Mechanism of Inhibition by C-terminal α-Helices of the ε Subunit of Escherichia coli F$_{0}$F$_{1}$-ATP Synthase*

Received for publication, January 28, 2009, and in revised form, April 3, 2009. Published, JBC Papers in Press, May 1, 2009, DOI 10.1074/jbc.M109003798

Ryota Iino$^{1}$, Rie Hasegawa, Kazuhito V. Tabata, and Hiroyuki Noji$^{2}$

From the Institute of Scientific and Industrial Research, Osaka University, 567-0047 Osaka, Japan

The ε subunit of bacterial F$_{0}$F$_{1}$-ATP synthase (F$_{0}$F$_{1}$), a rotary motor protein, is known to inhibit the ATP hydrolysis reaction of this enzyme. The inhibitory effect is modulated by the conformation of the C-terminal α-helices of ε, and the “extended” state is responsible for inhibition. Although the inhibition of ATP hydrolysis by the C-terminal domain of ε has been extensively studied, the effect on ATP synthesis is not fully understood. In this study, we generated Escherichia coli F$_{0}$F$_{1}$ (EF$_{0}$F$_{1}$) mutant in which the ε subunit lacked the C-terminal domain (F$_{0}$F$_{1}$εΔC), and ATP synthesis driven by acid-base transition (ΔpH) and the K$^{+}$-valinomycin diffusion potential (ΔΨ) was compared in detail with that of the wild-type enzyme (F$_{0}$F$_{1}$εWT). The turnover numbers (k$_{cat}$) of F$_{0}$F$_{1}$εWT were severalfold lower than those of F$_{0}$F$_{1}$εΔC. F$_{0}$F$_{1}$εWT showed higher Michaelis constants (K$_{m}$) of the activities of F$_{0}$F$_{1}$εWT and F$_{0}$F$_{1}$εΔC on various combinations of ΔpH and ΔΨ was similar, suggesting that the rate-limiting step in ATP synthesis was unaltered by the C-terminal domain of ε. Solubilized F$_{0}$F$_{1}$εWT also showed lower k$_{cat}$ and higher K$_{m}$ values for ATP hydrolysis than the corresponding values of F$_{0}$F$_{1}$εΔC. These results suggest that the C-terminal domain of the ε subunit of EF$_{0}$F$_{1}$ slows multiple elementary steps in both the ATP synthesis/hydrolysis reactions by restricting the rotation of the γ subunit.

F$_{0}$F$_{1}$-ATP synthase (F$_{0}$F$_{1}$)$^{3}$ is an enzyme that is responsible for ATP synthesis during oxidative phosphorylation and photosynthesis (1–3). F$_{0}$F$_{1}$ is a complex of two rotary motors F$_{1}$ and F$_{0}$, and the ATP synthesis/hydrolysis reaction that is reversibly catalyzed by F$_{1}$ is coupled with proton transport across membrane-embedded F$_{0}$ (4–6). The subunit composition of bacterial F$_{1}$ and F$_{0}$ is α$_{10}$β$_{3}$γε and α$_{2}$β$_{2}$γεδ respectively, and the γεδ$_{10}$ complex rotates against the α$_{2}$β$_{2}$σ$_{15}$ complex in F$_{0}$F$_{1}$. Among these subunits, ε is known to be an endogenous inhibitor of the ATP hydrolysis reaction catalyzed by F$_{1}$ and F$_{0}$F$_{1}$ (7–10). The inhibition of ATP hydrolysis by the ε subunit of Escherichia coli F$_{1}$ (EF$_{1}$) and F$_{0}$F$_{1}$ (EF$_{0}$F$_{1}$) has been extensively studied. Addition of ε to ε-depleted EF$_{1}$ showed noncompetitive inhibition of ATP hydrolysis (7). The affinity of MgATP and MgADP to the high affinity site of the three catalytic β subunits of EF$_{1}$ was decreased by ε (11). It has been reported that the ε subunit of EF$_{1}$ had no effect on the equilibrium between ATP and ADP-P, but inhibited product release under unsisite catalysis conditions (12). Inhibition of EF$_{0}$F$_{1}$-mediated ATP hydrolysis by ε was also demonstrated in experiments involving partial digestion by a protease (13). The inhibitory effect of the ε subunit of thermophilic Bacillus PS3 F$_{1}$ (TF$_{1}$) and F$_{0}$F$_{1}$ (TF$_{0}$F$_{1}$) has also been studied extensively. Slow binding and hydrolysis of TNP-ATP, a fluorescent ATP analog, under unsisite catalysis conditions have been reported (14). However, in contrast to EF$_{1}$, inhibition by the ε subunit of TF$_{1}$ was relieved slowly and apparently disappeared at high ATP concentrations ([ATP]). This is not due to the dissociation of ε from the F$_{1}$ complex, because disappearance of inhibition at high [ATP] was also reported in TF$_{0}$F$_{1}$ in which ε is indispensable for stable complex formation (15). However, in contrast to ATP hydrolysis, there has been no detailed analysis of the effect of ε on ATP synthesis.

The ε subunit has a molecular mass of 14 kDa and a two-domain structure consisting of an N-terminal 10-stranded β-sandwich and two C-terminal α-helices. Of these two domains, the C-terminal domain is responsible for inhibiting ATP hydrolysis, and the ε subunit in which this domain is absent does not have any inhibitory effect (15, 16). Structural studies on isolated ε and its complex with the truncated γ subunit have shown that the C-terminal domain of ε adopts two different conformations, the “hairpin-folded” and “extended” states (Fig. 1) (17–20). These conformations are also found in the crystal structure of the bovine mitochondrial homolog of F$_{1}$ and in the low resolution crystal structure of EF$_{1}$ (21, 22). Chemical modification of the ε subunit of EF$_{0}$F$_{1}$ indicated that the C-terminal domain is intrinsically flexible (23). Cross-linking and fluorescence resonance energy transfer experiments supported the existence of multiple conformations of the ε subunit of EF$_{0}$F$_{1}$ and TF$_{0}$F$_{1}$ (24–30). These studies have shown that the extended state inhibits ATP hydrolysis, whereas the hairpin-folded state does not.

