Stepping Rotation of F$_1$-ATPase with One, Two, or Three Altered Catalytic Sites That Bind ATP Only Slowly*

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Takayuki Ariga‡, Tomoko Masaikë§§, Hiroyuki Noji***, and Masasuke Yoshida‡‡‡

From the §Chemical Resources Laboratory, Tokyo Institute of Technology, 4259 Nagatsuta, Yokohama, 226-8503, Japan, Institute of Industrial Science, University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo, 153-8505, Japan, **Precursory Research for Embryonic Science and Technology, Japan Science and Technology Corporation (JST). 32-0012 and §ATP System Project, Exploratory Research for Advanced Technology, Japan Science and Technology Corporation (JST), 5800-2 Nagatsuta, Yokohama, 226-0026, Japan

F$_1$-ATPase is an ATP hydrolysis-driven motor in which the $\gamma$ subunit rotates in the stator cylinder $\alpha_3\beta_3$. To know the coordination of three catalytic $\beta$ subunits during catalysis, hybrid F$_1$-ATPases, each containing one, two, or three “slow” mutant $\beta$ subunits that bind ATP very slowly, were prepared, and the rotations were observed with a single molecule level. Each hybrid made one, two, or three steps per 360° revolution, respectively, at 5 $\mu$m ATP where the wild-type enzyme rotated continuously without step under the same observing conditions. The observed dwell times of the steps are explained by the slow binding rate of ATP. Except for the steps, properties of rotation, such as the torque forces exerted during rotary movement, were not significantly changed from those of the wild-type enzyme. Thus, it appears that the presence of the slow $\beta$ subunit(s) does not seriously affect other normal $\beta$ subunit(s) in the same F$_1$-ATPase molecule and that the order of sequential catalytic events is faithfully maintained even when ATP binding to one or two of the catalytic sites is retarded.

The F$_0$F$_1$-ATP synthase found in mitochondria, bacteria, and chloroplasts couples proton translocation in the F$_0$ portion and ATP synthesis/hydrolysis in the F$_1$ portion through physical rotation of the central shaft subunits (1–5). The isolated F$_1$ portion has ATPase activity, thus often called F$_1$-ATPase, and is composed of five different subunits with a stoichiometry of $\alpha_3\beta_3\gamma_6\epsilon$. The $\alpha_3\beta_3\gamma$ subcomplex is the minimum ATPase-active complex that has catalytic features similar to F$_1$-ATPase. In the crystal of the $\alpha_3\beta_3\gamma$ structure, $\alpha$ and $\beta$ subunits are arranged alternately to form an $\alpha_3\beta_3$ cylinder around the coiled-coil structure of the $\gamma$ subunit (6). Catalytic sites reside mainly on $\beta$ subunits, whereas some residues of $\alpha$ subunits also contribute.

The isolated F$_1$-ATPase by itself is a motor enzyme; the biochemical supporting result of sophisticated subunit-exchange experiments of Escherichia coli F$_1$-ATPase (7) was secured by the direct demonstration of ATP hydrolysis-driven rotation of the fluorescent actin filament attached to the $\gamma$ subunit of an $\alpha_3\beta_3\gamma$ subcomplex of thermophilic F$_1$-ATPase immobilized on a glass surface (8). At nanomolar ATP concentrations, the $\gamma$ subunit rotates in discrete 120° steps (9). The analysis indicates that the dwell time of the step corresponds to the waiting time for the ATP molecule to occupy the next catalytic site. Further study with a smaller rotation marker and higher time resolution has revealed that a 120° step is split into a 90° substep, triggered by ATP binding, and a 30° substep, probably driven by dissociation of ADP (or phosphate) from the enzyme (10).

