Correlation between the conformational states of F₁-ATPase as determined from its crystal structure and single-molecule rotation

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F₁-ATPase is a rotary molecular motor driven by ATP hydrolysis that rotates the γ-subunit against the αβγ ring. The crystal structures of F₁, which provide the structural basis for the catalysis mechanism, have shown essentially 1 stable conformational state. In contrast, single-molecule studies have revealed that F₁ has 2 stable conformational states: ATP-binding dwell state and catalytic dwell state.

In single-molecule rotation assay, chimera F₁ showed a catalytic dwell pause in every rotation when viewed from the Fo (7). Fo is also a rotary motor that is incorporated into a single copy of the mutant F₁, the corresponding residues in the ADP-bound F₁, and single-molecule experiments where F₁ rotation was inhibited by adenosine-5'-diphosphate (AMP-PNP) and sodium azide (Na₃N₅) (10). This structure, referred to as the “reference structure,” revealed that 3 α-subunits and 3 β-subunits are alternately aligned to form a hetero hexamer ring and the γ-subunit is set into the central cavity of the αβγ ring. The catalytic sites are located at each αβ interface, mainly on the β-subunit. Each β is in a different conformational state depending on the bound substrate; one binds to AMP-PNP (βTP), another to ADP (∆βTP), and the third to none (∆βempty). Both βTP and βDp are in the closed conformation where the C-terminal domain swings toward the nucleotide-binding domain to close the cleft between these domains. As a result, these β-subunits wrap the bound nucleotide tightly. In contrast, ∆βempty adopts an open conformation to weaken the affinity to the nucleotide. These structural features agree well with the binding change mechanism (5), which assumes that each catalytic site is in a different catalytic state and the interconversion of catalytic states drives the rotary motion of the γ-subunit, although there are some inconsistencies (11). Since this work, many crystal structures of MF₁ with different chemical inhibitors have been reported. The crystal structure that differs most from the reference structure is that which has ADP-AlF₄, where AlF₄ binds to ADP to adopt the half-closed conformation and the γ is twisted by −20° (12). However, all other MF₁ structures have very similar conformations to the reference structure. Thus, the crystal structures of MF₁ essentially represent a certain stable conformational state of F₁ except for the ADP-AlF₄-bound MF₁ structure.

In contrast, single-molecule studies on the γ rotation of F₁ have revealed that F₁ has 2 distinct stable conformations. Since the observation that F₁ performs a 120° step rotation of γ upon hydrolysis of 1 ATP, intensive attempts have been made to resolve the 120° step into multiple substeps to allow better understanding of how the elementary catalytic steps are coupled with the mechanical rotation. In many of these studies including this one, F₁ E190D from thermophilic Bacillus PS3 (TF₁) has been used because TF₁ is stable under the harsh conditions of the single-molecule experiments and can be genetically modified. Thus far, 2 substeps have been found: the 80° and 40° substeps. High-speed imaging of the rotation (13) and a study of a mutant F₁ having a noticeably low ATP hydrolysis rate (14) revealed that the 80° substep is induced by ATP binding and that the 40° substep was initiated after hydrolysis.

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of bound ATP. Hereinafter, the 2 conformational states before the 80° or 40° substeps are referred to as the “binding dwell state” and “catalytic dwell state,” respectively. Recent studies have suggested that ADP release and Pi release occur at the binding dwell angle and catalytic dwell angle, respectively (15, 16). More recently, a temperature-sensitive reaction was found at the binding dwell angle in a rotation assay at low temperature (16).

Thus, single-molecule studies have revealed that F1 has 2 stable conformational states, whereas F1 adopts essentially 1 specific stable conformation in the crystal structure. Which conformational state do the crystal structures of F1 represent, binding dwell state, catalytic dwell state, or another new state? On this issue, there are some interesting results. The analysis of fluorescence resonance energy transfer (FRET) between fluorescent probes attached to β and γ suggests that the crystal structure probably corresponds to the catalytic dwell state (17). However, FRET measurement provides the relative distance between the fluorophores, but not the precise position in the 3D structure. Therefore, this result is not conclusive.

