ATP-driven stepwise rotation of F₀F₁-ATP synthase

Hiroshi Ueno*, Toshiharu Suzuki*, Kazuhiko Kinosita, Jr., and Masasuke Yoshida*

*Chemical Resources Laboratory, Tokyo Institute of Technology, Nagatsuta 4259, Yokohama 226-8503, Japan; †ATP System Project, Exploratory Research for Advanced Technology, Japan Science and Technology Agency, Nagatsuta 5800-3, Yokohama 226-0026, Japan; and ‡Okazaki Institute for Integrative Bioscience, National Institutes of Natural Sciences, Higashiyama 5-1 Myodaiji, Okazaki 444-8787, Japan

ATP hydrolysis | binding change mechanism | membrane protein | single-molecule imaging

F₀F₁-ATPase/synthase (F₀F₁) is a large protein complex (~500 kDa) that catalyzes ATP synthesis/hydrolysis coupled with a transmembrane H⁺ (proton)-translocation in bacteria, chloroplasts, and mitochondria (1–5). The enzyme is easily and reversibly separated into two portions, termed F₁ and F₀. In its simplest prototype bacterial enzyme, a water-soluble F₁ portion consists of five different subunits, α₁β₁γ₁δ₁ε₁, and catalyzes ATP hydrolysis (hence, often called F₁-ATPase). Three α-subunits and three β-subunits are arranged alternately, forming a hexagonal cylinder around the coiled-coil structure of the γ-subunit (6). Membrane-integrated F₀ portion has three different subunits, α₁β₁γ₁ (n; variable among species) and mediates proton transport across the membrane. The c-subunits forms a ring structure, and αβ₂ associates with the c-subunit ring peripherally (7–10). F₀F₁ is a motor enzyme. When the magnitude of electrochemical potential of protons is large enough, downhill proton flow through F₀ causes rotation of the rotor subunits (c₂–γε relative to the stator subunits (αβ₂–αεβδ), and rotation of the γ-subunit forces the β-subunits of F₁ to change conformations sequentially that result in ATP synthesis. In the reverse reaction, ATP hydrolysis at F₁ causes the reverse rotation of the rotor subunits that drives F₀ to pump protons (Fig. 1) (11).

We have been studying rotation of thermophilic Bacillus F₁ since the first direct visualization of ATP-driven rotation of F₁ immobilized on the glass surface (12). A rotation probe attached on the γ-subunit rotates unidirectionally counterclockwise when viewed from membrane side. It repeats a pause and a 120° step rotation when medium ATP concentration ([ATP]) is low (13). The duration of the pause becomes shorter as [ATP] increases and is finally invisible beyond the limit of the observation system. This [ATP]-dependent pause corresponds to the period during which the enzyme waits for medium ATP to come into the empty catalytic site, and, hence, is called the ATP-waiting dwell. With high-speed imaging, it was found that the 120° step of rotation is further split into 90° and 30° substeps (14). A pause between two substeps, ~2 ms at 23°C, is not influenced by [ATP]. This [ATP]-independent nature of the pause means that catalytic events after substrate binding should occur during this pause, and we call this pause the catalytic dwell. The histogram of durations of the catalytic dwells did not obey a single exponential but did obey double exponentials, indicating that two catalytic reactions of ~1 ms occur in the catalytic dwell (14). More recent experiments using a slowly hydrolyzable ATP analog, adenosine 5'-[γ-thio]triphosphate (ATPγS) and a slow mutant in ATP hydrolysis, clarified that one of the two 1-ms events is cleavage of a bound ATP at a catalytic site (15). Also, previous 90° and 30° substeps were recently revised to be 80° and 40° substeps (15, 16). Thus, F₁ rotates by repeating four stages; ATP-waiting dwell, rapid 80° substep rotation upon ATP binding, catalytic dwell in which ATP hydrolysis occurs, and rapid 40° substep rotation, probably upon the release of the last product.

