The regulator of the F₁ motor: inhibition of rotation of cyanobacterial F₁-ATPase by the ϵ subunit

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The chloroplast-type F₁ ATPase is the key enzyme of energy conversion in chloroplasts, and is regulated by the endogenous inhibitor ϵ , tightly bound ADP, the membrane potential and the redox state of the γ subunit. In order to understand the molecular mechanism of ϵ inhibition, we constructed an expression system for the $\alpha_3\beta_3\gamma$ subcomplex in thermophilic cyanobacteria allowing thorough investigation of ϵ inhibition. ϵ Inhibition was found to be ATP-independent, and different to that observed for bacterial F₁-ATPase. The role of the additional region on the γ subunit of chloroplast-type F₁-ATPase in ϵ inhibition was also determined. By single molecule rotation analysis, we succeeded in assigning the pausing angular position of γ in ϵ inhibition, which was found to be identical to that observed for ATP hydrolysis, product release and ADP inhibition, but distinctly different from the waiting position for ATP binding. These results suggest that the ϵ subunit of chloroplast-type ATP synthase plays an important regulator for the rotary motor enzyme, thus preventing wasteful ATP hydrolysis.

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Introduction

F₀F₁ ATP synthase synthesizes ATP from ADP and inorganic phosphate by way of the proton motive force (*pmf*) generated across the cytoplasmic membranes of bacteria, thylakoid membranes of chloroplasts and inner membranes of mito-

chondria (Senior, 1990; Boyer, 1997; Yoshida *et al*, 2001). This enzyme can transport protons to generate *pmf* when it hydrolyzes ATP. The enzyme consists of the membrane-embedded portion F₀ and the water-soluble portion F₁. F₀ is composed of three different subunits, *a*, *b* and *c* with a stoichiometry of *a*₁*b*₂*c*_{10–14} (Senior, 1990; Stock *et al*, 1999; Seelert *et al*, 2000; Jiang *et al*, 2001; Mitome *et al*, 2004; Meier *et al*, 2005), and constitutes the proton translocation device. F₁ is composed of five different subunits designated α to ϵ with a stoichiometry of $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ (Yoshida *et al*, 1979). The minimum catalytic core, which maintains the property of F₁-ATPase, is $\alpha_3\beta_3\gamma$ (Kaibara *et al*, 1996; Hisabori *et al*, 1997; Matsui *et al*, 1997; Du *et al*, 2001), and the catalytic sites reside on each of the three β subunits at the interface with the α subunits (Abrahams *et al*, 1994). The rotary catalysis mechanism was first proposed by PD Boyer and co-workers based on detailed analysis of the kinetics of F₁-ATPase activity (Grubmeyer *et al*, 1982). Following determination of the central axis structure of the γ subunit in the $\alpha_3\beta_3$ hexagon (Abrahams *et al*, 1994), the rotation of the γ subunit during ATP hydrolysis was examined by biochemical cross-linking (Duncan *et al*, 1995) and polarized fluorescence experiments (Sabbert *et al*, 1996). Finally, by single molecule observation, the rotation of the γ subunit was conclusively determined to be coupled with ATP hydrolysis (Noji *et al*, 1997, 1999; Hisabori *et al*, 1999; Omote *et al*, 1999). Thorough analysis of rotation of the γ subunit revealed that the γ subunit rotates with discrete 120° step per single molecule ATP consumption and this 120° step consists of further 80° and 40° substeps (Yasuda *et al*, 1998, 2001). This 80° substep was recently revealed to be induced by ATP binding, whereas ATP cleavage and product release result in a subsequent additional 40° substep (Yasuda *et al*, 2001; Shimabukuro *et al*, 2003).

As ATP synthesis is a key reaction required to maintain a number of metabolic pathways, the F₀F₁ complex must be subject to various regulatory mechanisms required to modulate its activity, in order to optimally accommodate changes in environmental conditions. ADP inhibition of the ATP hydrolysis activity is a common regulatory mechanism that has been found to occur in most species; in such a mechanism, the ATP hydrolysis reaction is inhibited by tight binding of ADP-Mg to the catalytic site(s) (Minkov *et al*, 1979; Bar-Zvi and Shavit, 1982; Vasilyeva *et al*, 1982; Malyan and Vitseva, 1983; Feldman and Boyer, 1985; Zhou *et al*, 1988; Digel *et al*, 1996; Matsui *et al*, 1997). Recently, this type of inhibition has been assigned to the pause of the rotation motion at 80° within the 120° catalytic step (Hirono-Hara *et al*, 2001, 2005). Another regulatory mechanism for the ATP hydrolysis reaction is brought about by the intrinsic inhibitory function of the ϵ subunit (ϵ inhibition), which is well characterized in bacteria and in chloroplasts (Nelson *et al*, 1972; Richter *et al*, 1984; Aggeler and Capaldi, 1996; Kato *et al*, 1997; Nowak

