Eur. J. Biochem. 249, 134–141 (1997)

Cross-linking of chloroplast F_0F_1-ATPase subunit ε to γ without effect on activity
ε and γ are parts of the rotor

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(Received 3 June 1997) – EJB 97 0795/3

Cys residues were directed into positions 17, 28, 41 and 85 of a Cys6→Ser mutant of subunit ε of spinach chloroplast F_0F_1 ATP synthase. Wild-type and engineered ε were expressed in *Escherichia coli*, purified in the presence of urea, refolded and reassembled with spinach chloroplast F_1 lacking the ε subunit [$F_1(-\varepsilon)$]. Cys-containing ε variants were modified with a sulfhydryl-reactive photolabile cross-linker. Photocross-linking of ε to $F_1(-\varepsilon)$ yielded the same SDS gel pattern of cross-link products independent of the presence or absence of Mg^{2+} · ADP, phosphate and Mg^{2+} · ATP. ε (wild type) [Ser6,Cys28]ε and [Ser6,Cys41]ε were cross-linked with subunit γ. With chloroplast F_0F_1 the same cross-link pattern was obtained, except for one extra cross-link, probably between [Ser6,Cys28]ε and F_0 subunit III. [Ser6, Cys17]ε and [Ser6,Cys85]ε did not produce cross-links. Cross-linking of ε, [Ser6,Cys28]ε, [Ser6,Cys41]ε to γ in soluble chloroplast F_1 impaired the ability of ε to inhibit Ca^{2+}-ATPase activity. The Mg^{2+}-ATPase activity of soluble F_1 (measured in the presence of 30% MeOH) was not affected by cross-linking ε with γ. Functional reconstitution of photophosphorylation in F_1-depleted thylakoids was observed with F_1 in which γ was cross-linked to [Ser6,Cys28]ε or [Ser6,Cys41]ε but not with wild-type ε. In view of the intersubunit rotation of γ relative to $(\alpha\beta)_3$, which is driven by ATP hydrolysis, γ and ε would seem to act concertedly as parts of the 'rotor' relative to the 'stator' $(\alpha\beta)_3$.

Keywords: F_0F_1 ATP synthase cross-linking; mutation; photolabeling; subunit ε.

ATP synthases synthesize ATP at the expense of proton-motive [1–10] or sodium-motive force [11, 12]. The enzyme is composed of the membrane-embedded proton (sodium) channel F_0 and the extrinsic, water-soluble F_1. F_1 of chloroplasts consists of five subunits, α (56 kDa), β (54 kDa), γ (36 kDa), δ (21 kDa) and ε (15 kDa), in the stoichiometric proportion 3:3:1:1:1. According to the crystal structure [13], six nucleotide-binding sites are present at the α–β interfaces, three catalytic sites mainly on β and three non-catalytic sites on α. The mechanism by which ATP synthases may operate is beginning to emerge. The binding-change model of ATP synthesis [2, 6] envisages a functional cycling between three cooperative catalytic sites. Its structural correlate is a rotation of some subunits relative to $(\alpha\beta)_3$. This concept is supported by the structure of F_1 from bovine heart mitochondria at 2.8-Å resolution [13], and by electron-microscopy [14] and cross-linking data on *Escherichia coli* F_1 [15, 16]. Recently the functional rotation of subunity γ relative to $(\alpha\beta)_3$ has been time resolved by polarized spectrophotometry [17, 18]. In an elegant approach it has been visualized directly by polarized fluorescence microscopy [19]. By application of a theory of stepped molecular motors [20], the data in [17, 18] were shown to be compatible with F_1 acting like a three-stepped motor in ATP hydrolysis [18]. Published cross-linking data [15, 16, 21–30] give hints on the attribution of other subunits to the 'rotor' and the 'stator' portion of the enzyme.

Subunits γ, δ and ε function at the interfaces 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 [1–10, 31]. Surprisingly reconstitution and cross-linking experiments have led to the conclusion that δ is placed at the periphery of F_1, distal to the membrane [32]. Moreover, the cross-linking of δ to $(\alpha\beta)_3$ did not impair the hydrolysis activity of CF_1 [32]. In the context of intersubunit rotation this would identify δ as part of the stator.

