Rotary catalysis of bovine mitochondrial F1-ATPase studied by single-molecule experiments

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The reaction scheme of rotary catalysis and the torque generation mechanism of bovine mitochondrial F1 (bMF1) were studied in single-molecule experiments. Under ATP-saturated concentrations, high-speed imaging of a single 40-nm gold bead attached to the γ subunit of bMF1 showed 2 types of intervening pauses during the rotation that were discriminated by short dwell and long dwell. Using ATPγS as a slowly hydrolyzing ATP derivative as well as using a functional mutant βE188D 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 +50° to +80° from the ATP-binding angle, mostly consistent with other F1s. The position of short dwell was found at 50° to 60° from catalytic dwell, that is, +10 to 20° from the ATP-binding angle. This is a distinct difference from human mitochondrial F1, which also shows intervening dwell that probably corresponds to the short dwell of bMF1, at +65° from the binding pause. Furthermore, we conducted “stall-and-release” experiments with magnetic tweezers to reveal how the binding affinity and hydrolysis equilibrium are modulated by the γ rotation. Similar to thermophilic F1, 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.

The atomic structures of F1 have been intensively studied by X-ray crystallography since the first report on bovine mitochondrial F1, bMF1, in 1994 (3). The first crystal structure revealed most of the basic structural features of F1, which were repeatedly confirmed in later structural studies on bMF1 and other F1s (4–6). F1 is composed of the αβ3 stator ring and the central rotor complex of the γε in bacterial types and the γε sub in mammalian types. In the αβ3 stator ring, the α and the β subunits are arranged alternately. The catalytic sites reside on one side of the αβ interface, while the other side of the αβ interface binds to ATP; however, it is catalytically impotent and thereby termed the noncatalytic site. The catalytic residues are mostly located on the β subunit, except for the catalytically critical arginine residue termed the “arginine finger” on the α subunit (3, 7–9). Among the 3 β subunits, 2 β subunits bind to nucleotides: one β binds to the ATP analog AMP-PNP and the other binds to ADP [later revealed to also bind to azide (5)]. These β subunits, termed βD and βD′, respectively, adopt so-called closed conformation, in which the C-terminal helical domain rotates inwardly to the rotor γ subunit. The third subunit, P 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 β subunit, which is a major power-stroking motion. The conformational transition of the β 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 F1-ATPase has been bovine mitochondrial F1 (bMF1), but its rotational dynamics remain elusive. This study analyzes rotational characteristics of bMF1, bMF1 showed 3 distinct dwell 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 F1 (hMF1), bMF1 shows short dwell at a distinctly 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.

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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 the catalytically active conformational state for cleavage of bound AMP-PNP (4) or a transition-state analog (13), while typical rotation assay, the been well studied by single-molecule rotation assay (18). In a (6), thiophosphate (16), with the implication that typical rotation upon catalysis during catalytic dwell that is too small to be detected in conventional image analysis, suggesting a function of rotary angle that formed a basis for following evidence of ATP binding than hydrolysis revealed that TF1 generates as a function of rotary angle that formed a basis for following 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$_i$-release dwells. The split of the catalytic dwell was also proposed in studies on the rotation of yeast mitochondrial F$_1$ (28) and human mitochondrial F$_1$ (hMF$_1$) (29). The work on hMF$_1$ showed that the 120° rotation was resolved into 3 substeps: 65°, 25°, and 30°. Each step was initiated by ATP binding, presumably P$_i$ release and hydrolysis. Therefore, the dwells before the 65°, 25°, and 30° substeps are referred to as the binding dwell, P$_i$-release dwell, and catalytic dwell, respectively. The reaction scheme of hMF$_1$ was proposed as shown in Fig. 1B. Due to the close sequence homology of hMF$_1$ and bMF$_1$ [~99% in the α and β subunits and ~95% in the γ subunit (16)], it is expected that the reported rotation behavior of hMF$_1$ is similar to bMF$_1$. From the viewpoint of the structure–function relationship of F$_1$, the correlation between dwells and conformational states found in crystal structures is important to determine. Assays with inhibitors suggest that the P$_i$-release dwell corresponds to the state found in the majority of bMF$_1$ 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 hMF$_1$ and bMF$_1$, and the investigation of the exact correlation between rotary dynamics and atomic structure of F$_1$ requires a rotation assay with F$_1$ from the same species used in the crystal structure analysis. Although a preliminary study on bMF$_1$ was reported (30), basic characteristics of bMF$_1$ have not been analyzed.

