Inhibition of $F_1$-ATPase Rotational Catalysis by the Carboxyl-terminal Domain of the $\epsilon$ Subunit*

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Background: The $\epsilon$ subunit inhibits $F_1$-ATPase activity. Substitution of helix 2 or a point mutation in loop 2 of the $\epsilon$ subunit decreased its inhibitory effects on subunit rotation. Truncation of helix 2 or a point mutation in loop 2 of the $\epsilon$ subunit decreased its inhibitory effects on subunit rotation. Truncation of helix 2 or a point mutation in loop 2 of the $\epsilon$ subunit decreased its inhibitory effects on subunit rotation. The $\epsilon$ subunit, an endogenous inhibitor, lowers $F_1$-ATPase activity by decreasing the rotation speed and extending the duration of the inhibited state (Sekiya, M., Hosokawa, H., Nakanishi-Matsui, M., Al-Shawi, M. K., Nakamoto, R. K., and Futai, M. (2010) Single molecule behavior of inhibited and active states of *Escherichia coli* ATP synthase $F_1$ rotation. *J. Biol. Chem.* 285, 42058–42067). In this study, we constructed a series of $\epsilon$ subunits truncated successively from the carboxyl-terminal domain (helix 1/loop 2/helix 2) and examined their effects on rotational catalysis (ATPase activity, average rotation rate, and duration of inhibited state). As expected, the $\epsilon$ subunit lacking helix 2 caused about 1/2-fold reduced inhibition, and that without loop 2/helix 2 or helix 1/loop 2/helix 2 showed a further reduced effect. Substitution of $\epsilon$Ser108 in loop 2 and $\epsilon$Tyr114 in helix 2, which possibly interact with the $\beta$ and $\gamma$ subunits, respectively, decreased the inhibitory effect. These results suggest that the carboxyl-terminal domain of the $\epsilon$ subunit plays a pivotal role in the inhibition of $F_1$ rotation through interaction with other subunits.

Escherichia coli ATP synthase ($F_0F_1$) couples catalysis and proton transport through subunit rotation. The $\epsilon$ subunit, an endogenous inhibitor, lowers $F_1$-ATPase activity by decreasing the rotation speed and extending the duration of the inhibited state (Sekiya, M., Hosokawa, H., Nakanishi-Matsui, M., Al-Shawi, M. K., Nakamoto, R. K., and Futai, M. (2010) Single molecule behavior of inhibited and active states of *Escherichia coli* ATP synthase $F_1$ rotation. *J. Biol. Chem.* 285, 42058–42067). In this study, we constructed a series of $\epsilon$ subunits truncated successively from the carboxyl-terminal domain (helix 1/loop 2/helix 2) and examined their effects on rotational catalysis (ATPase activity, average rotation rate, and duration of inhibited state). As expected, the $\epsilon$ subunit lacking helix 2 caused about 1/2-fold reduced inhibition, and that without loop 2/helix 2 or helix 1/loop 2/helix 2 showed a further reduced effect. Substitution of $\epsilon$Ser108 in loop 2 and $\epsilon$Tyr114 in helix 2, which possibly interact with the $\beta$ and $\gamma$ subunits, respectively, decreased the inhibitory effect. These results suggest that the carboxyl-terminal domain of the $\epsilon$ subunit plays a pivotal role in the inhibition of $F_1$ rotation through interaction with other subunits.

ATP synthase ($F_0F_1$) plays a central role in biological energy transduction; this enzyme synthesizes most of the cellular ATP from ADP and phosphate ($P_i$), coupling with the electrochemical proton gradient through subunit rotation (for reviews, see Refs. 1–7). It is composed of membrane extrinsic catalytic sector $F_1$ ($\alpha_3\beta_3\gamma_6\epsilon$) and trans-membrane proton pathway sector $F_0$ ($ab_2$ stator and c-ring formed from multiple $c$ subunits) (5–7). Protons are transported through a pathway formed by the Asp or Glu residue in the rotating c-ring and the Arg residue of the stator a subunit (4, 5).

We have established an experimental system for observing the high-speed rotation of the *Escherichia coli* $\gamma$ subunit located at the center of the $\alpha_3\beta_3$ hexamer (8–10) or the $\gamma e/c$-ring in the entire ATP synthase (11). To observe the $\gamma$ subunit rotation, the $\epsilon$ subunit-depleted $F_1$ sector was fixed on a glass surface, and a small gold bead was introduced to the $\gamma$ subunit (8, 9). In the presence of a high concentration of ATP, the bead showed continuous rotation defined as the “active state” and entered stochastically into a period of long pauses ($\geq$100 ms), defined as the “inhibited state” (9, 10). Both states last for $\sim$1 s on average (9). The rotation rate during the active state is $\sim$400 revolutions/s (rps), and the overall rate, including the inhibited state, is $\sim$160 rps, which is comparable with that estimated from the bulk phase ATPase activity, assuming that three ATP molecules are hydrolyzed upon 360° revolution (9). A high data collection rate (4,000–8,000 frames/s) allows us to detect the short pauses between the 120° rotation steps during the active state (9, 10). We concluded that ATP is hydrolyzed during the short pauses (0.2 ms), which are thus defined as “catalytic dwells” (9). The 120° rotation steps are further divided into 40° and 80° rotation steps, and the short pause between the two substeps corresponds to the ATP-binding dwell (ATP-waiting dwell), during which ATP binds to one of the three $b$ subunits, and ADP is released from another $\beta$ subunit (6, 7, 9, 12).

