Catalysis and rotation of F₁ motor: Cleavage of ATP at the catalytic site occurs in 1 ms before 40° substep rotation

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F₁, a water-soluble portion of F₉F₁-ATP synthase, is an ATP hydrolysis-driven rotary motor. The central γ-subunit rotates in the αβ₃ cylinder by repeating the following four stages of rotation: ATP-binding dwell, rapid 80° substep rotation, interim dwell, and rapid 40° substep rotation. At least two 1-ms catalytic events occur in the interim dwell, but it is still unclear which steps in the ATPase cycle, except for ATP binding, correspond to these events. To discover which steps, we analyzed rotations of F₁ subcomplex (αβ₃γδε) from thermophilic Bacillus PS3 under conditions where cleavage of ATP at the catalytic site is decelerated: hydrolysis of ATP by the catalytic-site mutant F₁ and hydrolysis of a slowly hydrolyzable substrate ATP-S (adenosine 5’-[γ-thio]triphosphate) by wild-type F₁. In both cases, interim dwell were extended as expected from bulk phase kinetics, confirming that cleavage of ATP takes place during the interim dwell. Furthermore, the results of ATP-S hydrolysis by the mutant F₁ ensure that cleavage of ATP most likely corresponds to one of the two 1-ms events and not some other faster undetected event. Thus, cleavage of ATP on F₁ occurs in 1 ms during the interim dwell, and we call this “interim dwell catalytic dwell.”

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Abbreviations: ATP-S, adenosine 5’-[γ-thio]triphosphate; fps, frames per second; rps, revolutions per second; LDAO, N,N-dimethyldodecylamine-N-oxide.

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tained from Pierce. Carboxylate bead (diameter, 0.2 μm) was acquired from Polysciences. N,N-dimethyldecylamine-N-oxide (LDAO) was obtained from Fluka.

**Protein Preparation.** Subcomplex (αβγ) of F1 from thermophilic *Bacillus* strain PS3 (called F1 unless otherwise specified) was expressed in *Escherichia coli* JM103Δunc(uncB-D) by using an expression plasmid, pKAGB1/HC95, that carries genes for the α(C193S), χ(g107C/I210C), and β(10 His-tag at N terminus) (18). Although several residues in the expressed F1 (called HC95) were altered from the original F1, it functions in the same manner as the original one, and we consider HC95 as a wild type in this article. Glu-190 of β-subunit was mutated to Asp by the Kunkel method (24) with *E. coli* strain JM109 as a host. The mutant (called F1(E190D)) was expressed in JM103Δunc(uncB-D) and purified as described (25, 26). The purified F1 was further applied to gel-filtration HPLC (Superdex 200; Amersham Biosciences) equilibrated with 100 mM KPi, pH 7.0, containing 100 mM KCl and 2 mM EDTA, and the peak fractions were used for the experiments. The above purification procedures were carried out at room temperature. The concentration of F1 was estimated by absorbance at 280 nm (ε = 154,000 M⁻¹ cm⁻¹).

**Hydrolysis of ATP and ATPγS.** All reactions were carried out at 25°C. ATPase activity was measured in the presence of an ATP-regenerating system containing 0.2 mM NADH, 2.5 mM phosphoenolpyruvate, 50 μg/ml pyruvate kinase, 25 μg/ml lactate dehydrogenase, and an indicated amount of ATP in solution A (50 mM KCl/2 mM MgCl₂/10 mM 3-[N-morpholino]propanesulfonic acid-KOH, pH 7.0) (27). To examine the phosphate or thiophosphate inhibitions, 0.1% LDAO was added to the assay solution to avoid effects of F1 reactivation from the ADP-inhibited form by phosphate or thiophosphate (28). The reaction was initiated by the addition of F1 to 1.2 ml of assay solution, and the hydrolysis rate was determined from the absorbance decrease at 340 nm. The hydrolysis rate of ATPγS was measured without an ATP-regenerating system because it allows long-time data collection and is easy to handle. ATPγS was produced by the addition of F1 to 1.2 ml of assay solution, and the hydrolysis rate was determined from the absorbance decrease at 340 nm.

