Functional Consequences of N- or C-Terminal Deletions of the δ Subunit of Chloroplast ATP Synthase†

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ABSTRACT: Mutagenesis was used to generate seven truncation mutants of the spinach (*Spinacia oleracea*) chloroplast ATP synthase δ subunit lacking 5, 11, 17, or 35 amino acid residues from the N-terminus or 3, 9, or 15 residues from the C-terminus. Interactions between these mutants and all other subunits of the chloroplast ATPase were investigated by a yeast two-hybrid system. The results indicate that the N-terminal deletions mainly affected interactions between the δ subunit and the other part of CF_1, but did not significantly affect interactions with the CF_0 sector. In contrast, C-terminal truncations of the δ subunit mainly affected its interaction with the CF_0 sector and caused little impairment in interactions with the other part of CF_1. The conformation of the δ subunit C-terminal domain seems to be more sensitive to the truncations, as shown by minimal expression driven by C-terminal deleted (nine residues) mutants. Further studies showed C-terminal truncations of the δ subunit greatly impaired its ability to restore cyclic photophosphorylation in NaBr vesicles, whereas N-terminal truncations had little effect on the ability of δ to plug the CF_0 channel. None of the mutants impaired ATP hydrolysis by CF_1.

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 cross-membrane proton motive force generated by the respiratory chain or by photosynthetic electron transport (for recent reviews on ATPase in *Escherichia coli*, see refs *1* and *2*; for a review on ATP synthase in chloroplasts, see ref *3*). The general structural features of the enzyme are highly conserved among different organisms. In chloroplasts, ATP synthase consists of two parts: CF_0 and CF_1.[1] CF_0, a membrane-spanning complex, conducts proton flux through the thylakoid membrane and provides an affinity binding site for the CF_1 complex. CF_0 is comprised of four types of subunits: I, II, III, and IV (homologous to subunits b, b′, c, and a in *E. coli*). The stoichiometry of these subunits is $I_1II_1III_{12}IV_1$ (*4*), although 14 subunit III monomer rings have also been observed (*5*). In contrast, CF_1 is extrinsic to the membrane, contains nucleotide-binding and catalytic sites, can hydrolyze ATP at high rates after appropriate treatment (*6*), and consists of five types of subunits in a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$. The catalytic core of the enzyme is $\alpha_3\beta_3\gamma$, consisting of a hexagon of alternating α and β subunits with helices of γ subunit in the center (*7*).

Based on kinetic analysis of ATP hydrolysis and synthesis (*8*, *9*), the alternating catalytic site model was proposed (*8*). This was very soon reinforced by the X-ray crystallographic analysis of $\alpha_3\beta_3\gamma$ structure (*10*). Although there is some evidence that γ rotates within the F_1 core, the structural basis for this remains unclear, especially in terms of the connection between F_1 and F_0 (*11*). The ε subunit, which is part of the stalk region of the enzyme, contains a C-terminal domain and an N-terminal domain (*12*, *13*). The C-terminus is a helix−loop−helix capable of interacting with the α and β subunits (*14*, *15*), and the N-terminus is a strand β-sandwich capable of interacting with γ (*16*, *17*). The δ subunit, another subunit of F_1 (CF_1), is important for the interaction between F_1 (CF_1) and F_0 (CF_0) (*18*, *19*).

The topological arrangement of the δ subunit in the F_1F_0 complex is less well-known than that of the ε subunit. The atomic structure of the δ subunit N-terminus (residues 0−134) of *E. coli* was resolved by NMR, but the C-terminal domain of the δ subunit has not been well defined at this point (*20*). In *E. coli*, it is generally accepted that δ is located outside and near the top of F_1, making it a component of the second stalk (δb_2), which is responsible for connecting F_1 to F_0 (*20*−*22*). Thus, the δ subunit is necessary for the binding of F_1 to F_0 via the b subunit (*23*−*25*). However, the δ subunit was positioned near the bottom of the $\alpha_3\beta_3$ hexamer using X-ray solution scattering, which allows the determination of the overall structure under nearly physiological conditions, by Svergun (*26*). They proposed that there are two structural arrangements or conformations for subunit δ and the subunit undergoes those processes in the EF_1 during ATP hydrolysis. Earlier, Moritani et al investigated the interactions of the F_1-ATPase subunits from *E. coli* using the two-hybrid system and detected interactions between α and δ, β and δ, γ and δ, and δ and ε (*27*). Recent results obtained by Weber demonstrated that helices 1 and 5 in the N-terminal domain of the δ subunit provide the F_1-