The addition of an excess amount of the $\epsilon$ subunit extends the duration time of the inhibited state but has no effect on the time length of the active state (8, 9). We found that the $\epsilon$ subunit significantly decreased the activation energy required for subunit rotation (9).

The $\epsilon$ subunit consist of two domains, an amino-terminal $\beta$-sandwich (amino-terminal domain (eNTD)) and two carboxyl-terminal $\alpha$-helices (carboxyl-terminal domain (eCTD)) (Fig. 1a) (15–17). The eNTD binds to the $\gamma$ subunit and forms a rotor together with the c-ring and $\gamma$ subunit. Growing evidence suggests that the eCTD, comprising helix 1/loop 2/helix 2 (Fig. 1b, left), plays regulatory roles in ATP synthesis and hydrolysis (18–21).

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2 The abbreviations used are: rps, revolutions per second; eCTD, carboxyl terminal domain of the $\epsilon$ subunit; eNTD, amino terminal domain of the $\epsilon$ subunit; ATPγS, adenosine 5’-O-(thiotriphosphate); H1 and H2, helix 1 and 2, respectively; L2, loop 2.
The recently determined high-resolution crystal structure of the *E. coli* F_{1} sector revealed that the εCTD adopts a highly extended conformation, with helix 2 inserted deeply between the β and γ subunits (Fig. 1a) (15). This structure should correspond to a transiently inhibited conformation because the γ rotor may be connected non-covalently to the stator (α_{ε}β_{ε}) by the ε subunit, thereby preventing γ rotation. In contrast, the isolated ε subunit adopts a compact conformation in which the two α-helices interact with each other and form a hairpin structure (Fig. 1b, right) (19–21). An ε subunit with a compact structure was also observed in the F_{0}F_{1} complex (22–25) and may exhibit less inhibitory activity because the εCTD including helix 2 cannot interact with stator subunits.

In this study, we prepared a series of truncated ε subunits lacking part of the εCTD and ones with amino acid replacements and examined their effects on ATPase activity, rotation speed, and duration of the inhibitory state. These studies indicated the importance of the εCTD interaction with the β and γ subunits. The concerted effect of loop 2 and helix 2 was also suggested.

**EXPERIMENTAL PROCEDURES**

**Preparation and Materials**—A recombinant plasmid carrying the F_{0}F_{1} gene was used as the wild type throughout this study. *E. coli* F_{1}, with six histidine residues and cysteine substitutions (γS193C and γK108C) introduced into the α subunit amino terminus and γ subunit, respectively, was constructed from pBUR17 by replacing the Csp45I-RsrII segment with that containing the γK108C substitution (26).

The F_{1} sector was prepared from *E. coli* strain DK8 harboring the plasmid as described previously (26) with minor modifications. Briefly, membrane vesicles were prepared from the cells, suspended in 50 mM Tris-HCl buffer (pH 8.0) containing 0.5 mM dithiothreitol, 140 mM KCl, 1 mM EDTA, and 10% (w/v) glycerol, and then centrifuged at 160,000 × g for 1 h at 15 °C to remove the δ subunit. The following procedures were performed at room temperature. The precipitate was incubated in 2 mM Tris-HCl buffer (pH 8.0) for 10 min. After centrifugation, 0.5 M MOPS-NaOH buffer (pH 7.0) and 1 M Na_{2}SO_{4} were added to the supernatant (final concentrations of 20 and 50 mM, respectively), and the mixture was incubated with 100 μM biotin-PEAC_{5}-maleimide (Dojindo, Kumamoto, Japan) for 1.5 h. The ε subunit was removed from F_{1} by binding the biotinylated F_{1} (5 mg of protein) to a nickel-nitrilotriacetic acid resin column (Qiagen, Hilden, Germany) (0.5 ml bed volume), followed by extensive washing with 30 ml of 10 mM Tris- H_{2}SO_{4} buffer (pH 8.0) containing 10% glycerol and 1 mM ATP (pH adjusted to 8.0 with HCl). Bound F_{1} was eluted with 3 ml of 50 mM Tris- H_{2}SO_{4} buffer (pH 8.0) containing 200 mM imidazole, 50 mM Na_{2}SO_{4}, 10% glycerol, and 1 mM ATP. The elution speed was ~5 ml/h. Imidazole was removed by dialysis against 50 mM Tris- H_{2}SO_{4} buffer (pH 8.0) containing 50 mM Na_{2}SO_{4}, 25% glycerol, and 1 mM ATP. Purified enzyme (~1 mg/ml protein) was quickly frozen in liquid nitrogen and stored at ~80 °C until use. Protein concentrations were determined by the method of Bradford using bovine serum albumin (Sigma, Fraction V) as a standard (27). The concentration of F_{1} (α_{ε}β_{ε}γε) was determined assuming its molecular weight to be 365,000. Gel electrophoresis analysis indicated that >80% of the ε subunit was removed from F_{1}, as estimated from the densities of the ε band before and after the nickel column chromatography using recombinant ε as a standard. The small amount of remaining ε subunit was dissociated during the course of the ATPase or rotation assay (8).

