Effects of Sequential Deletions of Residues from the N- or C-Terminus on the Functions of ϵ Subunit of the Chloroplast ATP Synthase†

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ABSTRACT: Ten truncated mutants of chloroplast ATP synthase ϵ subunit from spinach (*Spinacia oleracea*), which had sequentially lost 1−5 amino acid residues from the N-terminus and 6−10 residues from the C-terminus, were generated by PCR. These mutants were overexpressed in *Escherichia coli*, reconstituted with soluble and membrane-bound CF_1, and the ATPase activity and proton conductance of thylakoid membrane were examined. Deletions of as few as 3 amino acid residues from the N-terminus or 6 residues from the C-terminus of ϵ subunit significently affected their ATPase-inhibitory activity in solution. Deletion of 5 residues from the N-terminus abolished its abilities to inhibit ATPase activity and to restore proton impermeability. Considering the consequence of interaction of ϵ and γ subunit in the enzyme functions, the special interactions between the ϵ variants and the γ subunit were detected in the yeast two-hybrid system and in vitro binding assay. In addition, the structures of these mutants were modeled through the SWISS-MODEL Protein Modeling Server. These results suggested that in chloroplast ATP synthase, both the N-terminus and C-terminus of the ϵ subunit show importance in regulation of the ATPase activity. Furthermore, the N-terminus of the ϵ subunit is more important for its interaction with γ and some CF_o subunits, and crucial for the blocking of proton leakage. Compared with the ϵ subunit from *E. coli* [Jounouchi, M., Takeyama, M., Noumi, T., Moriyama, Y., Maeda, M., and Futai, M. (1992) *Arch. Biochem. Biophys. 292*, 87−94; Kuki, M., Noumi, T., Maeda, M., Amemura, A., and Futai, M. (1988) *J. Biol. Chem. 263*, 4335−4340], the chloroplast ϵ subunit is more sensitive to N-terminal or C-terminal truncations.

The proton-translocating F_1F_o ATP synthase serves the central catalytic function of ATP synthesis in most organisms. The enzymes are located in the inner membrane of mitochondria, in thylakoid membranes of chloroplast, and in cytoplasmic membranes of bacteria. F_1F_o ATP synthases generate ATP using the electrochemical gradient across these energy-transducing membranes (*1*−*3*). In chloroplast, this enzyme consists of two parts: CF_o and CF_1.[1] CF_o is located in the thylakoid membrane and comprises four types of subunits, which were designed as subunit I, II, III and IV (homologous to subunits b, b, c, and a in *Escherichia coli*). The stoichiometry of these subunits is $I_1II_1III_{12}IV_1$ (*3*). A ring of 14 subunit III monomers was observed more recently (*4*). The CF_o part conducts protons flux through the thylakoid membrane and provides the affinity site for CF_1. CF_1 consists of five types of subunits with the stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$. CF_1 contains the nucleotide-binding and catalytic sites, and can hydrolyze ATP at high rates after appropriate treatment (*5*).

The crystallization of F_1 from bovine mitochondria has let to a high-resolution structure of the $\alpha_3\beta_3$ hexamer, plus part of the γ subunit in the central core (*6*). Electron cryomicorscopic images have also contributed to the understanding of subunit arrangement (*7*) and of the movement of ϵ and γ subunits in F_1 (*8*). Direct visualization of rotation of γ subunit (*9*) during ATP hydrolysis has confirmed the hypothesis of rotation γ relative to $\alpha_3\beta_3$ (*10*), which was previously supported by cross-linking and fluorescence spectroscopy studies (*11*). The three-dimensional structures of two other F_1 subunits have also been determined: δ (*12*) and ϵ (*13*, *14*). NMR and X-ray analysis have given a detailed structure of the ϵ subunit of EF_1. It is composed of two distinct domains: the N-terminal β-sandwich domain containing two antiparallel β-sheets and the C-terminal domain comprising an antiparallel coil of two α-helices (*13*, *14*). There is an apparently stable hydrophobic interface between these two domains, which leaves some room for rotational motions of the two domains. In *E. coli*, ϵ binds to γ and subunit c through its N-terminal domain (*15*) and interacts with subunits α and β through its C-terminal domain (*16*).

The ϵ subunit is a potent inhibitor of ATPase in both the soluble and bound forms, and is necessary for the formation of proton gate in *E. coli* (*17*) and in chloroplast (*5*). Mutants with deletions of residues from both the N-terminus (*18*) and C-terminus (*19*) of ϵ subunit from *E. coli* have been constructed and analyzed. A truncated form containing only the first 93 amino acid residues, plus 2 serine residues, was capable of partial inhibition of ATP hydrolysis and of promoting the binding of F_1 to F_o (*19*). Deletion of up to 15

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[1] Abbreviations: CF_1, catalytic portion of the chloroplast ATP synthase; $CF_1(-\epsilon)$, CF_1 deficient in ϵ subunit; IPTG, isopropyl-D-thiogalactopyranoside; ACMA, 9-amino-6-chloro-2-methoxyacridine.