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[1] Abbreviations: CF_0, the hydrophobic portion of chloroplast ATP synthase; CF_1, coupling factor one; $CF_1(-\delta)$, CF_1 deficient in δ subunit; IPTG, isopropyl-D-thiogalactopyranoside; PCR, polymerase chain reaction; PMS, phenazine methosulfate.

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Table 1: Amino Acid Sequences of N- and C- Terminal δ Truncation Mutants[a]

plasmid	amino acid sequences	proteins
pGAD424-δ/pET-δ	VDSTASRYASALADVADVTGTLEATNSDVEKLIRIFSEEP-41...	δWT
pGAD424-ΔδN5/pET-ΔδN5	SRYASALADVADVTGTLEATNSDVEKLIRIFSEEP-41...	ΔδN5
pGAD424-ΔδN11/pET-ΔδN11	LADVADVTGTLEATNSDVEKLIRIFSEEP-41...	ΔδN11
pGAD424-ΔδN17	VTGTLEATNSDVEKLIRIFSEEP-41...	
pGAD424-ΔδN35	FSEEP-41...	
pGAD424-δ/pET-δ	...157-EGSKLVDMSVKKQLEEIAAQLEMDDVTLAV	δWT
pGAD424-ΔδC3/pET-ΔδC3	...157-EGSKLVDMSVKKQLEEIAAQLEMDDVT	ΔδC3
pGAD424-ΔδC9/pET-ΔδC9	...157-EGSKLVDMSVKKQLEEIAAQL	ΔδC9
pGAD424-ΔδC15	...157-EGSKLVDMSVKKQLE	

[a] Listed are the amino acid sequences of N- and C-terminal fragments of the δ subunit of chloroplast ATP synthase. The numbers in the plasmids indicate the numbers of residues deleted preceded by N or C, designating N-terminus or C-terminal truncations, respectively. The products are the recombinant proteins of the wild-type δ and its mutants overexpressed in *E. coli*.

binding surface of δ (*28*). In chloroplast, subunits γ, δ, and ε are thought to function at the interface between the membrane-embedded CF_0 and the extrinsic CF_1 (*29, 30*). Evidence from cross-linking studies showed that a zero-length cross-link between δ and CF_0-I could be formed (*31*) and that δ was exclusively cross-linked to α and β (*32*). It has been proposed that the δ subunit acts to plug open CF_0 channels and is necessary for blockage of proton leaks and for high degrees of photophosphorylation restoration (*33*−*35*). Recent studies in our lab showed that there were interactions between the δ subunit and all other CF_1 and CF_0 subunits, except for the α subunit (*36*).

Here, we generated N- and C-terminal truncation mutants of the δ subunit and examined their interactions with all the other subunits of chloroplast ATP synthase, using the yeast two-hybrid system. We observed that the N- and C-terminal domains of the δ subunit functioned differently within interactions with the CF_1 and CF_0 subunits. We additionally investigated the effects of these deletions on restoration of PMS-dependent photophosphorylation in NaBr vesicles and on ATP hydrolysis by soluble CF_1. We found that C-terminal removal of nine residues greatly impaired the ability of the δ subunit to restore cyclic photophosphorylation but that the mutated δ recombinants did not impair ATP hydrolysis by CF_1.