Gold beads (60-nm diameter) were obtained from British BioCell International and coated with biotinylated bovine serum albumin (8). Other materials used were of the highest grade commercially available.

**Plasmid Construction and Mutagenesis**—To generate plasmids encoding truncated εCTD mutants (Fig. 1b), DNA fragments were amplified by means of polymerase chain reaction (PCR) using a plasmid encoding the ε subunit with a histidine tag and a recombinant tobacco etch virus protease site at the amino terminus (28) as a template. The sense primer used was the T7 promoter primer, and the antisense primers contained sequences encoding a truncated εCTD and an EcoRI site inserted downstream of a stop codon (Table 1). Insertion of the PCR products into the same plasmid (28) using NdeI and EcoRI yielded recombinant plasmids encoding a series of εCTD-truncated ε subunits. A plasmid encoding ΔH2/L2/H1 ε (Fig. 1b) containing the εA39C substitution was constructed by the same method using the plasmid containing the εA39C substitution (28) as a template. To construct a plasmid encoding εCTD (εGlu118→εMet138), a DNA fragment was amplified using a sense primer containing an Ncol site and an antisense primer containing the ε carboxyl terminus and an EcoRI site (carboxyl terminus/EcoRI primer) (Table 1) and then subcloned into the plasmid (28) using Ncol and EcoRI.

For alanine-scanning mutagenesis, 10 sets of sense and antisense complementary primers encoding the ε subunit with a single amino acid residue to alanine substitution were synthesized (Table 1). These antisense primers and the T7 promoter primer and the sense primers and carboxyl terminus/EcoRI primer sets were used to amplify the amino-terminal ~500 base pairs and carboxyl-terminal ~100 base pairs, respectively. The resulting PCR products were annealed and then used as templates with the T7 promoter and carboxyl terminus/EcoRI primers. The obtained DNA fragments encoding ε subunits with an alanine substitution were subcloned into the plasmid (28) using NdeI and EcoRI. A εSer108→Lys, Asn, or Asp replacement was also introduced by the same method using primers encoding the ε subunit with a single amino acid substitution (Table 1). All mutant sequences were confirmed by DNA sequencing.

**Expression and Purification of Mutant ε Subunits**—The wild type and mutant ε subunits having a histidine tag and a recombinant tobacco etch virus protease site at the amino terminus were expressed in *E. coli* cells, BL21(DE3)pLysS, harboring the corresponding recombinant plasmid, as described previously (28); cells were cultured in minimal medium supplemented with 1.1% glucose, and the ε subunits were expressed by incubation with isopropyl β-D-thiogalactopyranoside to the mid-log phase for 18 h at 18 °C. Cells were harvested and disrupted in buffer A (20 mM Tris-HCl (pH 8.0) and 150 mM NaCl) containing protease inhibitors, 1 μg/ml DNase I, and 1 mM dithiothreitol, passed through a French press twice, and then centri-
fuged at 12,000 g for 12 min twice. The supernatant (~150 mg of protein) was applied to a nickel-nitritotriacetic acid resin column (1-ml bed volume). The column was washed with 6 ml of buffer A containing 45 mM imidazole, and the ε subunit was eluted with 3 ml of the same buffer containing 300 mM imidazole. The elution speed was ~10 ml/h. After the removal of imidazole by dialysis against buffer A, the histidine tag was digested with recombinant tobacco etch virus protease (Invitrogen); dithiothreitol and the protease were added (final concentration with recombinant tobacco etch virus protease (Invitrogen); and the mixture was centrifuged, and then the phosphate released was determined. To examine the effect of the ε subunit, 100 nM ε subunit in rotation buffer was incubated with 4 nM F₁ for 10 min before the addition of ATP. These assays were repeated at least three times.

The rotation assay was carried out as described previously (8): two sizes of coverglasses (18 × 18 mm and 24 × 32 mm) were washed extensively with 0.1 M KOH and used to construct a flow cell with spacers (~30 μm deep). To observe the rotation of a gold bead attached to the γ subunit, the flow cell was filled with rotation buffer containing 200 nM biotinylated F₁ and then incubated for 10 min. After washing off unbound F₁ with rotation buffer, streptavidin (4 μl) in the same buffer was introduced to the cell, followed by incubation for 5 min and then extensive washing with the buffer. The biotinylated gold beads (0.2%) were introduced and washed similarly. To examine the effect of the ε subunit, 100 nM ε subunit in rotation buffer was introduced into the cell, followed by incubation for 10 min. Rotation buffer containing 2 mM ATP, 1 mM phosphoenolpyruvate, 50 μg/ml pyruvate kinase, and 100 nM ε subunit was introduced to start the rotation.