The reference structure was recently revised to show that βDP binds to Nβ7 at the γ-phosphate binding position (18), implying that F1 in the crystal structure is in the azide-stabilized form, while the feature of this structure was later revealed to be almost consistent with that of the catalytic ground state MF1 (19). Taking into account that F1 pauses at the catalytic angle when in the ADP-inhibited form (20), it seems plausible that the crystal structure is in the catalytic dwell state. However, there are no experimental results that clarify this point. In fact, a common view on the nature of crystal structure of the F1 has not been established yet: some postulate it to be in the binding dwell state (21, 22) and others think it is the catalytic dwell state (23, 24).

With an aim to addressing this issue, we generated a mutant F1γ from *Bacillus* PS3 in which cysteine residues were introduced at βE391 and γR84, respectively (25). In the crystal structures of bovine MF1, corresponding residues in the ADP-bound β (βDP) and the γ are in direct contact (18, 19, 22, 26). The residue of γR84 is a part of the “ionic track” (25, 27), which is the distinctive zonation of positively-charged residues around the axis of γ. It is postulated that β bends and unbinds its conformation, tracing the ionic track with the negative charges of βDP394 and βE395 (for MF1) so as to convert the bending motion of β into the rotary motion of γ (27). The importance of the ionic track is supported by the analysis of mutant F1γ in which βE391 and γR84 are substituted with cysteine (25). Thus, the direct contact between βE391 and γR84 is the representative βDP–γ interaction in the conformational state of the crystal structure. In this study, F1γ with βE391C and γR84C mutations was investigated in a single-molecule rotation assay to observe where it pauses when the introduced cysteine residues form a disulfide bond, thereby fixing the motor in the conformational state corresponding to the conformational state of the crystal structures. In addition to the cross-link experiment, the correlation between the crystal structures and the single-molecule experiment was studied by investigating at which angle F1γ stops rotation in the

### Table 1. Distance between γ-carbons of βE395 and γR75 in crystal structures of MF1

<table>
<thead>
<tr>
<th>Protein Data Bank ID code</th>
<th>Ref.</th>
<th>Distance, Å</th>
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<tbody>
<tr>
<td>1e79</td>
<td>26</td>
<td>βTP 11.2</td>
</tr>
<tr>
<td>1h8e</td>
<td>12</td>
<td>βDP 11.1</td>
</tr>
<tr>
<td>1woj</td>
<td>22</td>
<td>βempty 11.2</td>
</tr>
<tr>
<td>2ck3</td>
<td>18</td>
<td>11.0</td>
</tr>
<tr>
<td>2jdi</td>
<td>19</td>
<td>11.0</td>
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<table>
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<tr>
<th></th>
<th></th>
<th>βTP 11.0 ± 0.3</th>
<th>βDP 5.9 ± 0.2</th>
<th>βempty 22.6 ± 4.1</th>
</tr>
</thead>
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ND, not determined because E395 in βempty was not visible in these structures.

*Average ± SD.
presence of AMP-PNP and/or NaNO₃, the most commonly used chemicals for the crystallization of F₁.

