F1-ATPase is a rotary motor enzyme in which a single ATP molecule drives a 120° rotation of the central γ subunit relative to the surrounding αββ′ ring. Here, we show that the rotation of F1-ATPase spontaneously lapses into long (~30 s) pauses during steady-state catalysis. The effects of ADP-Mg and mutation on the pauses, as well as kinetic comparison with bulk-phase catalysis, strongly indicate that the paused enzyme corresponds to the inactive state of F1-ATPase previously known as the ADP-Mg inhibited form in which F1-ATPase fails to release ADP-Mg from catalytic sites. The pausing position of the γ subunit deviates from the ATP-waiting position and is most likely the recently found intermediate 90° position.

ATP synthase of mitochondria, chloroplasts, and bacteria catalyzes ATP synthesis coupled with a transmembrane proton flow (1–4). The enzyme consists of a membrane-embedded, proton-conducting portion (F0) and a protruding portion (F1) in which catalytic sites for ATP synthesis/hydrosynthesis exist. The isolated F1 portion has ATPase activity; hence, it is often called F1-ATPase. It is composed of five different subunits with a stoichiometry of α3β3γ6ε. The αβγ subcomplex is the minimum ATPase-active complex, which has catalytic features similar to F1-ATPase. In the crystal structure (5), the central γ subunit is surrounded by an αββ′ cylinder where three α and three β subunits are arranged alternately, and the six nucleotide binding sites are located at the α/β subunit interfaces. Three of the binding sites are catalytic, and the β subunits provide most of the catalytic residues. The other three are noncatalytic, and the α subunits provide most residues contributing nucleotide binding.

It has been postulated that the energy of the proton flow liberated at F0 is transformed into the energy of ATP synthesis at F1 through rotation of the central γ subunit and vice versa—the energy of ATP hydrolysis can be converted into the energy of proton pumping through reverse rotation of the γ subunit (6). By using an αββ′ subcomplex of thermophilic F1-ATPase (F1-ATPase) immobilized on a glass surface, we have observed ATP hydrolysis-driven rotation of the fluorescent actin filament attached to the γ subunit (7).

At nanomolar ATP concentration, F1-ATPase binds and hydrolyzes a single ATP molecule, makes a 120° rotation, and waits for the next ATP molecule. As the ATP concentration increases, the ATP-waiting period becomes shorter until it is finally undetectable, and rotation of the actin filament becomes apparently continuous over hundreds of revolutions (8). However, when the rotation was observed for long periods, occasional pauses of rotation were recognized, even at high ATP concentrations (7, 9). Here, we show that these pauses occur at an intermediate step of rotation and mostly correspond to the ADP-Mg inhibition, which has been observed in bulk-phase kinetics as a general feature of the F1-ATPases (and ATP synthases). Slow interconversion between rotating and pausing states thus contributes to the attenuation of ATPase during steady-state catalysis.
by applying buffer A or buffer B (100 mM potassium phosphate buffer, pH 7.0) containing 2 mM MgCl₂, 0.2 mM–2 mM ATP, and the ATP-regenerating system (0.2 mg/ml creatine kinase and 2.5 mM creatine phosphate). In the presence of 0.1% lauryl dimethyl amine oxide (LDAO), the rotation of the F₁ particles were observed in the presence of buffer B. The rotation of the DNC₉ mutant also was observed in buffer B (17). Buffer exchange was performed as described. To visualize induction of ADP-Mg inhibition, after the observation of the rotation in ATP-containing buffer A (1 mM ATP-Mg), an ADP-containing buffer A (1 mM ATP-Mg, 1 mM ADP or 0.1 mM ADP) was infused into the same chamber for rotation observation (see Fig. 2 A and B). To visualize release from the ADP-Mg inhibited state, the ADP buffer was exchanged to ATP+re buffer (ATP-containing buffer and ATP-regenerating system; see Fig. 2C).