Five ε, each with a single Cys residue, were expressed in *E. coli*, namely wild-type ε (containing Cys at position 6) [Ser6, Cys17]ε, [Ser6,Cys28]ε, [Ser6,Cys41]ε, [Ser6,Cys85]ε. Cys residues were modified with photo-activable cross-linkers. Cross-linked products between ε and F_1 lacking the ε subunit [$F_1(-\varepsilon)$] or $F_0F_1(-\varepsilon)$ were analysed with the activities of the cross-linked enzyme. In agreement with cross-linking data obtained with *E. coli* F_1 [28, 30, 33] we found that ε containing single Cys residues at positions 6, 28 or 41 were cross-linked with subunit γ under all conditions tested. With F_0F_1, [Ser6,Cys28]ε probably was cross-linked with subunit III of CF_0. Cross-linking did not impair ATP hydrolysis by soluble F_1 (Mg^{2+}-ATPase activity) but it interfered with the ability of ε to inhibit Ca^{2+}-ATPase activity. More importantly, ATP synthesis by cross-linked F_0F_1 was not completely inhibited. In view of the recently shown intersubunit rotation of γ relative to $(\alpha\beta)_3$ [15–19, 34] these findings identify ε as part of the rotor of ATP synthases.

MATERIALS AND METHODS

Materials. Enzymes and reagents for molecular biology were obtained from AMS Biotechnology, Bethesda Research

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Abbreviations. F_0F_1, F_0F_1-ATPase; F_0, proton channel (membrane-embedded); F_1, ATPase (soluble); $F_1(-\delta)$, F_1 lacking the δ subunit; $F_1(-\varepsilon)$, F_1 lacking the ε subunit; TFPAM-3, *N*-(4-azido-2,3,5,6-tetrafluorobenzyl)-3-maleimidopropionamide; TIDM/3, maleimidopropionic acid-(2-iodo-4-(trifluoromethyl-3H-diazirin-3-yl)benzylester.

Laboratories, Boehringer-Mannheim, and New England Biolabs. Chromatographic media were from Merck and Pharmacia Biotech, ultrafiltration membranes (YM 10) from Amicon, electrophoresis equipment from Pharmacia Biotech (PhastSystem). 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) was obtained from Pierce, *N*-(4-azido-2,3,5,6-tetrafluorobenzyl)-3-maleimidopropionamide (TFPAM-3) [35] from Molecular Probes/MoBiTec and maleimidopropionic acid-(2-iodo-4-(trifluoromethyl-3H-diazirin-3-yl) benzylester (TIDM/3) [36] from Photoprobes.

Plasmids, bacterial strains and molecular genetics. We have cloned the gene for spinach ε into pET-3d [37] and expressed the protein in *E. coli* strain BL21(DE3) [38]. Mutant recombinant ε subunits were obtained by synthesizing mutagenesis primers followed by two consecutive PCR cycles, one to introduce the mutations into the nucleotide sequence and the other to obtain full-length genes, followed by transformation and expression [39]. Prior to introducing further Cys residues, the one occurring in wild-type ε was changed to Ser. Upon expression, recombinant spinach ε precipitated into inclusion bodies and was further purified in the presence of 8 M urea, 5 mM dithiothreitol, 50 mM Mes/NaOH pH 5.5, by cation-exchange chromatography on Toyo Soda TSK CM-650(S) [38]. Chromatographic behaviour and yields were similar for the six proteins (12−24 mg purified protein/500 ml culture). The electrophoretic mobilities of the ε variants were the same. Nucleotide sequencing [40] proved the expected sequences.

Chemical modification. Chemical modification with TFPAM-3 or TIDM/3 was carried out after gel filtration of ε (100 μM) against 8 M urea, 50 mM Mops, pH 7, immediately followed by addition of 200 μM TFPAM-3. The reaction proceeded at room temperature over night in the dark. The reaction was terminated by addition of 1 mM *N*-acetyl-cysteine, and excess reagent was removed by gel filtration against 8 M urea, 25 mM Tris/HCl, pH 7.8. Initial cross-linking trials were carried out by diluting ε (dissolved in 8 M urea) directly into solutions containing F_1. Residual urea up to 1 M did not interfere with ATP hydrolysis activity.

Refolding of ε and enrichment of cross-linked F_1. ε was refolded prior to binding to $F_1(-\varepsilon)$. This followed the procedure developed by Cruz et al. [41]. ε (dissolved in 8 M urea) was diluted tenfold into 33% glycerol, 25% ethanol, 16 mM Tris/HCl, 6.25 mM 2-mercaptoethanol, pH 8.0. $F_1(-\varepsilon)$ was added (molar ratio of ε/CF = 5:1), and the resulting solution was gel filtered (Sephadex G-50) to decrease the concentrations of glycerol and ethanol and subjected to anion-exchange chromatography [Toyo Soda TSK DEAE-650(S), 25 mM Tris/HCl, pH 7.8]. This served to concentrate the sample and to remove surplus ε. After illumination, the samples were reduced by addition of 10 mM dithiothreitol, 1 mM MgATP was added and after 2 h the sample was chromatographed [Toyo Soda TSK DEAE-650(S), 25 mM Tris/HCl, 22 mM Mega 9, pH 7.8]. Elution was carried out until the salt concentration of the linear gradient (0−400 mM NaCl) reached 150 mM. At this concentration the buffer was changed to the same composition but without the detergent and the gradient was continued. This procedure largely removed subunits δ and ε [42] except those ε molecules that were cross-linked with γ. Subunit δ was substituted thereafter by addition of 1 mol δ/mol $F_1(-\delta)$.

