The N-terminal region of the ε subunit from cyanobacterial ATP synthase alone can inhibit ATPase activity

Kosuke Inabe‡,‡‡, Kumiko Kondo‡,‡‡, Keisuke Yoshida‡, Ken-ichi Wakabayashi†‡§, and Toru Hisabori†‡§

From the ‡Laboratory for Chemistry and Life Science, Tokyo Institute of Technology, Nagatsuta-cho 4259-R1-8, Midori-ku, Yokohama 226-8503, Japan and the §School of Life Science and Technology, Tokyo Institute of Technology, Nagatsuta-cho 4259, Midori-ku, Yokohama 226-8501, Japan

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ATP hydrolysis activity catalyzed by chloroplast and proteobacterial ATP synthase is inhibited by their ε subunits. To clarify the function of the ε subunit from phototrophs, here we analyzed the ε subunit–mediated inhibition (ε-inhibition) of cyanobacterial F1-ATPase, a subcomplex of ATP synthase obtained from the thermophilic cyanobacterium Thermosynechococcus elongatus BP-1. We generated three C-terminal α-helix null ε-mutants; one lacked the C-terminal α-helices, and in the other two, the C-terminal conformation could be locked by a disulfide bond formed between two α-helices or an α-helix and a β-sandwich structure. All of these ε-mutants maintained ATPase-inhibiting competency. We then used single-molecule observation techniques to analyze the rotary motion of F1-ATPase in the presence of these ε-mutants. The stop angular position of the γ subunit in the presence of the ε-mutant was identical to that in the presence of the WT ε. Using magnetic tweezers, we examined recovery from the inhibited rotation and observed restoration of rotation by 80° forcing of the magnetic tweezers, we examined recovery from the inhibited rotation when the membrane potential is insufficient for ATP synthase (11–13).

Because ATP synthase can potentially catalyze ATP hydrolysis when the membrane potential is insufficient for ATP synthesis, regulation of the activity should be important for living cells to avoid futile ATP hydrolysis reactions. One of the most well-known regulatory mechanisms is MgADP inhibition (ADP inhibition), which is induced by occupation of the catalytic site with MgADP and prevents the ATP hydrolysis reaction (14). The γ subunits of chloroplast-type ATP synthases, including the cyanobacterial one, possess an insertion region composed of 30–40 amino acids between the Rossmann-fold domain and the C-terminal domain (CTD)² of α-helices, which is not observed in the γ subunit of other F₁; this region has a function in regulating F₁-ATPase (15, 16). In addition, the γ subunit of the chloroplast ATP synthase has an additional nine-amino acid insertion containing a pair of Cys residues at this insertion region, and this Cys pair is key for the redox control by thioredoxin (17, 18). Under light conditions, this pair of Cys, which forms a disulfide bond under dark conditions, is reduced by thioredoxin, and consequently the catalytic activity is accelerated (12, 19, 20).

In addition, F₁-ATPases from chloroplasts and proteobacteria adopt the ε subunit as an intrinsic inhibitor for the ATP hydrolysis reaction (21, 22). The ε subunit is composed of two domains, the N-terminal domain (NTD) with a β-sandwich structure and the CTD containing two tandem α-helices (23–25). The inhibitory mechanism by the ε subunit (ε-inhibition) has been proposed as follows: the ε subunit changes its conformation of two C-terminal α-helices from the retracted to the extended form in the enzyme complex in a manner dependent on a change in the microenvironment (26). In the case of the bacterial ε subunit, this conformational change is caused by the binding and release of the ATP molecule at the CTD of α-helices (27) and/or the change in membrane potential (28).

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1 To whom correspondence should be addressed: Laboratory for Chemistry and Life Science, Tokyo Institute of Technology, Nagatsuta-cho 4259-R1-8, Midori-ku, Yokohama 226-8503, Japan. E-mail: thisabor@res.titech.ac.jp.