The fluorescent actin filament was observed with a fluorescence microscope (IX70; Olympus, New Hyde Park, NY). Photobleaching of the fluorescent actin filament was minimized by the use of a filter (ND20). The rotation of the actin filament could be observed for 40–50 min. The 440-nm and 517-nm beads were observed with a transmission light microscope. Images from actin- or bead-labeled F₁-ATPase were recorded with an intensified charge-coupled device (350 F; Videoscope, Dallas) camera on an 8-mm videotape. Analysis of rotational angle was performed as described (7).

**Materials.** LDAO (30% aqueous solution) was purchased from Calbiochem.

**Results**

**Pauses of Rotation of F₁-ATPase at High ATP.** Fig. 1A shows the typical time courses of the rotation of a fluorescent actin filament attached to the γ subunit of immobilized F₁-ATPase in the presence of 2 mM ATP. At this ATP concentration, ATP binding should take place within 0.1 ms (8) and does not result in a pause of rotation. However, each F₁-ATPase molecule made several distinct pauses during 500 s, some of which were longer than 60 s. Noticeably, paused filaments always stayed within one of three angular positions, consistent with the pseudo-3-fold symmetrical structure of F₁-ATPase. There is no obvious preference among the three angular positions for pauses to occur.
Sometimes, the time-averaged centers of three positions deviated slightly from the exact 3-fold symmetry, probably because of the oblique attachment of the F1-ATPase molecule to the glass surface. To confirm that these pauses are not due to obstruction by nearby proteins or surface, we observed the single molecule for a long period by using plastic beads of diameter 440 nm or 517 nm as a rotation probe under transmission light microscopy. Despite the difference in the observation system, the pauses in the rotation of a pair of biotin-coated beads attached to the γ subunit also occurred at three positions with a frequency and duration similar to those observed in the rotation of actin filament (Fig. 1B).

The duration of all pauses longer than 10 s observed in the rotation of actin filaments and beads were plotted as a function of the length of the probes (Fig. 2). Values are scattered, but, on average, pauses continue for \( \approx 30 \) s irrespective of the length of the probes. If pauses were caused by nearby obstacles, they should occur at random positions, and longer probes would generate more pauses because of an increased chance of encountering obstacles. Thus, the observed properties of pauses, the three angular-pause positions, and load-independence all suggest that the pause is not due to accidental obstruction by nearby proteins or by the surface, but that it reflects intrinsic properties of the F1 motor.

**State of F1-ATPase Corresponding to the Pauses.** It is natural to assume that the F1-ATPase in the pausing phase should be in an
incomplete form that is not hydrolyzing ATP. Because rotation resumes after awhile, this incomplete form should be activated again in the presence of ATP-Mg. The candidate for the incomplete form of F₁-ATPase that shows the above features is the ADP-Mg inhibited form, a state commonly found in F₁-ATPases from mitochondria (19), chloroplasts (20), and bacteria (13, 18, 21). The ADP-Mg inhibited form is generated in a stochastic manner during catalytic turnover of ATP hydrolysis by stable entrapment of ADP-Mg at a catalytic site. The ADP-Mg can be either an immediate hydrolysis product that is left bound to the enzyme or it can be picked up from the bulk phase medium (21). If pausing F₁-ATPase is really in the ADP-Mg inhibited form, inclusion of ADP-Mg in the solution during the rotation assay should result in an increase in the number of pauses. At first, rotation was observed in the presence of 1 mM ATP, and then a solution containing 0.1 mM ADP + 1 mM ATP (Fig. 3A) or 1 mM ADP + 1 mM ATP (Fig. 3B) was infused into the chamber. As expected, rotation was frequently (0.1 mM ADP) or almost completely (1 mM ADP) prevented after the infusion. Physical damage of the immobilized F₁-ATPase by the infusion is unlikely to be the reason for stopping rotation, because the reverse order of addition produced the opposite result—the molecules were unable to rotate in the presence of 1 mM ADP + 1 mM ATP and began rotation after the ADP was removed (Fig. 3C). Further, the rotating molecules in 1 mM ATP lapsed into a pause after the infusion of the ADP-containing solution, and started rotation again after the second infusion by the ADP-free solution (Fig. 3D).