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Table 1: Amino Acid Sequences and Primer Sequences of Truncated Mutants[a]

Plasmid	Amino Acid Sequences	Forward primers (5'-3')	Reverse primers (5'-3')
PJLA503-εΔN1	MLNLCVLTP-10	CGCTCATATGTTAAATCTTAGTG	
PJLA503-εΔN2	MNLCVLTP-10	CGCTCATATGAATCTTAGTGTAC	AAGGAGCTGA
PJLA503-εΔN3	MLCVLTP-10	CGCTCATATGCTTAGTGTACTG	CTGGGTTGAA
PJLA503-εΔN4	MCVLTP-10	CGCTCATATGAGTGTACTGAC	GGCTCTC
PJLA503-εΔN5	MVLTP-10	CGCTCATATG GTACTGACTCCG'	
PJLA503-εΔC6	120-RRARTRVEA	CGAAGGGATCCTATTTCAAGCCTCGAC	GCTCCATATG
PJLA503-εΔC7	120-RRARTRVE	CGGATCCCTTCACTCGACTCGTGTTCTG	ACCTTAAATC
PJLA503-εΔC8	120-RRARTRV	CGGATCCCTAGCCTAGACTCGTGTTC	TTAGTGTACT
PJLA503-εΔC9	120-RRARTR	ATTGGGATCCTCTTATCGTGTTCTAGC	GAC
PJLA503-εΔC10	120-RRART	TGGGATCCTCGACTCATGTTCTAGC	

[a] List below are the amino acid sequences and PCR primers of truncation mutants (Δ) of ϵ subunit of spinach chloroplast ATP synthase. The numbers in the plasmids indicate the numbers of residues deleted followed by N or C designating N-terminus or C-terminus truncation, respectively. The truncations begin with deletion of 1 residue with successive deletion of 1 residue up to 5 residues from the N-terminus, and 6 residues up to 10 residues from the C-terminus.

residues at the N-terminus did not seriously impair function (*18*). For chloroplast ϵ subunit, it has been shown that truncation of 10 residues from the C-terminus is much more deleterious to its functions than the similar truncation in *E. coli* (*20*), and that truncations from the N-terminus are even more deleterious (*21*). These results show that the chloroplast ϵ subunit is more impressible to N-terminal or C-terminal truncations than the ϵ subunit from *E. coli*.

To identify which amino acid residues in the C-terminus and N-terminus of the chloroplast ϵ subunit are essential for its abilities to inhibit ATPase and to restore proton impermeability, we constructed 10 truncated mutants with sequential deletions of 1−5 residues from the N-terminus or 6−10 residues from the C-terminus. Each of the mutants was overexpressed in *E. coli* and was reconstituted with soluble and membrane-bound CF_1. The ATPase-inhibiting effects and proton conductance-blocking activities of these ϵ variants were detected. In addition, the interactions of γ subunit with these ϵ variants were analyzed in vivo and in vitro.

EXPERIMENTAL PROCEDURES

Plasmid Construction. Plasmid pJLA503-pchlϵ containing the *atpE* genes of spinach was a gift from Dr. Sigfried Engelbrecht (*22*). Mutagenic primer 5′-GCTCCATATGAC-CTTAAATC-TTAGTGTACTGAC-3′ and 5′-AAGGAGCT-GACTGGGTTGAA-GGCTCTCAAG-3′ were used to introduce a site-specific mutation, Cys6 into Ser, in polymerase chain reaction. The PCR products were digested with *Nde*I and *Bam*HI and subcloned into pJLA503 to form pJLA503ϵ1. The following mutations were performed with pJLA503ϵ1 as a template.

Truncation Mutagenesis. Ten mutants of ϵ subunit that sequentially lack 1−5 residues from the N-terminus or 6−10 residues from the C-terminus were generated by PCR (*23*) using *Pfu* DNA polymerase (Pharmacia). The cycling parameters were 94 °C for 1 min, 55 °C for 1 min, 72 °C for 0.8 min in a total of 30 cycles. The primers used to generate these mutants were designed as in Table 1.

Solubilization and Folding of Overexpressed ϵ Mutants. The plasmids listed in Table 1 were transformed into the competent DH5α cells. The induction of expression and the purification of recombinant ϵ proteins were performed as described by Cruz et al. (*20*).

Assembly of ϵ Subunit and Its Mutants with ATP synthase. CF_1 and CF_1 deficient in ϵ subunit [$CF_1(-\epsilon)$] were prepared as previously described (*5*, *10*) from the fresh market spinach leaves and stored as ammonium sulfate precipitates. Prior to use, the proteins were desalted on Sephadex G50 centrifuge columns (Pharmacia) (*24*). The urea-solubilized ϵ proteins were diluted with ice-cold dilution buffer to the concentrations that would give appropriate molar ratio of ϵ:$CF_1(-\epsilon)$. Various amounts of the ϵ proteins were reconstituted with 20 μg of $CF_1(-\epsilon)$ in solution. Prior to assay, the mixture was incubated for at least 20 min at room temperature (*20*).

CF_1-deficient thylakoid membranes were prepared according to the method of Nelson and Eytan (*25*). $CF_1(-\epsilon)$ was incubated with thylakoid membrane deficient in CF_1 for 30 min at a mass ratio of 4 mg of $CF_1(-\epsilon)$/mg of Chl. ATP and $MgCl_2$ were added to a final concentration of 2mM and 5mM. The membrane was pelleted by centrifugation at 4 °C, 30000*g* for 5 min, and resuspended in cold STN buffer (0.4 M sucrose, 50 mM Tris-HCl, and 10 mM NaCl, pH 8.0). Reconstitution of ϵ-deficient membrane with recombinant ϵ proteins was performed as previously described (*20*).

