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$F_oF_\textsubscript{1}$-ATPase/synthase is geared to the synthesis mode by conformational rearrangement of $\epsilon$ subunit in response to proton motive force and ADP/ATP balance.

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Running title: Two states of $\epsilon$ subunit in ATP synthase

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SUMMARY

The ε subunit in FₐF₁-ATPase/synthase undergoes drastic conformational rearrangement, which involves transition of C-terminal two helices between a hairpin “down”-state and an extended “up”-state, and the enzyme with the up-fixed ε cannot catalyze ATP hydrolysis but can ATP synthesis [Tsunoda, S. P., et al. (2001) Proc. Natl. Acad. Sci. USA 98, 6560-6564]. Here, using cross-linking between introduced cysteine residues as a probe, we have investigated the causes of the transition. Findings are follows. (1) In the up-state, the two helices of ε are fully extended to insert the C-terminus into a deeper position in the central cavity of F₁ than was thought previously. (2) Without nucleotide, ε is in the up-state. ATP induces the transition to the down-state and ADP counteracts the action of ATP. (3) Conversely, the enzyme with the down-state ε can bind an ATP analogue, 2',3'O-(2,4,6-trinitrophenyl)-ATP, much faster than the enzyme with the up-state ε. (4) Proton motive force stabilizes the up-state. Thus, responding to the increase of proton motive force and ADP, FₐF₁-ATPase/synthase would transform the ε subunit into the up-state conformation and change gear to the mode for ATP synthesis.
\( \text{F}_o\text{F}_1 \)-ATPase/synthase (\( \text{F}_o\text{F}_1 \)) catalyzes ATP synthesis/hydrolysis coupled with a transmembrane \( \text{H}^+ \)-(proton)-translocation in bacteria, chloroplasts and mitochondria (1-5). The enzyme is composed of two portions, a water-soluble \( \text{F}_1 \), which has catalytic sites for ATP synthesis/hydrolysis, and a membrane-integrated \( \text{F}_o \), which mediates proton translocation. The bacterial enzyme has the simplest subunit structure, \( \alpha\beta_3\gamma_1\delta_1\epsilon_1 \) for \( \text{F}_1 \) and \( a_1b_2c_{10,11} \) for \( \text{F}_o \). \( \text{F}_1 \) is reversibly detached from \( \text{F}_o \) and is by itself a rotary motor driven by ATP hydrolysis (6-8), in which a central stalk made of \( \gamma \) and \( \epsilon \) subunits rotates relative to the surrounding \( \alpha\beta_3 \) hexamer ring where hydrolysis occurs (9,10). Remaining \( \text{F}_o \) portion in the membrane acts as a proton channel that mediates passive proton translocation across membrane (11).

The \( \epsilon \) subunit is known as an endogenous inhibitor of ATPase activity of \( \text{F}_1 \) and \( \text{F}_o\text{F}_1 \) (12,13). Structures of the isolated \( \epsilon \) from \textit{Escherichia coli} determined by X-ray crystallography (14) and NMR spectroscopy (15,16) show that \( \epsilon \) consists of two distinct domains; a C-terminal helical hairpin domain of about 50 residues lies on an N-terminal 10-stranded \( \beta \) sandwich domain of about 80 residues (Fig. 1A). The \( \delta \) subunit (equivalent to bacterial \( \epsilon \) subunit) in the crystal structure of bovine mitochondrial \( \text{F}_1 \) has also two-domain conformation that is very similar to that of the isolated bacterial \( \epsilon \), and is associated with the “bottom” globular part of the \( \gamma \) subunit (we refer this conformational state of \( \epsilon \) as “down”-state hereafter) (Fig. 1B) (17). However, the down-state \( \epsilon \) does not exhibit inhibitory effect on ATPase activity because when down-state conformation is locked by cross-linking between the two domains, the inhibitory effect of \( \epsilon \) is lost and apparent activation of ATP hydrolysis is observed (18,19). Actually, in the structure of mitochondrial \( \text{F}_1 \), the \( \delta \) subunit does not have any contact with the \( \alpha\beta_3 \) (Fig. 1B). Another conformation of \( \epsilon \) was suggested from observations that the residue (\( \epsilon \)S108, \textit{E. coli} numbering) in the C-terminal domain of \textit{E. coli} \( \epsilon \) subunit have interactions with the residues (\( \beta \)E381) in the “DELSEED” region of the \( \beta \) subunit (and homologous region of the \( \alpha \) subunit) (9,20-22). Also, it was shown that positive residues in the C-terminal domain of the \( \epsilon \) subunit of \( \text{F}_1 \) from thermophilic \textit{Bacillus} PS3 would make electrostatic interaction with the “DELSEED” region of the \( \beta \) subunit (23). Dynamic and flexible nature of the \( \epsilon \) subunit has been also reported for chloroplast \( \text{F}_o\text{F}_1 \) (24). In accordance with these biochemical results, a new conformation of \( \epsilon \) was found in the crystal structure of the complex of truncated-\( \gamma (\gamma') \) and \( \epsilon \) of \textit{E. coli} \( \text{F}_1 \) (Fig. 1C) (25). In this \( \gamma'\epsilon \) complex, a helical hairpin in the previous structures of \( \epsilon \) is opened and the helices are lifted up. Such a
location of the ε subunit could be an obstacle for the rotation of γ subunit and, indeed, FₐF₁ with the ε locked to this lifted-up conformation by γ-ε cross-linking did not show ATP hydrolysis activity. Interestingly, however, the activity of ATP synthesis of this cross-linked enzyme was fully retained (26). Thus, it has been established that ε can adopt at least two conformational states; “down”-state in which C-terminal helices form a hairpin, and “up”-state in which the helices are extended. Only the ε subunit in the up-state can exert inhibitory effect on ATPase activity.