The cross-linker was photoactivated either by 10 min illumination in an ultraviolet-transilluminator (≈312 nm) or by exposure to 1000 flashes at 50 Hz, 20 s, 500 mJ/cm² from Nd-YAG laser (Coherent) at 357 nm. Monochromatic laser excitation was instrumental in avoiding non-specific protein breakdown caused by the spectrally wider ultraviolet illumination.

SDS electrophoresis was carried out with the Pharmacia Phast System in gradient gels (from 8% to 25% polyacrylamide) stained with silver/silicotungstic acid [43]. Western blots were carried out in the Pharmacia Phast System described previously [42]. Secondary antibodies were peroxidase-carrying anti-rabbit IgG from Boehringer, which were detected by chemiluminescence. Monospecific polyclonal primary antibodies directed against spinach chloroplast F_1 subunits were obtained in cooperation with J. Buschmann and Prof. Dr R. J. Berzborn, Ruhr Universität Bochum, with recombinant α, β, γ and ε pET3a/d expressions in *E. coli* [38, 44] as antigens.

Preparation of thylakoids [45], of F_1-depleted thylakoids by EDTA treatment [46] and of NaBr-treated thylakoids [47] were performed according to published procedures. F_1 and F_1 lacking δ and ε [$F_1(-\delta\varepsilon)$] were obtained by chromatography as described [48, 49]. Reconstitution of F_0F_1 in F_1-depleted thylakoids, ATP synthesis by phenazine-methosulfate-mediated cyclic photophosphorylation and measurement of ATP were carried out as described [50].

The procedure to obtain cross-linked F_0F_1 was as follows. $F_1(-\varepsilon)$ was substituted with a 20-fold molar excess of TFPAM-3-modified ε. NaBr-treated spinach chloroplasts ([47], 300 μg chlorophyll) were reconstituted with 300 μg of $F_1(-\varepsilon)$ + ε in the presence of 10 mM $MgCl_2$. After washing to remove unbound protein, the samples were illuminated for 10 min at 312 nm on a transilluminator followed by extraction of the reconstituted membranes with 0.4 M sucrose, 20 mM Tricine/NaOH, 5 mM $MgCl_2$, 0.4 M $(NH_4)SO_4$, 0.2 mM ATP, 1% (mass/vol.) sodium cholate, 50 mM dithiothreitol, 60 mM n-octyl-glucoside, pH 8.0 [51, 52].

Measurement of Ca^{2+}-ATPase and Mg^{2+}-ATPase activities of soluble F_1, phosphate assays [42, 49, 53], and protein determination [54] were performed according to published procedures. Protein determinations [54] overestimated concentrations of ε by 18% (as determined by amino acid composition) and were corrected accordingly.

Homology-building and graphical manipulations ('docking') were carried out with the programs WhatIf [55] and O [56] on DEC α and Evans & Sutherland ESV workstations.

RESULTS

Cross-linking of ε with F_1 and F_0F_1. The ε proteins were modified with TFPAM-3 [35] or with TIDM/3 [36] in the presence of urea. Since we suspected the high concentration of urea to interfere with chemical modification, the number of Cys residues was titrated with Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)] before and after modification with the cross-linking reagent. Table 1 shows the results and reveals that all ε variants (except wild-type ε) contained the expected single Cys residue, which reacted with TFPAM-3 to a large extent.

All ε variants (including wild-type ε) inhibited the Ca^{2+}-ATPase activity of soluble CF_1 to the same extent, before and after modification with the cross-linking reagents. A 20-fold molar excess of ε was required to achieve greater than 90% inhibition of Ca^{2+}-ATPase activity if ε was dissolved in 8 M urea and diluted directly into the F_1 solution. Prefolding ε yielded around 40% soluble ε, which inhibited Ca^{2+}-ATPase activity by greater than 90% at fourfold molar excess.