Expression of GST-γ Fusion Protein in E. coli. The coding sequence of γ subunit was amplified by PCR, digested with *Bam*HI and *Eco*RI, and then subcloned into pGEX-2T (Pharmacia) to construct the fusion protein expression vector pGEX-2T-γ. The coding sequence of γ protein was confirmed to be in frame with the GST cassette.

pGEX-2T-γ was transformed into *E. coli* BL21(DE3) to express the GST-γ fusion protein. After 3 h of IPTG induction, the cells were harvested by centrifugation and resuspended in 10 mL of TNE buffer (50 mM Tris-HCl, 150 mM NaCl, and 1 mM EDTA, pH 8.0). After incubation with

lysozyme on ice for 20 min, 100 μL of 1 M DTT and 1.4 mL of 10% sarkosyl were added and the cells were sonicated for 1 min. Then 4 mL of 10% Triton ×100 were added and the total volume were adjusted to 20 mL to keep the final concentrations of sarkosyl and Triton X-100 at 0.7 and 2%, respectively. The cells were incubated at room temperature for 30 min and the lysates were centrifuged.

In Vitro Binding Assays of γ with ϵ Subunit and Immunoblotting Analysis. Two micrograms of the *E. coli* cell lysates containing ϵ subunit proteins was incubated with 2 μg of GST-γ fusion protein in 200 μL of binding buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, and 10% glycerol, pH 8.0). The mixture was gently agitated for 1 h at 4 °C, and then 20 μL of glutathione-Sepharose 4B beads (bed volume, Pharmacia) was added and rotation was continued for another 2 h at 4 °C. The beads were then washed three times with 0.5 mL of binding buffer, once with 0.5 mL of PBS buffer. Then the beads were boiled in 2× SDS loading buffer, and the proteins were analyzed on 15% SDS−polyacrylamide gel (*23*). The proteins were transferred to nitrocellulose membrane and detected by western immunoblot analysis using ECL Western Blotting Detection System (Amersham) using the anti-ϵ antiserum.

In Vivo Binding Assay of γ with ϵ Subunit in Yeast Two-Hybrid System. The coding sequences of the ϵ subunit and its mutants were digested with *Nde*I, end-filled with Klenow fragment and then cleaved with *Bam*HI. The sequences thus generated were inserted into the unique *Sma*I and *Bam*HI sites in two-hybrid vector pGBT9 (Clontech). The coding sequence of γ subunit was digested with *Bam*HI and *Sal*I and subcloned into pGAD424. The plasmids of pGBT9 and pGAD424 derivatives constructed above were cotransformed into yeast SFY526 (*26*). The β-galactosidase assays were performed as described previously (*27*). The specific enzyme activity is presented in Miller Unit (*28*).

Other Procedures. For measurement of Ca^{+}-ATPase activity, 100 μL of samples were mixed with 1 mL of reaction buffer (50 mM Tris-HCl, 5 mM ATP, of 5 mM CaCl, pH 8.0) and incubated at 37 °C for 5 min. The reaction was stopped by adding 100 μL of 20% trichloroacetic acid and the concentration of inorganic phosphate was determined (*29*). The quenching of ACMA (Sigma) fluorescence was measured as described previously (*30*). Chlorophyll concentration of NaBr-treated thylakoid membrane was determined according to Arnon (*31*). Protein concentration was measured by the method of Brandford (*32*). A chemical blank containing an amount of urea equivalent to the amount present in the samples was used to correct for the background absorptance by urea (*20*). The three-dimensional structure of wild and mutated ϵ proteins constructed in the present study were modeled through the web page of http://www.expasy.ch/spdbv/ (*33*).

RESULTS

(1) Overexpression of the Spinach Chloroplast atpE Gene in E. coli. All the plasmids listed in Table 1 were transformed into the *E. coli* DH5α. The spinach chloroplast *atpE* gene constructed in pJLA503 expression vector had a high expression level in *E. coli*. More than 100 mg of recombinant ϵ proteins could be obtained in each liter of culture medium.

