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### Subunit interactions of chloroplast ATP synthase from higher plant

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### Introduction

The chloroplast ATP synthase is made up of nine subunits and is physically and functionally composed of two parts, $CF_o$ and $CF_1$. The chloroplast $F_oF_1$ most closely resembles the bacterial enzyme in its subunit composition, overall structure and amino acid sequence homology of some subunits. But the chloroplast ATP synthase is unique among the ATP synthases in several respects (Richter et al. 2000). In this study, we detected the subunit interactions among the chloroplast ATP synthase subunits using the yeast two-hybrid system, and the important roles of interaction between γ and ε subunits in the functions of enzyme were also examined by mutagenesis analysis.

### Materials and Methods

1.1 $CF_1$-deficient thylakoid membranes were prepared according to the method of Nelson and Eytan from the fresh market spinach leaves (Nelson and Eytan 1979). $CF_1$, $CF_1(-\varepsilon)$ were prepared as previously described (Wang et al. 2000).

1.2 ε mutants were generated by PCR. The overexpression in *E.coli* and purification of ε proteins was performed as described previously (Wang et al. 2000).

1.3 *In vivo* binding assay was performed using yeast two-hybrid system(Shi et al. 2000). *In vitro* coimmuno-precipitation assay was performed following the previously described procedure (Shi et al. 2001).

1.4 ATPase activity was measured as described previously (Wei et al. 1987).

1.5 CD spectra measurement was performed according to reference (Wang et al.2000).

### 2. Results and Discussion

*Subunit interactions of chloroplast ATP synthase in the yeast two-hybrid system*

The coding sequences for the α, β, γ, δ and ε subunits of spinach $CF_1$ and for the I, II, III and IV subunits of $CF_o$ were cloned into two-hybrid vectors pGBT9 and pGAD424. The combinations of the derivatives of the two plasmids pGBT9 and pGAD424 containing the coding sequences of the nine subunits were cotransformed into yeast SFY526 and HF7c, and subunit interactions were detected by analyzing the expression of β-galactosidase, and by selecting transformants grown in SD medium without Leu, Trp and His supplementation. Of all the $CF_1$ subunits, the combination of γ and ε subunits yielded expression of high level of the reporter gene, indicating that the subunit interaction is strong in two-hybrid system. Stable

expressions of reporters were also observed for combinations of α and β, α and ε, β and ε, and β and δ subunits. Weak expressions of reporter genes were observed for combination of γ and δ and ε and δ subunits (Table 1). Subunit interactions in $CF_o$ section and between the subunits of $CF_1$ and $CF_o$ were also examined. The results are in consistent with the functions of these subunits in the holoenzyme (data not shown). For example, the subunits I and II of $CF_o$, which function as a "stator" in ATP synthase, could bind the subunits III, IV of $CF_o$ and the δ subunit of $CF_1$ in the two-hybrid assay. The subunit III of $CF_o$, which play an important role in proton translocation in ATP synthase, could interact with ε, γ, IV and other III subunits in this assay.

**Table 1.** Interactions between subunits of spinach $CF_1$ detected by the yeast two-hybrid system.

| Fusion proteins | β-galactosidase activity U |
|---|---|
| GAL4 bd-γ + GAL4 ad-ε | 2.9±0.2 |
| GAL4 bd-α + GAL4 ad-β | 2.4±0.3 |
| GAL4 bd-β + GAL4 ad-ε | 2.3±0.3 |
| GAL4 bd-β + GAL4 ad-α | 2.2±0.2 |
| GAL4 bd-α + GAL4 ad-ε | 1.9±0.2 |
| GAL4 bd-β + GAL4 ad-δ | 1.8±0.2 |
| GAL4 bd-ε + GAL4 ad-δ | 1.0±0.2 |
| GAL4 bd-γ + GAL4 ad-δ | 0.6±0.3 |
| GAL4 bd-ε + GAL4 ad-γ | 0.5±0.2 |
| GAL4 bd + GAL4 ad | 0.1 |

bd, DNA binding domain; ad, DNA activation domain. Only those combinations that could interact in the system were presented.

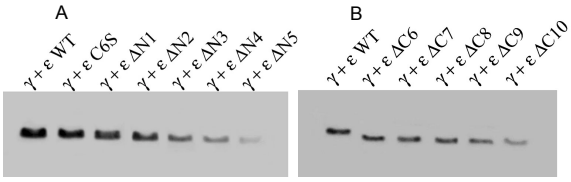


**Fig.1.** Interactions of wild-type ε and mutants with γ protein *in vitro.* (A), Interactions of GST-γ with N-terminal truncated mutants. (B), Interactions of GST-γ with C-terminal truncated mutants.

*Interactions of γ subunit with ε mutants in vivo and in vitro*

The interaction between ε and γ subunit plays an important role in the regulation of the functions of the $CF_1CF_o$ (Capaldi and Schulenberg 2000). For this, we studied the interactions of γ subunit with ε mutants *in vivo* and *in vitro*. Ten truncated mutants of chloroplast ATP synthase ε subunit from spinach, which had sequentially lost 1 to 5 amino acid residues from the N-terminus and 6 to 10 residues from the C-terminus, were generated by PCR. The coding sequences of γ subunit and ε mutants were cloned in pGAD424 and pGBT9, respectively, and the subunit interactions were analyzed in the yeast two-hybrid system. As shown in table 2, C-terminal deletions of ε have little effects on its ability to interact with γ, and as to the N-terminal deletions, relatively strong interactions remained between γ subunit and εΔN1(ε subunit truncated with 1 residues from the N-terminus), εΔN2 and εΔN3, but the interactions

between εΔN4 and εΔN5 and γ subunit were lower (data not shown). The interactions of all the ε mutants with γ subunit were also detected by *in vitro* binding assays. Results shown in Figure 2 are consistent with those of *in vivo* binding assay.