Figs 1 and 2 document the cross-link products of wild-type and engineered ε after modification with the first function of the cross-linker, incorporation into soluble $F_1(-\varepsilon)$ and photolysis to activate the second function of the cross-linker. TFPAM-3 and TIDM/3 yielded the same results. Fig. 1 shows a silver-stained SDS gel (bands of the the cross-linked product are barely visible

Table 1. Titration of Cys residues in subunit ε with Ellman's reagent before and after modification with TFPAM-3. ε samples were gel filtered against 6 M guanidinium hydrochloride, 50 mM Tris/HCl, pH 8.0, before mixing with reagent. After incubation for 5 min, the absorption increase at 412 nm was determined spectrophotometrically. The amount of liberated thionitrobenzoate (TNB) was calculated using an ε_{412} of 13700 m^{-1} cm^{-1}.

Protein	Amount of ε	Before TFPAM-3 treatment		After TFPAM-3 treatment	
		amount of TNB liberated	ratio of TNB/ε	amount of TNB liberated	ratio of TNB/ε
	nmol			nmol	
Wild-type ε	10.2	9.8	0.96	1.1	0.11
[Ser6]ε	12.2	0	0	0	0
[Ser6,Cys17]ε	4.7	4.2	0.89	0.5	0.11
[Ser6,Cys28]ε	15.0	13.0	0.87	1.7	0.11
[Ser6,Cys41]ε	7.7	6.0	0.78	0.6	0.08
[Ser6,Cys85]ε	11.9	8.2	0.69	0.9	0.08

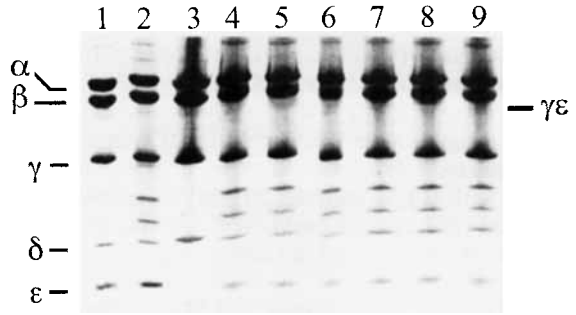

Fig. 1. SDS electrophoresis of chloroplast F_1(-ε) complemented with TFPAM-3-modified ε proteins and photolyzed on a transilluminator at 312 mn. Pharmacia Phast gel (8% to 25% polyacrylamide) stained with silver/silicotungstic acid [43]. 0.3 μg protein was applied/lane. Lane 1, F_1 (not illuminated); lane 2, F_1 (illuminated); lane 3, F_1(-ε) (not illuminated); lanes 4–9, illuminated samples of F_1(-ε) substituted with wild-type ε, [Ser6]ε, [Ser6,Cys17]ε, [Ser6,Cys28]ε, [Ser6,Cys41]ε and [Ser6, Cys85]ε, respectively.

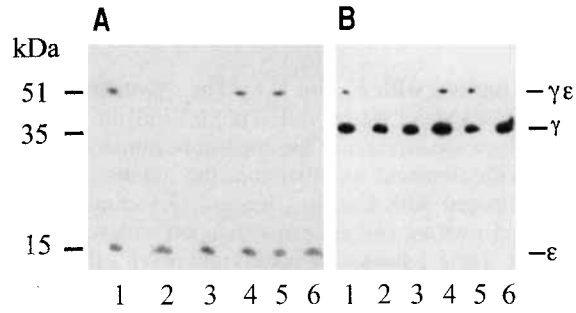

Fig. 2. Western blot of illuminated substituted chloroplast F_1(-ε) substituted with TFPAM-3–labeled ε. 0.3 μg protein/lane were applied. (A) Primary antibodies directed against spinach F_1 ε (diluted 1:5000); (B) primary antibodies directed against spinach chloroplast F_1 γ (1:1000). Visualization of IgG was by peroxidase-carrying secondary antibodies and chemiluminescences (exposure for 30 s). Lanes 1–6, F_1(-ε) substituted with wild-type ε, [Ser6]ε, [Ser6,Cys17]ε, [Ser6, Cys28]ε, [Ser6,Cys41]ε and [Ser6,Cys85]ε, respectively.

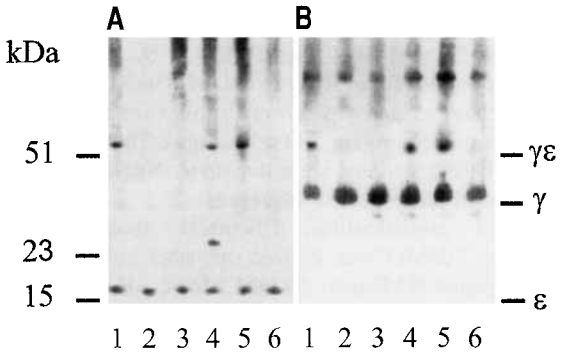

Fig. 3. Western blot of illuminated chloroplast F_0F_1 samples (prepared as outlined in Materials and Methods). 0.3 μg protein/lane was applied. (A) Primary antibodies directed against spinach F_1 ε (diluted 1:5000); (B) primary antibodies directed against spinach F_1 γ (1:1000). Visualization of IgG was by peroxidase-carrying secondary antibodies and chemiluminescences (exposure for 30 s). Lane 1–6, F_1(-ε) substituted with wild-type ε, [Ser6]ε, [Ser6,Cys17]ε, [Ser6,Cys28]ε, [Ser6, Cys41]ε and [Ser6,Cys85]ε, respectively.