The mechanism of how the ATP hydrolysis drives the rotation of F$_1$-ATPase is intriguing. The cooperative nature of substrate binding and catalysis of F$_0$F$_1$-ATP synthase and F$_1$-ATPase has long been recognized and explained by a model, the binding change mechanism proposed by Boyer (11), which assumed the rotational participation of three catalytic sites during catalysis. In support of this model, three $\beta$ subunits in F$_1$-ATPase crystals are in the three different nucleotide binding states: one $\beta$ subunit with bound AMP-PNP$^1$ at catalytic site, another with ADP, and the third without nucleotide. The $\beta$ subunits with bound nucleotides take a “closed” conformation, but the $\beta$ subunit with an empty catalytic site takes an “open” conformation (6). Thus, it is likely that the rotation of the $\gamma$ subunit is caused by alternative, sequential conformational transition of three $\beta$ subunits in F$_1$-ATPase that is accompanied by each catalytic step in ATP hydrolysis. Then, the mechanism that conducts coordination of three $\beta$ subunits needs to be clarified. To analyze this, the experimental system may have a great value that enables observation of the role of the individual $\beta$ subunit during rotation. In this report, we describe a novel method to prepare the hybrid F$_1$-ATPase$^2$ containing one or two copies of mutant $\beta$ subunit(s) that can bind ATP only slowly. Characteristic rotations of the hybrid F$_1$-ATPases were compared with those of wild-type F$_1$-ATPase.

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‡‡‡ To whom correspondence should be addressed: Chemical Resources Laboratory, R-1, Tokyo Institute of Technology, 4259 Nagatsuta, Yokohama, 226-8503, Japan. Tel.: 81-45-924-5233; Fax: 81-45-924-5277; E-mail: myoshidas@res.titech.ac.jp.

1 The abbreviations used are: AMP-PNP, adenosine 5′-[(β,γ-imino)triphosphate; FEFE mutations, F414E and F420E mutations of $\beta$ subunit of F$_1$-ATPase; F$_1$(0xFEFE), a mutant (C193S)3$\beta$8(His-tagged/I386C)3$\gamma$(S109C/I212C)3 subcomplex of F$_1$-ATPase; F$_1$(1xFEFE), a mutant (C193S)3$\beta$8(His-tagged/I386C)3$\gamma$(S109C/I212C)3 subcomplex of F$_1$-ATPase; F$_1$(2xFEFE), a mutant (C193S)3$\beta$8(His-tagged/I386C)3$\gamma$(S109C/I212C)3 subcomplex of F$_1$-ATPase; F$_1$(3xFEFE), a mutant (C193S)3$\beta$8(His-tagged/F414E/F420E)3$\gamma$(S109C/I212C)3 subcomplex of F$_1$-ATPase; F$_1$x(FEFE), a mutant (C193S)3$\beta$8(His-tagged/F414E/F420E)3$\gamma$(S109C/I212C)3 subcomplex of F$_1$-ATPase; F$_1$-ATPase, complex of F$_1$-ATPase; MOPS, 4-morpholinopropanesulfonic acid; biotin-PEAC5-maleimide, 6-N-[2-(N-maleimido)ethyl]-N-piperazinyldiamidohexyl-biotinamide; Ni-NTA, nickel nitrilotriacetic acid; HPLC, high pressure liquid chromatography; BSA, bovine serum albumin; pN, piconewtons.