**Bead Preparation.** Beads for rotation experiments were biotinylated as follows. The carboxylate beads (diameter, 0.2 μm) were washed twice by centrifugation with solution B (50 mM 2-morpholinoethanesulfonic acid-KOH, pH 5.5) and resuspended in solution B containing 200 μM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and 2 mM (N+)-biotinyl-3, 6-dioxaoctane diamine. After a 2-h incubation at room temperature for biotinylation, the beads were centrifuged, washed six times, suspended in solution B, and stored at 4°C.

**Rotation Assay.** For rotation assay, cysteines of γ-subunit were biotinylated as follows. F1 and 6-[(W’-2-[N-maleimide]ethylyl-N-piperazinylamidyl)]hexyl D-biotinamide were reacted at a molar ratio of 1:3 overnight at room temperature, and unreacted biotin was removed by PD-10 column (Amersham Biosciences). A flow chamber was made of a cover glass and a slide glass with a spacer of 50-μm thickness. Biotinylated F1 (0.2–0.5 μM) in solution A containing 10 mg/ml BSA was loaded into the flow chamber first, and, after 2 min, F1 unattached to the glass surface was washed away with 100 μl of solution A. Then, 2 μM NeutrAvidin in solution A was loaded. After 2 min, unbound NeutrAvidin was removed with 100 μl of solution A, and 2 mM biotinylated beads in solution A containing 10 mg/ml BSA were loaded. After a 12-min incubation, unbound beads were removed with 10 μl of solution A containing indicated amount of ATP or ATPγS. In the case of ATP, solution A was complemented by an ATP-regenerating system. Rotating duplex beads were observed with dark-field microscopy (IX-70; Olympus, Tokyo) with a ×100 objective lens (numerical aperture, 1.35; Olympus). The images of rotating beads were recorded directly to a hard disk of a computer as an 8-bit .avi file with a fast-framing charge-coupled device camera (Hi-Dcam; nac Image Technology, Tokyo) at the indicated frame rate. Custom software (created by R.Y.) was used for analyses of the bead movements and dwelling times of steps. With this experimental setup, the wild-type F1 rotated at a rate of 1.6 ± 2.6 revolutions per second (rps) (mean ± SD; n = 5) at 2 mM ATP, far below the rate observed by the 8,000-frames per second (fps) charge-coupled device camera with 40-nm bead (~130 rps) where viscous load did not impede the rotation (18). Therefore, the 2-ms interim dwell observed in the previous study was not seen. Nonetheless, this setup is good for observation of rotations slower than ~10 rps because it allows long-time data collection and is easy to handle.

**Results and Discussion**

**Bulk Phase Enzymatic Properties of F1(E190D).** To obtain a mutant F1 in which a chemical cleavage of ATP at the catalytic site is greatly decelerated while other catalytic steps are less affected, we replaced Glu-190 of β-subunit with Asp, and F1(E190D) was generated. Glu-190 was suggested to be an essential residue for catalysis of F1 (29), and, indeed, the mutant F1(E190D) with the replacement of this critical Glu with Gln has lost ATPase activity completely (22, 30). Crystal structure of mitochondrial F1 suggests that the carboxyl group of Glu-188 of the β-subunit (equivalent to Glu-190 of β-subunit of thermophilic F1) acts as a general base to polarize a water molecule to make nucleophilic attack on the γ-phosphorus of ATP (Fig. 1A). Crystal structure also shows that βGlu-188 of mitochondrial F1 does not directly contribute to binding AT(D)P to the catalytic site. Indeed, it was shown that a mutant *E. coli* F1 equivalent to F1(E190D) had binding affinities to ATP and ADP similar to those of the wild-type F1 (31). The F1(E190D), as expected, retained only very low ATP hydrolytic activity. Hydrolysis of stoichiometric amount of ATP (unisite catalysis) by F1(E190D) (time constant, 126 s) was 1,800 times as slow as the wild-type F1 (time constant, 6.9 ± 10⁻² s) (32). Steady-state ATPase activity of F1(E190D) showed simple Michaelis–Menten-type dependence on [ATP], and the Vₘₐₓ and Kₘ values (mean ± SD) were determined to be 2.4 ± 0.0 s⁻¹ and 1.4 ± 0.1 μM, respectively (Fig. 1B). This Vₘₐₓ value is ~100 times smaller than that of the wild-type F1 (~250 s⁻¹). This Kₘ value is one order smaller than that of the wild-type F1 (19 ± 1 μM) measured in the presence of LDAO, a suppressor of the MgADP inhibition (18).

