Cross-linking of Engineered Subunit δ to (αβ)₃ in Chloroplast F-ATPase*

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Ser → Cys mutations were introduced into subunit δ of spinach chloroplast F₆F₅-ATPase (CF₆CF₅) by site-directed mutagenesis. The engineered δ subunits were overexpressed in Escherichia coli, purified, and reassembled with spinach chloroplast F₆F₅-ATPase (CF₆) lacking the δ subunit (CF₆(-δ)). By modification with eosin-5-maleimide, it was shown that residues 10, 57, 82, 160, and 166 were solvent-accessible in isolated CF₁ and all but residue 166 also in membrane-bound CF₀CF₁. Modification of the engineered δ subunit with photolabile cross-linkers, binding of δ to CF₁(-δ), and photolysis yielded the same SDS gel pattern of cross-link products in the presence or absence of ADP, phosphate, and ATP and both in soluble CF₁ and in CF₀CF₁. By chemical hydrolysis of cross-linked CF₁, it was shown that δ₁₁₀C was cross-linked within the N-terminal 62 residues of subunit β. δ₁₀₅₇C, δ₈₂C, and δ₁₆₀C were cross-linked within the N-terminal 192 residues of subunit α. Cross-linking affected neither ATP hydrolysis by soluble CF₁ nor its ability to reassemble with CF₆ and to structurally reconstitute ATP synthesis. Functional reconstitution, however, seemed to be impaired.

F-ATPases synthesize ATP at the expense of protonmotive force (1–7) or sodium motive force (8, 9). F-ATPase is composed of the membrane-embedded proton (sodium) channel (F₀) and force (1–7) or sodium motive force (8, 9). F-ATPase is composed however, seemed to be impaired.

Subunits γ, δ, and ε are thought to function at the interface between the membrane-embedded F₀ and the extrinsic F₁. They are instrumental for the coupling between ion movements through F₀ and ATP release from F₁ (1–5, 7, 14). Purified subunit δ enhances the reconstitutitional activity of CF₁ lacking subunit δ (CF₁(-δ)) in partially CF₁-depleted thylakoids. This enhancement has been attributed to the plugging of open CF₀ channels. Because of the reduced proton leak, the proton motive force was restored, which activated both the reconstituted and remaining CF₀CF₁ (15–17).

Five Ser → Cys point mutants of chloroplast δ were overexpressed in Escherichia coli. The engineered single Cys residues were modified with a sulfhydryl-specific dye or with heterobifunctional and photoactivatable cross-linking reagents. We studied the topological and functional consequences of cross-linking δ to other F₁ subunits. All mutant δ subunits were cross-linked to either the α or β subunit under all conditions employed. Cross-linking did not impair ATP hydrolysis by soluble CF₁. ATP synthesis by CF₀CF₁ seemed to be impaired.

EXPERIMENTAL PROCEDURES

Materials—Enzymes and reagents for molecular biology were obtained from AMS Biotechnology (Bioggio-Lugano, Switzerland), Life Technologies, Inc., Boehringer Mannheim, and New England Biolabs Inc. Chromatographic media were from Merck and Pharmacia Biotech Inc.; ultrafiltration membranes (YM-10) were from Amicon, Inc.; and electrophoresis equipment (Phast system) was from Pharmacia Biotech Inc. Tentoxin was supplied by Dr. B. Liebermann (Institut für Pharmazie, Friedrich-Schiller-Universität Jena, Jena, Germany). Ellman’s reagent (5,5'-dithiobis(2-nitrobenzoic acid)) and N-(4-(azido-salicylamidobuty)-3’-(2’-pyridyldithio)propionamidine (APDP) were purchased from Pierce; 2-nitro-5-thiocyanatobenzoate was from Sigma (München, Germany); N-(4-azido-2,3,5,6-tetrafluorobenzyl)-3-maleimidopropionamide (TFPAM-3) (18) and eosin-5-maleimide were from Molecular Probes/MbiTec (Göttingen, Germany); and maleimidopropionic acid-2-iodo-4-(trifluoromethyl-3H-diazirin-3-yl)benzyl ester (TIDM3) (19) was from Photoprobes (Knonau, Switzerland).