EXPERIMENTAL PROCEDURES

Materials. Restriction endonucleases, T4 DNA ligase, Klenow fragment, and *Pfu* and *Taq* DNA polymerases were purchased from Takara and Promega. *O*-Nitrophenylgalactoside was purchased from Amresco. Other reagents were all standard AR grade.

E. coli, Yeast Strains, Plasmids and Culture Media. E. coli DH5α and BL21(DE3)/pLysS, and yeast SFY526 (*MATa, gal4-542, gal80-538, URA3::GAL1-lacZ, can*^{r}*, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112*) and HF7c (*MATa, gal4-542, gal80-538, URA3::(GAL4 17-mers)*$_3$*-CYC1-lacZ, LYS2::GAL1-HIS3, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112*) were used (from Clontech). Positive control plasmids pVA3, pTD1, pLAM5′, and pCL1 were kindly provided by Dr. Wen Hong. Yeast cells were grown in YPD medium or minimal SD medium at 30 °C.

Construction of Plasmids for Yeast Two-Hybrid System. The coding sequences for spinach chloroplast ATP synthase CF_1 subunits α, β, γ, δ, and ε (*37*) and those for CF_0 subunits I, II, III, and IV (*36*) were PCR-subcloned as described. The coding sequence for the δ subunit was derived from expression vector pJLA502-pchlδ (*38*), which was generously provided by Dr. Sigfried Engelbrecht. Seven truncation mutants of the δ subunit were generated by PCR (Table 1). The PCR products of the C-terminal truncations were digested with *Eco*R I and *Pst* I and those of the N-terminal truncations were digested with *Eco*R I. These fragments were then subcloned into two-hybrid vectors and confirmed by direct sequencing.

Yeast Two-Hybrid Assay of Protein Interactions. Plasmids containing the pGBT9 and pGAD424 derivatives constructed above were introduced into yeast SFY526 or HF7c as described previously (*37*). Transformants were selected in SD medium without leucine and tryptophan supplementation (for SFY526 transformants) or in SD medium without leucine, tryptophan, and histidine supplementation (for HF7c transformants). For liquid β-galactosidase assays, the selected transformants were grown in 5 mL of SD liquid medium and harvested at the logarithmic phase of growth. The harvested cells were suspended in Z buffer (60 mM sodium phosphate, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$) and disrupted by freeze−thawing. An aliquot of this extract was used as the substrate for β-galactosidase assay, and the specific enzyme activity was calculated in Miller units (*39*).

Generation of δ Subunit Mutants. The wild-type δ fragment was PCR-amplified from pJLA502-pchlδ. Four truncation mutants were PCR-generated with the mutagenesis method. The PCR products were then inserted into the *Nde* I and *Bam*H I sites of the pET11b expression vector (Table 1). The resulting plasmids (pET-δ, pET-ΔδN5, pET-ΔδN11, pET-ΔδC3, and pET-ΔδC9) were transformed into the expression strain *E. coli* BL21(DE3)/pLysS.

Expression and Purification of Recombinant δ Subunits. E. coli cells containing the *atpD* gene and its mutants were grown at 37 °C in LB medium containing L-ampicillin (100 μg/mL). Cells were induced with 0.4 mM IPTG in midexponential phase, incubation was continued for 7 h, and then cells were harvested as described previously (*40*). These inclusion bodies were solubilized into a urea solution (6 M urea, 50 mM Tris-HCl, pH 8.0) and recovered by stepwise dialysis. The preparations were then chromatographed on a DEAE-52 anion exchange column equilibrated with 50 mM Tris-HCl (pH 8.0). The purified recombinants were stored at −80 °C.

