Principal Role of the Arginine Finger in Rotary Catalysis of F$_1$-ATPase

Yoshihito Komoriya$^\ddagger$, Takayuki Ariga$^\ddagger$, Ryota Iino*, Hiromi Imamura$^\ddagger$, Daichi Okuno$^\ddagger$, and Hiroyuki Noji$^{1\dagger}$

From the $^\ddagger$Graduate School of Frontier Bioscience, Osaka University, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan, the $^\ddagger$Department of Applied Physics, School of Engineering, The University of Tokyo, Tokyo 113-8656, Japan, and the $^{1\dagger}$Department of Applied Chemistry, School of Engineering, The University of Tokyo, Tokyo 113-8656, Japan

F$_1$-ATPase (F$_1$) is an ATP-driven rotary motor wherein the $\gamma$ subunit rotates against the surrounding $\alpha_3 \beta_3$ stator ring. The 3 catalytic sites of F$_1$ reside on the interface of the $\alpha$ and $\beta$ subunits of the $\alpha_3 \beta_3$ ring. While the catalytic residues predominantly reside on the $\beta$ subunit, the $\alpha$ subunit has 1 catalytically critical arginine, termed the arginine finger, with stereo-geometric similarities with the arginine finger of G-protein-activating proteins. However, the principal role of the arginine finger of F$_1$ remains controversial. We studied the role of the arginine finger by analyzing the rotation of a mutant F$_1$ with a lysine substitution of the arginine finger. The mutant showed a 350-fold longer catalytic pause than the wild-type; this pause was further lengthened by the slowly hydrolyzed ATP analog ATP$\gamma$S. On the other hand, the mutant F$_1$ showed highly unidirectional rotation with a coupling ratio of 3 ATPs/turn, the same as wild-type, suggesting that cooperative torque generation by the 3 $\beta$ subunits was not impaired. The hybrid F$_1$ carrying a single copy of the $\alpha$ mutant revealed that the reaction step slowed by the mutation occurs at $+200^\circ$ from the binding angle of the mutant subunit. Thus, the principal role of the arginine finger is not to mediate cooperativity among the catalytic sites, but to enhance the rate of the ATP cleavage by stabilizing the transition state of ATP hydrolysis. Lysine substitution also caused frequent pauses because of severe ADP inhibition, and a slight decrease in ATP-binding rate.

F$_1$-ATPase (F$_1$)$^2$ is a water-soluble portion of the F$_1$F$_0$-ATP synthase, and a rotary motor protein driven by ATP hydrolysis (1–5). Bacterial F$_1$ is composed of $\alpha$, $\beta$, $\gamma$, $\delta$, and $\epsilon$ subunits with a stoichiometry of 3:3:1:1:1. The minimum complex that functions as a rotary motor is the $\alpha_3 \beta_3 \gamma$ subcomplex. Three $\beta$ subunits and 3 $\alpha$ subunits form the stator ring, in which these subunits are alternately arranged. The rotary shaft, namely, the $\gamma$ subunit, is accommodated in the central cavity of the $\alpha_3 \beta_3$ stator ring. The catalytic reaction centers are located at the $\alpha$/$\beta$ interfaces. Although most of the residues forming the catalytic site are on the $\beta$ subunits, the $\alpha$ subunits contain 1 catalytically critical and well-conserved arginine residue, termed the arginine finger, which is the focus of the present study.

The rotation of F$_1$ can be visualized by optical microscopy (6–9). The $\gamma$ subunit of F$_1$, from various species studied to date, rotates in a counterclockwise direction. The unidirectionality of the rotation is supported by the intrinsic cooperative torque generation of the 3 $\beta$ subunits (10). The step size of the rotation is 120°, each coupled with single turnover of ATP hydrolysis (8). The 120° step is further divided into 80° and 40° substeps. The 80° substep is triggered by ATP binding and ADP release, each of which occurs on different $\beta$ subunits. The 40° substep is initiated by ATP hydrolysis and release of inorganic phosphate (P$_i$), which also occurs on different $\beta$ subunits. The angular positions of the dwell before the 80° and 40° substeps are referred to as the ATP-binding (ATP-waiting) angle and the catalytic angle, respectively. The basic reaction scheme for the rotation and catalysis of F$_1$ has been recently established (11), although several uncertainties do remain (12). Each $\beta$ subunit binds to ATP when the $\gamma$ subunit is oriented at a specific angle, and the binding angles for the individual $\beta$ subunits differ by 120°. Each $\beta$ subunit catalyzes ATP hydrolysis at 200° from its ATP-binding angle (13). Subsequently, ADP and P$_i$ are released at 240° (14) and 320° (11), respectively. When the $\gamma$ subunit returns to the original position, the $\beta$ subunits initiate the next round of catalysis by binding to a new ATP.

