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Cross-linking of Engineered Subunit δ to $(\alpha\beta)_3$ in Chloroplast F-ATPase*

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Ser → Cys mutations were introduced into subunit δ of spinach chloroplast F_0F_1-ATPase (CF_0CF_1) by site-directed mutagenesis. The engineered δ subunits were overexpressed in *Escherichia coli*, purified, and reassembled with spinach chloroplast F_1-ATPase (CF_1) lacking the δ subunit ($CF_1(-\delta)$). By modification with eosin-5-maleimide, it was shown that residues 10, 57, 82, 160, and 166 were solvent-accessible in isolated CF_1 and all but residue 166 also in membrane-bound CF_0CF_1. Modification of the engineered δ subunit with photolabile cross-linkers, binding of δ to $CF_1(-\delta)$, 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 CF_1 and in CF_0CF_1. By chemical hydrolysis of cross-linked CF_1, it was shown that δ_{S10C} was cross-linked within the N-terminal 62 residues of subunit β. δ_{S57C}, δ_{S82C}, and δ_{S166C} were cross-linked within the N-terminal 192 residues of subunit α. Cross-linking affected neither ATP hydrolysis by soluble CF_1 nor its ability to reassemble with CF_0 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 sodiummotive force (8, 9). F-ATPase is composed of the membrane-embedded proton (sodium) channel (F_0) and the extrinsic, water-soluble F_1. CF_1 [1] consists of five different subunits, α (56 kDa), β (54 kDa), γ (36 kDa), δ (21 kDa), and ε (15 kDa), in stoichiometric proportion of 3:3:1:1:1. According to the crystal structure (10), six nucleotide-binding sites are present between subunits α and β, three catalytical mainly on β and three noncatalytical on α. The detailed mechanism by which F-ATPases synthesize ATP and couple ATP hydrolysis to proton pumping is still unknown, but it is generally accepted that two to three of the six nucleotide-binding sites participate cooperatively in the reaction and that ion flux through the F_0 portion of the enzyme causes conformational changes that are relayed into F_1 to drive ATP liberation. The binding change model of ATP synthesis (reviewed in Refs. 2 and 6) envisages a functional cycling between these catalytic sites. Its structural correlate may be a rotation of γ relative to $(\alpha\beta)_3$. This concept is supported by the recently published structure of F_1 from bovine heart mitochondria at 2.8-Å resolution (10), by electron microscopy (11), and by cross-linking data (12). Most recently, intersubunit rotation of subunit γ in $(\alpha\beta)_3$ was time-resolved by polarized spectrophotometry (13). The identity and linkage of the other "stator" or "rotor" elements of F_1 with their counterparts in F_0, however, remain to be elucidated.

Subunits γ, δ, and ε are thought to function at the interface between the membrane-embedded F_0 and the extrinsic F_1. They are instrumental for the coupling between ion movements through F_0 and ATP release from F_1 (1–5, 7, 14). Purified subunit δ enhances the reconstitutional activity of CF_1 lacking subunit δ ($CF_1(-\delta)$) in partially CF_1-depleted thylakoids. This enhancement has been attributed to the plugging of open CF_0 channels. Because of the reduced proton leak, the protonmotive force was restored, which activated both the reconstituted and remaining CF_0CF_1 (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 photoactivable cross-linking reagents. We studied the topological and functional consequences of cross-linking δ to other F_1 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 CF_1. ATP synthesis by CF_0CF_1 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-(*p*-azidosalicylamido)butyl)-3′-(2′-pyridyldithio)propionamide (APDP) were purchased from Pierce; 2-nitro-5-thiocyanatobenzoate was from Sigma (Munich, Germany); *N*-(4-azido-2,3,5,6-tetrafluorobenzyl)-3-maleimidopropionamide (TFPAM-3) (18) and eosin-5-maleimide were from Molecular Probes/MoBiTec (Göttingen, Germany); and maleimidopropionic acid-(2-iodo-4-(trifluoromethyl-3*H*-diazirin-3-yl)benzyl ester (TIDM/3) (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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[1] The abbreviations used are: CF_1, chloroplast F_1-ATPase (soluble part); CF_0, chloroplast proton channel (membrane-embedded); $CF_1(-\delta)$, CF_1 lacking the δ subunit; CF_0CF_1, chloroplast F_0F_1-ATPase; APDP, *N*-(4-(*p*-azidosalicylamido)butyl)-3′-(2′-pyridyldithio)propionamide; TFPAM-3, *N*-(4-azido-2,3,5,6-tetrafluorobenzyl)-3-maleimidopropionamide; TIDM/3, maleimidopropionic acid-(2-iodo-4-(trifluoromethyl-3*H*-diazirin-3-yl)benzyl ester; MOPS, 4-morpholinepropanesulfonic acid; MF_1, mitochondrial F_1-ATPase; EF_1, *E. coli* F_1-ATPase.

