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Substitutions of the conserved Thr42 increased the roles of the ε-subunit of maize CF_1 as CF_1 inhibitor and proton gate

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Abstract

The conserved residue Thr42 of ε-subunit of the chloroplast ATP synthase of maize (*Zea mays* L.) was substituted with Cys, Arg, and Ile, respectively, through site-directed mutagenesis. The over-expressed and refolded ε-proteins were purified by chromatography on DEAE-cellulose and FPLC on mono-Q column, which were as biologically active (inhibiting Ca^{2+}-ATPase activity and blocking proton gate) as the native ε subunit isolated from chloroplasts. The εT42C and εT42R showed higher inhibitory activities on the soluble $CF_1(-\varepsilon)$ Ca^{2+}-ATPase than the εWT. The εT42I inhibited the Ca^{2+}-ATPase activity of soluble CF_1 and restored photophosphorylation activity of membrane-bound CF_1 deficient in ε the most efficiently. Far-ultraviolet CD spectra showed that the portions of α-helix and β-sheet structures of the three mutants were somewhat different from εWT. Thus the conserved residue Thr42 may be important for maintaining the structure and function of the ε-subunit and the basic functions of the ε-subunit as far as an inhibitor of Ca^{2+}-ATPase and the proton gate are related.

Additional key words: ATP synthase; circular dichroism; coupling factor 1; ε-subunit; photophosphorylation; *Zea mays*.

Introduction

The chloroplast ATP synthase (CF_1-CF_0 complex or H^+-ATPase), like its closely related counterparts in bacteria and mitochondria, is a multi-subunit complex and consists of two portions: CF_0 and CF_1. CF_0 is an integral membrane-spanning proton transport protein complex, which conducts proton flux through the thylakoid membrane and provides the affinity site for CF_1. CF_1 is a hydrophilic membrane protein complex of ATP synthase and consists of five kinds of subunits with the stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$. CF_1 contains the nucleotide-binding and catalytic sites, and can hydrolyse ATP at high rates after appropriate treatment (McCarty and Richer 1987). Rotation of γ inside the centre cavity of $\alpha_3\beta_3$ in F_1 during ATP hydrolysis was supported by cross-linking and fluorescence spectroscopy studies (Duncan *et al.* 1995) and directly observed with fluorescence microscope (Noji *et al.* 1997). The three-dimensional structure of δ-subunit was also determined (Wilkens *et al.* 1997). Based on kinetic analysis of ATP hydrolysis and synthesis (Penefsky and Cross 1991, Boyer 1993) together with X-ray crystallographic analysis of αβγ-structure (Abrahams *et al.* 1994), an alternating catalytic site model was proposed (Boyer 1993).

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 on CF_1 (McCarty and Richer 1987). NMR and X-ray analysis of ε-subunit of F_1 from *E. coli* have given a detailed structure of the ε-subunit (Wilkens *et al.* 1995, Uhlin *et al.* 1997). The ε-subunit from *E. coli* is composed of two distinctive domains: the β-sandwich N-terminal domain containing two, anti-parallel β-sheets and the C-terminal domain comprising an anti-parallel coil of two α-helices. There is an apparently stable hydrophobic interface between the two domains, which leaves some room for a rotational motion of the two domains. The ε-subunit of CF_1 from spinach chloroplast is more impressible to N-terminal or C-terminal truncation on its structure and function than the ε-subunit from *E. coli*. The N-terminus of the ε-subunit is more important for its interaction with γ and some CF_0

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Abbreviations: CD – circular dichroism; CF_0 – chloroplast coupling factor 0; CF_1 – chloroplast coupling factor 1; $CF_1(-\varepsilon)$ – CF_1 deficient in subunit ε; DTT – dithiothreitol; FeCy – potassium ferricyanide; FPLC – fast protein liquid chromatography; PMS – phenazine methosulfate.
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subunits, indicating that there might be some differences between *E. coli* and chloroplast in conformation of two termini of the ε-subunit of ATP synthase (Shi *et al.* 2001).

The ε-subunit shows a low degree of amino acid sequence homology between different sources. The conserved amino acids are Gly29, Gly32, His37, Pro39, Thr42, Gly47, Gly65, Gly66, Leu78, and Ala123. Substitutions of some amino acids at special positions affect the structure and function of the ε-subunit in *E. coli* (Skakoon and Dunn 1993, Sawada *et al.* 1997, Xiong *et al.* 1998) and in chloroplasts (Cruz *et al.* 1995). In this study, we constructed some mutants of the ε-subunit of maize chloroplast CF_1 by site-directed mutagenesis and further verified the effect of point mutations at the conserved residue Thr42 on the structure and function of ε-subunit from maize chloroplast CF_1.

