction scheme of hMF1 was proposed as shown in Fig. 1B. Due to the close sequence homology of hMF1 and bMF1 [-99% in the α and β subunits and ~95% in the γ subunit (16)], it is expected that the reported rotation behavior of hMF1 is similar to bMF1. From the viewpoint of the structure–function relationship of F1, the correlation between dwells and conformational states found in crystal structures is important to determine. Assays with inhibitors suggest that the P release dwell corresponds to the state found in the majority of bMF1 crystal structures, including the first crystal structure (3), ground-state structure (4), and thiophosphate-bound structure (16). However, there are still differences in amino acid sequences between hMF1 and bMF1, and the investigation of the exact correlation between rotary dynamics and atomic structure of F1 requires a rotation assay with F1 from the same species used in the crystal structure analysis. Although a preliminary study on bMF1 was reported (30), basic characteristics of bMF1 have not been analyzed.

A single-molecule rotation assay of F1 enabled not only detailed kinetic analysis of stepping rotation but also manipulation experiments when combined with a magnetic tweezers system. The manipulation experiment of TF1 was first conducted for the direct demonstration of ATP synthesis upon the reverse rotation of the γ subunit (31, 32). After that, it has become a major focus how F1 modulates the rate and equilibrium constants of elementary reaction steps: binding, hydrolysis, and product releases. To assess this issue, a stall-and-release experiment was conducted to determine the rate constant and equilibrium constant of ATP binding or hydrolysis of ATP bound on the catalytic site as a function of rotary angle that formed a basis for following theoretical studies (17, 33–38). Significantly larger angle dependence of ATP binding than hydrolysis revealed that TF1 generates larger torque in the ATP binding step than in the hydrolysis step. However, the stall-and-release experiments have been conducted only for TF1 (39) and the generality of these findings remains unclear.

In this study, we investigated the γ rotation of bMF1 and found several differences in rotation dynamics between bMF1 and hMF1, from which we propose the reaction scheme for bMF1 shown in Fig. 1C. Based on the reaction scheme, we also analyzed the angle dependence of ATP affinity change as well as the modulation of the equilibrium constant of ATP hydrolysis by

Fig. 1. Chemomechanical coupling rotation schemes of TF1 (A), hMF1 (B), and bMF1 (C). Each circle and arrow represents the catalytic state of the β subunit and the angular positions of the γ subunit, respectively. 0° is defined as the position of γ subunit where a β subunit (orange) binds to ATP. The asterisks following “ATP*” represent the catalytically active state to undergo hydrolysis of bound ATP.
conducting a stall-and-release experiment. The single-molecule manipulation analysis revealed the general features of angle dependence of binding and catalysis are well-conserved across the species, suggesting that the torque generation mechanism is common among F1s, although the stepping behaviors have some variations.

**Results**

**Construct of bMF1 for Rotation Assay.** Recombinant bovine mitochondrial F1 composed of α, β, γ, δ, and ε subunits was coexpressed with assembly factors, AF1 and AF2, and purified according to a previous report (30) with slight modifications (Materials and Methods). Two cysteines were introduced in the protruding part of the γ subunit at A99 and S191. They were specifically biotinylated to attach 40-nm gold nanoparticles or magnetic beads with ~200-nm diameter as an optical probe. For immobilization, 9 histidine residues (His-tag) were introduced at the N terminus of the β subunit. The resultant bMF1 (His-tag, γA99C, γS191C) showed normal ATPase as shown below and was referred to as the wild type. In order to ensure the complex stability of bMF1, the bMF1 solution was diluted down to 10 nM and concentrated with a 100-kDa ultrafiltration filter to remove dissociated small subunits such as the δ and ε subunits. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed the genuine subunit composition was retained after dilution, showing the complex of bMF1 is stable at the nanomolar condition where the single-molecule rotation assay is to be conducted (SI Appendix, Fig. S1).