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 cystine-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 δ_{S10C}, δ_{S57C}, δ_{S82C}, and δ_{S166C} were as expected; δ_{S160C} contained a second mutation causing position 2 of the amino acid sequence (Asp) to be changed to His. Considering the unchanged chromatographic, electrophoretic, functional, and cross-linking behavior of $\delta_{D2H,S160C}$ (see "Results"), this second mutation was without relevance in the context of this study.

Two additional mutants (δ_{S112C} and δ_{T141C}) were prepared along with the described mutants, but were discarded. Cys-112 was not accessible in nondenatured δ (it was titratable with Ellman's reagent only under denaturing conditions), and δ_{T141C} did not bind to $CF_1(-\delta)$ after modification of Cys-141 with TFPAM-3, presumably due to steric hindrance.

Chemical Modification—Chemical modifications were carried out after gel filtrating δ_{SXC} (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-3 or 50 μM TIDM/3). 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/cm_2) 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 CF_1 was carried out after cyanylation of cysteine residues with 2-nitro-5-thiocyanatobenzoate in 6 M guanidinium chloride/Tris, pH 8, followed by acidification and then by incubation at 37 °C for 24 h in 6 M guanidinium chloride/sodium borate, pH 9 (25). SDS electrophoresis was carried out with the Pharmacia Phast system on 8–25% gradient gels stained with silver/silicotungstic acid (26).

Western blotting was carried out in the Pharmacia Phast system as previously (27). Secondary antibodies were either alkaline phosphatase-conjugated anti-rabbit IgG (Sigma), which was visualized by 5-bromo-4-chloro-3-indolyl phosphate/4-nitro blue tetrazolium, or peroxidase-conjugated anti-rabbit IgG (Boehringer Mannheim), which was visualized by chemiluminescence. Monospecific polyclonal primary antibodies directed against spinach CF_1 subunits were obtained, in cooperation with J. Buschmann and Prof. Dr. R. J. Berzborn (Ruhr Universität, Bochum, Germany), with recombinant α, β, γ, and ε from pET-3a/d expression in *E. coli* (21, 28) as antigen. Antibodies against δ (obtained with electro-eluted δ) were a kind gift of Prof. Berzborn.

Preparation of thylakoids (29), of CF_1-depleted thylakoids by EDTA treatment ("EDTA vesicles") (30), of EDTA- and tentoxin-treated thylakoids ("tentoxin vesicles") (31), and of NaBr-treated thylakoids ("NaBr vesicles") (32) was performed according to published procedures. CF_1 and $CF_1(-\delta)$ were obtained by chromatography as described (27). Reconstitution of CF_0CF_1 in CF_1-depleted thylakoids, ATP synthesis by phenazine methosulfate-mediated cyclic photophosphorylation, and measurement of ATP were carried out as described (16, 27, 31). Measurement of Ca^{2+}- and Mg^{2+}-ATPase activities of soluble CF_1 and phosphate assays (16, 27, 33) and protein determination (34) were all performed according to published procedures.

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 nondenaturing conditions (Table I), all Cys residues were exposed to this reagent in isolated δ.