Plasmids, Bacterial Strains, and Molecular Genetics—We have cloned the gene for spinach δ into pet-3d (20) and expressed the protein in E. coli strain BL21(DE3) (21). Mutant recombinant δ subunits were obtained by synthesizing mutagenesis primers, followed by two consecutive polymerase chain reaction cycles, one to introduce the mutations into the nucleotide sequence and the other one to obtain full-length genes, followed by transformation and expression (22). Recombinant spinach δ was purified from the cytoplasmic fraction by anion-exchange

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chromatography followed by hydrophobic interaction chromatography as described earlier (23). Chromatographic behavior and yields were similar as for wild-type δ (14–18 mg of purified protein/500-ml culture volume). To suppress formation of cysteine-linked δ dimers, 5 mM dithiothreitol was included in all buffers. The electrophoretic mobility of all five mutant δ subunits was indistinguishable from that of wild-type δ. Nucleotide sequencing (24) showed that the sequences of δS10C, δS15C, δS20C, and δS30C were as expected; δS160C contained a second mutation causing position 2 of the amino acid sequence (Asp to Ser) to be changed to Thr. Considering the unchanged chromatographic, electrophoretic, functional, and cross-linking behavior of δS160C (see "Results"), this second mutation was without relevance in the context of this study.

Two additional mutants (δS112C and δS141C) were prepared along with the described mutants, but were discarded. Cys-112 was not accessible in non-denaturing δ (it was titratable with Ellman's reagent only under denaturing conditions), and δS141C did not bind to CF1δ after modification of Cys-141 with TFPAM-3, presumably due to steric hindrance.

**Chemical Modification**—Chemical modifications were carried out after gel filtration δS30C (19 μM) against 50 mM MOPS, pH 7, immediately followed by the addition of the respective modifying reagent (100 mM eosin-5-maleimide, APDP, or TFPAM-5 or 50 mM TIDM3). The reaction was allowed to proceed at room temperature for 1 h in the dark. Excess reagent was removed by a second gel filtration against 25 mM Tris-HCl, pH 7.8. Photoactivation was achieved by 20 min of illumination in a UV transilluminator shielded by an optical filter (Schott KG2, short wavelength cutoff at 340 nm) or by exposure to 20 flashes (300 mJ/cm2) from a frequency-doubled Ruby laser at 347 nm. Laser excitation was superior in avoiding nonspecific protein breakdown as caused by the continuous UV illumination.

Chemical cleavage of X-Cys peptide bonds in cross-linked CF1 was carried out after cyanation of cysteine residues with 2-nitro-5-thio-

| Protein | δ | DTNB bound | Ratio DTNB/δ |
|---------|---|------------|-------------|
|         | μmol | μmol |          |
| δS10C   | 28.0 | 0.5 | 0.02      |
| δS15C   | 35.0 | 31.3 | 0.89      |
| δS20C   | 11.0 | 10.5 | 0.95      |
| δS30C   | 11.4 | 11.8 | 1.04      |
| δS160C  | 7.6  | 7.1  | 0.85      |
| δS166C  | 25.6 | 25.2 | 0.98      |

 Samples comprising identical amounts of protein were prepared as outlined under "Results" and separated by SDS gel electrophoresis. Gels were illuminated with UV light and photographed by digital image processing unit. Pixel (rgb) values for the band representing δ were measured with the public domain program xev (available from ftp.cis.upenn.edu/pub/xev).

**RESULTS**

Accessibility of Engineered Cys Residues in δ—The titration of engineered Cys residues in spinach δ with Ellman's reagent (35) gave ratios of close to 1 for each of the five single point mutants. Under non-denaturing conditions (Table I), all Cys residues were exposed to this reagent in isolated δ.