Binding of ATP to the noncatalytic nucleotide-binding site on the α subunit stimulates the recovery of F₁-ATPase from the ADP-Mg inhibited form (14). Therefore, the mutant F₁-ATPase that has defective noncatalytic sites (ΔNC; ref. 13) is soon converted completely to the ADP-Mg inhibited form during catalysis. We examined whether the ΔNC mutant could rotate but were unable to find any rotating molecules. A second mutation, β-T165S, was introduced into the ΔNC mutant. The single β-T165S mutant is less susceptible to attaining the ADP-Mg inhibited form (14). The propensity of the ΔNC + β-T165S mutant (ΔNC’ mutant) to achieve the ADP-Mg inhibited form was intermediate, as it was greater than the wild-type F₁-ATPase but less than the ΔNC mutant. When we observed rotation of the ΔNC’ mutant, the number of actively rotating molecules was much smaller than in wild-type F₁-ATPase; even if they rotated, they stopped readily and stayed in the pausing phase for a long time (Fig. 3E). The inclusion of LDAO, which is known to be a potent activator of the ADP-Mg inhibited form (13, 21), resulted in a sharp decrease in the length of the long pauses (Fig. 3F). The same effect of LDAO was observed for the wild-type F₁-ATPase (data not shown). These results support the contention that the state of F₁-ATPase during the pause is the ADP-Mg inhibited form.

Kinetic Analysis of the Pause. We collected the data from all pauses longer than 1 s from many rotating molecules at 2 mM ATP. The duration of each pause was plotted as the time-dependent decay of the number of pausing F₁-ATPase, which remained in the pause and had not yet resumed rotation (Fig. 4A). The pauses observed for the rotation of probes of various lengths were analyzed together because the load, as shown in the previous section, does not affect the pausing (Fig. 2). The plot could not be fitted with a single exponential but was well fitted with the sum of two exponentials, suggesting that there are at least two kinds of pauses: the short-lived pause with a life-time (τsp) of 1.7 s, and the long-lived pause with a life-time (τlp) 32.3 s. On average, actively working F₁ in the presence of 2 mM ATP spent 33.5% of its time in rotation, 4.5% in the short pause, and 62% in the long pause. A curve calculated from the long pause alone (the solid line in Fig. 4A) indicates that most (96% of the total) of the observed pauses longer than 10 s belong to the category of the long pauses. The frequency of the incidence for the rotating F₁-ATPase to pause also was examined. For the short pauses, however, the incidence did not seem to be statistically random, and simple analysis was impossible. For the long pauses, the periods between one long pause (>10 s) and the next long pause (>10 s) were collected and analyzed. They were plotted as the time-dependent decay of the number of the “rotating” F₁-ATPases that escaped from one long pause but had not lapsed into the next long pause yet (Fig. 4B). The data were fitted with a single exponential with a 34 s life-time (τlp). This value was obtained for the 72 pauses longer than 10 s and should be corrected by the uncounted long pauses shorter than 10 s (calculated to be 43). The corrected value of τlp is 22 s; that is, rotating F₁-ATPase lapes into the long pauses after 22 s, on average.