The crystal structure of F$_1$ reveals the structural basis of the chemomechanical coupling of F$_1$ (15). The 3 $\beta$ subunits have distinct ligand-bound states and conformations: one $\beta$ is bound...
The Arginine Finger and Rotary Catalysis of F$_1$-ATPase

The Arginine Finger and Rotary Catalysis of F$_1$-ATPase

To an ATP analog, AMP-PNP; a second is bound to ADP and azide (16); and the third binds to none. These 3 states of $\beta$ are referred to as $\beta_{TP}$, $\beta_{DP}$, and $\beta_{empty}$, respectively. A cross-linking experiment in a single-molecule rotation assay established that the crystal structure represents the catalytic dwell state (17), and that $\beta_{TP}$, $\beta_{DP}$, and $\beta_{empty}$ represent the 80°, 200°, and 320° states of the above mentioned reaction scheme, respectively (18). Both the $\beta_{TP}$ and $\beta_{DP}$ states assume a closed conformation in which the C-terminal domain rotates inward toward the $\gamma$ subunit and enclose the bound nucleotide. On the other hand, $\beta_{empty}$ assumes an open conformation by the outward rotation of the C-terminal domain. Therefore, the widely accepted view is that the $\beta$ subunit undergoes a conformational transition between the open and closed states during nucleotide binding/disassociation, inducing unidirectional rotation of the central $\gamma$ subunit (19, 20).

The $\beta_{TP}$ and $\beta_{DP}$ conformations closely resemble each other. Both $\beta$ subunits bind to the ATP analog MgADP-BeF$_3$ (21) or the transition state analog MgADP-AlF$_4$ (22), simultaneously. A recent single-molecule experiment has indicated that $\beta_{DP}$ is the catalytically active state for ATP cleavage (18). The conformations of the catalytic sites on $\beta_{TP}$ and $\beta_{DP}$ are different at the $\alpha/\beta$ interfaces. $\beta_{TP}$ forms a relatively open interface with the neighboring $\alpha$ subunit ($\alpha_{TP}$), whereas $\beta_{DP}$ forms a closed interface with $\alpha_{DP}$, suggesting this is the highest affinity site. However, based on nucleotide titration experiments, it was shown that $\beta_{TP}$ has the highest affinity for nucleotide (23). This positional rearrangement of the $\alpha$ subunit induces proximity of the well-conserved arginine residue to the $\beta$- and $\gamma$-phosphates of the bound ATP (Fig. 1). The stereogeometric position of this arginine residue is similar to that of the catalytically crucial arginine residue found in the $\alpha$ subunit of trimeric G-proteins and in GTPase-activating proteins (GAPs) for small G-proteins (15, 24, 25). The latter is termed the “arginine finger,” and upon binding to small G-proteins, GAPs present this residue to the $\beta$- and $\gamma$-phosphate of bound GTP, and remarkably accelerate their hydrolysis by stabilizing the transition state.

In a genetic screen, the conserved arginine of Escherichia coli F$_1$ (EF$_1$) was originally found to be one of the catalytically important residues on the $\alpha$ subunit (26). Biochemical studies then confirmed a critical role for this arginine (26–29). In particular, a nucleotide titration study with a transition state analog, MgADP-fluoroaluminate, showed that this arginine stabilizes the transition state of hydrolysis and accelerates the rate of hydrolysis (28). Several theoretical studies also support the view that this arginine contributes to lowering the energy level of the post-hydrolysis state compared with the pre-hydrolysis state (30, 31). A crystal structure of mitochondrial F$_1$ (MF$_1$) revealed that the guanidinium group of the arginine finger is positioned at 1 Å closer to the $\beta$-phosphate in the $\beta_{DP}$ than that in the $\beta_{TP}$ (Fig. 1) (21). This positional rearrangement is proposed to initiate ATP hydrolysis. The arginine finger of EF$_1$ is $\alpha$R376, and corresponds to $\alpha$R373 in MF$_1$, and $\alpha$R364 in the thermophilic Bacillus PS3 F$_1$ (TF$_1$).

