Rotational Catalysis of Escherichia coli ATP Synthase F₁ Sector
STOCHASTIC FLUCTUATION AND A KEY DOMAIN OF THE β SUBUNIT*

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A complex of γ, ε, and c subunits rotates in ATP synthase (FoF₁) coupled with proton transport. A gold bead connected to the γ subunit of the Escherichia coli F₁ sector exhibited stochastic rotation, confirming a previous study (Nakanishi-Matsui, M., Kashiwagi, S., Hosokawa, H., Cipriano, D. J., Dunn, S. D., Wada, Y., and Futai, M. (2006) J. Biol. Chem. 281, 4126–4131). A similar approach was taken for mutations in the ε subunit key domain; consistent with its bulk phase ATPase activities, F₁, with the Ser-174 to Leu substitution and the corresponding Ser-174 to Phe substitution (S174F) exhibited a slower single revolution time (time required for 360° degree revolution) and paused almost 10 times longer than the wild type at one of the three 120° positions during the stepped revolution. The pause positions were probably not at the “ATP waiting” dwell but at the “ATP hydrolysis/product release” dwell, since the ATP concentration used for the assay was ~30-fold higher than the Km value for ATP. A βGly-149 to Ala substitution in the phosphate binding P-loop suppressed the defect of βS174F. The revertant (βG149A/βS174F) exhibited similar rotation to the wild type, except that it showed long pauses less frequently. Essentially the same results were obtained with the Ser-174 to Leu substitution and the corresponding revertant βG149A/βS174L. These results indicate that the domain between β-sheet 4 (βSer-174) and P-loop (βGly-149) is important to drive rotation.

A ubiquitous ATP synthase (FoF₁) synthesizes ATP coupled with an electrochemical proton gradient formed by a respiratory chain (for reviews, see Refs 1–5). FoF₁ consists of a catalytic sector, F₁ (αβγδε), and a membrane-embedded proton pathway, Fo (ab,c₁₀), and can reversibly transport protons coupled with ATP hydrolysis. The α and β subunits form a catalytic hexamer (α₃β₃), the central space of which is occupied by the γ subunit α-helices. ATP is synthesized or hydrolyzed cooperatively at a catalytic site in each β subunit as the binding change mechanism predicts (2). The γ subunit rotation in αβγ3 has been supported by biochemical studies (1, 6, 7), a crystal structure of the αβγ3 complex (8), and video recorded using an actin filament as a probe (9, 10). Consistent with ATP-dependant proton translocation, a γετ₁₀ complex rotated relative to the αβδεβ3γγ complex in the purified FoF₁ (11–14) or its membrane-bound form (15, 16). The rotation of FoF₁ in liposomes has been revealed by means of single molecule fluorescence resonance energy transfer (17).

Counterclockwise rotation of the γ subunit has been studied more recently with probes giving low viscous drag such as colloidal gold (14, 18, 19). The three 120° steps in one revolution of Bacillus F₁ were first observed using an actin filament (20), and later the single 120° step was further subdivided into two substeps (80° and 40°) using gold beads that allow finer observation and analysis (14, 19, 21). The sub-steps with larger displacement angles (80°) and smaller sub-steps (40°) are assigned to ATP binding and hydrolysis/product release steps, respectively (19, 21, 22). We have observed that the rotation speed of beads attached to the Escherichia coli γ subunit varied, reflecting stochastic fluctuations (18, 23). Although the average speeds were dependent on the diameter of beads, 40- and 60-nm diameter beads rotated with essentially the same rate (18), suggesting that their rotation speeds were close to that of the γ subunit without a probe attached.

The mechanism underlying the chemistry and energy coupling of FoF₁ has been studied by introducing mutations (1, 10, 13, 16, 24). One of the most interesting mutations is the substitution of the BSer-174 residue (24–27) located in β-sheet 4, thus being distant from the bound ATP in the β subunit (8) (see Fig. 1). The size of the residue at this position is pertinent as to the activity (25); the larger the side-chain volume of the residue introduced, the lower the ATPase activity became. The βS174F (βSer-174 to Phe substitution) or βS174L (βSer-174 to Leu) F₁ sector exhibited ~10% of the wild-type activity (25). The defect of βS174F was suppressed by the replacement of βGly-149 (βGly-149 to Ala, Ser, or Cys) in the phosphate binding P-loop near α-helix B (26, 27). However, rotation of an actin filament connected to γ was not proportional to the ATPase activity; generated torque for βS174F and βS174L were 40–100% that of the wild-type level (24). Thus, it became of interest to analyze mechanical revolution of the γ subunit in these mutants using a probe smaller than an actin filament.