Although the importance of conformational transition of ε has been thus recognized, critical questions on this transition remain unanswered. (i) What is the actual up-state conformation of ε in native FₐF₁? The present knowledge on the up-state conformation of ε is largely based on the crystal structure of the γ'ε complex. However, it is obvious that truncated γ' imposes artificial constraint on the conformation of ε (as well as γ) in the γ'ε structure. Indeed, if the extreme C-terminal helix of ε were to have the same conformation as in the γ'ε, it would clash sterically with the closest β subunit. In addition, in the model reconstituted from α₃β₃γ part of mitochondrial F₁ structure and the γ'ε structure, εS108 in the γ'ε is apparently too far from βE381 to account for efficient cross-linking (Fig. 1C). In 4.4-Å resolution electron density map of *E. coli* F₁, the first α helix of ε subunit in the extended conformation was barely seen as continuous density but the second α helix was unable to be traced (27). Therefore, the conformation and arrangement of the up-state ε in intact FₐF₁ is yet unclear. (ii) What is the effect of ATP and ADP on the conformational transition of ε in FₐF₁? In *E. coli* F₁, depending on whether added nucleotide is ATP or ADP, the same residue of the ε (E108C) changes the cross-linking partner subunit; ε-α in Mg²⁺+ATP state (in the presence of MgCl₂+5'-adenylyl-β,γ-imidodiphosphate) and ε-β in Mg²⁺+ADP state (20,28). However, the individual roles of ATP and ADP were not obvious for F₁ from thermophilic *Bacillus* PS3 in our previous paper (19). The distinct role of ATP and ADP in the conformational transition of the ε must be clarified. (iii) Does the enzyme with the up-state ε and the enzyme with down-state ε have different affinity to ATP and ADP? If ATP and ADP have the different effect on the conformational transition of ε, binding affinity to ATP and ADP, conversely, might be different between the enzyme with the up-state ε and the enzyme with the down-state ε. (iv) Does the proton motive force affect the transition of ε? Because the enzyme with the up-state ε can apparently catalyze ATP synthesis but not ATP hydrolysis, the enzyme with the up-state ε can be regarded as the enzyme species geared to the ATP synthesis mode. If so, it is natural to expect that
proton motive force would facilitate the down-to-up transition of the ε subunit. To address these questions, we generated a new set of mutant F\textsubscript{o}F\textsubscript{1} from thermophilic Bacillus PS3\textsuperscript{1} that enabled us to detect and fix the down- and up-states of ε in the working enzyme.

**EXPERIMENTAL PROCEDURES**

*Preparation of the enzymes* — Plasmids for three mutant F\textsubscript{o}F\textsubscript{1}, γε\textsubscript{-}-F\textsubscript{o}F\textsubscript{1} (γSer3Cys, εCys134), γε\textsubscript{-}-F\textsubscript{o}F\textsubscript{1} (γSer3Cys, εAla85Cys, εCys134), and ε\textsubscript{cc}-F\textsubscript{o}F\textsubscript{1} (εAla85Cys, εCys134), were constructed from the plasmid pTR19-ASDS (29) by the Mega-primer method (30). Sequences of the regions amplified by PCR were verified by nucleotide sequencing. These plasmids were used for transformation of an *E. coli* strain DK8 [bgIR, thi-1, rel-1, HfrP01, Δ(uncB-uncC), ilv::Tn10] that lacked whole F\textsubscript{o}F\textsubscript{1} genes. F\textsubscript{1} (α\textsubscript{3}β\textsubscript{3}γδε complex) of thermophilic Bacillus Strain PS3 were purified as follows. *E. coli* cells (DK8/pTR19-ASDS) expressing thermophilic F\textsubscript{o}F\textsubscript{1} were disrupted in PA3-buffer (10 mM HEPES/KOH, pH 7.5, 5 mM MgCl\textsubscript{2}, 10 % glycerol) (29), and cytosol fraction was obtained by removing a membrane fraction with centrifugation (150 k×g for 20 min). The supernatant was incubated at 67 °C for 15 min, and aggregated *E. coli* proteins were removed by centrifugation (150 k×g for 20 min). An yellow supernatant was supplemented with 2 volumes of 20 mM KPi buffer, pH 7.5, containing 100 mM KCl and 50 mM imidazole, and applied on a Ni-NTA superfllow column (Qiagen, Germany) equilibrated with the same buffer. After washing the column with 10 volumes of the buffer, F\textsubscript{1} was eluted with 20 mM KPi buffer, pH 7.5, containing 100 mM KCl and 200 mM imidazole, and DTT (final concentration, 50 mM) was added to the eluted fraction. After incubation for 60 min at 25 °C, ammonium sulfate was added to the solution (final concentration, 1 M) and the solution was applied to a Phenyl-TOYOPEARL 650M column (TOSOH, Japan) equilibrated with 20 mM KPi buffer, pH 7.5, containing 0.5 mM EDTA and 1 M ammonium sulfate. The column was washed with 20 volumes of 100 mM KPi buffer, pH 7.5, containing 4 mM EDTA and 1 M ammonium sulfate to remove endogenously bound nucleotides, and a linear reverse gradient of ammonium sulfate (1-0 M) was applied. Fractions containing F\textsubscript{1} were collected, precipitated with ammonium sulfate, and further purified with a Superdex 200HR column (Pharmacia, Sweden) in 20 mM HEPES/KOH buffer, pH 7.5, containing 100 mM KCl. The purified protein was frozen with liquid N\textsubscript{2} and stored at –80 °C until use. The
purified $\gamma_c\varepsilon_cF_1$, $\gamma_c\varepsilon_ccF_1$ and $\varepsilon_ccF_1$ contained 0.096±0.01, 0.18±0.02, and 0.18±0.02 mol ADP per mol $F_1$, respectively.