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et al, 2002). Both ADP inhibition and ϵ inhibition may not affect the ATP synthesis activity of F₀F₁ (Bald *et al*, 1998; Tsunoda *et al*, 2001). However, the ϵ subunit of F₁ from thermophilic *Bacillus* PS3 (TF₁) is known to rotate with the γ subunit and the rotation speed of the ϵ subunit attached to the γ subunit is not significantly different to that of the γ subunit without ϵ (Kato-Yamada *et al*, 1998), and inhibition of rotation of the γ subunit caused by ϵ inhibition has not been characterized in depth at the single molecule level. Furthermore, rotation of the ϵ subunit in *Escherichia coli* F₀F₁ has been observed by FRET measurement (Zimmermann *et al*, 2005). Recently, Nakanishi-Matsui *et al* (2005) reported the inhibition of rotation of the γ subunit by the ϵ subunit in *E. coli* F₁-ATPase (EF₁). They observed a distinct increase in the pausing period of the γ subunit during rotation caused by addition of the ϵ subunit. However, thorough analysis of inhibition by the bacterial ϵ subunit appears difficult because inhibition shows a complicated kinetic profile based on the conformational change of the ϵ subunit by ATP during inhibition of the catalytic reaction (Aggeler and Capaldi, 1996; Kato-Yamada *et al*, 2000; Suzuki *et al*, 2003; Iino *et al*, 2005).

In contrast, ϵ inhibition of the chloroplast-type F₁-ATPase is independent of ATP concentration, and ATP hydrolysis activity is inhibited by addition of ϵ subunit alone to the ϵ -free complex (Richter *et al*, 1984). However, the effect of ϵ binding on the CF₁ complex has not been studied from the point of view of rotation because of the lack of an enzyme complex that can be suitably manipulated. For the study of CF₁ complex at the molecular level, cyanobacterial F₁-ATPase is a suitable alternative, as the active enzyme complex can be reconstituted from the recombinant individual subunits α , β , γ , δ and ϵ (Steinemann *et al*, 1995). In addition, insertion of a 'chloroplast-like' regulatory segment responsible for thiol modulation into the γ subunit of F₀F₁-ATPase of the cyanobacterium *Synechocystis* sp. PCC6803 at the chromosome level can be used to generate a redox-sensitive F₀F₁, which is one of the main distinguishing features of the chloroplast-type ATP synthase *in vivo* (Werner-Grüne *et al*, 1994; Krenn *et al*, 1995).

In order to carry out a detailed study on the effect of the ϵ subunit on γ subunit rotation, we prepared a 'purpose-built' F₁-ATPase $\alpha_3\beta_3\gamma$ complex using an *E. coli*-based expression system for synthesis of the thermophilic cyanobacterial ATP synthase complex, which possesses similar properties to the chloroplast ATPase.

Results

Inhibition of ATPase activity of thermophilic cyanobacterial $\alpha_3\beta_3\gamma$ complex by the ϵ subunit

The $\alpha_3\beta_3\gamma$ complex of *Thermosynechococcus elongatus* was over-expressed in *E. coli* BL21(DE3)*uncΔ702* strains and purified as described (see Materials and methods). Using this complex, we first determined whether the cyanobacterial F₁ subcomplex has similar properties to the chloroplast-type F₁-ATPase in terms of ϵ inhibition. In the previous study, we found that the ATPase activity of the chimeric complex comprised of the thermophilic bacterial α and β subunits and the spinach chloroplast γ subunit became insensitive to ϵ subunit inhibition upon deletion of the specific additional region of the chloroplast-type γ subunit that contains the two

Figure 1 Effect of the ϵ subunit on the ATP hydrolysis activities of $\alpha_3\beta_3\gamma_{\text{wild}}$ and $\alpha_3\beta_3\gamma_{\Delta198-222}$. $\alpha_3\beta_3\gamma$ subcomplexes (400 nM) were incubated with (closed bar) or without (open bar) 1 μ M ϵ subunit for 6 min at 40°C, and the ATP hydrolysis activity was measured in the presence of ATP regenerating system at 40°C. For the assay, 27 nM wild-type complex and 9 nM $\alpha_3\beta_3\gamma_{\Delta198-222}$ were used.

regulatory cysteines (Hisabori *et al*, 1998). As this result demonstrated the significance of the contribution of additional region of the γ subunit on ϵ inhibition, we hereby prepared a similar mutant of the cyanobacterial $\alpha_3\beta_3\gamma$ complex, $\alpha_3\beta_3\gamma_{\Delta198-222}$ (Figure 1B in the Supplementary data). The ATPase activity of $\alpha_3\beta_3\gamma_{\Delta198-222}$ was inhibited by 20% by the ϵ subunit when the enzyme activity was measured in the presence of 5 mM ATP, whereas the extent of inhibition was slightly higher when 5 μ M ATP was used (Figure 1). In contrast, the wild-type $\alpha_3\beta_3\gamma$ complex was completely inhibited by the ϵ subunit irrespective of the ATP concentrations. This strong inhibitory effect under high ATP concentration was very different from that observed for the thermophilic bacterial enzyme (Kato-Yamada *et al*, 1999), but similar to that observed for the chloroplast-type ATPase (Richter *et al*, 1984).

