Stochastic High-speed Rotation of Escherichia coli ATP Synthase F1 Sector
THE ε SUBUNIT-SENSITIVE ROTATION*

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The γ subunit of the F1 sector rotates at the center of the αβ3 hexamer during ATP hydrolysis. A gold bead (40–200 nm diameter) was attached to the γ subunit of Escherichia coli F1, and then its ATP hydrolysis-dependent rotation was studied. The rotation speeds were variable, showing stochastic fluctuation. The high-speed rates of 40- and 60-nm beads were essentially similar: 721 and 671 rps (revolutions/s), respectively. The average rate of 60-nm beads was 381 rps, which is ∼13-fold faster than that expected from the steady-state ATPase turnover number. These results indicate that the F1 sector rotates much faster than expected from the bulk of ATPase activity, and that ∼10% of the F1 molecules are active on the millisecond time scale. Furthermore, the real ATP turnover number (number of ATP molecules converted to ADP and phosphate/s), as a single molecule, is variable during a short period. The ε subunit inhibited rotation and ATPase, whereas ε fused through its carboxyl terminus to cytochrome b562 showed no effect. The ε subunit significantly increased the pausing time during rotation. Stochastic fluctuation of catalysis may be a general property of an enzyme, although its understanding requires combining studies of steady-state kinetics and single molecule observation.

The biological energy currency ATP is synthesized by a ubiquitous ATP synthase (F0F1) conserved in mitochondria, chloroplasts, and bacteria (see Refs. 1–5, for reviews). Escherichia coli F0F1 has a basic subunit structure consisting of catalytic F1 (αβ3γεδ) and transmembrane F0 (ab2c10) sectors with a distinct stoichiometry of eight subunits. The α and β subunits form a catalytic hexamer, αβε, the γ subunit being located at its center. Ten molecules of the ε subunit form a membrane-embedded ring for continuous proton transport through its interface with subunit a (6–8). ATP at a catalytic site in each β subunit is synthesized or hydrolyzed cooperatively, as predicted by the binding change mechanism or rotational catalysis (2), and supported by the crystal structure of the αβ2γε complex (9). The γ subunit rotation has been shown biochemically by photobleaching of a probe attached to the carboxyl terminus of the γ subunit (10), chemical cross-linking between the α and β ε subunit (11), cryoelectron microscopy (12), and finally video recording of the ATP-dependent anti-clockwise rotation of an actin filament attached to the ε subunit (13–15). Consistent with tight interaction between the γ and ε subunits, a filament connected to ε rotated together with the γ subunit (16).

For proton translocation, rotation of the γ subunit is transmitted to the ε subunit ring, as shown by rotation of the γεc10 complex relative to the αβαβε2 (17). Mechanical rotation of purified F1F1 (17–21) and its membrane-bound form have been shown experimentally (22, 23). The rotation of γεc10 was consistent with cross-linking experiments (21, 24–26). The γ subunit and γεc10 rotated in F1 and F1F1, respectively, essentially at the same rate, generating a frictional torque of ∼40 piconewtons/nm.

The maximal rotational rates of an actin filament were ∼10 s−1, these values being much lower than those expected from ATPase turnover, although rotation and turnover were assayed under different conditions (13–15, 18–20). Furthermore, the ε subunit had no effect on the rotation, although it is known to inhibit F1-ATPase activity (27–29).

Rotation has been studied recently using gold beads (30, 31). The rates were variable depending on bead size, but much faster than those expected from ATP hydrolysis. On the other hand, single molecule fluorescence resonance energy transfer (FRET) revealed that membrane-bound F10F1 in liposomes was rotated, as expected from the turnover number (32–35). Thus, further studies are required to establish the rotation rates and their sensitivity using a probe that exhibits lower viscous drag.

In this study, we have evaluated the turnover number of E. coli F1, and studied the ATP-dependent rotation of a series of gold beads attached to the γ subunit. We analyzed the rotation rates of single beads, and observed stochastic fluctuation of rotation rates, high-speed rotation being ∼700 rps, when 40- or 60-nm beads were attached. Analysis of the average rotation rates and the ATPase turnover number, under the same conditions, suggested that ∼10% of the F1 molecules are active on the millisecond time scale. The ε subunit increased pausing of mechanical rotation leading to the lower rotation rate.

