N-terminal deletion of the γ subunit affects the stabilization and activity of chloroplast ATP synthase

Zhang-Lin Ni, Hui Dong and Jia-Mian Wei

Shanghai Institute of Plant Physiology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China

ATP synthase occurs ubiquitously on energy-transducing membranes such as chloroplast thylakoid membranes, mitochondrial inner membranes, and bacterial plasma membranes. This enzyme catalyzes ATP synthesis by a proton motive force across the membrane formed by the respiratory chain or photosynthetic electron transport (ATPase in *Escherichia coli* recently reviewed in [1,2], and ATP synthase in chloroplasts in [3,4]). The general structural features of the enzyme are highly conserved among different organisms. The enzyme in chloroplasts consists of two parts: CF0 and CF1. CF0, a membrane-spanning complex, conducts proton flux through the thylakoid membrane and provides affinity sites for the CF1 complex. CF1, extrinsic to the membrane, contains the nucleotide-binding and catalytic sites, and can hydrolyze ATP at high rates after appropriate treatment [4,5]. The CF1 complex consists of five types of subunit with the stoichiometry α3β3γ3δ3ε.

Five truncation mutants of chloroplast ATP synthase γ subunit from spinach (*Spinacia oleracea*) lacking 8, 12, 16, 20 or 60 N-terminal amino acids were generated by PCR by a mutagenesis method. The recombinant γ genes were overexpressed in *Escherichia coli* and assembled with αβ subunits into a native complex. The wild-type (WT) αβγ assembly i.e. αβγWT exhibited high Mg2+-dependent and Ca2+-dependent ATP hydrolytic activity. Deletions of eight residues of the γ subunit N-terminus caused a decrease in rates of ATP hydrolysis to 30% of that of the αβWT assembly. Furthermore, only ≈6% of ATP hydrolytic activity was retained with the sequential deletions of γ subunit up to 20 residues compared with the activity of the αβWT assembly. The inhibitory effect of the ε subunit on ATP hydrolysis of these αβγ assemblies varied to a large extent. These observations indicate that the N-terminus of the γ subunit is very important, together with other regions of the γ subunit, in stabilization of the enzyme complex or during cooperative catalysis. In addition, the *in vitro* binding assay showed that the γ subunit N-terminus is not a crucial region in binding of the ε subunit.

The first high-resolution X-ray structure of ATP synthase was of bovine mitochondrial F1 in 1994 [6]. The structure is essentially unchanged in X-ray studies of bovine F1 inhibited by N,N'-dicyclohexylcarbodiimide (Fig. 1) [7]. Nucleotide bound to all three catalytic sites in the aluminum fluoride-inhibited form of bovine F1 [8]. The X-ray structures show that the α and β subunits alternate with each other to form a hexamer surrounding a central cavity, where a coiled-coil structure formed by the N-terminal and C-terminal helices of the γ subunit penetrates. The three catalytic sites of F1 are located on the β subunits, where the sites interface subunit α in three different conformational states. The importance of the γ subunit in the catalytic cycle has been demonstrated previously, showing that it is probably related to the sequential conformational changes in the αβ pairs in addition to being responsible for the generation of a high-affinity nucleotide-binding site on the β subunits.

**Abbreviations**

CF0, the hydrophobic portion of chloroplast ATP synthase; CF1, coupling factor one; GST, glutathione S-transferase; WT, wild-type.
by its rotation within the $\alpha_3\beta_3\gamma$ core [1–4, 6–8]. In $F_1$, rotation of the $\gamma$ subunit coupled with ATP hydrolysis was confirmed by its direct observation in the movement of a fluorescence-labeled actin filament, which was attached to the $\gamma$ subunit of the thermophilic bacterial $F_1$ subcomplex, $\alpha_3\beta_3\gamma$ [9], $\varepsilon$ subunit $F_1$ [10, 11] and CF$_1$ [12].