In order to determine the cause of the reduction in the extent of the ϵ inhibition in the $\alpha_3\beta_3\gamma_{\Delta198-222}$ complex, we calculated the apparent K_D values of the interaction between ϵ and the $\alpha_3\beta_3\gamma$ complex based on the extent of inhibition of ATP hydrolysis activity of the complex in the presence of various concentrations of the ϵ subunit (Figure 2A). The estimated K_D values were 2.1 ± 0.3 nM for the wild type and 4.2 ± 0.9 nM for the mutant complex. To confirm the difference in affinity, we then examined the binding of the ϵ subunit to this $\alpha_3\beta_3\gamma_{\Delta198-222}$ complex using fluorescence correlation spectroscopy (FCS), a valuable method that allows the measurement of the affinity between two protein molecules in solution. For this FCS measurement, a Cys residue was introduced into the ϵ subunit at the position of Q100 (ϵ_{Q100C}) and was labeled with Alexa Fluor 488-C₅-maleimide. This mutation and the fluorescent dye used did not affect ϵ inhibition (see the Supplementary Figure 2). This direct binding measurement showed that the affinity between the ϵ subunit and the complex decreased 3.5-fold by deletion of the additional region of the γ subunit (Figure 2B). The K_D values obtained were 12.5 ± 3.2 nM for the wild type and 44 ± 7.2 nM for the mutant complex. As ϵ inhibition of the $\alpha_3\beta_3\gamma_{\Delta198-222}$ complex did not increase even in the presence of 1 μ M ϵ subunit (a much higher concentration than the observed K_D value), we concluded that the deleted portion of the γ subunit must play a significant role in ϵ -induced

Figure 2 Binding of the ϵ subunit to the $\alpha_3\beta_3\gamma_{\text{wild}}$ and $\alpha_3\beta_3\gamma_{\Delta 198-222}$ complexes. (A) The extent of inhibition of the ATPase activity of the $\alpha_3\beta_3\gamma_{\text{wild}}$ ('Wt', open symbol) and $\alpha_3\beta_3\gamma_{\Delta 198-222}$ (' $\gamma_{\Delta 198-222}$ ', closed symbol) complexes were measured in the presence of various concentrations of the ϵ subunit. The results from three independent experiments (circle, triangle and square) are plotted against the concentration of free ϵ subunit (see 'Materials and methods'). Lines were calculated based on the K_D values obtained from the independent measurements above for $\alpha_3\beta_3\gamma_{\text{wild}}$ ('Wt', solid line) and for $\alpha_3\beta_3\gamma_{\Delta 198-222}$ (' $\gamma_{\Delta 198-222}$ ', dotted line). (B) Binding of the ϵ subunit to the $\alpha_3\beta_3\gamma$ complex was measured by FCS method as a function of the concentration of $\alpha_3\beta_3\gamma_{\text{wild}}$ ('Wt', open symbol) and $\alpha_3\beta_3\gamma_{\Delta 198-222}$ (' $\gamma_{\Delta 198-222}$ ', closed symbol). For the measurements, the fluorescence-labeled ϵ was fixed at 5 nM. The complex and the ϵ subunit were incubated for 60 min at 40°C, and the FCS measurement was carried out at 37°C. The results from three independent experiments (circle, triangle and square) are plotted. The titration curves for $\alpha_3\beta_3\gamma_{\text{wild}}$ ('Wt', solid line) and for $\alpha_3\beta_3\gamma_{\Delta 198-222}$ (' $\gamma_{\Delta 198-222}$ ', dotted line) were fitted as described (see Materials and methods).

inhibition, although the reduced inhibitory effect of the ϵ subunit on the mutant complex might be in part due to decreased binding of the ϵ subunit. To further dissect the properties of the mutant γ subunit, we measured the enzyme activity of the wild type and $\alpha_3\beta_3\gamma_{\Delta 198-222}$ complex in the presence and absence of the nonionic detergent lauryldimethylamine oxide (LADO) (Figure 3). LADO is a useful indicator of the susceptibility of an enzyme to ADP inhibition (Paik *et al*, 1993). The mutant was found to be less activated in the presence of LADO, suggesting that, in comparison to the wild type, the $\alpha_3\beta_3\gamma_{\Delta 198-222}$ complex may be less prone to ADP inhibition. These results suggest that the additional region of the chloroplast-type γ subunit is an important determinant of the susceptibility of the complex to both ϵ inhibition and ADP inhibition.

