

Characterization of the Relationship between ADP- and ϵ -induced Inhibition in Cyanobacterial F_1 -ATPase^{*S}

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Hiroki Konno, Atsuko Isu, Yusung Kim, Tomoe Murakami-Fuse, Yasushi Sugano, and Toru Hisabori¹

From the Chemical Resources Laboratory, Tokyo Institute of Technology, Nagatsuta, 4259-R1-8, Midori-ku, Yokohama 226-8503, Japan

The ATPase activity of chloroplast and bacterial F_1 -ATPase is strongly inhibited by both the endogenous inhibitor ϵ and tightly bound ADP. Although the physiological significance of these inhibitory mechanisms is not very well known for the membrane-bound F_0F_1 , these are very likely to be important in avoiding the futile ATP hydrolysis reaction and ensuring efficient ATP synthesis *in vivo*. In a previous study using the $\alpha_3\beta_3\gamma$ complex of F_1 obtained from the thermophilic cyanobacteria, *Thermosynechococcus elongatus* BP-1, we succeeded in determining the discrete stop position, $\sim 80^\circ$ forward from the pause position for ATP binding, caused by ϵ -induced inhibition (ϵ -inhibition) during γ rotation (Konno, H., Murakami-Fuse, T., Fujii, F., Koyama, F., Ueoka-Nakanishi, H., Pack, C. G., Kinjo, M., and Hisabori, T. (2006) *EMBO J.* 25, 4596–4604). Because γ in ADP-inhibited F_1 also pauses at the same position, ADP-induced inhibition (ADP-inhibition) was assumed to be linked to ϵ -inhibition. However, ADP-inhibition and ϵ -inhibition should be independent phenomena from each other because the ATPase core complex, $\alpha_3\beta_3\gamma$, also lapses into the ADP-inhibition state. By way of thorough biophysical and biochemical analyses, we determined that the ϵ subunit inhibition mechanism does not directly correlate with ADP-inhibition. We suggest here that the cyanobacterial ATP synthase ϵ subunit carries out an important regulatory role in acting as an independent “braking system” for the physiologically unfavorable ATP hydrolysis reaction.

F_0F_1 -ATP synthase synthesizes ATP from ADP and inorganic phosphate using the proton motive force (*pmf*),² which is generated across the cytoplasmic membranes of bacteria, thylakoid membranes of chloroplasts, and inner membranes of mitochondria by respiratory or photosynthetic electron transport systems (1–3). The complex consists of the membrane-embedded portion F_0 and the water-soluble portion F_1 . F_0 , the proton translocation device, is composed of three different sub-

units, *a*, *b*, and *c* with a stoichiometry of $a_1b_2c_{10-15}$ (1, 4–6). F_1 is composed of five different subunits designated α to ϵ with a stoichiometry of $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ (7). The minimum catalytic core as F_1 -ATPase is $\alpha_3\beta_3\gamma$ (8–10). As a prevailing enzyme catalysis mechanism, the rotary catalysis mechanism was first proposed by Boyer and co-workers (11). Following the x-ray crystal structure analysis of mitochondrial F_1 (12), the rotation of the γ subunit during ATP hydrolysis was studied by the biochemical cross-linking experiment (13), the polarized spectroscopic technique (14). Finally, rotation of the γ subunit was visualized at a single-molecule level using a microprobe attached to the rotating γ subunit (15). Thorough analysis of rotation of the γ subunit revealed that the γ subunit rotates with a discrete 120° step per single-molecule ATP consumption and that this 120° step consists of further 80° and 40° substeps (16, 17). The 80° substep rotation of γ occurs by ATP binding, whereas ATP cleavage and the release of one of its products, phosphate, result in a subsequent 40° substep (17, 18).

Activity of the ATP synthase is highly regulated *in vivo* and *in vitro*. Among them, ADP-inhibition of the ATPase activity is a common regulatory mechanism that has been found to occur in all ATP synthase examined so far. The ATP hydrolysis reaction of F_1 -ATPase is strongly inhibited by tight binding of ADP-Mg to the catalytic site(s) (9, 19–21). Single-molecule analysis showed that F_1 often lapses into an inactive ADP-induced inhibition (ADP-inhibition) state during rotation, and the inhibition has been assigned to the pause of rotation at 80° (22).

The intrinsic inhibitory function of ϵ , which is well characterized in bacteria and chloroplasts, is known as further regulation of the ATP hydrolysis reaction (23–25). The C-terminal α -helical domain of the ϵ subunit has been suggested to be important in conferring its inhibitory function (26, 27) and can adopt two distinct conformations: the extended conformation and the retracted conformation (28). In the extended conformation, the ϵ subunit inhibits the ATPase activity of F_0F_1 but has no significant effect on ATP synthesis (28, 29). Transition from the retracted conformation to the extended conformation is thought to act as an intrinsic regulation system (29, 30).

The significance of the C-terminal domain of the ϵ subunit on the inhibition is especially remarkable in the case of CF_1 obtained from spinach thylakoids. When the effect of the structural mutant ϵ subunit containing a deleted C-terminal domain ($\epsilon_{\Delta C}$) was examined, the ATPase activity of CF_0CF_1 was found to be 6-fold higher than that of the enzyme inhibited by the wild-type ϵ (31), whereas the ATPase activity of EF_0EF_1 in the presence of $\epsilon_{\Delta C}$ was only 1–2-fold higher compared with EF_0EF_1 with the wild-type ϵ subunit (32).

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^S The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S3 and Table S1.