Results

A Chimera F₁⁺βγ, αβββ(E190D/E391C)γ(R84C). A mutant F₁⁺βγ from Baccillus PS3 was prepared in which cysteine residues were introduced at βE391 and γR84, which correspond to the βE395 and γR75 of bovine MF₁, respectively. In the crystal structure of bovine MF₁, these 2 residues are in direct contact (Fig. 1). Although the whole structure of TF₁ has not been solved, it is most likely that TF₁ structure is very similar to MF₁, and that βE391 and γR84 of TF₁ are also in a very close proximity when TF₁ takes the conformational state corresponding to the crystal structure of MF₁. This is because F₁ from other species shows structural features essentially identical to that of MF₁ even if the amino acid sequence is not highly homologous to that of MF₁, as seen in the γ subunit of F₁ from Escherichia coli (EF₁). The structure of the γ of EF₁ is very similar to that of MF₁ (28), although the sequence homology against the γ of MF₁ is low (only ~30%) the same as the γ of TF₁. Furthermore, the sequences around βE391 and γR84 are highly conserved in many species, suggesting the high structural conservation of these regions (Figs. S1 and S2). When βE391 and γR84 of F₁⁺βγ from Baccillus PS3 were replaced with cysteine residues, they efficiently (~90%) formed a disulfide bond under oxidizing conditions within only a few minutes (25). Thus, it is reasonable to assume that TF₁ has the essentially identical structural features to MF₁. In the MF₁ crystal structure, the β that has direct contact with γR75 adopts the β₁DP form. The γ-carbons of these residues that are in equivalent positions to the sulfur atoms of cysteine residue are only 5.9 Å away (Table 1), which is sufficiently close for disulfide bond formation. In contrast, the distances in the β₁DP or β₁empt–γ pairs are 11 and 23 Å, respectively. Thus, the disulfide bond would be formed in the β₁DP–γ pair. In the single-molecule rotation assay, γ was actually cross-linked to β only when the β was in a specific catalytic state, revealed to be the catalytic dwell state (see below). Furthermore, E190D mutation was introduced to the β-subunit in addition to E391C mutation. The mutant β(E190D/E391C)* was reconstituted with the wild-type β to build a chimera F₁⁺βγ, αβββ(E190D/E391C)γ(R84C), which has a single copy of β(E190D/E391C) (Fig. 2A). Here, the β(E190D) mutation was used as an angular position marker to determine the pause angle of cross-linked F₁⁺βγ. Shimatani et al. (14) showed that the βE190D mutation severely slows the hydrolysis step and causes the long pause (τ = 320 ms) at the catalytic angle. When a single copy of β with E190D mutation is incorporated in F₁⁺βγ, reconstituted chimera F₁⁺βγ exhibits a transient pause at the catalytic dwell angle of βE190D that is ~200° from the angle at which the βE190D subunit binds to ATP (Fig. 2B) (29). Thus, a chimera F₁⁺βγ with a single β(E190D) enables us to determine the pause angle of the cross-linked F₁⁺βγ relative to the catalytic angle of β(E190D). It should be noted that a chimera F₁⁺βγ with 1 β(E190D) was reported to exhibit another short pause at ~120° from the catalytic dwell angle of β(E190D) (29). In this

*The designation subunit name (mutation name) indicates the subunit with the indicated mutations. The designation without parentheses indicates a mutation.
3-carboxy-4-nitrophenyl disulfide (DTNB) was injected to the flow experiment. First, the free rotation of a study (33 ms per frame). Then rotated back to the inversed angle of the original magnetic field (θ); and then rotated back to the inversed angle of the original magnetic field (θ) or some paused at irregular positions (indicated by a mark in dwell angles (Fig. 3). Among selected molecules, 50% showed the clear pausing at a catalytic angle and were identified as the chimera, which carry 2, 3, or 0 of cysteine residue was modified with a biotin that blocks the disulfide bond formation. After observing a pause, a pausing F1 cross-linked F1 was manipulated with magnetic tweezers to confirm the cross-linkage (Fig. 4). Unlike ADP-inhibited F1 cross-linked F1 never resumes rotation, even when forcibly rotated more than +80°, which is sufficient to reactivate ADP-inhibited F1 with nearly 100% efficiency (Fig. 4 A) (30). Instead, cross-linked F1 behaved as a twisted spring: when forcibly rotated and released, it just stopped rotation as a twisted spring: when forcibly rotated and released, it just returned to the original pausing position. The angular velocity of return was always very fast (3.8 revolutions per s) and comparable with the ATP-driven rotation velocity at high ATP concentration (∼5 revolutions per s). In rare cases, F1 exhibited irregular behaviors such as large fluctuations at an irregular position (Fig. 4 B–D). Such irregular behaviors were observed in some cases where cross-linked F1 was rotated more than ±120° with the magnetic tweezers. This phenomenon is probably caused by the partial unfolding of the γ- or β-subunits. When the buffer was exchanged (Fig. 3 B and E). The remaining would be molecules in which cysteine residue was modified with a biotin that blocks the disulfide bond formation.
The proposed reaction scheme of F₁ and correlation with the crystal structure. State A represents the binding dwell state. After ATP binding and ADP release, F₁ makes an 80° substep. Thereafter, F₁ hydrolyzes the tightly bound ATP (denoted by *) in the state transition from B to C. These states correspond to the crystal structure of F₁. After releasing P, F₁ makes a 40° substep to complete a cycle of ATP hydrolysis reaction coupled with 120° rotation. State D is the next binding dwell state.