We studied their accessibility after incorporation of δ into CF1δ(–δ). To this end, CF1δ(–δ) (250 nM) was complemented with δS30C (500 nM); unbound δS30C was removed by anion-exchange chromatography; and labeling was carried out with eosin-5-maleimide (100 μM). For CF1δS10C samples, NaBr vesicles (300 μM chlorophyll) were incubated with CF1δ(–δ) + δS10C (1.5 μg, respectively). After 1 h of incubation, excess label was removed by centrifugation, and the reconstituted vesicles were labeled with eosin-5-maleimide (100 μM). After washing to remove excess label, they were again treated with NaBr to remove the labeled CF1 from the membrane. The isolated CF1 was run on SDS gels and photographed under UV illumination. The relative intensities of the bands of labeled δ and γ were measured, and their ratios were calculated; the results are shown in Table II. It is evident that all introduced cysteines were labeled both in CF1δ and in CF1δS10C. In CF1δS10C, however, the labeling yield of Cys-160 and Cys-166 was decreased. This pointed toward an exposed location of Cys-10, Cys-57, and Cys-82 both in CF1δ and in CF1δS10C. Cys-160 and Cys-166 were less accessible than the other engineered Cys residues in CF1δS10C.

Cross-linking of δ with CF1δ and with CF1δS10C—δS30C was modified with one of three heterobifunctional cross-linkers, APDP, TFPAM-3, or TIDM3. APDP is a cleavable, rather long (1.9 nm) cross-linker specifically reacting with sulfhydryl groups by a diazirine exchange reaction; the photoactivatable group is an azide. TFPAM-3 was introduced by Capaldi and co-workers (18) in cross-linking studies of E. coli F-ATPase. It reacts by its maleimide function with sulfhydryl groups; the spacer is 0.9 nm long, and the photolabile group is a perfluorophenylazide. TIDM3 is also 0.9 nm long, with a maleimide as the sulfhydryl-reactive group and with a diazirine as the photolabile group.

Figs. 1 and 2 document the cross-link products of engineered δ after its modification with the first function of the respective cross-linker, reincorporation into CF1δ(–δ), and photolysis to activate the second function of the cross-linker. Figs. 1 and 2 show the results for the cross-linker TIDM3. Fig. 1 shows a silver-stained SDS gel, and Fig. 2 the respective Western blots with monospecific rabbit antisera directed against spinach CF1δ, CF1δS10C, CF1δS15C, and CF1δS160C. δS10C was cross-linked to subunit α, the other four mutant δ subunits were cross-linked to subunit β. Additional bands in Fig. 1 (protein stain) represent degradation products caused by UV illumination. These were always observed at slightly different positions depending on the cross-linking reagent running between subunits γ and δ. These breakdown products probably originated from subunit β since bands at corresponding spots were visible only in the Western
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Cross-linking by photolysis of chemically derivatized proteins relies both on the specificity of the cross-linker and on the specific re-binding of the modified subunit to its “host.” The specificity of the derivatization reaction was ensured by the chosen pH (7), which favors the attack of maleimides at sulfhydryl groups over the reaction with primary amines by a factor of 1000 (36). Since cysteine-free wild-type δ did not yield cross-link products after UV illumination, nonspecific side reactions could be ruled out.

Specific re-binding of the derivatized δ subunits to CF₁(−δ) was checked by titrating CF₁(−δ) with chemically modified, engineered δ. We observed the same cross-link products (δ ↔ α or δ ↔ β) irrespective of the molar ratio of δXXC over CF₁(−δ) (2:1). Moreover, the binding of wild-type δ followed by the addition of derivatized δXXC completely prevented the formation of cross-links (Fig. 3). This implied that the engineered subunits bound to the same domain on α and β as their wild-type counterpart.