**Assays of membrane vesicles** ---- Inverted membrane vesicles from *E. coli* cells expressing thermophilic $F_oF_1$ were prepared by the procedures described previously (29) except for a modification that 5 mM DTT was supplemented to the cell extract just after disruption of the cells. Thermophilic $F_oF_1$ used in this work has a histidine-tag of 10 residues at N-terminus of the $\beta$ subunit. Prior to use, the membrane vesicles were washed twice with PA3-buffer to remove DTT. ATPase activities of the membrane vesicles containing the mutant $F_oF_1$’s were inactivated by $N,N'$-dicyclohexylcarbodiimide down to < 20 % of the initial activities, which were almost the same as the case of the wild-type (15-20 %). ATP-driven proton pump activity of membrane vesicles was assayed with fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine at 40 °C in PA4-buffer (10 mM HEPES/KOH, pH 7.5, 100 mM KCl, 5 mM MgCl$_2$) as described previously (29). The reaction was started by addition of 1 mM ATP and terminated by addition of 1 $\mu$M carbonyl cyanide $p$-trifluoromethoxyphenylhydrazone (FCCP). ATP synthesis activity was measured at 50 °C in PA4-buffer containing 1 mM ADP, 25 mM KPi, pH 7.5, and membrane vesicles (4.5 $\mu$g protein/ml). Oxidized and reduced membrane vesicles were prepared by treating with 20 $\mu$M CuCl$_2$ for 30 min and with 10 mM DTT for 30 min, respectively. EDTA (final concentration, 1 mM) was added to the oxidized vesicle solution prior to the assay of ATP synthesis to chelate free Cu$^{2+}$. EDTA was also added to the reduced vesicle solution to adjust the conditions. After a 5 min-preincubation, the reaction was initiated by adding 5 mM NADH and terminated at 2, 4, 6, 8, 10 and 12 min by adding 2.5 % trichloroacetic acid. The solution was neutralized to pH 7.7 with 0.25 M Tris/acetate (pH 9.5) and the amount of synthesized ATP was determined with ATP Bioluminescence Assay Kit CLSII (Roche, Germany).

**Other assays** ---- ATPase activity was monitored in triplicate in 50 mM HEPES/KOH, pH 7.5, containing 100 mM KCl, 5 mM MgCl$_2$, and 3 mM ATP with an ATP-regenerating system (31) and average hydrolysis rates in a time period from 3 to 6 min after initiation of the reactions at 40 °C were measured. The activity that hydrolyzed one $\mu$mole of ATP per min was defined as one unit. $2',3'-O$-(2,4,6-trinitrophenyl)-ATP (TNP-ATP) and $2',3'-O$-(2,4,6-trinitrophenyl)-ADP (TNP-ADP) were purchased from Molecular Probe (USA). Fluorescence change induced by the binding of TNP-nucleotide to the enzyme was monitored in a Spectrofluorometer model FP-6500 (JASCO, Japan) as performed.
RESULTS

Mutants ---- We generated three mutants. To obtain the enzyme with the up-fixed ε by cross-linking between γ and ε, one cysteine residue was introduced into N-terminal region of γ subunit (γS3C) and another was added to the C-terminal end of ε subunit (εC134) (Fig. 1D). To obtain the enzyme with the down-fixed ε by cross-linking two domains within ε, a mutant that had εA85C and εC134 was used (Fig. 1E). To assess the relative population of enzymes with up- and down-state ε under various conditions, three mutations, γS3C, εA85C and εC134 were introduced. The εA85 is located in a region between two domains of ε, and abuts on εC134 (Cα-distance is 9.0 Å) when the C-terminal domain adopts a hairpin structure (14). Therefore, ε134C are expected to make the cross-link with γS3C when the ε is in the up-state or with εA85C when the ε is in the down-state (Fig. 1F). These mutations were termed γεc, εcc, and γεcc, respectively. The enzymes have one endogenous cysteine residue in F,o subunit. This cysteine is buried inside of the transmembrane region and do not respond to the CuCl₂ and 5,5′-dithiobis-(2-nitro)benzoic acid (DTNB) treatment employed in this report. The enzymes containing γεc, εcc and γεcc was as active as the wild-type enzyme in their reduced forms (Table I). Also ATPase activities of the inverted membrane vesicles prepared from the cells expressing the wild-type and mutant F,F₁’s were similar each other under the reducing conditions. SDS-PAGE analysis of the membrane vesicles showed almost identical band patterns for the three mutants and the wild-type (not shown). Therefore, the amounts of expressed mutant F,F₁ in the inverted membranes are similar to that of the wild-type F,F₁.

Cross-linking of ε in the up-state ---- In the previous experiments using E. coli F,F₁ (26), cysteine residues were introduced at positions γ99 and ε118 (E. coli numbering) to fix the conformation of ε in the up-state by a γ-ε cross-link. These positions were chosen based on the crystal structure of the γ'ε complex, in which γ99 is located in the globular domain of γ' and ε118 is in the extreme C-terminal helix of ε. In this structure, two helices do not fully extend but rather entwine globular domain of γ' (Fig. 1C). Expecting that helices of the up-state ε in the native enzyme could extend more straight, we introduced cysteine
residues to a near N-terminal position of γ (γS3), atop the central helical coiled-coil of the γ subunit, and to C-terminus of ε (ε134) (Fig. 1D). The membrane vesicles of E. coli expressing γεc-FoF1 were oxidized in 20 µM CuCl2 for 20 min at 25 °C and analyzed with non-reducing SDS-PAGE (Fig. 2A). Compared with a control γεc-FoF1 which was treated with 50 mM DTT prior to electrophoresis (lane 1), a new band appeared just below the band of β subunit (lane 4, indicated by an arrow). Peptide sequencing of this band gave two kinds of amino-acid sequences corresponding to the N-terminus sequences of γ and ε, indicating that this band is a cross-link product of these two subunits. Consistently, band intensities of γ and ε decreased. The same γ-ε cross-link product was also readily generated in the purified γεc-F1 under the same oxidizing conditions (lane 5). Cross-linking yields in the F1 and FoF1 were estimated from the band intensities to be 80-85 %. It is worth noting that γ-ε cross-link was generated spontaneously in ~40 % of F1 during the purification (2-days) that was carried out without DTT and EDTA. Also, ~60 % of FoF1 in membrane vesicles were spontaneously oxidized during preparation. The efficient cross-linking between γS3C and εC134 suggests their proximal location in the up-state conformation of ε in F1 and FoF1, and that the C-terminal helix of ε inserts itself deep into the central cavity of the α3β3. Because the isolated γεc-F1 used in the above experiments was mostly free from endogenous nucleotide, the ε mostly adopts the up-state in the absence of bound nucleotide.  