**ATP-Driven Rotation.** To observe genuine rotation of bMF1, 40-nm gold colloid attached to the γ subunit of bMF1 was observed under various [ATP]s at 23 ± 2 °C (Fig. 2A) with a dark-field microscope (20). Images were recorded at 125 to 45,000 frames per second (fps) (22 to 8,000 μs per frame), depending on [ATP]. Red data points in Fig. 2B show the Michaelis–Menten curve of the rotation rate, in which the maximum rotation rate (V\text{max}) and Michaelis constant (K\text{m}) are 707 revolutions per second (rps) and 77 μM, respectively. The maximum rotation rate of 707 rps was comparable with that of human mitochondrial F1 (hMF1), 741 rps, and remarkably faster than those of bacterial F1s: 129 rps for thermophilic *Bacillus* PS3 (TF1) (19) and 449 rps for *E. coli* F1 (EF1) (40). Considering the coupling ratio of 3 ATPs per turn, the maximum rotation rate corresponded to the ATP hydrolysis rate of 2,121 per s. We also measured the ATP hydrolysis rate of bMF1 in solution with ATP-regeneration system (blue data points in Fig. 2B) and determined V\text{ATPase} and K\text{ATPase} as 1,037 per s and 218 μM, respectively. The ATPase rates measured in solution were lower than the estimated catalytic rate from the rotation rate at all [ATP]s. Significantly lower catalytic rates than expected from the rotation rate were often reported in other F1s (19, 28, 41, 42). This is due to ADP inhibition, which is an inactive state of F1 transiently halting catalysis and rotation. The ADP-inhibited state lowers the time-averaged rotation rate in the single-molecule rotation assay and ATP hydrolytic activity, determined as an ensemble average of molecules in solution.

Fig. 2. ATP-driven rotation of bMF1. (A) A schematic image of the single-molecule rotation assay of bMF1. The αβδ-ring is immobilized on a glass surface, and a detection probe is attached to the γ subunit via biotin–streptavidin interaction. (B) [ATP] versus the rate of rotation (red) or ATPase/3 (blue). The mean value and the SD for each data point are shown with circles and error bars, respectively (n = 20 to 25 for measurement of rotation rate, n = 3 for measurement of ATPase). Solid lines represent Michaelis–Menten fittings; V\text{max} = 707 ± 5 rps, K\text{m} = 77 ± 2 μM for rotation rate; V\text{ATPase} = 346 ± 11 μM/s, k\text{ATPase} = 218 ± 26 μM for ATPase/3 (fitted parameter ± fitting error). (C and D) x–y plot, angular histogram, and time course of rotation found at 300 nM ATP (C) and at 3 mM ATP (D). The recording rate was 500 and 45,000 fps, respectively.

**ATP\text{γS}-Driven Rotation.** To resolve the rotation and dwells more clearly, we observed rotation in the presence of ATP\text{γS}, which is a slowly hydrolyzable ATP analog. The previous rotation assays showed that ATP\text{γS} slows the ATP hydrolysis on TF1 (22) and also presumably release of thiophosphate on hMF1 (29). Rotation rates of bMF1 were determined at various [ATP\text{γS}]s to draw the Michaelis–Menten curve (SI Appendix, Fig. S3A). V\text{max} and K\text{m} were determined to be 20.3 rps and 2.2 μM, respectively. As expected, V\text{max} was largely suppressed at about 35 times slower than that of the ATP-driven rotation. The binding constant of ATP\text{γS}, k\text{ATP\text{γS}}, was estimated from 3 × V\text{max}/K\text{m} to be 3.0 × 10^{-7} M^{-1} s^{-1}, which was almost identical to k\text{ATP}.

At high [ATP\text{γS}]s over K\text{m}, bMF1 showed distinct pauses separated by 120° steps, corresponding to 3 dense clusters in the x–y
plot of rotation (Fig. 3A, Inset). A closer look at the time courses showed additional short pauses during a 120° step (Fig. 3A), showing that bMF1 makes 2 types of dwells, hereafter referred to as long dwell (blue in Fig. 3A) and short (orange) dwell. (Inset) x–y plot. (B) Histograms for dwell position analysis. (Upper) The conventional histogram of angle positions from all data points from the time course. (Lower) The histogram of angle positions of dwells detected by CP analysis. (C) Dwell-time analysis of long and short dwells at 1 mM ATP (n = 6). Values are fitted parameter ± fitting error. (D) Time course of rotation at 1 μM ATP recorded at 1,000 fps. (Inset) x–y plot. (E) Histograms of angle positions. (Upper) The conventional histogram from all data points. (Lower) The histogram of the angle positions of long dwell from binding dwell (A), defined in the upper panel. Values are mean ± SD (n = 45, 15 molecules). (F) Dwell time analysis of long and binding dwell at 1 μM (n = 4). Values are fitted parameter ± fitting error.