To observe the rotation of a gold bead attached to the ε subunit, 25 nM unbiotinylated F₁ was incubated with 100 nM biotinylated ε for 10 min and then introduced to the flow cell. After streptavidin and the biotinylated gold beads were sequentially applied to the flow cell as described above, the rotation buffer containing 2 mM ATP and its regenerating system was introduced to start rotation. The rotation assay was performed at 24 °C.

Images of beads were observed after the addition of 2 mM ATP at 24 °C by dark field microscopy (BX51WI-CDEVA-F, Olympus, Tokyo), and recorded with an intensified charge-
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Results

Effects of the εCTD on F₁-ATPase Activity—The ε subunit inhibits F₁-ATPase catalysis by decreasing the rotation rate and extending the inhibited state (8, 9). Considering the two conformations of the ε subunit (Fig. 1b), we can assume that the εCTD of the extended ε subunit interacts with other subunits, as shown in the recent E. coli F₁ structure (Fig. 1a). This structure should lead to inhibition of rotational catalysis.

To address the role of the εCTD, we prepared truncated ε subunits lacking the domains of the εCTD successively from the carboxyl terminus (Fig. 1b, left). The truncated subunits prepared were as follows: ΔH2, lacking helix 2 (residues 115–138); ΔH2/L2, lacking helix 2/loop 2 (residues 106–138); and ΔH2/L2/H1, lacking helix 2/loop 2/helix 1 (residues 95–138) (Fig. 2a).

The bulk phase ATPase activity of ε-depleted F₁ was 20.0 ± 1.2 μmol/mg/min, i.e. slightly higher than the previous result (8) because of extensive washing of the ε subunit. The inhibitory effects of the wild type and mutant ε subunits were expressed as relative values, taking the control without the ε subunit as 100%. As shown in Fig. 2b, the bulk phase activity decreased with increasing ε concentration and reached a plateau level with more than 30 nM, indicating that the ε subunit concentration used (100 nM) is enough to examine the inhibitory effects.

About 80 ± 0.2% of the bulk phase activity was inhibited by 100 nM wild type ε subunit (Fig. 2c), consistent with the previous results (18, 29). The inhibition decreased to 36 ± 1.3% with the ΔH2 mutant, indicating that helix 2 plays a pivotal role in the inhibitory effect. On the other hand, the inhibition decreased to 20 ± 5.0% with the further truncated ΔH2/L2/H1 mutant. Thus, helix 1 contributes slightly to the inhibition. The ΔH2/L2 mutant caused almost the same inhibition as ΔH2 (Fig. 2c), suggesting that the loop 2 region does not have a significant effect. It is noteworthy that the shortest mutants, ΔH2/L2/H1 and ΔH2/L2/H1(A39C), retained weak inhibitory effects. The εCTD protein (between εGlu₉⁸ and εMet¹³⁹) without the amino-terminal β sandwich domain caused no inhibition (Fig. 2c), suggesting that the β sandwich domain is required for the binding of the ε subunit to F₁, which is consistent with the previous result (30).

To determine the relative affinities of F₁ for the wild type and mutant ε subunits, competition assays were carried out: we added various amounts of the mutant ε subunit to the ATPase reaction mixture together with the wild type ε subunit. As shown in Fig. 2d (1), 100 nM wild type and ΔH2 mutant ε subunit inhibited ~80 and 16% of the ATPase activity, respectively (Fig. 2d (1), closed and open bars, respectively). When increasing amounts of the ΔH2 mutant were added together with 100 nM wild type ε subunit, the inhibitory effect of the wild type decreased (Fig. 2d (1), gray bars). The addition of 100 nM ΔH2 mutant decreased the inhibition to 44%, which corresponded to the value when half of the F₁ molecules were inhibited by the wild type and the rest by the ΔH2 mutant ((80 + 16%)/2 = 48%) (Fig. 2d (1), horizontal dotted line). These results suggest that the amounts of F₁ molecules with ΔH2 and ones with the wild type ε subunit were essentially the same.
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FIGURE 2. Effects of εCTD-truncated mutants on F₁-ATPase activity. a, purification of the wild type (WT) and truncated mutant ε subunits. The purified proteins (0.4 μg) were subjected to polyacrylamide gel electrophoresis in the presence of SDS and then stained with Coomassie Blue. The arrows indicate the positions of 10- and 15-kDa molecular markers. b, effects of the wild type and CTD-truncated ε subunits on F₁-ATPase activity. F₁ was incubated with the indicated amounts of the wild type (circles), ΔH2 (squares), ΔH2/L2 (triangles), or ΔH2/L2/H1 (diamonds) mutant ε for 10 min, and then ATPase activity was assayed at 24 °C. Each relative activity is shown with the S.D. value, taking the control without ε (21.8 ± 1.0 μmol/mg/min) as 100%. c, inhibitory effect by the wild type and mutant ε subunits on F₁-ATPase activity. After incubation with 100 nm wild type or a mutant ε subunit for 10 min, the ATPase activity was assayed at 24 °C, and inhibition is shown as the relative value, taking the control without ε (21.8 ± 1.0 μmol/mg/min) as 100%. ΔH2/L2/H1(A39C) and eCTD are the ΔH2/L2/H1 mutant with the εAla¹⁹² to Cys substitution and the εCTD protein (between εGlu⁹¹ and εMet¹⁵⁶) without the NTD, respectively. d, competition of εCTD-truncated mutants with the wild type ε. Varying concentrations of a mutant ε subunit were incubated with F₁ in the presence of 100 nm wild type ε for 10 min, and then ATPase activity was assayed. The relative inhibition is shown with the S.D. value, taking the control without ε (22.4 ± 2.1 μmol/mg/min) as 100%. Closed bars, with 100 nm wild type ε; open bars, with 100 nm each mutant ε; gray bars, with increasing amounts of each mutant ε together with 100 nm wild type. Horizontal dotted lines indicate the estimated values when half of the F₁ molecules were inhibited by the wild type and the rest by the mutant ε. e, effect of temperature on the ATPase activity with or without the wild type or ΔH2 ε subunit. Activities are shown as relative values, taking that without ε at 24 °C (19.2 ± 0.8 μmol/mg/min) as 100%. Circles, without ε; squares, ΔH2 ε; triangles, wild type ε.