In contrast to ATP hydrolysis, the correlation between the

$^{*}$This work was supported in part by Grants-in-aid for Scientific Research 18770134 and 18074005 (to R. I. and H. N., respectively) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

$^{1}$To whom correspondence may be addressed. Tel: 81-6-6879-8481; Fax: 81-6-6875-5724; E-mail: iino@sanken.osaka-u.ac.jp.

$^{2}$To whom correspondence may be addressed. Tel: 81-6-6879-8481; Fax: 81-6-6875-5724; E-mail: hnoji@sanken.osaka-u.ac.jp.

$^{3}$The abbreviations used are: F$_{0}$F$_{1}$, ATP synthase; F$_{1}$, ATPase; EF$_{0}$F$_{1}$, F$_{0}$F$_{1}$ from Escherichia coli; EF$_{1}$, F$_{1}$ from Escherichia coli; TF$_{0}$F$_{1}$, F$_{0}$F$_{1}$ from thermophilic Bacillus PS3; TF$_{1}$, F$_{1}$ from thermophilic Bacillus PS3: F$_{0}$F$_{1}$εΔC, F$_{0}$F$_{1}$ lacking the two α-helices at the C terminus of the ε subunit; F$_{0}$F$_{1}$εWT, wild-type F$_{0}$F$_{1}$; C$_{12}$E$_{8}$ octaethylene glycol mono-n-dodecyl ether; PAB, 4-aminobenzamidine dihydrochloride; TNP-ATP, 2’-(3’)-O-(2,4,6-trinitrophenyl)ladenosine 5’-triphosphate.

$^{4}$Another kind of “extended” state was proposed in TF$_{0}$F$_{1}$ (27); however, for simplicity, we did not distinguish between the two extended forms in this manuscript.
Inhibitory Mechanism of the C Terminus of \( \epsilon \) Subunit of \( F_{o}F_{1} \)

conformation of \( \epsilon \) and its effect on ATP synthesis has not been fully understood. In both \( E_{o}F_{1} \) and \( T_{o}F_{1} \), when \( \epsilon \) was fixed in an extended state that inhibits ATP hydrolysis, no change was observed in the ATP synthesis rate driven by the proton motive force (\( \Delta \mu \)) generated by the respiratory chain in the inverted membrane (26, 27). Based on these results, it has been proposed that \( \epsilon \) functions as a ratchet that inhibits only ATP hydrolysis. However, Masaike \emph{et al.} (31) reported that truncation of the C-terminal domain of \( \epsilon \) increased the ATP synthesis activity of \( T_{o}F_{1} \), suggesting that the C-terminal domain of \( \epsilon \) suppresses the ATP synthesis activity.

In this study, we generated an \( E_{o}F_{1} \) mutant with a truncated \( \epsilon \) subunit that did not contain the C-terminal \( \alpha \)-helices (\( F_{o}F_{1}{ }^{\alpha\psi\epsilon} \)). This mutant was purified and reconstituted into a liposome, and the ATP synthesis rate was measured by the acid-base transition (\( \Delta \phi \)) and K\(^+\)-valinomycin diffusion potential (\( \Delta \Psi \)) methods. The rate of ATP hydrolysis by solubilized \( E_{o}F_{1} \) was also measured. The activities of \( F_{o}F_{1}{ }^{\alpha\psi\epsilon} \) under various conditions were investigated and compared in detail with those of the wild-type enzyme (\( F_{o}F_{1}{ }^{\psi\epsilon} \)). The results indicated that the ATP synthesis and hydrolysis activities of \( F_{o}F_{1}{ }^{\alpha\psi\epsilon} \) were much higher than those of \( F_{o}F_{1}{ }^{\psi\epsilon} \). The inhibitory mechanism of the C-terminal \( \alpha \)-helices of the \( \epsilon \) subunit of \( E_{o}F_{1} \) was discussed.

**EXPERIMENTAL PROCEDURES**

\textbf{Construction and Expression of the \( E_{o}F_{1} \) Mutant—}A wild-type \( E_{o}F_{1} \) expression vector (pRA100) (32) was provided by Prof. R. A. Capaldi, University of Oregon. Based on this vector, a 1.3 S subunit of \emph{Propionibacterium shermanii} transcarboxylase (33) was fused to the N terminus of the \( \beta \) subunits, and three histidine residues were introduced at the C terminus of the C subunits. This mutant is referred to as the wild-type (denoted by \( F_{o}F_{1}{ }^{\psi\epsilon} \)) hereafter. \( F_{o}F_{1} \) lacking the two \( \alpha \)-helices in the C terminus of the \( \epsilon \) subunit (denoted by \( F_{o}F_{1}{ }^{\alpha\psi\epsilon} \)) was generated by introducing a stop codon at the position of Asp-91.

RA1 strain \emph{E. coli} (unc \( ^{-} \) cyo \( ^{-} \)) (34) was transformed with the \( F_{o}F_{1} \) mutant plasmid. After preculture in 5 ml of LB (1% tryptone, 0.5% yeast extract, and 1% NaCl) containing 30 \( \mu \)g/ml chloramphenicol for 8–9 h at 37 °C, the cells were inoculated in 1.2 liters of TB (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol, 1.25% \( K_{2}HPO_{4} \), and 0.23% \( KH_{2}PO_{4} \)) containing 30 \( \mu \)g/ml, wet weight of inverted membrane/volume of buffer) in a 1.5-ml tube containing 1 mm dithiothreitol. The purified \( E_{o}F_{1} \) fractions were run on 15% SDS-PAGE to select the purest fractions that contained all the subunits and the least contaminants. The chosen fractions were pooled and passed through a NAP-5 column (GE Healthcare) that had been pre-equilibrated with 5 ml of buffer D (20 mm HEPES-KOH (pH 7.5), 500 mm NaCl, 5 mm MgCl\(_{2}\), 200 \( \mu \)M ADP, 50 mm imidazole, 20% (w/v) glycerol, 1X protease inhibitor mixture, and 5 mm PAB) and 2% (w/v) octaethylene glycol mono-n-dodecyl ether (C\(_{12}E_{8}\), Wako) were added to the pellet, and the mixture was incubated for 15 min on ice. It was then centrifuged, and the supernatant was collected. The collected supernatant was injected into a HisTrap HP column (GE Healthcare) pre-equilibrated with 5 ml of buffer D (20 mm HEPES-KOH (pH 7.5), 500 mm NaCl, 5 mm MgCl\(_{2}\), 200 \( \mu \)M ADP, 50 mm imidazole, 20% (w/v) glycerol, 1X protease inhibitor mixture, 5 mm PAB, 0.3% (w/v) C\(_{12}E_{8}\), and 0.1% (w/v) \emph{E. coli} total lipid). The column was washed twice with 5 ml of buffer D. His-tagged \( E_{o}F_{1} \) was eluted with 3 ml of buffer E (20 mm HEPES-KOH (pH 7.5), 500 mm NaCl, 5 mm MgCl\(_{2}\), 200 \( \mu \)M ADP, 50 mm imidazole, 20% (w/v) glycerol, 1X protease inhibitor mixture, 5 mm PAB, 0.3% (w/v) C\(_{12}E_{8}\), and 0.1% (w/v) \emph{E. coli} total lipid). Six drops of the eluate were collected in each 1.5-ml tube containing 1 mm dithiothreitol. The purified \( E_{o}F_{1} \) was eluted with 1 ml of buffer F. The eluate was concentrated and further purified in an Amicon Ultra-4 100,000 centrifugal filter device (Millipore). The protein concentration of the sample was determined by the BCA assay (Pierce) using bovine serum albumin (Sigma) as the standard. Purified \( E_{o}F_{1} \) was immediately used for reconstitution into the liposome or to measure the ATP hydrolysis activity.