*ATPase-Inhibitory activities of the truncated ε mutants to $CF_1$(-ε)*

Recombinant ε proteins were incubated with soluble $CF_1$ and thylakoid membrane deficient in ε subunit. As shown in figure 3, when reconstituted with $CF_1$ in solution, the εC6S mutant inhibited ATPase activity as potently as the εWT. Deletions of 1 or 2 amino acids from the N-terminus of the ε subunit had only marginal effects on the maximum inhibitory activities. Deletion of 3 residues from the N-terminus of the ε protein decreased its inhibitory activity significantly, and εΔN4 and εΔN5 mutants lost most of the inhibitory activities in solution (Fig 2A). 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. 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.

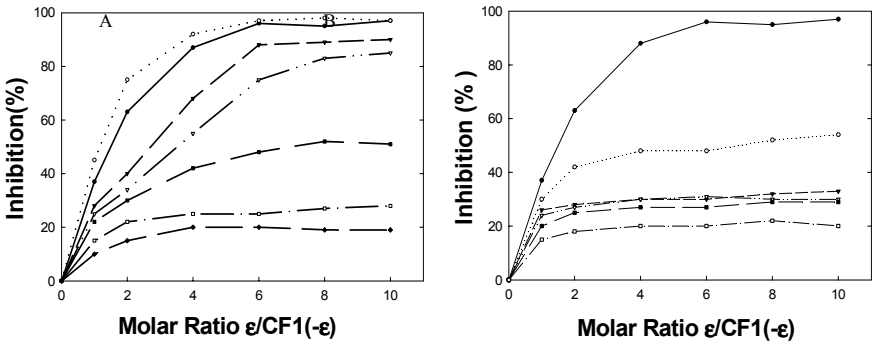


**Fig.2.** Inhibition of ATP hydrolysis in solution. (A), wild-type ε (circles), εC6S (open circles), εΔN1 (filled triangles), εΔN2 (open triangles), εΔN3 (filled squares), εΔN4 (open squares), εΔN5 (filled diamonds). (B), wild-type ε (filled circles), εΔC6 (open circles), εΔC7 (filled triangles), εΔC8 (open triangles), εΔC9 (filled squares), εΔC10 (open squares).

The role of C-terminus in the function of ε subunit has also been addressed by analyzing five C-terminal truncated mutants. Our results are largely consistent with previous conclusions on *E.coli* ε subunit that the C-terminal helical domain of *E.coli* ε subunit was unnecessary for the functional coupling but important for the inhibitory effect (Kato-Yamada et al 1999, 2000, wilkens and Capaldi 1998). In the present study, 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. Deletions of 7, 8 and 9 residues from the C-terminus have additional effects on the inhibitory activity of ε to soluble $CF_1$ (Fig 2B). The decrease of the inhibiting activity of these C-terminal truncated mutants should be the results of shortening of the C-terminus, because from the structural models of these C-terminal truncations, it can be seen that deletions of 6 to 10 residues from the C-terminus only shorten the length of the second α helix and have no obvious effects on the N-terminal sandwich structure(data not shown). *In vivo* and *in vitro* binding assays also showed that C-terminal truncations of ε subunit have no effects on its interactions with γ subunit. Cross-linking experiments showed that the C-terminal helix ε-$\alpha_2$ spans and interacts with two β subunits simultaneously through Ser108 and Met138 (wilkens and Capaldi 1998), indicating that the length of the C-ternimal α helix, which is about 40 Å

normally, is important for the regulation of the structure of the nucleotide acid binding sites in the β subunit (Capaldi and Schulenberg 2000).

*Secondary structure and ATPase-inhibitory activities of site-direct ε mutants*

Thr42 of ε subunit was substituted by Cys, Arg and Ile, respectively, through site-directed mutagenesis. Recombinant ε proteins were incubated with soluble $CF_1$ and the ATPase activities were measured. The mutants εT42C and εT42R show a little higher efficiency than that of the εWT, and εT42I inhibit the ATPase activity the most effectively (Fig. 3A). The difference in the secondary structure of the wild-type and three mutated ε subunits were analyzed through far-ultraviolet CD spectra measure-ment. As shown in fig3B and table 3, the portions of α helix and β sheet of the three mutants are much different from that of the εWT, indicating that the conservative Thr42 play an important role in maintaining the secondary structure of ε subunit.

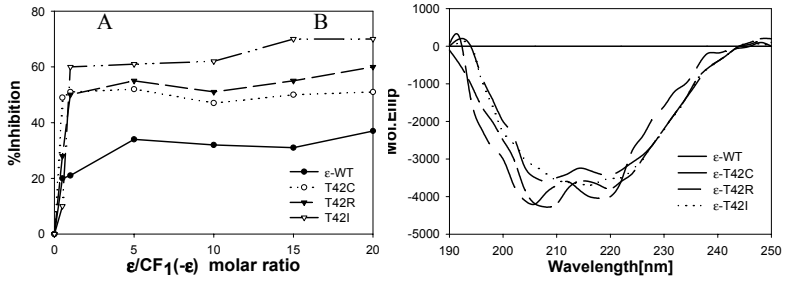


**Fig.3.** CD spectra and inhibitory activities of εWT and three site-directed mutants.

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