The cysteine mutant of the arginine finger of F$_1$ was severely impaired in multisite catalysis (26), which is the highly active mode where 3 catalytic sites sequentially hydrolyze ATP to reach the maximum hydrolysis rate. However, this mutation only slightly impaired unisite catalysis, a slow catalysis mode of F$_1$ that proceeds only when one of the catalytic sites is occupied by a nucleotide and the others are unoccupied. Unisite catalysis is considered indicative of the intrinsic catalytic ability of F$_1$ that is independent of the allosteric interactions with other catalytic sites. Subsequent biochemical research showed that alanine or lysine mutations of the arginine finger also caused almost complete loss of multisite catalysis (by a factor of 10$^2$), although unisite catalysis was not affected (27). From these observations, it was concluded that the arginine finger is not directly involved in the enhancement of ATP hydrolysis rate of F$_1$, but it has a critical role in mediating cooperativity among the 3 catalytic sites to facilitate multisite catalysis.

Thus, although the importance of the arginine finger in catalysis is well recognized, its principal role is still unclear. In this study, we investigated the role of the arginine finger of TF$_1$ by analyzing the rotational behavior of the lysine mutant. This allowed us to elucidate the impact of this mutation on the intrinsic catalytic power and the efficiency of allosteric cooperative communication among the catalytic sites of F$_1$.

Experimental Procedures

Preparation of Proteins—The expression plasmid for the mutant $\alpha_{TP}\beta_{TP}\gamma$ subcomplex of F$_1$-ATPase ($\alpha_{TP}\beta_{TP}\gamma$ subcomplex was referred to as F$_1$ in this study) was generated using conventional gene manipulation techniques. The $\alpha$ (His$_6$ at N terminus/C193S)$_3\beta$ (His$_{10}$ at N terminus)$\gamma$ (S108C/I211C) subcomplex of F$_1$ from the thermophilic Bacillus PS3, which was modified for the rotation assay, and the $\alpha$ (His$_6$ at N terminus/C193S/R364K)$\beta$ (His$_{10}$ at N terminus)$\gamma$ (S108C/I211C) subcomplex were expressed in Escherichia coli, purified, and biotinylated as described previously (32). For simplicity, the former was referred to as wild-type F$_1$, because the effect of the mutations on the hydrolysis of ATP was minor, and the latter was referred to as F$_1$($\alpha$R364K). Proteins used for preparation of hybrid F$_1$, $\alpha$ (C193S)$\beta$ (S108C/I211C) subcomplex and monomer $\alpha$ (His$_6$ at N terminus/C193S/R364K), were prepared as described previously (13). The former and latter are referred to as the His-tag-less wild-type F$_1$ and $\alpha$ (R364K), respectively.

Depletion of ADP Bound to F$_1$.—The ADP tightly bound to purified F$_1$ was removed by a method described previously (33), with modification. EDTA in the buffer solution for the purified F$_1$ was removed by exchanging with another buffer (50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)-KOH, 50 mM NaCl, pH 8.0) by using a PD10 column (GE Healthcare). Then, calf intestine alkaline phosphatase (Wako, Japan) was added to the F$_1$ solution (140 units of alkaline phosphatase per 1 mg of F$_1$) and incubated overnight at room temperature. Tightly bound ADP is released into the medium at a slow rate and is re-bound at a fast rate. Alkaline phosphatase cleaves released ADP to AMP, and then, to adenosine. Therefore, alkaline phosphatase promotes the removal of bound ADP from F$_1$. After incubation, F$_1$ was separated from calf intestine alkaline phosphatase and adenosine by size exclusion chromatography (Superdex 200 10/300 GL; GE Healthcare) for use in experiments. The ATP hydrolysis activity of the wild-type F$_1$ and F$_1$($\alpha$R364K) was measured with an ATP-regenerating system at 25 ± 2 °C (18).
The Arginine Finger and Rotary Catalysis of F1-ATPase

Rotation Assay of F1(αR364K)—The rotation assays of F1(αR364K) was carried out as described previously (18). A streptavidin-coated magnetic bead (<0.3 μm) was prepared from a commercially available one with large variations in size (nominal diameter = 0.76 μm; Thermo Scientific). The original magnetic bead suspension was sonicated for 5 s with a bath-type sonicator, and centrifuged at 3000 × g for 2 min at 4 °C to remove large beads. Then the supernatant, containing small beads, was collected and centrifuged at 20,000 × g for 5 min at 4 °C. The pellet of the small beads was suspended into a buffer and used as a rotation probe attached to the rotor γ subunit, and Ni-nitritolatriacetic acid-modified glass was used as a surface immobilizing stator αβ3 ring. Because the F1(αR364K) easily lapsed into the long inhibited state, the rotation assay was difficult to perform. This problem was avoided using magnetic beads that can be manipulated with magnetic tweezers to mechanically activate the inhibited F1 (34). The images of rotating beads were recorded at a rate of 30 frames/s and analyzed as previously described (18).