\textbf{Liposome Preparation and Reconstitution of \( E_{o}F_{1} \) into the Liposome—}\( \alpha \)-Phosphatidylcholine from soybean (type II-S, Sigma) was suspended in buffer G (10 mm HEPES-NaOH, 5 mm MgSO\(_{4}\), and 1 mm KCl (pH 7.5)) by vigorous vortexing. The liposome suspension was then repeatedly freeze-thawed three times. The final concentration of the lipid was 40 mg/ml. Solubilized \( E_{o}F_{1} \) was reconstituted into the liposomes using the freeze-thaw method. The \( E_{o}F_{1} \) solution (~1 mg/ml, 100 \( \mu \)l) was added to the liposome suspension (40 mg/ml, 1 ml), and the mixture was frozen in liquid nitrogen and stored at ~80 °C prior to further use. Reconstitution efficiency of \( E_{o}F_{1} \) into liposome was assessed by SDS-PAGE of \( E_{o}F_{1} \) and liposome suspension and the supernatant after centrifugation of \( E_{o}F_{1} \) into liposome at 75,000 rpm for 20 min at 4 °C.

\textbf{Measurement of the ATP Synthesis Activity—}The ATP synthesis activity was measured as described previously with slight variations. The supernatant containing the inverted membrane was transferred to a new tube and centrifuged at 75,000 rpm for 20 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in buffer B at 250 mg/ml. The inverted membrane was stored at ~80 °C prior to further use.

\textbf{Purification of \( E_{o}F_{1} \)—}The membrane suspension (250 mg/ml, wet weight of inverted membrane/volume of buffer) in 0.8% (w/v) n-octyl β-D-glucopyranoside (Sigma) was centrifuged at 75,000 rpm for 20 min at 4 °C. The supernatant was transferred to a new tube on ice. Buffer C (20 mm HEPES-KOH (pH 7.5), 500 mm NaCl, 5 mm MgCl\(_{2}\), 200 \( \mu \)M ADP, 50 mm imidazole, 20% (w/v) glycerol, 1X protease inhibitor mixture, and 5 mm PAB) and 2% (w/v) octaethylene glycol mono-n-dodecyl ether (C\(_{12}E_{8}\), Wako) were added to the pellet, and the mixture was incubated for 15 min on ice. It was then centrifuged, and the supernatant was collected. The collected supernatant was injected into a HiTrap HP column (GE Healthcare) pre-equilibrated with 5 ml of buffer D (20 mm HEPES-KOH (pH 7.5), 500 mm NaCl, 5 mm MgCl\(_{2}\), 200 \( \mu \)M ADP, 50 mm imidazole, 20% (w/v) glycerol, 1X protease inhibitor mixture, 5 mm PAB, 0.3% (w/v) C\(_{12}E_{8}\), and 0.1% (w/v) \emph{E. coli} total lipid). The column was washed twice with 5 ml of buffer D. His-tagged \( E_{o}F_{1} \) was eluted with 3 ml of buffer E (20 mm HEPES-KOH (pH 7.5), 500 mm NaCl, 5 mm MgCl\(_{2}\), 200 \( \mu \)M ADP, 50 mm imidazole, 20% (w/v) glycerol, 1X protease inhibitor mixture, 5 mm PAB, 0.3% (w/v) C\(_{12}E_{8}\), and 0.1% (w/v) \emph{E. coli} total lipid). The ATP synthesis and hydrolysis activities of \( E_{o}F_{1} \) were measured by the acid-base transition (\( \Delta \phi \)) and K\(^+\)-valinomycin diffusion potential (\( \Delta \Psi \)) methods.
modifications (35). To acidify the interior of the proteoliposomes, 10 µl of the proteoliposome suspension was mixed with 50 µl of acidification buffer (300 mM MES-NaOH or HEPES-NaOH) and incubated for 3 min at room temperature. Following incubation, the pH of the acidified liposome suspension was measured by a glass electrode, and this value was regarded as the pH inside the liposome (pH_{in}). The ATP synthesis reaction was initiated by injecting 60 µl of the acidified proteoliposomes into 1 ml of base buffer containing 100 mM Tricine-Na (pH 8.8) or HEPES-NaOH (pH 7.5), 2.5 mM MgSO_{4}, 0.1–50 mM phosphate, 2.2 mg of luciferin/luciferase mixture from ATP Bioluminescence Assay Kit CLS II (Roche Applied Science), 0.001–1 mM ADP, 36 nM valinomycin, and 1–300 mM KCl. The amount of ATP generated was measured as the increase in the luminescence intensity of the luciferin/luciferase reaction at 550 nm in an FP-6500 spectrofluorometer (Jasco, Japan). The ATP synthesis rate was calculated using the initial slope of the increase in the luminescence intensity. To calibrate the system for measuring the amount of generated ATP, a known amount of ATP was injected into the base buffer after measurement. To measure the dependence of the ATP synthesis rate on the ADP or phosphate concentration, the phosphate and ADP concentrations were fixed at 10 and 1 mM, respectively. The pH of the mixture of acidified proteoliposome suspension and base buffer (pH_{out}) was directly measured using a glass electrode to determine ΔpH (= pH_{out} - pH_{in}). The ΔpH and ΔΨ dependences were measured by changing the pH and [K^+] of the acidification and base buffers. ΔΨ was calculated by the Nernst equation using the difference in [K^+] inside and outside the liposome, i.e., 2.3(k_B T/ε) log([K^+]_{out}/[K^+]_{in}), where k_B is the Boltzmann constant, and ε is the elementary charge. Measurements were carried out at 24–25 °C.