The catalytic core of the enzyme is $\alpha_3\beta_3\gamma$, despite $\alpha\beta$ exhibiting lower rates of ATP hydrolysis [13]. It is generally accepted that ATP synthase generates coupling between cooperative catalysis and proton translocation during hydrolysis/synthesis processes. However, the precise catalytic mechanism of $F_1$-ATPase is still unknown [1, 14]. With respect to CF$_1$, it is also proposed that isolated CF$_1$ operates through a full 360° rotation, like other $F_1$-ATPases [12]. CF$_1$ is unique in that thiol modulation, the structural basis of which is an insert of about 20 amino acids including a regulatory disulfide bond, is reversibly oxidized and reduced. The N-terminal and C-terminal of $\gamma$ subunits from different organisms are highly conserved [15]. Deletion of the 20 amino acids in the C-terminus of the $\gamma$ subunit resulted in an active chloroplast enzyme [16]. Crystal structures (Fig. 1) reveal that the N-terminal domain of the $\gamma$ subunit makes contact with the $\beta_E$ subunit C-terminal domain containing the conserved DELSEED motif, which is thought to be important for energy-coupling rotation of the $\gamma$ subunit by steric interaction. This indicates the possible importance in the $\gamma$ N-terminal domain during catalytic cooperativity [4, 7, 8]. $\gamma$Ser8 substitution with a Cys residue resulted in it being cross-linked with a different $\beta$ region in the presence of $\text{Mg}^{2+}$-ADP or $\text{Mg}^{2+}$-ATP [17]. $\gamma$Met23 substitution caused ATPase uncoupling [18], which was suppressed by amino-acid replacements between 269 and 280 in the C-terminal domain [19]. It has been demonstrated by fluorescence mapping [20] and crosslinking [21] that the $\varepsilon$ subunit is in close proximity to the $\gamma$ subunit. The $\varepsilon$ subunit interacts directly with the $\gamma$ subunit [22–24], but does so with a higher affinity when the $\gamma$ subunit is assembled with the $\alpha_3\beta_3$ core [25, 26]. The hybrid enzyme from the $\alpha\beta$ subunits of a thermophilic bacterium and the mutant CF$_1$ $\gamma$ subunit ($\Delta$194–230) was insensitive to added $\varepsilon$ subunit [25].

Studies by Gao et al. [26], who developed an in vitro reconstitution system by assembling the $\alpha\beta$ complex with an isolated $\gamma$ subunit, showed that this complex was able to obtain the reconstituted core enzyme complex as effectively as the native $\alpha_3\beta_3\gamma$. Recently, a hybrid $F_1$-ATPase from Rhodospirillum rubrum or chloroplast subunits was used to study the mechanism of photosynthetic $F_1$-ATPase [27, 28]. In the present study, we examined the importance of the CF$_1$ N-terminal of the $\gamma$ subunit during hydrolytic turnover using this reconstitution system and the binding of $\varepsilon$ to $\gamma$ through a glutathione S-transferase (GST) pull-down assay. To do this, we selectively deleted 8, 12, 16, 20 or 60 residues from the N-terminus of the $\gamma$ subunit. The reconstituted $\alpha\beta\gamma$ assemblies were tested for ATP hydrolytic activity. The results show that the $\gamma$ subunit’s N-terminus is very important for stabilization of the enzyme complex.