Similar experiments suggested that the numbers of F₁ molecules with the ΔH2/L2 mutant and ones with the wild type were the same when 30–100 nm ΔH2/L2 and 100 nm wild type ε were included in the assay mixture (Fig. 2d (2)). These results suggest that the two mutant ε subunits bound to F₁ similarly to the wild type.

On the other hand, 0.3–1 μM ΔH2/L2/H1 mutant was required to obtain similar results when 0.1 μM wild type ε was present in the assay mixture (Fig. 2d (3)), indicating that the mutant’s affinity to F₁ is 3–10-fold lower than that of the wild type. Because the dissociation constant (Kₐ) of the wild type ε is ~1 nM (31, 32), Kₐ for this mutant should be 3–10 nM. We performed ATPase and rotation assays in the presence of 100 nM ε subunit, which was ~10-fold higher than the Kₐ for the mutant. These results suggest that at least 90% of the F₁ bound to the mutant ε subunits under experimental conditions and that the reduced inhibition by the mutants is mainly due to the truncation of the εCTD.

Next we examined the effect of temperature on ATPase activity (Fig. 2e) and calculated the activation energy of F₁ with and without the ε subunit. The wild type ε subunit lowered the activation energy, confirming the previous results (9, 33); the activation energy values calculated for ATP hydrolysis by F₁ with and without ε were 4.6 and 35.8 kJ/mol, respectively (Fig. 2e, triangles and circles, respectively). Truncation of helix 2 affected the temperature dependence (Fig. 2e, squares); the activation energy value was 48.8 kJ/mol, i.e. significantly higher than that with the wild type. Other truncated mutants showed high activation energy values similar to ΔH2, i.e. 49.1 and 39.1 kJ/mol for ΔH2/L2 and ΔH2/L2/H1, respectively. These results clearly indicated that helix 2 is essential for lowering of the activation energy for ATP hydrolysis.

Effects of the εCTD on F₁ γ Subunit Rotation—Because the wild type ε subunit causes a decreased rotation rate of the γ subunit in the active state and an extended duration time of the inhibited state, we determined the effects of the εCTD on the rate and the duration of the inhibited state. We observed the rotation of the F₁ γ subunit for 2 s with or without a super-stoichiometric concentration (100 mM) of the ε subunit or one of its truncated derivatives (Fig. 3a). Rotation rates were calculated as reciprocal values of the geometric means of the single revolution time (time required for 360° revolution). The rotation rate (without ε, ~530 rps) decreased to about one-half (~310 rps) upon the addition of the wild type ε subunit (Fig.
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Effects of the $\epsilon$CTD-truncated mutants on F$_{1}$ subunit rotation—As shown previously (34), a proper rotor-stator interaction through $\beta$Glu$^{381}$ of $\beta_{1}$ corresponding to bovine $\beta_{2}$ (ADP-bound $\beta$ subunit) (35) and $\gamma$Met$^{23}$ of the $\gamma$ subunit is essential for efficient rotation; the rotation rate of the $\gamma$Met$^{23}$ to Lys mutant F$_{1}$ became $\sim$2-fold slower and recovered to the wild type level upon introduction of the $\beta$Glu$^{381}$ to Asp substitution, indicating that a hydrogen

$\Delta H_{2}/L_{2}$, consistent with the previous results (8, 9). As expected, the $\Delta H_{2}$ and $\Delta H_{2}/L_{2}$ mutants were less inhibitory, giving rotation rates of 370 and 420 rps, respectively (Fig. 3b). The $\Delta H_{2}/L_{2}/H_{1}$ mutant gave a similar speed to $\Delta H_{2}/L_{2}$. These results suggest that helix 2 and loop 2 are involved in the decrease in the rotation rate, although helix 1 plays only a minor role.