Measurement of the ATP Hydrolysis Activity—The ATP hydrolysis activity of solubilized EF_{o}F_{1} was measured with an ATP regeneration system using a UV-visible spectrophotometer (VP-550, Jasco). Various concentrations of ATP (Sigma) were added to the assay mixture (10 mM HEPES-NaOH (pH 7.5), 5 mM MgSO_{4}, 1 mM KCl, 0.3% (w/v) C_{12}E_8, 2.5 mM phosphoenolpyruvate, 0.1 mg/ml pyruvate kinase, 0.1 mg/ml lactate dehydrogenase, and 0.2 mM NADH) at 0 s, and this was followed by the addition of various concentrations of EF_{o}F_{1} at 30 s. The NADH absorbance at 340 nm was monitored for 1200 s. The ATP hydrolysis rate was calculated from the time course of the change in [NADH] using a molecular extinction coefficient value of 6220 at 340 nm. Because the ATP hydrolysis activity gradually increased in the initial state after the addition of EF_{o}F_{1}, the maximum activity in the steady state at around 1000–1200 s was used for data analysis. Measurements were carried out at 24–25 °C.

RESULTS

ATP Synthesis by the F_{o}F_{1}^{εβC} Mutant of EF_{o}F_{1} Reconstituted into the Liposome—The C-terminal α-helices of the ε subunit of EF_{o}F_{1} were truncated by introducing a stop codon at the position of Asp-91.

Measurement of the ATP Hydrolysis Activity—The ATP hydrolysis activity of solubilized EF_{o}F_{1} was measured with an ATP regeneration system using a UV-visible spectrophotometer (VP-550, Jasco). Various concentrations of ATP (Sigma) were added to the assay mixture (10 mM HEPES-NaOH (pH 7.5), 5 mM MgSO_{4}, 1 mM KCl, 0.3% (w/v) C_{12}E_8, 2.5 mM phosphoenolpyruvate, 0.1 mg/ml pyruvate kinase, 0.1 mg/ml lactate dehydrogenase, and 0.2 mM NADH) at 0 s, and this was followed by the addition of various concentrations of EF_{o}F_{1} at 30 s. The NADH absorbance at 340 nm was monitored for 1200 s. The ATP hydrolysis rate was calculated from the time course of the change in [NADH] using a molecular extinction coefficient value of 6220 at 340 nm. Because the ATP hydrolysis activity gradually increased in the initial state after the addition of EF_{o}F_{1}, the maximum activity in the steady state at around 1000–1200 s was used for data analysis. Measurements were carried out at 24–25 °C.

RESULTS

ATP Synthesis by the F_{o}F_{1}^{εβC} Mutant of EF_{o}F_{1} Reconstituted into the Liposome—The C-terminal α-helices of the ε subunit of EF_{o}F_{1} were truncated by introducing a stop codon at the position of Asp-91 (Fig. 1). The ATP synthesis activity of the truncated mutant (F_{o}F_{1}^{εβC}) was compared in detail with that of the wild-type enzyme (F_{o}F_{1}^{WT}). The expression level of F_{o}F_{1}^{εβC} was similar to that of F_{o}F_{1}^{WT} in the inverted membrane of RA1 strain E. coli (unc^-/cyo^-), and it was purified as a stable EF_{o}F_{1} complex using the His tags introduced into the ε subunits (Fig. 2A). No differences in concentration determination by the BCA assay and purity were observed between F_{o}F_{1}^{εβC} and F_{o}F_{1}^{WT}, CBB, Coomassie Brilliant Blue. B, SDS-PAGE of F_{o}F_{1}^{εβC} and F_{o}F_{1}^{WT} were performed. The protein bands were visualized with CBB and enhanced with CBB stain. The C-terminal α-helices of the ε subunit (enclosed by black lines) of EF_{o}F_{1} were truncated by introducing a stop codon at the position of Asp-91.
were measured. Several methods have been proposed for reconstituting EFoF1 into liposomes, including the following: 1) reconstitution by detergent removal from a mixture of solubilized EFoF1 and liposome using Bio-Beads SM (36); 2) dialysis; and 3) reconstitution by diluting the detergent in solubilized EFoF1 with an excess amount of liposome and subsequent freeze-thaw. Because the last method resulted in the highest ATP synthesis activity (data not shown), we employed this method throughout this study. The reconstitution efficiencies were very high, and no differences were observed between F0,F1WT and EFoF1 WT (11 s⁻¹, blue line) and EFoF1 WT (11 s⁻¹, blue line), as reported earlier for TFoF1 in an inverted membrane (31). The observed differences were actually due to the removal of C-terminal α-helices of the e subunit and not to variations in concentration determination, purity, and reconstitution efficiency between EFoF1 WT and EFoF1 WT (Table 2). This result indicates that the C-terminal domain of e suppresses the ATP synthesis activity. As a control, the time course of the liposome without EFoF1 is shown (<1 s⁻¹, Fig. 3B, black line), and the results indicate that the ATP was actually generated by EFoF1.