¹ To whom correspondence should be addressed. Tel.: 81-45-924-5234; Fax: 81-45-924-5268; E-mail: thisabor@res.titech.ac.jp.

² The abbreviations used are: *pmf*, proton motive force; LDAO, lauryl dimethylamine oxide; TF_1 and TF_0TF_1 , F_1 and F_0F_1 from *Bacillus PS3*; ADP-inhibition, ADP-induced inhibition; ϵ -inhibition, ϵ -induced inhibition.

Regulator of Photosynthetic F_1 -ATP Synthase

In a previous study, we showed that the pausing angular position of γ in ϵ -inhibition is identical to that observed for ADP-inhibition, but distinctly different from the waiting position for ATP binding (33). Based on this result, we suggested that ϵ -inhibition is closely related to ADP-inhibition. Feniouk *et al.* also reported that the ADP-inhibition of TF_0TF_1 is enhanced by the ϵ subunit (34) and vice versa, inhibitory ADP-induced ϵ -inhibition in $\alpha_3\beta_3\gamma$ from *Bacillus* PS3 was confirmed by the single-molecule experiment (35). However, at their origin, ADP-inhibition and ϵ -inhibition must be independent phenomena from each other because even the $\alpha_3\beta_3\gamma$ subcomplex lapses into the ADP-inhibition state without the ϵ subunit. Hence, the relationship between these two inhibition systems is still unclear. In this study, we aimed to determine whether ϵ -inhibition can act independently of ADP-inhibition or whether ϵ -inhibition requires ADP-inhibition. The kinetic difference of the inhibited enzymes derived by these inhibition mechanisms is critical in understanding the regulation of the rotary motor enzyme when physiological conditions fluctuate.

EXPERIMENTAL PROCEDURES

Materials—Biotin-PEAC₅-maleimide was purchased from Dojin (Kumamoto, Japan). ATP, phosphoenolpyruvate, and BSA were obtained from Sigma. Pyruvate kinase, lactate dehydrogenase, and NADH were purchased from Roche Diagnostics. Other chemicals were of the highest grade commercially available.

Preparation of Proteins—In the previous single-molecule study of the cyanobacterial F_1 , we used a mutant, $\alpha_3\beta_3\gamma_{rot}$, for the rotation study, which consisted of α C144S/C194S, His₁₀- β C53S, and γ C90S/G112C/A125C (33). In this study, we prepared a new mutant complex, $\alpha_3\beta_3\gamma_{Arot}$ using His₁₀- α C144A/C194A, His₁₀- β C53A, and γ C90A/G112C/A125C. In addition, a His₁₀ tag was introduced onto α to strengthen the binding of the complex to the glass plate during the single-molecule experiments, and the cysteines on the original complex were substituted into alanine, conferring higher structural stability to the complex compared with $\alpha_3\beta_3\gamma_{rot}$. The stability of the complex was confirmed by gel filtration as described elsewhere. The higher structural stability of $\alpha_3\beta_3\gamma_{Arot}$ compared with $\alpha_3\beta_3\gamma_{rot}$ is mainly due to His₁₀ onto α (supplemental Fig. S1). All cysteines on the complex were substituted with alanine by the Mega-primer method (36) using the following mutation primers: 5'-CCCCTGCACCTGGTATTGTCCAGCGCAAATCTGTGGCGGAGCCATTGCAAACGGG-3' for α C144A; 5'-CAATCTTGAACAAAAGGGCCAAGACGTGATTGCGGTGTATGTGGCCATTGGTCAAAGCC-3' for α C194A; 5'-CGCGCCCGGCTTAGACGTGGCTGTAACCGCGAA-GTGCAACAACCTCCTTGGCG-3' for β C53A; and 5'-GGC-GCTGCTGGTGGTAAACAGGCGATCGCGGGCTGGCGG-GCGGTTACAACACTAATGTCATTCGCC-3' for γ C90A. His₁₀ was introduced to the N terminus of α using the following primer: 5'-CGCGCCCATGGAATCTAAGAAG-GAGATATACATATGCATCACCATCATCACCATCAC-CATCACCATATGGTAAGTATCCGACCCG-3'. The sub-complexes $\alpha_3\beta_3\gamma_{Arot}$ and the ϵ subunit were expressed and purified as described (33) and stored at -80°C . The yield of the $\alpha_3\beta_3\gamma_{Arot}$ complex was 3.0 mg/g cells, which was much

higher than that of the former complex $\alpha_3\beta_3\gamma_{rot}$ (0.3 mg/g cells). This higher yield should be accomplished by alanine mutation and the additional histidine tag on the α subunit. The ATPase activity and the sensitivity for ϵ -inhibition of the $\alpha_3\beta_3\gamma_{Arot}$ were almost the same as that of $\alpha_3\beta_3\gamma_{rot}$, but demonstrated slightly higher sensitivity to lauryl dimethylamine oxide (LDAO) (supplemental Table S1).

Restoration of the Rotation of Inhibited Complex by Magnetic Tweezers—Magnetic tweezers were constructed and manipulated as described (37). The magnetic field was measured with a gaussmeter (421 Gaussmeter; Lake Shore), and the measuring magnetic field 10 mm above the sample was about 200 G. The rotation assay was carried out as described (33) with slight modifications. Rotation of the attached 250-nm diameter duplex magnetic beads (nanomag-D streptavidin; Micromod) on the γ subunit was monitored with a conventional optical microscope type IX71 (Olympus, Tokyo, Japan) with a 100 \times objective lens, and the images were recorded by digital video recorder. Recorded images were analyzed by custom software, Trans viewer, prepared by Yusung Kim.