**Rotation of F1(E190D).** We observed rotation of F1(E190D) at various [ATP]. All [ATP], the rotation rate averaged over 30 s roughly agreed with the ATP hydrolysis rate measured in the bulk divided by 3 (Fig. 1B). Some 20% of the higher rate of the rotation than ATP hydrolysis can be explained by MgADP inhibition in the latter condition. In bulk phase kinetics, some fraction of enzyme is always in the state of MgADP inhibition. On the other hand, rotation rate was measured for only actively rotating molecules and hardly was affected by MgADP inhibition. A rotation vs. [ATP] curve could be fitted with a simple Michaelis–Menten-type kinetics, where Vₘₐₓ and Kₘ values (mean ± SD) were calculated to be 0.9 ± 0.1 rps (for 2.7 s⁻¹ ATP hydrolysis) and 1.0 ± 0.4 μM, respectively. Unlike the wild-type F1, F1(E190D) rotated with discrete 120° steps even at 2 mM ATP (Fig. 2A). As will be discussed later, these dwell steps are not the ATP-binding dwell but the interim dwells. At low [ATP] (<2 μM), the 120° steps were further divided into two substeps, and...
the steps at six regular positions were observed in one revolution (Fig. 2B and Inset). The angles of the two substep rotations were \( \approx 80 \, ^\circ \) (80.9 ± 1.8\(^\circ\); mean ± SE; \( n = 15 \)) and \( \approx 40 \, ^\circ \) (39.1 ± 1.7\(^\circ\)). The dwelling time before the 80\(^\circ\) substep rotation depended on [ATP], therefore, this dwell is the ATP-binding dwell. On the other hand, the dwelling time before the 40\(^\circ\) substep rotation was independent of [ATP], and this dwell is the interm dwell.

The 80\(^\circ\) and 40\(^\circ\) substeps described here correspond to the previously reported 90\(^\circ\) and 30\(^\circ\) substeps (18). The apparent discrepancy in the angles seems to arise from the difference between the observation methods. In the previous study, a 40-nm single bead obliquely attached to the \( \gamma \)-subunit was used as a probe of rotation, whereas we used 0.2-\( \mu \)m duplex beads in the present study. With a larger probe, better angular resolution was attained in the present study. The study on MgADP inhibition also presented 37.6 ± 2.5\(^\circ\) as an angle of substep rotation that precedes the ATP-binding dwell (33). Therefore, although the very accurate angles of substep rotations should be determined in future studies, the terms 80\(^\circ\) and 40\(^\circ\) substeps are more appropriate at present than the previous 90\(^\circ\) and 30\(^\circ\) substeps.

### Dwelling Times of Rotation of F1\(_{102}\) - E190D

Histogram of the ATP-binding dwell before 80\(^\circ\) substeps obeyed an exponential function (data not shown), and the second-order rate constant for ATP binding to F1 (\( k_{\text{on}} \)) was estimated to be \( (3.1 \pm 0.1) \times 10^6 \) M\(^{-1}\)s\(^{-1}\) (mean ± SE). This \( k_{\text{on}} \) value of F1\(_{102}\) - E190D is approximately one order smaller than that of the wild-type ((3.0 ± 0.1) \( \times 10^7 \) M\(^{-1}\)s\(^{-1}\)) (18), probably because of the subtle change of environments near \( \gamma \)-phosphate of ATP caused by replacing Glu with Asp. If we apply a simple Michaelis–Menten scheme, the 10-fold decrease in \( k_{\text{on}} \) is consistent with the observed 10-fold decrease in \( k_{\text{on}} \) and 100-fold decrease in \( k_{\text{off}} \) on the assumption that \( k_{\text{off}} \) is negligible compared with \( k_{\text{on}} \). Histogram of the interm dwell has a distinct peak, which can be fit with double exponentials with two time constants of 321 ± 22 ms and 19.9 ± 0.1 ms (mean ± SE) (Fig. 2C). Thus, the interm dwell of F1\(_{102}\) - E190D is comprised of at least two kinds of successive dwell:

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\text{dwell time before the 40° substep rotation on ATP} = \text{ATP-binding dwell. On the other hand, the dwelling time before the 40° substep rotation was independent of [ATP], and this dwell is the interm dwell.}
\]

The 80° and 40° substeps described here correspond to the previously reported 90° and 30° substeps (18). The apparent discrepancy in the angles seems to arise from the difference between the observation methods. In the previous study, a 40-nm single bead obliquely attached to the γ-subunit was used as a probe of rotation, whereas we used 0.2-μm duplex beads in the present study. With a larger probe, better angular resolution was attained in the present study. The study on MgADP inhibition also presented 37.6 ± 2.5° as an angle of substep rotation that precedes the ATP-binding dwell (33). Therefore, although the very accurate angles of substep rotations should be determined in future studies, the terms 80° and 40° substeps are more appropriate at present than the previous 90° and 30° substeps.