Dependence of ATP Synthesis and Hydrolysis Rates on the Substrate Concentration—The ATP synthesis rate was measured at various concentrations of ADP and P_i. The [substrate]-velocity plots for ADP and P_i showed that the ATP synthesis rates of F0,F1 e^WT (blue) and F0,F1 e^AC (red) and liposome without F0,F1 (black), determined by linear fitting of the initial slope after addition of the F0,F1 liposome, were 11, 49, and 87 mV, respectively. The concentrations of ADP and P_i were 1 and 10 mM, respectively. Measurements were carried out at 24 –25 °C. Final concentrations of EFoF1 were ~0.5 mM, and final concentrations of ATP added as standard were 200 nM.
**TABLE 1**

Kinetic parameters for ATP synthesis of $F_{o}F_{1}^{WT}$ and $F_{o}F_{1}^{\Delta C}$ reconstituted into liposomes

| Substrates varied | $k_{cat}$ | $K_{m}(\text{ADP})$ | $k_{cat}/K_{m}(\text{ADP})$ | $k_{cat}$ | $K_{m}(\text{Pi})$ | $k_{cat}/K_{m}(\text{Pi})$ |
|-------------------|-----------|---------------------|----------------------------|-----------|-------------------|---------------------------|
| $F_{o}F_{1}^{WT}$ | 16 ± 0.6  | (1.0 ± 0.12) $\times 10^{-4}$ | 1.6 $\times 10^{5}$ | 20 ± 1.2 | (4.2 ± 0.92) $\times 10^{-3}$ | 4.8 $\times 10^{3}$ |
| $F_{o}F_{1}^{\Delta C}$ | 55 ± 3.2  | (2.5 ± 0.58) $\times 10^{-5}$ | 2.2 $\times 10^{6}$ | 66 ± 5.0 | (3.2 ± 0.86) $\times 10^{-3}$ | 2.1 $\times 10^{4}$ |
| Ratio$^{a}$       | 0.29      | 4.0                 | 0.073                       | 0.30      | 1.3               | 0.23                       |

$^{a}$ The values for $F_{o}F_{1}^{WT}$ were divided by those for $F_{o}F_{1}^{\Delta C}$.

**TABLE 2**

Kinetic parameters for ATP hydrolysis of solubilized $F_{o}F_{1}^{WT}$ and $F_{o}F_{1}^{\Delta C}$

| Substrates varied | $k_{cat}$ | $K_{m}(\text{ATP})$ | $k_{cat}/K_{m}(\text{ATP})$ |
|-------------------|-----------|---------------------|----------------------------|
| $F_{o}F_{1}^{WT}$ | 285 ± 4.4 | (7.8 ± 0.54) $\times 10^{-5}$ | 3.8 $\times 10^{6}$ |
| $F_{o}F_{1}^{\Delta C}$ | 539 ± 15.0 | (5.2 ± 0.68) $\times 10^{-5}$ | 1.0 $\times 10^{6}$ |
| Ratio$^{a}$       | 0.53      | 1.5                 | 0.38                       |

$^{a}$ The values for $F_{o}F_{1}^{WT}$ were divided by those for $F_{o}F_{1}^{\Delta C}$.

**Dependence of the ATP Synthesis Rate on $\Delta pH$ and $\Delta \Psi$**—In the chemiosmotic theory first proposed by Mitchell (37), $\Delta \mu$ consists of $\Delta pH$ and $\Delta \Psi$, i.e. $\Delta \mu = 2.3(k_{B}T/e)\Delta pH + \Delta \Psi$. Several studies on $F_{o}F_{1}$ from *E. coli*, *Propionigenium modestum*, and the chloroplast have reported that the dependence of the ATP synthesis rate on the amplitude of $\Delta pH$ and $\Delta \Psi$ is kinetically different (38–40). Previous results have suggested the possibility that elementary steps of the ATP synthesis reaction are affected in different ways by $\Delta pH$ and $\Delta \Psi$, although the mechanism is still unclear. Therefore, we measured the ATP synthesis rates using various combinations of $\Delta pH$ and $\Delta \Psi$. Fig. 5A shows contour plots of the ATP synthesis rate as a function of $\Delta pH$ and $\Delta \Psi$. Although the absolute values of the rates of $F_{o}F_{1}^{WT}$ and $F_{o}F_{1}^{\Delta C}$ differed, their dependences on $\Delta pH$ and $\Delta \Psi$ were similar. Applying $\Delta pH$ resulted in a higher ATP synthesis rate than when the same amplitude of $\Delta \Psi$ was applied in both $F_{o}F_{1}^{WT}$ and $F_{o}F_{1}^{\Delta C}$. This result was apparently inconsistent with previous studies reporting that $\Delta \Psi$ was more effective than $\Delta pH$ in driving ATP synthesis by EF$_{1}$F$_{1}$ (38–40). So far, we have no clear idea why $\Delta pH$ was more effective in our experiment. Because we did not use the dicarboxylic acid such as maleic acid and succinic acid as the acidification buffer, concomitant formation of $\Delta \Psi$ was not plausible (38, 40). Other factors such as different compositions of the liposome and the reaction solution might affect the dependence of $\Delta pH$ and $\Delta \Psi$.

Under our experimental conditions, [ADP] and [P] were 1 and 10 mM, respectively, and ATP contamination in ADP was $\sim 0.5\%$. By using the standard Gibbs free energy value of 14.9 $k_{B}T$ (37 kJ/mol) (41), this corresponds to Gibbs free energy for ATP hydrolysis of $-11.9k_{B}T$ ($= -14.9 k_{B}T + k_{B}T \ln(10^{-3} \times 10^{-2}/5 \times 10^{-7})$) or $-306$ mV. Thus, if we assume that 3 ATP molecules are synthesized and 10 protons are transported per turn (42), the potential difference that counteracts single proton translocation, or the value of $\Delta \mu$ at the ATP synthesis/hydrolysis equilibrium point, will be 92 mV ($= (306 \text{ mV} \times 3)/10$). We next plotted the dependence of the ATP synthesis rate on $\Delta pH$ and $\Delta \Psi$ when $2.3(k_{B}T/e)\Delta pH$ and $\Delta \Psi$ were fixed around this equilibrium value (89 and 87 mV, respectively) (Fig. 5B, corresponds to the dashed lines in Fig. 5A). As expected, no ATP synthesis was observed around the equilibrium value. When $\Delta pH$ increased, the ATP synthesis rate increased nonlinearly in the $\Delta pH$ range from 5.5 to 8.5 (Fig. 5B, circles). The $\Delta \mu$ has a dimension equivalent to energy. Therefore, the nonlinear increase can be explained if we assume that the ATP synthesis rate changes according to the equation that resembles the Arrhenius equation in which the rate increases exponentially as the activation energy decreases.
Inhibitory Mechanism of the C Terminus of \( \epsilon \) Subunit of \( \text{FoF}_1 \)