We asked whether the same cross-link pattern as with isolated CF₁(−δ), namely δS10C ↔ β and δS57C/δS82C/δS160C/δS166C ↔ α (cf. Fig. 2), also holds for membrane-bound CF₀CF₁(−δ)+δXXC. Fig. 4 shows Western blots with δ-specific antibodies. The same pattern as in Fig. 2 for CF₁(−δ) was obtained with membrane-bound CF₀CF₁(−δ)+δXXC. The spots of cross-linked and non-cross-linked δ after integration yielded relative amounts of 80, 100, 77, 58, and 18% of cross-linked δS10C, δS57C, δS82C, δS160C, and δS166C, respectively. Thus, the pattern was unchanged, but the yield of cross-link products decreased in the order δS10C ≈ δS57C ≈ δS82C > δS160C >> δS166C, in line with the finding that the yield of labeling with eosin-5-maleimide was decreased with CF₀CF₁/δS160C/δS166C in comparison with CF₀CF₁/δS160C/δS166C (cf. Table II).

In the light of the supposed role of δ at the interface between CF₁ and CF₀, it was surprising to find δ ↔ α/β cross-links exclusively. We attempted to locate the contact region. In view of the rather low yield of cross-links, we chose a chemical method of cleaving peptide bonds in order to pinpoint cross-linked residues. The cyanylation of Cys residues with 2-nitro-5-thiocyanatobenzoate followed by alkaline treatment (25) cleaves polypeptide chains at the amino group of modified Cys residues. Both spinach chloroplast α and β contain one single Cys residue each at positions 194 and 63, respectively. Cleavage at these residues generates two fragments each, with molecular masses of 21 and 35 kDa for subunit α and of 7 and 48 kDa for β. One would then expect 42- or 56-kDa fragments with δ ↔ α and 28 or 69 kDa with δ ↔ β. Modification of subunit δ with maleimides modifies the sulfhydryl group of Cys residues such that the cyanylation with 2-nitro-5-thiocyanatobenzoate becomes impossible, thus preventing a cleavage of cross-linked δ.

Fig. 5 shows Western blots of SDS gels containing the cleavage products of cross-linked CF₁. Monospecific primary antibodies directed against subunit δ (left panel) or against subunit α (right panel) were used. Samples were generated as outlined above. The pretreatment of the samples generated many anti-δ antibody-reactive fragments, cf. wt* and wt (non-illuminated and illuminated CF₁(−δ)+δXXC (where wt is wild type), respectively). Western blots with anti-δ antibody showed that there was only one band at 28 kDa attributable to a δ ↔ β cross-link. It appeared only with δS10C. To expose this band, both the amount of protein loaded onto the SDS gel and the exposure time for recording chemiluminescence were so high that regions of interest with the δ ↔ α cross-links (42 and 56 kDa)
enzymatic activity of the residual CF1 that remained on the b-domain active. A differentiation between both types of reconstituted CF1 were largely unaffected by the formation of cross-links. These data pointed to the "top" of the F1 molecule (10), these data showed the expected unmodified subunit δ of wt, wild type; 10, 57, 82, 160, and 166, the respective δΔX mutants. 150 μg of CF1(δ−δ + δΔX) were incubated for 20 min at room temperature and then cross-linked (δΔX) was previously labeled with TFPAM-3 by a 10-min UV illumination. Samples were gel-filtrated through BioSpin 30 columns (Bio-Rad) in order to remove unbound δ and reduced with 0.5 mM dithiothreitol at 37 °C for 30 min, and then diluted with 0.1 m guanidinium Cl and 0.1 m Tris acetate, pH 8, followed by a 30-min incubation at 37 °C in the presence of 1 mM 2-nitro-5-thiocyanatobenzoate. Samples were acidified by the addition of acetic acid and then gel-filtrated (Pharmacia NAP-5) against 6 m guanidinium Cl and 0.1 m sodium borate, pH 9.0, followed by a 24-h incubation at 37 °C. Thereafter, they were gel-filtrated after modification with the cross-linking reagent. This holds for subunit δ and illuminated for 5 min at room temperature at 340 nm. The activity of the control (CF1(δ−δ + δwt)) before illumination was 17 units/mg of Mg2+-ATPase and 1.5 units/mg of Ca2+-ATPase.