### Dwelling Times of Rotation of F1\(_{102}\) - E190D

Histogram of the ATP-binding dwell before 80° substeps obeyed an exponential function (data not shown), and the second-order rate constant for ATP binding to F1 (\( k_{\text{on}} \)) was estimated to be \( (3.1 \pm 0.1) \times 10^6 \) M\(^{-1}\)s\(^{-1}\) (mean ± SE). This \( k_{\text{on}} \) value of F1\(_{102}\) - E190D is approximately one order smaller than that of the wild-type ((3.0 ± 0.1) \( \times 10^7 \) M\(^{-1}\)s\(^{-1}\)) (18), probably because of the subtle change of environments near γ-phosphate of ATP caused by replacing Glu with Asp. If we apply a simple Michaelis–Menten scheme, the 10-fold decrease in \( k_{\text{on}} \) is consistent with the observed 10-fold decrease in \( k_{\text{on}} \) and 100-fold decrease in \( k_{\text{off}} \) on the assumption that \( k_{\text{off}} \) is negligible compared with \( k_{\text{on}} \). Histogram of the interm dwell has a distinct peak, which can be fit with double exponentials with two time constants of 321 ± 22 ms and 19.9 ± 0.1 ms (mean ± SE) (Fig. 2C). Thus, the interm dwell of F1\(_{102}\) - E190D is comprised of at least two kinds of successive dwell:

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twice as high as that measured in the bulk solution, presumably because some of the $F_1$ molecules in the solution were in the MgADP-inhibited form. The difference was greater than in the case of ATP because we could not use an ATP-regenerating system for ATP$\gamma$S. The dwells between the steps are independent of [ATP$\gamma$S], indicating that they are the interim dwells. This finding also excluded a possibility that rotation was driven by contaminated ATP that was <0.1%, as assessed with an anion-exchange HPLC. At 0.5 $\mu$M ATP$\gamma$S,** rotations with 80° and 40° substeps were observed (Fig. 4B). From analysis of ATP$\gamma$S-binding dwells, the second-order rate constant for ATP$\gamma$S binding to $F_1$ ($k_{on}$) was estimated to be $(2.6 \pm 0.1) \times 10^7$ M$^{-1}$s$^{-1}$ (mean ± SE), which is similar to that of ATP $[(3.0 \pm 0.1) \times 10^7$ M$^{-1}$s$^{-1}]$ (18) and agrees well with the value determined from a biochemical experiment $[(2.8 \pm 0.2) \times 10^7$ M$^{-1}$s$^{-1}]$ (32). Histogram of the interim dwells showed an upward convex shape passing through the origin (Fig. 4C), which can be fit by double exponentials with two time constants of 61.0 ± 0.6 ms and 8.20 ± 0.6 ms (mean ± SE). Biochemical studies suggest that ATP$\gamma$S decreases the cleavage rate by 30-fold (23). Thus, the longer dwell most likely corresponds to the slow cleavage of ATP$\gamma$S and the short one corresponds to the product release, as discussed later.

**At low ATP$\gamma$S concentrations (<2 $\mu$M), rotating beads were rarely seen in the observation field, probably because of the MgADP inhibition. Usually the ATP-regenerating system eliminates ADP, but it cannot be used in these experiments because it replaces ATP$\gamma$S with ATP.