Materials and methods

Construction of expression plasmids and expression of *atp*E gene in *E. coli*: The expression plasmid pJLA505 containing chloroplast gene *atp*E of maize was constructed as described by Shi *et al.* (1998). The plasmids listed in Table 1 were transformed into competent DH5α cells. After 6 h of induction, cells were harvested by centrifugation at 10 000×*g* and 4 °C for 10 min. Aliquots of the samples were applied on SDS-PAGE electrophoresis (not shown).

Solubilisation and re-folding of expressed product: Recombinant ε-proteins over-expressed in *E. coli* were insoluble within inclusion bodies. Cells were lysed by three cycles of freezing and thawing, and then disrupted by sonication. Undisrupted cells were removed by gentle centrifugation. Insoluble recombinant ε was washed three times with TNE buffer (consisting of 25 mM Tris-HCl, 1 mM EDTA, and 100 mM NaCl, pH 8.0) and then collected by centrifugation at 12 000×*g* and 4 °C for 25 min. Pellets were re-suspended in 20 cm^3 of buffer containing 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 8 M urea. Solubilised ε-proteins were dialysed stepwise against buffers containing decreasing amounts of urea to no urea for 10 h at 4 °C followed by incubation of the re-natured ε-proteins at room temperature for at least total of 2 h to refold completely.

Purification of ε-subunit protein: Refolded ε-subunit proteins were preliminarily purified by anion-exchange chromatography on two continuous DEAE-cellulose *DE52* columns (25.0×2.8 cm) as described by Gu *et al.* (1998). The preliminarily purified ε-proteins were applied to *FPLC* mono-Q column (*Pharmacia*, Rockford, IL, USA) equilibrated with TE buffer (25 mM Tris-HCl and 1 mM EDTA, pH 8.0). After loading of samples, the column was eluted with a linear gradient of NaCl (0-500 mM) in the TE buffer at flow rate of 8.33 $mm^3\ s^{-1}$. The effluent was monitored at 280 nm. The needed fractions of eluates of each protein were pooled and subjected to SDS-PAGE.

Assay of the ATPase activity: CF_1 was isolated from maize chloroplasts. Reconstitution of recombinant ε with soluble $CF_1(-\varepsilon)$ and assay of the ATPase activity were carried out according to Cruz *et al.* (1995).

Photophosphorylation activity was measured according to Wei *et al.* (1998). Chloroplast photophosphorylation was performed in 1 cm^3 reaction mixtures containing 50 mM Tris-HCl (pH 8.0), 5 mM NaCl, 5 mM $MgCl_2$, 2 mM Na_2HPO_4, 1 mM ADP, 0.3 M sucrose, 1 mM FeCy (potassium ferricyanide), or 0.05 mM PMS (phenazine methosulfate), and chloroplasts containing 20 μg of chlorophyll. ATP content was determined by the luciferin/luciferase luminescence assay (Allnutt *et al.* 1991, Wang *et al.* 2000). Preparations of thylakoid membrane deficient in ε and reconstitution of ε were carried out by the method of Cruz *et al.* (1995). Before addition of reconstituted membrane into the reaction mixture, membranes were incubated on ice for 10 min with 20 μg of recombinant proteins.

Circular dichroism (CD) spectra of the ε-subunit proteins were measured by *Jasco J715* spectropolarimeter (*JASCO*, Tokyo, Japan). Samples were placed in 1-mm path-length quartz cuvette (*Helma Cells*, Jamaica, NY, USA). CD spectra from 250 to 190 nm were recorded at constant rate of 10 nm min^{-1} and resolution of 0.1 nm. All measurements were made at room temperature. The CD spectra were expressed as molar ellipticity based on the following equation:

$$[\theta] = (\theta MRM/(10\ l\ c)\ [\text{deg cm}^2\ \text{dmol}^{-1}]$$

using the measured ellipticity (θ, deg), protein concentration (c, kg m^{-3}), cuvette path length (l, cm), and mean residue mass (MRM) of 110.4 g mol^{-1}. The secondary structure percentages were determined from the corrected molar ellipticities by using a multi-linear least-square program based on the method of Yang *et al.* (1986), considering four conformational states (α-helix, β-sheet, β-turn, and unordered structure). Because of the uncertainties in protein concentration, unconstrained analysis was used to report the final percentages of secondary structure.