2 The abbreviations used are: CTD, C-terminal domain; NTD, N-terminal domain; AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonate.
The e-inhibition of cyanobacterial F₁-ATPase

Inhibition of the ATP hydrolysis activity of F₁-ATPase was examined in the presence of the WT e subunit (εWT) or its mutants (Fig. 2, A and B). The extent of the inhibition by εWT was very similar to that reported previously (22). In contrast, one of our interesting findings is that εN clearly inhibited F₁-ATPase activity even at 300 nm, which was comparable with the findings for εWT (Fig. 2A). This result was unexpected because CTD of the e subunit was thought to be a key for the inhibition of F₁-ATPase activity, as mentioned previously (28, 29), and to date no reports of the inhibition of F₁-ATPase activity by NTD of the e subunit have been published. In addition, we found that εCC-SS and εNC-SS can also inhibit F₁-ATPase activity irrespective of their redox states (Fig. 2, B and C). These results also contradict the previous findings that the e subunit mutant, which is incapable of conformational change at CTD, cannot inhibit the activity of F₁-ATPase obtained from proteobacteria (34, 35). The apparent dissociation constant (Kd(app)) values between F₁-ATPase and the e subunit or its mutants were determined based on the e-dependent decrease of ATP hydrolysis activity (Table 1). The Kd(app) value for εWT was slightly lower than the previously reported value, 2.1 ± 0.3 nm (36), but comparable with those in other reports (13, 34). The Kd(app) value for εN (21 ± 6.3 nm) was about 10 times greater than those for εWT and εCC-SS_Ox (2.5 ± 0.1 nm) and εNC-SS_Ox (2.3 ± 2.2 nm).

Previously, we showed that the level of e-inhibition of F₁-ATPase containing the mutant γΔ198–222 subunit, which lacks the insertion region from Leu198 to Val222, apparently decreased to 20% (22). We therefore applied this mutant ATPase complex (F₁-ATPaseΔins) to investigate the inhibitory properties of εN. Neither εWT nor εN inhibited the ATP hydrolysis activity of the mutant ATPase complex (Fig. 2D), indicating that εN does not affect the activity of PK or LDH, which are used for our coupling assay system, and does not affect the α or β subunit of the complex as well.

The Kd(app) values of εSS under various oxidation or reduction conditions are shown in Table 1. No significant differences between the values that we obtained for εCC-SS or εNC-SS and those from the previous study on εWT were observed (36).

The properties of the e-mutants

To confirm that εN maintains the β-sheet structure, we measured the CD spectrum of the protein. εN showed a negative peak at around 220 nm, which indicates the formation of the typical β-sheet (Fig. 3A) (37), and is different from the possible unfolded structure, because the latter shows a positive peak at 220 nm and negative peak at 200 nm (38). The CD spectra of εCC-SS suggested that the folding of εCC-SS is identical to that of εWT irrespective of its redox state (Fig. 3B). In addition, εCC-SS_Ox inhibited F₁-ATPase activity, like εWT and εN (Fig. 2B). These results imply that the introduced Cys residues on the εCC-SS and the formation and dissociation of the disulfide bond in the CTD do not affect the affinity of εCC-SS to F₁-ATPase. As shown in Table 1, Kd(app) values of εWT were weaker than those of εWT and the other mutants. To examine the binding of εN to F₁-ATPase, we then tested the co-migra-

Results

**e-Mutants at the C-terminal domain**

To study the molecular mechanism of the e-inhibition in cyanobacterial ATP synthase, three e subunit mutants were prepared: εN, which consists of 83 amino acid residues at the NTD and lacks the CTD part containing α-helices; εCC-SS, whose Ala99 and Phe122 were substituted to Cys to allow disulfide bond formation between two α-helices (Fig. 1A); and εNC-SS, whose Thr16 and Arg124 were substituted with Cys to allow disulfide bond formation between the β-sandwich structure in the NTD and the α-helix in the CTD. We then determined the oxidant concentration that is sufficient for disulfide bond formation in εCC-SS and εNC-SS, and the reduced concentration to completely reduce these disulfide bonds (Fig. 1B). In the presence of more than 300 μM aldriothiol-2, disulfide bonds were formed between the two α-helices of εCC-SS and between the β-sandwich structure in the NTD and the α-helix in the CTD of εNC-SS. The disulfide bond in εCC-SS was cleaved in the presence of 300 μM DTT. In contrast, the disulfide bond in εNC-SS was cleaved when 1 mM DTT was added. We therefore used 300 μM aldriothiol-2, and 300 μM or 1 mM DTT to control the disulfide bond formation in the mutants for further experiments. Hereafter, we describe εCC-SS_Ox as the oxidized εCC-SS, εCC-SS_Red as the reduced εCC-SS, and εCC-SS.Non as the untreated εCC-SS. In addition, εNC-SS_Ox as the oxidized εNC-SS, εNC-SS_Red as the reduced εNC-SS, and εNC-SS.Non as the untreated εNC-SS were used.
The ε-inhibition of cyanobacterial F₁-ATPase