ATP-driven rotation of F₀F₁ was demonstrated for Propionigenium modestum F₀F₁ with single-fluorophore polarization (17), and for Escherichia coli F₀F₁ with disulfide cross-linking (18, 19), direct visualization (20), and fluorescence resonance energy transfer (21). In general, ATP-driven rotation of F₀F₁ can differ from that of F₁ because proton transport through F₀ and interaction between the c-subunit ring (rotor) and αβ₂ (stator) during rotation may modify the rotation. It was reported that the central rotor rotates counterclockwise when viewed from membrane side, and there are three pauses (each ~19–30 ms) in one revolution, likely corresponding to the catalytic dwell (21, 22). The rotation of F₀F₁ driven by proton flow was also observed, and the direction of the rotation is opposite of that of the ATP-driven rotation (22). However, in general, knowledge on rotation of F₀F₁ has yet been very limited. For example, even the following basic motor natures of F₀F₁ remain unknown: (i) whether ATP-binding dwell appears at low [ATP], (ii) how the rotation depends on [ATP], (iii) whether the observed rotation consists of the sequence of 80° and 40° substep rotation or other new substep(s) exists at different angular position(s), (iv) whether catalytic dwell is composed of two events, as observed for F₁, and (v) how rotation changes when a reversible F₀ inhibitor is present. To address these questions, we isolated thermophilic F₀F₁, which kept structural integrity in a detergent, immobilized it on a glass surface through the β-subunits, attached a small (80 nm) bead to the c-subunit ring as a rotation probe whose viscous friction was low enough to allow full-speed rotation, and observed ATP-driven rotation with a fast camera. The results reveal that basic natures of ATP-driven rotation of...
ethylene glycol monododecyl ether and 100 mM Ni$_2^+$-NTA for FoF1 complex of thermophilic Bacillus PS3. The purification was finished within 4 h, and the purified sample was used within 1 day. The purified FoF1 contained 0.9 mol of ADP and 0.8 mol of ATP per mol of FoF1 as endogenously bound nucleotides. Specific biotinylation of the F$_{10}$-subunit was confirmed by immunoblotting using streptavidin-alkaline phosphatase conjugates (Promega).

**Materials and Methods**

**Isolation of F$_{0}$F$_{1}$.** A plasmid pTR19-ASDS-CNCR3 for mutant F$_{0}$F$_{1}$ (cSer2Cys/$\beta$-His$_{10}$ tags) was made by the Mega-primer method (23) using a plasmid, pTR19-ASDS (24), an expression vector for FoF1 complex of thermophilic Bacillus PS3. The mutant F$_{0}$F$_{1}$ was expressed constitutively in Bacillus PS3. Inversion of ATPase activity was measured at 37°C with an ATP regeneration system, and ATP at indicated concentrations, and observation of rotation was started. When indicated, ATP$_{5}$S was added instead of ATP, and an ATP-regeneration system was omitted in this case. The number of the beads at the glass surface depended on the concentrations of biotinylated FoF1 infused to the flow cell. When nonbiotinylated enzyme was infused, it was almost the same as the case when no protein was infused, and we could not find any rotating beads in this case. Inhibitory effect of DCCD was assessed by observing the rotation of F$_{0}$F$_{1}$ incubated with 50 mM DCCD for 30 min at room temperature before infusion into the flow cell. Beads were observed with a dark-field microscopy (IX-70, Olympus) with a ×100 objective lens (numerical aperture of 1.35, Olympus) and a dark-field condenser (numerical aperture of 1.2–1.4, Olympus). Both the objective lens and the condenser were warmed by lamp (condenser) heater (Tokai Hit) to maintain temperature of the flow cell at 37°C. Bead images were recorded as an eight-bit AVI file with a fast-framing charge-coupled device camera (Hi-Dcam, NAC Image Technology) at the indicated frame rate. To analyze the acquired image data, custom software (created by R. Yasuda; Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) was used. Rotation rate was obtained from the average of 20 continuous revolutions without unnatural interruption.