Other procedures: Hundreds of microlitres of protein were desalted through a *Sephadex G-25* as described by Penefsky (1997). Chlorophyll content of NaBr-treated thylakoid membranes was determined spectrophotometrically according to Arnon (1949). Protein amount was measured by the method of Bradford (1976). SDS-PAGE

on 15 % polyacrylamide gels was performed on the *Bio-Rad* mini-Gel system and then stained with *Coomassie Brilliant Blue R250*. The proteins were transferred to nitrocellulose membrane and detected by Western immunoblot analysis using ECL Western Blotting Detection System (*Amersham*) with the anti-ε antiserum.

Results

Over-expression and purification of maize chloroplast *atp*E gene in *E. coli*: All plasmids listed in Table 1 were transformed into *E. coli* DH5α. The maize chloroplast *atp*E gene constructed in the vector pJLA 505 was over-expressed in *E. coli*. Large amount of non-ε-proteins was removed by preliminary purification through DEAE-cellulose *DE52* columns twice. A prominent peak of the recombinant proteins was recorded and further purified by FPLC on a mono-Q column (Fig. 1*A*). On SDS-PAGE, the εWT and all mutated peptides migrated the same distance as the native ε-subunit from purified CF_1 (Fig. 1*A*). Each of the mutants cross-reacted with an anti-ε-antiserum in immunoblots (Fig. 1*B*)

Table 1. Plasmids used and notation for each re-natured recombinant ε-protein.

Plasmid	Mutation	Product
pJLA505εWT	-	εWT
pJLA505εTC	Thr^{42} to Cys^{42}	εT42C
pJLA505εTR	Thr^{42} to Arg^{42}	εT42R
pJLA505εTI	Thr^{42} to Ile^{42}	εT42I

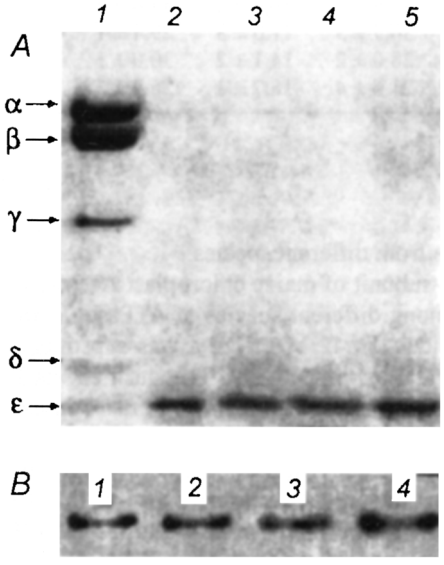

Fig. 1. (*A*) SDS-PAGE of preparations of the εWT and the variants after subsequent purification on DE52 cellulose and FPLC mono-Q column and dialysis against a decreasing gradient of 8M urea to no urea. (*B*) Western-blotting of over-expressed proteins. *A*: lane *1*, purified CF_1; lane *2*, εWT; lane *3*, εT42C; lane *4*, εT42R; lane *5*, εT42I. *B*: lane *1*, εWT; lane *2*, εT42C; lane *3*, εT42R; lane *4*, εT42I.

Inhibition of ATPase activity of $CF_1(-\varepsilon)$ in solution: The CF_1 deficient in ε [$CF_1(-\varepsilon)$] is an active Ca^{2+}-ATPase. The presence of the ε-subunit inhibits the ATPase activity of $CF_1(-\varepsilon)$. Recombinant ε-proteins were incubated with soluble $CF_1(-\varepsilon)$. The inhibitory potency of the εWT and the mutant variants was determined. As shown in Fig. 2, ε of the wild type inhibited Ca^{2+}-ATPase activity by about 50 % whereas the three mutants inhibited Ca^{2+}-ATPase activity to a higher degree. Especially the εT42I inhibited the Ca^{2+}-ATPase activity most effectively by about 75 % and showed the maximal inhibitory activity at a molar ratio of ε/$CF_1(-\varepsilon)$ 1 : 1 or higher.