We studied their accessibility after incorporation of δ into $CF_1(-\delta)$. To this end, $CF_1(-\delta)$ (250 nM) was complemented with δ_{SXC} (500 nM); unbound δ_{SXC} was removed by anion-exchange chromatography; and labeling was carried out with eosin-5-maleimide (100 μM). For CF_0CF_1 samples, NaBr vesicles (300 μM chlorophyll) were incubated with $CF_1(-\delta)+\delta_{SXC}$ (1.5 + 1.5 μM, respectively). After 1 h of incubation, excess CF_1 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 CF_1 from the membrane. The isolated CF_1 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 CF_1 and in CF_0CF_1. In CF_0CF_1, 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 CF_1 and in CF_0CF_1. Cys-160 and Cys-166 were less accessible than the other engineered Cys residues in CF_0CF_1.

TABLE I
Titration of engineered Cys residues in subunit δ with Ellman's reagent

Protein samples were mixed with reagent under nondenaturing conditions. After incubation for 5 min, the absorption increase at 412 nm was determined spectrophotometrically. The amount of bound reagent was calculated using ϵ_{412} = 14,150 M^{-1} cm^{-1} (47).

Protein	δ	$DTNB_{bound}$[a]	Ratio DTNB/δ
	μmol	*μmol*	
δ_{WT}	28.0	0.5	0.02
δ_{S10C}	35.0	31.3	0.89
δ_{S57C}	11.0	10.5	0.95
δ_{S82C}	11.4	11.8	1.04
δ_{S160C}	8.6	7.1	0.83
δ_{S166C}	25.6	25.2	0.98

[a] DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

TABLE II
Labeling efficiency of engineered Cys residues in subunit δ

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 a digital image processing unit. Pixel (rgb) values for the band representing δ were measured with the public domain program *xv* (available from ftp.cis.upenn.edu:/pub/xv).

Sample	δ_{wt}	δ_{S10C}	δ_{S57C}	δ_{S82C}	δ_{S160C}	δ_{S166C}
	%	%	%	%	%	%
CF_1	0	100	79	89	98	110
CF_0CF_1	0	100	90	71	36	12

Cross-linking of δ with CF_1 and with CF_0CF_1—δ_{SXC} was modified with one of three heterobifunctional cross-linkers, APDP, TFPAM-3, or TIDM/3. APDP is a cleavable, rather long (1.9 nm) cross-linker specifically reacting with sulfhydryl groups by a disulfide exchange reaction; the photoactivable 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. TIDM/3 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 $CF_1(-\delta)$, and photolysis to activate the second function of the cross-linker. Figs. 1 and 2 show the results for the cross-linker TIDM/3. Fig. 1 shows a silver-stained SDS gel, and Fig. 2 the respective Western blots with monospecific rabbit antisera directed against spinach CF_1-α, CF_1-β, and CF_1-δ. δ_{S10C} was cross-linked to subunit β; the other four mutant δ subunits were cross-linked to subunit α. 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

blot with anti-β antibodies. Blots with primary antibodies directed against subunits γ and ϵ revealed only bands at the expected positions of monomeric γ and ϵ (data not shown). It was noteworthy that identical cross-link products were observed under rather different conditions, namely in the absence and presence of ADP, phosphate, and ATP. TFPAM and TIDM/3 gave identical results upon cross-linking, whereas APDP cross-linked δ to both large subunits, α and β (data not shown).