Assay of ATPase and Photophosphorylation Activity. Mg^{2+}-ATPase activities were measured in soluble CF_1 as described (*32, 38*). Reconstitution of photophosphorylation

Table 2: Interactions between δ Mutants and the Other Subunits of CF_1 as Detected by the Yeast Two-Hybrid System[a]

A. Interactions between N-Terminal-Truncated δ Proteins and Other Subunits of CF_1

	the wild-type δ subunit and its mutants				
CF_1 subunits	GAL4ad-δ	GAL4 ad-ΔδN5	GAL4ad-ΔδN11	GAL4ad-ΔδN17	GAL4ad-ΔδN35
GAL4 bd-α	0.2	0.1	0.1	0.1	0.1
GAL4 bd-β	1.8 ± 0.2	0.7 ± 0.2	0.6 ± 0.2	0.3 ± 0.1	0.1
GAL4 bd-γ	0.6 ± 0.2	0.6 ± 0.3	0.3 ± 0.1	0.1	0.1
GAL4 bd-ε	0.9 ± 0.3	0.4 ± 0.1	0.2	0.1	0

B. Interactions between C-Terminal-Truncated δ Proteins Missing 15 Residues and Other CF_1 Subunits

	β-galactosidase activity	
fusion proteins	growth on SD (−W,−L,−H)[b]	liquid assay (U)
GAL4 ad-ΔδC15 + GAL4 bd-α	−	0.2
GAL4 ad-ΔδC15 + GAL4 bd-β	+++	1.7 ± 0.2
GAL4 ad-ΔδC15 + GAL4 bd-γ	+	0.5 ± 0.1
GAL4 ad-ΔδC15 + GAL4 bd-ε	+	0.5 ± 0.1
GAL4 bd + GAL4 ad	−	0
GAL4 bd-δ[c]	*d*	24.0 ± 2
GAL4 bd-δ + GAL4 ad[c]	*d*	25.0 ± 2

[a] The yeast reporter strain SFY526 was cotransformed with pairs of recombinant plasmids as listed in the table. The level of β-galactosidase activity, presented in Miller units, was determined as described in Experimental Procedures. Values are the means of triple determinations with standard deviations. bd = DNA binding domain; ad = DNA activation domain. [b] The pairs were simultaneously cotransfected into HF7c. Transformants were selected by plating onto SD medium lacking tryptophan, leucine, and histidine. Transformants capable of growth on this plate are followed by "+". [c] The δ subunit fused to bd alone could induce high levels of β-galactosidase expression. [d] Not determined.

and ATP determination was carried out as previously described, using PMS (phenazine methosulfate) as a mediator of electron transport (*34*, *41*, *42*).

Other Procedures. CF_1 and CF_1 lacking the δ subunit were prepared as previously described (*33*, *43*) and stored as ammonium sulfate precipitates. Prior to use, the proteins were desalted on a Sephadex G-50 centrifuge tube (*44*). Spinach chloroplast thylakoids depleted of CF_1 ("NaBr vesicles") were prepared by treatment with 2 M NaBr as described (*45*). Protein concentrations were measured by the Bradford method (*46*).

RESULTS

Interactions of Mutant δ Subunits with the Other Part of CF_1. Interactions between pairwise combinations of the recombinant δ subunits and the other subunits of CF_1 were tested by the yeast two-hybrid system. When the δ subunit was fused with the DNA-binding domain of GAL4 in pGBT9, the β-galactosidase reporter gene was expressed strongly with the δ subunit alone (Table 2, section B). Hence, we chose to use chimeric pGAD424 plasmids carrying the mutated δ fragments fused to the activation domain and test these against the other possible binding partners. Combinations of the wild-type δ and all other subunits of CF_1 (except the α subunit) yielded expression of the reporter gene, which is consistent with previous results (*37*). The combination of β and δ yielded strong expression of the reporter gene, indicating that this subunit interaction is stable (Table 2, section A). Relative weak signals were also observed for the combinations of δ and γ and δ and ε, which were also detected in ATPase from *E. coli* (*27*). Although δ was reported to form a cross-link with α in vitro (*30*), we did not observe this interaction in the two-hybrid system. When 5, 11, or 17 amino acids were deleted from the N-terminus of δ, reporter gene expression was strongly impaired. When 35 residues were deleted from the N-terminus of δ, there was almost no expression of the reporter gene. To check the effect of C-terminal δ deletions on protein interactions, we cotransformed combinations of C-terminal deleted mutant δ with the other subunits of CF_1 into yeast. To our surprise, δ with 15 residues deleted from its C-terminus was able to combine with CF_1 subunit β to yield significant expression of the reporter gene, and weak expression of the reporter gene was observed when tested against γ or ε (Table 2, section B). Further, plasmids pGBT9 and pGAD424, which contained the coding sequences of all the other subunits of CF_1 and the mutated δ, were cotransformed into yeast HF7c. Transformants were selected in SD medium without leucine, tryptophan, and histidine supplementation, and only those combinations in which the two proteins interacted with each other could grow on the selective SD medium. The results (Table 2, section B) were consistent with those obtained by measurement of β-galactosidase activity in SFY526. Taken together, our results indicated that the δ subunit interacts with the other part of CF_1 chiefly via its N-terminal domain.