Kinetic Analysis of the ADP-Mg Inhibited Form. To compare the kinetic parameters of the long pauses to those of the ADP-Mg inhibited form, we estimated from the bulk-phase experiments the rates of conversion from the active to the ADP-Mg inhibited form (ka→i) and those of reverse conversion (ki→a) during catalysis, with the equation:

\[ y = N_{lp} \cdot exp(-y / \tau_{lp}) + N_{sp} \cdot exp(-y / \tau_{sp}) \]

where \( N_{lp} \) and \( \tau_{lp} \) are the number of the long pauses, short pause and long pause, in the following manner: \( y = N_{lp} \cdot exp(-y / \tau_{lp}) \) and \( N_{sp} \cdot exp(-y / \tau_{sp}) \). The decay of the number of the rotating F₁-ATPase in 2 mM ATP-Mg was fitted with a single exponential function (red line): const. \cdot exp (–kapp \cdot t), where kapp (the apparent rate constant for inhibition) = 0.054 s⁻¹.

Fig. 4. Kinetic analyses of the rotation of single molecules. (A) The decay of the number of the pausing F₁-ATPases in 2 mM ATP-Mg. The data are fitted with a double exponential function (dashed line) assuming two kinds of pauses, short pause and long pause, in the following manner: \( y = N_{lp} \cdot exp(-y / \tau_{lp}) + N_{sp} \cdot exp(-y / \tau_{sp}) \), where \( N_{lp} \) (number of the long pauses) = 340, \( N_{sp} \) (number of the long pauses) = 115, \( \tau_{lp} \) (life-time of the short pause) = 1.7 s, \( \tau_{lp} \) (life-time of the long pause) = 32.3 s, with an error of about ±15%. The solid line shows a single exponential curve calculated from \( N_{lp} = 115 \) and \( \tau_{lp} = 32.2 \) s. (B) The decay of the number of the rotating F₁-ATPase in 2 mM ATP-Mg. The rotation between pauses longer than 10 s were measured. The solid line shows a single-exponential function with \( k_{-\psi} = 1 / \tau_{lp} \) (rate of conversion from rotation to the long pause) = 0.029 s⁻¹.

Fig. 5. Kinetic analyses of ADP-Mg inhibition as derived from the ATPase activity measurements. (A) The time course of ATP hydrolysis catalyzed by wild-type F₁-ATPase (black line) in Buffer A at 2 mM ATP-Mg. Dashed line, the initial rate; blue line, steady-state rate. (B) Inactivation of ATPase activity (black line) was fitted with a single exponential function (red line): const. \cdot exp (–kapp \cdot t), where kapp (the apparent rate constant for inhibition) = 0.054 s⁻¹.
When the ATP hydrolysis assay was started by the addition of F1-ATPase to the solution, the initial rapid hydrolysis decelerated within 1 min and reached a slow steady-state of hydrolysis (Fig. 5A). This time course suggests that the initial rapid hydrolysis is catalyzed by the F1-ATPase free from ADP-Mg inhibition, and that the ADP-Mg inhibited form gradually accumulates, causing a decrease in the rate of hydrolysis. The final, steady-state hydrolysis at a stable rate is catalyzed by the F1-ATPase free from ADP-Mg inhibition (Fig. 5A). This time course suggests that the initial rapid hydrolysis is catalyzed by the F1-ATPase free from ADP-Mg inhibition, and that the ADP-Mg inhibited form gradually accumulates, causing a decrease in the rate of hydrolysis. The final, steady-state hydrolysis at a stable rate is catalyzed by the F1-ATPase free from ADP-Mg inhibition. Based on the above scheme, the dependency of the rates of ADP-Mg inhibition were ATP-concentration dependent with an apparent $K_i$ for ATP of 4 $\mu$M (13) or 13 $\pm$ 7 $\mu$M (22). By the same procedures, life-times in the presence of 100 mM potassium phosphate (Pi) and those of ΔNC mutant in 100 mM Pi with or without LDAO also was obtained. It is clear that the values of $\tau_i$ and $\tau_p$ change in parallel and are always in the same range.