EXPERIMENTAL PROCEDURES

Materials and Preparations—E. coli F1, with six histidine residues and cysteine substitutions (γS193C, yK108C) introduced into the α subunit amino terminus and γ subunit, respectively, were constructed from pBUR17 (14) by replacing the Csp451-RsR2I segment with that having

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‡ The abbreviations used are: FRET, fluorescence resonance energy transfer; MOPS, 3-(N-morpholino)propanesulfonic acid; LDAO, lauryl dimethyl amine N-oxide; rps, revolutions/second.

1 Y. Sambongi, S. D. Dunn, and M. Futai, unpublished observation.

2 Y. Sambongi, S. D. Dunn, and M. Futai, unpublished observation.
yK108C substitution (14). The engineered F₁, was purified by glycerol gradient centrifugation (15), and gel electrophoresis showed the presence of about 1 mol of ε, but no detectable δ subunit (data not shown). The ε subunit and the same subunit fused with cytochrome b₅₆₂ at the carboxyl terminus were purified as described previously (29). Gold beads (40, 60, 80, 100, or 200 nm diameter) were obtained from British BioCell International and coated with biotinylated bovine serum albumin (40, 60, 80, 100, or 200 nm diameter) were obtained from British BioCell International and coated with biotinylated bovine serum albumin (30). Fluorescently labeled actin filaments were prepared as described previously (14).

**Assay Procedures**—ATPase activity was assayed as described previously (36) with a minor modification: the buffer for rotation was used to compare the results with the rotation rate. The reaction was started by the addition of 2 mM ATP, and terminated with trichloroacetic acid immediately after a 10-min incubation at 24 °C. Each mixture was centrifuged, and then the phosphate released was determined. Protein concentrations were determined by the method of Bradford (37) using bovine serum albumin (Sigma, Fraction V) as a standard. The concentration of F₁ (α₂β₃γε) was determined assuming its molecular weight = 365,000.

**Observation of Rotation**—To observe rotation, a gold bead was connected to the γ subunit of F₁ immobilized on a glass surface. A flow cell (~30 μm deep) was constructed from cover glass washed extensively with 0.1 N KOH, filled with Buffer A (10 mg/ml bovine serum albumin, 10 mM MOPS/KOH, pH 7.0, 50 mM KCl, and 2 mM MgCl₂) containing 200 mM engineered F₁, and then incubated at 24 °C for 10 min. After washing off unbound F₁ with Buffer A, streptavidin (4 μM) in Buffer A was introduced into the cell, followed by incubation for 5 min and then extensive washing with 100 μl of Buffer A. Gold beads (0.2%) in the same buffer were introduced and washed similarly. Buffer A containing 2 mM ATP, 1 mM phosphoenolpyruvate, and 50 μg/ml pyruvate kinase was introduced to start the reaction. To examine the effect of the ε subunit, 100 mM ε in Buffer A was introduced into the flow cell after the gold beads, followed by incubation for 10 min and then the introduction of Buffer A containing 100 mM ε subunit, ATP, and its regenerating system. To test the effects of LDAO, Buffer A containing 0.5% LDAO, ATP, and an ATP regenerating system was introduced.

Images of gold beads illuminated with laser light (532 nm) were observed at 24 °C by dark field microscopy (BX51WI-CDEVA-F, Olympus, Tokyo) with a ×100 objective and ×2 relay lenses, and recorded with an intensified charge-coupled device camera (Photon Co.). Camera speeds used were 2,000–4,000 frames/s. The temperature of the assay mixture was determined by introducing a probe into the immersion oil (~50 μm deep) between the objective lens and the flow cell. The laser light was generated using JUNO EX (Showa Optronics Co.), and power levels between 0.3 and 0.02 watts gave essentially the same results. The bead centroid was analyzed, and rotation results were obtained using MetaMorph (Molecular Device).