**DISCUSSION**

Correlation between the Conformation of the \( \epsilon \) Subunit of \( \text{EF}_\text{F}_1 \) and Catalytic Activity—Truncation of the C-terminal domain of the \( \epsilon \) subunit of \( \text{EF}_\text{F}_1 \) (\( \text{FoF}_1^{\text{WT}} \)) resulted in increased ATP synthesis and hydrolysis activities (Fig. 4 and Tables 1 and 2). The enhancement was observed under all conditions studied. This indicates that the C-terminal domain of the \( \epsilon \) subunit of \( \text{EF}_\text{F}_1 \) suppresses both ATP synthesis and hydrolysis. As described above, \( \epsilon \) can adopt either of two different conformations, hairpin-folded or extended (Fig. 1). The crystal structure of \( \text{FoF}_1 \) with the hairpin-folded \( \epsilon \) shows that \( \epsilon \) does not have any direct interaction with the catalytic \( \alpha_3\beta_3 \) ring, whereas in the \( \text{EF}_1 \) crystal structure with the extended \( \epsilon \), the C-terminal helix of \( \epsilon \) interacts with the \( \alpha_3\beta_3 \) ring. Therefore, the extended form is thought to be responsible for the inhibitory effect of \( \epsilon \). Previous experiments involving the cross-linking of \( \text{EF}_\text{F}_1 \) and \( \text{TF}_\text{F}_1 \) showed that the ATP hydrolysis activity was inhibited when \( \epsilon \) was fixed in the extended state (26, 27). These results are consistent with the present results. On the other hand in the previous studies, there was no significant change in the ATP synthesis activity when \( \epsilon \) was fixed in the extended state. This observation can be explained by assuming that \( \epsilon \) mostly adopts the extended form under conditions of ATP synthesis, as suggested earlier (27). Thus, although the apparent inhibitory effect of \( \epsilon \) differs during ATP hydrolysis and synthesis, probably as a result of the different ratios of the extended versus hairpin-folded forms, the extended form of \( \epsilon \) is the primary inhibitory state under both ATP synthesis and hydrolysis conditions, and the C-terminal domain is responsible for inhibition.

**Mechanism of Inhibition of ATP Synthesis by the C-terminal Domain of the \( \epsilon \) Subunit of \( \text{EF}_\text{F}_1 \) in the Extended State**—During both ATP synthesis and hydrolysis, \( \text{FoF}_1^{\text{WT}} \) exhibited lower \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \) values than those of \( \text{FoF}_1^{\text{ΔC}} \) (Fig. 4 and Tables 1 and 2). Higher \( K_m \) and lower \( k_{\text{cat}}/K_m \) values of \( \text{FoF}_1^{\text{WT}} \) in comparison with those of \( \text{FoF}_1^{\text{ΔC}} \) indicate decreased substrate affinity and rate of substrate binding, respectively. Furthermore, the lower \( k_{\text{cat}} \) value of \( \text{FoF}_1^{\text{WT}} \) in comparison with that of \( \text{FoF}_1^{\text{ΔC}} \) indicates a decreased rate of covalent bond formation/cleavage or product release.

With respect to ATP hydrolysis, our results are consistent with that of a previous study in which the decreased affinity of MgATP in the presence of the \( \epsilon \) subunit was demonstrated (11). A previous study also reported that the \( \epsilon \) subunit of \( \text{EF}_1 \) had no effect on the equilibrium between ATP and ADP-Pi, but reduced the rate of product release under unisite ATP hydrolysis conditions (12). If this is also the case in multisite catalysis, \( \epsilon \) will not affect covalent bond formation/cleavage, and product release will be slowed. In the case of ATP synthesis, a previous study proposed that the energy of \( \Delta \mu \) is mainly used for product release (43), suggesting that the product release step is rate-limiting. This is consistent with the assumption that it is the product release step that is slowed in \( \text{FoF}_1^{\text{WT}} \) and not the covalent bond formation/cleavage step. In our study, the dependence of the ATP synthesis rate on various combinations of \( \Delta \psi \) and \( \Delta \mu \) was similar in both \( \text{FoF}_1^{\text{WT}} \) and \( \text{FoF}_1^{\text{ΔC}} \) (Fig. 5A). This suggests that the C-terminal domain of \( \epsilon \) in the extended state does not change the rate-limiting step of ATP synthesis. Furthermore, it is consistent with the notion that multiple elementary steps of the ATP synthesis reaction are slowed. Thus, during both ATP synthesis and hydrolysis, the rates of substrate binding and product release appear to be suppressed by the C-terminal domain of \( \epsilon \).

However, when the reversibility of the reaction is considered, there is a problem in explaining our results. To examine this, we discuss our results based on our recently proposed model of mechanochemical coupling of \( \text{F}_1 \) (Fig. 6) (44–48). During ATP hydrolysis, the lower \( k_{\text{cat}} \) of \( \text{FoF}_1^{\text{WT}} \) in comparison with that of \( \text{FoF}_1^{\text{ΔC}} \) corresponds to a lower rate of product (ADP or Pi) release (Fig. 6, B → C or E → F). Slow ADP or Pi release generally results in high affinity, but this is inconsistent with the higher \( K_m \) value of \( \text{FoF}_1^{\text{WT}} \) for ADP or Pi in comparison with the corresponding values of \( \text{FoF}_1^{\text{ΔC}} \) during ATP synthesis. Similarly, although a lower \( k_{\text{cat}} \) value of \( \text{FoF}_1^{\text{WT}} \) in comparison with that of \( \text{FoF}_1^{\text{ΔC}} \) during ATP synthesis corresponds to a
lower ATP release rate (Fig. 6, $B \rightarrow A$), this result also appears to be inconsistent with the higher $K_m$ value of $F_o F_1^{WT}$ for ATP in comparison with that of $F_o F_1^{\Delta C}$ during ATP hydrolysis.

From a structural point of view, there is another problem in explaining our results. As shown in Fig. 6, each $\beta$ subunit sequentially executes elementary steps of the reaction and is always in a different chemical state from the other subunits (49, 50). In the low resolution crystal structure of $EF_1$, (Fig. 1), the $C$-terminal domain of $\epsilon$ in the extended state interacts with only one $\beta$ subunit bound to the ATP analog (Fig. 6, $\beta_{TP}$ in crystal structure), although there must be some displacement due to steric hindrance in the structural model (22). We recently proved that the crystal structures of $F_1$ correspond to states $D$–$F$ in Fig. 6 (states in catalytic dwell), and $\beta_{TP}$ corresponds to the $\beta$ subunit immediately after ATP binding (51). Thus, $\epsilon$ would affect only ATP binding and release by direct interaction with $\beta_{TP}$. Even if we consider states A to C in Fig. 5 (states in binding dwell), it seems difficult for $\epsilon$ to simultaneously interact with multiple $\beta$ subunits and to directly suppress other elementary steps of the reaction such as ADP and $P_i$ binding/release that occur in other $\beta$ subunits.