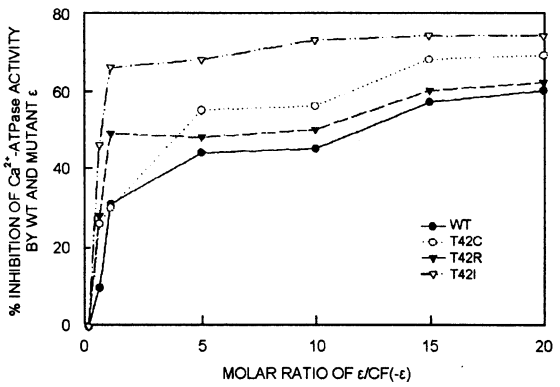

Fig. 2 Comparison of inhibitory potency of εWT and mutant variants of chloroplast ATPase from maize. The activity of each variant was expressed as percent inhibition of maximal ATPase activity, which is the Ca^{2+}-ATPase activity of $CF_1(-\varepsilon)$ preincubated with control buffer. $CF_1(-\varepsilon)$ from maize was reconstituted with increasing amounts of εWT, εT42C, εT42R, and εT42I.

Photophosphorylation activity of ε-deficient thylakoid membrane reconstituted with the WT and recombinant proteins: Using PMS as the cofactor for the cyclic electron transport or FeCy as the electron acceptor for non-cyclic electron transport in the reaction mixture, we compared the effects of ε variants on the photophosphorylation activity of chloroplasts. Table 2 shows that both cyclic and non-cyclic photophosphorylation activities of reconstituted membranes were much lower than the activities of chloroplasts isolated from fresh spinach leaves. But the residue membranes reconstituted with the ε-mutants restored greater photophosphorylation activity than those reconstituted with εWT. The ability of εT42I to restore photophosphorylation activity was the highest (about 20 % higher than εWT), similar to the inhibitory potency to $CF_1(-\varepsilon)$ Ca^{2+}-ATPase.

Table 2. Photophosphorylation activities [mmol(ATP) kg^{-1}(Chl) s^{-1}] of reconstituted thylakoid deficient in ε with the εWT and the mutants.

		Cyclic photophosphorylation		Non-cyclic photophosphorylation	
Chloroplast		69.4	63.9	50.0	45.3
Thylakoid membrane	(–ε)	0	0	0	0
	+ εWT	24.3	22.5	19.4	20.9
	+ εT42C	25.9	24.0	21.1	21.5
	+ εT42R	27.6	24.9	25.9	22.1
	+ εT42I	29.2	30.7	27.7	25.1

Spectroscopic analysis of secondary structures: In order to know the conformational differences in the secondary structure of εWT protein and the mutant proteins, CD spectra were measured. Far-ultraviolet CD spectra of εWT and the mutants proteins showed (Fig. 3) that the portions of α-helix and β-sheet of the three mutants are slightly different from those of εWT. A decomposing program was designed on the basis of the method of Yang *et al.* (1986), which considered four secondary structures (α-helix, β-sheet, β-turn, and unordered structure) and was based on the data derived from the linear analysis of CD spectra of 16 globular proteins with high-resolution crystal structures. Unconstrained analysis of CD spectrum for all the preparations given is summarised in Table 3. Although the program was not constrained (see Materials and methods), the sum of the proportions of four secondary structures of each ε-subunit was close to 100 % (Table 3), which suggests that the results of analysis were reliable.

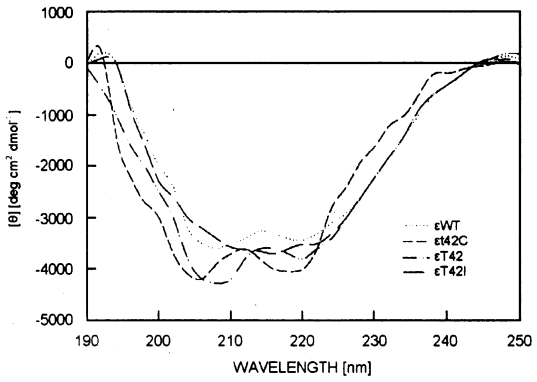

Fig. 3. CD spectra of purified preparations of the εWT(·····), εT42C(– – –), εT42R(–··–), and εT42I(——) of CF_1 from maize. All samples were in 25 mM Tris-HCl and 5 mM EDTA.

Table 3. Percentage of secondary structures of WT and mutant ε-protein preparations of CF_1 from maize.