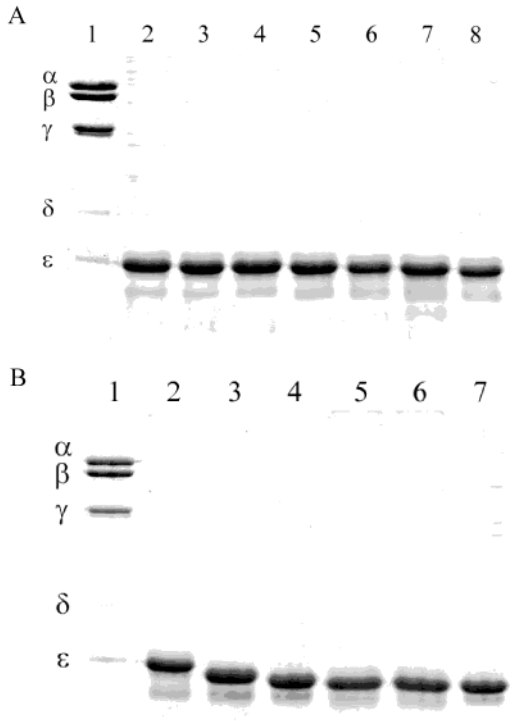

FIGURE 1: Gel electrophoresis profiles of preparations of ϵWT and mutants overexpressed in *E. coli*. Inclusion body preparations were analyzed by SDS−PAGE on an 15% polyacrylamide gels and proteins were stained with Coomassie Brilliant Blue R. Each lane contained about 10 μg of proteins. (A) Lane 1, partially purified spinach chloroplast CF_1; lane 2−8, inclusion body preparations of ϵWT, ϵC6S, ϵΔN1, ϵΔN2, ϵΔN3, ϵΔN4, and ϵΔN5, respectively. (B) Lane 1, partially purified spinach chloroplast CF_1; lane 27, inclusion body preparations of ϵWT, ϵΔC6, ϵΔC7, ϵΔC8, ϵΔC9, and ϵΔC10, respectively.

On SDS−PAGE, wild-type ϵ protein and the mutated peptides migrate for distances consistent with the extent of truncation (Figure 1). Each of the ϵ variants cross-reacted with the ϵ antisera on immunoblots (data not shown). Overexpression of the cloned polypeptides in *E. coli* resulted in accumulations in insoluble inclusion bodies. In the initial purification attempts, the overexpressed polypeptides were solubilized from inclusion bodies into 8 M urea solution and recovered by stepwise dialysis. The soluble proteins thus obtained had higher electrophoretic mobility on SDS−PAGE than the purified ϵ proteins from chloroplast, and they lost the ATPase-inhibiting activities (data not shown). It may be the results of site-specific proteolysis during the stepwise dialysis or due to some other reasons (*20*). In later preparations, the denatured inclusion bodies were directly diluted with dilution buffer. The diluted polypeptides were soluble and the recombinant wild ϵ proteins were as active as the ϵ subunit purified from spinach chloroplast (data not shown).

(2) Reconstitution of Recombinant ϵ Proteins with CF_1(-ϵ) and ϵ-Deficient Thylakoid Membrane. Previous study showed that deletion of as few as five residues from the N-terminus of ϵ subunit resulted in an obvious decrease in its ability to inhibit the ATP hydrolysis of the holoenzyme. Ten residues deleted from the C-terminus got similar results (*20*). It is interesting to test the inhibitory activity of ϵ subunit mutants of which the residues in the corresponding region were truncated one by one. For this, each of the ϵ mutants that sequentially miss 1−5 residues from the N-terminus and

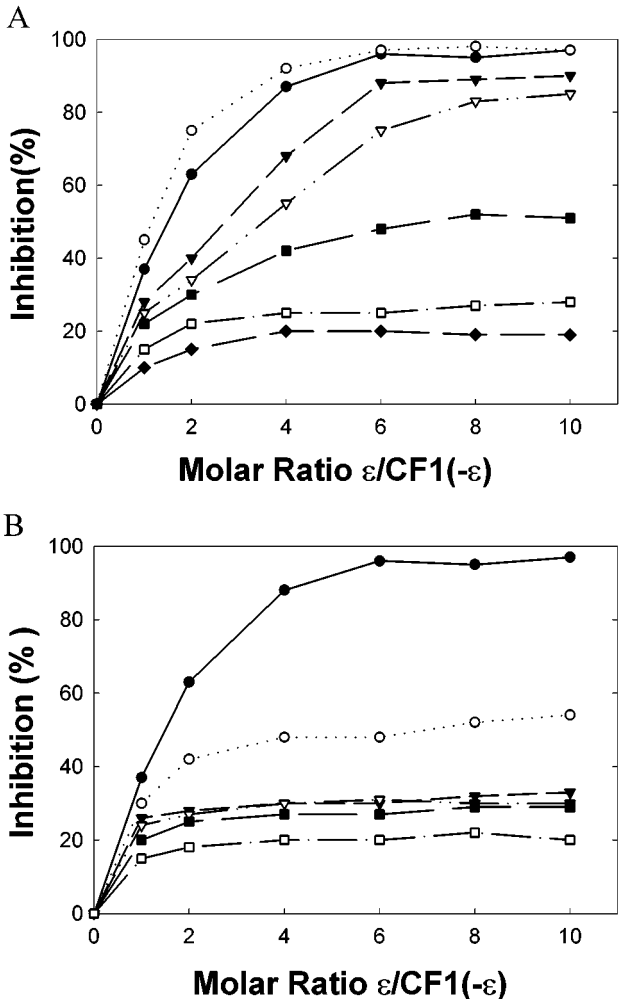

FIGURE 2: Inhibition of ATP hydrolysis in solution. $CF_1(-\epsilon)$ was reconstituted with increasing amounts of ϵWT and mutants in solution and the equivalent of 20 μg of $CF_1(-\epsilon)$ from each reconstitution were assayed for Ca^+-ATPase activity as described in the Experimental Procedures. The inhibitory activity of ϵ is given as percentage inhibition of the maximal ATPase activity in the absence of epsilon (100%). In each case, the maximal activity was the activity of $CF_1(-\epsilon)$ preincubated with an equivalent amount of control buffer. The maximal activity varied between 9 and 12 μmol of P_i released min^{-1} mg^{-1} protein. (A) wild-type ϵ (filled circles), ϵC6S (open circles), $\epsilon\Delta$N1 (filled triangles), $\epsilon\Delta$N2 (open triangles), $\epsilon\Delta$N3 (filled squares), $\epsilon\Delta$N4 (open squares), $\epsilon\Delta$N5 (filled diamonds). (B) wild-type ϵ (filled circles), $\epsilon\Delta$C6 (open circles), $\epsilon\Delta$C7 (filled triangles), $\epsilon\Delta$C8 (open triangles), $\epsilon\Delta$C9 (filled squares), $\epsilon\Delta$C10 (open squares).