2 Actually, an $\alpha_3\beta_3\gamma$ subcomplex of thermophilic F$_1$-ATPase was used for experiments in this study. However, hereafter in this report, we also call this subcomplex F$_1$-ATPase for simplicity unless confusion occurs.
and mutant F₂-ATPase with three mutant β subunits. The results show that the binding of ATP to one β subunit in F₂-ATPase is rate-limiting for a corresponding single 120°-step rotation in these mutants and that the sequence of catalytic events in F₂-ATPase is not confused even if one of the ATP binding steps is delayed.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Protein Preparation—**E. coli strains used were JM109 for preparation of plasmids, C3236 for generation of uracil-containing single-stranded plasmids for site-directed mutagenesis, and JM107 (λamp-E. coli) for expression of the mutant αβγ, mutant αβ, and mutant β subunits of F₂-ATPase of thermophilic *Bacillus* strain PS3. Plasmids pUC-β, which carried genes for the β subunit of thermophilic F₂-ATPase, and pKAGB1, which carried genes for the α, β, and γ subunits of thermophilic F₂-ATPase (12), were used for mutagenesis and for gene expression. The plasmid pkk-hc95 (pKAGB1α/εα/ε9265/γ/S109C/I212C/β(His tag at the N terminus)) has been described previously (10). The plasmid pkk-c95 (pKAGB1α/ε/ε9253/γ/S109C/I212C/β), which is a non-His-tagged version of pkk-hc95, was prepared by ligating a 7.4-kb *NheI-PmaCl* fragment of pkk-hc95 and a 1.3-kb fragment of pkk-b1. The αβγ subcomplexes expressed from either pkk-hc95 or pkk-c95 were considered the wild-type F₂-ATPase in this report. 4) The released orange-colored band of the 1.4-kb *MluI-PstI* fragment of the β subunit of thermophilic F₂-ATPase were used for site-directed mutagenesis to introduce "FEFE" mutations (β(F414E/F420E)) by the Kunkel method (13) with the synthetic oligonucleotide 5′-GTA CGA GCC CGG TTG ACC GCT TTC GTC CGC CAC GTC TTC GTT TCG GAA CAA GAA C-3′ where bold letters were changed for the mutations Phe to Glu, and italic letters indicate silent mutations that introduce an AgeI site used to identify the mutant. The 1.4-kb *MluI-PstI* fragment of this plasmid containing the FEFE mutations was ligated with the 7.4-kb *MluI-PstI* fragment of pkk-hc95 to produce pkk-hc95/FEFE and that of pkk-c95 to produce pkk-c95/FEFE. The final plasmids used for expression of the mutant products were proved correct by direct nucleotide sequence determination. The expressed αβγPε subcomplex and β subunits were purified as described previously (12, 14) and stored in 70% ammonium sulfate.

**Preparation of Hybrid Complexes—**It has been known that the dimer of β subunits is formed in F₂-ATPase through a disulfide bond between cysteines identified at the position of β-Ile-386 (15). We have adopted this mutation to obtain a β dimer that can reconstitute F₂-ATPase with other subunits. The outline of the procedure to isolate Pε (1×FEFE) is as follows (see Fig. 1A). 1) The β(ε/ε66C/His tag) dimers were generated from monomers and isolated. The β(ε/ε66C/His tag) subunits in ammonium sulfate suspension were centrifuged and dissolved in 40 mM Tris-HCl, pH 8.0, and 100 mM NaCl. To dimerize β subunits, the solution was treated with 100 μM CuCl₂ for 2 h at room temperature. The solution was concentrated to 200 μl by ultracentrifugation and was subjected to a gel filtration HPLC column Superdex 200 HR (Amersham Biosciences) that had been equilibrated with potassium phosphate, pH 7.0, 2 mM EDTA. The peak fraction of the β(ε/ε66C/His tag) dimer was collected and was treated with 100 mM dithiothreitol for 1 h at room temperature and was used for assays within a day. 2) The flow cell was washed with 10 μl of 0.5 M HEPES-KOH (pH 7.0, 100 mM KCl, 10 mM MgCl₂, and 100 mM bovine serum albumin). The flow cell was inoculated with 20 μl of 0.5 μM ATP buffer (H₂O, 7.0, 50 mM KCl, 2 mM MgCl₂, and 100 mM bovine serum albumin). The flow cell was treated with a 20-fold molar excess of biotin-PEAC 5-maleimide for 2 h at room temperature, washed with 20 μl of TAP buffer, and incuated for 2 min at room temperature to fix them on the glass surface. Unbound molecules of F₂-ATPase were washed out with 20 μl of a BSA buffer (10 mM MOPS, pH 7.0, 50 mM KCl, 2 mM MgCl₂, and 10 μg/ml bovine serum albumin). The flow cell was inoculated with 10 μl of 0.5 M HEPES-KOH buffer containing 0.1 mg/ml creatine kinase, 1 mM creatine phosphate, and indicated concentrations of Mg-ATP, and rotation was observed. Then, 40 μl of 5 μM ATP buffer was infused into the flow cell, and the rotation was observed again. Finally, the solution was exchanged with 10 μl of BSA buffer to remove unbound beads. The flow cell was rinsed with 20 μl of 100 μM ATP buffer (BSA buffer containing 0.1 mg/ml creatine kinase, 1 mM creatine phosphate, and indicated concentrations of Mg-ATP), and rotation was observed. This procedure was repeated twice. Analysis of rotational angle was performed as described previously (8, 9).