Interaction of Mutant δ Subunits with the Subunits of CF_0. Next, we observed the expression of the reporter gene when the δ subunit constructs were tested against all CF_0 subunits. We found strong expression generated by the combination of δ and CF_0-I or CF_0-II, indicating stable interactions. Weak expression of the reporter gene was also observed between δ and III or IV (Table 3, section A). In contrast, deletions of as few as three residues from the C-terminus of δ caused significant decreases in reporter gene expression. When nine residues were deleted, the reporter gene was hardly expressed. However, the reporter gene was expressed following cotransformations including N-terminal deletions of as many as 17 or 35 residues of the δ subunit. Combinations of these δ mutants and subunits I or II yielded strong reporter gene expression; for these combinations, the growth of cotransformants on selective SD medium was consistently strong as well (Table 3, section B).

Table 3: Interactions between δ Mutants of CF_1 and CF_0 Subunits Detected by the Yeast Two-Hybrid System

A. Interactions between C-Terminal-Truncated δ Proteins and CF_0 Subunits

	the wild-type δ subunit and its mutants			
CF_0 subunits	GAL4 ad-δ	GAL4 ad-ΔδC3	GAL4 ad-ΔδC9	GAL4 ad-ΔδC15
GAL4 bd-I	1.2 ± 0.2	0.5 ± 0.1	0.1	0.1
GAL4 bd-II	1.1 ± 0.3	0.2	0.2	0.1
GAL4 bd-III	0.4 ± 0.2	0.4 ± 0.1	0.2	0.1
GAL4 bd-IV	0.3 ± 0.1	0.2	0.1	0.1

B. Interactions between N-Terminal-Truncated δ Proteins Missing 17 or 35 Residues with CF_0 Subunits

	β-galactosidase activity	
fusion proteins	growth on SD (−W,−L,−H)[a]	liquid assay (U)
GAL4 ad-ΔδN17 + GAL4 bd-I	++	1.0 ± 0.2
GAL4 ad-ΔδN17 + GAL4 bd-II	++	0.8 ± 0.3
GAL4 ad-ΔδN17 + GAL4 bd-III	+	0.3 ± 0.1
GAL4 ad-ΔδN17 + GAL4 bd-IV	−	0.2
GAL4 ad-ΔδN35 + GAL4 bd-I	++	0.9 ± 0.2
GAL4 ad-ΔδN35 + GAL4 bd-II	++	0.8 ± 0.2
GAL4 ad-ΔδN35 + GAL4 bd-III	−	0.2
GAL4 ad-ΔδN35 + GAL4 bd-IV	−	0.2

[a] The pairs were simultaneously cotransfected into HF7c. Transformants were selected by plating onto SD medium lacking tryptophan, leucine, and histidine. Transformants capable of growth on this plate are followed by "+".