**Other Assays.** ATPase activity was measured at 37°C with an ATP regeneration system (25). The assay solution was composed of buffer R containing 5 mM MgCl$_2$, 1 mM ATP-Na, 2 mM phosphoenolpyruvate, 100 µg/ml lactate dehydrogenase, 100 µg/ml pyruvate kinase, and 0.2 mM NADH. DCCD inhibition of ATP hydrolyzing activity was measured as described for the inverted membrane vesicles (24), or after a 30-min preincubation
with DCCD (50 μM) for the purified enzymes. To measure inhibitory effect of tributyltin chloride (TBT-Cl) on ATP hydrolyzing activity, indicated concentrations of TBT-Cl was added to the assay solution before the start of the reaction. Because the inhibitory effect of TBT-Cl on ATPase activity, as well as on rotation, tended to be relieved gradually as time passed (for an unknown reason), we collected rotation data quickly after the initiation of measurements. Reconstitution of FoF1 into liposomes was performed by the method described (24). Protein concentrations were determined by the BCA protein assay kit (Pierce) with BSA as a standard.

Results

Intact FoF1 in Detergent. In this study, we used FoF1 of thermophilic Bacillus PS3 expressed in the plasma membranes of an F1-deficient E. coli strain, DK8. A detergent octaethylen glycol monododecyl ether was used to solubilize FoF1 from the membrane vesicles and it was substituted with LPC at the next step of purification. We have tested various detergents, but this combination of detergents gave the most efficient solubilization, and the FoF1 preparation had the most stable, intact coupling properties. The FoF1 has His10 tags at the N terminus of the β-subunits to immobilize onto a glass surface and has a cysteine residue at the second position from N terminus of the c-subunit for biotinylation to attach the beads. The enzyme was purified with a Ni²⁺-NTA column and a Soft-Link avidin column (Fig. 1 Inset, lane 1). These procedures should help to remove free F1 and F0, if any. Specific biotinylation of the c-subunit was confirmed by immunoblotting (Fig. 1 Inset, lane 2). The purified FoF1 in LPC comprised eight kinds of subunits and exhibited DCCD-sensitive ATPase activity; ≈85% of the activity was inhibited by DCCD in the solution used for observation of rotation (Tables 1 and 2). This degree of inhibition is similar to that of the intact FoF1 embedded in membranes, either in the membrane vesicles, or in the reconstituted vesicles. It has been known that DCCD covalently labels an essential carboxyl residue of the c-subunit, blocks proton transport, and consequently, if FoF1 is intact, prevents ATP hydrolysis/synthesis. Unlike FoF1, ATPase activity of the isolated F1 was not affected by DCCD at pH values of >7.5. (data not shown). Therefore, high sensitivity to DCCD inhibition is a good indication of the intactness of our FoF1 preparation. The reconstituted vesicles containing purified FoF1 showed substantial ATP-driven proton-pumping activity, comparable with that of the authentic wild-type thermophilic FoF1 (24). Based on these observations, we concluded that the purified FoF1 in LPC had intact ATPase activity that coupled proton transport. This conclusion was further confirmed by TBT-Cl sensitivity of FoF1 as described later.

Table 1. ATPase activity sensitivity of FoF1 to DCCD inhibition

<table>
<thead>
<tr>
<th>Samples</th>
<th>Residual activity +DCCD, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane vesicles</td>
<td>25</td>
</tr>
<tr>
<td>Reconstituted vesicles</td>
<td>20</td>
</tr>
<tr>
<td>FoF1</td>
<td>15</td>
</tr>
</tbody>
</table>

The samples were pretreated with 50 μM DCCD for 30 min and subjected to the assays. Membrane vesicles were prepared from Escherichia coli cells expressing FoF1 from thermophilic Bacillus PS3. Purified FoF1 was incorporated into reconstituted vesicles. ATPase activity was measured in the same solution used for rotation observation. Other experimental details are described in Materials and Methods.

Table 2. Effect of DCCD on rotation of FoF1

<table>
<thead>
<tr>
<th>Trial</th>
<th>No. of rotating beads +DCCD /−DCCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 / 10</td>
</tr>
<tr>
<td>2</td>
<td>1 / 10</td>
</tr>
<tr>
<td>3</td>
<td>0 / 9</td>
</tr>
<tr>
<td>4</td>
<td>1 / 11</td>
</tr>
<tr>
<td>5</td>
<td>1 / 8</td>
</tr>
<tr>
<td>6</td>
<td>1 / 8</td>
</tr>
<tr>
<td>Total</td>
<td>7 / 60</td>
</tr>
</tbody>
</table>

Rotating beads were looked for in 120 optical fields of a unit area (~45 × 55 μm²) in 10 min in one trial. Other experimental details are described in Materials and Methods.
and proton transport through F$_0$, does not limit rotation rate of F$_{0}$F$_{1}$, at least in the absence of the electrochemical gradient of protons.