Measurement of ATPase Activity—ATPase activity was measured in the presence of an ATP-regenerating system (38) in 50 mM HEPES-KOH, pH 8.0, 100 mM KCl, 2.5 mM MgCl₂, 100 μ M ATP, 50 μ g/ml pyruvate kinase, 50 μ g/ml lactate dehydrogenase, 2 mM phosphoenolpyruvate, and 0.2 mM NADH. The assay was carried out at 25 $^\circ\text{C}$. The rate of ATP hydrolysis after addition of the enzyme was determined by monitoring the decrease in NADH absorption at 340 nm using a spectrophotometer.

Apparent Binding Affinity of the ϵ Subunit to the Complex—The proportion of the complex-bound ϵ subunit was estimated from the extent of the inhibition of the ATPase activity of the $\alpha_3\beta_3\gamma$ complex. ATP hydrolysis was initiated by addition of the complex to the reaction mixture, monitored for 4.5 min, and various concentrations of the ϵ subunit added. The final concentration of the $\alpha_3\beta_3\gamma$ complex in the reaction mixture was fixed at 1 nM. The rate of ATP hydrolysis in the steady state was determined from 350 to 400 s after addition of the ϵ subunit. The observed K_d value of the ϵ subunit for the $\alpha_3\beta_3\gamma$ complex was calculated from the inhibition curve as described (33).

Estimation of the Stiffness of the $\alpha_3\beta_3\gamma$ Complex—Fluctuation of the γ subunit was monitored as fluctuation of 340-nm diameter duplex streptavidin-coated beads attached to the γ subunit using a dark-field microscope (IX 70; Olympus, Tokyo, Japan) equipped with a mercury lamp and a high speed camera (Hi-DcamII; NAC Image Technology, Tokyo) at 500 frames/s. Recorded images were analyzed using our original software as described above. Torsional stiffness of the γ subunit was calculated from the rotary fluctuations as described (39).

Removal of the Bound Nucleotides on the $\alpha_3\beta_3\gamma_{Arot}$ and $\alpha_3\beta_3\gamma_{Arot}\epsilon$ Complexes—Removal of the bound nucleotides on the $\alpha_3\beta_3\gamma_{Arot}$ and $\alpha_3\beta_3\gamma_{Arot}\epsilon$ complexes was carried out as described (40), and the amount of bound nucleotides was determined by the method reported (41). The stability of the complexes was then analyzed by gel filtration chromatography. To investigate structural recovery of $\alpha_3\beta_3\gamma_{Arot}$, 0.3 mg of the nucleotide-depleted $\alpha_3\beta_3\gamma_{Arot}$ complex was incubated with 2 mM ADP or with the wild-type ϵ subunit (10 times molar excess

of the $\alpha_3\beta_3\gamma_{Arot}$ complex) or with the C-terminal α -helices truncated ϵ subunit at room temperature for 2 h. The incubated complex was then loaded onto the Superdex 200 HPLC column (GE Healthcare) equilibrated with 50 mM HEPES-KOH, pH 8.0, and 100 mM KCl. The elution was monitored by absorbance at 280 nm.

RESULTS

Observation of the Restoration of Rotation of F_1 -ATPase in ADP-inhibition and ϵ -inhibition with Magnetic Tweezers—The restoration of rotation of the ADP-inhibited TF_1 by pushing the γ subunit to the direction of ATP hydrolysis with magnetic tweezers has already been reported (37), and this method appeared useful for studying the relevance between ADP-inhibition and ϵ -inhibition. By using the newly prepared $\alpha_3\beta_3\gamma_{Arot}$ complex, we first investigated the frequency of restoration of rotation of ADP-inhibited F_1 (Fig. 1, A–C). The γ subunit of ADP-inhibited $\alpha_3\beta_3\gamma_{Arot}$ was forced at arbitrary angles from the position of the ADP-inhibition (80°) for 5 s (Fig. 1A) and then released from the tweezers. When rotation of F_1 -ATPase was not restored by forcing, the beads attached to the γ subunit returned to the original position of ADP-inhibition. When rotation of $\alpha_3\beta_3\gamma_{Arot}$ was observed at 250 nM ATP, discrete 120° steps were detected as an oscillating trace (Fig. 1B). In addition, the $\alpha_3\beta_3\gamma_{Arot}$ complex spontaneously lapsed into the ADP-inhibited form at 80° forward from that for ATP binding. We then investigated the frequency of restoration of rotation of the ADP-inhibited $\alpha_3\beta_3\gamma_{Arot}$ by 40° forcing (Fig. 1B) and 80° forcing (Fig. 1C), respectively. The frequencies of restoration of γ rotation were 64% for 40° forcing, and 86% for 80° forcing (Table 1). Frequencies of the restoration of rotation at these forcing angles were almost same as those observed for TF_1 (37). We then examined the restoration of rotation of the ϵ -inhibited F_1 by 80° forcing. Because the frequency of the restoration of rotation of the ADP-inhibited F_1 by 80° forcing was high (86%), we expected that the restoration of rotation of the ϵ -inhibited F_1 must be observed by the same 80° forcing, if ϵ -inhibition requires ADP-inhibition. Rotation of $\alpha_3\beta_3\gamma_{Arot}$ was observed for 30 s at 250 nM ATP, and the buffer containing $3 \mu\text{M}$ ϵ subunit was infused into the objective chamber. As observed in our previous experiment, rotation of the γ subunit completely stopped at 80° forward from the angular position for ATP binding (Fig. 1D) by infusion of the ϵ subunit. However, no restoration of rotation was observed by 80° forcing in ϵ -inhibition, which was very different from that of ADP-inhibition. The results obtained for the restoration of γ rotation with magnetic tweezers are summarized in Table 1. These results strongly suggest that ϵ -inhibition does not require ADP-inhibition.