Results

Overexpression and assembly of the $\gamma$ truncated mutants

All the plasmids listed in Table 1 were transformed into the expression strain E. coli BL21 (DE3)/pLysS. The spinach chloroplast apC gene constructed in the pET11b expression vector had a high expression level in E. coli. More than 100 mg recombinant $\gamma$ protein was obtained per litre of culture medium. Overexpression of the cloned polypeptides in E. coli resulted in the accumulation of insoluble inclusion bodies. The inclusion bodies were solubilized in 4 M urea and recovered by slow dialysis as described previously [16]. Like the wild-type and native $\gamma$ subunits [16], all the $\gamma$ mutants tended to aggregate during dialysis when protein concentration was high. On SDS/PAGE, wild-type $\gamma$ protein and the truncated polypeptides migrated for distances consistent with the extent of truncation.
Table 1. Amino-acid and primer sequences of truncated mutants. Listed below are the amino-acid sequences and PCR primers of truncation mutants (Δ) of the γ subunit of spinach chloroplast ATP synthase. The numbers in the plasmids indicate the numbers of residues deleted followed by N designating N-terminus truncation, respectively. The truncations begin with deletion of eight residues with successive deletion of four residues up to 20 residues from the N-terminus, and N-terminal mutants with deletion of 60 residues (ΔN60).

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Amino-acid sequences</th>
<th>Forward primers (5'→3')</th>
<th>Reverse primers (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET11–γWT</td>
<td>ANLRELDRIGSVKNTQKITEMKLVAAXK–31</td>
<td>TTTGTCATATGCAAACCTCCCGTACGGC</td>
<td>ATTCGGAC</td>
</tr>
<tr>
<td>pET11–γΔN8</td>
<td>…RIGSVKNTQKITEMKLVAAXK–31</td>
<td>TCCGGCATATGCTCAAAACCGGAGGAAGGGATCCCAT</td>
<td>ATGGAAC</td>
</tr>
<tr>
<td>pET11–γΔN12</td>
<td>…12VKNTQKITEMKLVAAXK–31</td>
<td>TGGCCCATATGCTCAAAACCGGAGGAAGGGATCCCAT</td>
<td>ATGGAAC</td>
</tr>
<tr>
<td>pET11–γΔN16</td>
<td>…16QKITEAMKLVAAXK–31</td>
<td>TGCCGATATGCAGAAGATCCGAGGCAATTAAATTC</td>
<td>ATGGAAC</td>
</tr>
<tr>
<td>pET11–γΔN20</td>
<td>…20 EAMKLVAAXK–31</td>
<td>TCCGGCATATGCGAGCAATTGAAGCTCGTC</td>
<td>ATGGAAC</td>
</tr>
<tr>
<td>pET11–γΔN60</td>
<td>…60 TE–62</td>
<td>TCCGGCATATGCGAGCAATTGAAGCTCGTC</td>
<td>ATGGAAC</td>
</tr>
</tbody>
</table>

(Found on page 2A). Each of the γ constructs reacted with γ antisera on immunoblots (data not shown). The truncated polypeptides were designated γΔN8 to γΔN60 according to the number of amino-acid residues deleted from the γ subunit N-terminus. The N-terminus amino-acid sequence of γ subunit from chloroplasts is shown in Table 1.

**ATP hydrolytic activity of the mutant assemblies**

We tested ATP hydrolytic activity of the reconstituted assemblies. Incubation of the native γ protein from chloroplasts with isolated αβ subunits (Fig. 2B) resulted in their assembly into a stable, highly active αβγ complex under optimal conditions. The cloned γ polypeptide was identical with the native γ subunit in its ability to form a fully active core enzyme complex [16,26]. Mg$^{2+}$-ATPase activity was measured in the presence of sodium sulfite, a strong stimulator of ATP hydrolysis [16]. The relative rates of ATP hydrolysis of these reconstituted assemblies in the presence of either Ca$^{2+}$ or Mg$^{2+}$ as the bivalent cation substrate were compared (Fig. 3). The wild-type assembly exhibited the maximum activity [14.4 μmol P$_1$/(mg protein)$^{-1}$min$^{-1}$], consistent with previous results showing that assembling the cloned γ polypeptide with the isolated αβ subunits resulted in a fully active core enzyme complex [26], although there were some differences among the hydrolytic rates. Deletion of eight residues from the γ subunit N-terminus impaired the ATP hydrolytic ability, despite differences between Mg$^{2+}$-ATPase and Ca$^{2+}$-ATPase. The deletion of 12 residues resulted in a greater decrease in Ca$^{2+}$-ATPase activity. About 6% of ATP hydrolytic activity retained on deletion of 20 residues. When 60 residues were deleted, the rate of ATP hydrolysis of the reconstituted assembly was similar to the αβ complex containing no γ subunit.