Fig. 3. ATPγS-driven rotation of bMF1. From Michaelis–Menten fitting, Vₘₐₓ and Kₘ were estimated to be 20.3 ± 1.0 rps and 2.2 ± 0.5 μM (fitted parameter ± fitting error), respectively (SI Appendix, Fig. S3A). (A) Time course of rotation at 1 mM ATP recorded at 10,000 fps. CP analysis detects long (blue) and short (orange) dwell. (Inset) x–y plot. (B) Histograms for dwell position analysis. (Upper) The conventional histogram of angle positions from all data points from the time course. (Lower) The histogram of angle positions of dwells detected by CP analysis. (C) Dwell-time analysis of long and short dwells at 1 mM ATP. Values are fitted parameter ± fitting error. (D) Time course of rotation at 1 μM ATP recorded at 1,000 fps. (Inset) x–y plot. (E) Histograms of angle positions. (Upper) The conventional histogram from all data points. (Lower) The histogram of the angle positions of long dwell from binding dwell (A), defined in the upper panel. Values are mean ± SD (n = 45, 15 molecules). (F) Dwell time analysis of long and binding dwell at 1 μM ATP. Values are fitted parameter ± fitting error.
of long dwell was constant at 11 to 13 ms, consistent with the abovementioned value (14 ms). The length of binding dwell depended on [ATP] as expected, giving the rate constants of ATP binding ($k_{\text{on}}^{\text{ATP}}$), $2.9 \times 10^7$ M$^{-1}$s$^{-1}$, well consistent with $k_{\text{on}}^{\text{ATP}}$ (3.0 $\times 10^7$ M$^{-1}$s$^{-1}$) determined from the Michaelis–Menten analysis. In the rotation assay at low [ATP]s, a distinctively long pause attributable to ADP inhibition was again observed at the angle of long dwell (SI Appendix, Fig. S7 D–F), suggesting that long dwell corresponds to catalytic dwell. Short dwells were not detected throughout the rotation assay with low [ATP]s, probably because short dwells of ATP rotation are too short to be detected with the recoding frame rate (1 ms per frame).

**Rotation of bMF1(βE188D).** To confirm that long dwell is +80° from binding angle, we tested a mutant F1 (βE188D) in the rotation assay. This glutamic acid is highly conserved in primary sequences among all F1s. In crystal structures, this glutamic acid interacts with the γ phosphate via a coordinated water molecule. A quantum mechanics/molecular mechanics study revealed that this glutamic acid accelerated the ATP cleavage reaction, promoting the rate-limiting proton relay (7). When βE190 of TF1 (equivalent to βE188 of bMF1) was replaced with aspartic acid (D), the rate constant of ATP cleavage step was greatly slowed over 320-fold (22).

**SI Appendix, Fig. S3B** shows the rotation rates of bMF1(βE188D) at various [ATP]. The data points were well fitted with the Michaelis–Menten curve with $V_{\text{max}}$ and $K_m$ of 1.2 rps and 1.2 μM, respectively. As expected, $V_{\text{max}}$ was largely suppressed, which was about 600 times lower than that of wild-type bMF1. The ATP binding constant, $k_{\text{on}}^{\text{ATP}}$, was estimated to be $3.2 \times 10^6$ M$^{-1}$s$^{-1}$, which was 10 times lower than $k_{\text{on}}^{\text{ATP}}$ of the wild type.