Cross-linking by photolysis of chemically derivatized proteins relies both on the specificity of the cross-linker and on the specific rebinding 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 rebinding of the derivatized δ subunits to $CF_1(-\delta)$ was checked by titrating $CF_1(-\delta)$ with chemically modified, engineered δ. We observed the same cross-link products ($\delta \leftrightarrow \alpha$ or $\delta \leftrightarrow \beta$) irrespective of the molar ratio of δ_{SXC} over $CF_1(-\delta)$ (2:1). Moreover, the binding of wild-type δ followed by the addition of derivatized δ_{SXC} 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 $CF_1(-\delta)$, namely $\delta_{S10C} \leftrightarrow \beta$ and $\delta_{S57C}/\delta_{S82C}/\delta_{S160C}/\delta_{S166C} \leftrightarrow \alpha$ (*cf.* Fig. 2), also holds for membrane-bound $CF_0CF_1(-\delta)+\delta_{SXC}$. Fig. 4 shows Western blots with δ-specific antibodies. The same pattern as in Fig. 2 for $CF_1(-\delta)$ was obtained with membrane-bound $CF_0CF_1(-\delta)+\delta_{SXC}$. 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 δ_{S10C}, δ_{S57C}, δ_{S82C}, δ_{S160C}, and δ_{S166C}, respectively. Thus, the pattern was unchanged, but the yield of cross-link products decreased in the order $\delta_{S10C} \approx \delta_{S57C} \approx \delta_{S82C} > \delta_{S160C} \gg \delta_{S166C}$, in line with the finding that the yield of labeling with eosin-5-maleimide was decreased with $CF_0CF_1/\delta_{S160C/S166C}$ in comparison with $CF_1/\delta_{S160C/S166C}$ (*cf.* Table II).

In the light of the supposed role of δ at the interface between CF_1 and CF_0, it was surprising to find $\delta \leftrightarrow \alpha/\beta$ 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-thiocyanobenzoate 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 $\delta \leftrightarrow \alpha$ and 28 or 69 kDa with $\delta \leftrightarrow \beta$. 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 CF_1. 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 $CF_1(-\delta)+\delta_{wt}$ (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 $\delta \rightarrow \beta$ cross-link. It appeared only with δ_{S10C}. 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 $\delta \rightarrow \alpha$ cross-links (42 and 56 kDa)

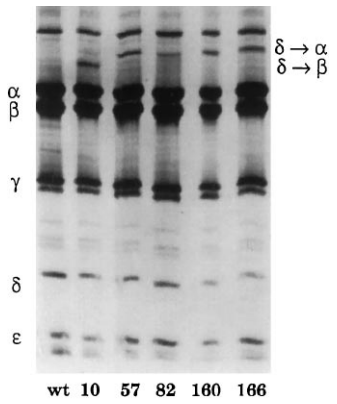

FIG. 1. **SDS electrophoresis of $CF_1(-\delta)$ complemented with TIDM/3-modified δ_{SXC}.** TIDM/3 was photolyzed by nanosecond flashes at a wavelength of 347 nm from a frequency-doubled Ruby laser (300 mJ/cm^2). Pharmacia Phast gel (8–25%) was stained with silver/silicotungstic acid. *wt*, $CF_1(-\delta)+\delta_{wt}$; *10*, $CF_1(-\delta)+\delta_{S10C}$; *57*, $CF_1(-\delta)+\delta_{S57C}$; *82*, $CF_1(-\delta)+\delta_{S82C}$; *160*, $CF_1(-\delta)+\delta_{S160C}$; *166*, $CF_1(-\delta)+\delta_{S166C}$. 0.3 μg of protein were applied per lane.