For further confirmation, we connected gold particles to the mutant $\epsilon$ subunit and examined whether or not they rotated at a rate similar to that of those attached to the $\gamma$ subunit; the $\epsilon$Ala$^{39}$ to Cys substitution was introduced into the $\Delta H_{2}/L_{2}/H_{1}$ mutant, which was then biotinylated and attached to a gold bead. The $\Delta H_{2}/L_{2}/H_{1}(A39C)$ mutant showed the same effect on ATPase activity as the $\Delta H_{2}/L_{2}/H_{1}$ mutant (Fig. 2c). The rotation rate of beads attached to the $\Delta H_{2}/L_{2}/H_{1}(A39C)$ mutant was 430 rps, i.e. essentially the same rate as for the ones attached to the $\gamma$ subunit (Fig. 3b). These results indicate that even the shortest mutant can bind to the F$_{1}$ sector and rotate together with the $\gamma$ subunit.

Effects of $\epsilon$CTD on the Durations of the Inhibited and Active States—As shown in Fig. 3a, F$_{1}$ stochastically enters into an inhibited state (>100 ms) during continuous rotation (active state) (9, 10). We followed the rotation for 32 or 16 s with or without the $\epsilon$ subunit. $\Delta H_{2}/L_{2}/H_{1}(A39C)$ indicates the $\Delta H_{2}/L_{2}/H_{1}$ mutant with substitution of $\epsilon$Ala$^{39}$ to Cys, to which residue a gold bead was introduced. $\delta$, rotation rates of the $\gamma$ subunit in F$_{1}$ with or without the wild type or an $\epsilon$CTD-truncated $\epsilon$ subunit. A gold bead was introduced to the $\gamma$ subunit, and rotation was followed for 32 or 16 s. The duration times of the inhibited (pink) and active (blue) states are shown with S.E. (error bars) values. None, without $\epsilon$; WT, wild type $\epsilon$. Because the time courses of F$_{1}$ with the $\Delta H_{2}$ mutant did not show clear inhibited states for an unknown reason, the duration of the states could not be estimated. $\delta$, comparison of the ATPase activity in the bulk phase and the average total revolutions. We estimated the average total revolutions of 10 rotating beads and expressed them as relative values, taking the control without the $\epsilon$ subunit (296 rps) as 100% (open bars). The relative ATPase activity in the bulk phase (closed bars) was cited from Fig. 2b, 100 nM mutant $\epsilon$. None, without $\epsilon$; WT, wild type $\epsilon$.

Effects of Substitutions of $\epsilon$CTD on the Durations of the Inhibited and Active States—As shown previously (34), a proper rotor-stator interaction through $\beta$Glu$^{381}$ of $\beta_{1}$ corresponding to bovine $\beta_{2}$ (ADP-bound $\beta$ subunit) (35) and $\gamma$Met$^{23}$ of the $\gamma$ subunit is essential for efficient rotation; the rotation rate of the $\gamma$Met$^{23}$ to Lys mutant F$_{1}$ became $\sim$2-fold slower and recovered to the wild type level upon introduction of the $\beta$Glu$^{381}$ to Asp substitution, indicating that a hydrogen
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FIGURE 4. Effects of amino acid replacements of εSer108 in loop 2 on F1 inhibition. a, interaction between the ε (magenta), β, (dark gray), and γ (light green) subunits. εSer108 and εTyr114 in the extended conformation are close enough to interact with βGlu381 and γLys23, respectively. The position of γMet23 is also shown. b, purification of the wild type and the mutant ε subunits with substitution of εSer108. a, εSer108A; b, εSer108K; c, εSer108D; d, εSer108N. The purified proteins (2 μg) were subjected to SDS-PAGE and then stained with Coomassie Blue. The arrows indicate the positions of 10- and 15-kDa molecular markers. c, effects of the ε subunit with substitution of Ser108 on ATPase activity. S(WT), εSer108ε(WT) (wild type); K, εSer108K; N, εSer108N; A, εSer108A; D, εSer108D. The wild type F1 (ε381E1(WT)) or F1, with amino acid substitution of βGlu381 to Asp (ε381D) was used. Inhibition is shown as the relative value, taking the control without ε(19.6 ± 0.9 μmol/min) as 100%. The open bar indicates inhibition by the wild type ε subunit. d, durations of the inhibited and active states with or without the wild type (WT) or ε108D mutant. F1 rotation was followed for 32 or 16 s, and the duration times of the inhibited (pink) and active (blue) states are shown with S.E. values (error bars). None, without ε; WT, wild type; S108D, the mutant ε subunit with substitution of εSer108. The durations for without ε and the wild type are cited from Fig. 3c.