Of F₁-ATPase and H₉₂₈₀ WT or H₉₂₈₀ N by gel-filtration chromatography and analyzed the subunit composition in the peak by Western blotting (Fig. 4A–D). When only F₁-ATPase was subjected to the gel-filtration chromatography, a single peak was observed (Fig. 4A, peak 1). The H₉₂₅₂ subunit was detected by Western blotting as indicated (Fig. 4D, lane 1). In contrast, two peaks were obtained when F₁-ATPase was incubated with H₉₂₈₀ WT or H₉₂₈₀ N (Fig. 4B, peaks 2, 3, 5, and 6). After collecting these peak fractions, co-migrations of the ε subunits with F₁-ATPase were examined by anti-ε subunit antibody (Fig. 4D, lanes 2, 3, 5, and 6). These protein bands showed the protein mass at around 15 and 10 kDa, which correspond to the molecular mass of H₉₂₅₂ WT and H₉₂₅₂ N, respectively. As a control, ε₉ was subjected to gel-filtration chromatography (Fig. 4C, peak 7) solely, and the collected peak was analyzed by Western blotting (Fig. 4D, peak 7). Based on these results, we concluded that ε₉ can directly bind to the F₁-ATPase complex.

Inhibition of F₁-ATPase by εcc,ss at the single-molecule level

To understand the molecular mechanism behind the inhibition by NTD of the ε subunit, the inhibition of rotation of the γ subunit by one of the CTD null ε-mutants εcc,ss,ox was analyzed at the single-molecule level (Fig. 5). In this assay system, 3 μM ε₉₅₂ WT or εcc,ss,ox was used. Although we examined 3 μM ε₉ in this system, the marked change of rotation of the γ subunit was not observed. This must be due to the low affinity of ε₉ to the γ subunit, which was lower than ε₉₅₂ WT or εcc,ss,ox. We therefore tried to prepare 10 times higher concentrations of ε₉ for this experiment. However, we failed to handle the higher concentration of ε₉ in this study due to the low solubility of this
The \(\epsilon\)-inhibition of cyanobacterial \(F_\gamma\)-ATPase

To distinguish the inhibition of \(F_\gamma\)-ATPase by ADP and that by \(\epsilon\), restoration of rotation of the \(\gamma\) subunit was thoroughly studied using the magnetic tweezer technique (36, 39). For this purpose, a magnetic bead was attached to the \(\gamma\) subunit instead of the polystyrene beads, and the \(\gamma\) subunit stopped by the inhibition was forced 80° in the counterclockwise direction using the magnetic tweezers (Fig. 6A). ADP inhibition is a common way to inhibit \(F_\gamma\)-ATPase caused by the tightly bound ADP at the catalytic site(s) of the enzyme, and is conserved among the ATPases from mitochondria, proteobacteria, and chloroplasts. In the case of cyanobacterial \(F_\gamma\)-ATPase (36) and thermophilic bacterial \(F_\gamma\)-ATPase (39), restoration by 80° forcing was observed at the ADP inhibition state, whereas the \(\epsilon\)-inhibition was not recovered by this procedure. We therefore applied this technique to the \(\epsilon_{CC,SS,\text{Ox}}\)-inhibited \(F_\gamma\)-ATPase. This time, restoration of rotation was observed in ADP-inhibited \(F_\gamma\)-ATPase by 80° forcing (Fig. 6B and C), and the frequency of restoration was about 70% (Table 3). This value is fairly similar to that reported previously (86% in Ref. 36). In contrast, \(\epsilon_{WT}\)-inhibited \(F_\gamma\)-ATPase did not restore the rotation after 80° forcing (Fig. 6D). Restoration was also not observed in the case of \(\epsilon_{CC,SS,\text{Ox}}\)-inhibited \(F_\gamma\)-ATPase (Fig. 6E). These results are summarized in Table 3 and suggest that the mechanism of inhibition of \(\epsilon_{CC,SS,\text{Ox}}\)-inhibited \(F_\gamma\)-ATPase is identical to that of \(\epsilon_{WT}\).