Preparation of Hybrid F1—Hybrid F1, containing one α(364K), α(His6 at N terminus/C193S/R364K)α(C193S)2β3γ(S108C/I211C), termed F1(1×αR364K), was prepared as previously described (13) except for the introduced mutation. The biotinylated His tag-less wild-type F1 was mixed with the purified monomer of the His-tagged α(364K) at a 3:1 molar ratio (F1;α) in the presence of 2 mM urea, where F1 was disassembled into subunits. The hybrid F1, including the His-tagged α(R364K), was then reconstituted by dilution with urea. The remaining non-His-tagged F1 and subunits were removed by purifying the desired complex via Ni-NTA affinity chromatography (Qiagen). His-tagged monomers were also removed by size exclusion chromatography (Superdex 200 10/300 GL; GE Healthcare). This preparation could be a mixture of the hybrid F1 containing 1, 2, and 3 α(R364K) subunits. However, an F1(1×αR364K)-enriched sample was obtained by the mixing ratio described above. Previously, we had obtained a sample with ~90% of the hybrid F1 carrying one βE190D mutant at the same mixing ratio (13). Here, we obtained a sample containing almost the same percentage of F1(1×αR364K), which was confirmed by observing its rotation (See “Results”), although the theoretical probability according to the Poisson statistics predicted the slightly lower percentage of the F1(1×αR364K) in the population.

Rotation Assay of Hybrid F1—Rotation assays of the F1(1×αR364K) were carried out using duplex of polystyrene beads (0.2 μm; Polysciences) coated with NeutrAvidin (Thermo Scientific) as a rotation probe attached to the rotor γ subunit, and silane-coated coverglass as a surface immobilizing stator αβ3 ring, as previously described (13). Because the F1(1×αR364K) had only one His-tagged α subunit, fixation to the Ni-nitritolatriacetic acid-modified glass surface via His tags in the 3 α and 3 β subunits, as used in the rotation assay of F1(αR364K) (18), was not possible. To achieve adequate observation of the rotation, the F1(1×αR364K) molecules were fixed to the glass surface via nonspecific hydrophobic adsorption, and polystyrene beads were used as a rotation probe to avoid undesirable absorption to the surface directly. The images of rotating beads were recorded at 500 or 1,000 frames/s, and analyzed as previously described (35).

RESULTS

ATP Hydrolysis Activity—The arginine finger of TF1, αR364, was substituted with lysine to produce the mutant F1(αR364K) (Fig. 1). Unlike wild-type TF1, F1(αR364K) tightly holds bound ADP, and hence, nucleotide-depleted F1 was not obtained using the conventional purification procedure. Similarly, it was previously reported that the bound nucleotide on arginine-finger mutants of EF1 was resistant to the conventional nucleotide-
The Arginine Finger and Rotary Catalysis of F<sub>1</sub>-ATPase

depletion procedures (28). Therefore, in this study, F<sub>1</sub>(αR364K) was treated with alkaline phosphatase to completely degrade the nucleotide to nucleoside. After this treatment, bound nucleotides were not detected (considerably <0.1 molecule/F<sub>1</sub>) in the measurement of bound nucleotides (36). Fig. 2A shows the time course of ATP hydrolysis for F<sub>1</sub>(αR364K) and wild-type F<sub>1</sub>, measured using an ATP-regenerating system. After an initial burst, the activity exponentially decayed until it reached a steady state. This is typical of the time-dependent inactivation observed in TF<sub>1</sub>. Initially, all the F<sub>1</sub> molecules in the assay mixture are fully active and show strong ATPase activity. Subsequently, some fraction of the molecules stochastically lapses into an inactive state, called the ADP-inhibited form, in which the catalytically produced ADP tightly binds to F<sub>1</sub> and inhibits catalysis and rotation (37). Because the inhibited F<sub>1</sub> spontaneously regains its catalytic activity by releasing the bound ADP into the solution, the apparent ATPase activity is not completely diminished, and a certain level of steady-state activity is retained. The hydrolysis rate at the initial phases measured at 1 mM ATP was 1.0 ± 0.04 s<sup>-1</sup> (mean ± S.D., n = 3), which is 350 times slower than that of the wild-type TF<sub>1</sub> (8). This corroborates the previous findings on EF<sub>1</sub>, indicating that the lysine substitution at the arginine finger caused suppression of ATPase activity by a factor of ~10<sup>3</sup> (27, 28). By fitting the time course of ATPase activity, the rate constants for inactivation and activation of F<sub>1</sub>(αR364K) were determined to be 0.091 s<sup>-1</sup> and 0.0092 s<sup>-1</sup>, respectively, 2 times faster and slower than those of wild-type, respectively. The resultant equilibrium constant of the active state was four times lower than that of the wild-type TF<sub>1</sub> (8).