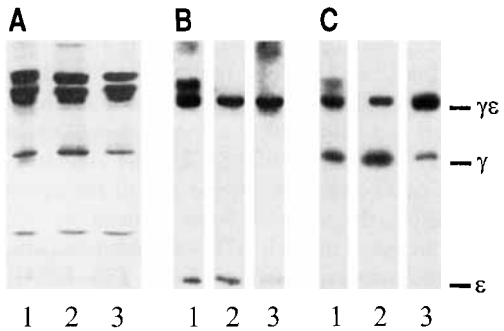

Fig. 4. SDS electrophoresis and western blot of enriched, cross-linked chloroplast F_1. (A) Silver-stained samples; (B), western blot with anti-ε IgG; (C) western blot with anti-γ IgG. Conditions were as described in Figs 1 and 2. Lane 1–3, F_1 containing γ cross-linked to wild-type ε, [Ser6,Cys28]ε and [Ser6,Cys41]ε, respectively.

underneath the band of subunit β at ≈54 kDa) and Fig. 2 the respective western blots with monospecific rabbit antisera directed against spinach chloroplast F_1 γ and ε. Wild-type ε, [Ser6, Cys28]ε and [Ser6,Cys41]ε were cross-linked exclusively with subunit γ and [Ser6,Cys28]ε and [Ser6,Cys85]ε were not cross-linked at all. This pattern was the same in the absence and presence of ADP, phosphate and ATP. Fig. 3 shows western blots obtained with F_0F_1. Modified ε was bound to solubilized F_1(-ε). The complemented F_1 was rebound to F_1-depleted thylakoids, followed by ultraviolet illumination of the reconstituted membranes to activate the cross-linker. Except for an additional band

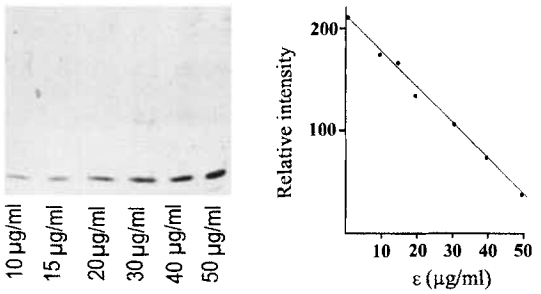

Fig. 5. Correlation between silver-staining intensity of isolated subunit ε and pixel (RGB) values (0, 0, 0 = black; 255, 255, 255 = white). 0.3-µl samples were subjected to SDS electrophoresis in a Pharmacia PhastSystem gel (8% to 25% polyacrylamide) and silver stained [43].

Table 2. Composition of cross-linked F_1 after enrichment by chromatography. SDS gels were stained [43] and photographed by a digital-image processing unit. Relative intensity values for the the bands representing ε were measured with the public domain program *xv* (available from ftp.cis.upenn.edu:/pub/xv).

F_1 γ cross-linked to	Relative intensity of		
	cross-linked sample	non-cross-linked sample	$F_1(-\varepsilon)$
wild-type ε	48	44	8
[Ser6,Cys28]ε	48	44	8
[Ser6,Cys41]ε	70	24	6

at around 25 kDa in the anti-ε-western blot with [Ser6,Cys28]ε (Fig. 3), the cross-link pattern was unchanged in comparison with that obtained with soluble F_1. The location of the additional spot indicated an [Ser6,Cys28]ε-III cross-link product. Attempts to prove this interpretation, however, were unsuccessful due to the lack of suitable antibodies.

The activity of cross-linked F_1. The activity of $F_1(-\varepsilon)$ reconstituted with engineered or wild-type ε followed by cross-linking was studied by the ability of soluble, cross-linked F_1, to hydrolyse ATP, and by the degree of reconstitution of ATP synthesis in F_1-depleted thylakoid membranes, which were recombined with cross-linked F_1. Since the cross-link yields were low, cross-linked F_1 was enriched by chromatography. Fig. 4 shows the resulting preparations of F_1 in which γ is cross-linked to wild-type ε, [Ser6,Cys28]ε or [Ser6,Cys41]ε as seen in SDS electrophoresis after silver staining, and in western blots with antibodies directed against spinach chloroplast F_1 γ and ε. The cross-link product wild-type ε with γ migrated in two distinct bands, which were reactive with antibodies against ε and γ but not with those against α or β.