### TABLE 1

| F₁ preparation | ATPase activity | Turnover number | Rotation rates estimated from ATPase | ε Inhibition |
|----------------|----------------|-----------------|--------------------------------------|-------------|
|                | units/mg protein |                | s⁻¹                                  | %           |
| 1              |                |                |                                      |             |
| F₁; non-engineered | 14.7 ± 0.3     | 90              | 30                                   |             |
| + ε            | 7.3 ± 0.2      | 45              | 15                                   | 50          |
| + ε-Cytochrome b₅₆₂ | 15.9 ± 0.1     | 97              | 32                                   | 0           |
| 2              |                |                |                                      |             |
| F₁; engineered for rotation | 14.9 ± 0.1     | 91              | 30                                   |             |
| + ε            | 6.9 ± 0.2      | 42              | 14                                   | 54          |
| + ε-Cytochrome b₅₆₂ | 15.4 ± 0.0     | 94              | 31                                   | 0           |

Rotation of the fluorescent actin filaments connected to the γ subunit of F₁ was observed using a Zeiss Axiovert 135 with a charge-coupled device camera as described previously (14). The rotating filaments were subjected to video recording and centroid analysis.

### RESULTS

**ATPase Activity of F₁ Engineered for Rotation**—The F₁ engineered for rotation has an NH₂-terminal His, tag and Cys substitutions introduced into the α and γ subunits, respectively. The ATPase activity of the engineered F₁ was assayed essentially under the same conditions as those used for rotation. The engineered and non-engineered wild-type F₁ showed similar ATPase activity (Table 1). The differences from previous results (14, 38, 39) may be because of the assay conditions, including the buffers used. The present turnover number (91 s⁻¹ at 24 °C) corresponds to the γ subunit rotation rate of 30 rps, estimated assuming that three ATP molecules were hydrolyzed during one rotation. It became of interest to compare the ATPase turnover number with the single molecule rotation using probes exhibiting lower viscous drag.

**ATP-dependent Rotation of Gold Beads Attached to the γ Subunit**—ATP-dependent rotation of a gold bead (40–200 nm diameter) was examined under laser light illumination for less than 0.2 s (Fig. 1a). Beads rotating continuously could be detected by inspecting images on the monitor screen (Fig. 1b). We selected the laser light power allowing quick and clear observation, although essentially the same rates were obtained when the power was reduced to 1/10 or increased 1.5-fold. The frequency of beads rotating continuously for more than 20 revolutions was ~1% when ~1000 beads were examined on the monitor screen. The rotation rates of 100- and 200-nm beads were apparently slower than those of 40- and 60-nm beads (Fig. 1c). Because rotation speeds were apparently variable, the rates were estimated every 10 ms for each randomly selected bead. The resulting histograms indicate stochastic fluctuation of the rotation speeds showing a Gaussian distribution (Fig. 1d). The fluctuation of the rotation speed was clearly observed with smaller gold beads, but decreased with larger beads (compare 40- and 200-nm beads). These results indicate that the fluctuation is an intrinsic property of γ subunit rotation that may be masked when probes with large viscous drag are used to observe rotation.

**High-speed Rotation of Gold Beads**—Stochastic fluctuation of the rotation speed prompted us to determine high-speed and average rotation rates. To estimate high-speed rates, we randomly selected beads showing more than 20 continuous rotations, and estimated the fastest rates (maximal rotation rates) (Fig. 2a). As expected, rotation became slower with an increase in the bead diameters (Fig. 2b). The high-speed rates for 200-, 100-, 80-, 60-, and 40-nm beads were 146, 389, 506, 671, and 721 rps, respectively, with standard deviations of 7–17% of these values.
Gold beads rotated much faster than actin filaments connected to F₁ (Fig. 2b). The 40- and 60-nm beads rotated at essentially the same speed, indicating that their rates were close to those that would be exhibited without viscous friction. It should be noted that the high-speed rate of the 40-nm beads was about 24-fold faster than that estimated from the ATPase turnover number (Table 1).