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FIGURE 1. Models of the $\beta$-sheet 4/loop-$\alpha$-helix B/P-loop domain in $E$. coli ATP synthase $\beta$ subunit. a, the structure of $\alpha_\beta_\gamma$ is shown with a region discussed in this study (squares): blue, $\beta$ subunit; green, $\alpha$ subunit; gray, $\gamma$ subunit. $b$ and $c$, the $\beta$-sheet 4/loop-$\alpha$-helix B/P-loop domain structure of $E$. coli $\beta$ subunit was modeled after the bovine structure (8). The sequences identity of the $\beta$ subunit between cow and $E$. coli is 71.7% (24). The nomenclature for $\alpha$-helix and $\beta$-sheet is cited from Abrahams et al. (8). The domains of empty ($b$) and ATP-bound ($c$) $\beta$ subunits are shown together with amino acid residues discussed in under "Results and Discussion." The positions of $\beta$Ser-174 and $\beta$Gly-149 residue are shown (red line).

In this study we confirmed stochastic fluctuation of the $\gamma$ subunit rotation by analyzing the single revolution time (time required for 360° revolution). The $\beta$S174F mutant paused at 120° steps longer than the wild type, giving an ~6 times lower revolution time. The rotation of second-site revertant $\beta$G149A/$\beta$S174F was similar to that of the wild type. Essentially the same results were obtained for $\beta$S174L mutant and its second-site revertant $\beta$G149A/$\beta$S174L. We discuss a possible role(s) of the domain including $\beta$-sheet 4 and the phosphate binding P-loop, where $\beta$Ser-174 and $\beta$Gly-149 are located, respectively (Fig. 1).

EXPERIMENTAL PROCEDURES

Preparation and Materials—$E$. coli strain DK8 ($\Delta$uncB-C) was used as a host for recombinant plasmids and grown at 37 °C in a synthetic medium containing 0.5% glycerol as a carbon source. A plasmid carrying the unc operon introduced six His residues at the $\alpha$ subunit amino terminus, and $\gamma$S193C and $\gamma$K108C substitutions in the $\gamma$ subunit were described previously (18). Mutations $\beta$S174F and $\beta$S174L and related substitutions of the $\beta$ subunit were introduced into $F_1$ engineered for rotation (24). The wild-type and mutant $F_1$ sectors were purified on a glycerol gradient (24). Gel electrophoresis showed the presence of about 1 mol e/mol $F_1$ but no $\delta$ subunit in any of the preparations analyzed. Gold beads (60-nm diameter) were obtained from British Bio Cell International and were coated with biotinylated bovine serum albumin (18). The cover glass used for constructing a cell for observing rotation was soaked in purity water (18).

Assay Procedures—Mutant and wild-type ATPase activities were assayed as described previously (10) under the conditions used for the rotation assay (18). The third (highest) $K_m$ values for ATP were obtained with the Mg$^{2+}$ concentrations that gave the maximal activities. When varying the ATP concentration, the Mg$^{2+}$:ATP ratio was maintained at 1:1, except that the Mg$^{2+}$ concentration was 0.5 mM when ATP concentration was lower than 0.5 mM. For the $\beta$G149A mutant, the ratio was maintained at 2:1, except that the Mg$^{2+}$ concentration was 4 mM for ATP lower than 0.5 mM. Protein concentrations were determined using bovine serum albumin (Sigma, Fraction V) as a standard (28).

Observing $\gamma$ Subunit Rotation—Rotation was assayed essentially as described previously (18). Briefly, gold beads were connected to the immobilized $F_1$ sector in a flow cell (~30-μm deep) filled with buffer A (10 mg/ml bovine serum albumin, 10 mM MOPS/KOH, pH 7.0, 50 mM KCl, and 2 mM MgCl$_2$). Immediately after the introduction of buffer A containing 2 mM ATP and its regenerating system, images of the beads illuminated with laser light (JUNO EX, Showa Optronics Co.) were obtained on dark field microscopy (BX51WI-CDEVA-F, Olympus, Tokyo) and recorded with a charge-coupled device camera for data analysis using a Metamorph (Molecular Devices Corp.). The proper camera speeds (1000~4000 frames/s) were selected depending on the mutations of the $F_1$ sectors; wild-type and the revertants, 4000 frames/s; $\beta$S174F, $\beta$S174L, and $\beta$G149A, 1000 frames/s. They were also assayed at a different camera speed when necessary. Other methods, including construction of glass cells for rotation and laser-light illumination, were described previously (18). We occasionally observed apparent clockwise movements of less than two revolutions, although they were not actual rotations. The beads analyzed were those showing such movements amounting to less than 5% of the total counterclockwise revolutions.