Activities of F1 and FoF1 with the up-fixed ε ---- The ATPase activity of γεc-F1 was severely inhibited by oxidation, residual activity of only 21 % of that of the reduced γεc-F1, while the ATPase activity of the wild-type F1 was hardly affected by whether it was oxidized or reduced (Fig. 2B, left panel). The degree of inhibition by oxidation (79 %) agreed well with the yield of cross-link by oxidation (81%). Similarly, the ATPase activity of the γεc-FoF1 contained in the vesicles was inhibited (77 %) in proportion to the yield of cross-linking (80 %) (Fig. 2B, right panel). Since ATP hydrolysis was blocked, oxidized γεc-FoF1 was unable to mediate ATP-driven proton translocation while reduced γεc-FoF1 was fully capable of it (Fig. 2C). ATP synthesis activities of membrane vesicles containing reduced or oxidized γεc-FoF1 were also measured. Membrane vesicles containing the wild-type and γεc-FoF1 treated with DTT catalyzed ATP synthesis at 44.5±2.8 and 34.7±2.4 nmol ATP/min/mg membrane protein, respectively. Oxidized vesicles showed 71 % (wild-type) and 75 % (γεc-FoF1) of the ATP synthesis activity of the vesicles treated with DTT (Fig. 2D). Thus, ATP synthesis activity was retained after the formation of the γ-ε cross-
link to lock the $\varepsilon$ in the up-state. These results are consistent with the previous reports, demonstrating remarkable asymmetric inhibition by the up-state $\varepsilon$ toward ATP hydrolysis (18,26,33).

**Effect of ATP and ADP on the conformational state of $\varepsilon$**

To assess the distribution of the $\varepsilon$ either in the up-state or down-state, $\gamma\varepsilon_{cc}-F_1$ and $\gamma\varepsilon_{cc}-F_0F_1$ were used. With oxidation procedures, the down-state $\varepsilon$ can be detected as a band corresponding to an internally cross-linked $\varepsilon$ ($\varepsilon$A85C and $\varepsilon$134C) and the up-state $\varepsilon$ as a $\gamma$-$\varepsilon$ band ($\gamma$S3C and $\varepsilon$C134). The $\varepsilon$ subunit behaved very similarly in $\gamma\varepsilon_{cc}-F_1$ and in $\gamma\varepsilon_{cc}-F_0F_1$ (Fig. 3A, 3B). In the absence of nucleotide, the $\varepsilon$ in $F_1$ and in $F_0F_1$ was mostly in the up-state (Fig. 3A, 3B, lane 2’s) and the up-state conformation was stabilized when 3 mM ADP was present (lane 3’s). Further addition of 5 mM Pi caused no significant change (not shown). However, the $\gamma$-$\varepsilon$ band disappeared and the internally cross-linked $\varepsilon$ band (arrowheads) appeared when ADP was converted into ATP by pyruvate kinase (lane 4’s). Also, the internally cross-linked $\varepsilon$ band appeared when ATP was added from the beginning (lane 5’s). Addition of hexokinase and glucose to the sample of lane 5 resulted in appearance of the $\gamma$-$\varepsilon$ band (lane 6’s). Thus, it is clear that the $\varepsilon$ subunit in $F_1$ and $F_0F_1$ adopts reversibly the up-state conformation in the presence of ADP and the down-state in the presence of ATP. As shown previously (19), hydrolysis of ATP is not necessary to stabilize the down-state $\varepsilon$ because 3 mM AMP-PNP also stabilized the down-state conformation of $\varepsilon$ (not shown).

**TNP-AT(D)P binding to $F_1$ with up- or down-state $\varepsilon$**

It has been known that a nucleotide analogue, TNP-AT(D)P, increases its fluorescence upon binding to $F_1$ (32). Taking advantage of this, we compared initial kinetics of nucleotide binding to the enzymes that contained the up- or down-state $\varepsilon$. To measure the binding to the nucleotide binding site with the highest affinity, substoichiometric amount of TNP-AT(D)P was mixed with $\gamma\varepsilon_{cc}-F_1$ or $\varepsilon_{cc}-F_1$ and fluorescence changes were monitored. Time courses of TNP-ADP binding were almost the same for $\gamma\varepsilon_{cc}-F_1$ and $\varepsilon_{cc}-F_1$ irrespective of whether they were reduced or oxidized (Fig. 4A, 4B). The wild-type $F_1$, with or without oxidizing treatment, also showed the same kinetics of TNP-ADP binding (not shown). These results indicated that TNP-ADP binding to $F_1$ was not affected by the conformational states of the $\varepsilon$ subunit. Time course of TNP-ATP binding to reduced $\gamma\varepsilon_{cc}-F_1$ was also the same as that of $\varepsilon_{cc}-F_1$ (Fig. 4C) and wild-type $F_1$ (not shown), ensuring no significant effect of the introduced cysteines on TNP-ATP binding kinetics of $F_1$. The time course of TNP-ATP binding to the oxidized $\gamma\varepsilon_{cc}-F_1$ (Fig. 4D, lower curve) was similar to that of TNP-ADP binding to the oxidized $\gamma\varepsilon_{cc}$-
F₁, indicating that TNP-ATP and TNP-ADP bind to the same site of F₁ with the up-fixed ε. The oxidized εcc-F₁, on the contrary, bound TNP-ATP much faster and fluorescence reaches higher magnitude than the oxidized γεc-F₁. (Fig. 4D, upper curve). Thus, F₁ with the down-state ε binds TNP-ATP quickly while F₁ with the up-state ε binds it slowly. Accordingly, results of TNP-ATP binding to the reduced γεc-F₁ and εcc-F₁ in Fig. 4C are well interpreted as a mixture of F₁’s with the up- and down-state ε.