**Rotation Rate Versus ATPase Activity.** The bulk-phase steady-state ATPase activities of the purified F$_{0}$F$_{1}$ in the same solution used for observation of rotation were only $\approx 10\%$ at 2 mM ATP, and even $<1\%$ at 2 $\mu$M ATP of the expected values from the rotation rates (Fig. 2, c). This result indicates that $>90\%$ population of F$_{0}$F$_{1}$ molecules are not working at a given moment, or, in other words, a single molecule spends $\approx 90\%$ of time in inactive state(s). Indeed, we noticed that rotating molecules usually stopped rotation after several seconds of continuous rotation. Sometimes, the same molecule resumed rotation after a while. We also often saw, under the microscopic field, that a previously nonrotating bead started rotating. The real reason why such high fractions of F$_{0}$F$_{1}$ molecules are in inactive state(s) is not known, but it should be noted that F$_{1}$ (a$\alpha$$\beta$$\gamma$$\delta$ subcomplex) also shows similar large discrepancy of rates between rotation and bulk-phase steady-state ATPase activity. In the case of F$_{1}$, the nonrotating bead started rotating. The real reason why such high fractions of F$_{0}$F$_{1}$ molecules are in inactive state(s) is not known, but it should be noted that F$_{1}$ (a$\alpha$$\beta$$\gamma$$\delta$ subcomplex) also shows similar large discrepancy of rates between rotation and bulk-phase steady-state ATPase activity. In the case of F$_{1}$, the responsibility for the discrepancy is thought to be the ADP-Mg inhibition, which is caused from the nonturnover retention of ADP-Mg at a catalytic site (27–29). The steady-state ATPase activity of F$_{1}$ is largely suppressed by the ADP-Mg inhibition, but, uninhibited activity that is almost constant with the rotation rate is, estimated from the initial burst activity that appears upon initiation of ATPase assays (14). We speculate that ADP-Mg inhibition occurring in F$_{0}$F$_{1}$ can explain, at least partly, the discrepancy between rotation and bulk-phase ATPase activity, although we cannot estimate its quantitative contribution because F$_{0}$F$_{1}$ does not show the uninhibited initial-burst activity. Pronounced inhibition of ATPase activity of F$_{0}$F$_{1}$ at low [ATP] in Fig. 2 might be due to the inhibition by the endogenous inhibitor, $\varepsilon$-subunit, that exhibits inhibition at low [ATP] (30).

**Stepwise Rotation.** We analyzed the time course of rotation at 2 mM ATP (Fig. 3A). Under $V_{\max}$ conditions, it is expected that ATP binds to F$_{0}$F$_{1}$ very quickly, and the catalytic events occurring in the enzyme determine the rates of ATP hydrolysis and rotation. Therefore, the rotation would show only the catalytic dwell. Indeed, the histogram of angular distribution of centroid of bead (Fig. 3A Inset) indicated the presence of three favorable positions for a bead to make a brief pause. However, the expanded time course of rotation did not always show clear steps (see Fig. 6, which is published as supporting information on the PNAS web site), and we could not define each dwell time with certainty.