Discussion

In this study, we aimed to clarify the mechanism of \(\epsilon\)-inhibition of cyanobacterial ATP synthase in detail. First, we found that three CTD null mutants, \(\epsilon_{WT}, \epsilon_{CC,SS},\) and \(\epsilon_{NC,SS}\), inhibited \(F_\gamma\)-ATPase activity based on the enzymatic analysis (Fig. 2A–C). This was unexpected because the significance of CTD of the \(\epsilon\) subunit for the inhibition of ATP hydrolysis has already been reported (29, 30). Single-molecule observation revealed that \(\epsilon_{CC,SS,\text{Ox}}\) stopped rotation of the \(\gamma\) subunit at around 80° (Fig. 5, I–L). The restoration of rotation of the \(\gamma\) subunit by forcing the \(\gamma\) subunit was not observed when it was inhibited by \(\epsilon_{CC,SS,\text{Ox}}\) (Fig. 6E), although ADP-inhibited \(F_\gamma\)-ATPase was easily restored by 80° forcing. We therefore concluded that the \(\epsilon_{CC,SS}\)-inhibitory mechanism was identical to that of \(\epsilon_{WT}\) inhibi-
The \(\epsilon\)-inhibition of cyanobacterial \(F_1\)-ATPase

### Table 1

| Type of \(\epsilon\) | \(\epsilon_{\text{WT}}\) | \(\epsilon_N\) | \(\epsilon_{\text{CC,SS}}\) | \(\epsilon_{\text{NC,SS}}\) |
|---------------------|----------------|---------------|----------------|-----------------|
|                     |               | Non | Red | Ox | Non | Red | Ox |
| \(K_{\text{dissym}}\) (nM) | 0.7 ± 0.3 | 21 ± 6.3 | 2.2 ± 0.5 | 2.8 ± 0.7 | 2.5 ± 0.1 | 1.1 ± 0.5 | 1.4 ± 0.7 | 2.3 ± 2.2 |

**Figure 3. Spectroscopic analysis of the \(\epsilon\)-mutants structure.** A, CD spectra of \(\epsilon_{\text{WT}}\) or \(\epsilon_N\). Solid line, \(\epsilon_{\text{WT}}\); dotted line, \(\epsilon_N\). B, CD spectra of \(\epsilon_{\text{CC,SS,Non}}\), \(\epsilon_{\text{CC,SS,Red}}\), \(\epsilon_{\text{CC,SS,Ox}}\). The concentrations of the samples were 0.1 mg/ml.

This result indicates that \(\epsilon\)-inhibition of cyanobacterial ATP synthase can occur irrespective of the conformation of CTD of the \(\epsilon\) subunit, although CTD of the \(\epsilon\) subunit is required for the tight binding to the \(\gamma\) subunit (Table 1). To our knowledge, all previous studies on the proteobacterial \(\epsilon\) subunit indicated that CTD is indispensable for \(\epsilon\)-inhibition (40–42), and no reports have described that only NTD of the \(\epsilon\) subunit inhibited the ATP hydrolysis activity of \(F_1\)-ATPase or \(F_0F_1\)-ATPase.

Cyanobacteria are believed to be the origin of chloroplasts, which were incorporated into ancestor cells during symbiosis. Consequently, many metabolic processes in chloroplasts are very similar to those in cyanobacteria. Therefore, the \(\epsilon\) subunit of cyanobacterial ATP synthase may also have inhibitory mechanisms similar to those of chloroplast ATP synthase. However, Nowak et al. (30) reported that the mutant \(\epsilon\) subunit of chloroplast ATP synthase from spinach, which lacks CTD, cannot inhibit \(F_1\)-ATPase activity. In contrast, our results clearly indicate that \(\epsilon_N\) inhibits the activity (Fig. 2), although the affinity to the complex was lower than that for \(\epsilon_{\text{WT}}\) (Fig. 2 and Table 1). There is therefore a possibility that the \(\epsilon\) subunit that lacks CTD did not sufficiently associate with the \(\gamma\) subunit of chloroplast ATP synthase in their experimental conditions (30) and did not inhibit \(F_1\)-ATPase activity very well. Recently, the whole molecular structure of chloroplast ATP synthase was determined by cryo-EM (43), and our group also determined the X-ray crystal structure of the cyanobacterial \(\gamma\)–\(\epsilon\) subcomplex (16). In both structures, CTD of the \(\epsilon\) subunit showed a retracted form, although CTDs of \(\epsilon\) subunits from *Escherichia coli* and *Geobacillus stearothermophilus* (formerly known as thermophiliic *Bacillus* PS3) were extended in the crystal structures (44, 45). Recently, the structure of \(F_1\)-ATPase from *Caldalkalibacillus thermarum* was reported, and the \(\epsilon\) subunit was found as a retracted form in this complex, whereas CTD of the \(\epsilon\) subunit exerted the inhibitory effect on the ATPase activity (46, 47). This might be another example of inhibition by the retracted form CTD of the \(\epsilon\) subunit.