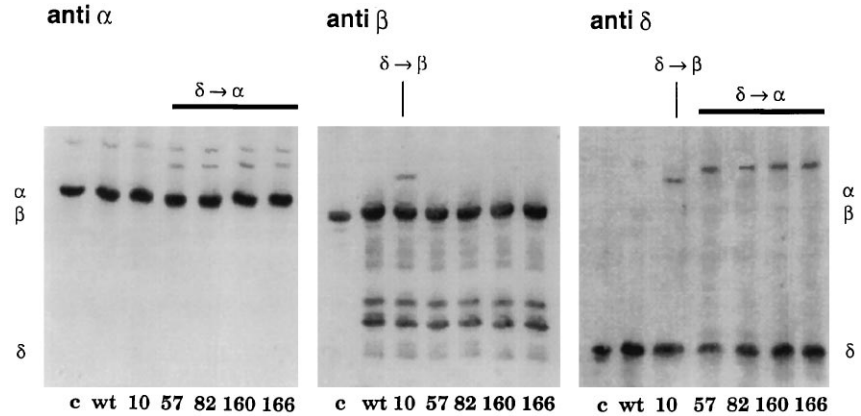

FIG. 2. **Western blot of the samples shown in Fig. 1.** *Left panel*, primary antibodies directed against spinach CF_1-α (diluted 1:1000); *center panel*, primary antibodies directed against spinach CF_1-β (1:5000); *right panel*, primary antibodies directed against spinach CF_1-δ (1:6000). Order of samples is (from left to right) as follows: control (*c*; CF_1), $CF_1(-\delta)+\delta_{wt}$, $CF_1(-\delta)+\delta_{S10C}$, $CF_1(-\delta)+\delta_{S57C}$, $CF_1(-\delta)+\delta_{S82C}$, $CF_1(-\delta)+\delta_{S160C}$, and $CF_1(-\delta)+\delta_{S166C}$. Visualization of IgG was by alkaline phosphatase-conjugated secondary antibodies with 5-bromo-4-chloro-3-indolyl phosphate/4-nitro blue tetrazolium as substrate. *wt*, wild type.

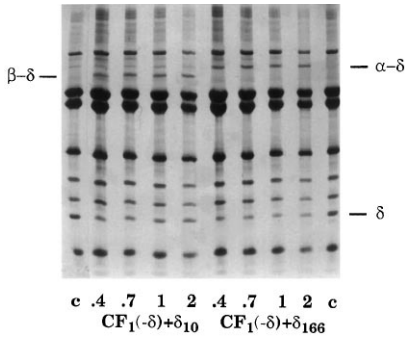

FIG. 3. **SDS electrophoresis of $CF_1(-\delta)$ complemented first with wild-type δ (molar ratio of 1:1), followed by δ_{S10C} (molar ratio of 0.4:1; *left lane c*) or by δ_{S160C} (molar ratio of 0.4:1; *right lane c*), and $CF_1(-\delta)$ complemented with TFPAM-3-modified δ_{S10C} or with δ_{S160C} at molar ratios of δ to CF_1 = 0.4:1, 0.7:1, 1:1, and 2:1.** Following photolysis, 0.3 μg of protein were applied per lane. Pharmacia Phast gel (8–25%) was stained with silver/silicotungstic acid. Labeling of subunits was according to Western blots (*cf.* Fig. 2).

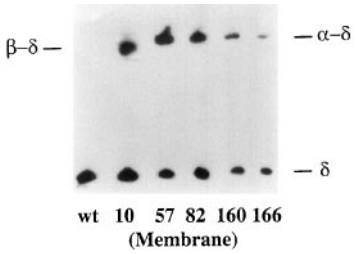

FIG. 4. **Western blot of NaBr vesicles reconstituted with $CF_1(-\delta)$ complemented with δ_{SXC}.** *wt*, wild type; *10*, *57*, *82*, *160*, and *166*, the respective δ_{SXC} mutants. 150 μg of chlorophyll were reconstituted with $CF_1(-\delta)$ and with TFPAM-3-modified δ_{SXC} (300 + 30 μg). Unbound protein was removed by centrifugation. The reconstituted vesicles were resuspended, illuminated by UV for 10 min, pelleted, run on SDS electrophoresis (~2 μg of chlorophyll/lane), blotted, and reacted with anti-δ antibodies (1:4000). Visualization of IgG was by peroxidase-conjugated secondary antibodies and chemiluminescence; exposure was for 30 s.