6−10 residues from the C-terminus was tested for its abilities to inhibit the ATP hydrolysis and to restore the proton impermeability.

Recombinant ϵ proteins were incubated with soluble CF_1 and thylakoid membrane deficient in ϵ subunit. As shown in Figure 2, when reconstituted with CF_1 in solution, the ϵC6S mutant inhibited ATPase activity as potently as the ϵWT. Both of them could inhibit about 97% of the ATPase activity when given in a ratio of $\epsilon/CF_1(-\epsilon)$ higher than 6:1. Cruz reported that ϵWT did not inhibit ATPase as potently as ϵ purified from CF_1 while ϵC6S did (*21*). They attributed this to the dimerization of ϵWT via Cys6 that prevented its binding to $CF_1(-\epsilon)$. In the present study, the ϵWT could inhibit the ATPase as much as the ϵC6S in the presence of DTT. To eliminate the need for reducing agents, pJLA503ϵC6S was used for further mutagenesis since the C6S mutation had no effect on the function of the ϵ subunit. Deletions of 1 or 2 amino acids from the N-terminus of the ϵ subunit had only marginal effects on the maximum inhibitory activities. Both could inhibit the ATPase by more than 85%, though high $\epsilon/CF_1(-\epsilon)$ ratios were needed to attain the maximum. Deletion of as few as 3 residues from the N-terminus of the ϵ protein decreased its inhibitory activity significantly, and $\epsilon\Delta$N4 and $\epsilon\Delta$N5 mutants lost most of the inhibitory activities in solution (Figure 2A).

It has been reported that deletion of a large portion of the C-terminus has no significant effect on ϵ activity in *E. coli* (*19*). But in chloroplast ATP synthase, deletion of 6 residues from the C-terminus of ϵ subunit resulted in about 40% decrease of its inhibitory potency, showing that the C-terminus is important for its inhibitory activity (*20*). Deletions of 7, 8, and 9 residues from the C-terminus have additional effects on the inhibitory activity of ϵ to soluble CF_1. All these three mutants inhibited ATPase by only about 30%, and $\epsilon\Delta$C10 held less than 20% of the inhibitory potency of the wild ϵ protein (Figure 2B).

In addition, we also tested the effects of these truncated ϵ proteins on their abilities to inhibit the membrane ATPase and to restore the proton gradient through the thylakoid membrane deficient in ϵ subunit. When the ϵ variants were added to the ϵ-deficient thylakoid membrane, the inhibitory potencies were much higher than that of the corresponding ϵ mutants binding to soluble CF_1 (Figure 3, black column). At a molar ratio of $\epsilon/CF_1(-\epsilon)$ of 16:1, the ϵWT and ϵC6S could inhibit the membrane-bound ATPase by almost 100%, and the $\epsilon\Delta$N1 and $\epsilon\Delta$N2 could also inhibit membrane ATPase by more than 90%. The extents of inhibiting ATPase of membrane-bound $CF_1(-\epsilon)$ by $\epsilon\Delta$N3, $\epsilon\Delta$N4, and $\epsilon\Delta$N5 were as high as 70, 55, and 50%, respectively (Figure 3A), whereas these mutants could only inhibit ATPase of soluble $CF_1(-\epsilon)$ by about 50, 28, and 19%, respectively (Figure 2A). These results indicated that the membrane-bound CF_o might stabilize the interactions between the ϵ subunit and other subunits of CF_1. The same conclusion could also be reached from the reconstitution of C-terminal-truncated mutants of ϵ subunit with membrane-bound CF_1. For example, when they were reconstituted with soluble CF_1, $\epsilon\Delta$C7, $\epsilon\Delta$C8, and $\epsilon\Delta$C9 inhibited the ATPase by about 33, 30, and 29%, respectively (Figure 2B), while binding to CF_o could enhance their degrees of inhibition by up to 70, 65, and 56%, respectively (Figure 3B). But to the $\epsilon\Delta$C10 mutant, this kind of CF_o-binding enhancement was not significant.

Membrane ATPase inhibition studies are usually performed in conjunction with ACMA fluorescence quenching assays, which is closely connected with the proton gradient across the thylakoid membrane, and can be used to monitor the restoration of proton gradient formed across the ϵ-deficient thylakoid membrane reconstituted with ϵ variants (*30*). Results in Figure 3 (white column) show the extents of ACMA quenching of variant ϵ mutants reconstituted with ϵ-deficient membrane. All C-terminal-truncated mutants, except the $\epsilon\Delta$C10, were efficient at blocking the proton conductance more than 50% of that of ϵWT. On the contrary, though $\epsilon\Delta$N1 and $\epsilon\Delta$N2 could block the proton flux by about 85 and 70% of the ϵWT, respectively, $\epsilon\Delta$N4 and $\epsilon\Delta$N5 could attain only 28 and 12% of the ϵWT, respectively, even at a molar ratio of $\epsilon/CF_1(-\epsilon)$ as high as 16:1. These results showed