	% of secondary structure			
	α-helix	β-sheet	β-turn	Unordered
εWT	26.7 ± 4	27.4 ± 3	12.4 ± 1	33.6 ± 7
εT42C	29.1 ± 2	30.7 ± 3	11.0 ± 3	29.4 ± 1
εT42R	27.8 ± 1	28.0 ± 2	14.1 ± 2	30.0 ± 1
εT42I	32.4 ± 3	21.6 ± 4	18.7 ± 1	26.4 ± 5

Discussion

In *E. coli*, the conserved residue His39 of the ε-subunit is a surface amino acid residue on this peptide that can slightly affect interaction with the γ-subunit (Skakoon *et al.* 1993). Point mutations M102T and D141V caused increases of membrane-bound F_1-ATPase activity and proton pumping activity with decreased γ-ε interaction (Sawada *et al.* 1997) and E70A, S65A, and E31A showed somewhat higher affinities and extents of inhibition than the εWT of *E. coli* (Xiong *et al.* 1998). In spinach chloroplast ATPase, substitution of His37 with Arg can fully inhibit the ATPase activity of membranes deficient in ε but cannot fully restore proton impermeability. This suggests that the mutation perturbs the interaction between the ε-subunit and the rest of CF_1 that are required for restoration proton impermeability and are not necessary for inhibition (Cruz *et al.* 1995). In the present study the ε-subunit of maize ATP synthase was chosen to investigate the role and to identify possible differences of the ε-subunit of the ATPase from different species.

Thr42 of the ε-subunit of maize chloroplast ATPase is well conserved among different species of ATPase. Here, the conserved residue Thr42 of maize ε was substituted with Cys, Arg, and Ile, respectively. The purified εWT was biologically active as well as the native one. Compared with the ability of εWT of spinach ATP synthase to inhibit the Ca^{2+}-ATPase activity, the ability of the εWT of maize CF_1-ATPase was much weaker, possibly due to species differences. Of the three mutant ε tested, εT42I was the most potent inhibitor of soluble CF_1, inhibiting the Ca^{2+}-ATPase activity much more effectively (about 75 %) than εWT (about 50 %) at ratios (just 1 : 1) of ε : CF_1(–ε). Previous studies proposed that the C-terminal helical domain of the ε-subunit is mainly responsible for the inhibitory function in *E. coli* ε (Kuki *et al.* 1988, Jounouchi *et al.* 1992) and chloroplast (Allnutt *et al.* 1991, Cruz *et al.* 1995, Shi *et al.* 2001). Taken together, it

is possible that the substitution of the conserved residue Thr42 leads to some conformational changes of the C-terminus of the peptide, which influences the interaction of the ε-subunit with the CF_1 core.

The addition of phosphate to thylakoids in light results in significant decrease of the proton gradient that is the driving force for ATP formation. For thylakoid membranes from plant chloroplasts under identical treatment, the photophosphorylation activity may be proportional to the proton gradient (Shen *et al.* 1963). In this study, the photophosphorylation activity of reconstituted thylakoid membrane was much lower than that of native chloroplasts (Table 2), showing much higher degree of integrity of native thylakoid membranes than of the reconstituted thylakoid membranes. However, the restoring degree of photophosphorylation activity of the reconstituted residue membrane by the ε-mutant εT42I was 10-20 % greater than that by the wild type (Table 2), indicating that the proton gradient was enhanced after reconstitution of residue membrane with mutant ε-proteins. Taken together, all the results suggest that the two basic roles of the ε-subunit of chloroplast ATP synthase, *i.e.* the proton gate and inhibitor of the ATPase activity, may be closely linked. In most cases, the abilities to inhibit CF_1-ATPase activity by mutated variants of ε-subunits, such as the εC6S, C-terminal truncation of 10 or 45 amino acid residues (εΔ10C, εΔ45C), paralleled the abilities to restore proton impermeability (Sawada *et al.* 1997). The results reported here also provide another evidence supporting that the two basic functions of the ε-subunit are related (Wei *et al.* 1998).

The CD spectra of proteins containing α- and β-structures usually have negative ellipticity minima at 208 and 222 nm. Parthasarathy and Johnson (1983) showed that the 222 nm peak was high in proteins in which the α- and β-domains were mixed, whereas the 208 nm peak was high in proteins in which the α- and β-domains were separated. The CD spectra of all preparations showed that the 208 nm peaks were higher than the 222 nm peaks (Fig. 3). This indicates that the α-helix and β-strand domains of ε-subunit may be separated. Decomposition of the CD spectra of all preparations shows that the proportions of α-helix and β-strand domains of the mutant protein have changed to some extent, indicating that the studied substitutions of the conserved Thr42 of maize chloroplast ATPase result in conformational changes of the ε-subunit. Moreover, inhibition of the ATPase activity and restoration of photophosphorylation activity of reconstituted residue thylakoid membrane can be attributed to the conformational changes of the ε-subunit caused by a substitution of the conserved residue Thr42.

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