The procedure resulted in nearly quantitative separation of $F_1(-\varepsilon)$ from F_1, but it did not fully resolve cross-linked F_1 and non-cross-linked F_1 still containing ε. To determine the composition of the samples, they were serially diluted and subjected to SDS electrophoresis, stained and photographed. The resulting pictures were evaluated with respect to residual subunit ε (using pure ε as a standard) and with respect to γ−ε (using γ as a standard) by measuring staining intensines. Fig. 5 shows a typical set of data obtained with pure ε. The good correlation between intensity and actual amount is evident. This allowed us to determine the proportion of cross-linked F_1 and non-cross-linked F_1 (still containing ε) with sufficient confidence. Table 2 shows

Table 3. Mg^{2+}-ATPase activities of soluble $F_1(-\varepsilon)$ substituted with TFPAM-3-modified ε, illuminated and chromatographed as outlined in Materials and Methods. Illumination was by a 312-nm transilluminator or by a 357-nm Nd-YAG laser. The Mg^{2+}-ATPase activities of F_1 and of $F_1(-\varepsilon)$ were 28 µmol ATP hydrolysed · min^{-1} · mg^{-1} (U/mg), the Ca^{2+}-ATPase activities were less than 2 U/mg for F_1 and 22 U/mg for $F_1(-\varepsilon)$.

Sample	Illumination method	Mg^{2+}-ATPase activity	Ca^{2+}-ATPase activity
		%	
$F_1(-\varepsilon)$ + ε	none	100	9
$F_1(-\varepsilon)$	none	100	100
$F_1(-\varepsilon)$ + ε	transillumination	30	26
$F_1(-\varepsilon)$ + ε	laser	100	100
$F_1(-\varepsilon)$ + ε	laser	92	39
$F_1(-\varepsilon)$ + [Ser6,Cys28]ε	laser	100	61
$F_1(-\varepsilon)$ + [Ser6,Cys41]ε	laser	100	39

Table 4. Reconstitution of photophosphorylation. NaBr-treated thylakoids were reconstituted with $F_1(-\varepsilon)$, which was substituted with TFPAM-3−labeled [Ser6,Cys41]ε before illumination and after illumination and enrichment. Cyclic photophosphorylation was measured in the presence of 50 µM phenazinemethosulfate. For further details see text.

Addition	Concentration	Activity
	µg/10 µg chlorophyll	µmol ATP · mg chlorophyll $^{-1}$ · h^{-1}
None		0
$F_1(-\varepsilon)$ + [Ser6,Cys41]ε before illumination	1	12
	5	109
	10	161
	20	257
$F_1(-\varepsilon)$ + [Ser6,Cys41]ε after illumination	1	11
	5	104
	10	155
	20	196
$F_1(-\varepsilon)$	1−20	0
$F_1(-\varepsilon)$ + wild-type ε	20	206

the results for F_1 containing γ cross-linked to wild-type ε, [Ser6, Cys28]ε or [Ser6,Cys41]ε.

Table 3 summarizes the Ca^{2+}-ATPase and Mg^{2+}-ATPase activities of soluble, cross-linked F_1. In view of the composition of the three samples (i.e. more than 90% ε present, cross-linked to γ or not cross-linked) one would have expected Ca^{2+}-ATPase activities around 15% instead of 39% or 61%. The inhibitory activity of ε upon Ca^{2+}-ATPase activitiy was abolished to some extent after cross-linking ε to γ. The Mg^{2+}-ATPase activity was unaffected however.

Table 4 shows the reconstitution of photophosphorylation by $F_1(-\varepsilon)$ that was substituted with TFPAM-3−labeled [Ser6, Cys41]ε and of the enriched, cross-linked F_1 containing γ cross-linked to [Ser6,Cys41]ε. Reconstitution of photophosphorylation (≈80%) was not affected by the ε−γ cross-link to an extent correlating with the extent of cross-linking (70%). Similar experiments with enriched samples containing γ cross-linked to wild type ε or [Ser6,Cys28]ε showed that the reconstitutive ef-