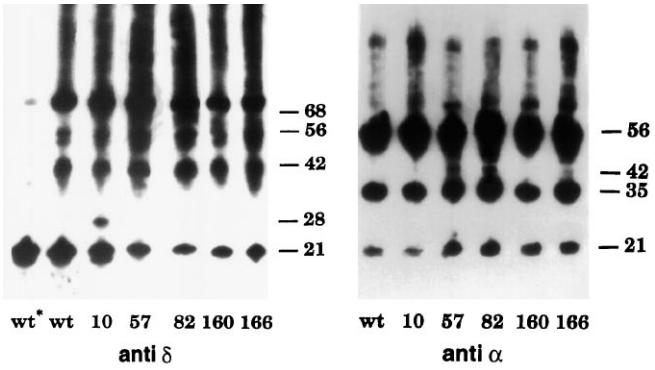

FIG. 5. **Western blot with primary antibodies directed against subunit δ (*left panel*) or subunit α (*right panel*) of spinach chloroplast CF_1.** *wt**, wild type (non-illuminated); *wt*, wild type (illuminated); *10*, *57*, *82*, *160*, and *166*, the respective δ_{SXC} mutants. 100 μg of $CF_1(-\delta)$ + 5 μg of δ_{SXC} were incubated for 20 min at room temperature and then cross-linked (δ_{SXC} 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 8 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 against 10% (v/v) formic acid and precipitated by trichloroacetic acid. SDS electrophoresis was carried out on an 8–25% Pharmacia Phast gel with 1.5 μg of protein/lane. Samples were blotted semi-dry in the Pharmacia Phast system and reacted with anti-δ antibodies (1:4000) or with anti-α antibodies (1:2000). Visualization of IgG was by peroxidase-conjugated secondary antibodies and chemiluminescence (Boehringer Mannheim); exposure was for 5 min (*left panel*) and 1 min (*right panel*).

were overexposed. The Western blots with anti-α antibody showed the expected unmodified α fragments (21 and 35 kDa), along with substantial amounts of uncleaved α (56 kDa) (*right panel*). In addition, there were bands at 42 kDa for δ_{57}, δ_{82}, and (very weak) δ_{166}. As the pertinent two smaller (N-terminal) fragments (21 kDa with α and 7 kDa with β) both are located at the "top" of the F_1 molecule (10), these data pointed to the N-terminal β-sheets of both α and β as contact regions for δ.

Activity of Cross-linked CF_1—The activity of $CF_1(-\delta)$ reconstituted with engineered or wild-type δ and before and after chemical modification and cross-linking was studied 1) by the ability of soluble $CF_1(-\delta)+\delta_{SXC}$ to hydrolyze ATP and 2) by the degree of reconstitution of ATP synthesis in CF_1-depleted thylakoid membranes recombined with $CF_1(-\delta)+\delta_{SXC}$. Table III shows that the Ca^{2+}- and Mg^{2+}-ATPase activities of soluble CF_1 were largely unaffected by the formation of cross-links.

Reconstitution of ATP synthesis by the addition of CF_1 to EDTA-treated and thereby partially CF_1-depleted thylakoids can be simply due to the rebinding of CF_1 and plugging of the proton channel CF_0 (without formation of fully functional CF_0CF_1 pairs). This "structural reconstitution" relies on the enzymatic activity of the residual CF_0CF_1 that remained on the membrane upon EDTA treatment. "Functional reconstitution," on the other hand, implies that the reassembled CF_0CF_1 pairs are active. A differentiation between both types of reconstitution is possible after irreversible blocking of residual CF_1 molecules by tentoxin, followed by the addition of untreated CF_1 (31).

TABLE III

Mg^{2+}- and Ca^{2+}-ATPase activities of soluble $CF_1(-\delta)$ complemented with TIDM/3-modified δ and illuminated for 5 min at room temperature at 340 nm

The activity of the control ($CF_1(-\delta)+\delta_{WT}$) before illumination was 17 units/mg of Mg^{2+}-ATPase and 1.5 units/mg of Ca^{2+}-ATPase.

Sample	Mg^{2+}-ATPase activity	Ca^{2+}-ATPase activity
	units/mg	*units/mg*
$CF_1(-\delta)+\delta_{WT}$	13.6	1.3
$CF_1(-\delta)+\delta_{S10C}$	14.2	1.1
$CF_1(-\delta)+\delta_{S57C}$	14.5	1.3
$CF_1(-\delta)+\delta_{S82C}$	13.6	1.7
$CF_1(-\delta)+\delta_{D2H,S160C}$	13.2	1.1
$CF_1(-\delta)+\delta_{S166C}$	13.4	1.0