Effect of proton motive force on the state of ε --- The inverted membrane vesicles containing γεcc·F₀F₁ were incubated for 3 min in the varying amounts of ATP and ADP and conformational states of the ε subunit were analyzed with non-reducing SDS-PAGE after fixing the conformation by cross-linking (Fig. 5A, lanes 1-6). As ATP increased and ADP decreased, intensity of the γ-ε band decreased as is expected from the results above mentioned. However, when the incubation was continued for another 5 min after addition of NADH to impose proton motive force, intensity of the γ-ε band did not significantly decrease even at high ATP concentrations (Fig. 5A, lanes 7-12). When FCCP, an uncoupler that dissipates proton motive force, was added in addition to NADH, the intensity of the γ-ε band was decreased as ATP increased, similar to lanes 1-6 (lanes 13-18). These results suggest that when proton motive force is provided the ε subunit in F₀F₁ strongly favors the up-state conformation irrespective of ADP/ATP balance. In other words, proton motive force counteracts the effect of ATP in the conformational transition of the ε subunit.

DISCUSSION

C-terminus of the ε subunit reaches the center of F₁. --- The questions listed in the INTRODUCTION were mostly answered by the present study. Concerning the question (i), it becomes evident that C-terminus of ε in the up-state is located near the N-terminus of the γ subunit. To reach this position, referring to the structure of mitochondrial F₁, the C-terminal helices of ε have to extend ~70 Å from the exit (εA85) of the N-terminal β sandwich domain. Considering the length of α-helix per residue (1.5 Å/residue) (34), a peptide stretch of 48 residues from εA85 to εK133 can extend by 72 Å as an α-helix or longer as two helices with a connecting segment. Previous cross-linking results of E. coli F₁ between βE381 and εS108 (9,20-22) are explained by this new arrangement rather than by the γε structure (compare Fig. 1C and 1D) (25). Probably, the γε structure represents an
intermediate conformation that appears during the transition of ε from the down-state to the up-state. Our study suggests that three helices, coiled coil of the γ subunit and C-terminal helix of the ε subunit, rather than two as previously thought, rotate as a body within the α₃β₃ ring when F₀F₁ with the up-state ε is synthesizing ATP.

*ATP and ADP have opposite effect on the conformational states of the ε subunit.*---- As to question (ii), it is now clear that the ε subunit, either in F₁ or F₀F₁, is in the up-state conformation in the absence of nucleotide or in the presence of ADP and it is in the down-state conformation in the presence of ATP. Thus, ATP and ADP counteract each other (Fig. 6). Reciprocally, an ATP analogue, TNP-ATP binds to F₁ with down-state ε quickly but to F₁ with up-state ε slowly. An ADP analogue, TNP-ADP does not show binding preference between F₁’s with up- and down-state ε. Therefore, if TNP-AT(D)P mimics the AT(D)P correctly in binding to F₁, answer to question (iii) will be that F₁ with down-state ε indeed prefer ATP to ADP while F₁ with up-state ε binds both ATP and ADP in the same slow kinetics. The results are consistent with the previous observation that the α₃β₃γ complex binds TNP-ATP quickly but the reconstituted α₃β₃ε complex does slowly (32), because without previous exposure to nucleotide, the ε subunit in the reconstituted α₃β₃ε must be in the up-state.

*The ε subunit transits between two states depending on proton motive force and ADP/ATP.*---- This study has revealed that proton motive force counteracts the effect of ATP by stabilizing the up-state ε (answer to question (iv)). Therefore, the two conformational states of ε in F₀F₁ are alternated by two factors; proton motive force and ADP/ATP balance (Fig. 6). At high proton motive force and low ATP, ε is predominantly in the up-state and F₀F₁ is geared to the ATP synthesis mode. At low proton motive force and high ATP, ε adopts the down-state and F₀F₁ hydrolyzes ATP to pump out protons, generating proton motive force with enough magnitude to drive uptake of nutrients and flagella motion.

*Role of C-terminal helices of the ε subunit* ---- In some bacteria, such as *Chlorobium limicola* (35) and *Thermotoga neapolitana* (36), the native ε subunit lacks the C-terminal helical domain. Without C-terminal helical domain, the ε subunit cannot adopt the up-state arrangement and should be always in the state that is functionally similar to the down-state. These bacteria grow in anaerobic environments and F₀F₁ should work as an ATP hydrolysis-driven proton pump. Since the F₀F₁ with up-state ε is unable to mediate ATP hydrolysis-driven proton pumping, these bacteria do not need, or even had better delete, the
C-terminal domain of the ε subunit. F₀F₁ with down-state ε can catalyze both ATP synthesis and ATP hydrolysis (26). Therefore, it is not surprising that a mutant E. coli F₀F₁ containing the ε subunit with deleted C-terminal helical domain or with an artificially fused protein at C-terminus can support aerobic growth by oxidative phosphorylation (37,38). A similar observation was reported recently for chloroplast F₀F₁ (33). Then, a critical question should be asked: what is the essential function of the F₀F₁ whose ε subunit is in the up-state? Probably, the F₀F₁ with the up-state ε plays an important role under starving conditions rather than rich nutritional environments. In E. coli cells, total concentration of cellular adenine nucleotides is maintained to be about 3 mM (39) but the fraction of ATP in total adenine nucleotide pool varies from 3 to 0.3 mM in parallel with growth rate (40-42) through ribosome synthesis (43) and transcription (44). As ATP concentration decreases from 3 to 0.3 mM in the absence of proton motive force, population of the F₀F₁ with up-state ε increases about 3-fold (Fig. 5B) so that hydrolysis of the precious ATP by F₀F₁ is suppressed. For any organisms, regulation of ATP synthesis/hydrolysis to meet physiological demand in quickly changing nutritional conditions is a critical matter and conformational transition of the ε subunit in F₀F₁ might constitute a part of an elaborately integrated regulatory system that awaits further study.