Overexpression of the Spinach Chloroplast atpD Gene in E. coli. The yeast two-hybrid system is a yeast-based genetic assay system used to detect protein−protein interaction in vivo. To further test the functional consequence of N- or C-terminal deletions of the δ subunit on ATP hydrolysis by CF_1 and photophosphorylation restoration, we constructed chimeric pET11b plasmids carrying the genes for wild-type δ, truncated δ subunits with N-terminal deletions of 5 or 11 residues, and those with C-terminal deletions of 3 or 9 residues (Table 1). All chimeric plasmids were transformed into BL21(DE3)/pLysS. The spinach chloroplast *atpD* gene, contained in the pET11b expression vector, had a high expression level in *E. coli*; more than 40 mg of recombinant δ proteins could be obtained in per liter of culture medium, and we observed accumulations of the recombinant protein in insoluble inclusion bodies. All recombinants were applied to SDS−PAGE, which revealed that wild-type δ protein and the mutated peptides migrated at the expected molecular weights (Figure 1).

Restoration of Photophosphorylation in NaBr-Treated Thylakoids (NaBr Vesicles). NaBr vesicles are nearly devoid of CF_1; they show almost no cyclic-photophosphorylation but evidence restoration of cyclic-photophosphorylation when reconstituted with CF_1 (*43*). Restoration of cyclic-photophosphorylation in NaBr vesicles was determined using PMS as a mediator of cyclic electron transport. Table 4 shows the successful reconstitution of photophosphorylation when we added recombinant δ mutants plus spinach $CF_1(-\delta)$ to CF_1-deficient spinach thylakoid membranes. Addition of wild-type δ greatly improved the degree of restoration of cyclic-photophosphorylation, and addition of N-truncated δ recombinants was as effective as wild-type δ. In contrast, C-truncated mutants were unable to restore cyclic-photophosphorylation activity well.

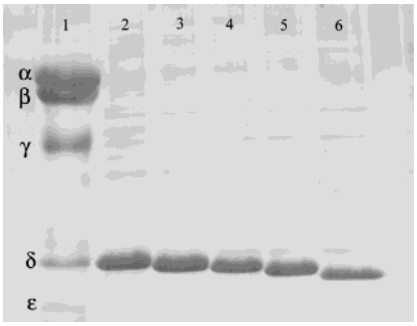

FIGURE 1: Gel electrophoresis profiles of purified δWT and mutants overexpressed in *E. coli*. Preparations were analyzed by SDS−PAGE on 15% polyacrylamide gels, and proteins were stained with Coomassie Brilliant Blue R. Each lane contained about 4 μg of proteins: lane 1, partially purified spinach chloroplast CF_1; lanes 2−6, purified δWT (wild-type) and truncation mutants ΔδC3, ΔδN5, ΔδC9, and ΔδN11, respectively.

Table 4: Comparison of the Abilities of N- and C-Truncated δ Recombinant Proteins to Restore PMS-Dependent Photophosphorylation in NaBr Vesicles[a]

	ATP synthase (μmol of ATP·mg·chl^{-1}·h^{-1})		
sample	expt 1	expt 2	expt 3
thylakoids	875	830	922
NaBr vesicles	0	0	0
NaBr vesicles + $CF_1(-\delta)$	90	85	84
NaBr vesicles + $CF_1(-\delta)$ + δWT	318	330	310
NaBr vesicles + $CF_1(-\delta)$ + ΔδN5	300	334	320
NaBr vesicles + $CF_1(-\delta)$ + ΔδN11	310	261	256
NaBr vesicles + $CF_1(-\delta)$ + ΔδC3	159	128	168
NaBr vesicles + $CF_1(-\delta)$ + ΔδC9	101	118	140

[a] PMS served as the mediator of cyclic electron transport. The reconstitution experiment was performed as previously described (*30*). NaBr vesicles (equivalent to 10 μg of chlorophyll) were recombined with 20 μg of $CF_1(-\delta)$ and $CF_1(-\delta)$ that was preincubated with 1 μg of δ recombinant protein for about 20 min. For further experimental details, see Experimental Procedures.