On the other hand, the Ala and Asp substitutions decreased the inhibitory effect to 61 ± 1.2 and 47 ± 0.3%, respectively (Fig. 4c). These results suggest that the replaced Ala or Asp residue interacts with βGlu381 only weakly, and thus the effect decreases. The carboxyl group of substituted ε108D may induce repulsion against βGlu381 and decrease the interference with smooth rotation. Therefore, we substituted βGlu381 with Asp to reduce the repulsion between ε108D and βGlu381. As expected, the inhibitory effect of ε108D recovered from 47 ± 0.3 to 56 ± 1.3% with this mutation (Fig. 4c). The βE381D substitution showed no effect on the F1-ATPase activity without the ε subunit (34).

Furthermore, we observed γ subunit rotation in the presence of the ε108D mutant. Compared with the rotation rate with the wild type ε subunit (~310 rps), that with the ε108D mutant increased to ~430 rps, i.e. it was almost the same as that with the ΔH2L2H1 mutant. In addition, the duration of the inhibited states with the ε108D subunit was 1.3 s, i.e. more than ~2-fold shorter than that with the wild type (Fig. 4d). In summary, the interaction between εSer108 and βGlu381 is important to the inhibitory effect of the ε subunit on F1-ATPase activity through a decreased rotation rate and an extended duration of the inhibited state.

Identification of a Key Amino Acid Residue between εSer108 and εTyr114—The region between εSer108 and εTyr114 contains loop 2 and the first three amino acids of helix 2 (Fig. 1b). As shown in the E. coli x-ray structure (15), this region interacting with multiple subunits (α, β, and γ) (Fig. 4a) may contain amino acid residues involved in F1 inhibition. We substituted amino acid residues in the region one by one with alanine, purified the mutants (Fig. 5a), and then examined their effects on F1-ATPase activity. Among the 10 substitution mutants, the εY114A mutant decreased the inhibition to 41 ± 3.1% (Fig. 5b), suggesting that this residue in helix 2 is involved in the ε subunit function. Because εTyr114 is close to γLys23 (2.91 Å) (Fig. 4a) (15), a hydrogen bond could be formed between the hydroxyl group of the εTyr114 side chain and the amide group of the γLys23 main chain. Interaction between εTyr114 and γLys23 is possibly important for holding the ε subunit in a certain conformation for F1 inhibition.

To determine the relative affinities of F1 for the wild type and mutant ε subunits, competition assays were performed. The wild type and ε108D mutant ε (both 100 nM) inhibited ATPase activity ~80 and ~30%, respectively (Fig. 5c (1), closed and open bars, respectively). With an increase in the mutant ε subunit relative to 100 nM wild type, the inhibition decreased (Fig. 5c (1), gray bars), and the addition of 300 nM ε108D mutant decreased the inhibition to 52%, which corresponded to the value when equal populations of F1 were inhibited by the wild type and ε108D mutant subunits. These results indicate that the affinity of the mutant is ~3-fold lower than that of the wild type. 0.3–1 μM εY114A mutant was required to attain equal inhibition (Fig. 5c (2)), indicating that the mutant has 3–10-fold lower affinity. We estimated that the Kd values of these mutants were less than 3 and 10 nM, respectively. Therefore, at least 97 and 90% of the F1 molecules should bind to ε108D and εY114A under our assay conditions, respectively.
As described above, the $\epsilon$ subunit decreases the activation energy of F$_1$ ATP hydrolysis and participates in the effective energy coupling (9). Interactions between the $\epsilon$ and other subunits through $\alpha$Ser$^{108}$ and $\epsilon$Tyr$^{114}$ should be responsible for lowering of the activation energy. We measured ATPase activities with the $\epsilon$S108D or $\epsilon$Y114A mutant at various temperatures (Fig. 5d) and calculated the activation energies. The estimated values were 45.9, 18.7, 38.4, and 29.5 kJ/mol for without $\epsilon$, the wild type, $\epsilon$Y114A, and $\epsilon$S108D, respectively, indicating that both the $\alpha$Ser$^{108}$ and $\epsilon$Tyr$^{114}$ residues contribute to lowering of the activation energy, at least partly. Interactions between the $\epsilon$ and other subunits through these residues may be required for efficient energy coupling.

**DISCUSSION**

In this study, we have shown that the carboxyl-terminal domain of the $\epsilon$ subunit, $\epsilon$CTD, especially loop 2/helix 2, plays pivotal roles in inhibition of F$_1$-ATPase activity. The inhibition occurs through a decreasing rotation rate and an extending duration of the inhibited state, as shown in this and previous studies (8). Moreover, we have identified important amino acid residues, $\alpha$Ser$^{108}$ in loop 2 and $\epsilon$Tyr$^{114}$ in helix 2, for the $\epsilon$ subunit inhibitory function. Because the $\epsilon$ subunit rotates with the $\gamma$ subunit in the $\alpha_3\beta_3$ hexamer, interactions of the $\epsilon$ subunit with other subunits, possibly with the $\alpha$ and $\beta$ ones, are essential to inhibit ATPase activity. Through these interactions, the $\epsilon$ subunit maintains an extended conformation, and thereby interferes with smooth rotation of the $\gamma$ subunit.