RESULTS

Stochastic Rotation of Gold Beads Attached to Wild-type $F_1$—Gold beads connected to $F_1$ rotated with various speeds and often paused for a short period (~ms) as shown previously (18). In the previous paper, we recorded their rotations for 0.25 s, estimated rates every 10 ms, and combined those from different beads (18). Histograms of the rotation rates showed stochastic fluctuation. However, previous analysis may emphasize the frequencies of rates close to 0 rps if beads paused a long time (>10 ms). Furthermore, these histograms may include variation between beads together with fluctuation of the rotation speeds. The previous observation time (18) was not enough to detect long pauses (>0.25 s). Thus, we were prompted to study longer time courses and single rotation events (360° rotations). The present analysis was more appropriate than estimating rates every 10 ms (18), because all revolutions could be included.

In this study the time course of each bead was followed for 2 s. We occasionally observed long pauses (>0.1 s) (Fig. 2a), which were not found in previous time courses (18); a bead rotated about 500 revolutions and paused (yellow curve in Fig. 2a), and others paused after 300 revolutions (green and pink curves). Sometimes we observed beads that started rotating after a long pause (gray curve). On average, about 3 long pauses appeared when we recorded 1000 revolutions. These pauses were possibly because of Mg-ADP inhibition (29), as discussed below. We assumed that the long dwells.

2 The abbreviation used is: MOPS, 3-(N-morpholino) propanesulfonic acid.
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**FIGURE 2.** Stochastic rotations of gold beads attached to $F_1$. a, time courses of 60-nm beads attached to wild-type $F_1$, were followed in the presence of 2 mM ATP. Different colors represent rotations of 10 individual gold beads. b, time courses of apparent smooth rotations were expanded to show dwells and ~120° stepping. c, examples of histograms of single revolution times (time required for 360° revolution) obtained for four single beads are shown. The colors correspond to those in a. d, histograms combined for multiple beads. The single revolution times obtained for 10 randomly selected beads are combined and shown as histograms.

are Mg-inhibited states of the enzyme and analyzed single rotation events.

When time courses were expanded, apparent smooth rotations (Fig. 2a) exhibited dwells (short pauses, ~ms) and ~120° stepping (Fig. 2b). Therefore, we analyzed the single revolution time, i.e. the time required for 360° of revolution, to evaluate stochastic fluctuation of rotation. This parameter could follow all rotations of a bead in a time course regardless of the length of the pauses. As expected from the various speeds observed in a time course, each bead clearly showed stochastic fluctuation of the single revolution time (for examples, see Fig. 2c). The histograms for the individual beads and those for multiple beads (Fig. 2d) are closely similar, indicating that fluctuation tendency is an intrinsic property of the $F_1$ molecule. The geometric mean of single revolution time was ~2.3 ms (2.0, 2.3, 2.3, and 2.6 ms, for four different beads) (Fig. 2c), and the average rotation rate (reciprocal of single revolution time) was 440 rps, i.e. slightly higher than the reported value, 380 rps (18). A 360° rotation including the long pause was observed with lower frequency and shown in the histograms of single rotation times (see >15, horizontal axis in Fig. 2, c and d).

**ATPase Activity of Mutant $F_1$**—It became of interest to analyze the mutant $F_1$ sector to understand the mechanisms underlying rotation and its stochastic fluctuation. βS174F (Fig. 1b) and related mutations were introduced into the engineered $F_1$ to observe rotation as previously described (18, 24). Steady state ATPase activities were assayed under the same conditions as those for rotation observation. The relative activities (% of wild-type level) were essentially similar to previous results: βS174F, 8%; βS174L, 9%; G149A/βS174F, 314%; G149A/βS174L, 200%; G149A, 75%. We also estimated the third (highest) $K_m$ for ATP to assay rotation in the presence of excess ATP. βS174F, βS174L, and the second-site revertant G149A/βS174F exhibited $K_m$ values for ATP of 69, 60, and 64 μM, respectively, i.e. essentially the same as that of the wild type (71 μM). G149A exhibited a $K_m$ value (0.3 mM) of ~4-fold higher than that of the wild type, possibly because of the location of βGly-149 near bound-Mg-ATP at the catalytic site (Fig. 1c). Based on these biochemical properties of the mutant enzymes, we assayed rotations of βS174F, βS174L, and the corresponding second-site revertants in the presence of 2 mM ATP, ~30-fold higher than the $K_m$ values to compare steady state ATP hydrolysis and rotation.