The \(\gamma\) subunits of chloroplast-type (cyanobacteria and chloroplast-type) ATP synthase equip the insertion region (Fig. 7), which also functions to inhibit ATP hydrolysis activity (16). We therefore investigated the involvement of this region with the \(\epsilon\)-inhibition of cyanobacterial ATP synthase in Fig. 2C. We then prepared a \(\gamma\) subunit mutant of \(F_1\)-ATPase lacking the insertion region of the \(\gamma\) subunit (\(F_1\)-ATPase\(_{\Delta\text{ins}}\)). The inhibition of the ATP hydrolysis activity of \(F_1\)-ATPase\(_{\Delta\text{ins}}\) by the \(\epsilon\) subunit and its mutants (Fig. 2D) was not remarkable compared with that of the WT \(F_1\)-ATPase. In the crystal structure of the cyanobacterial \(\gamma\)–\(\epsilon\) subcomplex, the \(\beta\) strand at the proximal end of \(\epsilon\)-NTD appeared to form a mixed parallel \(\beta\)-sheet with the \(\beta\)-strand of the \(\gamma\) subunit to provide tight coupling between the \(\gamma\) and \(\epsilon\) subunits (see Fig. 1C of Ref. 16). Therefore, both the insertion region of the \(\gamma\) subunit of cyanobacterial ATP syn-
The \( \varepsilon \)-inhibition of cyanobacterial \( F_\gamma \)-ATPase

In addition, the \( \varepsilon \)-subunit may affect the conformation of the insertion region of the \( F_\gamma \)-subunit, which was found to be a \( \beta \)-hairpin structure (16, 43), to control the ATP hydrolysis activity in cyanobacteria and have an impact on the redox state of the \( \gamma \) subunit from chloroplast ATP synthase as well (48).

Only from these *in vitro* analyses, we could not draw a definitive conclusion on whether CTD of the \( \varepsilon \) subunit of cyanobacterial ATP synthase can change the conformation in the \( F_\gamma F_\delta \) complex and exert the inhibitory effect on ATP hydrolysis. However, it should be noted that there are some organisms whose \( \varepsilon \) subunit of ATP synthase lacks CTD (42, 49).

Table 2
Stop angular position of rotation of the \( \gamma \) subunit inhibited by the \( \varepsilon \)WT or \( \varepsilon \)CC,SS, Ox
The most stopped angular positions in Fig. 5 were averaged on the indicated number of the particles.

| Type of \( \varepsilon \) | Pausing angular position of \( \gamma \) (mean ± S.E.) |
|-----------------------|--------------------------------------------------|
| - \( \varepsilon \)     | 122 ± 4.3° (n = 12)                              |
| + \( \varepsilon \)WT   | 86.1 ± 4.1° (n = 10)                             |
| - \( \varepsilon \)CC,SS, Ox | 79.0 ± 3.1° (n = 12)                           |

Figure 5. Direct observation of the \( \gamma \) subunit rotation and its inhibition. Shown are total revolution numbers before and after the exchange of the assay buffer (A), those of \( \varepsilon \)WT (E), and those of \( \varepsilon \)CC,SS, Ox (I). The histograms of the stop angular position of the \( \gamma \) subunit before and after the exchange of assay buffer (B and C), those of \( \varepsilon \)WT (F and G), and those of \( \varepsilon \)CC,SS, Ox (U and K) were then calculated. The insets indicate the trajectories of each stop angular position of the \( \gamma \) subunit. D, H, and L, merged images of B and C, F and G, and J and K, respectively.