Data from such reconstitution experiments revealed the following. The reconstitutional 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 δ_{S10C}, δ_{S57C}, and δ_{S82C}. δ_{S160C} and δ_{S166C} were not quite as efficient as the other δ_{SXC} mutants, especially after modification with the cross-linking reagent (59–79%). This agreed with the observation that residues δ_{160} and δ_{166} were partially shielded in CF_0CF_1, but not in CF_1, 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 tentoxin vesicles for all five δ_{SXC} 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

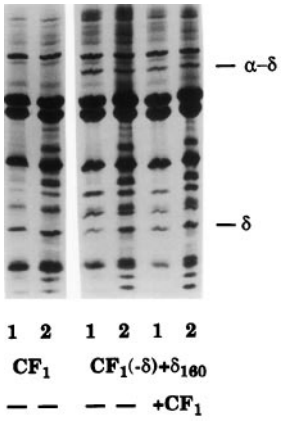

FIG. 6. **SDS electrophoresis of unbound and bound re-extracted fractions from reconstituted vesicles.** NaBr vesicles (150 μg of chlorophyll) were incubated with CF_1 (300 μg; *left panel*) or cross-linked $CF_1(-\delta)+\delta_{160}$ (300 μg;, *right panel*). The supernatant containing unbound CF_1 was removed (*lanes 1*), and the reconstituted vesicles were re-extracted with NaBr (*lanes 2*). *Lanes +CF_1* indicate an additional incubation of NaBr vesicles with 300 μg of CF_1 after prior incubation with cross-linked $CF_1(-\delta)+\delta_{160}$. 0.5 μg of protein were applied per lane.

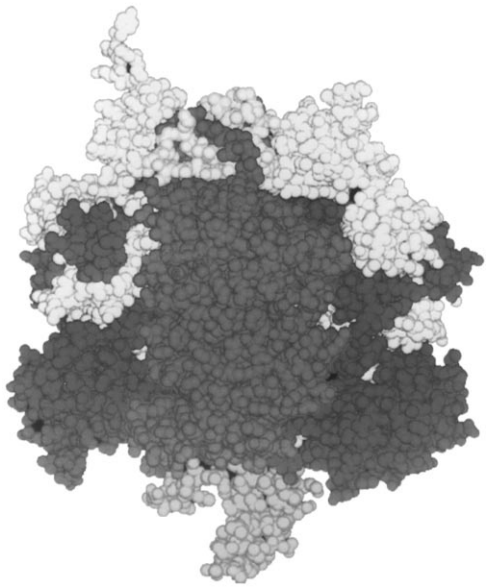

FIG. 7. **Corey-Pauling-Koltun model of parts of CF_1 showing regions of subunits α and β that can be cross-linked to δ_{SXC} in *light gray*.** Shown are two α subunits at the outside with one β subunit in the center and part of γ sticking out at the bottom. CF_1 was homology-modeled (WHATIF (42)) into the published bovine MF_1 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 CF_1 bound preferentially to CF_0. We checked whether cross-linked CF_1 *per se* rebound to CF_0 as follows. NaBr vesicles were incubated with $CF_1(-\delta)+\delta_{SXC}$; the supernatant containing unbound CF_1 was removed; and the reconstituted vesicles were re-extracted with NaBr. This "second" NaBr extract contained those CF_1 molecules that had rebound to CF_0. Fig. 6 shows the results of such an experiment with $CF_1(-\delta)+\delta_{160}$. *Lanes 1* show SDS electrophoretic separations of the unbound fractions; *lanes 2* show the re-extracted fractions. The *left panel* (CF_1) shows controls, in which NaBr vesicles (150 μg of chlorophyll) were incubated with 300 μg of CF_1; the *right panel* shows the same experiment with cross-linked $CF_1(-\delta)+\delta_{160}$. It was evident that even the $\delta \leftrightarrow \alpha$ cross-linked CF_1 was bound to CF_0. Its characteristic band appeared in both the unbound and the re-extracted fractions. We chased bound cross-linked $CF_1(-\delta)+\delta_{160}$ with CF_1 (300 μg). It did not change the result (*cf. lanes +CF_1*). This implied that both cross-linked CF_1 and native CF_1 bound to CF_0 with similar affinity.