Rotatory motion of F<sub>1</sub>(αR364K) was observed in the single-molecule assay by using a magnetic bead as a rotation probe (38). At 1 mM ATP, the F<sub>1</sub>(αR364K) showed continuous rotation in the counterclockwise direction (Fig. 2B), the same as the wild-type. This suggested that F<sub>1</sub>(αR364K) retained the intrinsic cooperativity among the 3 catalytic sites, contradicting the previous finding that mutation of the arginine finger impairs multisite catalysis (26, 27). However, the rotation rate of the mutant was only 0.37 rps (see Fig. 3A), which is much...
The Arginine Finger and Rotary Catalysis of F$_1$-ATPase

lower than that of the wild-type (5–10 rps under the same conditions) (39). This decrease in the rotation rate was not due to slow angular velocity of the mechanical rotation process, i.e. lower rotary torque. As seen in Figs. 2D and 3, B and C, F$_1$(αR364K) rotated with 120° steps, each separated by a pause. This means that the rotation rate is determined by a slow chemical step that causes a pause. Under the same conditions, the wild-type does not show a clear stepping rotation, because the catalytic dwell of ~2 ms is too short to be resolved in the rotation assay performed using a magnetic bead that obscures the catalytic pause due to the large viscous friction of water. Another remarkable feature of F$_1$(αR364K) was that its continuous rotation was terminated by a long pause after several turns, as suggested from the bulk ATPase assay (Fig. 2B). The histogram of the rotating time until the long pause is shown in Fig. 2C. The distribution of the rotating time exponentially decayed with a time constant of 13 s. This value is in agreement with the time constant of inactivation (11 s) determined in the bulk ATPase assay. As reported for the ADP inhibition of wild-type F$_1$ (34), F$_1$(αR364K) also resumed its activity and the continuous stepwise rotation when the magnetic beads were forcibly rotated over 90° in the forward direction. Furthermore, the angular position of F$_1$(αR364K) during the long pause (Fig. 2, D and E) coincided with that during the short pause, the latter of which was identified as the catalytic pause (see below). These findings indicated that the long-paused state represented the ADP inhibited form. The average number of rotation steps of 120° before lapsing into the ADP inhibited form is only 14.4, suggesting a strong tendency of F$_1$(αR364K) to lapse into the inhibited form. In following experiments, we analyzed the active state of F$_1$(αR364K). When molecules lapsed into the ADP inhibited form, they were reactivated using magnetic tweezers.

Michaelis-Menten Kinetics of Rotation—The rotation rate was determined at several ATP concentrations ranging from 500 nM to 10 mM. At <500 nM ATP, it was extremely difficult to finding rotating particles, probably because of severe ADP inhibition. The rotation rate follows a Michaelis-Menten-type curve against ATP concentration (Fig. 3A), yielding a maximum rotation rate ($V_{\text{max}}$) of 0.37 rps and a Michaelis constant ($K_m$) of 0.75 μM. As discussed above, the $V_{\text{max}}$ is not suppressed by the viscous friction of water on the bead, but by the interfering pauses (Fig. 3, B and C). Therefore, this value represents the maximum catalytic rate of F$_1$(αR364K). Even when 40-nm colloidal gold was used as a non-frictional probe, the rotation rate remained the same (data not shown). The ATP hydrolysis rate (1.1 s$^{-1}$) expected from the $V_{\text{max}}$, assuming a coupling ratio of 1 ATP per 120° step, is largely consistent with that at 2 mM ATP (1.1 ± 0.06 s$^{-1}$, mean ± S.D., n = 3) as determined in the bulk ATPase assay. Thus, the coupling ratio is also not impaired in F$_1$(αR364K).

The $K_m$ of the mutant was less than the genuine $K_m$ of the wild-type, i.e. 15 μM, that was determined using 40-nm colloidal gold as a non-frictional probe (B). The binding rate constant for ATP, that was determined from $3 \times V_{\text{max}}/K_m$ was 1.5 $\times 10^6$ M$^{-1}$s$^{-1}$. Although this value is distinctly lower than that of the wild-type (2.6 $\times 10^7$ M$^{-1}$s$^{-1}$), the impact of the αR364K mutation is not very large compared with the effect on $V_{\text{max}}$, which is 350 times lower than that of the wild-type.