**Interaction of subunits γ and ε in vitro**

GST pull-down assays were used to detect γ–ε interaction. The full-length cDNA encoding the ε subunit was fused to the C-terminus of the GST gene in the expression plasmid, and the GST-fusion protein was over-expressed in _E. coli_. As shown in Fig. 4, GST alone did not bind to the wild-type γ subunit (γWT); in contrast, GST–ε was able to bind directly to all the γ constructs. It was also able to bind to the γΔN8, γΔN12
and cDN16 molecules with similar affinity to cWT. Deletion of 20 residues resulted in slight impairment of the binding activity.

Inhibitory effects of the ε subunit

Given that the N-terminal deletions of the γ subunit barely inhibited the interaction between γ and ε, further studies were performed to confirm the responses of the mutant assemblies to the inhibitory ε subunit. The inhibitory responses of the Ca2+-ATPase activity of the different mutant assemblies were examined after the addition of the ε subunit (Fig. 5). The γβγWT assembly that exhibited the highest Ca2+-ATPase activity was inhibited by 84%. The inhibitory effects of the ε subunits on hydrolytic rates in the mutant (γΔN8, γΔN12, γΔN16 and γΔN20) assemblies varied greatly, ranging from 35% to 63% reduction in hydrolysis.

Discussion

The studies presented here focused on the importance of the N-terminus of the γ subunit during ATP hydrolysis in addition to examining the role of binding and inhibition of the ε subunit. A schematic representation of bovine heart mitochondria F1 is shown in Fig. 1. The conserved N-terminal region of the γ subunit forms an antiparallel left-handed coiled coil with the C-terminal part, which penetrates into a cavity formed by the αβ3 hexamer. The γ subunit’s N-terminus makes contact with the C-terminal domain of the βE subunit, which contains the conserved DELSEED motif [7,8]. It is generally accepted that the γ subunit confers the asymmetric properties of the catalytic sites by interacting with the α and β subunits, resulting in catalytic cooperativity. The permanent asymmetry of
isolated CF$_1$ was found in labeling experiments with Lucifer Yellow [29], which possibly indicated that the N-terminal part of subunit $\gamma$ remains in contact with the $\alpha$$_{E}$ and $\beta$$_{E}$ subunits during the complete catalytic turnover without a full 360° rotation [4]. However, direct observation of the movement of subunit $\gamma$ in isolated CF$_1$ was also determined recently to resemble that of $E$. coli $F_1$ or thermophilic bacterial $F_1$ subcomplex, thereby favoring multisite catalysis with a full 360° rotation.

The data presented here reveal that deletion of 60 residues eliminated almost all hydrolytic activity of the reconstituted assembly; moreover, the removal of eight residues abolished most of the activity. When up to 20 residues were deleted, very low ATP hydrolytic activity was retained (Fig. 3). There are two possible explanations for the above results. (a) The deletions impaired the stability of the reconstituted assemblies and the efficient assembly of the $\gamma\beta$ complex with recombinant $\gamma$ constructs; or (b) the structural change in the truncated $\gamma$ construct altered the asymmetry conformation of $\alpha_3\beta_3\gamma$, thereby affecting transmission of conformational signals between catalytic sites, which resulted in impaired normal catalytic cooperativity. These observations indicate that the N-terminal part of subunit $\gamma$ is indispensable and functions with other regions of $\gamma$ during stabilization of the $\gamma\beta\gamma$ complex and rotational catalysis. Consistent with previous studies, we also found that Ca$^{2+}$-dependent and Mg$^{2+}$-dependent ATP hydrolytic activities were different, indicating different catalytic mechanisms [28].