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REFERENCES

1. Boyer, P. D. (1997) *Annu. Rev. Biochem.* **66**, 717-749
2. Yoshida, M., Muneyuki, E., and Hisabori, T. (2001) *Nat. Rev. Mol. Cell. Biol.* **2**, 669-677
3. Pedersen, P. L. (2002) *J. Bioenerg. Biomemb.* **34**, 327-332
4. Capaldi, R. A., and Aggerler, R. (2002) *TIBS* **27**, 154-160
5. Senior, A. E., Nandanaciva, S., and Weber, J. (2002) *Biochim. Biophys. Acta* **1553**, 188-211
6. Duncan, T. M., Bulygin, V. V., Zhou, Y., Hutcheon, M. L., and Cross, R. L. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 10964-10968
7. Noji, H., Yasuda, R., Yoshida, M., and Kinosita, K. Jr. (1997) *Nature* **386**, 299-302
8. Ren, H., and Allison, W. S. (2000) *Biochim. Biophys. Acta* **1458**, 221-233
9. Aggerler, R., Ogilvie, I., and Capaldi, R. A. (1997) *J. Biol. Chem.* **272**, 19621-19624
10. Schulenberg, B., Wellmer, F., Lill, H., Junge, W., and Engelbrecht, S. (1997) *Eur. J. Biochem.* **249**, 134-141
11. Fillingame, R. H., and Dmitriev, O. Y. (2002) *Biochim. Biophys. Acta* **1565**, 232-245
12. Laget, P. P., and Smith, J. B. (1979) *Arch. Biochem. Biophys.* **197**, 83-89
13. Sternweis, P. C., and Smith, J. B. (1980) *Biochemistry* **19**, 526-531
14. Uhlin, U., Cox, G. B., and Guss, J. M. (1997) *Structure* **5**, 1219-1230
15. Wilkens, S., Dahlquist, F. W., McIntosh, L. P., Donaldson, L. W., and Capaldi, R. A. (1995) *Nat. Struct. Biol.* **2**, 961-967
16. Wilkens, S., and Capaldi, R. A. (1998) *J. Biol. Chem.* **273**, 26645-26651
17. Gibbons, C., Montgomery, M. G., Leslie, A. G. W., and Walker, J. E. (2000) *Nat. Struct. Biol.* **7**, 1055-1061
18. Schulenberg, B., and Capaldi, R. A. (1999) *J. Biol. Chem.* **274**, 28351-28355
19. Kato-Yamada, Y., Yoshida, M., and Hisabori, T. (2000) *J. Biol. Chem.* **275**, 35746-35750
20. Aggerler, R., and Capaldi, R. A. (1996) *J. Biol. Chem.* **271**, 13888-13891
21. Aggerler, R., Chicas-Cruz, K., Cai, S. X., Keana, J. F. W., and Capaldi, R. A. (1992) *Biochemistry* **31**, 312956-312961
22. Grüber, G., and Capaldi, R. A. (1996) *J. Biol. Chem.* **271**, 32623-32628
23. Hara, K. Y., Kato-Yamada, Y., Kikuchi, Y., Hisabori, T., and Yoshida, M. (2001) J. Biol. Chem. 276, 23969-23973
24. Komatsu-Takaki, M. (1993) Eur. J. Biochem. 214, 587-591
25. Rodgers, A. J., and Wilce, M. C. (2000) Nat. Struct. Biol. 7, 1051-1054
26. Tsunoda, S. P., Rodgers, A. J. W., Aggeler, R., Wilce, M. C. J., Yoshida, M., and Capaldi, R. A. (2001) Proc. Natl. Acad. Sci. USA 98, 6560-6564
27. Hausrath, A. C., Capaldi, R. A., and Matthews, B. W. (2001) J. Biol. Chem. 276, 47227-47232
28. Aggeler, R., Haughton, M. A., and Capaldi, R. A. (1995) J. Biol. Chem. 270, 9185-9191
29. Suzuki, T., Ueno, H., Mitome, N., Suzuki, J., and Yoshida, M. (2002) J. Biol. Chem. 277, 13281-13285
30. Landt, O., Grunert, H. P., and Hahn, U. (1990) Gene 96, 125-128
31. Suzuki, T., Suzuki, J., Mitome, N., Ueno, H., and Yoshida, M. (2000) J. Biol. Chem. 275, 37902-37906
32. Kato, Y., Matsui, T., Tanaka, N., Muneyuki, E., Hisabori, T., and Yoshida, M. (1997) J. Biol. Chem. 272, 24906-24912
33. Nowak, K. F., Tabidze, V., and McCarty, R. E. (2002) Biochemistry 41, 15130-15134
34. Dunn, S. D., McLachlin, D. T., and Revington, M. (2000) Biochim. Biophys. Acta 1458, 356-363
35. Xie, D., Lill, H., Hauska, G., Maeda, M., Futai, M., and Nelson, N. (1993) Biochim. Biophys. Acta 1172, 267-273
36. Iida, T., Inatomi, K., Kamagata, Y., and Maruyama, T. (2002) Extremophiles 6, 369-375
37. Kuki, M., Noumi, T., Maeda, M., Amemura, A., and Futai, M. (1988) J. Biol. Chem. 263, 17437-17442
38. Daniel, J., Cipriano, D. J., Bi, Y., and Dunn, S. D. (2002) J. Biol. Chem. 277, 16782-16790
39. Neuhard, J., and Nygaard, P. (1987) in Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology (Neidhardt, F. C., ed), 1 Ed., pp. 445-73, American Society for Microbiology, Washington, DC
40. Franzen, J. S., and Binkley, S. B. (1961) J. Biol. Chem. 236, 515-519
41. Smith, R. C., and Maaløe, O. (1964) *Biochim. Biophys. Acta* **86**, 229-234
42. Bagnara, A. S., and Finch, L. R. (1973) *Eur. J. Biochem.* **36**, 422-427
43. Gaal, T., Bartlett, M. S., Ross, W., Turnbough, C. L. Jr., and Gourse, R. L. (1997) *Science* **278**, 2092-2097
44. Alper, S., Dufour, A., Garsin, D. A., Duncan, L., and Losick, R. (1996) *J. Mol. Biol.* **260**, 165-177
45. Koradi, R., Billerter, M., and Wuthrich, K. (1996) *J. Mol. Graphics* **14**, 51-55
FOOTNOTES

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1 Abbreviation used are: $F_oF_1$ and $F_1$, we use these terms as the general names of $F_oF_1$-ATPase/synthase and $F_1$-ATPase but also as particular $F_oF_1$ and $F_1$ from thermophilic Bacillus PS3 for simplicity in this article as far as the context is clear; DTNB, 5,5′-dithiobis-(2-nitro)benzoic acid; FCCP, carbonyl cyanide $p$-trifluoromethoxyphenylhydrazone; non-reducing SDS-PAGE, SDS-PAGE without prior reducing treatment; TNP-AT(D)P, 2′,3′-$O$-(2,4,6-trinitrophenyl)-AT(D)P.