At 2 $\mu$M ATP, F$_{0}$F$_{1}$ rotated with discrete 120° steps (Fig. 3B), and the dwell time of the pauses between adjacent step-rotations was apparently dependent on [ATP], indicating that the observed pauses were the ATP-waiting dwells. In addition, very short dwells were often seen in the intermediate position in a 120° rotation. They are not obvious in the bead-centroid distribution plots (Fig. 3B Lower Inset) because these dwells would be buried behind spreading distribution of the long ATP-waiting dwells. Usually, the starting time points of the short dwells could be recognized in the rotation time course as an interruption of 120° rotation, but the end points were mostly unclear; the dwells transited to the next ATP-waiting dwell without showing discrete step rotation. Therefore, the total dwell time from the start of the short dwell to the end of the ATP-waiting dwell was analyzed. The histogram of the total dwell times at 2 $\mu$M ATP showed a distinct peak, and was fitted by the sum of two exponential components that assumed two rate-limiting reactions (Fig. 3B Upper Inset). One of the time constants was [ATP]-dependent and corresponds to the ATP-waiting dwell. From the time constant, the ATP-binding rate ($k_{\text{on-ATP}}$) of F$_{0}$F$_{1}$ was estimated to be 3.6 ± 0.1 $\times$ 10$^{-7}$ M$^{-1}$s$^{-1}$. This value is close to that of F$_{1}$ at 23°C ($3.0 ± 0.1 \times 10^7$ M$^{-1}$s$^{-1}$) (14). The other time constant ($\approx 0.58$ ms) was [ATP]-independent and corresponds to the catalytic dwell. This value is consistent with that ($\approx 0.95$ ms) obtained from the $V_{\max}$ rotation.

From these results, we learned that F$_{0}$F$_{1}$ rotates by repeating ATP-waiting dwell and catalytic dwell. However, the very short lifetime of the catalytic dwell did not allow for its further analysis, and the angular position of the catalytic dwell and the number of events occurring in the catalytic dwell were not determined. To learn these answers, we extended the duration of the catalytic dwell by adopting a slowly hydrolyzable ATP analog, ATP$\gamma$S, as a substrate (15).

**Rotation Driven by ATP$\gamma$S.** F$_{0}$F$_{1}$ rotated in 1 mM ATP$\gamma$S at $\approx 20$ rps, $>10$ times slower rate compared with the rate in 1 mM ATP ($\approx 355$ rps), and the discrete 120° steps were observed (Fig. 4A Lower Inset). The rotation in 100 $\mu$M ATP$\gamma$S was apparently very similar to that observed in 1 mM ATP$\gamma$S; rotation rate of $\approx 18$ rps and 120° step rotation (data not shown). The dwell time between steps was not changed in two ATP$\gamma$S concentrations and should be the catalytic dwell. The histogram of the catalytic dwell of the rotation in 1 mM ATP$\gamma$S showed a peak, and was fitted with the sum of two exponential components that provided two time constants, $\approx 12.4$ and $\approx 2.3$ ms (Fig. 4A Upper Inset). Taking the F$_{1}$ study on hydrolysis of ATP$\gamma$S as a reference (15), the long time constant is likely the one for cleavage of ATP$\gamma$S, and the short time constant for the release of the last product. The total of these time constants ($\approx 14.7$ ms) agrees well with that ($\approx 16.7$ ms) obtained from $V_{\max}$ (20 rps). In 10 $\mu$M ATP$\gamma$S, the 120° step was further split into two substeps (Fig. 4B Lower Inset). The angles of the two substep rotations were roughly 80° and 40° that are the same as observed for F$_{1}$. The pauses between adjacent 40° and 80° substep rotations depended on ATP$\gamma$S concentrations, and, hence, the ATP$\gamma$S-waiting dwell. The histogram of the ATP$\gamma$S-waiting dwell was fitted well with a single-exponential component (Fig. 4B Upper Inset), and the ATP$\gamma$S-binding rate ($k_{\text{on-ATP$\gamma$S}}$) of F$_{0}$F$_{1}$ was estimated to be
We speculate that TBT-Cl interferes with the rotation of Fo because the pauses before 40° substeps (catalytic dwell) in rotation at 1 mM ATP are not observed. However, the rotating bead did not show any obvious pause at certain angular position (Fig. 5A). No inhibition was observed in the presence of TBT-Cl (Fig. 5B). The pauses between adjacent 80° and 40° substep rotations corresponded to the catalytic dwell, and the step-wise rotation obscure. The pauses between adjacent 20° and 40° substep rotations corresponded to the catalytic dwell, and the step-wise rotation obscure. The pauses between adjacent 80° and 40° substep rotations corresponded to the catalytic dwell, and the step-wise rotation obscure. The pauses between adjacent 20° and 40° substep rotations corresponded to the catalytic dwell, and the step-wise rotation obscure.