with a reducing one, the disulfide bond was cleaved and F₁∅∅∅ molecules resumed active rotation (Fig. 3 C and F). Molecules that did not resume rotation or significantly changed their binding dwell angles were omitted from our data analysis. The angle differences of the cross-link angle (Fig. 3E) from the nearest binding angle on the clockwise side (Δθ₁ in Fig. 3D) or the catalytic angle of β(E190D/E391C) (Δθ₂ in Fig. 3D) were determined. These values were also determined by the comparison with the binding and catalytic dwell angles after reduction (Δθ₁ and Δθ₂ in Fig. 3F). For this analysis, the set of the experiments was repeated a total of 72 times with 36 molecules. The histograms of Δθ₁ and Δθ₂ showed a single peak and gave mean values of 82.7 ± 15.9° for Δθ₁ and 2.0 ± 11.0° for Δθ₂ (Fig. 3G). The histograms of Δθ₁ and Δθ₂ also gave essentially the same values, 81.3 ± 17.0° for Δθ₁ and 1.4 ± 13.5° for Δθ₂, showing the reproducibility of the experiment (Fig. 3H). These results show that the cross-linked F₁∅∅∅ pauses at the catalytic dwell angle of β(E190D/E391C). This means that the reference structure of MF₁ represents the conformational state of F₁ in the catalytic dwell state, and that β∅∅∅∅ conformation represents the catalytically active state that executes ATP hydrolysis. Considering that the standard deviations are much smaller than the magnitude of the 40° substep, cross-linked F₁∅∅∅ will not be stable at the binding angle.

Other chimera F₁∅∅∅ molecules, αβ(E190D)β(E391C)γ(R84C) and αβ(E190D)β(E391C)γ(R84C), were also examined to confirm that γR84C can form a disulfide bond with βE391C, which does not have the βE190D mutation. The rotation of αβ(E190D)β(E391C)γ(R84C) was rarely seen because of the low reconstitution efficiency of β(E190D) as described above. The cross-linked chimera paused at either of the 2 catalytic angles that did not correspond to the catalytic angle of βE190D (Fig. S3). Thus, it was verified that βE190D mutation does not affect the pause position of cross-linked F₁∅∅∅.

Discussion

The crystal structure of F₁ was experimentally shown to represent the conformational state of catalytic dwell state found in single-molecule rotation assay. This means each β-subunit is in the βTP, βTP∅, or β∅∅∅∅ conformation during the catalytic dwell state. The β-gas cross-linking using a chimera F₁∅∅∅ carrying 1 copy of β(E190D)/E391C showed that the catalytic angle of β(E190D/E391C) coincides with the pause position of F₁∅∅∅ where β∅∅∅∅ is cross-linked with γ. This finding means that the β∅∅∅∅ corresponds to the conformational state of β that executes ATP hydrolysis reaction, consistent with theoretical works on quantum mechanics and molecular mechanics (31) or free-energy difference simulations (32). The crystal structure of BeF₃/F₁, which is thought to mimic the catalytic intermediate state, also supports this result (22).