2 We previously reported that ADP, though less effective than ATP, induced the down-state conformation of the $\epsilon$ subunit in the $\alpha_3\beta_3\gamma\epsilon$ complex (19). When we used ADP pretreated with hexokinase and glucose in the experiment, the down-state conformation was not detected. Therefore, the contaminated ATP in the commercial ADP might be the reason of previous result.

3 Very recently, we have succeeded in determining crystal structure of an $\alpha_3\beta_3\gamma\epsilon$ complex of thermophilic $F_1$ (unpublished results by Y.S., M.Y. and T.S.). In the structure, C-terminal helices of the $\epsilon$ subunit indeed extend straight and the C-terminus of the $\epsilon$ subunit is close to the N-terminus of the $\gamma$ subunit.
FIGURE LEGENDS

FIG. 1. Conformations of the ε subunit. A. Crystal structure of the isolated E. coli ε subunit (16). N-terminal and C-terminal domains were respectively shown with green and red/yellow colors. B. Crystal structure of the down-state conformation of δ subunit (equivalent to the ε subunit in bacterial F₁) observed in bovine mitochondrial F₁ (17). Only subunits of βTP, γ, δ and ε (no equivalent subunit in bacterial F₁), are depicted in the figure. A loop which contains DELSEED sequence was colored with purple. C. Crystal structure of γε complex of E. coli F₁ (25) superimposed with βTP of bovine F₁. Blue spheres indicate βE395 residue of DELSEED region (second Glu residue). Cα-distance between βE381 and εS108 is 24-27 Å, too far to be cross-linked. D, E, F. Schematic diagrams of cross-link formation in γεc-F₁ (D), εcc-F₁ (E), γεcc-F₁ (F). In the up-state, εC134-γS3C is to be cross-linked. In the down state, εC134-εA85C is to be cross-linked. These figures were prepared by using a program package, MOLMOL (45).

FIG. 2. Cross-link between N-terminus of the γ subunit and C-terminus of the ε subunit. A. non-reducing SDS-PAGE analysis. Lanes 1 and 4, membrane vesicles containing γεc-F₀F₁; lanes 2 and 5, isolated γεc-F₁; lane 3, isolated wild-type F₀F₁. All samples except for lane 3 were treated with 20 µM CuCl₂ for 30 min at 25 °C. Then, samples of lanes 1 and 2 were reduced with 50 mM DTT for 1 h. A band of γ-ε cross-linked product appeared just below the band of β subunit. B. Effect of the γ-ε cross-linking on ATPase activities of the isolated γεc-F₁ (left panel) and membrane vesicles containing γεc-F₀F₁ (right panel). ATP hydrolysis by the reduced (white bars) and oxidized (black bars) γεc-F₁ and membrane vesicles containing γεc-F₀F₁ were assayed at 40 °C. The same procedures were applied to the wild-type F₁ and F₀F₁. C. Effect of the γ-ε cross-linking on proton pump activity. Proton pump activities of the reduced or oxidized membrane vesicles were analyzed by monitoring the fluorescence of 9-amino-6-chloro-2-methoxyacridine at 40 °C. Prior to the analysis, 1 mM EDTA was added to the solutions to remove free Cu²⁺. At the indicated times, pumping was initiated by adding 1 mM ATP and terminated by 1 µg/ml FCCP. D. Effect of the γ-ε cross-linking on ATP synthesis activity. The reactions were started by addition of 5 mM NADH to the membrane vesicle solutions containing reduced or oxidized wild-type F₀F₁ and γεc-F₀F₁. The reactions were carried out at 50 °C and the amount of generated ATP was measured with luciferase.
FIG. 3. Effect of ATP and ADP on the conformational state of the ε subunit. A. Analysis of γεcc-F$_1$. γεcc-F$_1$ was incubated with indicated components for 2 min. Concentrations of ADP (lanes 3 and 4) and ATP (lanes 5 and 6) were 3 mM. For the sample of lane 4, 125 µg/ml pyruvate kinase and 9.4 mM phosphoenolpyruvate (final concentrations) were added and incubated for 1 min. For the sample of lane 6, 9.4 units/ml hexokinase and 38 mM glucose (final concentrations) were added and incubated for 1 min. The samples were reduced with 50 mM DTT (lane 1) or oxidized with 20 µM CuCl$_2$ (lanes 2-6) for 10 min and applied to non-reducing SDS-PAGE. Lanes 1 and 2, no nucleotide. Lane 3, ADP. Lane 4, ADP followed by pyruvate kinase treatment. Lane 5, ATP. Lane 6, ATP followed by hexokinase treatment. Arrowheads in lanes 4 and 5 indicate the position of ε subunit with internal cross-link. All the reactions were carried out in 50 mM HEPES/NaOH, pH 7.5, containing 100 mM NaCl and 5 mM MgCl$_2$ at 50 °C. A distorted band above of the α subunit band in lane 4 was pyruvate kinase. B. Analysis for γεcc-F$_o$F$_1$. Membrane vesicles containing γεcc-F$_o$F$_1$ were used for the analysis. Lane 1, reduced γεcc-F$_o$F$_1$, no nucleotide. Lanes 2-6, membrane vesicles containing γεcc-F$_o$F$_1$ treated with the same procedures as A. Lane 7, γεcc-F$_1$ incubated with ATP (the same sample as A lane 5) to show the band position of the ε subunit with internal cross-link. The samples were reduced with 50 mM DTT (lane 1) or oxidized with 100 µM CuCl$_2$ (lanes 2-6) and applied to non-reducing SDS-PAGE.