These apparent difficulties can be explained on the basis of the following model in which we assume that the rates of elementary steps of the reaction executed in each $\beta$ subunit are modulated not by direct interaction with $\epsilon$ but by the rotary angle of $\gamma$ (rotor). The $C$-terminal domain of $\epsilon$ in the extended state increases the diameter of the rotor ($\gamma$ and $\epsilon$) inserted into the $\alpha_3\beta_3$ ring and restricts its rotation. This results in lower rates of multiple elementary steps during both ATP synthesis and hydrolysis in multiple $\beta$ subunits. Our assumption that the rate of the elementary step is dependent on the rotary angle of $\gamma$ is supported by our previous single-molecule experiments in which we reported that the rate of ADP release from the MgADP-inhibited $\alpha_3\beta_3\gamma$ subcomplex of $TF_1$ strongly depended on the rotary angle of $\gamma$ (52). This suggests that the rate of ADP release (Fig. 6, $B \rightarrow C$) is active $F_1$ is also dependent on the angle of $\gamma$. Furthermore, the rates of ATP binding/release (Fig. 6, $A \leftrightarrow B$) are strongly dependent on the rotary angle of $\gamma$ in an actively rotating $F_1$ $\alpha_3\beta_3\gamma$ subcomplex.3 Interestingly, our unpublished results5 also indicate that the rates of covalent bond formation/cleavage (Fig. 6, $D \leftrightarrow E$) are less dependent on the angle of $\gamma$. Taking this into consideration, the results of a previous study in which it was reported that this step was unaffected by $\epsilon$ (12) are consistent with our assumption that $\epsilon$ restricts the rotation of $\gamma$.

Our model predicts that the dwell times before the 80° and 40° substeps will become longer when the rotation of $EF_1$ is assayed in the presence of $\epsilon$. Previous single molecule observations of $EF_1$, rotation driven by ATP hydrolysis have shown that $\epsilon$ increases the frequency and duration of the transient pause and results in decreased rotation speed (53). However, in the previous study, the pause angles were not resolved. Detailed analysis of pause angles and dwell times will provide further insights into the mechanism of inhibition by the $\epsilon$ subunit.

3 R. Watanabe, D. Okuno, S. Sakakihara, K. Shimabukuro, R. Iino, M. Yoshida, and H. Noji, unpublished results.

Effect of the C-terminal Domain of $\epsilon$ on the Efficiency of the Mechanochemical Coupling during ATP Synthesis—In this study, $F_o F_1^{\Delta C}$ showed a higher rate of ATP synthesis than $F_o F_1^{WT}$. However, we could not directly assess the coupling efficiency between the mechanical rotation of $\gamma$ and each elementary step of the reaction. A high ATP synthesis rate does not necessarily indicate high coupling efficiency. As proposed previously (54), we can expect a situation in which the rotation rate is faster but the coupling efficiency of $F_o F_1^{\Delta C}$ is lower than that of $F_o F_1^{WT}$ during ATP synthesis. We previously reported that reconstitution of $\epsilon$ into the $\alpha_3\beta_3\gamma$ subcomplex of $TF_1$ improved the coupling efficiency of ATP synthesis when $\gamma$ was forcibly rotated with magnetic tweezers (55, 56). The role of the $C$-terminal domain of $\epsilon$ in the coupling efficiency of ATP synthesis can be directly investigated by single molecule manipulation of $F_o F_1^{\Delta C}$ or the $\alpha_3\beta_3\gamma$ subcomplex of $F_1$ reconstituted with C-terminal truncated $\epsilon$.

Acknowledgments—We thank Drs. Nobushito Sone, Masatoshi Toei, Masahiro Nakano, and Ken Yokoyama for technical advice and members of the Noji laboratory for helpful discussions.

REFERENCES
1. Boyer, P. D. (1997) Annu. Rev. Biochem. 66, 717–749
2. Senior, A. E., Nadanaciva, S., and Weber, J. (2002) Biochim. Biophys. Acta 1553, 188–211
3. Capaldi, R. A., and Aaggeler, R. (2002) Trends Biochem. Sci. 27, 154–160
4. Noji, H., Yasuda, R., Yoshida, M., and Kinoshita, K., Jr. (1997) Nature 386, 299–302
5. Yoshida, M., Muneyuki, E., and Hisabori, T. (2001) Nat. Rev. Mol. Cell Biol. 2, 669–677
6. Kinosita, K., Jr., Adachi, K., and Itoh, H. (2004) Annu. Rev. Biophys. Biomol. Struct. 33, 245–268
7. Sternweis, P. C., and Smith, J. B. (1980) Biochemistry 19, 526–531
8. Capaldi, R. A., and Schulenberg, B. (2000) Biochim. Biophys. Acta 1458, 263–269
9. Vik, S. B. (2000) J. Bioenerg. Biomembr. 32, 485–491
10. Feniouk, B. A., Suzuki, T., and Yoshida, M. (2006) Biochim. Biophys. Acta 1757, 326–338
11. Weber, J., Dunn, S. D., and Senior, A. E. (1999) J. Biol. Chem. 274, 19124–19128
12. Dunn, S. D., Zadorozny, V. D., Tozer, R. G., and Orr, L. E. (1987) Biochemistry 26, 4488–4493
13. Mendel-Hartvig, J., and Capaldi, R. A. (1991) Biochemistry 30, 10987–10991
14. Kato, Y., Matsui, T., Tanaka, N., Muneyuki, E., Hisabori, T., and Yoshida, M. (1997) J. Biol. Chem. 272, 24906–24912
15. Kato-Yamada, Y., Bald, D., Koike, M., Motohashi, K., Hisabori, T., and Yoshida, M. (1999) J. Biol. Chem. 274, 33991–33994
16. Xiong, H., Zhang, D., and Vik, S. B. (1998) Biochemistry 37, 16423–16429
17. Wilkens, S., Dahanquist, F. W., McIntosh, L. P., Donaldson, L. W., and Capaldi, R. A. (1995) Nat. Struct. Biol. 2, 961–967
18. Uhlin, U., Cox, G. B., and Guss, J. M. (1997) Structure 5, 1219–1230
19. Wilkens, S., and Capaldi, R. A. (1998) J. Biol. Chem. 273, 26645–26651
20. Rodgers, A. J., and Wilce, M. C. (2000) Nat. Struct. Biol. 7, 1051–1054
21. Gibbons, C., Montgomery, M. G., Leslie, A. G., and Walker, J. E. (2000) Nat. Struct. Biol. 7, 1055–1061
22. Haurath, A. C., Capaldi, R. A., and Matthews, B. W. (2001) J. Biol. Chem. 276, 47227–47232
23. Ganti, S., and Vik, S. B. (2007) J. Bioenerg. Biomembr. 39, 99–107
24. Schulenberg, B., and Capaldi, R. A. (1999) J. Biol. Chem. 274, 28351–28355
25. Kato-Yamada, Y., Yoshida, M., and Hisabori, T. (2000) J. Biol. Chem. 275,
Inhibitory Mechanism of the C Terminus of ε Subunit of F₀F₁