DISCUSSION

We engineered cysteines into subunit δ of spinach chloroplast CF_0CF_1 at sequence positions (Ser) 10, 57, 82, 160, and 166. The cysteines served as anchors for the maleimide function of photoactivable cross-linking reagents. The aim was 2-fold: 1) to gain information on the location of subunit δ in the complex and 2) to reveal the functional consequences of cross-linking on ATP hydrolysis with CF_1 and ATP synthesis with CF_0CF_1.

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 CF_1. All but Cys-160 and Cys-166 were just as reactive when δ was incorporated into CF_0CF_1. 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 $(CF_0)CF_1$. 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 dissociation of δ from the remainder of the complex. The hindered access of eosin-5-maleimide to Cys-166 in CF_0CF_1 makes the first assumption plausible, and the fact that δ was isolated together with CF_1 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 CF_0CF_1. δ participates in the coupling of proton translocation through F_0 and substrate conversion in F_1. It functionally interacts both with F_0 and with F_1 (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 MF_1 (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 CF_1? The cross-linked fragments generated by cleavage at Cys residues contained the span β_{1-62} with $\delta_{S10C} \rightarrow \beta$ and the span α_{1-192} with $\delta_{S57C} \rightarrow \alpha$, $\delta_{S82C} \rightarrow \alpha$, and $\delta_{S166C} \rightarrow \alpha$. These segments are shown in *light gray* at the top of CF_1 in Fig. 7. This implied that the amino-terminal portions of both subunits α and β were within ~10 Å of δ_{10}, δ_{57}, δ_{82}, and δ_{166}.

According to the atomic structure of F_1 (10), most of these portions of α and β are located at the periphery and at the top third of F_1 (*cf.* Fig. 7). Taking the $\delta \leftrightarrow CF_0$-I cross-link (38) into account, this would imply the following. Subunit I (α in *E. coli*) protrudes from the membrane around the outside of F_1 up to the point where it contacts δ. In view of the $\delta_{S10C} \leftrightarrow \beta$ cross-link, Cys-10 is expected rather at the top of the F_1 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 CF_0, 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 F_0 and F_1.

The data presented here are compatible with and expand results from other laboratories. Studies with *E. coli* F_1 revealed an $\alpha \leftrightarrow \delta$ disulfide cross-link (39, 40) involving $\delta_{Cys\text{-}140}$ (equivalent to Cys-141 in spinach δ) (41). *E. coli* α contains Cys residues at positions 47, 90, 193, and 243. These residues correspond to positions 48, 91, 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-193 and Cys-243 are both buried in the central domain not far from the nucleotide-binding region. The disulfide cross-link between EF_1-$\delta_{Cys\text{-}140}$ and EF_1-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 EF_1-α (43) and MF_1-α (44) results in loss of the capability to bind EF_1-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 $(\alpha\beta)_3$ assembly contrasts with conclusions inferred from immunological and proteolysis studies. A rather hidden location of δ within intact CF_0CF_1 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 CF_0CF_1 is compatible both with the recently published 2.8-Å structure of mitochondrial F_1 (10), which leaves little if any space inside the $(\alpha\beta)_3\gamma$ 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 MF_0MF_1 (46).

Outlook—The yield of cross-linking between δ and α or β was dependent to a small degree on the state of CF_1, solubilized or bound to CF_0, with or without added nucleotides. When engineered and chemically modified δ was added to $CF_1(-\delta)$, the activities of isolated CF_1 were unaffected. The hydrolysis activity by solubilized and chemically modified CF_1 was not impaired even after photolysis of the second function of the cross-linker. Cross-linked CF_1 rebound to CF_0 with about the same affinity as native CF_1, and it restored photophosphorylation in partially CF_1-depleted thylakoids (EDTA vesicles) to the same extent as non-cross-linked CF_1. 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 $(\alpha\beta)_3$ of spinach CF_1 was demonstrated (13). In terms of a rotatory mechanism of catalysis (2, 6) with γ acting as a rotor relative to $(\alpha\beta)_3$, we visualize δ together with subunits I and II rather as elements of the stator or counterbearing.

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

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