Effect of LDAO on ϵ -Inhibition—LDAO has been reported to stimulate the ATP hydrolysis activity of F_1 or F_0F_1 (42), and the effect is thought to promote the release from ADP-inhibition (43). In addition, several biochemical studies on EF_1 or EF_0EF_1 showed that the restoration from ADP-inhibition by LDAO obviously decrease the extent of ϵ -inhibition (42, 44, 45) without dissociation of the ϵ subunit (46), and therefore these two inhibitions were regarded as closely linked systems. To confirm the relationship between ϵ -inhibition and ADP-inhibition on cyanobacterial F_1 , we investigated the effect of LDAO on ϵ -in-

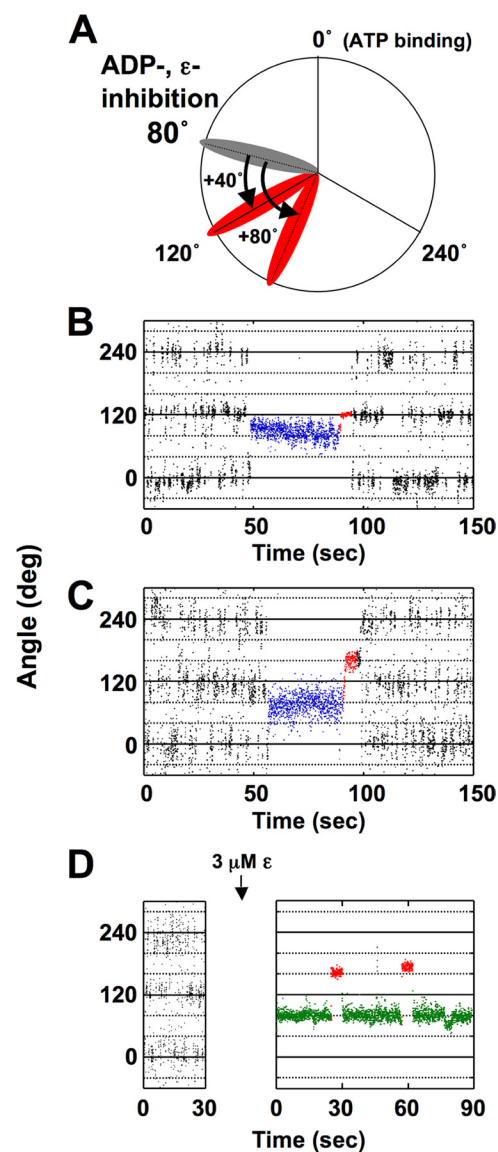


FIGURE 1. Manipulation of rotation of the γ subunit in the ADP-inhibited and the ϵ -inhibited $\alpha_3\beta_3\gamma_{Arot}$ complex with magnetic tweezers. A, scheme of mechanical manipulation of the γ subunit with magnetic tweezers. During the observation of rotation, $\alpha_3\beta_3\gamma_{Arot}$ spontaneously fell into ADP-inhibition. When the ATP binding position prior to ADP-inhibition was settled as 0° , the position of ADP-inhibition was 80° (gray). The bead attached to the γ subunit of this $\alpha_3\beta_3\gamma_{Arot}$ complex was then forced to 40° or 80° from the angle for the ADP-inhibition with magnetic tweezers, and the position of the beads reached to 120° or 160° (red; we designated this manipulation as 40° or 80° forcing). B, time course of the restoration of rotation of the ADP-inhibited $\alpha_3\beta_3\gamma_{Arot}$ complex by 40° forcing with magnetic tweezers. The γ subunit in the $\alpha_3\beta_3\gamma_{Arot}$ complex rotated with 120° steps at 250 nM ATP, which can be seen as an oscillating trace. During the observation of rotation, $\alpha_3\beta_3\gamma_{Arot}$ spontaneously fell into ADP-inhibition (blue colored traces). Red traces show the manipulation time (5 s) using the magnetic tweezers. C, time course of the restoration of the rotation of the ADP-inhibited $\alpha_3\beta_3\gamma_{Arot}$ complex by 80° forcing with magnetic tweezers. ADP-inhibition (blue traces) was forced by using the magnetic tweezers (red traces). D, time course of the 80° forcing of the ϵ -inhibited $\alpha_3\beta_3\gamma_{Arot}$ with magnetic tweezers. The ϵ -inhibition (green traces) was forced by using the magnetic tweezers (red traces).

hibition of $\alpha_3\beta_3\gamma_{Arot}$. At first, the extent of stimulation of ATPase activity of $\alpha_3\beta_3\gamma_{Arot}$ by LDAO was measured using the ATP-regenerating system at 100 μM ATP. LDAO stimulated the ATPase activity of $\alpha_3\beta_3\gamma_{Arot}$ up to 4-fold, and the stimulation effect was saturated at 0.1% LDAO (Fig. 2A). Next, the

TABLE 1**Frequency of mechanical restoration of the ADP-inhibited and ϵ -inhibited $\alpha_3\beta_3\gamma$ complex**

The $\alpha_3\beta_3\gamma$ complex in ADP-inhibition or ϵ -inhibition was stalled at the angle indicated from its inhibition with magnetic tweezers. The number of the particles that showed rotation at each of the stall angles was counted and is shown as "Activated." Those that did not show rotation were defined as "Not activated." ND, not detected.