**Assay of ATPase Activity—**F₂-ATPases were conjugated with streptavidin and immobilized on the glass surface of the flow cell. The flow cell was inoculated with 10 μl of 1 mM fluorescent actin filament instead of beads, incubated for 10 min at room temperature, washed with 20 μl of BSA buffer, and incubated with 100 μl of 0.5 μM ATP buffer. The fluorescent actin filament was observed with a fluorescence microscope (IX70; Olympus). Observed images were recorded with an intensified charge-coupled device camera (ICCD-350F; Videoscope) on a digital video tape at 30 frames/s. Analysis of rotational angle was performed as described previously (8, 9).

**Measurements of Torque Force—**The biotinylated F₂-ATPases were conjugated with streptavidin and immobilized on the glass surface of the flow cell (9). The flow cell was incubated with 10 μl of 1 mM fluorescent actin filament instead of beads, incubated for 10 min at room temperature, washed with 20 μl of BSA buffer, and incubated with 20 μl of 5 mM ATP buffer. The fluorescent actin filament was observed with a fluorescence microscope (IX70; Olympus). Observed images were recorded with an intensified charge-coupled device camera (ICCD-350F; Videoscope) on a digital video tape. Analysis of rotational angle and computation of torque force were performed as described previously (9).

**RESULTS**

**FEFE Mutant—**To generate mutants that can bind substrate ATP slowly, we replaced two phenylalanine residues of β subunits, Phe-414 and Phe-420, which are located at the adenine-binding pocket (6), with glutamic acid residues. These mutations of the β subunit are called FEFE in this report, and according to the number of copies of β(FEFE) subunits contained, we designate the mutant F₂-ATPase as F₂(0xFEFE), F₂(1xFEFE), F₂(2xFEFE), and F₂(3xFEFE), respectively.
FIG. 1. Isolation of hybrid F1-ATPase. A, the procedures to isolate hybrid complexes. 1) The His-tagged β dimer, cross-linked between Cys-386 of two β subunits, and F1(3xFEFE) were mixed, denatured, renatured, and reassembled. 2) Hybrid F1(1xFEFE) was purified with Ni-NTA and gel filtration columns. B, elution profile of the gel filtration HPLC monitored by absorbance at 280 nm. The His-tagged F1(1xFEFE) was eluted at 21 min as indicated by an arrow. The peaks at 26 and 43 min correspond to β dimer and ATP, respectively. C, polyacrylamide gel electrophoresis of F1(1xFEFE) in the presence of sodium dodecyl sulfate. The purified F1(1xFEFE) was incubated at room temperature for 1 h (+) with or (−) without 100 mM dithiothreitol (DTT) and applied on the 13% gel.