Average Rotation Rates of Gold Beads—As described above, the rotation rates were variable during the observation, and the high-speed rates were much faster than those expected from the ATPase turnovers (Fig. 2b, Table 1). Thus, it was of interest to estimate the average rotation rates. Rotating 60-nm beads were selected randomly, and their rates were estimated every 10 ms (Fig. 2c). Paused beads (0 rps in 10 ms) were often found, and included for calculation, assuming that paused F₁ molecules are present in the steady-state (bulk phase) ATPase assay. The overall average rate was 381 ± 33 rps, i.e. close to 13 times faster than expected from the turnover number obtained for steady-state ATPase.

As seen with high-speed rates, 40- and 60-nm beads gave essentially the same average rates (Fig. 2b), confirming that their rates were close to those without viscous friction.

Effect of the ε Subunit on Steady-state ATPase—The ε subunit is known to inhibit F₁-ATPase activity, although its effect on rotation was not observed previously. The ATPase activities of wild-type and engineered F₁ were inhibited about 50% with excess ε subunit when assayed with 2 nM F₁ (Table 1), indicating that the genetic modifications introduced to observe the rotation had no effect on the ε inhibition. The ε with cytochrome b₅₆₂ connected to the carboxyl terminus was not inhibitory, confirming previously published results (29). These results prompted us to study the effects of the ε subunit on gold bead rotation.

Effect of the ε Subunit on F₁ Rotation—For estimating the ε effect under conditions similar to ATPase assay, we estimated the concentration of F₁ bound to the glass surface. The fraction of added F₁ immobilized on the glass surface was ~8%, as estimated by ATPase activity, when we introduced 200 nM F₁. Thus, addition of 100 nM ε to the rotation assay provides an excess ε similar to that used in testing its effect on bulk ATPase activity. As described above for gold beads connected with F₁, rotation in the presence of ε exhibited various rates. However, the time courses with ε were slower than those without ε (Fig. 3a). Histograms of the rotation speed in the presence of the ε subunit also showed stochastic fluctuation similar to the distribution curve without ε (Fig. 3b). It is noteworthy that the histogram of rates in the presence of ε showed two populations with one peak at less than 40 rps and another at 200–240 rps. Most of the former population are paused beads. The second peak is slower than that at ~380 rps without the ε subunit, indicating that ε inhibited F₁ rotation. These results indicate inhibition of rotation by the ε subunit.

We also estimated high-speed and average rates of rotation from apparent rotation of beads with the ε subunit. As shown for the ATPase activity, the average rotation rate of 60-nm beads by the enzyme in the presence of 100 nM ε subunit was ~50% of that observed without ε (Fig. 3c).

No inhibition was observed when the ε subunit connected with cytochrome b₅₆₂ was introduced, or when immobilized F₁ containing ε was washed with Buffer A right before the rotation assay (Fig. 3, b and c). The slight shift of the peak in the ε-cytochrome b₅₆₂ histograms may be because of the interaction of ε-cytochrome b₅₆₂ with F₁. These results indicate that the inhibitory effect of ε was reversible and that most ε was released from F₁ during the washing step. With excess ε, high-speed rotation was also about 40% of that observed for F₁ without ε (Fig. 3c).

Because one of the effects of LDAO is to eliminate inhibition of F₁-ATPase activity by ε (40), we compared the effects that LDAO had on rotation rates and ε inhibition. However, no rotating beads were observed when more than 6,000 beads were tested in the presence of 0.5% LDAO.

Effect of the ε Subunit on Pausing—As described above, histograms of rotating beads clearly indicated that a substantial population of beads were paused in the presence of the ε subunit (Fig. 3b). They were apparently paused at one of the three positions of stepped rotation (30) (data not shown). One possibility was that the ε subunit increased pauses to slow the average rate. Thus, we estimated pausing time, assigning beads to a paused state when they moved anticlockwise less than 0.06 rounds in a 250-ms rotation period. Paused beads were observed when more than 6,000 beads were tested in the presence of 0.5% LDAO.

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were also analyzed in each 10 ms and total 10-ms pausing times are shown as histograms (Fig. 4b). The pausing times in the absence of ε, the average being 2–6 ms, the average being 4.4 ms within 10 ms. The pauses became significantly longer in the presence of ε, the average time being 6.0 ms, i.e. about 1.6 ms longer than in the case of F1 without ε addition (Fig. 4b). Increased pausing was also consistent with about 3-fold lower frequency of observed bead rotation when the ε subunit was added (data not shown). It should be noted that the method to estimate pausing time is different from that used to obtain rates for the histograms in Fig. 3b. As described above, rates were estimated for 10 ms from the linear segment of the time course. These results indicate that the ε subunit increases pausing times, explaining at least partially the ε inhibition of rotation.