A single-molecule rotation assay of F$_1$ enabled not only detailed kinetic analysis of stepping rotation but also manipulation experiments when combined with a magnetic tweezers system. The manipulation experiment of TF$_1$ 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 F$_1$ 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 TF$_1$ generates larger torque in the ATP binding step than in the hydrolysis step. However, the stall-and-release experiments have been conducted only for TF$_1$ (39) and the generality of these findings remains unclear.

In this study, we investigated the γ rotation of bMF$_1$ and found several differences in rotation dynamics between bMF$_1$ and hMF$_1$, from which we propose the reaction scheme for bMF$_1$ 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
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 via biotin–streptavidin interaction (SDS-PAGE) analysis showed the genuine subunit composition was retained after dilution, showing the complex of bMF₁ in solution with ATP-regeneration system (blue data points in Fig. 2B) and determined V₅⁰/ATPase and K₅⁰ATPase 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.

Rotation trajectories projected on the x–y plane showed stepping rotation of bMF₁ (Fig. 2 C and D). At low [ATP]s well below Kₘ bMF₁ showed 3 distinctive pauses with 120° intervals (Fig. 2C) that should correspond to ATP-binding dwell. The histograms of the dwell time for ATP binding showed a single exponential decay function (SI Appendix, Fig. S2A). The rate constants determined from the dwell-time histograms were proportional to [ATP] as expected, giving the rate constant of ATP binding (k_on) of 3.4 × 10⁷ M⁻¹·s⁻¹. This is mostly comparable to that of TF₁ (3.0 × 10⁷ M⁻¹·s⁻¹) (19) and hMF₁ (2.7 × 10⁷ M⁻¹·s⁻¹) (29). At high [ATP]s over Kₘ, several bMF₁ molecules showed 6 pauses as found in the rotation of hMF₁ (29), although many of the molecules did not show 6 clear pauses. Subpauses were detected in the angle histograms by eye in 4 of 23 molecules. Fig. 2D showed x–y projections of a trajectory and the corresponding histogram of angular position observed at 3 mM ATP. The time course also shows multiple pauses within one revolution. Three of the 6 pauses should correspond to catalytic dwell as found in TF₁ and hMF₁. The estimated time constant of ATP binding at 3 mM should be less than 10 μs, too short to be detected. Thus, the intervening pause is not binding dwell. These suggest that bMF₁ makes an intervening pause in addition to catalytic dwell. Note that the response time of the 40-nm gold nanoparticle was ~0.1 ms, and thereby submillisecond events are principally blurred and difficult to resolve. Therefore, the dwell-time analysis at high [ATP] was impractical.

ATP₅S-Driven Rotation. To resolve the rotation and dwells more clearly, we observed rotation in the presence of ATP₅S, which is a slowly hydrolyzable ATP analog. The previous rotation assays showed that ATP₅S slows the ATP hydrolysis on TF₁ (22) and also presumably release of thiophosphate on hMF₁ (29). Rotation rates of bMF₁ were determined at various [ATP₅S]s to draw the Michaelis–Menten curve (SI Appendix, Fig. S3A). V₅⁰max and Kₘ were determined to be 20.3 rps and 2.2 μM, respectively. As expected, V₅⁰max was largely suppressed at about 35 times slower than that of the ATP-driven rotation. The binding constant of ATP₅S, kₐp₅S, was estimated from 3 x V₅⁰max/Kₘ to be 3.0 × 10⁷ M⁻¹·s⁻¹, which was almost identical to kₐp₅.

At high [ATP₅S]/Kₘ, bMF₁ showed distinct pauses separated by 120° steps, corresponding to 3 dense clusters in the x–y

Fig. 2. ATP-driven rotation of bMF₁. (A) A schematic image of the single-molecule rotation assay of bMF₁. The αβγ-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; V₅⁰max, 707 ± 5 rps; Kₘ, 77 ± 2 μM for rotation rate; V₅⁰ATPase, 346 ± 11 s⁻¹; kₐp₅ATPase, 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.

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plot of rotation (Fig. 3A, Inset). A closer look at the time courses showed additional short pauses during 120° steps (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 (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 (40), 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, V_{max}, and K_{m} 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 (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 (40), 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.