FIG. 2. Stepping rotations of the mutant F1-ATPases. Time course of the rotations of the beads attached to the γ subunits of F1(3xFEFE) (A), F1(2xFEFE) (B), and F1(1xFEFE) (C) at 200 μM ATP (left), 5 μM ATP (center), and 10 mM ATP (right). Rotation of the same molecule of F1-ATPase was observed at three ATP concentrations that were changed by infusion. The dashed line in panel A is the rotation of the wild-type F1-ATPase at 5 μM ATP. Insets show histograms (number of events) of the angular positions (degree) of the beads at 5 μM ATP at each frame. These histograms are taken from the rotations observed at 0–140 s (A), 0–66 s (B), and 0–30 s (C).

TABLE I

| F1-ATPase     | Vmax (turnover/sec) | Km (mM) |
|---------------|---------------------|---------|
| F1(0xFEFE)    | 71                  | 0.016   |
| F1(1xFEFE)    | 38                  | 0.45    |
| F1(2xFEFE)    | 85                  | 0.90    |
| F1(3xFEFE)    | 62                  | 1.55    |

* Obtained from the steady-state ATPase activity.

F1(1xFEFE) and F1(2xFEFE) were purified by similar procedures (see “Experimental Procedures”). F1(3xFEFE) was isolated by gel filtration HPLC (Fig. 1B). The analysis of the arrows peak fraction in Fig. 1B with non-reducing SDS-PAGE showed the bands of β dimer and β monomer with consistent band intensities (Fig. 1C, right lane). The β dimer band disappeared and the intensity of the β monomer band increased after dithiothreitol treatment (Fig. 1C, left lane). The disulfide cross-link in F1(1xFEFE) was reduced just prior to the rotation assay. F1(2xFEFE) and F1(0xFEFE) were prepared by similar procedures (see “Experimental Procedures”).

Bulb Phase Kinetics of the Mutants—The initial rates of ATP hydrolysis by F1(0xFEFE), F1(1xFEFE), and F1(2xFEFE) were measured and analyzed by non-linear regression analysis assuming simple Michaelis-Menten kinetics (Table I). For F1(3xFEFE), since ATP hydrolysis started with a lag phase, the steady-state rate of ATP hydrolysis, instead of the initial rate, was used for analysis. As expected, FEFE mutations resulted in the dramatic decrease of ATP affinity, and the Km value of F1(3xFEFE) was almost 100 times larger than that of F1(0xFEFE). The Vmax values of F1(1xFEFE) and F1(2xFEFE) are approximately one-third and two-third of the Vmax value of F1(3xFEFE), respectively, reflecting the occurrence of one or two times of slow ATP binding in a catalytic turnover for each mutant.4 In contrast, the Vmax values of the mutants remain in the same order with the value of F1(1xFEFE) being the smallest. It should be added that the Vmax value of F1(0xFEFE) is similar to that of the wild-type F1-ATPase (0.027 μM), whereas the Vmax value of F1(0xFEFE) is about 40% of that of the wild type (162 s⁻¹). The decreased Vmax value of F1(0xFEFE) is 4 Assuming that three catalytic sites have the same kcat and independent, different kATPs and koff of ATP hydrolysis (V) at steady-state catalysis is calculated as V = Vmax · S/(S + (Km1 + Km2 + Km3)/3); S = concentration of ATP, Pm = Km1, 2, or 3 = (RATP, 1, 2, or 3 + Km)/S. Because kcat/ATP = Km1, 2, or 3 ≫ kcat/ATP, Km1, 2, or 3 of F1(1xFEFE) = (Km1 + Km2 + Km3)/(3/3); Km1, 2, or 3 of F1(1xFEFE) = (Km1 + Km2 + Km3)/(3/3); and Km1, 2, or 3 of F1(2xFEFE) = (Km1 + Km2 + Km3)/(3/3).
probably due to the presence of two β-I886C mutations that differentiate F₁(0xFEFE) from the wild-type F₁-ATPase. The turnover rate of F₁(1xFEFE) is about half the rates of other hybrids. The reason is not known, but possibly the prepared 27 molecules of F₁(0xFEFE) that we observed at 5 μM ATP (Fig. 2C and inset). The remaining five molecules rotated like F₁(0xFEFE), that is, without step. The stepping rotations described above are all dependent on ATP concentrations. At 200 μM ATP, rotations of all mutants were rather smooth, but a rotation trajectory was slightly rugged by occasional brief steps (<0.1 s). At 10 mM ATP, all rotations were smooth and fast. It should be worth noting that even during a long dwell time, obvious single backward rotation was not observed throughout the whole experiment.