Enzyme preparation for single molecule observation and its inhibition by the ϵ subunit

To observe ϵ inhibition of rotation of the γ subunit in detail, two cysteine residues were introduced at the surface of the γ

Figure 3 Effect of LADO on the ATP hydrolysis activity of the $\alpha_3\beta_3\gamma_{\text{wild}}$ and $\alpha_3\beta_3\gamma_{\Delta 198-222}$ complexes. ATP hydrolysis of $\alpha_3\beta_3\gamma_{\text{wild}}$ and $\alpha_3\beta_3\gamma_{\Delta 198-222}$ was measured as described in Figure 1 in the presence (closed bar) and the absence (open bar) of 0.1% (w/v) LADO. Steady-state activity was determined from the slope from 180 to 200 s after addition of the enzyme solutions, and the results of three independent experiments were averaged. The extent of the activation by the addition of LADO is indicated on the bars.

subunit. These two cysteines were used as an attachment site for the beads that are used as a probe to observe the movement of γ . After screening the γ subunit for mutations that would not affect ϵ -induced inhibition, amino-acid positions corresponding to Gly-112 and Ala-125 were used as recipients for introduction of the required cysteine residues, in addition to the substitution of Lys-221 to Ser, which is located in the additional region. Lys-221 appears to be a critical residue of this region of the enzyme, because substitution of this residue to Ser resulted in an increase in ATPase activity, and reduced the susceptibility of the enzyme to ADP inhibition state as observed for the $\alpha_3\beta_3\gamma_{\Delta 198-222}$ complex. However, the remarkable difference between the K221S mutation and $\gamma_{\Delta 198-222}$ was that the former showed no significant difference in the extent of ϵ inhibition (see Figure 1 in contrast to Table I of the Supplementary data). The complex containing the three γ subunit mutations G112C, A125C and K221S (designated $\alpha_3\beta_3\gamma_{\text{rot}}$) showed 10-fold higher activity than the wild-type complex (Table I of the Supplementary data), and was used for the rotation experiments. Specific biotinylation of the introduced cysteines was confirmed by Western blotting using a streptavidin-conjugated alkaline phosphatase (Figure 3 in the Supplementary data).

Having prepared the experimental system described above, we sought to investigate the kinetic properties of $\alpha_3\beta_3\gamma_{\text{rot}}$ ATPase activity and its inhibition by the ϵ subunit (Figure 4A). The K_M and V_{max} values obtained for $\alpha_3\beta_3\gamma_{\text{rot}}$ were the following: 85.8 μM and 44.3 s^{-1} in the absence of the ϵ subunit, 52.2 μM and 32.1 s^{-1} in the presence of 3 nM ϵ subunit, and 66.5 μM and 21.4 s^{-1} in the presence of 5 nM ϵ subunit. The ϵ subunit was thus found to inhibit the ATPase activity noncompetitively. Figure 4B shows a typical time course for the ϵ -induced inhibition. The decrease in the activity following addition of ϵ occurred within a few minutes and the obtained curve fitted to a single exponential function. The gradual decrease in activity indicates binding of the ϵ subunit to the complex, and the binding rate constant, k_{obs} , was calculated from these curves. Based on the observed linear relation of k_{obs} against the ϵ concentrations, a k_{on} value of $4.9 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ was obtained (Figure 4C). Although the extrapolated intercept of Figure 4C gives the theoretical k_{off}

Figure 4 Inhibition of ATP hydrolysis activity of the $\alpha_3\beta_3\gamma$ -rot complex by the ϵ subunit. **(A)** S/v-S plots of the rates of ATP hydrolysis with 5–2500 μ M ATP are indicated. ATP hydrolysis activity was measured at 25°C. The reaction was initiated by addition of 6 nM $\alpha_3\beta_3\gamma$ -rot and the activity was monitored for 1 min. 0 nM (triangle), 3 nM (square) and 5 nM (circle) of ϵ subunit was then added and the activity in the steady state was determined from the slope from 175 to 200 s following ϵ subunit addition. **(B)** ATP hydrolysis activity of $\alpha_3\beta_3\gamma$ -rot was measured with 5 mM ATP at 25°C. The reaction was initiated by addition of 1.5 nM $\alpha_3\beta_3\gamma$ -rot and the activity monitored for 1 min. The indicated concentrations of the ϵ subunits were then added. **(C)** Time course of the inhibition after addition of the ϵ subunit shown in (B) was used for calculation of k_{on} of the ϵ subunit to the complex. The curves are fitted to a single exponential equation: $y = a \times e^{-k_{\text{obs}} \times t} + b$, where a and b are constants, and k_{obs} is the observed binding rate constant. The association rate constant k_{on} was then obtained from the slope based on the function, $k_{\text{obs}} = k_{\text{on}} \times [\epsilon \text{ subunit}] + k_{\text{off}}$.

value, owing to large experimental fluctuations, it was deemed unsuitable for calculation of the K_D value. Instead, the K_D value of 3.6 nM obtained from the inhibition of steady-state ATPase activity by the ϵ subunit was used, allowing determination of the k_{off} value, which was found to be $1.8 \times 10^{-3} \text{ s}^{-1}$.