Stall angle	Activated	Not activated	Frequency of activation %
40° (ADP-inhibition)	9	5	64 ($n = 14$)
80° (ADP-inhibition)	12	2	86 ($n = 14$)
80° (ϵ -inhibition)	ND	10	ND ($n = 10$)

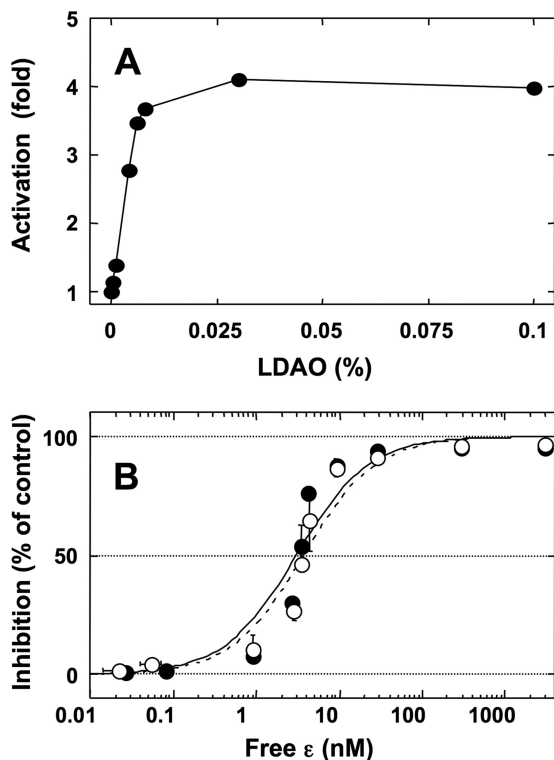


FIGURE 2. Effect of LDAO on the inhibition of ATPase activity of the $\alpha_3\beta_3\gamma_{Arot}$ complex by the ϵ subunit. A, ATPase activity of $\alpha_3\beta_3\gamma_{Arot}$ was measured in the presence of various concentrations of LDAO at 25 °C. The measurement was initiated by the addition of 1 nM $\alpha_3\beta_3\gamma_{Arot}$ to the reaction mixture containing 100 μ M ATP. Steady-state activities were then determined from the slope from 350 to 380 s after addition of the enzyme, and the results obtained from two independent experiments were averaged. The ATP hydrolysis activity in the absence of LDAO was 3.6 units/mg for $\alpha_3\beta_3\gamma_{Arot}$. B, extent of inhibition of ATP hydrolysis activities of $\alpha_3\beta_3\gamma_{Arot}$ by the ϵ subunit in the presence (filled circles) and the absence (open circles) of LDAO measured at various concentrations of the ϵ subunit. The concentration of LDAO used was 0.1%. ATP hydrolysis was initiated by the addition of the complex to the reaction mixture and monitored for 4.5 min, and various concentrations of the ϵ subunit were then added. Steady-state activities were determined from the slope from 400 to 450 s following ϵ addition. The percentages of inhibition of ATPase activity of $\alpha_3\beta_3\gamma_{Arot}$ by the ϵ subunit were plotted against the concentration of free ϵ subunit. The results of three independent experiments were averaged. The vertical and horizontal error bars in the figure indicate the S.D. for the extent of ϵ -inhibition and the concentration of free ϵ , respectively. The titration curves in the presence (solid line) and the absence (dotted line) of LDAO were fitted with the hyperbolic equation, $y = (A \times ([\epsilon]_{free}) / (K_d + [\epsilon]_{free}))$, where y represents the percentage of inhibition, A is the maximum inhibition (%), and K_d is the equilibrium dissociation constant for the ϵ subunit.

effect of LDAO on ϵ -inhibition was investigated by measuring the change of the apparent K_d values for the interaction between ϵ and $\alpha_3\beta_3\gamma_{Arot}$. The apparent K_d values were determined from the extent of inhibition of ATPase activity of the

complex at various concentrations of the ϵ subunit in the presence or the absence of LDAO (33). If ϵ -inhibition requires ADP-inhibition, release from ADP-inhibition by LDAO must increase the apparent K_d of the ϵ subunit to the $\alpha_3\beta_3\gamma_{Arot}$. The apparent K_d values of the ϵ subunit obtained for $\alpha_3\beta_3\gamma_{Arot}$ in the presence and absence of LDAO were, however, 3.0 ± 0.3 nM and 3.7 ± 0.4 nM, respectively (Fig. 2B). In contrast to the results from $EF_1 \alpha_3\beta_3\gamma$, LDAO did not affect ϵ -inhibition in cyanobacterial $\alpha_3\beta_3\gamma$. However, as the theoretical curve obtained from these K_d values did not fit to the experimental data very well (Fig. 2B), there may be a further undefined relationship between the ATPase activity and ϵ binding. Because the data obtained in the presence and in the absence of LDAO showed a similar tendency, these apparent discrepancies must be not due to ADP-inhibition or LDAO effects. Again, the result in this study indicates that ϵ -inhibition does not require ADP-inhibition.