**Rotation of Mutant $F_1$ with βSer-174 Replacement**—The time course of a gold bead attached to βS174F was followed for 2 s. The mutant rotated with variable rates and exhibited long pauses similar to the wild type (Fig. 3, a and b). The total revolutions (in 2 s) were much less than those of the wild type (Fig. 3b). Thus, we compared the wild type and mutants by analyzing single rotation events. The mutant apparently exhibited longer single revolution times than the wild type, as shown for the histograms of multiple beads (Fig. 3c). They are similar to those for single beads (data not shown). The peaks of the wild-type and mutant histograms from multiple beads were at 1.75 and 5 ms, respectively, and their geometric means were 2.3 and 14 ms, respectively. Most (~80%) of the mutant single revolution times were longer than 5 ms, and ~50% of them were longer than 10 ms (Fig. 3c), whereas ~90% of those of the wild type were ~5 ms. The geometric mean of the βS174L single revolution times was ~26 ms, i.e. ~11 times longer time than that of the wild type, and >20% of the mutant times were longer than 100 ms, as shown by the histograms (Fig. 3d). These results were consistent with the low ATPase activities of the mutants.

**Pausing of the γ Subunit in the Presence of a High Concentration of ATP**—The single revolution times of βS174F and βS174L were significantly longer than those of the wild type in the presence of 2 mM ATP (Fig. 3, c and d), possibly because the mutants exhibited a high tendency for longer
dwell. Thus, we compared dwells of the wild type and mutants. As shown by expanded time courses, the mutant paused longer at 1/3, 2/3, or 3/3 of a 360° revolution compared with the wild type (Fig. 4a, note the time scale). The angular distributions of the centroids showed three peaks at about 120°, 240°, and 360°/0° (Fig. 4b), indicating that beads paused after 120° revolutions, as shown previously with low ATP concentrations (20). However, the pausing observed in the present study may not be at the ATP waiting dwell (time for a catalytic site waiting for ATP binding), since it was not affected by a further increase in the ATP concentration (data not shown), and rotations were assayed in the presence of a ~30-fold higher ATP concentration than the $K_m$.

The pausing dwell contributed significantly to a single revolution time since the stepping (0 → 120°, 120 → 240°, or 240 → 360°/0°) speeds were high (mostly ≈0.25 and ≈1 ms per 120° step for wild-type and mutant, respectively). About 80% of the pausing dwells of the wild type and mutant were between 0.5 and 5 ms, respectively (Fig. 4c), indicating that mutant paused at least ~10 times longer than the wild type. Essentially the same results were obtained for the BS174L mutant. Thus, the longer pausing dwells caused the slow rotation speeds and low ATPase activities of mutants.

**Rotation of the Second-site Revertant**—As shown previously, a defect of the BS174F enzyme was suppressed by the second-site mutations, βGly-149 to Ser, Cys, or Ala (26, 27), giving apparently wild-type ATPase activity. The time course of the revertant βG149A/BS174F and βG149A single mutant was analyzed (Fig. 5, a and b) and shown together with those of the wild type and BS174F mutant for comparison (Fig. 5c). Beads attached to the revertant F1 rotated similar to those of the wild type, except that they rarely exhibited pauses longer than 0.1 s. The histogram of the single revolution times for the revertant showed a peak at 2.5 ms and a geometric mean of 2.9 ms (Fig. 5d), i.e. similar to the wild-type values (Fig. 2d).

The average rotation rates were estimated as reciprocals of the geometric means for single revolution times, assuming that F1 sectors exhibiting various speeds were present in the assay mixture; those for the wild type, BS174F, revertant, and βG149A were 440, 70, 340, and 70 rps, respectively. The results for the two single mutants and the revertant indicate that the two mutations (BS174F and βG149A) suppressed each other and gave similar rates as the wild type. Essentially the same results were obtained for βG149A/BS174L (Fig. 5e), the second-site revertant of BS174L.

**DISCUSSION**

We have previously observed that a gold bead attached to the γ subunit rotated at various rates, indicating stochastic fluctuation of F1 rotation (18). However, the reported results may have included variations between F1 molecules because data for multiple beads were combined to obtain histograms. In this study we analyzed single revolution times that include the pausing dwell and stepping velocity. As expected from the time courses, individual beads exhibited stochastic fluctuations, and their histograms were similar, indicating that the variation among F1 molecule was not significant.

The observed stochastic fluctuation was probably because of the intrinsic properties of the γ subunit driven by catalysis in α3β3 hexamer, since they were essentially independent of the bead sizes (40–200-nm diameter) (18, 23), lengths of histidine tag (6 or 10 histidine residues) introduced into the α subunit,3 and enzyme preparations (F1 or FoF1) (data not shown). The fluctuations were mainly because of the varying pausing dwells (~ms) because stepping was fast. Stochastic fluctuation was also observed in *Bacillus* F1 with the fluorophore Cy3, attached to the γ subunit (30). Careful definition of wild-type rotation was the basis of further studies on mutant F1.