Inhibition of rotation of the γ subunit by the ϵ subunit

The rotation of the γ subunit in the $\alpha_3\beta_3\gamma$ -rot complex was observed using a 0.2- μ m duplex beads as a probe. The

Figure 5 Effect of the ϵ subunit on the rotation of the γ subunit in the $\alpha_3\beta_3\gamma$ complex. Rotations of the beads attached to the γ subunit in the presence or the absence of the ϵ subunit were recorded at 250 nM ATP at 25°C. The buffer without **(A)** or with **(B)** 3 μ M ϵ subunit was infused into the chamber following an initial 5 min observation of rotation. In general, solution exchange in the flow-chamber took 1–2 min. The total number of observed particles was 17 for A (without ϵ) and 13 for B (with ϵ).

exchange of the buffer was found to have little effects on the rotation behavior of the γ subunit (Figure 5A). In contrast, significant inhibition of rotation was observed when the ϵ subunit was injected into the objective chamber (Figure 5B). No continuous rotation was observed on the 13 glass plates examined after injection of the ϵ subunit, suggesting very tight binding of the ϵ subunit to the immobilized ATPase molecule and efficient inhibition of the rotation of the γ subunit. From the k_{off} value obtained for the ϵ subunit, the half-lifetime of the binding of the ϵ subunit to the complex was calculated as 9.3 min, and the ϵ subunit could easily rebind to the complex when the concentration of the ϵ subunit was 3 μ M. Therefore, the occasional detachment of the ϵ subunit from the γ subunit and subsequent resumption of rotation within this experimental period was deemed to be highly unlikely.

Analysis of the pause position of the γ subunit caused by the ϵ inhibition

When a very low ATP concentration (250 nM) was used for the rotation assay, the movement of the beads attached to the γ subunit showed three discrete pauses, each separated by 120° (Figure 6A). This stepping rotation was not observed when 10 μ M ATP was used (Figure 4 in the Supplementary data). The observed 120° pauses can be attributed to ATP binding (Yasuda *et al*, 1998). The angular position of this 120° pause was not significantly affected by the buffer exchange (Figure 6A–C), indicating that the immobilized ATPase complex does not easily undergo a change in position or direction on the glass surface. The average change in pausing angle of the γ subunit from a prior ATP waiting after buffer exchange was $125.3 \pm 17.9^\circ$ (Table I). We next determined the angular position of the pause when rotation was inhibited by the

Figure 6 Stop angular position of the γ subunit inhibited by the ϵ subunit. Histograms of angular positions of the beads at 250 nM ATP before (A, D) and after (B, E) buffer exchanges are indicated. (C, F) are superimposed histograms of (A, B) and (D, E), respectively. Histograms were taken from rotations observed for 5 min. One of three peak positions of the histogram was set as 0° , and was indicated as the position of the arrow in the traces of the centroid of the beads in the *inset* figures. In the case of the experiments shown in (D–F), buffer containing $3 \mu\text{M}$ ϵ subunit was used.

Table I Stop angular position of rotation of the γ subunit inhibited by the ϵ subunit

Buffer exchange	Pausing angular position of the γ subunit (mean $\pm$ s.d.)
– ϵ	$125.3 \pm 17.9^\circ$ ($n = 15$)
+ ϵ	$79.0 \pm 20.2^\circ$ ($n = 5$)

The most common location of the bead during rotation is shown as peaks in Figure 6. Each of the peaks was fitted by Gaussian distributions, and the center of the angular position of the γ subunit from the previous ATP binding position (see the peaks in Figure 6B and E) was averaged on the indicated number of the particles.

ϵ subunit. The location of the beads after the ϵ injection was clearly limited (Figure 6D–F), and the pausing angle was approximately 80° forward from that for the ATP binding (Table I).

To characterize this 80° pausing position induced by the ϵ inhibition, we analyzed the slow rotation of the γ subunit of the $\alpha_3\beta_3\gamma$ -rot in the presence of 250 nM ATP in detail, and could observe the long pauses (5–45 s) of rotation at 80° from the ATP binding position frequently (Figure 7). These long

Figure 7 Long pause of the rotation of the γ subunit at 80° . Pausing angular position of the beads from the ATP waiting position to the next ATP waiting position (A) or to the ADP inhibition (B) were overlaid ($n = 10$), when 250 nM ATP was used for the assay.

pauses at 80° seem to be nearly identical to that observed for ADP inhibition state on TF_1 (Hirono-Hara *et al*, 2001), suggesting that the pausing angular position by the ϵ inhibition was identical to that observed for ATP hydrolysis, product release and that for ADP inhibition, but distinctly different from the waiting position for ATP binding.

Discussion

Why can $\text{CF}_1\text{-}\epsilon$ behave as a strong intrinsic inhibitor?

Based on the observation of the recovery of the ϵ inhibition of TF_1 , the ϵ subunit is thought to exist in two different states in the complex known as the inhibitory state and the non-inhibitory state (Kato-Yamada *et al*, 1999). Based on structural analysis, these two states are considered to be the consequence of the two different ϵ subunit conformations: the extended conformation and the contracted conformation, respectively (Suzuki *et al*, 2003). Therefore, the transition between these two conformations is thought to be a mode of regulation of the $\text{F}_1\text{-ATPase}$ complex (Suzuki *et al*, 2003; Iino *et al*, 2005). ATP hydrolysis is strongly inhibited in the F_0F_1 complex as well, when the ϵ subunit assumes the extended conformation, in which the C-terminal α -helices of the ϵ subunit extend into the $\alpha_3\beta_3$ hexagon ring, whereas the contracted ϵ subunit does not inhibit ATP hydrolysis (Tsunoda *et al*, 2001). The apparent insensitivity of the TF_1 or TF_0TF_1 to ϵ inhibition observed in the presence of high concentrations of ATP has therefore been attributed to the contracted conformation of the ϵ subunit, which is induced by ATP (Kato-Yamada *et al*, 2000, 2005). The ATP-dependent conformational change of the C-terminal α -helical part of ϵ has also been shown in EF_0EF_1 (Schulenberg and Capaldi, 1999).