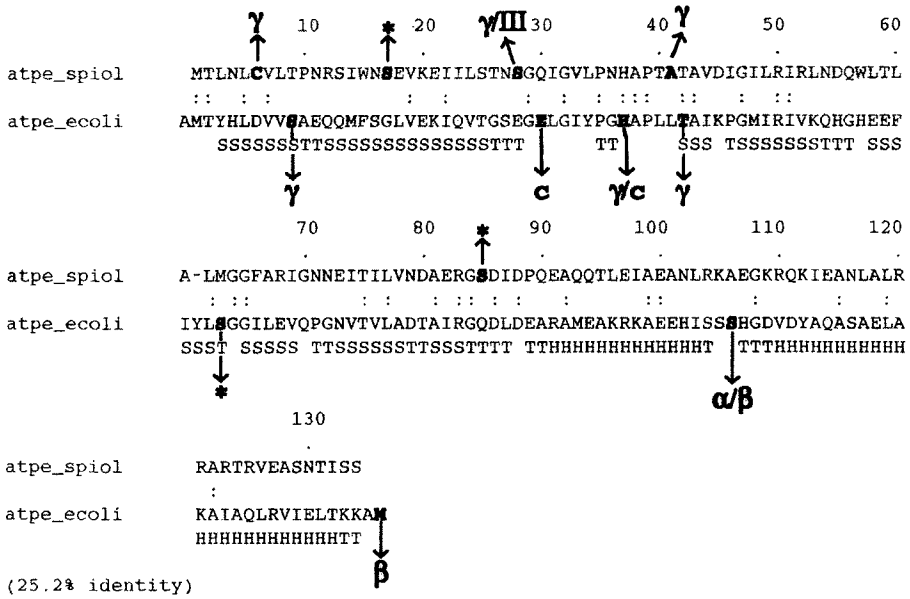

Fig. 6. Summary of cross-link data for spinach chloroplast and *E. coli* subunit ε. The amino acid alignment was obtained with WhatIf [55]; identical residues are indicated by colons. The rows below the *E. coli* sequence show the secondary structure of *E. coli* ε as calculated by the program DSSP [65] from the ε coordinates [57]. Residues that have been used in cross-linking are indicated with their target subunit. Asterisks indicate residues that were modified, but not cross-linked. The following two residues of *E. coli* ε were disulfide bridged with the following residues of subunits α, β and *c* of F_0F_1: ε Cys108 with α Cys411 and β Cys381 [27, 29]; and ε Cys31 with Cys40, Cys42 and Cys43 of subunit *c* [21, 22]. All other cross-links were obtained after (photo)chemical modification of the respective residues [23, 27, 28].

fectivity of $F_1(-\varepsilon)$ +[Ser6,Cys28]ε was in the same range as with F_1 containing γ cross-linked to [Ser6,Cys41]ε before, and after cross-linking and enrichment. The reconstitutive effectivity of F_1 containing γ cross-linked to wild-type ε amounted to only 58% of the effectivity of the non-cross-linked control, which in view of the content of non-cross-linked F_1 within the sample (44%) indicated that F_1 probably had lost its ability to functionally reconstitute photophosphorylation.

DISCUSSION

We engineered one cysteine at a time into a Cys-free mutant of subunit ε of spinach chloroplast F_1 at positions 17, 28, 41 and 85. These cysteines, plus the one in wild-type ε at position 6, served as anchors for the maleimide function of photo-activable cross-linking reagents. The aim was to gain information on the location of subunit ε within F_1 and to reveal the functional consequences of cross-linking on ATP hydrolysis by F_1 and on ATP synthesis by F_0F_1.

The studies were complicated by that recombinant ε precipitated into inclusion bodies upon overexpression and had to be modified with the cross-linker in the presence of urea since the refolded, soluble protein precipitated upon modification. In addition, very low cross-link yields were obtained, which necessitated an enrichment of cross-linked F_1. This was possible to some extent by chromatography of irradiated samples. Anion-exchange chromatography of the reduced enzyme in the presence of a detergent removes ε from F_1 [42, 48, 63]. F_1 and $F_1(-\varepsilon)$ were base-line separated under these conditions.

As a prerequisite for the presented studies, the biological activities of the ε variants before and after modification with the cross-linking reagent were tested. Inhibition of Ca^{2+}ATPase activity and reconstitution of photophosphorylation [in the presence of $F_1(-\varepsilon)$ were not affected by the modification of ε, but without photoactivation of the cross-linker. This was taken as indication of the native-like rebinding of modified ε to $F_1(-\varepsilon)$.