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**Fig. 2.** (A and B) Typical time course of the stepwise rotations of $F_{1(\beta-E190D)}$ at 2 mM (A) and 2 $\mu$M (B) ATP at 30 fps. (Insets) The trace of the centroid of the beads images. Arrows indicate the positions at which 40° substeps were observed. (C) The histogram of dwelling times between steps in the presence of 2 mM ATP at 500 fps. Total counts are 2,068. (Inset) The time course of rotation at 500 fps. Black line shows fit with two exponentials assuming there were two rate-limiting reactions: constant $\times (\exp(-k_1 t) - \exp(-k_2 t))$, where $k_1 = 50.3 \pm 0.2$ s$^{-1}$ (mean ± SE; time constant, 19.9 ± 0.1 ms) and $k_2 = 3.12 \pm 0.22$ s$^{-1}$ (time constant, 321 ± 22 ms).

**Fig. 3.** (A) ATP$\gamma$S hydrolysis rate as a function of ATP$\gamma$S concentration. The solid curve is the fit with two $K_m$ values: $V = (V_{max1} \times K_{m2} \times [ATP] + V_{max2} \times [ATP])/(V_{max1} + K_{m1} \times [ATP] + K_{m2} \times [ATP] + K_{m3} \times [ATP])$, where $V_{max1} = 3.0 \pm 0.2$ s$^{-1}$, $V_{max2} = 5.8 \pm 0.3$ s$^{-1}$, $K_{m1} = 1.0 \pm 0.3$ $\mu$M, and $K_{m2} = (2.4 \pm 1.0) \times 10^2$ $\mu$M. (Inset) The time courses of ATP$\gamma$S hydrolysis. Black circles, in the presence of 200 $\mu$M ATP$\gamma$S and 0.5 $\mu$M $F_1$ (wild type); gray squares, in the absence of $F_1$. (**Rotations with 80° and 40° substeps were observed (Fig. 4B). From analysis of ATP$\gamma$S-binding dwells, the second-order rate constant for ATP$\gamma$S binding to $F_1$ ($k_{on}$) was estimated to be $(2.6 \pm 0.1) \times 10^7$ M$^{-1}$s$^{-1}$ (mean ± SE), which is similar to that of ATP $[(3.0 \pm 0.1) \times 10^7$ M$^{-1}$s$^{-1}]$ (18) and agrees well with the value determined from a biochemical experiment $[(2.8 \pm 0.2) \times 10^7$ M$^{-1}$s$^{-1}]$ (32). Histogram of the interim dwells showed an upward convex shape passing through the origin (Fig. 4C), which can be fit by double exponentials with two time constants of 61.0 ± 2.6 ms and 8.20 ± 0.6 ms (mean ± SE). Biochemical studies suggest that ATP$\gamma$S decreases the cleavage rate by 30-fold (23). Thus, the longer dwell most likely corresponds to the slow cleavage of ATP$\gamma$S and the short one corresponds to the product release, as discussed later.

**Fig. 3.** (A) ATP$\gamma$S hydrolysis rate as a function of ATP$\gamma$S concentration. The solid curve is the fit with two $K_m$ values: $V = (V_{max1} \times K_{m2} \times [ATP] + V_{max2} \times [ATP])/(V_{max1} + K_{m1} \times [ATP] + K_{m2} \times [ATP] + K_{m3} \times [ATP])$, where $V_{max1} = 3.0 \pm 0.2$ s$^{-1}$, $V_{max2} = 5.8 \pm 0.3$ s$^{-1}$, $K_{m1} = 1.0 \pm 0.3$ $\mu$M, and $K_{m2} = (2.4 \pm 1.0) \times 10^2$ $\mu$M. (Inset) The time courses of ATP$\gamma$S hydrolysis. Black circles, in the presence of 200 $\mu$M ATP$\gamma$S and 0.5 $\mu$M $F_1$ (wild type); gray squares, in the absence of $F_1$. (**Rotations with 80° and 40° substeps were observed (Fig. 4B). From analysis of ATP$\gamma$S-binding dwells, the second-order rate constant for ATP$\gamma$S binding to $F_1$ ($k_{on}$) was estimated to be $(2.6 \pm 0.1) \times 10^7$ M$^{-1}$s$^{-1}$ (mean ± SE), which is similar to that of ATP $[(3.0 \pm 0.1) \times 10^7$ M$^{-1}$s$^{-1}]$ (18) and agrees well with the value determined from a biochemical experiment $[(2.8 \pm 0.2) \times 10^7$ M$^{-1}$s$^{-1}]$ (32). Histogram of the interim dwells showed an upward convex shape passing through the origin (Fig. 4C), which can be fit by double exponentials with two time constants of 61.0 ± 2.6 ms and 8.20 ± 0.6 ms (mean ± SE). Biochemical studies suggest that ATP$\gamma$S decreases the cleavage rate by 30-fold (23). Thus, the longer dwell most likely corresponds to the slow cleavage of ATP$\gamma$S and the short one corresponds to the product release, as discussed later.