Activity of Cross-linked CF1−The activity of CF1(δ−δ) reconstituted with engineered or wild-type δ and before and after chemical modification and cross-linking was studied 1) by the ability of soluble CF1(δ−δ + δΔX) to hydrolyze ATP and 2) by the degree of reconstitution of ATP synthesis in CF1-depleted thylakoid membranes recombined with CF1(δ−δ + δΔX). Table III shows that the Ca2+- and Mg2+-ATPase activities of soluble CF1 were largely unaffected by the formation of cross-links.

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**FIG. 5.** Western blot with primary antibodies directed against subunit δ (left panel) or subunit α (right panel) of spinach chloroplast CF1, wt*, wild type (non-illuminated); wt, wild type (illuminated); 10, 57, 82, 160, and 166, the respective δΔX mutants. 100 μg of CF1(δ−δ + δΔX) were incubated for 20 min at room temperature and then cross-linked (δΔX) was previously labeled with TFPAM-3 by a 10-min UV illumination. Samples were gel-filtrated through BioSpin 30 columns (Bio-Rad) in order to remove unbound δ, reduced with 0.5 mM dithiothreitol at 37 °C for 30 min, and then diluted with 0.1 m guanidinium Cl and 0.1 m Tris acetate, pH 8, followed by a 30-min incubation at 37 °C in the presence of 1 mM 2-nitro-5-thiocyanatobenzoate. Samples were acidified by the addition of acetic acid and then gel-filtrated (Pharmacia NAP-5) against 6 m guanidinium Cl and 0.1 m sodium borate, pH 9.0, followed by a 24-h incubation at 37 °C. Thereafter, they were gel-filtrated after modification with the cross-linking reagent. This holds for subunit δ and illuminated for 5 min at room temperature at 340 nm. The activity of the control (CF1(δ−δ + δwt)) before illumination was 17 units/mg of Mg2+-ATPase and 1.5 units/mg of Ca2+-ATPase.

| Sample | Mg2+-ATPase activity | Ca2+-ATPase activity |
|--------|----------------------|----------------------|
|        | units/mg             | units/mg             |
| CF1(δ−δ + δwt) | 13.6 | 1.3 |
| CF1(δ−δ + δ85C) | 14.2 | 1.1 |
| CF1(δ−δ + δ57C) | 14.5 | 1.3 |
| CF1(δ−δ + δ82C) | 13.6 | 1.7 |
| CF1(δ−δ + δ160C) | 13.2 | 1.1 |
| CF1(δ−δ + δS160C) | 13.4 | 1.0 |

Data from such reconstitution experiments revealed the following. The reconstitutinal activity (ATP synthesis) of engineered δ was in the range of 94–100% in comparison with wild-type δ both before and after modification with the cross-linking reagent. This holds for δS10C, δS57C, and δS82C. δS160C and δS166C were not as efficient as the other δΔX mutants, especially after modification with the cross-linking reagent (59–79%). This agreed with the observation that residues δ160 and δ166 were partially shielded in CF1, but not in CF1, and might indicate steric hindrance caused by the cross-linker. Photolysis-induced cross-linking did not affect structural reconstitution in EDTA vesicles in comparison with the effectiveness of the modified, but not yet photolyzed samples. The average loss of functional reconstitution of toxin vesicles for all five δΔX mutants ranged between 14 and 28% and thus grossly matched the estimated yield of cross-linking in the range of 10–20%.

Titration of engineered δ with Ellman’s reagent before and after the reaction with maleimides yielded a ratio of >10:1, thus indicating a nearly quantitative derivatization of Cys...
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FIG. 6. SDS electrophoresis of unbound and bound re-extracted fractions from reconstituted vesicles. NaBr vesicles (150 μg of chlorophyll) were incubated with CF1 (300 μg; left panel) or cross-linked CF1 (−δ)+δ160 (300 μg; right panel). The supernatant containing unbound CF1 was removed (lanes 1), and the reconstituted vesicles were re-extracted with NaBr (lanes 2). Lanes +CF1 indicate an additional incubation of NaBr vesicles with 300 μg of CF1 after prior incubation with cross-linked CF1 (−δ)+δ160. 0.5 μg of protein were applied per lane.