It is well established that the $\varepsilon$ subunit, a regulatory protein of ATPase, binds to $\gamma$ directly and rotates with the $\gamma$ and c (homologous to CF$_0$-III in chloroplasts) subunits as a part of a rotor (Fig. 1) [1,2]. The interaction of $\gamma$ and $\varepsilon$ increases when $\gamma$ penetrates into a $\alpha_3\beta_3$ hexamer [26]. The $\varepsilon$ subunit binds to CF$_1$ with an apparent dissociation constant of $< 10^{-10}$ M [20]. In the $\varepsilon$ subunit, the sites of $\gamma$ interaction with $\varepsilon$ were mapped to between R49 and R70, and the C-terminal part beyond K199 [24]. The regulatory $\gamma$ regions of CF$_1$ seem to be very important for $\varepsilon$ subunit binding [30]. Our results also show that the $\varepsilon$ subunit stably binds to $\gamma$, which is consistent with earlier studies [23-25]. Deletion of 20 residues from the N-terminal region did not markedly decrease the binding affinity between the $\gamma$ and $\varepsilon$ subunits in vitro (Fig. 4).

The engineered $\gamma$ subunit bound to the $\varepsilon$ subunit with almost identical affinity, although the inhibitory effects of $\varepsilon$ subunit varied with the number of residues removed. The high binding affinity of $\alpha_3\beta_3\gamma$ for the $\varepsilon$ subunit is essential for inhibition of ATPase catalysis, which is much higher than that of binding $\varepsilon$ to individual $\gamma$ [26]. The truncation seemed not to change the binding affinity between $\gamma$ and $\varepsilon$ (Fig. 5), but the altered asymmetrical conformation of $\alpha_3\beta_3\gamma$ resulting from deletions in $\gamma$ is enough to decrease the binding of $\varepsilon$ to $\alpha_3\beta_3\gamma$. Meanwhile, impaired catalytic cooperativity may not be efficiently inhibited by the $\varepsilon$ subunit. Both of the above may partially explain the varied inhibitory effect of $\varepsilon$ on ATP hydrolysis.

Taken together, we have shown that the N-terminal region of the $\gamma$ subunit is important for stabilization of $\alpha_3\beta_3\gamma$ or cooperative catalysis of isolated CF$_1$. The N-terminal region of subunit $\gamma$ in CF$_1$ is not crucial for binding of subunit $\varepsilon$ in vitro. Further studies are needed to determine candidate residues participating in transmission of conformational signals and efficient energy coupling of the $\gamma$ subunit N-terminus.

**Experimental procedures**

**Materials**

Restriction endonucleases, T4 DNA ligase, Klenow fragment, and Pfu and Taq DNA polymerase were purchased from Takara (Dalian, China) and Promega (Shanghai, China). Sephadex G-50 was purchased from Pharmacia (Uppsala, Sweden). DEAE-cellulose was obtained from Whatmann (Uppsala, Sweden) and hydroxypapitate from Bio-Rad (Hercules, CA, USA). Other reagents were all standard AR grade.

**Generation of $\gamma$ truncation mutants and GST-fusion protein**

Plasmid pJLA503-pchl$\gamma$, a gift from S. Engelbrecht (University of Osnabruck, Germany), contains the atpC genes encoding the ATP synthase $\gamma$ subunit [31]. The wild-type $\gamma$ fragment was PCR amplified from pJLA503-pchl$\gamma$, and five truncation mutants were PCR generated with the mutagenesis primers (Table 1). The PCR products were digested with NdeI and BamHI, and subsequently subcloned into the pET11b expression vector. The resulting plasmids were confirmed by DNA sequencing. For construction of the GST-$\varepsilon$ fusion protein, full-length cDNA encoding the $\varepsilon$ subunit was amplified by PCR using pJLA-$\varepsilon$WT [23] as a template with the following primers: 5'-GACGGATCC CCATGACCTTTAATCTTTGT-3' as the 5' primer and 5'-ATAGTCGACCTGGTTACGAAGAAATCG-3' as the 3' primer. The PCR products were cleaved with BamHI and EcoRI, and cloned into plasmid pGEX-5X-1 (Amer sham-Pharmacia Biotech, Shanghai, China). The resulting plasmid pGEX-5X-$\varepsilon$ was confirmed to be inframe with the GST cassette by DNA sequencing.
Solubilization and folding of overexpressed γ mutants and GST-fusion protein