**Rate Constant of ATP Binding**—The dwell times at 5 μM ATP of all rotating molecules examined for F₁(3xFEFE) were collected and plotted (Fig. 3, top). The histogram is well fitted by a single exponential curve, suggesting that the dwell time corresponds to the waiting time of the enzyme for the next ATP and that binding of a single ATP drives one 120° rotation (9). The rate constant for ATP binding (k_{ATP}^{\text{on}}) is obtained from the fitting as 1.8 × 10^{5} M\(^{-1}\) s\(^{-1}\). Similar analyses were carried out for a two-step rotation of F₁(2xFEFE) and a single-step rotation of F₁(1xFEFE) (Fig. 3, middle and bottom), and k_{ATP}^{\text{on}} values of 1.6 × 10^{5} M\(^{-1}\) s\(^{-1}\) and 3.7 × 10^{5} M\(^{-1}\) s\(^{-1}\), respectively, were obtained. These values of mutants were 2 orders of magnitude smaller than the k_{ATP}^{\text{on}} value of the wild type (3 × 10^{7} M\(^{-1}\) s\(^{-1}\)) (10). As described, the Kₘ value of F₁(3xFEFE) is 2 orders of magnitude larger than that of F₁(0xFEFE) (and the wild-type F₁-ATPase). Therefore, the increase in Kₘ value of FEFE mutations can be explained solely by the decrease in the k_{ATP}^{\text{on}} value.

The fact that k_{ATP}^{\text{on}} values of F₁(1xFEFE), F₁(2xFEFE), and F₁(3xFEFE), obtained from the above analysis are similar to each other implies that ATP binding to β (βFEFE) in F₁-ATPase occurs at the same rate regardless of how many β (βFEFE) and normal β subunits are present in the F₁-ATPase. In F₁(2xFEFE), for example, the ATP binding to the first β (βFEFE) during steady-state catalysis is neither decelerated by the presence of the second β (βFEFE) nor accelerated by the third normal β subunit. It seems that the effect of slow ATP binding to the first β (βFEFE) is restricted to the corresponding 120° rotation driven by that ATP binding.

**Torque Force of Rotation**—Instead of beads, an actin filament was attached to the γ subunit, and rotation velocities

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5 The k_{ATP}^{\text{on}} value of F₁(1xFEFE) is about double of the values of other mutants. However, we do not take this difference seriously at this stage because k_{ATP}^{\text{on}} values obtained from dwell time observed for each of rotating molecules varied from one molecule to another. The k_{ATP}^{\text{on}} values calculated for F₁(1xFEFE), F₁(2xFEFE), and F₁(3xFEFE) are (3.4 ± 0.4) × 10^{5} M\(^{-1}\) s\(^{-1}\) (23 molecules), (1.2 ± 0.6) × 10^{5} M\(^{-1}\) s\(^{-1}\) (8 molecules), and (1.8 ± 0.8) × 10^{5} M\(^{-1}\) s\(^{-1}\) (8 molecules), respectively. The reason for this scattering is not known, but it may arise from either molecular individuality or uncontrolled difference of experimental conditions.
were measured for F1(3xFEFE) at 5 mM ATP. As shown in Fig. 4, the rotational velocities decreased as the length of the actin filament increased. The observed rotations were scattered mostly between two theoretical lines that assumed the constant torque forces, 20 and 40 pN·nm. Higher velocities are thought to be more reliable because any obstructions against rotation would reduce the velocity. Therefore, we conclude that F1(3xFEFE), similar to the wild-type F1-ATPase, exerted constant torque of ~40 pN·nm (9).