Analysis of Stepping Rotation—Fig. 3B shows the time-course of the rotation observed at several ATP concentrations. Each trace shows the stepping behavior. As shown in the histogram of the angular position during the continuous stepping rotation (Fig. 3C), F$_1$(αR364K) made intervening pauses at 3 positions, at a distance of 120° from each other, at ATP concentrations ranging from 10 μM to 10 mM. Around the $K_m$ region where the ATP-waiting dwell becomes comparable with the duration of the intervening pause found at saturating ATP concentration, the histogram showed 6 peaks (1 μM in Fig. 3C). This indicates that F$_1$ makes substeps of 80° and 40°, pausing at the binding and catalytic angles. This finding suggests that the intervening pauses found at saturating ATP concentration correspond to the catalytic dwell, not to the ATP-waiting dwell. The distribution of the duration time at the catalytic angle (Fig. 3D) also supports this notion; the histograms of the duration time showed almost identical exponential decay across all ATP concentrations tried.

Buffer Exchange Experiment—To further confirm that the intervening pauses observed at saturating ATP concentration correspond to the catalytic dwell, we conducted a buffer exchange experiment where the ATP concentration was changed from 1 mM to 500 mM. A set of representative time courses is shown in Fig. 4A. At 1 mM ATP, F$_1$(αR364K) showed a 120° stepping rotation. When the buffer was changed to 500 mM ATP, the mutant showed substeps, pausing at the binding and catalytic angles. This finding suggests that the intervening pauses found at saturating ATP concentration correspond to the catalytic dwell, not to the ATP-waiting dwell. The histogram of the angular position showed additional peaks at +40° from the original pausing angles. These peaks disappeared after ATP concentration was returned to 1 mM. For statistical analysis, we compared the relative angular position ($\Delta\theta_i$) of the peaks that appeared at 500 nM ATP to the
original pauses at 1 mM ATP in the buffer exchange experiment. For comparison, the relative angular position of the original pause (Δθ2) at the second set of 1 mM condition was also analyzed. As expected, Δθ2 was 38 ± 6.2°, and Δθ1 was −8.9 ± 9.4° (n = 21, Fig. 4C). Thus, we confirmed that F1(R364K) pauses at the catalytic angle.

ATPγS-driven Rotation—The recently established reaction scheme of catalysis and rotation suggests that F1 undergoes 2 reactions at the catalytic angle: cleavage of the bound ATP on β at the 200° state, and release of P1 from β at the 320° state. Therefore, there are (at least) 2 possibilities for the reduction in the reaction rate by the αR364K mutation: slow ATP hydrolysis and slow P1 release. Considering that several studies suggest that the arginine finger is responsible for the hydrolysis step (21, 28, 30, 31), we assessed the possibility that αR364K drastically reduces the hydrolysis rate. For this, we used an ATP analog (ATPγS), which is known to be slowly hydrolyzed—and found that it specifically decelerated the hydrolysis step by 60 times compared with that of ATP (Fig. 5) (40). In the presence of 1 mM ATPγS, F1(αR364K) showed an extremely slow rotation of around 0.008 rps. To our knowledge, this is the slowest unidirectional rotation of F1 reported thus far. As evident from the time courses of the rotation (Fig. 5A), F1 makes long pauses at 3 positions, suggesting that ATPγS lengthens the catalytic dwell. However, the histogram of the duration times showed a convex distribution (Fig. 5B), which can be explained by a consecutive two-reaction model. Fitting of the distribution with the consecutive reaction model yielded time constants of 47 and 4.7 s. The longer time constant is consistent with the expected time constant for the ATPγS hydrolysis of F1(αR364K); 60 × the ATP hydrolysis dwell of F1(αR364K), i.e. 0.7 s, is 42 s. This finding supports the notion that F1(αR364K) slows the ATP cleavage step. The time constant of the second reaction was also lengthened, i.e. 4.7 s, possibly indicating that the mutation affects P1 release because the arginine finger is also involved in P1 binding/release (41–43).

**FIGURE 5. Rotation of F1(αR364K) driven by ATPγS.** A, examples of time course of rotation. Each trace represents rotation of different single molecules. B, distribution of duration times of the pauses. Solid line shows fit of the double-exponential functions with time constants of 47 ± 2.1 s and 4.7 ± 0.58 s (mean ± S.E.), assuming the two consecutive reactions. Measurements were carried out at 1 mM ATPγS.