**Rotation of $F_{1(\beta-E190D)}$ by ATP$\gamma$S.** We assume that both the 320-ms event of $F_{1(\beta-E190D)}$ in hydrolysis of ATP and the 61-ms event of $F_1$ in hydrolysis of ATP$\gamma$S correspond to the same rate-limiting reaction, that is, cleavage of terminal (thio)phosphate moiety of ATP or ATP$\gamma$S. To further confirm this point, we analyzed the rotation of $F_{1(\beta-E190D)}$ driven by the hydrolysis of ATP$\gamma$S. At 2 mM ATP$\gamma$S, very slow rotation $(2.3 \times 10^{-2} \pm 0.3$ rps; mean ± SE; $n = 5$) was observed, and rotation proceeded with discrete 120° steps (Fig. 5A). Histogram of the dwelling times showed an apparent single-exponential decay, and a value of 12.5 ± 0.4 s
was obtained as the time constant†† (Fig. 5B). The very long time constant observed in the combination of the mutation and ATP/βS is consistent with the contention that the main effect of βE190D mutation and ATP/βS is to retard the same step of ATP(βS) cleavage. Furthermore, if we assume that their effects are energetically additive, the very long time constant leads a conclusion that one of the two 1-ms events in the interim dwell of the wild-type F1 is the cleavage of ATP. Actually, if we assume that one of the two 1-ms events in the interim dwell of the wild-type F1 is the cleavage of ATP at the catalytic site, the mutation βE190D and ATP/βS slowed down the time constant of this event 320-fold and 61-fold, respectively. Then, if modification of a catalytic residue and a substrate might exhibit a dual effect on the catalytic rate (rotation rate), the time constant of ATP/βS cleavage by F1(βE190D) is predicted to be 19.5 s (for 1 ms \( \times 320 \times 61 \)). The value obtained from the experiment, 12.5 s, is fairly close to this predicted value and strongly suggests that these assumptions are valid. This agreement also exclude the possibility that a third, unidentified event with a faster time constant corresponds to the ATP cleavage. For example, if an event of 0.1 ms corresponded to the ATP cleavage at the catalytic site, the mutation βE190D and ATP/βS would retard the step by 3,200-fold and 610-fold, respectively. Then, the combination of the mutant and ATP/βS would cause 1,952,000-fold deceleration, resulting in a time constant as long as 195.2 s. This value is far larger than the experimental value observed, and the above possibility is proved to be unlikely. Thus, cleavage of ATP on F1 occurs in 1 ms during the interim dwell, and we call this interim dwell the catalytic dwell. The present results also indicate that the 80° substep rotation takes place before the cleavage of ATP into ADP and Pi and the 40° substep rotation requires completion of the cleavage.

**Conclusion**

In the function of F1, catalysis and rotation seems to be tightly coupled. Therefore, altered catalysis, either by mutation or different substrate, results in altered rotation, and analysis of the latter with good reference of the former can bring a insight into the mechanism of F1 motor. We have adopted this approach and obtained results leading to the conclusion that the ATP cleavage reaction at the catalytic site is one of the 1-ms events in the

†† A short dwell with a time constant <0.2 s might exist but could not be seen in the present observations.
catalytic dwell. Contrary to the previous hypothesis (21), it is clearly indicated that the 80° substep rotation takes place before the cleavage of ATP into ADP and P_i. It also is established that the 40° substep rotation requires completion of the cleavage. This conclusion was to be expected but needed to be examined experimentally because the two 1-ms events can be any events (except ATP binding) that occur in F_1 during catalysis. Even a conformational isomerization of F_1 without changes of chemical species of bound substrate/product cannot be excluded as a candidate. Now that one of the 1-ms events is determined to be an ATP cleavage, the next challenge will be the identification of another 1-ms event and clarification of the sequence of the events.‡‡

‡‡If the two catalytic events occur at the same β-subunit, ATP hydrolysis must occur first followed by release of products. But in the case where the two events occur at different β-subunits, ATP hydrolysis could occur after product release from another β-subunit.

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