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$_1$(-ε) 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 α$_3$β$_3$γδε. CF$_1$ contains the nucleotide-binding and catalytic sites, and can hydrolyze ATP at high rates after appropriate treatment (McCarty and Richer 1987). Rotation of γ inside the centre cavity of α$_3$β$_3$ 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 (Penevsky 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 impressive 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$_1$(-ε) – 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* (Skaakoon 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 CF₁ 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 CF₁.

### Materials and methods

#### Construction of expression plasmids and expression of atPE gene in *E. coli*

The expression plasmid pJLAs05 containing chloroplast gene atPE 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 cm³ 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 mm³ 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

CF₁ was isolated from maize chloroplasts. Reconstitution of recombinant ε with soluble CF₁(ε) and assay of the ATPase activity were carried out according to Cruz et al. (1995).

#### Photophosphorylation activity

Photophosphorylation activity was measured according to Wei et al. (1998). Chlorophyll photophosphorylation was performed in 1 cm² reaction mixtures containing 50 mM Tris-HCl (pH 8.0), 5 mM NaCl, 5 mM MgCl₂, 2 mM Na₂HPO₄, 1 mM ADP, 0.3 M sucrose, 1 mM FeC₅ (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

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:

\[
[θ] = (θ/10) (l/c) [deg cm² dmol⁻¹]
\]

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

Hundred 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 atpE gene in E. coli:** All plasmids listed in Table 1 were transformed into E. coli DH5α. The maize chloroplast atpE 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. 1A). On SDS-PAGE, the εWT and all mutated peptides migrated the same distance as the native ε-subunit from purified CF1 (Fig. 1A). Each of the mutants cross-reacted with an anti-ε-antiserum in immunoblots (Fig. 1B).

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

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Mutation</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJLA505εWT</td>
<td></td>
<td>εWT</td>
</tr>
<tr>
<td>pJLA505εTC</td>
<td>Thr42 to Cys42</td>
<td>εT42C</td>
</tr>
<tr>
<td>pJLA505εTR</td>
<td>Thr42 to Arg42</td>
<td>εT42R</td>
</tr>
<tr>
<td>pJLA505εTI</td>
<td>Thr42 to Ile42</td>
<td>εT42I</td>
</tr>
</tbody>
</table>

**Inhibition of ATPase activity of CF1(ε−) in solution:** The CF1 deficient in ε [CF1(ε−)] is an active Ca2+-ATPase. The presence of the ε-subunit inhibits the ATPase activity of CF1(ε−). Recombinant ε-proteins were incubated with soluble CF1(ε−). The inhibitory potency of the εWT and the mutant variants was determined. As shown in Fig. 2, ε of the wild type inhibited Ca2+-ATPase activity by about 50% whereas the three mutants inhibited Ca2+-ATPase activity to a higher degree. Especially the εT42I inhibited the Ca2+-ATPase activity most effectively by about 75% and showed the maximal inhibitory activity at a molar ratio of ε/CF1(ε−) 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 Ca2+-ATPase activity of CF1(ε−) preincubated with control buffer. CF1(ε−) 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 CF1(ε−) Ca2+-ATPase.
Table 2. Photophosphorylation activities [mmol(ATP) kg⁻¹(Chl) s⁻¹] of reconstituted thylakoid deficient in ε with the εWT and the mutants.

<table>
<thead>
<tr>
<th>Cyclic photophosphorylation</th>
<th>Non-cyclic photophosphorylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroplast</td>
<td>69.4</td>
</tr>
<tr>
<td>Thylakoid membrane</td>
<td>63.9</td>
</tr>
<tr>
<td>εWT</td>
<td>24.3</td>
</tr>
<tr>
<td>εT42C</td>
<td>25.9</td>
</tr>
<tr>
<td>εT42R</td>
<td>27.6</td>
</tr>
<tr>
<td>εT42I</td>
<td>29.2</td>
</tr>
</tbody>
</table>

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 summarized 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.

Table 3. Percentage of secondary structures of WT and mutant ε-protein preparations of CF₁ from maize.

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

Discussion

In E. coli, the conserved residue His39 of the ε-subunit is a surface acidic 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 F₁-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 CF₁ 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 Ca²⁺-ATPase activity, the ability of the εWT of maize CF₁-ATPase was much weaker, possibly due to species differences. Of the three mutant ε tested, εT42I was the most potent inhibitor of soluble CF₁, inhibiting the Ca²⁺-ATPase activity much more effectively (about 75%) than εWT (about 50%) at ratios (just 1:1) of ε : CF₁(— 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, Joumouchi 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 CF₁ 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 CF₁-ATPase activity by mutated variants of ε-subunits, such as the εC6S, C-terminal truncation of 10 or 45 amino acid residues (εA10C, εA45C), 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.

References

Shi, J., Wei, J.M., Shen, Y.K.: Site-directed mutagenesis of ε subunit of ATP synthase of maize chloroplast. – Acta bio-