**DISCUSSION**

**F1-ATPase Does Not Easily Confuse the Order of Catalytic Sequence**—This study reports that when one, two, or three β subunits in F1-ATPase become slow in binding of substrate ATP, an obvious consequence is the retardation of initiating one, two, or three 120° rotations. During a long period in which one slow β subunit waits for ATP, other β subunits are also just waiting without letting the next catalytic event start. The principle underlying this seemingly natural consequence is the faithfulness of F1-ATPase in keeping the order of sequential catalytic events. Also confirmed is the assumption that although three catalytic sites exist in F1-ATPase, catalytic events of all three sites are coordinated essentially as a single, linear chain reaction pathway, excluding possibilities of other diverging-converging or parallel reaction pathways. This is another expression of Boyer’s binding change mechanism, and this report provides a support for it from the rotation kinetics. Previously, we reported that when one of the β subunits in F1-ATPase was replaced with an incompetent β subunit, catalytic turnover of ATP hydrolysis was completely blocked (14). Two other active β subunits cannot exert their catalytic ability until a preceding catalytic event, which is impossible for the incompetent β subunit, finishes.

Probably related to the above contention is the absence of the backward step during prolonged dwell time. Previously, occasional backward steps, about once per 20 forward steps, were observed and were supposed to be caused by the ATP binding to the wrong empty catalytic site (9). If so, under the situation in which the next correct ATP binding site is on the β(FEFE) subunit and the wrong binding site is on the normal β subunit, chances for the wrong site to bind ATP (and thus cause a backward step) should have increased drastically because ATP binding to the correct site is retarded about 100 times. However, such frequent backward steps were not observed for F1(1xFEFE) and F1(2xFEFE), and therefore, ATP binding to the wrong site does not account for the backward steps. The above argument is based on the bi-site catalysis model (18–20) in which two catalytic sites are empty at the moment just before a new ATP binds to the enzyme. If the tri-site catalysis is assumed (21–24), only one catalytic site is available for the next ATP binding and wrong ATP binding cannot occur. In this context, faithful order of catalysis of F1-ATPase revealed in this study is explained more easily by the tri-site model than the bi-site model.

**Energy of ATP Binding and Torque**—It has been known that a 120°-step rotation is composed of 90- and 30°-substep rotations (10). The 90°-substep rotation is likely driven by the energy liberated when F1-ATPase binds (but not hydrolyzes) ATP, and subsequent 30°-substep rotation occurs in ~2 ms. The method employed here did not resolve the substeps, and we observed a 120°-step rotation triggered by ATP binding. The magnitude of energy to be liberated upon ATP binding varies depending on the binding affinity of ATP to F1-ATPase. Then kATP values of β(FEFE) in F1-ATPase mutants are 2 orders of magnitude smaller than the wild type, and therefore, the affinity of ATP to the mutants is also very low. Nonetheless, the observed torque force of F1(3xFEFE) is not significantly changed from that of the wild-type F1-ATPase. In the converse combination of catalytic site and substrate, that is, when wild-type F1-ATPase binds GTP or ITP, whose binding affinity was lower than ATP, the observed torque forces were not significantly changed from those observed for ATP (25). These apparent contradictions are explained by the “binding zipper” model proposed by Oster et al. (26, 27). In this model, the process of ATP binding to F1-ATPase is divided into two subprocesses: the docking of ATP and zipping of hydrogen bonds between phosphate moiety of ATP and residues of the catalytic site. The torque force is not generated at the docking process but during the zipping process. The FEFE mutations contain mutations at the adenine-binding pocket rather than at the phosphate binding residues in F1-ATPase. It is plausible that this mutation might reduce the chance for the docking of ATP (or the chance to enter the zipping process), but once the ATP-binding mode can transit into the zipping process by chance, the same torque force is exerted.

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