thase and \( \varepsilon \)-NTD must be involved in the regulation of ATP hydrolysis by the tight interaction between the \( \gamma \) and \( \varepsilon \) subunits. In addition, the \( \varepsilon \) subunit may affect the conformation of the insertion region of the \( \gamma \) subunit, which was found to be a
The e-inhibition of cyanobacterial F₁-ATPase

Figure 6. Molecular manipulation of the γ subunit in the ADP- or e-inhibited F₁-ATPase with magnetic tweezers. A, scheme of the mechanical manipulation of the γ subunit. ADP- or e-inhibited γ subunit was forced by 80°, which was maintained for 5 s. Black dots, angular position of the γ subunit. Blue dots, angular position of the ADP-inhibited γ subunit. Red dots, angular position of the γ subunit, which was manipulated by magnetic tweezers. D and E, time-course data of the angle of the γ subunit under the mechanical manipulation of the ADP-inhibited γ subunit. Blue dots, angular position of the γ subunit. Red dots, angular position of the γ subunit, which was manipulated by magnetic tweezers.

The possibility that the conformational change of CTD may occur in the F₁,F₃ complex. Cyanobacterial ATP synthase works for both the photophosphorylation and the oxidative phosphorylation reaction, and this unique feature might be the origin of the unique regulatory system.

Experimental procedures

Materials

Biotin-PEAC₉-maleimide was purchased from Dojindo (Kumamoto, Japan). ATP, phosphoenolpyruvate, and BSA were obtained from Sigma. Pyruvate kinase, lactate dehydrogenase, and NADH were purchased from Roche Diagnostics (Basel, Switzerland). Other chemicals were of the highest grade commercially available.

Protein preparation

In this study, the α₃β₃γ subcomplex from a thermophilic cyanobacterium (T. elongatus BP-1) (36) was used as a WT F₁-ATPase. The expression and purification of the F₁-ATPase complex were described previously (36). e₃γ was generated by the PrimeSTAR Mutagenesis Basal kit (Takara, Shiga, Japan) using the mutation primers shown in Table 4, and e₃γ was generated by an infusion method (Takara) using the mutation primers shown in Table 4.

The e subunit and its mutants were expressed in E. coli and purified as described previously (22) with some modification. For purification of the e subunits, the combination of anion-exchange chromatography using DEAE Sephadex (GE Healthcare) and hydrophobic interaction chromatography using a Phenyl-Toyopearl column (Tosoh, Tokyo, Japan) was used. For further purification, size-exclusion chromatography using a Superdex 75 column (GE Healthcare) equilibrated with 50 mM HEPES-KOH (pH 8.0) and 100 mM KCl (Buffer A) was performed. All proteins were stored at −80 °C until use.

Oxidation or reduction of e₃γ

e₃γ or e₃γmutant was incubated with various concentrations of Aldrithiol-2 or DTT in Buffer A for 60 min at room temperature to obtain the oxidized form e₃γ or e₃γmutant (e₃γOx) or the reduced form e₃γ or e₃γmutant (e₃γRed). To remove oxidants or reductants, the protein solution was loaded onto a Microcon column (10-kDa cut-off; Millipore) and centrifuged repeatedly. To confirm the oxidation and reduction state of e₃γ, the mutant proteins were precipitated by adding 5% (w/v, final concentration) TCA. After centrifugation, the supernatant was removed, and the remaining oxidant was washed away with 500 µl of acetone. After the removal of acetone by centrifugation, the pellet was dissolved in 50 mM Tris–HCl (pH 8.0), 1% (w/v) SDS, and thiol-modifying reagent 4-acetamido-4′-maleimidylstilbene-2′,2′-disulfonate (AMS). After labeling for 1 h at room temperature, protein samples were subjected to nonreducing SDS-PAGE, and the redox state of e₃γ or e₃γ was confirmed by determining the mobility in the gel.

ATP hydrolysis activity assay

ATP hydrolysis activity was measured in the presence of an ATP-regenerating system in 50 mM HEPES-KOH (pH 8.0), 100

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mm KCl, 2 mM MgCl₂, 2 mM ATP, 50 µg/ml PK, 50 µg/ml LDH, 2 mM phosphoenolpyruvate, and 0.2 mM NADH (53). The assay was carried out at 25 °C. The ATP hydrolysis rate after the addition of F₁-ATPase was determined by monitoring the decrease in NADH absorption at 340 nm using a spectrophotometer, V-550 (Jasco, Tokyo, Japan). The results of three independent experiments were averaged.