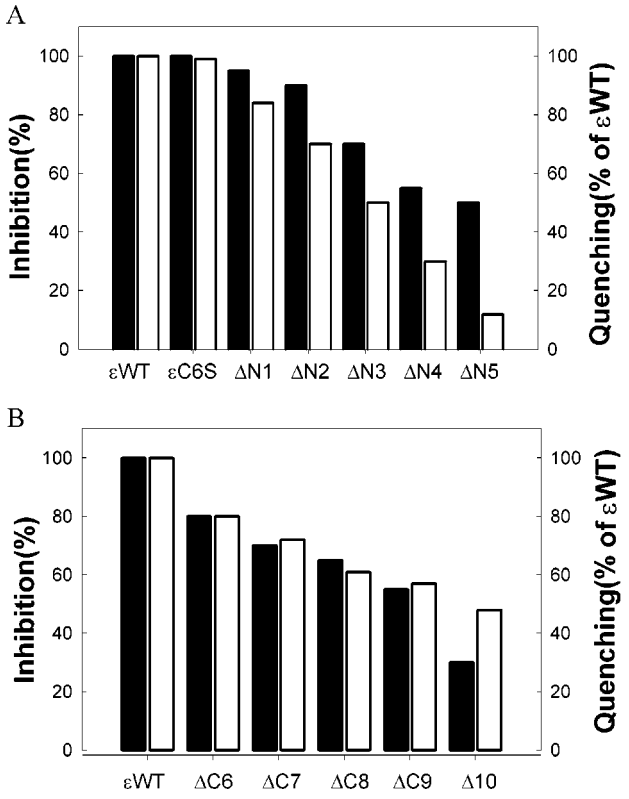

FIGURE 3: Reconstitution of e-WT and truncated mutants with membrane-bound CF1(-ϵ). Thylakoid membrane deficient in ϵ subunit (equivalent to 20 μg of chlorophyll) was reconstituted with 10 μg of wild-type ϵ, N-terminal (A)- and C-terminal (B)-truncated mutants. The Ca^{+}-ATPase activity (black columns) and the quenching of ACMA fluorescence (white columns) were analyzed as described in the Experimental Procedures. The inhibitory activity of ϵ is given as percentage inhibition of the maximal ATPase activity in the absence of epsilon (100%). The ability to restore the proton impermeability of the mutants is given as percentage of that of the ϵ-WT.

that deletions of residues from the N-terminus of ϵ subunit affected its ability to block the proton leaking more significantly than those from the C-terminus.

(3) Interactions of γ Subunit with ϵ Mutants in Vivo and in Vitro. Several lines of evidence suggest that the ϵ subunit is associated with the γ subunit in the central stalk of F_1F_o ATP synthase (*19*, *34*, *35*). In the soluble CF_1, reduction of the disulfide bond in γ subunit elicits the latent ATPase activity (*36*). Activation of CF_1 by organic solvents such as methanol, also involves dissociation of the ϵ subunit (*35*). These results indicate that the inhibitory activity to ATPase of ϵ subunit is related to its interaction with the γ subunit. For this, we studied the interactions of γ subunit with ϵ mutants in vivo and in vitro.

The coding sequences of γ subunit and ϵ mutants were cloned in pGAD424 and pGBT9, respectively. The vectors were cotransformed into the yeast strain SFY526, and the subunit interactions were analyzed through detecting the activity of the reporter β-galactosidase in the yeast two-hybrid system. The activities of β-galactosidase in Miller Unit can represent the potencies of protein interactions. C-terminal deletions of ϵ have little effects on its ability to interact with γ (Table 2A). As to the N-terminal deletions, relatively strong interactions remained between γ subunit and $\epsilon\Delta$N1, $\epsilon\Delta$N2, and $\epsilon\Delta$N3, but the interactions between $\epsilon\Delta$N4 and $\epsilon\Delta$N5 and γ subunit were lowered (Table 2B).

Table 2: Interactions between ϵ Mutants and γ Subunit in Yeast Two-Hybrid System[a]

A

fusion proteins	β-galactosidase activity: filter assay (blue/white)	β-galactosidase activity: liquid assay (U)
pGBT9-ϵWT+pGAD424-γ	blue	3.6 ± 0.3
pGBT9-ϵC6S+pGAD424-γ	blue	3.7 ± 0.2
pGBT9-ΔN1+pGAD424-γ	blue	3.4 ± 0.1
pGBT9-ΔN2+pGAD424-γ	blue	3.3 ± 0.1
pGBT9-ΔN3+pGAD424-γ	blue	2.9 ± 0.1
pGBT9-ΔN4+pGAD424-γ	blue	2.4 ± 0.2
pGBT9-ΔN5+pGAD424-γ	blue	1.5 ± 0.2
pGBT9+pGAD424	white	0.01

B

fusion proteins	β-galactosidase activity: filter assay (blue/white)	β-galactosidase activity: liquid assay (U)
pGBT9-ϵWT+pGAD424-γ	blue	3.6 ± 0.3
pGBT9-ΔC6+pGAD424-γ	blue	3.3 ± 0.2
pGBT9-ΔC7+pGAD424-γ	blue	3.2 ± 0.2
pGBT9-ΔC8+pGAD424-γ	blue	3.2 ± 0.2
pGBT9-ΔC9+pGAD424-γ	blue	3.0 ± 0.1
pGBT9-ΔC10+pGAD424-γ	blue	2.8 ± 0.2
pGBT9+pGAD424	white	0.01

[a] The yeast reporter strain SFY526 was cotransformed with pairs of recombinant plasmids as shown in the table. The level of β-galactosidase activity, presented in Miller Units, was determined as described in the Experimental Procedures. Values are means of triple determinations with standard deviations. (A) Interactions between γ and N-terminal-truncated ϵ proteins. (B) Interactions between γ and C-terminal-truncated ϵ proteins.