Torsional Stiffness of $\alpha_3\beta_3\gamma$ in ϵ -Inhibition Compared with That of ADP-Inhibition—Because the apparent K_d value of the ϵ subunit to $\alpha_3\beta_3\gamma_{Arot}$ was not affected very much by LDAO, it seems likely that ADP-inhibition and ϵ -inhibition are independent phenomena of each other. To explore further the relationship between them, we compared the torsional stiffness of $\alpha_3\beta_3\gamma_{Arot}$ in ϵ -inhibition with that of ADP-inhibition by the single-molecule analysis. Torsional stiffness of $\alpha_3\beta_3\gamma_{Arot}$ in ϵ -inhibition and ADP-inhibition was estimated by measuring thermal fluctuations of the beads attached to the $\alpha_3\beta_3\gamma_{Arot}$ complex (Fig. 3, A and B) as described (39). The fluctuations of the beads were observed for 30 s with 2-ms time resolution. The probability distributions of fluctuation of the beads in ϵ -inhibition and ADP-inhibition were then fitted using a single Gaussian function (Fig. 3C). We found that the resulting distribution for ADP-inhibition was broader than that for ϵ -inhibition, and the standard deviations, σ , were 14.8 for ADP-inhibition and 10.6 for ϵ -inhibition, respectively. The torsional stiffness of $\alpha_3\beta_3\gamma_{Arot}$ was calculated using variance, σ^2 , from the Gaussian distribution, as described in the legend to Fig. 3, and the determined stiffnesses, κ , were 119.6 pNnm for ϵ -inhibition and 61.4 pNnm for ADP-inhibition, respectively. The results clearly indicate that the ϵ subunit physically stiffens $\alpha_3\beta_3\gamma$ complex more strictly than ADP-inhibition.

Effect of ADP and the ϵ Subunit on the Structural Stability of Bound Nucleotide-depleted $\alpha_3\beta_3\gamma$ Complex—To examine how the ϵ subunit affects the nucleotide binding at the catalytic or noncatalytic sites, we depleted the bound nucleotides from $\alpha_3\beta_3\gamma_{Arot}$ and $\alpha_3\beta_3\gamma_{Arot}\epsilon$ as described (40). As shown in [supplemental Fig. S2, A and B](#), most of the bound nucleotides on $\alpha_3\beta_3\gamma_{Arot}$ and $\alpha_3\beta_3\gamma_{Arot}\epsilon$ were removed from the complex by this procedure. Stabilities of these nucleotide-depleted $\alpha_3\beta_3\gamma_{Arot}$ and $\alpha_3\beta_3\gamma_{Arot}\epsilon$ complexes were then analyzed as described in the legend of Fig. 4. Although depletion of bound nucleotides from CF_1 without the ϵ subunit did not result in structural instability of the complex (40), the cyanobacterial $\alpha_3\beta_3\gamma_{Arot}$ complex was highly unstable when the bound nucleotides were depleted (Fig. 4A). In contrast, the stability of $\alpha_3\beta_3\gamma_{Arot}\epsilon$ was in no way affected by the amount of bound nucleotides (Fig. 4B). These unexpected phenomena allowed us to investigate the effect of the ϵ subunit on the structural sta-

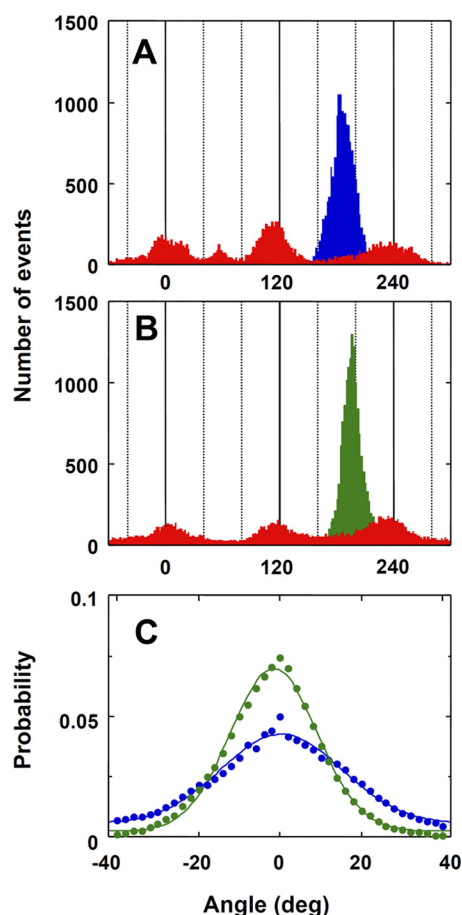


FIGURE 3. Stiffness of the F_1 in ADP-inhibition and ϵ -inhibition. *A* and *B*, histograms of angular position of the 340-nm duplex beads at 250 nm ATP (*red*), in ADP-inhibition (*blue* in *A*) or in ϵ -inhibition (*green* in *B*). Both the stop angular positions of the γ subunit inhibited at ADP-inhibited form and by the ϵ subunit were 80° forward from that for ATP binding. The fluctuations of the beads in ADP-inhibition or ϵ -inhibition were observed for 30 s with a 2-ms time resolution. *C*, probability distribution of the fluctuation of the beads in ADP-inhibition (*blue*) and ϵ -inhibition (*green*) fitted using a single Gaussian function. The stiffness of the γ subunit, κ , was calculated from the equation: $\sigma^2 = k_B T \kappa^{-1}$, where σ^2 is a variance from Gaussian distribution as described (39).