**Table 3**
Frequency of mechanical restoration of the ADP-γ, εWT-, or εCC-SS-Ox-inhibited γ subunit

| Stall angle | Activated | Not activated | Frequency of activation (%) |
|-------------|-----------|---------------|-----------------------------|
| 80° (ADP inhibition) | 7 | 3 | 70 (n = 10) |
| 80° (εWT- inhibition) | 0 | 8 | 0 (n = 8) |
| 80° (εCC-SS-Ox inhibition) | 0 | 8 | 0 (n = 8) |

**Table 4**
Sequence of primers used in this study to generate the ε subunit mutants

| ε mutation | Sequence of mutation primers |
|------------|-----------------------------|
| εN | 5'-CCAGCCGCTAGAGGCCGCGCGCA-3' |
| εA99C | 5'-GCCCATGCTGGCCGCACACCTTTG-3' |
| εf122C | 5'-GGCCGAAATACTCGTTGGGCT-3' |
| εT46E | 5'-CTCTCGACGATGCGTACGCTG-3' |
| εR124C | 5'-GCCGACGCGCTTTGGGCTGAGGCT-3' |

**Figure 7. Comparison of the amino acid sequence of the γ subunit from various species.** Partial amino acid sequence alignment of the γ subunits from various organisms was performed using the ESPript program (http://espript.ibcp.fr/ESPript/ESPript/index.php) (54). (Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.) The indicated numbers are the amino acid locations of the γ subunit of *T. elongatus* BP-1. Red box, strictly conserved amino acids; red letters, closely related amino acids. Black line, to Leu198–Val222 of the *T. elongatus* BP-1 γ subunit. B. taurus, Bos taurus; Bacillus PS3, thermophilic *Bacillus* PS3 (G. steathermophilus); S. oleracea, Spinacia oleracea; A. thaliana, Arabidopsis thaliana chloroplast-type 1; *Synechocystis* synechocystis sp. PCC6803; *T. elongatus* BP-1, Thermosynechococcus elongatus BP-1.

**CD spectrum**
The ε subunit or its mutants were diluted in 20 mM Tris-HCl (pH 8.0), and their CD spectra were obtained using a spectrophotometer, J-820 (Jasco, Tokyo, Japan), at room temperature. The concentration of the ε subunit or its mutants was 0.1 mg/ml.

**Estimation of ε subunit binding based on gel filtration chromatography**
The amount of εN bound to F₁-ATPase was estimated from the fraction of gel-filtration chromatography. 1 µM F₁-ATPase and 5 µM εWT or εN were incubated at room temperature for 10 min in Buffer B (50 mM HEPES-KOH (pH 8.0), 100 mM KCl, 0.1 mM MgCl₂, 0.1 mM ATP). Then the mixture was subjected to gel-filtration chromatography using a Superdex 200 increase column equilibrated with Buffer B, and the peaks were collected and analyzed (peaks 1–7). The proteins in the peak fractions were then precipitated by 5% (w/v, final concentration) TCA. After centrifugation, the supernatant was removed, and the remaining protein pellet was washed away with 500 µl of acetone. After the removal of acetone by centrifugation, the pellet was dissolved in 50 mM Tris-HCl (pH 8.0), 1% (w/v) SDS and subjected to SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane and detected by anti-β or -ε subunit antibodies.

**Single-molecule observation**
Rotation assays were carried out as reported previously (22) with some minor modifications. Streptavidin-coated beads with a diameter of 340 nm were used. Observation of rotation of the γ subunit was performed at room temperature. In general, solution exchange in the flow chamber took 1–2 min. The rotation...
tion was analyzed using custom software, Trans Viewer, prepared by Dr. Yusung Kim (12). Molecular manipulation using magnetic tweezers was performed as reported previously except for the magnetic beads (Sera-Mag Magnetic Streptavidin-Coated Particles, GE Healthcare) (36).

**Author contributions**—K. I. and T. H. conceived the study, and K. I., K. K., and K. Y. performed the experiments. K. W. and T. H. supervised the research. K. I., K. K., K. Y., K. W., and T. H. discussed the data. K. I. and T. H. wrote the paper, and K. K., K. Y., and K. W. commented on the manuscript.

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