The stepping behaviors of bMF1(βE188D) were well consistent with those found in the ATPγS-driven rotation of the wildtype bMF1. At high [ATP], we again observed long and short dwells during 120° steps (Fig. 4 A–C). The dwell position histogram based on CP analysis showed that short dwell was located at 48° between long dwells (Fig. 4 B, Lower and SI Appendix, Fig. S5B). Histograms of durations of long and short dwells showed a single exponential decay function with time constants of 220 to 280 ms and 6 to 12 ms, respectively (Fig. 4F and SI Appendix, Fig. S8). At low [ATP], bMF1(βE188D) showed that long dwell occurred at +84° from binding dwell (Fig. 4 D–F). Dwell-time histograms determined the time constants of long dwell to be 180 to 230 ms and binding dwell to be 251 ms for 1 μM ATP, 75 ms for 3 μM ATP, and 27 ms for 10 μM ATP. The rate constant of ATP binding was determined to be $4.3 \times 10^6$ M$^{-1}$s$^{-1}$, which is mostly consistent with that estimated from the abovementioned Michaelis–Menten analysis. Thus, rotation assay of ATPγS and bMF1(βE188D) confirmed that bMF1 makes long dwell at +80° from binding dwell and short dwell at +50° to 60° from long dwell, that is, +10° to 20° from binding dwell.

**Stall by AMP-PNP.** The rotation assays with ATPγS or bMF1(βE188D) showed that long dwell occurred at +80° from binding angle. In addition, the coincidence of long-dwell angle with the angle of the ADP-inhibited state suggested that long dwell represented catalytic dwell where F1 executes the cleavage reaction. To further confirm these findings, we investigated the pause positions of bMF1 by blocking rotation with AMP-PNP, a nonhydrolyzable ATP analog to stall rotation at the angle of cleavage.

The rotation of the γ subunit of bMF1 was visualized with magnetic beads as a rotation probe because AMP-PNP–inhibited bMF1 could be reactivatable with magnetic tweezers, which

---

Fig. 4. ATP-driven rotation of bMF1(βE188D). From Michaelis–Menten fitting, $V_{\text{max}}$ and $K_m$ were estimated to be 1.24 ± 0.03 rps and 1.15 ± 0.13 μM (fitted parameter ± fitting error), respectively (SI Appendix, Fig. S3B). (A) Time course of rotation at 1 mM ATP recorded at 2,000 fps. CP analysis detects long (blue) and short (orange) dwell. (Inset) $x$–$y$ plot. (B) Histograms for dwell position analysis. (Upper) The conventional histogram of angle positions from all data points of the wild type. (Lower) The histogram of angle positions of dwells detected by CP analysis. (C) Dwell-time analysis of long and short dwells at 1 μM (n = 5). Values are fitted parameter ± fitting error. (D) Time course of rotation at 1 μM ATP recorded at 500 fps. (Inset) $x$–$y$ plot. (E) Histograms of angle positions. (Upper) The conventional histogram from all data points. (Lower) The histogram of the angle positions of long dwell from binding dwell (a0), defined in the upper panel. Values are mean ± SD (n = 24, 8 molecules). (F) Dwell time analysis of long and binding dwell at 1 μM (n = 5). Values are fitted parameter ± fitting error.

Kobayashi et al.
allowed repeated experiments for the molecules. Rotation was observed at 100 nM ATP, where clear pauses at the ATP binding dwell were observed at 3 positions (Fig. 5A). Recording rate was 30 fps. After confirming the 3 pauses as binding dwell in a turn, the solution of 100 nM ATP plus 500 nM AMP-PNP was gently introduced into a flow cell to minimize interference of rotation by buffer flow. Typically, molecules stopped rotation within 3 min after buffer exchange. Once lapsed into AMP-PNP inhibition, bMF₁ molecules never resumed rotation unless forcibly rotated over +360° with magnetic tweezers. It should be noted that ADP inhibition is rarely observed at 100 nM ATP (SI Appendix, Fig. S9A). The mean duration time of ADP inhibition observed at 2 nM ATP was ~25 s, which is evidently shorter than the duration time of AMP-PNP inhibition, which is over 4 min (SI Appendix, Fig. S9B). In this experiment, after confirming that the pause lasted over 4 min, we defined the pause as an AMP-PNP stall. The pause angle of AMP-PNP inhibition was evidently different from the angles for binding dwell. The angular distance of the AMP-PNP stall from the nearest binding dwell on the left side was +76° (Fig. 5B), which is consistent with the position of long dwell. Thus, the angular position of ATP hydrolysis was confirmed at +80° from the angle of binding dwell, which is the same position as long dwell.