Orientation of γ Subunit in the ADP-Mg Inhibited Form. The γ subunit makes a 120° rotation by using a single ATP and waits at this position (ATP-waiting position) for the next ATP. At low-ATP concentrations, this stepping rotation is well observed (8). As described, the pausing caused by ADP-Mg inhibition takes place in three angular positions that are separated by 120° from each other. To compare the relative positions of the long pause and the ATP-waiting state, we recorded the stepping rotation at 20 nM ATP. Each discrete 120° step was caused by ATP binding. (B) The pausing positions at 2 mM ATP. A reaction mixture containing 2 mM ATP was slowly introduced into the same chamber. Instead of the stepwise rotations, we could detect both continuous rotation and long pauses, as in Fig. 1. The angles of the long pauses, which are assumed to be caused by ADP-Mg inhibition, are shown. The positions of probes during the pausing period are presented at each of three angular positions.

### Table 1. Lifetimes obtained from single-molecule analysis and ATPase activity measurements under several conditions

<table>
<thead>
<tr>
<th>Protein</th>
<th>ATP, $\mu$M</th>
<th>Single molecule pausing time ($t_a$) (s)</th>
<th>ATPase ADP-Mg inhibition time ($t_p$) (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>2000</td>
<td>32</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>31</td>
<td>71*</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>48</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>77</td>
<td>59</td>
</tr>
<tr>
<td>$\Delta$NC'</td>
<td>2000</td>
<td>694</td>
<td>1429</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>167</td>
<td>143*</td>
</tr>
</tbody>
</table>

*Buffer contains 100 mM potassium phosphate.
†Buffer contains 100 mM potassium phosphate and 0.1% LDAO.
nM ATP at first, then infused the buffer containing 2 mM ATP into the observation chamber and observed the long pauses of the same molecule. As seen in Fig. 6, the positions of ADP-Mg inhibition and the ATP-waiting positions are significantly different and separated by 37.6° ± 2.5°. The same particle started stepwise rotation again at 0°, 120°, and 240° after exchange of the buffer for 200 nM ATP.

At 200 nM ATP, both 120° steps caused by ATP-waiting and the pauses caused by the ADP-Mg inhibition were observed frequently enough for analysis (Fig. 7). Under these conditions, the ATP-waiting period is short (∼0.4 s), whereas ADP-Mg inhibition continues for a long period (∼30 min). Therefore, we can easily separate these two kinds of pauses: the result of the short ones define the ATP-waiting state, and long ones are the result of ADP-Mg inhibition. Moreover, the two paused states can be distinguished by the angle of rotation after the pause. Whereas steps between two adjacent ATP-waiting positions were 120°, the long pauses always started after ∼90° rotation.

**Discussion**

Here, we showed that individual single molecules of active F$_1$-ATPase alternate between at least three phases: continuous rotation, short pauses, and long pauses during catalysis at saturating ATP concentrations. The origin of the short pause is not yet known, but the long pause has been identified as the ADP-Mg inhibited state that has been recognized previously by bulk-phase kinetics. ADP-Mg inhibition is a common feature of the F$_1$-ATPases and ATP synthases from various sources and, hence, likely to have functional significance. ATP hydrolysis and ATP-driven proton-pumping activity by ATP synthase are susceptible to this inhibition. Conversely, the ADP-Mg inhibition is relieved when membrane potential exists (23); ATP synthesis by ATP synthase is completely free from this inhibition (24, 25).

F$_1$-ATPase rotates in discrete 120° steps, consistent with sequential ATP hydrolysis on the three β subunits. Recently, it was revealed that each 120° step is further divided to the 90° and 30° substeps, each taking only a fraction of a msec (15). ATP binding drives the 90° substep. The enzyme spent about 2 msec on average at the 90° position (the 90° dwell), irrespective of medium ATP concentrations. During the 90° dwell, at least two 1-msec events occur; the latter one, resetting the enzyme to start the next cycle, accompanies the 30° rotation. ADP-Mg inhibition arises at ∼83°, measured from the ATP-waiting position (Figs. 6 and 7), and therefore it is likely to be generated in a stochastic manner at the 90° dwell by some off-catalytic-pathway event.

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