The interactions of all the ϵ mutants with γ subunit were also detected by in vitro binding assays. The γ subunit was expressed as GST-fusion protein in *E. coli*, and the γ/ϵ interactions were detected by GST pull-down assays. Results shown in Figure 4 are consistent with those of in vivo binding assay: deletions of 6−10 residues from the C-terminus have little effects on the interactions between these ϵ variants and γ subunit (Figure 4B), and deletions of 4 and 5 residues from the N-terminus caused significant decreases in the extents of their interactions with GST-γ, while $\epsilon\Delta$N1, $\epsilon\Delta$N2, and $\epsilon\Delta$N3 did not (Figure 4A).

DISCUSSION

The present study offers insights into the functional importances of the N-terminus and C-terminus of ϵ subunit of chloroplast ATP synthase. Ten truncated mutants were generated, by reference to the studies of truncational analysis of ϵ subunit from *E. coli* (*19*) and chloroplast (*20*, *21*). Deletions of as few as 3 amino acid residues from the N-terminus or 6 residues from the C-terminus of ϵ subunit could weaken the inhibitory activity to ATPase. Deletion of 5 residues from the N-terminus abolished its abilities to inhibit the ATPase activity in solution and to restore proton impermeability.

The functional importance of the N-terminus of ϵ subunit of chloroplast ATP synthase is clearly demonstrated in our present study. The deletions of 1 or 2 N-terminal residues have only weak effects on the abilities to inhibit ATPase in solution. Truncation of 3 residues have moderate effects, whereas peptides with 4 or 5 residues deleted from the

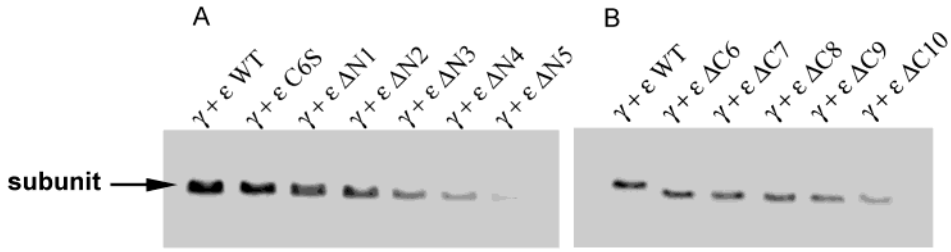

FIGURE 4: Interactions of wild-type ϵ and mutants with γ protein in vitro. GST pull-down assays were performed as described in the Experimental Procedures. Two micrograms of the bacterial cell lysates containing ϵ proteins were incubated with 2 μg of GST-γ fusion protein expressed in *E. coli*. (A) Interactions of GST-γ with N-terminal truncated mutants. (B) Interactions of GST-γ with C-terminal truncated mutants.

N-terminus almost lost all their abilities to inhibit ATPase in solution. The γ/ϵ interactions detected by the yeast two-hybrid system and GST pull-down assay are in agreement with the results of ATPase analysis of these N-terminal-truncated ϵ mutants. Cross-linking experiments showed that residues Ser10, His38, Thr43, Ser65, Glu70, Thr77, and Asp81 could be cross-linked with the γ subunit (*37*). The N-terminal 5 residues are located on the same side as these residues in the β "sandwich" structure of the ϵ subunit. These results indicated that the decrease in inhibitory ability was at least partially caused by the reduced affinities of these ϵ mutants with γ subunit. It has been reported that ϵ subunit can cross-link with c- and a-subunits in *E. coli* (*37*, *38*). Interactions of ϵ with subunit III were also detected in yesat two-hybrid system (X. B. Shi, unpublished data). In this study, when these N-terminal-truncated ϵ mutants were reconstituted with the thylakoid membrane deficient in ϵ subunit, their inhibitory abilities to ATPase were all significently enhanced, suggesting that the CF_o subunits may strengthen the interactions of ϵ with γ and other subunits of CF_1.

It is interesting to note that though the inhibitory abilities of the N-terminal-truncated ϵ proteins, compared with those of the polypeptides reconstituted with CF_1 in solution, were significantly enhanced when they were reconstituted with ϵ-deficient membrane, the $\epsilon\Delta$N4 and $\epsilon\Delta$N5 failed to diminish the proton conductance. Hermolin et al. reported that Cys at residues 26-33 of *E. coli* ϵ subunit could be cross-linked to Cys at positions 40, 42, and 44 in the polar loop region of the c subunit (*39*). They interpreted this as the formation of a turn of the β-sheet at residues 26−33, which packs between the polar loop regions of adjacent c subunits at the cytoplasmic surface of the c_{12} oligomer. The three-dimensional structure of wild-type and mutated ϵ proteins constructed in the present study were modeled through the SWISSMODEL server (*33*). As shown in Figure 5, the N-ternimus of $\epsilon\Delta$N5 mutant, which is shorter than that of the wild-type ϵ protein in the N-terminus, partially disturbs the second and third β strands from the N-terminus into irregular coils. So it is reasonable to deduce that the changes in three-dimensional structure in the third β strand, in which residues 26−33 are located, impair the interactions of the mutant ϵ proteins with subunit III and consequently result in an increase in proton impermeability.