FIG. 7. Corey-Pauling-Koltun model of parts of CF1, showing regions of subunits α and β that can be cross-linked to δNC in light gray. Shown are two α subunits at the outside with one β subunit in the center and part of γ sticking out at the bottom. CF1 was homology-modeled (WHATIF (42)) into the published bovine MF1 structure (10). The plot was generated with O (48).

(data not shown). The efficiency of photo-cross-linking, on the other hand, was low. It was conceivable that a subset of non-cross-linked CF1 bound preferentially to CF0. We checked whether cross-linked CF1 per se rebound to CF0 as follows. NaBr vesicles were incubated with CF1 (−δ)+δ160; the supernatant containing unbound CF1 was removed; and the reconstituted vesicles were re-extracted with NaBr. This “second” NaBr extract contained those CF1 molecules that had rebound to CF0. Fig. 6 shows the results of such an experiment with CF1 (−δ)+δ160. Lanes 1 show SDS electrophoretic separations of the unbound fractions; lanes 2 show the re-extracted fractions. The left panel (CF1) shows controls, in which NaBr vesicles (150 μg of chlorophyll) were incubated with 300 μg of CF1; the right panel shows the same experiment with cross-linked CF1 (−δ)+δ160. It was evident that even the δ ↔ α cross-linked CF1 was bound to CF0. Its characteristic band appeared in both the unbound and the re-extracted fractions. We chased bound cross-linked CF1 (−δ)+δ160 with CF1 (300 μg). It did not change the result (cf. lanes +CF1). This implied that both cross-linked CF1 and native CF1 bound to CF0 with similar affinity.

DISCUSSION

We engineered cysteines into subunit δ of spinach chloroplast CF0CF1 at sequence positions (Ser) 10, 57, 82, 160, and 166. The cysteines served as anchors for the maleimide functional group on ATP hydrolysis with CF1 and ATP synthesis with CF1 CF1.

Structural Considerations—The engineered cysteine residues reacted with the fluorescent reagent eosin-5-maleimide when δ was solubilized, but also when δ was incorporated into CF1. All but Cys-160 and Cys-166 were just as reactive when δ was incorporated into CF1 CF1. This points to a rather peripheral location of δ, with most of the engineered Cys residues remaining solvent-exposed even after binding of δ to (CF0)CF1. The largely α-helical structure of subunit δ (84%) (37) with many of the side chains pointing outwards and the expected exposed location of the polar hydroxyl group of Ser residues are compatible with this view. Our interpretation relied on two assumptions. 1) Eosin-5-maleimide does not penetrate into the protein interior; and 2) the reagent does not induce the disassociation of δ from the remainder of the complex. The hindered access of eosin-5-maleimide to Cys-166 in CF1 CF1 makes the first assumption plausible, and the fact that δ was isolated together with CF1 from reconstituted washed NaBr vesicles supports the second.

δ was cross-linked exclusively to α and β, and this allowed us to narrow down its position in CF1. δ participates in the coupling of proton translocation through F0 and substrate conversion in F1. It functionally interacts both with F0 and with F1 (reviewed in Ref. 14). Was δ cross-linked to one particular α/β pair, or was it “bridging” two or even all three α subunits, depending on the position of the engineered Cys residue? In view of the recently published structure of MF1 (10), the length of the cross-linker and the distribution of the cross-links (one between δ and β and four between δ and α) would seem to exclude the latter possibility. It is unlikely that δ traverses one complete β subunit, a distance of at least 50 Å (cf. Fig. 3 in Ref. 10). Instead, δ probably interacts with one single α/β couple.

Where is δ located in CF1? The cross-linked fragments generated by cleavage at Cys residues contained the span β1,62 with δS10C → β and the span α1,192 with δS57C → α, δS82C → α, and δS166C → α. These segments are shown in light gray at the top of CF1 in Fig. 7. This implied that the amino-terminal portions of both subunits α and β were within ~10 Å of δ10, δ57, δ82, and δ166.