Our recent findings also support the importance of interactions between $\epsilon$CTD and other subunits: the $\epsilon$ subunit fused with a globular protein, cytochrome $b_{562}$, at its carboxyl terminus does not inhibit the subunit rotation of F$_1$ (8). This finding suggests that the globular protein prevents $\epsilon$CTD from interacting with the $\alpha_3\beta_3$ hexamer, which results in a decreased inhibition.

The $\alpha$Ser$^{108}$ and $\epsilon$Tyr$^{114}$ residues involved in the $\epsilon$ subunit inhibitory function are in the region between $\alpha$Ser$^{108}$ and $\epsilon$Tyr$^{114}$ (Fig. 1b). However, truncation of this region did not have a significant effect on F$_1$-ATPase activity; the $\Delta$H2L2 mutant showed almost the same reduced inhibitory effect as $\Delta$H2 (Fig. 2c). These results indicate that the region is involved in the inhibitory function in a cooperative manner with helix 2. Helix 2 may be essential for holding $\alpha$Ser$^{108}$ at a position close to $\beta$Glu$^{361}$ to form a hydrogen bond that can inhibit high-speed subunit rotation.

We have studied the detailed mechanism of F$_1$ rotational catalysis by analyzing mutant enzymes, such as $\gamma$M23K and $\beta$S174F, and inhibitors (ATP$^\gamma$S and picteatannol) (9, 10, 13, 14, 34, 36), which extended the duration of the catalytic dwell but

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**FIGURE 5. Effects of alanine substitutions in the region between $\alpha$Ser$^{108}$ and $\epsilon$Tyr$^{114}$ on F$_1$-ATPase activity.**

- **a**. purification of the wild type (WT) and alanine-substituted mutant $\epsilon$ subunits. The amino acid residues in the region between $\alpha$Ser$^{108}$ and $\epsilon$Tyr$^{114}$ were substituted with alanine one by one. The purified proteins (2 $\mu$g) were subjected to SDS-PAGE and then stained with Coomassie Blue. The numbers and arrows indicate the positions of alanine substitutions and molecular markers, respectively.
- **b**. effects of the $\epsilon$ subunit with alanine substitutions on ATPase activity. Inhibitory effects of the mutants on F$_1$-ATPase activity are shown with S.E. values (error bars), taking the control without $\epsilon$ (20.2 $\pm$ 0.9 $\mu$mol/mg/min) as 100%. The amino acid sequence of the wild type $\epsilon$ subunit is shown as one-letter symbols with the corresponding motifs. The open bar indicates inhibition by the wild type $\epsilon$ subunit.
- **c**. competition of mutant $\epsilon$ with the wild type. Varying concentrations of a mutant $\epsilon$ subunit were incubated with F$_1$ in the presence of 100 nM wild type $\epsilon$ for 10 min, and then ATPase activity was assayed. The relative inhibition is shown with S.D. values (error bars), taking the control without $\epsilon$ (20.3 $\pm$ 0.1 $\mu$mol/mg/min) as 100%.
- **d**. effect of temperature on ATPase activity with or without the wild type or a mutant $\epsilon$ subunit. Activities are shown as relative values, taking that without the $\epsilon$ subunit at 24 °C (20.9 $\pm$ 0.1 $\mu$mol/mg/min) as 100%. Circles, without $\epsilon$; diamonds, $\epsilon$Y114A; squares, $\epsilon$S108D; triangles, wild type $\epsilon$.
not the duration of the 120° rotation. The catalytic dwell was observed more clearly with these mutations or in the presence of these inhibitors. As mentioned above, we have previously shown that the ε subunit increases the frequency and duration of short pauses (∼ ms) during continuous rotation, which results in a lowered rotation rate (8). These short pauses should be the catalytic dwell and/or ATP-binding dwell. Unlike other inhibitors and mutations, the catalytic dwell did not become clearer in the presence of the ε subunit (8). Moreover, the recently determined E. coli F₁ structure suggests that the ε subunit in its extended conformation traps the γ subunit in a rotary position close to the dwell before the ATP binding event (15). Therefore, at least a certain population of the increased short pauses may be ATP-binding dwells.

The temperature effect on ATPase activity with the wild type or mutant ε subunit revealed that helix 2 is essential for lowering of the activation energy. As discussed previously (9), lowering of the activation energy should be important for avoiding loss of energy due to ATP hydrolysis and for maintaining the efficient coupling between catalysis and rotation.

In conclusion, our results suggest that the εCTD plays a pivotal role in inhibitory regulation of subunit rotation in F₁ and, at the same time, contributes to efficient coupling by lowering the activation energy. Our structure-based studies on the ε subunit function will contribute to understanding of the mechanism underlying rotational catalysis.

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