35746–35750
26. Tsunoda, S. P., Rodgers, A. J., Aggeler, R., Wilce, M. C., Yoshida, M., and Capaldi, R. A. (2001) *Proc. Natl. Acad. Sci. U.S.A.* 98, 6560–6564
27. Suzuki, T., Murakami, T., Iino, R., Suzuki, J., Ono, S., Shirakihara, Y., and Yoshida, M. (2003) *J. Biol. Chem.* 278, 46840–46846
28. Bulygin, V. V., Duncan, T. M., and Cross, R. L. (2004) *J. Biol. Chem.* 279, 35616–35621
29. Iino, R., Murakami, T., Iizuka, S., Kato-Yamada, Y., Suzuki, T., and Yoshida, M. (2005) *J. Biol. Chem.* 280, 40130–40134
30. Zimmermann, B., Diez, M., Zarrabi, N., Gra¨ber, P., and Bo¨rsch, M. (2005) *EMBO J.* 24, 2053–2063
31. Masaike, T., Suzuki, T., Tsunoda, S. P., Konno, H., and Yoshida, M. (2006) *Biochem. Biophys. Res. Commun.* 342, 800–807
32. Aggeler, R., Chicas-Cruz, K., Cai, S. X., Keana, J. F., and Capaldi, R. A. (1992) *Biochemistry* 31, 2956–2961
33. Cronan, J. E., Jr. (1990) *J. Biol. Chem.* 265, 10327–10333
34. Aggeler, R., Ogilvie, I., and Capaldi, R. A. (1997) *J. Biol. Chem.* 272, 19621–19624
35. Toei, M., Gerle, C., Nakano, M., Tani, K., Gyobu, N., Tamakoshi, M., Sone, N., Yoshida, M., Fujiyoshi, Y., Mitsuoka, K., and Yokoyama, K. (2007) *Proc. Natl. Acad. Sci. U.S.A.* 104, 20256–20261
36. Fischer, S., Etzold, C., Turina, P., Deckers-Hebestreit, G., Altendorf, K., and Gräber, P. (1994) *Eur. J. Biochem.* 225, 167–172
37. Mitchell, P. (1961) *Nature* 191, 144–148
38. Kaim, G., and Dimroth, P. (1998) *FEBS Lett.* 434, 57–60
39. Fischer, S., and Gräber, P. (1999) *FEBS Lett.* 457, 327–332
40. Kaim, G., and Dimroth, P. (1999) *EMBO J.* 18, 4118–4127
41. Turina, P., Samoray, D., and Gräber, P. (2003) *EMBO J.* 22, 418–426
42. Jiang, W., Hermolin, J., and Fillingame, R. H. (2001) *Proc. Natl. Acad. Sci. U.S.A.* 98, 4966–4971
43. Boyer, P. D., Cross, R. L., and Momsen, W. (1973) *Proc. Natl. Acad. Sci. U.S.A.* 70, 2837–2839
44. Yasuda, R., Noji, H., Kinosita, K., Jr., and Yoshida, M. (1998) *Cell* 93, 1117–1124
45. Yasuda, R., Noji, H., Yoshida, M., Kinosita, K., Jr., and Itoh, H. (2001) *Nature* 410, 898–904
46. Shimabukuro, K., Yasuda, R., Muneyuki, E., Hara, K. Y., Kinosita, K., Jr., and Yoshida, M. (2003) *Proc. Natl. Acad. Sci. U.S.A.* 100, 14731–14736
47. Adachi, K., Iino, R., Yoshida, M., Kinosita, K., Jr. (2007) *Cell* 130, 309–321
48. Watanabe, R., Iino, R., Shimabukuro, K., Yoshida, M., and Noji, H. (2008) *EMBO Report* 9, 84–90
49. Ariga, T., Muneyuki, E., and Yoshida, M. (2007) *Nat. Struct. Mol. Biol.* 14, 841–846
50. Masaike, T., Koyama-Horibe, F., Iino, K., Yoshida, M., and Nishizaka, T. (2008) *Nat. Struct. Mol. Biol.* 15, 1326–1333
51. Okuno, D., Fujisawa, R., Iino, R., Hirono-Hara, Y., Imamura, H., and Noji, H. (2008) *Proc. Natl. Acad. Sci. U.S.A.* 105, 20722–20727
52. Hirono-Hara, Y., Ishizuka, K., Kinosita, K., Jr., Yoshida, M., and Noji, H. (2005) *Proc. Natl. Acad. Sci. U.S.A.* 102, 4288–4293
53. Nakanishi-Matsui, M., Kashiwagi, S., Hosokawa, H., Cipriano, D. J., Dunn, S. D., Wada, Y., and Futai, M. (2006) *J. Biol. Chem.* 281, 4126–4131
54. Cipriano, D. J., and Dunn, S. D. (2006) *J. Biol. Chem.* 281, 501–507
55. Rondelez, Y., Tresset, G., Nakashima, T., Kato-Yamada, Y., Fujita, H., Takeuchi, S., and Noji, H. (2005) *Nature* 433, 773–777
56. Iino, R., Rondelez, Y., Yoshida, M., and Noji, H. (2005) *J. Bioenerg. Biomembr.* 37, 451–454