The results presented here show that, in contrast to the reported ϵ inhibition of the bacterial F₁-ATPase, the inhibitory effect of the ϵ subunit on the cyanobacterial $\alpha_3\beta_3\gamma$ complex is not influenced by the concentrations of ATP (Figure 1B), suggesting that the drastic conformational changes of the ϵ subunit from the extended to the contracted conformation is not induced by ATP. This observation is in agreement with the reported inhibitory mechanism of the chloroplast CF₁ by the ϵ subunit (Richter *et al*, 1984). As stated, one of the remarkable differences between the bacterial F₁ and CF₁ is the existence of an additional ~40 amino acids located in the middle of the γ subunit. Although the γ subunit of the cyanobacterial F₁-ATPase is not redox sensitive, it also contains a 26 amino-acid addition whose sequences are very similar to those of CF₁- γ . The additional region of the cyanobacterial and chloroplast γ subunit (Hisabori *et al*, 2003) may interfere with the conformational change of the ϵ subunit, thus CF₁- ϵ can be thought of more as a stationary inhibitory subunit rather than a regulatory one like the bacterial type ϵ -subunit. In the case of CF₁, the thiol modulation system constitutes an additional regulatory mechanism of enzyme activity (Nalin and McCarty, 1984), and this regulation ability seemed to be conferred by the molecular evolution of the additional region of the cyanobacterial γ subunit. In plants, this kind of redox regulation mechanism is likely to be of greater importance because CF₁ activity can be linked both to the photochemical reactions and electron transport, thus constituting an effective mechanism for the efficient use of light as an energy source for ATP synthesis.

Relation between ϵ inhibition and ADP inhibition

The pausing period observed at 80° is considered to encompass the two sequential events; ATP hydrolysis (Shimabukuro *et al*, 2003) and product release (Yasuda *et al*, 2001). Nishizaka *et al* (2004) recently reported that ATP hydrolysis and/or phosphate release but not ADP release would occur at this 80° pause position. In addition, the TF₁ subcomplex showed extended pauses at this angular position caused by ADP inhibition (Hirono-Hara *et al*, 2001).

Feniouk and Junge (2005) recently proposed that the ϵ subunit acts as a stabilizer of the ADP inhibition state in the F₀F₁ complex. As the $\alpha_3\beta_3\gamma$ subcomplex also lapses into the ADP inhibition state, ADP inhibition and ϵ inhibition are independent phenomena. However, our results suggest that these two inhibitions may be tightly linked to each other. In this study, we succeeded in determining the discrete stop position caused by the ϵ -induced inhibition of rotation, and found it to be approximately 80° forward from the pause position for ATP binding (Figure 6, Table I), confirming that binding of the ϵ subunit may stabilize ADP inhibition. This conclusion is further supported by the results showing that enzyme activation by LADO was less marked following deletion of additional region of the γ subunit, and this deletion affects ϵ inhibition (Figure 3).

Materials and methods

Materials

Biotin-PEAC₅-maleimide was purchased from Dojindo (Kumamoto, Japan). ATP, phosphoenolpyruvate and bovine serum albumin (BSA) were obtained from Sigma. Pyruvate kinase, lactate dehydrogenase and nicotinamide adenine dinucleotide (reduced

form) (NADH) were purchased from Roche Diagnostics. Other chemicals were of the highest grade commercially available.

Strains and plasmid

E. coli strains used were JM 109 (TAKARA) for cloning and BL21(DE3)*uncΔ702* (Tc^r, ATPase mutant, BL21(DE3) *uncΔ702*, asnA::Tn10) (Joshi *et al*, 1989; Nichols and Harwood, 1997) for expression of *T. elongatus* $\alpha_3\beta_3\gamma$. *E. coli* BL21(DE3)*uncΔ702* strain was kindly gifted from Dr Harwood (University of Iowa). A plasmid pTR19, the expression vector for F₀F₁ of thermophilic *Bacillus* PS3 (Suzuki *et al*, 2002), was used as a template to construct the expression vector for *T. elongatus* $\alpha_3\beta_3\gamma$.