According to the atomic structure of F1 (10), most of these portions of α and δ are located at the periphery and at the top third of F1 (cf. Fig. 7). Taking the δ ↔ CF1 CF1 cross-link (38) into account, this would imply the following. Subunit I (α in E. coli) protrudes from the membrane around the outside of F1 up to the point where it contacts δ. In view of the δS10C ↔ β cross-link, Cys-10 is expected rather at the top of the F1 molecule. The mass of subunit δ (21 kDa) and its elongated shape (14) in comparison with known proteins of similar size may allow for a length of ~45 Å. δ then could reach down to just below the height of the nucleotide-binding sites. Beckers et al. (38) concluded that δ is cross-linked to the C-terminal end of subunit I of CF1, which is built from one transmembrane stretch plus a hydrophilic headpiece. If its hydrophilic head is stretched out,
subunit I would be long enough to contact δ. In our view, δ by itself is not part of the stalk linking F₀ and F₁.

The data presented here are compatible with and expand results from other laboratories. Studies with *E. coli* F₁ revealed an α ↔ δ disulfide cross-link (39, 40) involving Cys-140 (equivalent to Cys-141 in spinach δ) (41). *E. coli* α contains Cys residues at positions 47, 90, 194, and 244 in spinach chloroplast α and to positions 47, 90, 201, and 251 in mature bovine heart mitochondrial α, respectively. We homology-built the *E. coli* sequence of subunit α into the Leslie-Walker structure (10)²; modeling was performed with WHATIF 4.99 (42). The modeled positions of the four Cys residues in *E. coli* α are as follows. Residues 47 and 90 are close together and rather exposed at the β-sheet on top of α, with Cys-90 sticking out just a little bit farther than Cys-47; Cys-194 and Cys-243 are both buried in the central domain not far from the nucleotide-binding region. The disulfide cross-link between EF₁-Sys-140 and EF₁-α thus pins the reactive sites on α down to residue 47 or 90. In a different approach, it has been shown that proteolytic digestion of the amino-terminal portion of both EF₁-α (43) and MF₂-α (44) results in loss of the capability to bind EF₁-δ or oligomycin sensitivity-conferring protein. Taken together, these results identify the amino-terminal third of subunit α as the prime candidate for the binding of δ and oligomycin sensitivity-conferring protein.

The rather exposed position of δ on the outside of the upper half of the (αβ)₃ assembly contrasts with conclusions inferred from immunological and proteolysis studies. A rather hidden location of δ within intact CF₁CF₂ has been postulated (27, 45). It is conceivable, though, that antibodies are sterically or electrostatically excluded from the thylakoid membrane surface. An exposed location of δ within CF₁CF₂ is compatible both with the recently published 2.8-Å structure of mitochondrial F₁ (10), which leaves little if any space inside the (αβ)₃γ core, and with previous findings that oligomycin sensitivity-conferring protein is cross-linked to subunit β, but not to the other subunits that presumably form the stalk in MF₂MF₁ (46).

**Outlook**—The yield of cross-linking between δ and α or β was dependent to a small degree on the state of CF₁, solubilized or bound to CF₀, with or without added nucleotides. When engineered and chemically modified δ was added to CF₁(−δ), the activities of isolated CF₁ were unaffected. The hydrolysis activity by solubilized and chemically modified CF₁ was not impaired even after photolysis of the second function of the cross-linker. Cross-linked CF₁ rebound to CF₁ with about the same affinity as native CF₁, and it restored photophosphorylation in partially CF₁-depleted thylakoids (EDTA vesicles) to the same extent as non-cross-linked CF₁. Preliminary data suggested that the ability of modified δ to functionally reconstitute ATP synthesis in NaBr vesicles might be impaired by photo-cross-linking.

Recently, a large-scale (>200°) rotational motion in a few 10 ms of subunit γ relative to immobilized (αβ)₃ of spinach CF₁ was demonstrated (13). In terms of a rotatory mechanism of catalysis (2, 6) with γ acting as a rotor relative to (αβ)₃, we visualize δ together with subunits I and II rather as elements of the stator or counterbearing.

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² A. G. W. Leslie, personal communication.

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