Fig. 6 shows the present reaction scheme of F₁, in which there are 2 chemical states during the catalytic dwell: prehydrolysis (state B) and posthydrolysis states (state C). Both states have the corresponding crystal structures. The recently reported structures (19, 33), the so-called “ground state” structures, in which both βTP and βTP∅ bind with AMP-PNP correspond to the prehydrolysis state. However, the ADP-AlF₃-bound structure (34) would represent the posthydrolysis state. The azide-bound structures (10, 18) would also correspond to this state. Considering that β∅∅∅∅ represents the state that hydrolyzes ATP, β∅∅∅∅ corresponds to the β before and after executing hydrolysis (the β at the bottom in states B and C). Consequently, βTP conformations represent the ATP-bound state A, where F₁ hydrolyzes ATP (state A in Fig. 6), and β∅∅∅∅ corresponds to the state after ADP release. Thus, this study established the correlation between the conformational states of F₁ found in the crystal structures and the single-molecule rotation assay. However, there is one uncertain point in the reaction scheme, the timing of Pi release. The present model assumes that β∅∅∅∅ is the Pₐ-bound state, and after β∅∅∅∅ releases Pi, the 40° substep is triggered (state C to state D). This assumption is based on the recent study of the crystal structure of
yeast MF1 (33) in which P\textsubscript{i} binds to \(\beta_{\text{empty}}\). The P\textsubscript{i}-binding residues in the structure are consistent with those of biochemical studies that have identified the P\textsubscript{i} binding site (35), supporting our reaction mechanism. However, considering the other crystal structures that do not show obvious electron density of P\textsubscript{i} in the putative P\textsubscript{i}-binding site, an alternative model is still possible; P\textsubscript{i} is released from the \(\beta_{\text{psi}}\) immediately after hydrolysis. A clarification of this issue is the next challenging task in the study of the mechanochanical-coupling mechanism of F\textsubscript{1}-ATPase.

Another issue is the structure of the binding dwell state. Although the ADP-AF\textsubscript{1}-bound MF1 shows obvious structural differences, the direction of the twist in the \(\gamma\) is opposite to our expectations. Therefore, ADP-AF\textsubscript{1}-bound MF1 is not in the binding dwell state. Rather, it might represent the intermediate state between state A and B of our reaction scheme in Fig. 6. Thus, there are no structural data about the binding dwell state. Because it is crucial for the understanding of the mechanochanical coupling mechanism of F\textsubscript{1}, a crystallographic study of F\textsubscript{1} in the binding dwell state is one of the most important tasks for the understanding of the mechanism of F\textsubscript{1}-ATPase. However, the fact that the crystal structures identified so far are comparable with the reference structure implies that the crystallization of F\textsubscript{1} in the binding dwell state is very difficult. One possible way is to crystallize F\textsubscript{1} at low temperature (16) or F\textsubscript{1B} inhibited by tentoxin (36), where it spends most of the catalytic turnover time pausing at the binding dwell angle.

Materials and Methods

Preparation of F\textsubscript{1P}:

Throughout this work, \(\alpha\beta\gamma\) complex of F\textsubscript{1}-ATPase from thermophilic Bacillus PS\textsubscript{3} (TF) was used. For inhibition by AMP-PNP or NADH, wild-type F\textsubscript{1P} was modified for the rotation assay. \(\alpha\) (His\textsubscript{189}, A195, His\textsubscript{197} at N terminus); \(\beta\) (His\textsubscript{10}, A110, and His\textsubscript{312}) was used. For simplicity, this F\textsubscript{1P} was referred as wild-type F\textsubscript{1P} or \(\alpha\beta\gamma\). For the \(\beta\)-\(\gamma\) cross-link experiment, cysteine residues were introduced into \(\gamma\) and \(\beta\) of wild-type F\textsubscript{1P} and \(\alpha\beta\gamma\) (E190D)\textsubscript{3} (14) to construct 4 mutants: \(\alpha\beta\gamma\) (R84C), \(\alpha\beta\gamma\) (E190D)\textsubscript{3} (R84C), \(\alpha\beta\gamma\) (E190D)\textsubscript{3} (R84C), \(\alpha\beta\gamma\) (E190D)\textsubscript{3} (R84C). Mutagenesis to construct the expression vectors of these mutants was performed as per the previous report on \(\beta\)-\(\gamma\) cross-linking (25). The mutants of F\textsubscript{1P} were expressed in E. coli, purified, and biotinylated as reported (37). For the reconstitution of the chimera, \(\alpha\beta\gamma\) (E190D)\textsubscript{3} (R84C), solutions of \(\alpha\beta\gamma\) (R84C) and \(\alpha\beta\gamma\) (E190D)\textsubscript{3} (R84C) were mixed in a molar ratio of 2:1 and incubated for -2 days in the presence of 200 mM NaCl and 100 mM DTT at 4 °C and pH 7.0. The chimera \(\alpha\beta\gamma\) (E190D)\textsubscript{3} (R84C) was prepared by mixing solutions of \(\alpha\beta\gamma\) (E190D)\textsubscript{3} (R84C) and \(\alpha\beta\gamma\) (E190D)\textsubscript{3} (R84C) in a molar ratio of 2:1.