The presence of short dwells was confirmed in the CP analysis. Fig. 3B shows angular histograms of a representative molecule. Fig. 3B, Upper shows a conventional angular histogram prepared from all data points of a time course trajectory. Fig. 3B, Lower shows the histogram constructed from the CP intervals, denoted as a histogram of CP intervals, in which the angular positions between 2 successive CPs are represented by the median angle of the interval. Each CP interval gives a single count in the histogram regardless of the dwell length, contrary to the conventional angle histogram where a longer dwell provides more counts. It is evident from Fig. 3B that the histogram of CP intervals clearly showed 3 clusters corresponding to the short dwells (orange) between distinctly high peaks of long dwells (blue). The angular position of short dwell was 60° from a long dwell at the left side (clockwise side) (SI Appendix, Fig. S5A). The dwell time was analyzed for long and short dwells, both of which showed single exponential decay functions, giving time constants of 14 to 15 ms for long dwell and 0.7 to 0.9 ms for short dwell (Fig. 3C and SI Appendix, Fig. S6). It should be noted that in addition to long and short dwells, distinctively long pauses over 1 s were also observed occasionally (SI Appendix, Fig. S7A–C). We attributed the occasional long pause to ADP inhibition, considering that all characterized F_{1}\delta in the rotation assay showed long dwells during rotation by ADP inhibition on the order of seconds (40, 41, 45, 46). The position of the ADP-inhibition dwell coincided with the position of long dwell (SI Appendix, Fig. S7C), suggesting that long dwells correspond to catalytic dwell where F_{1} executes the ATP cleavage reaction (22).

Rotation was observed at [ATP\gamma S]s below K_{m}, which was expected to resolve the rotation into binding dwell and long dwell. The recording rate was set at 1,000 fps to preserve image data storage of the high-speed camera. This allowed long-time observations. As expected, 2 types of dwells were found during 120° rotations in both the angle histogram and time course (Fig. 3D and E). In the region below K_{m}, the binding dwell time was consistent with hMF1 (29) and TF1 (22). As expected, with ATP\gamma S]s decreased, the duration time of binding dwell was lengthened. Fig. 3F and SI Appendix, Fig. S6 show the dwell-time histogram for binding dwell and long dwell. The time constant
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]s as expected, giving the rate constants of ATP$^4$S binding ($k_{\text{on}}^{\gamma}$), $2.9 \times 10^7$ M$^{-1}$s$^{-1}$, well consistent with $k_{\text{on}}^{\gamma}$ (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$^4$S rotation are too short to be detected with the recording frame rate (1 ms per frame).

**Rotation of bMF$_1$ (βE188D).** To confirm that long dwell is $+80^\circ$ from binding angle, we tested a mutant F$_1$ (βE188D) in the rotation assay. This glutamic acid is highly conserved in primary sequences among all F$_1$s. In crystal structures, this glutamic acid interacts with the $\gamma$ 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 TF$_1$ (equivalent to βE188 of bMF$_1$) was replaced with aspartic acid (D), the rate constant of ATP cleavage step was greatly slowed over 300-fold (22).

SI Appendix, Fig. S3B shows the rotation rates of bMF$_1$ (βE188D) at various [ATP]s. 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 bMF$_1$. The ATP binding constant, $k_{\text{on}}^{\text{βE188D–ATP}}$, 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 bMF$_1$ (βE188D) were well consistent with those found in the ATP$^4$S-driven rotation of the wild-type bMF$_1$. At high [ATP]s, 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]s, bMF$_1$ (βE188D) showed that long dwell occurred at $+84^\circ$ 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 ATP$^4$S and bMF$_1$ (βE188D) confirmed that bMF$_1$ makes long dwell at $+80^\circ$ from binding dwell and short dwell at $+50^\circ$ to $60^\circ$ from long dwell, that is, $+10^\circ$ to $20^\circ$ from binding dwell.

**Stall by AMP-PNP.** The rotation assays with ATP$^4$S or bMF$_1$ (βE188D) showed that long dwell occurred at $+80^\circ$ 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 F$_1$ executes the cleavage reaction. To further confirm these findings, we investigated the pause positions of bMF$_1$ by blocking rotation with AMP-PNP, a nonhydrolyzable ATP analog to stall rotation at the angle of cleavage.

The rotation of the $\gamma$ subunit of bMF$_1$ was visualized with magnetic beads as a rotation probe because AMP-PNP–inhibited bMF$_1$ could be reactivatable with magnetic tweezers, which...
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 the rate 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 at 10 mM ATP in the presence of ATPyS, 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. 6A shows time courses of the probability of ATP binding, P_ON (binding), measured at the stall angles in the presence of 100 nM [ATP]. P_ON (binding) increased with the stall time and reached a plateau level, suggesting that the ATP binding reaction is reversible and in equilibrium with ATP release into solution. Assuming the reversible reaction scheme, F1 + ATP ⇄ F1-ATP, the time courses were fitted with the equation

\[
P_{\text{ON}}(\text{binding}) = \frac{k_{\text{ATP}}}{k_{\text{ATP}} + k_{\text{off}}} \cdot \left[1 - \exp\left(-\left(k_{\text{on}}^{\text{ATP}}[\text{ATP}] + k_{\text{ATP}}^{-} \cdot t\right)\right)\right]
\]

where \(k_{\text{ON}}^{\text{ATP}}\) and \(k_{\text{ATP}}^{-}\) represent the rate constants of binding and release, respectively.