**DISCUSSION**

The binding change mechanism predicted continuous rotation of the γ subunit together with the c subunit ring during ATP synthesis or hydrolysis (2). This mechanism ensures continuous energy coupling between chemistry and proton transport. The rotation of actin filaments connected to the γ subunit and γεc10 complex in F1 and F0F1, respectively, has been recorded under a microscope (13–16, 18–23). Because the rotation rates were lower than those estimated from the ATPase activity, it was of interest to determine the rotation rate without viscous drag.

In contrast to results with actin filaments, gold beads (40 or 60 nm) connected to the γ subunit rotated much faster than expected from the ATPase turnover number. Rotation speeds were variable, giving a Gaussian distribution of the histograms. The fluctuation may be because of the stochastic property of γ subunit rotation, because it was more prominent with the smaller beads. Consistent with this notion, larger beads showed less fluctuations.

The high-speed rate, estimated as described above, was 670 rps for the 60-nm bead during the 250-ms assay. As ATP is hydrolyzed by multiple F1 molecules through steady-state kinetics, the average rate for randomly selected beads should correspond to the rate expected from the ATPase turnover number. However, the average rotation rate (380
The rotation rate at 24 °C. The higher ATPase activity could not be estimated because F1 was not stable above 52 °C.

Yasuda et al. (30) reported that the rotation rate estimated from ATPase turnover was 30–60% of the rotation rate of the 40-nm beads in thermophilic F1 (30). Ueno et al. (31) observed more recently that the steady-state ATPase activity of thermophilic F0F1 was only ~10% of the value expected from the rotation. Although they did not determine stochastic properties of rotation, their results and our observations are consistent, showing that the rotation rate of a single molecule is significantly faster than that estimated from ATPase turnover.

These results suggest that ~90% of the F1 molecules are inactive or in an inhibited state at any given point in time. Another possibility, that the preparation contained 90% denatured F1, is unlikely, because transitions between long pausing and fast rotation were often observed. Furthermore, steady-state hydrolysis rates that were consistent with the observed rotation rates have not been reported, even when the enzyme was assayed at 37 °C (31). Why did we observe a significantly low population of F1 molecules that were rotating at the ~250-ms time scale? The mechanism underlying the inhibition is worth considering. One possibility is that much of the enzyme is inactive because of the binding of the inhibitory MgADP at the active site (41, 42). It is also possible that both active and inactive conformations of F1 may exist in a slow dynamic equilibrium.

Single molecule FRET showed the rotation of F0F1 in proteoliposomes during ATP synthesis and hydrolysis (34, 35). The rotation rates were close to the values expected from the ATPase turnover. The difference between the present and the FRET results may be partly because of the experimental systems. The FRET results were obtained from F0F1 in liposomes with a transmembrane pH gradient and membrane poten-
tial, whereas the present results were from the F₁ sector. It is also noted that apparent rotations for hydrolysis and synthesis direction were also observed without nucleotides in the FRET experiment (35). Further studies on the two systems are of interest for understanding the mechanism of rotational catalysis in F₁ and F₀F₁.

F₁-ATPase activity was inhibited ~50% in the presence of 100 nM ε subunit. In the previous study, excess ε inhibited the activity by 80–90% (27–29). The lower inhibitory effect in our case could be because of the turnover number is variable over time for a single enzyme. Moreover, kinetic analysis of the rotation indicated that the turnover number of the enzyme molecule. Furthermore, the present study indicated that this enzyme parameter, which is generally obtained from steady-state kinetics, does not necessarily correspond to the value for a single enzyme molecule. However, the present study indicated that this enzyme parameter, which is generally obtained from steady-state kinetics, does not necessarily correspond to the value for a single enzyme molecule. Furthermore, kinetic analysis of the rotation indicated that the turnover number is variable over time for a single enzyme.

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