Table 5: Mg^{2+}-ATPase Activities of Soluble $CF_1(-\delta)$ Reconstituted with the Wild-Type and Truncated δ Recombinant Proteins

	Mg^{2+}-ATPase activity[a]	
sample	expt 1	expt 2
$CF_1(-\delta)$	10.2	10.8
$CF_1(-\delta)$ + δWT	11.6	11.9
$CF_1(-\delta)$ + ΔδN5	10.9	11.6
$CF_1(-\delta)$ + ΔδN11	12.5	11.0
$CF_1(-\delta)$ + ΔδC3	11.1	10.9
$CF_1(-\delta)$ + ΔδC9	10.5	10.5

[a] The activities are expressed as μmol of Pi formed/mg of protein/min. Mg^{2+}-ATPase activities of CF_1 are 12.6.

Reconstitution of Recombinant δ Proteins with $CF_1(-\delta)$. We then studied the ATP hydrolysis abilities of soluble $CF_1(-\delta)$ reconstituted with mutants and wild-type δ. Recombinant δ proteins were incubated with soluble $CF_1(-\delta)$. As shown in Table 5, the Mg^{2+}-ATPase activities of $CF_1(-\delta)$ were similar to those of CF_1. There were few differences among Mg^{2+}-ATPase activities of soluble $CF_1(-\delta)$ + mutant δ, showing that ATPase activities were almost unaffected by N- and C-terminal truncations of the δ subunit. These results are in good accord with previous results (*32*) and suggest that δ may not be necessary for the hydrolysis of ATP by soluble CF_1.

DISCUSSION

The δ subunit and its equivalent in the mitochondrial ATPase, OSCP, one of the smaller subunits, has not been resolved from any of the available ATPase crystal structures till now, probably due to some disorder in it. A NMR study showed that the individual δ subunit from *E. coli* is a two-domain protein consisting of an N-terminal domain of around 104 residues, which contains a six α-helical bundle, and a less-ordered C-terminal domain (*20*). No information about the three-dimensional structure of the δ subunit in chloroplasts has been reported to date. Reports of cross-linking between δ and α or β have shown that there are interactions between δ and the other subunits of CF_1 (*32*). Here, we extended the previous work by providing additional information about the interactions of δ with the other subunits of CF_1 and those of CF_0. Our yeast two-hybrid results show that the N-terminal domain of δ interacts chiefly with CF_1; these results are consistent with previous reports in *E. coli* (*18*). Recent quantitative analyses of fluorescence signals have shown that helices 1 and 5 in the N-terminal domain of δ subunit in *E. coli* provide the F_1-binding surface of δ (*28*), indicating that the N-terminal domain of δ interacts with the F_1 core. Here, we showed that N-terminal truncations impaired the interactions of δ with the other part of CF_1, and in particular removal of the N-terminal 35 residues, which approximately correspond to helix 1 + 2 in *E. coli* δ, completely blocked the expression of the reporter gene. In addition, we did not observe significant expression of the reporter gene when we tested binding between δ and α, showing possible absence of stable interactions between them under in vivo conditions.

The C-terminal truncation of nine residues caused a great decrease in reporter gene expression when we tested δ against CF_0-I or CF_0-II. This may mean one of two things: (1) the C-terminus of δ interacts with CF_0-I and CF_0-II in vivo, or (2) the C-terminal domain of δ is more sensitive to truncation. In previous studies, zero-length cross-linking between δ and CF_0-I was formed by EDC treatment (*31*). This is consistent with our results in that both indicate stable interactions between δ and CF_0-I. However, the previous study (*31*) found that ∼15 C-terminal amino acids clipped by V8-protease did not affect cross-linking to CF_0-I, whereas we found that C-terminal truncations of nine residues significantly decreased this interaction. This may be explained by the possibility that interactions between two amino acids that are close enough to cross-link may not be observed after truncation due to the conformational damage from the truncation process.