**FIGURE 6. Rotation of a hybrid F1, F1(1×αR364K).** A, examples of time course of rotation at different ATP concentrations. Buffer was exchanged during observation and ATP concentration was increased from 500 nM (green-slow trajectory) to 10 mM (blue-fast trace). B, distribution of angles of the same observation in A, C, distribution of duration times of the ATP-waiting dwells at 500 nM ATP (top) and the catalytic dwells at 10 mM ATP (bottom). Solid lines show fits of the single-exponential decay function with time constants of 1.10 ± 0.02 s (top) and 606 ± 2.22 ms (bottom) (mean ± S.E.). Note that duration times at 10 mM ATP were counted from the observations without buffer exchange (data not shown), and ATP dependence of the waiting dwells were confirmed by the buffer exchange in each case. D, distributions of angular difference (Δθ1, or Δθ2 defined in B) between the pausing angles before and after buffer exchange. The averaged values of Δθ1 and Δθ2 are 160 ± 10° and 4.6 ± 10° (mean ± S.D., n = 20), respectively.

**Rotation of Hybrid F1, Carrying 1 αR364K Subunit and 2 Wild-type α Subunits—**To verify the above hypotheses, we attempted to determine the absolute angular position of the lengthened catalytic pause in the 360° reaction scheme by using a hybrid F1 that carries a single α(R364K) subunit and 2 wild-type α subunits: F1(1×αR364K). In this hybrid, if the hydrolysis were slowed by the mutation, the incorporated α(R364K) would cause a pause at 200° from its own ATP-waiting angle. In case of P1 release, the mutant would cause a pause at +320° (−40°) from the binding angle. To discriminate these two possibilities, we observed the rotation of F1(1×αR364K) prepared by mixing the His tag-less wild-type F1 with monomer α(R364K) at a 3:1 molar ratio. The fraction of F1(1×αR364K) expected from the mixing ratio is ~90% (13). As expected, the major fraction of the observed particles (~84%) showed a single pause of ~0.6 s per revolution at 10 mM ATP, corresponding to the lengthened catalytic pause of αR364K (data not shown, but very similar to the blue-fast trajectory in Fig. 6A).

Next, F1(1×αR364K) was subjected to a rotation assay at a low ATP concentration of 500 nM, where the αR364K should cause a distinctly longer ATP-waiting pause than that of wild-type, in addition to a lengthened catalytic pause. The typical rotation of F1(1×αR364K) showed 2 pauses per revolution (green-slow trajectory in Fig. 6A), and the angular difference between these pauses (Δθ1) was around 160° (top histogram in Fig. 6B). Note that a few molecules showed multiple pauses during the rotation, implying these were the hybrid F1's with 2

*The Arginine Finger and Rotary Catalysis of F1-ATPase*
or 3 mutant α subunits; the data for these molecules were, therefore, omitted from the analysis. After the rotating particles were noted, ATP concentration was changed to 10 mM (blue fast trajectory in Fig. 6A). While one pause corresponding to the ATP-waiting pause of the αR364K disappeared, the other pause persisted (bottom histogram in Fig. 6B). Hence, we could identify the angular position of the catalytic pause slowed by the αR364K mutation in relation to the ATP-waiting angle of the αR364K. The time constants for the ATP-waiting and catalytic pauses of the αR364K were 1.1 s and 0.61 s (Fig. 6C), respectively, both of which were almost identical to those determined in the Michaelis–Menten analysis of F₁(αR364K) (Fig. 3). Statistical analysis of the angular displacement of the catalytic pause from the ATP-waiting angle was 200° ± 10° (360° − Δθ), n = 20 molecules, whereas the positional shift of the catalytic pause angle before and after buffer exchange (Δθ₂) was only 4.6° ± 10° (Fig. 6D). The latter supports the validity of the experiment. Thus, it was confirmed that the mutation slowed the ATP cleavage step.

**DISCUSSION**

Our results verify the crucial role of the arginine finger of F₁ (Fig. 1) in the ATP cleavage step. The lysine mutation caused drastic suppression of the rate constant of the ATP cleavage step in the rotary catalysis of F₁. This is shown by the following results: F₁(αR364K) showed a 350-times longer pause than wild-type F₁ at the catalytic angle where ATP cleavage and Pᵢ release occur (Figs. 3 and 4). Moreover, this catalytic pause was lengthened by a slowly hydrolyzed ATP analog, ATPγS (Fig. 5), as expected from the impact of ATPγS on the catalysis of the wild-type. Hybrid F₁, carrying a single copy of the αR364K subunit, F₁(1×αR364K), was found to specifically induce a long catalytic pause only at +200° from the binding angle of the introduced αR364K, where the ATP cleavage step occurs (Fig. 6). On the other hand, the lysine mutation did not affect the unidirectionality of the rotation that is sustained by the sequential torque generated by the 3 β subunits. In addition, the mutation did not affect the coupling ratio of 3 ATPs per turn that is found in the wild-type. Thus, the present study establishes that the principal role of the arginine finger of F₁ is not cooperative signal transmission among catalytic sites, but acceleration of the ATP cleavage rate.