FIG. 4. TNP-ATP binding to F$_1$ with up- or down-fixed ε subunit. To obtain F$_1$ with up- or down-fixed ε subunit by γε cross-linking (γεcc-F$_1$) or internal cross-linking within ε (εcc-F$_1$), purified γεcc-F$_1$ and εcc-F$_1$ in 50 mM HEPES/KOH, pH 7.5, were oxidized with 20 µM (γεcc-F$_1$) or 100 µM (εcc-F$_1$) CuCl$_2$ for 1 h at 25 °C. Reduced samples were not subjected to this oxidation procedures. TNP-AT(D)P (50 nM) was incubated at 50 °C for 5 min in 50 mM HEPES/KOH, pH 7.5, containing 100 mM KCl, 5 mM MgCl$_2$, and 20 µl of F$_1$ (6 µM) were added into the cuvette (final F$_1$ concentration, 100 nM) at the time indicated with arrows (final volume, 1.2 ml). Fluorescence change was monitored at 548 nm with an excitation light at 410 nm. Red traces, γεcc-F$_1$. Blue traces, εcc-F$_1$. Apparent rate constants for the nucleotide binding were estimated by fitting single-exponential functions to the time courses, and shown in the figure.
FIG. 5. **Effect of proton motive force on the conformational state of ε subunit in FₐFₐ.**

A. Membrane vesicles containing γεcc-FₐFₐ were incubated for 3 min with mixtures of varying amounts of ATP and ADP (ATP+ADP = 3 mM). An aliquot of each reaction mixture was transferred to another tube, treated with 200 µM DTNB for 5 min to fix the conformational state of the ε subunit by cross-linking, and subjected to non-reducing SDS-PAGE analysis (lanes 1-6). For remaining reaction mixtures, 5 mM NADH (lanes 13-18) or 5 mM NADH + 3 µg/ml FCCP (lanes 6-12) was added and incubation was continued for 5 min. Then, the reaction mixtures were treated with 200 µM DTNB for 5 min and analyzed with non-reducing SDS-PAGE. DTNB was used instead of CuCl₂ as an oxidant because DTNB is more harmless to the membrane vesicles than CuCl₂. Concentrations of ATP and ADP were 0 mM and 3 mM (lanes 1, 7, and 13), 0.3 mM and 2.7 mM (lanes 2, 8, and 14), 0.5 mM and 2.5 mM (lanes 3, 9, and 15), 1 mM and 2 mM (lanes 4, 10, and 16), 2 mM and 1 mM (lanes 5, 11, and 17), and 2.7 mM and 0.3 mM (lanes 6, 12, and 18), respectively. The experiments were carried out at 50 °C in 50 mM HEPES/NaOH, pH 7.5, containing 100 mM NaCl and 5 mM MgCl₂. It was confirmed that NADH oxidation by respiratory chains of vesicles under these conditions was as active as that at 37 °C and could generate proton motive force. In this figure, γ–ε bands formed by cross-linking are shown. B. The relative staining intensities of the γ-ε bands in A were plotted against the ATP concentrations. Closed triangles, the control samples (lanes 1-6); closed circles, NADH (lanes 7-12); open circles, NADH + FCCP (lanes 13-18).

FIG. 6. **Schematic diagram of two forms of the FₐFₐ with up-state ε (left) and down-state ε (right).** The FₐFₐ with up-state ε can catalyze ATP synthesis but cannot ATP hydrolysis (ATP synthesis mode). This form is stabilized by ADP and proton motive force. The FₐFₐ with down-state ε can catalyze ATP synthesis as well as ATP hydrolysis (proton pump/ATP synthesis mode). This form is favored when ATP is present. Transition between two forms is determined by proton motive force and ADP/ATP balance.
TOP, Fig. 1 Suzuki et al.
TOP, Fig. 4, Suzuki et al.

Fluorescence at 548 nm

TNP-ADP

TNP-ATP

Reduced

Oxidized

\[ \gamma_c \epsilon_c \]

\[ \epsilon_{cc} \]

0.0085 s\(^{-1}\)

0.0074 s\(^{-1}\)

0.030 s\(^{-1}\)

0.032 s\(^{-1}\)

0.060 s\(^{-1}\)

0.012 s\(^{-1}\)

0.0082 s\(^{-1}\)

0.0074 s\(^{-1}\)

0.030 s\(^{-1}\)

0.032 s\(^{-1}\)

Fluorescence at 548 nm
Fig. 5, Suzuki et al.

**A**

- ADP
- ATP
- +NADH
- +FCCP+NADH

**B**

- Relative band intensity of γ-e
- ATP [mM]

The graph shows the changes in relative band intensity of γ-e as a function of ATP concentration in the presence of ADP and ATP, with and without NADH and FCCP+NADH.
ATP synthesis mode

H⁺-pump/ATP-synthesis mode
Table I  ATPase activity of the wild-type and mutant 
$F_1$ and $F_oF_1$.

| Enzymes \(a\) | ATPase activity \(b\) [Units/mg/min] |
|---------------|-------------------------------------|
| Purified $F_1$ |                                     |
| wild-type     | 10.6 ± 0.1                          |
| $\gamma_c\varepsilon_c$ | 9.4 ± 0.1                          |
| $\gamma_c\varepsilon_{cc}$ | 11.3 ± 0.5                          |
| $\varepsilon_{cc}$ | 9.9 ± 0.2                           |
| $F_oF_1$ (membrane vesicles) |                     |
| wild-type     | 0.99 ± 0.02                         |
| $\gamma_c\varepsilon_c$ | 0.89 ± 0.03                         |
| $\gamma_c\varepsilon_{cc}$ | 1.0 ± 0.02                          |
| $\varepsilon_{cc}$ | 1.0 ± 0.1                           |

\(a\) Reduced form of the enzymes was used for the analysis.

\(b\) ATPase activity was determined at 40 °C in the presence of 3 mM ATP. Values for $F_oF_1$ are the activity per 1 mg of membrane proteins.
FoF-ATPase/synthase is geared to the synthesis mode by conformational rearrangement of ε subunit in response to proton motive force and ADP/ATP balance

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