Rotation Assay.

The rotation motion of F\textsubscript{1P} was visualized by attaching a magnetic bead (\(0.2 \mu m\), Seradyn) onto the \(\gamma\)-subunit of F\textsubscript{1P} and immobilizing the \(\alpha\beta\gamma\) ring on a Ni-NTA-modified glass surface. Phase-contrast images of the rotating bead were obtained with an inverted optical microscope (IX-70, Olympus) equipped with magnetic tweezers (30). The image was captured with a CCD camera (FC300M, Takenaka) and recorded with a DV-CAM (DSR-11, Sony) at 30 fps. The recorded images were analyzed with image analysis software (Celery, Library) or a custom-made program (K. Adachi, Waseda University). The experimental procedures of the rotation assay for \(\beta\)-\(\gamma\) cross-linking were mostly the same as those reported (16), except for the content of the buffer. The basal buffer for the rotation assay contained 50 mM Hepes-KOH at pH 8.0, 50 mM KC\textsubscript{2}, 2 mM MgCl\textsubscript{2}, 1 mM phosphonoenoic acid, 0.1 mg/ml pyruvate kinase, 5 mg/ml BSA, and 1 mM DTT. For \(\beta\)-\(\gamma\) cross-linking, 200 mM DTNB was added to the basal buffer, from which DTT and BSA were omitted. The rotation assay for AMP-PNP and Na\textsubscript{2}H\textsubscript{2}PO\textsubscript{4} inhibition was carried out in the buffer containing 50 mM Mops-KOH at pH 7.0, 50 mM KC\textsubscript{2}, 2 mM MgCl\textsubscript{2}, 5 mg/ml BSA, and the indicated amount of nucleotides and Na\textsubscript{2}H\textsubscript{2}PO\textsubscript{4}.

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**Supporting Information**

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### \( \beta \) subunit

![Sequence alignment of the \( \beta \)-subunit of F1, fom bovine mitochondria (MF1), yeast mitochondria (yF1), *Escherichia coli* (EF1), and thermophilic Bacillus PS3 (TF1). A red arrow indicates the position of mutation into cysteine for cross-link. A black arrow indicates the mutated position of the \( \beta \)E190D. Sequence alignment was carried out by using ClustalX 2.0.9 software (1). Amino acid residues of precursor peptides were not included for alignment.](image)

Fig. S2. Sequence alignment of the γ-subunit of MF1, yF1, EF1, and TF1. A red arrow indicates the position of mutation into cysteine for cross-link. Blue arrows indicate the mutated positions to cysteine for biotinylation. Sequence alignment was carried out by using ClustalX 2.0.9 software (1).

Fig. S3. The pausing positions of chimera F1βγ, αβ(190D)β(391C)(R84C) or αβ(190D)β(391C)2γ(R84C). (A) Schematic images of the chimera F1βγ molecules. (B) Typical angle distribution of a αβ(190D)β(391C)(R84C) molecule. Red bars represent the angular position of free rotation before cross-link. Blue bars represent pausing angular position after cross-link. (Inset) The trace of centroid of the bead image. (Scale bar: 100 nm.) (C) Histogram of angle deviation of pausing position from the nearest binding dwell angle on the clockwise side (Δθ1). (D) Histogram of angle deviation of pause position from the catalytic angle of β(190D). Because αββ(190D)β(391C)2γ(R84C) paused at two catalytic angles that correspond to those of wild-type β-subunits, in other words at the position about ±120° from the catalytic angle of β(190D), distribution of absolute values is given. In C and D, data from two kind of chimera were combined. The total molecule and trial numbers were 11 (of them, two were αββ(190D)γβ(391C)2γ(R84C)) and 20, respectively.