**Angle-Dependent Modulation of Reaction Rates and Equilibriums.** Identification of rotation angles for ATP binding and ATP hydrolysis is fundamental to elucidate how F₁ interconverts chemical energy of ATP hydrolysis into mechanical rotation. One of the most distinctive features that discriminate F₁ from other molecular motor proteins is that F₁ largely modulates chemical equilibriums of catalytic reaction steps depending on rotary angle to achieve ATP synthesis upon reversed rotation (31, 32). In a previous study (39), we established a "stall-and-release" experiment with magnetic tweezers, which allows for measurements of both constant and equilibrium constant of ATP binding or ATP hydrolysis, as a function of rotary angle. This experiment revealed quantitative aspects of the "binding-change mechanism." It was shown that TF₁ exponentially increased affinity to ATP by 235-fold upon rotation by 60°, while it increased the equilibrium constant of ATP hydrolysis/synthesis only by 3-fold. From these results, the contribution of affinity change for torque generation was estimated to be 21 to 54 pN nm, while that of hydrolysis was only 4 to 17 pN nm (2).

To investigate the angle-dependent modulation of affinity change and hydrolysis equilibrium of bMF₁, we conducted a "stall-and-release" experiment. The experimental procedure was as follows. Rotation was observed under conditions where the target reaction, ATP binding or hydrolysis, was the rate-limiting step in the overall rotation rate. For ATP-binding, [ATP] was lowered to 100 nM, in which the mean waiting time for ATP binding was 0.9 s, while other reaction steps should be completed within 1 ms. For ATP-hydrolysis measurement, the intrinsic time constant for ATP hydrolysis, less than 0.5 ms, is too short for manipulation. Therefore, we observed rotation of bMF₁(ΔE188D) in the presence of ATPγS, in which the catalytic dwell was prolonged to 4.0 s. When F₁ paused to wait for the target reaction to occur, we stalled the rotation of bMF₁ at the targeted angle with magnetic tweezers. After the set time period lapsed, bMF₁ was released from the magnetic tweezers. Principally, bMF₁ showed 2 behaviors: returning to the original waiting angle or stepping to the next waiting angle. Returning indicated that F₁ had not executed the waiting reaction during the stall. We refer to that case as "OFF." Stepping indicated that F₁ had already executed the reaction and torque had been generated on the magnetic beads. That is referred to as an "ON" case. By determining the probability of ON cases (P_{ON}), we measured the probability of reaction as a function of rotary angle.

![Fig. 5](Fig. 5) Pause positions stalled by AMP-PNP. (A) Experimental procedure. After observing binding dwell at 100 nM [ATP] (Left), 500 nM AMP-PNP and 100 nM ATP was introduced into the reaction mixture (Right). Blue data points represent the positions when rotation was blocked with AMP-PNP. (B) The angular distance (Δθ) of AMP-PNP inhibition from the nearest binding dwell (pink) (n = 27). Values are mean ± SD.
Temperature Dependence of Maximum Rotation Rate. All of the abovementioned experiments were conducted at 23 ± 2 °C. In order to confirm that the reaction scheme is essentially not different in a wide range of temperatures including those near the physiological temperature of bMF₁, the rotation rate at the ATP-saturated condition (1 mM) was observed at temperatures ranging from 17 °C to 35.5 °C. The rotation of bMF₁(βE188D) or with ATP₇S was also analyzed. The resulting Arrhenius plot showed the clear linearity in the range of temperature examined in all conditions (Fig. 7), indicating that the catalytic dwell is the kinetically bottlenecked reaction determining the overall reaction rate from 17 °C to 35.5 °C. This suggests that the reaction scheme found at room temperature is valid at a wide range of temperatures.

The temperature dependence of the rotation rate of bMF₁ is essentially the same as those of TF₁. The Q₁₀ factor of the rotation rate of bMF₁ (1.3 to 1.9) is almost the same as the Q₁₀ factors of ATP hydrolysis (1.9) and Pi release (1.6) of TF₁ (47). As a result, the activation free energy, calculated from ΔG = ΔH − TΔS, was 56 to 72 kJ/mol, also well consistent with the values obtained previously for TF₁ (47), and EF₁ (48, 49). These results indicate that the transition states of the catalytic dwell of bMF₁ are the same as those for TF₁.