The resulting pET11b plasmids containing the atpC gene were transformed into the expression strain E. coli BL21(DE3)/pLysS. The resulting E. coli cells were grown at 37 °C in Luria–Bertani medium containing 1-ampicillin. Cells were induced with 0.4 mM isopropyl-thio-β-d-galactoside in mid-exponential phase, incubated for 7 h, and harvested as described previously [32]. Solubilization and folding of the insoluble γ polypeptide were performed according as described previously [16]. Overexpression and collection of GST or GST–ε fusion protein in E. coli were carried out as previously described [23].

Preparation and reconstitution of an αβ complex and γ mutants

An αβ complex was isolated from CF1 (– δε) as described previously [26]. Reconstitution of the complex and γ mutants was carried out as described previously [26]. The incubated mixture was assayed directly for ATP hydrolytic activity.

In vitro binding assays of ε with γ subunit and immunoblotting analysis

Equal amounts of GST or GST–ε fusion proteins were bound to glutathione–Sepharose 4B beads in binding buffer [50 mM Tris/HCl, 100 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride and 10% (v/v) glycerol, pH 8.0] at 4 °C for 2 h. The bound glutathione–Sepharose 4B beads were washed three times with binding buffer to remove unbound fusion proteins. These beads were incubated with equal amounts of the γ constructs for 4 h at 4 °C in binding buffer and washed four times with binding buffer to remove unbound proteins. Subsequently, the beads were suspended in 2× SDS loading buffer and boiled for 3 min. Proteins released from the beads were analyzed by SDS/PAGE (15% polyacrylamide gel) [23], transferred to nitrocellulose membrane, and detected by western immunoblot analysis using an ECL Western Blotting Detection System (Amersham) and γ antiserum.

CF1 and CF1 (–δε) preparation and measurement of ATPase activity

CF1 and CF1 (–δε) were prepared from fresh market spinach as described previously [33,34]. Before use, the proteins were desalted on Sephadex G-50 centrifuge columns [35]. ATPase activities were determined by measuring phosphate release for 5–10 min at 37 °C. The assay was performed in 1 mL volumes of assay mixture containing 50 mM Tricine/NaOH, pH 8.0, and 5 mM ATP. Ca^{2+}-ATPase was carried out in the presence of 5 mM CaCl₂, and Mg^{2+}-ATPase in the presence of 2 mM MgCl₂ and 20 mM Na₂SO₃. The reaction was stopped by adding 200 μL 20% trichloroacetic acid. γ Antiserum was raised by subcutaneous injections into rabbits. Inclusion bodies containing recombinant γ polypeptide were subjected to SDS/PAGE. Proteins were recovered from Coomassie Brilliant Blue R250-stained gel bands and used for the immunization of rabbits. Protein concentration was measured by the method of Bradford [36].

Acknowledgements

This work was supported by the National Natural Science Foundation of China (30170078) and State Key Basic Research and Development Plan (G1998010100).

References


17 Nagel R, Cai SX, Keana JF, Koike T & Capaldi RA (1993) The γ subunit of the *Escherichia coli* F1-ATPase can be cross-linked near the glycine-rich loop region of a β subunit when ADP + Mg2⁺ occupies catalytic sites but not when ATP + Mg2⁺ is bound. J Biol Chem 268, 20831–20837.


32 Ni ZL, Wang DF & Wei JM (2002) Substitutions of the conserved Thr42 increased the roles of the ε subunit of *Escherichia coli* CF1 as CF1 inhibitor and proton gate. Photosynthetica 40, 517–522.