The αR364K mutation also affected other aspects of F₁. The other remarkable effect was severe ADP inhibition (Fig. 2); the mutant sustained successive rotation for only 13 s and for up to 80 s observed. The reason for this severe inhibition is unknown. One possibility, considering that the arginine residue remarkably reduces F₁’s catalytic power for ATP cleavage, in addition to differences in chemistry between the primary amino group and the guanidium group. Nadanaciva et al. (28) previously investigated a glutamine substitution mutant of the arginine finger. They showed that the glutamine substitution completely impaired the ability to attain transition state stabilization, whereas the lysine substitution slightly retained this ability. This suggests that the positive charge at the arginine finger position is required for catalysis. We have also tried an alanine substitution of the arginine finger. However, the ATP hydrolytic activity was not detected in the bulk ATPase assay, and the rotary motion of F₁ was not found under the present conditions.

There is apparent discrepancy between the present work and the previous biochemical study by Le et al., who concluded that the principal role of the arginine finger is not rate enhancement of ATP cleavage, but sustenance of cooperative signal transmission among the 3 catalytic sites (27). Their conclusion was drawn on the basis of the observation that the arginine mutants diminished chase promotion, which assesses whether unisite catalysis is enhanced by the binding of ATP to other catalytic sites; thus, chase promotion is used as a barometer of the cooperative interactions among the catalytic sites. The apparent discrepancy between the present and previous conclusions is attributable to not only the largely slowed turnover rate of multisite catalysis, but also the severe level of ADP inhibition. The strong tendency of the mutant to be inhibited by bound ADP would mask the apparent chase promotion.

Unisite catalysis was reported not to be impaired by the lysine mutation (26, 27). On the other hand, the present study establishes that the same mutation remarkably lowers the ATP cleavage rate in multisite catalysis. Therefore, in conclusion, it is reasonable to assume that the arginine finger is not directly consistent with that in a biochemical study that showed KD is not affected by the mutation (28). This could be because the rotation assay evaluates the ATP-binding rate at the binding angle (0°), whereas the titration experiment in bulk likely assesses the binding at the catalytic angle of 320° under saturation conditions for measuring the third affinity site, KD₃ (44). Considering the angle-dependent modulation of the ATP-binding kinetics, the angular difference likely causes the quantitative difference of the αR364K mutation on the ATP binding process. A stall experiment (38) on the ATP binding of F₁(αR364K) would provide evidence for the role of the arginine finger in this process.

The arginine finger principally contributes to hydrolysis by accelerating the ATP cleavage step, corroborating the recent findings of structural, biochemical, and theoretical studies (21, 28, 30, 31). As suggested by these studies, the arginine finger likely stabilizes the transiently formed penta-coordinated state of γ-phosphate. Recently, the impact of the lysine mutation on ATP cleavage was studied using the quantum mechanics/molecular mechanics method.³ This theoretical data reproduced the present experimental data on the suppression of the ATP cleavage rate well, revealing that the shorter side chain of the lysine residue remarkably reduces F₁’s catalytic power for ATP cleavage, in addition to differences in chemistry between the primary amino group and the guanidium group. Nadanaciva et al. (28) previously investigated a glutamine substitution mutant of the arginine finger. They showed that the glutamine substitution completely impaired the ability to attain transition state stabilization, whereas the lysine substitution slightly retained this ability. This suggests that the positive charge at the arginine finger position is required for catalysis. We have also tried an alanine substitution of the arginine finger. However, the ATP hydrolytic activity was not detected in the bulk ATPase assay, and the rotary motion of F₁ was not found under the present conditions.

³ S. Hayashi, H. Ueno, A. R. Shaikh, M. Umemura, M. Kamiya, Y. Ito, M. Ikeguchi, Y. Komoriya, R. Iino, and H. Noji, unpublished data.
involved in unisite catalysis; the chemistry of unisite catalysis differs from that of the catalytically active site (200° state) in multisite catalysis, as thoroughly discussed previously (3).

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