Fig. 6B shows time courses of the probability of ATP hydrolysis, \(P_{\text{ON}}(\text{hydrolysis})\), that also shows typical saturation curves, reaching equilibrium levels. The time courses were fitted with

\[
P_{\text{ON}}(\text{hydrolysis}) = \frac{k_{\text{ATP}}^{\text{hydrolysis}}}{k_{\text{ATP}}^{\text{hydrolysis}} + k_{\text{off}}^{\text{hydrolysis}}} \cdot \left[1 - \exp\left(-\left(k_{\text{on}}^{\text{ATP}}[\text{ATP}] + k_{\text{ATP}}^{\text{hydrolysis}}^{-} \cdot t\right)\right)\right]
\]

where \(k_{\text{ATP}}^{\text{hydrolysis}}\) and \(k_{\text{ATP}}^{\text{hydrolysis}}^{-}\) represent the rate constants of hydrolysis and synthesis, respectively.

Fig. 6 C–E show the determined rate constants and equilibrium constants plotted against the rotary angle, where “0°” degree is defined as the mean angle for ATP binding or ATP hydrolysis. The directions for “forward” and “reverse” reactions are defined as that for ATP hydrolysis (counterclockwise) and ATP synthesis (clockwise), respectively. In both ATP binding and ATP hydrolysis, forward reactions are exponentially accelerated upon forward rotation, while the reverse reactions are exponentially decelerated. As a result, equilibriums are both exponentially changed to stabilize binding and hydrolysis states upon forward rotation. All rate constants and equilibrium constants were fitted with simple exponential functions as summarized in SI Appendix.
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 bMF, 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 bMFβ188D or with ATPγS was also analyzed. The resulting Arrhenius plot showed the clear change in the linearity 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 bMFβ188D is essentially the same as those of TF1. The Q10 factor of the rotation rate of bMFβ1 (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 ∆G = ∆H − T∆S, 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 bMFβ1 are the same as those for TF1.

Discussion

Catalytic Event in Long Dwell and Binding Dwell. This study investigated the fundamental features of bMFβ1 rotation under 3 conditions: in the presence of ATP, in the presence of ATPγS, and, by using a mutant F1, bMFβ1β188D). 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 Ksyn. Although the short dwell in the ATP-driven rotation was too short for analysis, the rotation assays with ATPγS or with bMFβ1β188D 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°.

ATPγS and the β188D 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. Actually, the short pause of bMF1 and the 1st dwell of hMF1 show a clear impact on the rotation behavior of bMF1 in current concentrations from $10^{-1}$ to $10^0$ M. However, the addition of phosphate or thiophosphate at concentrations $10^{-3}$ to $10^{-1}$ M decreases $V_{\text{max}}$ and $K_m$ for both bMF1 and hMF1. 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 $\beta_\gamma$ binds to thionucleotide, the γ subunit is positioned at $-30^\circ$ (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 $+65^\circ$ from the binding site to the hydrolysis waiting state at $+90^\circ$. However, the actual bMF1 does not show a dwell at approximately $-30^\circ$ 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 strong angle-dependent affinity change is a conserved characteristic of F1-ATPase among species.

Materials and Methods

Preparation of bMFs. The bMF1, plasmid, a gift from T. Suzuki, Tokyo Institute of Technology, Tokyo, was introduced into the ΔF1-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 bMF1 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 0.1 mg/mL lactate dehydrogenase). The bMF1 was eluted with 50 mL of elution buffer (40 mM potassium phosphate buffer, pH 7.5, 200 mM NaCl, 0.5 mM EDTA, pH 8.0, 10% glycerol, and 0.2 mM ATP). The fractions were collected and concentrated using a centrifugal concentrator (30-kDa, Centricron50, Millipore Corp.). The concentration of bMF1 was determined using 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 bMF1, 2 cysteines on a rotor γ subunit (γ99C → γ99C, γ191C → γ191C) were biotinylated to attach 40-nm gold nanoparticles or magnetic beads as an optical probe. The bMF1 rotation assay was performed in the microscope field 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 bMF1 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 MgCl2, 10 mM phosphoenolpyruvate, 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 bMF1 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 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.

Data Availability. Data are available in the Dryad Digital Repository (https://doi.org/10.5061/dryad.pfg4jkj).

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