Replacement of βSer-174 in β-sheet 4 lowered the ATPase activity to ~10% of the wild-type level (25). The means of

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3 M. Nakanishi-Matsui, S. Kashiwagi, T. Uebukata, A. Iwamoto-Kihara, Y. Wada, and M. Futai, unpublished observation.
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单个旋转时间的位点被附着到β174F和β174L中，而β174L则更长于野生型的单个旋转时间。这些差异可能意味着，尽管其稳定态ATPase活性相似，但单个旋转时间可能长于野生型的单个旋转时间。β174L的单个旋转时间短于β174F。β174F和β174L的ε亚基敏感性相似于野生型（未显示），表明了缓慢旋转的突变体不是因为ε亚基的原因，虽然它们在稳定态的ATPase活性相似，但单个旋转时间可能长于野生型的单个旋转时间。E. coli的酶在同一个twostep作为β174 mutant showed long pauses (Fig. 1, b, and c). Thus, the conformational changes of the P-loop during catalysis affect β-sheet 4 through α-helix B for the rotation. Substitution of βSer-174 possibly affected the conformational transition ($\beta_\text{D} \rightarrow \beta_\text{A}$ or $\beta_\text{A} \rightarrow \beta_\text{D}$) of the entire domain and increased the pausing dwell. The transition became similar to the wild type with the second mutation, βG149A.

It should be important to discuss which rotation step is related to the conformation transition. 120° revolution was observed initially for an actin filament connected to Bacillus F₁, when the ATP concentration was lowered (20). Using 40-nm gold beads, Yasuda et al. (19) further observed 90° and 30° substeps in each 120° step, which were later revised to 80° and 40°, respectively, by the same group (21). The dwell before the 80° revolution was dependent on the ATP concentration, whereas that before the 40° substep was not. These results indicated that the 80° and 40° substeps were driven by ATP binding and hydrolysis/product release, respectively. We assayed rotations with a high ATP concentration and observed pauses upon 120° revolution possibly at one of the two substeps. As discussed above, they paused not at the ATP waiting dwell but possibly at the ATP hydrolysis/product release dwell. Assuming that the E. coli enzyme has the same two substeps as Bacillus, the mutant F₁ paused longer before the 40° stepping. Present results of mutants and revertants indicate that the 40° stepping is driven by the conformation transition of the β-sheet4/loop/α-helix B/P loop domain. Hydrolysis in or product release from the catalytic site including P-loop apparently originates this transition.

We occasionally observed long pauses (>0.1 s), which apparently lowered bulk-phase ATPase activity. The revertant βG149A/β174F and βG149A mutant showed long pauses less often than the wild type. These pauses may be because of the Mg-ADP-inhibited form observed previously using duplex beads (440- and 517-nm diameter) (29). The wild-type ATPase activity is sensitive to Mg²⁺ (31), which stabilizes the Mg-ADP-inhibited form (32, 33). On the other hand, the ATPase activities of βG149A/β174F and βG149A were less sensitive to Mg²⁺ than that of the wild type (data not shown), similar to the revertant, βG149S/β174F (26). Thus, the long pauses of the revertant and βG149A occurred rarely because their Mg-ADP-inhibited forms were unstable. These results were consistent with higher steady state ATPase activities of the revertant and βG149A. In this

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**FIGURE 4. Pausing of the wild-type and mutant rotation.** a, rotation of the wild type, β174F, and β174L. Rotation was followed as described in Fig. 3, and expanded time courses of typical beads are shown: black, wild-type; dark gray, β174F; light gray, β174L. b, histograms of the angular distribution of the wild-type and mutant beads, c, histograms of the pausing duration of the wild-type, β174F, and β174L. Rotations of randomly selected beads were followed for 200 revolutions, and histograms of pausing duration are shown. Wild-type histograms are also shown on an expanded time scale (inset).
regard mutations in the corresponding domain of *Bacillus F*₁ changed the tendency to generate Mg-ADP-inhibited form (34).

In conclusion, using gold beads but not actin filaments, present studies clearly exhibited stochastic fluctuation of *F*₁ rotation and its defect in β subunit mutants. Mutation/suppression studies revealed that the β-sheet 4/loop/a-helix B/P-loop is an important domain to drive rotation and is at least partially responsible for Mg-ADP inhibition. Further single molecule analysis will provide a new insight in enzyme mechanism (35).

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