Construction of expression plasmids for $\alpha_3\beta_3\gamma$ and ϵ of *T. elongatus* BP-1

A 948 base pair DNA fragment containing *atpC* gene, coding for γ subunit of F₀F₁, was amplified by polymerase chain reaction (PCR) from genomic DNA of *T. elongatus* BP-1, (a kind gift from Masahiko Ikeuchi, University of Tokyo), using primers 5'-CCGCGGGAATTTCGCTTTGCTTAGGAGTTTAAATTACCATGGCCAATCTCAAAGC-3' (*EcoRI*) and 5'-CGCCGGCGGTACCGCTAGCGCAGAGCCTCAGCCCC-3' (*NheI*). The restriction sites for the enzyme shown in parentheses are underlined. A 1512 base pair DNA fragment containing *atpA* gene, coding for α subunit was amplified using primers 5'-GGCGGC GCCATGGAATCTAAGAAGGAGATATACATATGGTAAGTATCCGACCCG ACG-3' (*NcoI*) and 5'-CCGCGCCGAATTCTTAGGCAGTGAAGTAGCT TTGTAC-3' (*EcoRI*). A 1449 base pair DNA fragment containing *atpB* gene, coding for β subunit of F₀F₁, was amplified by PCR using primers 5'-GGCGGGCGCTAGCATTATGAAGGAGATTAAATCAAATGCA TCACCATCATCACCATCACCATCACCATTGTCATATCAGCAGACAACG AACCC-3' (*NheI*) and 5'-CCC CGCGGAAGCTTCTAAATCTCGACCA CACCCCTGCG-3' (*HindIII*). These three DNA fragments for α , β and γ subunits were then ligated into the pTR19 plasmids using the restriction enzymes indicated. Consequently, the genes for TF₀TF₁ subunits were removed from the plasmid and the plasmid was converted to the expression plasmid for the $\alpha_3\beta_3\gamma$ complex of *T. elongatus*. The obtained plasmid was named pTR19FW.

For the ϵ subunit expression plasmid, a 417 base pair DNA fragment containing *atpH* gene, coding for the ϵ subunit of F₀F₁, was amplified by PCR from genomic DNA of *T. elongatus* BP-1 using primers 5'-GGCGGGCATATGGTGACTGTCCGGGTAATTG CG-3' (*NdeI*) and 5'-CCC CGCGGAAGCTTCTAAATCTCGACCACTCCTGCG-3' (*HindIII*). The PCR fragment was digested with *NdeI* and *HindIII* and ligated into the plasmid pET21c (Novagen).

Mutant complex preparation

Additional region on the γ subunit corresponding to amino acids 198–222 was deleted from the γ subunit gene on the expression plasmid for $\alpha_3\beta_3\gamma$ using the Mega-primer method (Landt *et al*, 1990) with the following mutation primers: 5'-GCTCCCCCTCGATCCC CAAGGGACCTCGACGCTGCCGCTGCG-3'. The obtained plasmid was used for the expression of the $\alpha_3\beta_3\gamma_{\Delta 198-222}$ complex.

To use the $\alpha_3\beta_3\gamma$ complex for single molecule experiments, all cysteines on the complex were substituted with serine by the Mega-primer method using the following mutation primers: 5'-CCCTGTC ACCTGGTATTGTCCAGCGCAATCTGTGTCCGAGCCATTGCAAACG GG-3' for α Cys144Ser; 5'-CCAAAGGGCCAAAGACGTGATTTCCGTG TATGTGGCCATTGGTCAAAAAGCCTCC-3' for α Cys194Ser; 5'-GCGG CCGGCTTAGACGTGGCTGTAACTCCGAAGTGCAACAACCTCC-3' for β Cys53Ser and 5'-GGTGGTAACAGCGCATCGCGGCTGTCCGGCGG GTTACAACACTAATGTCTATTCGCGG-3' for γ Cys90Ser. Substitutions of Gly112 and Ala125 on γ to Cys for Biotin-PEAC₅-Maleimide labeling were carried out by the Mega-primer method using the following mutation primers: 5'-GGAACGTCTCCAAGAACTCGAAGCCGAGTGC CTCAAATACACCCCTAGT-3' for γ Gly112Cys and 5'-GGGATAGTCACG GCGCTGGAAATATTGGCATGCTTCGCCACCC-3' for γ Ala125Cys. In addition, Lys221 on γ was substituted with Ser using the following mutation primers: 5'-CATTTGGAAGTCAACCCGAGTCGGTAACCTC GACGCTGCCCCGCTCTGCCC-3'. The plasmid containing the above mutations was named pTR19FR and used for the expression of $\alpha_3\beta_3\gamma$ -rot.

Expression and purification of the $\alpha_3\beta_3\gamma$ complex and the ϵ subunit

E. coli BL21(DE3)*uncΔ702* strain was transformed with pTR19FW or pTR19FR, and cultured in 2 × YT medium containing 100 µg/ml ampicillin and 0.2 mM isopropyl- β -D-thiogalactopyranoside at 37°C

for 19 h. The desired proteins were purified and labeled with Biotin-PEAC₅-maleimide by using the same method as described (Ueoka-Nakanishi *et al*, 2004) and stored at -80°C . The ϵ subunit was expressed and prepared as described (Hisabori *et al*, 1997) and stored at -80°C .