To further reveal the functional consequences of truncations on ATP hydrolysis by CF_1 and cyclic-photophosphorylation activities that are in parallel to degrees of restoration of the proton motive force by the δ subunit, we examined these reactions using wild-type and four truncated δ recombinants. The truncations of δ had no effect on the ATP hydrolysis activities, confirming previous reports suggesting that δ is not necessary for ATP hydrolysis by CF_1 (*41*). As shown in Table 4, the amount of ATP production restored by $CF_1(-\delta)$ plus δWT was much higher than that by CF_1-$(-\delta)$ alone, demonstrating that δ blocked proton leaks and restored the proton motive force effectively since it has been proposed that δ acts to plug open CF_0 channels and is necessary for blockage of proton leaks through CF_0 (*33*−*35*). In contrast, δ with C-terminal truncations could not restore ATP synthesis efficiently through cyclic-photophosphorylation, indicating inefficient restoration of the proton motive force due to the C-terminal truncation. Thus, considering our two-hybrid results, we proposed that δ restored cyclic-photophosphorylation by coupling factors including remnants on NaBr vesicles or those added, resulting from the successful restoration of the proton motive force through the interaction between δ and CF_0 subunits via its C-terminal domain. Previous studies had shown that the degradative loss of about 10 amino acids at the C-terminus has no effect on the reconstitutional activity of δ (*34*). However, in those experiments, δ might not have been completely degraded, and the remaining intact δ may have been involved in the reconstitution. N-terminal truncations of δ had little effect on restoration of the cyclic photophosphorylation, indicating that the N-terminus of δ plays little part in this context.

It has been well established that the δ subunit of *E. coli* and its equivalent in the mitochondrial ATPase, OSCP, are involved in the interaction of F_1 with F_0. For example, EF_1 does not bind to F_0 in the absence of δ (*23*). The removal of as few as four to six C-terminal residues from either δ or OSCP prevents the rebinding of ECF_1 or MF_1, respectively, to F_0 (*23*, *24*). However, the δ subunit is not absolutely required for the binding of CF_1 to CF_0 (*43*). Cross-linking of δ with b in *E. coli* has no effect on its ability to catalyze ATP synthesis, whereas cross-linking of δ with subunits I and II in chloroplast ATP synthase resulted in the inhibition of photophosphorylation and the loss of ATPase activity in chloroplasts (*31*). These observations indicate that there are differences in structure and function among δ molecules from *E. coli*, chloroplasts, and their mitochondrial equivalents. Here, besides those strong binding interactions, we also observed weak signals when testing δ against γ, ε, CF_0-III, or CF_0-IV (Table 2, section A, Table 3, section A), which were compatible with previous results (*27*, *36*, *37*). As we know, the two-hybrid system provides a powerful technique for analysis of subunit interactions between two subunits under physiological analysis. However, we should not neglect limitations of the method such as weak signals obtained with this method that might indicate unspecific interactions. If excluding these possibilities, we might deduce that the δ subunit is flexible, allowing some conformational changes relative to the $\alpha_3\beta_3$ core and the CF_0-I and CF_0-II subunits during the catalytic reaction, which was compatible with the results obtained by Svergun (*26*). For this, further studies are urgently required for in-depth understanding for those possible bindings. This is shown by our observation that the δ subunit participated in the blocking of proton flux through the thylakoid membrane via its C-terminus, interacted strongly with CF_1-β via its N-terminus and with CF_0-I and -II via its C-terminus and by observation of weak signals for the δ with ε, γ, III, and IV subunits. All these observations may provide new insights into the subunit−subunit interactions of chloroplast ATP synthase.

In summary, we showed that the N-terminal domain of the chloroplast δ subunit binds to the other parts of CF_1, whereas its C-terminal domain interacts chiefly with the CF_0 sector. The C-terminal region of chloroplast δ seems to be more sensitive to truncations, which cause great decreases

in the ability of the δ subunit to restore photophosphorylation activity.

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