Discussion

Catalytic Event in Long Dwell and Binding Dwell. This study investigated the fundamental features of bMF₁ rotation under 3 conditions: in the presence of ATP, in the presence of ATP₇S, and, by using a mutant F₁, bMF₁(βE188D). The latter 2 conditions were employed to slow down the cleavage step for resolving rotation into clear substeps. In all conditions, we observed long and short dwells under substrate-saturated conditions and long dwell and binding dwell in the region below K₉₉₉. Although the short dwell in the ATP-driven rotation was too short for analysis, the rotation assays with ATP₇S or with bMF₁(βE188D) showed coincident angle assignments for short and long dwells: When the angular position for binding dwell was defined as 0°, long dwell was at +80° and short dwell at +10° to 20°.

ATP₇S and the βE188D mutation are known to specifically slow down the hydrolysis step, although several studies suggested that the release step of thiophosphate or phosphate was also
short pause of bMF1 remains to be elucidated. As the "1st dwell" position is the position of the pause between binding dwell and catalytic dwell: +65° from binding dwell in hMF1 and +10 to 20° in bMF1. As a result, substep size is also different: 65°, 25°, and 30° substeps for hMF1, while bMF1 makes 10 to 20°, 50 to 60°, and 40° substeps. Note while experimentally this had not been determined, careful data analysis based on a data-mining method found that TF1 also makes small substeps during catalytic dwell (27).

There are also some distinctive differences in the kinetics of rotation between bMF1 and hMF1, although overall kinetic parameters such as $V_{\text{max}}$ and $K_m$ are mostly the same. In the rotation of bMF1, the duration time of catalytic dwell was always longer than short dwell, although the reverse is true in the rotation of hMF1: The 1st dwell was longer than catalytic dwell in hMF1. The source of these differences found in substeps and kinetics between bMF1 and hMF1 is unknown. The amino acid sequences are overall quite similar between bMF1 and hMF1. The α and β subunits share mostly identical sequences (99%), whereas the γ subunit shows relatively lower homology, 93% (16). Therefore, the most probable explanation is that the structural difference of the γ subunit causes differences in the kinetics and stepping behavior.

Correlation with Crystal Structures. The present work reveals that bMF1 has at least 3 distinctive conformational states: binding dwell state, short dwell state, and catalytic dwell state. Obviously, the catalytic dwell state principally corresponds to the bMF1 crystal structures. It has been suggested that the current resolved crystal structures of bMF1 represent the catalytically active state, as supported by several studies, including the crystal structure with transient state analog, beryllium fluoride, and theoretical analysis (13). However, variations among crystal structures have been reported to date. They differ in bound nucleotides, inhibitors, inorganic ligands, and conformational states of subunits. Particularly, there is variety in rotational orientation of the γ subunit in crystal structures. Although it should depend on methods for structural alignment and analysis, the maximum difference in the angular orientation of the γ subunit has been reported to be over 30°. Particularly, when $\beta_b$ binds to thio- phosphate the γ subunit is positioned at −30° (16) from that found in crystal structures with mitochondrial inhibitor proteins (50, 51). This feature is almost consistent with the rotation scheme proposed for hMF1, where phosphate release triggers rotation from the phosphate-releasing state at +65° from the binding site to the hydrolysis waiting state at +90°. However, the actual bMF1 does not show a dwell at around -30° from the catalytic dwell position. Although it is possible to assign short pause as the phosphate-releasing state, the angular distance between short and catalytic dwells, 50 to 60°, is too large. Thus, it is still unclear which crystal structure(s) exactly correspond to the catalytic dwell state.

Angle Dependence of Catalytic Power of bMF1. One of the most remarkable features of F$_1$-ATPase that discriminates it from other molecular motors is that F$_1$-ATPase can reverse the catalytic reaction to synthesize ATP from ADP and phosphate when the rotation is reversed. This means that all catalytic reaction steps should be modulated with the rotation angle. To investigate this characteristic feature in detail, we developed a “stall-and-release" experiment. In the previous study on TF1, we found that the affinity to ATP was exponentially increased with forward rotation, while the equilibrium constant of hydrolysis was only slightly increased (39). The estimated free energy release upon...
the progressive affinity change of ATP was 21 to 54 pN nm. In contrast, upon equilibrium shift of hydrolysis, it was only 4 to 17 pN nm, where the lower-limit values represent the estimation directly from the angle dependence and the upper-limit values represent the corrected values with consideration of the torsional elasticity of the γ subunit (52). In this study, we confirmed the generality of the angle-dependent catalysis modulation: bMF₂ progressively and exponentially increased affinity to ATP while the hydrolysis equilibrium shifted slightly upon forward rotation.