Measurement of ATP hydrolysis activity

ATP hydrolysis activity was measured in the presence of an ATP-regenerating system (Stiggall *et al*, 1979) in 50 mM N-2-hydroxyl piperazine-*N'*-2-ethane sulfonic acid (HEPES)-KOH, pH 8.0, 100 mM KCl, 2.5 mM MgCl₂, 5 mM ATP, 50 $\mu\text{g}/\text{ml}$ pyruvate kinase, 50 $\mu\text{g}/\text{ml}$ lactate dehydrogenase, 2 mM phosphoenolpyruvate and 0.2 mM NADH. The assay was carried out at 25 and 40°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 V-550 (Jasco, Tokyo, Japan).

Estimation of the ϵ binding

The proportion of the complex-bound ϵ subunit was estimated from the extent of the inhibition of the ATPase activity of the complex. ATP hydrolysis was initiated by addition of the complex to the reaction mixture, monitored for 1 min, and various concentrations of the ϵ subunit added. The concentration of the $\alpha_3\beta_3\gamma$ complex was fixed at 5 nM. The rate of ATP hydrolysis in steady state was determined from 260 to 280 s after the addition of the ϵ subunit. The change in the extent of inhibition dependent on the ϵ concentrations were then fitted with the hyperbolic equation, $y = [(A \times [\epsilon]_{\text{free}}) / (K_D + [\epsilon]_{\text{free}})]$, where y represents the percentage of inhibition, A is the maximum inhibition (%) and K_D is the equilibrium dissociation constant for the ϵ subunit. To calculate the concentration of the free ϵ subunit, we assumed that the complex that bound an ϵ subunit is completely inhibited, thus $[\epsilon]_{\text{free}} = [\epsilon]_{\text{add}} - [\text{complex}] \times y$.

FCS measurement

ϵ_{Q100C} was labeled with Alexa Fluor 488-C₅-maleimide (Molecular Probes) with a molar ratio of 1:5 in 100 mM K-phosphate, pH 7.0, 5 mM TCEP and 100 mM KCl at 25°C for 2 h. Unbound fluorescent dye was removed from the solution with NAP5 column (Amersham) equilibrated with 50 mM HEPES-KOH, pH 8.0 and 100 mM KCl. The concentrations of the fluorescent dye bound to the ϵ subunit was determined from the absorption using excitation coefficient at 493 nm = $72\,000\text{ M}^{-1}\text{ cm}^{-1}$ for the dye. The concentrations of the ϵ subunit were determined by BCA protein assay. FCS measurements were performed using ConfoCor2 (Carl Zeiss, Jena, Germany) as described (Pack *et al*, 1999, 2000). The sample was excited with 62.5 μW of laser power. To prevent nonspecific adsorption of proteins on the surface of the cover glass, the sample chamber was treated with a protein blocker (N101, NOF Corporation, Japan) before measurement. The measured fluorescence autocorrelation functions were fitted by two-component model as described (Pack *et al*, 2000). In the titration experiment, the

duration of measurement was 60 s, and each point on the titration curve is represented by the average of three independent measurements. The titration curves obtained in this study were then fitted with a hyperbolic equation, $y = [(B \times [\epsilon]_{\text{free}}) / (K_D + [\epsilon]_{\text{free}})]$, where y represents the % of the binding of the complex to the ϵ subunit, B is the maximum occupancy of ϵ with the complex (%) and K_D is the dissociation constant for the ϵ subunit. As the concentrations of the complex that bound to the ϵ subunit are obtained directly from the FCS measurement, the concentration of the free ϵ subunit was calculated as $[\epsilon]_{\text{free}} = [\epsilon]_{\text{add}} - [\text{complex}]_{\text{bound}}$.

Rotation assay

Biotinylated complexes (10 μl) in 20 mM K-phosphate, pH 7.0, 100 mM KCl and 0.2% (w/v) BSA were infused into a flow chamber and were incubated for 2 min at room temperature. The flow chamber was then washed with 50 μl of 20 mM K-phosphate, pH 7.0 and 100 mM KCl to remove unattached complexes. A streptavidin beads (209 nm) in 20 mM K-phosphate, pH 7.0, 100 mM KCl and 0.2% (w/v) BSA were infused into the flow chamber and were incubated for 15 min. Rotation was initiated by addition of 30 μl of assay buffer (50 mM HEPES-KOH, pH 8.0, 100 mM KCl, 0.5 mM MgCl₂, 250 nM ATP, 100 $\mu\text{g}/\text{ml}$ pyruvate kinase and 2 mM phosphoenolpyruvate) after washing the flow chamber with 50 μl of 50 mM HEPES-KOH, pH 8.0 and 100 mM KCl. The rotation was monitored for 5 min after which the assay buffer containing 3 μM of the ϵ subunit was infused into the flow chamber to inhibit the enzyme. Rotation of the attached 209 nm duplex beads on the γ subunit was monitored with a conventional optical microscope type IX 71 (Olympus, Tokyo, Japan) with a $\times 100$ objective lens and the images were recorded into a digital video recorder. Recorded images were analyzed by custom software prepared by Yasuda *et al* (1998).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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