Discussion

In this study, we directly observed ATP-driven rotation of single-molecule FoF1 immobilized on a glass surface. To do so, FoF1 must be solubilized and purified without losing original structural integrity. It should be noted that the previous demonstrations of rotation of purified E. coli FoF1 immobilized on a glass surface were carried out by using the enzyme preparation, of which ATPase activity in the rotation buffer and the rotation itself were totally insensitive to DCCD (34, 35), and might have been the rotation of incomplete FoF1, as stated in the subsequent paper (36). We have spent considerable effort to establish the procedures to isolate intact thermophilic FoF1 from the recombinant E. coli cells, and found that extraction from membranes by octaethylene glycol monododecyl ether and isolation in LPC gave the best yield and preparation. Both bulk-phase ATPase activity in the rotation buffer and the observed rotations under microscopy were efficiently inhibited by DCCD and TBT-Cl. Combination of this intact FoF1 and the submillisecond fast camera enabled us to learn several features of ATP-driven rotation of FoF1.

Firstly, FoF1 rotates as fast as 350 rps (37°C). We observed rotations at 25°C and 45°C and obtained the $V_{\text{max}}$ rotation rates of ~230 and ~650 rps, respectively. Therefore, extrapolated rotation rate at 60°C, an optimum growth temperature of Bacillus PS3, can reach ~1,600 rps. Although reservation is needed concerning whether these enormous numbers are really the case, it is certain that the rotation rate of thermophilic FoF1 at 25°C is much faster than the rotation rate (19 ms/120°, that is, 17 rps, at 23°C) reported for E. coli FoF1 (37), yeast mitochondrial F1 (80, 81), and Bacillus PS3 (38, 39). Much lower ATPase activities corresponding to 120°, that is, 17 rps, at 23°C) reported for E. coli FoF1 (37), yeast mitochondrial F1 (80, 81), and Bacillus PS3 (38, 39). Much lower ATPase activities correspond to 10° of ATPase activity in the rotation buffer and the rotation itself were totally insensitive to DCCD (34, 35), and might have been the rotation of incomplete FoF1, as stated in the subsequent paper (36).
rotation rates can be much faster. It is intriguing to learn whether these rapid rotations are really occurring in living cells.

Second, FoF₁ rotates in the almost same manner as F₁. V_{\text{max}} rotation rate, [ATP] dependency, ATP-waiting dwell, and catalytic dwell are all very similar to those observed for F₁. Apparently, friction in Fo motor is negligible, that is, Fo does not impose significant drag during ATP-driven rotation of FoF₁. This finding means that Fo keeps the interaction between the c-subunit ring and ab$_2$ neither too strong nor too weak. The c-subunit ring seems to be able to pause rotation at the angular positions dictated by F₁. We recently proposed that b$_2$ in Fo act as a strong brake of ATP-driven rotation when the a-subunit is removed (40). The association of the a-subunit makes b$_2$ to be a proper anchor rail that allows the c-subunit ring to slide without breaking association during rotation.

Finally, an Fo-specific inhibitor, TBT-Cl, obscures the steps in ATP-driven rotation of FoF₁. Obviously, now the Fo$_2$ portion resists the rotary torque generated in F₁ portion. Because the rotor of Fo$_2$ in thermophilic FoF₁ is a decamer c-subunit ring (41), it is likely that resistance occurs at every 36°. As discussed in the recent paper (41), mismatch of the unit-rotation angle in F₁ (120°) and in Fo (36°) assumes a torsion-spring-like motion of the central rotor shaft (and/or the peripheral stalk) that makes the dwelling position of the c-subunit ring in ATP-driven rotation obscure. We don’t know whether this explanation is really the case, but we expect that further study on TBT-Cl inhibition will provide the answer.

We thank J. Suzuki for technical assistance; K. Shimabukuro, E. Muneyuki, R. Iino, T. Masaike, A. Araiga, and T. Akaishi for valuable discussion and technical advice; and H. Noji, R. Yasuda, and K. Adachi for creating and developing the single-molecule observation system. This work was supported by research fellowships from the Japan Society for the Promotion of Science for Young Scientists (to H.U.).