The free energy change upon rotation was 17 to 49 pN nm for binding change and 7 to 16 pN nm for equilibrium shift of hydrolysis. Although the estimated free energy change was lower in both reactions compared to TF₁, similar angle dependence was confirmed. Considering that thermophilic V₁-ATPase (TV₁) showed much weaker angle dependence of binding affinity (53), the strong angle-dependent affinity change is a conserved characteristic feature of F₁-ATPase among species.

Materials and Methods

Preparation of bMF₂. The bMF₂ plasmid, a gift from T. Suzuki, Tokyo Institute of Technology, Tokyo, was introduced into the Δε₁ deficient E. coli strain BL21. The recombinant E. coli strain was cultured in Super Broth medium containing 100 μg/mL carbenicillin and 25 μg/mL tetracycline for 24 h at 27 °C. To avoid dissociation of the bMF₂ complex, purification was performed throughout at room temperature, 23 ± 2 °C. Harvested cells were suspended in 50 mM imidazole-HCl (pH 7.2) containing wash buffer A (40 mM potassium phosphate buffer, pH 7.5, 100 mM K₂SO₄, 10% glycerol, and 0.2 mM ATP), disrupted using an ultrasound disintegrator, and subjected to ultracentrifugation (81,000 g, 20 min, 25 °C). The supernatant was introduced into Ni-Sepharose FF resin (GE Healthcare). After binding of bMF₂ to the resin, it was washed with more than 10 volumes of wash buffers. A and B (100 mM imidazole containing wash buffer A) to remove contaminating proteins. Subsequently, bMF₂ was eluted with 50 mL of elution buffer (400 mM imidazole containing wash buffer A). The eluted fraction was applied to a gel-filtration column (Superdex 200; GE Healthcare) that had been previously equilibrated with gel-filtration buffer (40 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.5 mM EDTA, pH 8.0, 10% glycerol, and 0.2 mM ATP). The columns were collected and concentrated using a centrifugal concentrator (30-kDa, Centricon50; Millipore Corp.). The concentration of bMF₁ was determined from bovine serum albumin (BSA) as a standard.

Measurement of ATPase Activity. ATPase activity was measured at 25 °C in 50 mM Hepes-KOH (pH 7.5) containing 50 mM KCl, various concentrations of MgCl₂, and ATP-regenerating system supplemented with 0.2 mM NADH and 0.1 mg/mL lactate dehydrogenase. The ATPase activity was calculated from the maximum slope of the absorbance of NADH during 5 s after the start of measurement.

Rotation Assay. To visualize the rotation of bMF₂, 2 cysteines on a rotor γ subunit (γ99C, γ191C) were biotinylated to attach 40-nm gold nanoparticles or magnetic beads as an optical probe. The bMF₂ rotation assay was performed in the same manner as previously described (25), except for slight modifications. The procedures were as follows. The flow cell was constructed from 2 cover glasses (18 × 18 mm² and 24 × 32 mm²; Matsunami Glass) using double-sided tape as a spacer. The bMF₂ of ~1 nM in the basal buffer was into the flow cell and incubated for 5 to 10 min. After that, unbound bMF₂ molecules were washed out with the basal buffer containing 10 mM BSA. Then, 40-nm gold nanoparticles or magnetic beads were infused and incubated for 5 to 10 min. Unbound beads were washed out with the basal buffer containing indicated concentrations of substrate. The basal buffer for ATP assay contained 50 mM Hepes-KOH (pH 7.5), 50 mM KCl, and various concentrations of MgCl₂. When ATP was used, an ATP-regenerating system (1 mM (S)-3-(3-picolinyl)oxaloacetate and 50 μg/mL pyruvate kinase) was added to the reaction mixture.