The role of C-terminus in the function of ϵ subunit has also been addressed by analyzing five C-terminal truncated mutants. Previous studies indicated that though the C-terminal helical domain of *E. coli* ϵ subunit was important for its inhibitory effect, it was unnecessary for the functional

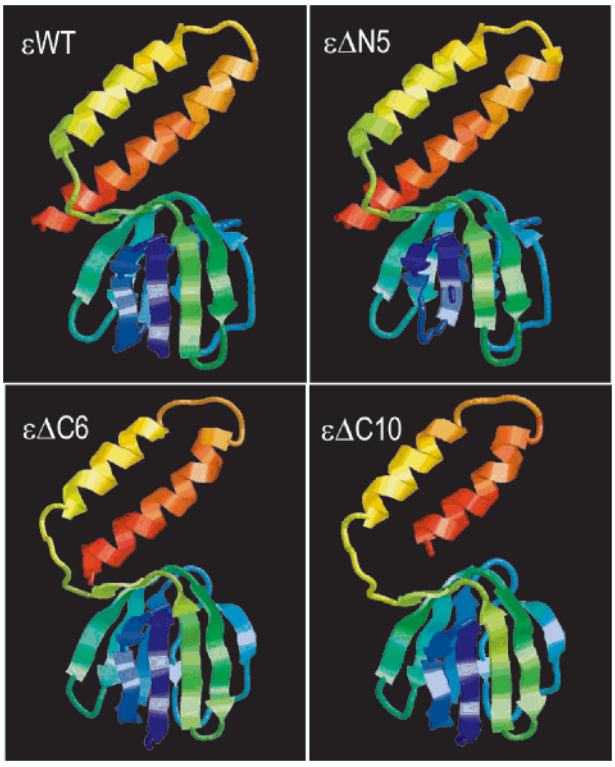

FIGURE 5: Structural models for wild-type ϵ, $\epsilon\Delta$N5, $\epsilon\Delta$C6, and $\epsilon\Delta$C10. The three-dimensional structures of wild-type and truncated ϵ subunits of spinach chloroplast ATP synthase were modeled through the webpage http://www.expasy.ch/spdbv/ (*33*). The figures were generated by the software of Ras-Mol.

coupling (*19*, *40*−*41*). In the present study, truncations of 6−10 residues from the C-terminus significently decreased the inhibitory potency of the ϵ subunit of chloroplast ATP synthase, and also impaired its ability to restore proton impermeability. The two C-terminal antiparallel α-helices are important in regulating the structure of the nucleotide acid binding sites in the β subunit (*37*). Cross-linking experiments showed that the C-terminal helix ϵ-α_2 spans and interacts with two β subunits simultaneously through Ser108 and Met138 (*42*). From the structural models of these C-terminal truncations, it can be seen that deletions of 6−10 residues from the C-terminus only shorten the length of the second α helix and have no marked effects on the N-terminal sandwich structure (Figure 5), indicating that the length of the C-ternimal α helix, which is about 40 Å normally, is important for the inhibitory functiuon of ϵ subunit. The crystal structures of ϵ and central domain of γ subunit (*43*, *44*) showed that following ATP hydrolysis at the catalytic sites, the C-terminal α helix of ϵ separated from one another

and from the β-sandwich, and wrapped the γ subunit, which was much different from the NMR and X-ray structures of the isolated ϵ protein (*13*, *14*). These results suggest that these helix have considerable freedom of movement during catalysis (*43*), and the C-terminus of ϵ may directly associated with the γ subunit. However, the alignment of the ϵ subunit helices with the F_1F_o complex is uncertain, since our study with CF_1 showed that deletion of up to 10 residues from the C-terminus has no significant effect on its interaction with γ subunit (Figure 4B), and cross-linking, which locks the two C-terminal α helices together or holds the $\epsilon-\alpha2$ against the β-sandwich, has little effects on enzyme functions in *E. coli* (*45*).

In conclusion, the results of this study have demonstrated the functional consequence of the N-terminus and the C-terminus of ϵ subunit of the chloroplast ATP synthase. In chloroplast ATP synthase, both the N-terminus and C-terminus of the ϵ subunit show importance in regulation of the ATPase activity. Furthermore, the N-terminus of the ϵ subunit is more important for its interaction with γ and some CF_o subunits, and crucial for the blocking of proton leakage. There results may argue against the opinion that regulation of ATPase and proton gating were related phenomena. Compared with the ϵ subunit from *E. coli* (*18*, *19*), the chloroplast ϵ subunit is more sensitive to N-terminal or C-terminal truncations, indicating that there were some structural and functional differences between the ϵ subunits from *E. coli* F_1 and from CF_1. Therefore, it seems to be meaningful and challenging to do further research on the structure and function of the ϵ subunit of chloroplast ATP synthase.

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