bility of the complex, which might be useful in differentiating between the impact of ϵ binding and nucleotide binding on stability of the complex structure. The nucleotide-depleted $\alpha_3\beta_3\gamma_{Arot}$ complex was incubated in the presence and absence of 2 mM ADP for 2 h at room temperature, and the stability of the complex was analyzed as described. The structural stability of the nucleotide-depleted $\alpha_3\beta_3\gamma_{Arot}$ was restored by incubation with ADP (Fig. 4C). Next, the nucleotide-depleted $\alpha_3\beta_3\gamma_{Arot}$ complex was incubated with the ϵ subunit or the mutant ϵ subunit lacking the C-terminal α -helical part, rendering it unable to inhibit ATPase activity (26, 27, 31). Recovery of the structural stability of the nucleotide-depleted $\alpha_3\beta_3\gamma_{Arot}$ complex by addition of the ϵ subunit was only observed when the full-length ϵ subunit was used (Fig. 4D), indicating that the C-terminal α -helical part of the ϵ subunit is important for both the inhibition of ATPase activity of $\alpha_3\beta_3\gamma_{Arot}$ and the structural stability of the complex. The structural stability of $\alpha_3\beta_3\gamma_{wt}$ was also measured (supplemental Fig. S3). The result for $\alpha_3\beta_3\gamma_{wt}$ was not much different from that for $\alpha_3\beta_3\gamma_{Arot}$, suggesting that the effect of the C-terminal α -helical part of the ϵ subunit on the

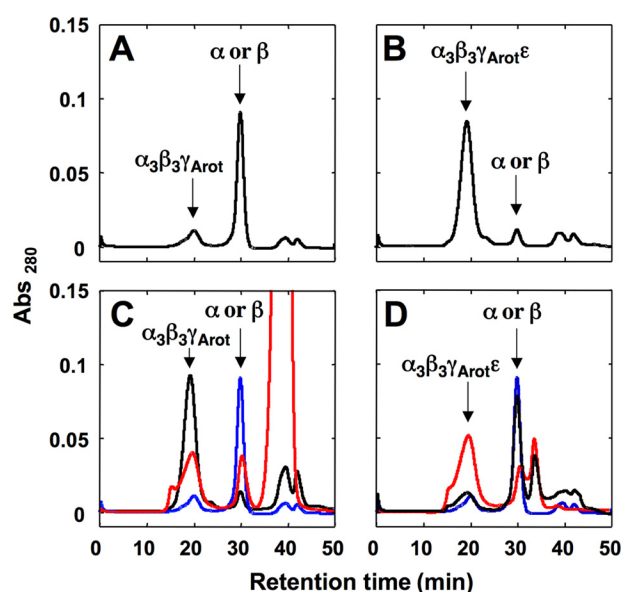


FIGURE 4. Effect of ADP and the ϵ subunit on the stability of the bound nucleotide-depleted $\alpha_3\beta_3\gamma_{Arot}$ complex. *A* and *B*, effects of depletion of the bound nucleotides on the stability of the $\alpha_3\beta_3\gamma_{Arot}$ (*A*) and the $\alpha_3\beta_3\gamma_{Arot}\epsilon$ (*B*) complexes were investigated as described under "Experimental Procedures." *C*, 0.3 mg of the nucleotide-depleted $\alpha_3\beta_3\gamma_{Arot}$ complex was incubated without (*blue*) or with (*red*) 2 mM ADP at room temperature for 2 h, and the incubated complexes were loaded onto the Superdex 200 HPLC column. *Black* shows the case of $\alpha_3\beta_3\gamma_{Arot}$ without nucleotide depletion. *D*, effect of the ϵ subunit on the stability of the nucleotide-depleted $\alpha_3\beta_3\gamma_{Arot}$ complex was also investigated. 0.3 mg of the nucleotide-depleted $\alpha_3\beta_3\gamma_{Arot}$ complex was incubated without ϵ (*blue*), with wild-type ϵ ($10 \times$ mol of the $\alpha_3\beta_3\gamma_{Arot}$ complex, *red*) or with C-terminal α -helices truncated ϵ ($10 \times$ mol of the $\alpha_3\beta_3\gamma_{Arot}$ complex, *black*) at room temperature for 2 h, and the stability of $\alpha_3\beta_3\gamma_{Arot}$ was investigated as described above.

stability of $\alpha_3\beta_3\gamma_{Arot}$ is not an experimental artifact of the mutation.

DISCUSSION

No restoration of rotation was observed by 80° forcing in ϵ -inhibition, which was very different from ADP-inhibition. Therefore, ϵ -inhibition may not require ADP-inhibition to inhibit the ATPase activity of F_1 . The frequency of restoration of rotation of the ADP-inhibited TF_1 increases with the stall angle from the position of ADP-inhibition, which was interpreted as a decrease in the binding affinity to the inhibitory ADP caused by the forced movement of the γ subunit (37). If the forced conformational change and the decrease in the binding affinity for ADP occur also in our cyanobacterial F_1 complex, there is a possibility that the ϵ subunit increases binding affinity of ADP to F_1 . In this case, ADP may not be released by 80° stalling or immediately rebind to the F_1 after release caused by the forced rotation, and no restoration of the rotation must be observed in the presence of the ϵ subunit. Consistent with this assumption, an increase of the binding affinity of Mg-ATP and Mg-ADP to the high affinity catalytic site(s) of $\alpha_3\beta_3\gamma$ of EF_1 caused by the addition of the ϵ subunit has already been reported (47). In contrast, the ϵ subunit weakened the binding affinity of Mg-ADP to each catalytic site, especially to the high affinity site(s) in the case of the $\alpha_3\beta_3\gamma$ complex of TF_1 (48). Hence, there must be a variety of ϵ effects on the nucleotide binding affinity based on the difference of the origin of the ϵ subunits (47, 48).