In the rotation assays with the 40-nm gold colloid, the rotating colloid particle that was attached to the γ subunit of bMF₂ was observed using a dark-field microscope with a 60x objective lens at the recording rate of 125 to 45,000 fps (FASTCAM-1024PCI; Photron). The localization precision was 1 to 2 nm with signal-to-noise ratio ranging from 60 to 100 (20). For observation of the magnetic beads, a phase-contrast microscope (IX-70 or IX-71; Olympus) with a 100x objective lens at 30 fps (FC300M; Takeux) was used. The rotation assay was performed at 23 ± 2 °C, room temperature, except for 17 °C, 30 °C, and 35.5 °C in the temperature-dependence experiment (Fig. 7). For assay at 17 °C, the microscope room was cooled with an equipped air conditioner, and temperature was monitored with a thermometer attached on the flow cell on the microscopic stage. For assay at 30 °C or 35.5 °C, an objective lens heater (MATRS-7FR, Tokai Hit Corp.) was used. Actual temperature of the sample was monitored the same as in the assay at 17 °C.

Data Analysis. To suppress the effect of focus drifts on analysis, we have corrected data using nonspecific binding molecules on a glass surface before analysis. To avoid undesired fluctuation, the median filter (±2 frames) was applied to the time course in Figs. 3A and 4A. To estimate time constants, the histograms of the dwell time were fitted by a single exponential decay function. Phase angles were determined by fitting the angle distribution with a Gaussian function in Figs. 3E, 4E, and 5B. For the visualization and estimation of short pause between long pauses, CP analysis was applied to the time traces shown in Figs. 3A and 4A, as described in SI Appendix, Supplementary Information Text.

Manipulation with Magnetic Tweezers. Magnetic tweezers were equipped on the microscope, induced by stage magnetic coils, observed by stage magnetic coils, observed by a polarized light microscope (DMI3000B, Leica). The position of magnetic tweezers was controlled by custom software (Celery) (39). In Fig. 6A and B, kinetic parameters were determined by using a single exponential function according to the reversible reaction scheme. The SD of P₀/ P₅₀ in Fig. 6A and B is given as \( \sqrt{P₀(P₀ - P₅₀)} / N \), where N is the number of trials for each experiment.

Data Availability. Data are available in the Dryad Digital Repository (https://doi.org/10.5061/dryad.pg4f4rkj).

ACKNOWLEDGMENTS. We thank T. Suzuki (Tokyo Institute of Technology) for the kind gift of the bMF₂ plasmid; M. Haru, R. Watanabe, and S. Mori (University of Tokyo) for technical support; and all members of the H.N. laboratory for valuable comments. This work was supported in part by Grant-in-Aid for Scientific Research 114600050 (H.N. and T.U.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and Bilateral Joint Research Projects from the Japan Society for the Promotion of Science (to H.N.).

27. T. Suzuki
28. B. C. Steel
31. H. Itoh
32. D. Spetzler, et al.
33. S. Volkán-Kacsó, R. A. Marcus, Theory for rates, equilibrium constants, and Brønsted slopes in F1-ATPase single molecule imaging experiments.
35. S. Volkán-Kacsó, R. A. Marcus, Theory of long binding events in single-molecule imaging and manipulation.
37. S. Mukherjee, A. Warshel, The rotation of the γ-subunit in the rotary-chemical coupling and torque generation of F1-ATPase.
39. S. Mukherjee, A. Warshel, Branched slopes based on single-molecule imaging data help to unveil the chemically coupled rotation in F1-ATPase.
41. K. Nam, J. Pu, M. Karpilus, Trapping the ATP binding state leads to a detailed understanding of the F1-ATPase mechanism.
43. R. Watanabe et al., Mechanical modulation of catalytic power on F1-ATPase.
45. T. Bilyard et al., High-resolution single-molecule characterization of the enzymatic states in Escherichia coli F1-ATPase.
49. D. Spetzler et al., Single molecule measurements of F1-ATPase reveal an interdependence between the power stroke and the dwell duration.
53. T. G. Terentyeva et al., Dynamic disorder in single-enzyme experiments: Facts and artifacts.
55. Y. Hirorono-Hara et al., Pause and rotation of F1-ATPase during catalysis.
57. T. Hishibori, A. Kondo, M. Yoshida, The γ-subunit in chloroplast F1-ATPase can rotate in a unidirectional and counter-clockwise manner.
60. Protein Sci. 23, 1773-1779 (2014).