In this study, the difference in nucleotide binding affinity both on $\alpha_3\beta_3\gamma$ and $\alpha_3\beta_3\gamma\epsilon$ could not be precisely determined because the nucleotide-depleted $\alpha_3\beta_3\gamma_{A_{rot}}$ complex of cyanobacteria was obviously unstable (Fig. 4A). When mechanical manipulation of the γ subunit in the ϵ -inhibited $\alpha_3\beta_3\gamma$ complex was investigated in the presence of the ATP-regenerating system in our experiments shown in Fig. 1D, inhibitory ADP released from the complex was likely immediately converted to ATP. Rebinding of ADP to $\alpha_3\beta_3\gamma\epsilon$ is therefore highly unlikely. This suggests that binding of the ϵ subunit to the complex only affects the off-rate of the inhibitory ADP, if the ϵ subunit increases binding affinity of ADP to the $\alpha_3\beta_3\gamma$ complex of the cyanobacteria. As mentioned, the restoration from ADP-inhibition by LDAO an obvious decrease in the extent of ϵ -inhibition (42, 44, 45) without dissociation of the ϵ subunit (46) was reported, and these findings might be good evidence that these two inhibitions are closely linked to each other. In contrast, LDAO-assisted restoration from ADP-inhibition had no effect on ϵ -inhibition of the ATPase activity of cyanobacterial F_1 (Fig. 2B), suggesting that these two inhibition mechanisms are not directly related. The γ subunit of the cyanobacterial F_1 , chloroplast one too, contains the inserted region (25~40 amino acids) that is absent from bacteria and mitochondria, and the region plays an important role in the sensitivity of ϵ -inhibition (33, 49). The cause of the different result of the LDAO effect on ϵ -inhibition between EF_1 and cyanobacterial F_1 in this study might be attributed to the specific inserted region on the γ subunit of cyanobacteria.

The other possible event inhibited at 80° by the ϵ subunit is thought to be the phosphate-releasing step (50). Indeed, it has been previously found that binding of the ϵ subunit to EF_1 inhibits ATPase activity by decreasing the off-rate of phosphate (51). Furthermore, a conformational change of the ϵ subunit dependent on the addition of phosphate has been reported (52). However, the recovery of the structural stability of the nucleotide-depleted complex by the ϵ subunit required the C-terminal α -helical part of the ϵ subunit but not nucleotide or phosphate (Fig. 4D). Although the above phenomena do not explain the effect of inhibition of ATPase activity by the ϵ subunit directly, there is a possibility that the ϵ -inhibition can act independently of ADP-inhibition or inhibition of phosphate release.

The C terminus of the ϵ subunit reaches to the central cavity of F_1 in the extended conformation (53). Our result in this study therefore indicates that ϵ -inhibition of ATPase activity must be simply caused by steric hindrance of rotation of the γ subunit, and this inhibition alone may be sufficient to stop rotation without other events, ADP-inhibited form and inhibition of phosphate release, which also occur at 80° from ATP binding in cyanobacterial enzyme. Indeed, the torsional stiffness of $\alpha_3\beta_3\gamma$ in ϵ -inhibition was stronger than that in ADP-inhibition (Fig. 3). However, the stiffness of $\alpha_3\beta_3\gamma$ containing the ϵ subunit (119.6 pNm) was too high to force the rotation of ATP synthase by the reported *pmf* value 200 mV for ATP synthesis, which is equivalent to 56~72 pNm on 10~14 c-ring. Recently, an increase of the stiffness of $\alpha_3\beta_3\gamma$ by the ϵ subunit was also shown for TF_1 -ATPase (54). However, the conformational change of the ϵ subunit in chloroplast CF_1 , and probably that in cyanobacteria too, is induced when the *pmf* is formed across the

thylakoid membranes (55), and the C-terminal α -helical part of the ϵ subunit would ultimately be exposed (56). This implies that the ϵ subunit in CF_0CF_1 may not induce higher stiffness of the enzyme during the ATP synthesis reaction in chloroplasts. This assumption is supported by the finding that ATP synthesis activity of CF_0CF_1 did not change significantly when the C-terminal domain of the ϵ subunit was deleted (53), although ATP synthesis activities of TF_0TF_1 ($\epsilon_{\Delta C}$) and EF_0EF_1 ($\epsilon_{\Delta C}$) are higher than that of the wild-type enzyme complex (57, 58). The cause of the conformational change of chloroplast-type ϵ required to overcome a high stiffness remains unclear.

The hypothesis, which shows that ϵ -inhibition can act independently of ADP-inhibition and inhibition of phosphate release in cyanobacterial enzyme, is further supported by the result that the recovery of the structural stability of the complex by the ϵ subunit does not require nucleotide or phosphate binding (Fig. 4). However, the steric hindrance caused by the ϵ subunit may not be sufficient to stop rotation of the γ subunit in other bacterial F_1 probably because of the lack of the inserted region on the γ subunit of chloroplast-type F_1 . Therefore, in bacterial F_1 , except for the cyanobacterial one, a greater opportunity to lapse into ADP-inhibition or inhibition of phosphate release caused by the binding of the ϵ subunit to γ must be originally required to stop rotation of the γ subunit to the ATP hydrolysis direction, which must confer a strong advantage to avoiding futile ATP consumption in the cells.

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