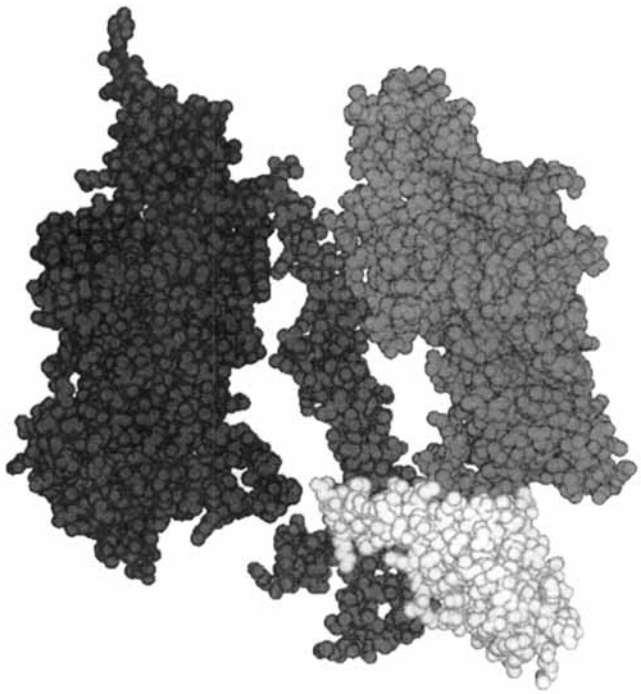

Fig. 7. Corey-Pauling-Koltun model of part of chloroplast F_1 showing subunit ε (light grey) proposed to be located within F_1. CF_1 was homology-modeled (WhatIf [55]) into the published bovine mitochondrial F_1 structure [13], chloroplast ε was homology-modeled [55] into *E. coli* ε [57]. The plot was generated with O [56].

Structural and functional implications. Single cysteine variants of subunit ε,after modification with a Cys-specific cross-linking reagent, incorporation into $F_1(-\varepsilon)$ and photoactivation of the second function of the cross-linker produced three classes of products: wild-type ε and [Ser6,Cys41]ε were cross-linked

exclusively to subunit γ, [Ser6,Cys28]ε was cross-linked to γ and probably also to subunit III of F_0, and [Ser6,Cys17]ε and [Ser6,Cys85]ε were not detectably cross-linked.

Related cross-link data were obtained previously for subunit ε of the *E. coli* enzyme [23–30]. Our data complement and extend these results (Fig. 6). Since the crystal structure of bovine heart F_1 [13] and a solution structure of *E. coli* ε [57] are known, a modeling of subunit ε into F_1 was attempted. To this end, the respective chloroplast polypeptide chains were homology-built into the known structures (with the program WhatIf [55] followed by 'docking' of ε to $(\alpha\beta)_3\gamma$ (with the program O [56]). Fig. 7 shows a Corey-Pauling-Koltun model of the resulting structure (a Protein Data Bank-type file is available on request and can be used for visual inspection of the structure, by public-domain programs such as RasMol [58]). Due to spacial restrictions, there are not many possibilities for such a venture. The cross-link data, the 'accessible' surface of γ (only ≈40% of the chloroplast γ structure can be disclosed currently by homology modeling) and the shape of ε only allow for two positions of ε within $(\alpha\beta)_3\gamma$, which are related by a C2 symmetry. The decisive experiment allowing the relative orientation of ε within F_1 to be restricted to these two possibilities was obtained with the *E. coli* enzyme: *E. coli* ε can be disulfide bridged via residue 108 to both α and β [26, 28]. In an attempt to satisfy this condition ε was placed such as to keep A106 close to S401, of chain a [13] and E412 of chain d (chloroplast numbering; the C2-related position would have placed A106 close to S401 of chain c [13].

Although quite a number of cross-links between subunits ε and γ are now known ([10, 28, 30], this work), the domain of γ that is cross-linked to ε is lacking in the picture. ε and γ must be intertwined to some extent, with a portion of γ (equal to the size of ε) covering most of one side of the large β-barrel domain of ε. Due to the length of the cross-linker (9 Å) the two surfaces may not be interacting *in situ*. The resulting model, however, is in full accordance with fluorescence-resonance-energy-transfer data on the location of ε within chloroplast F_1 [59].

The construction of the model shown in Fig. 7 was based upon the following assumptions: the solution structure of ε (obtained by NMR [57]) resembles the struture of ε bound to $F_1(\text{-}\varepsilon)$ (the validity of this assumption is open) and the structures of subunits α, β, γ and ε are very similar between *E. coli* mitochondria and chloroplasts.

The three enzymes differ in several aspects. In the mitochondrial enzyme the counterpart of *E. coli* ε and chloroplast ε probably comprises two proteins (mitochondrial subunits δ and ε). In comparison with mitochondrial F_1 and *E. coli* F_1, chloroplast F_1 γ contains additional stretches of amino acids, which are responsible for the redox regulation unique for the chloroplast enzyme [60]. In *E. coli* subunit ε is indispensable for binding F_1 to F_0 [61, 62] whereas in chloroplasts $F_1(\text{-}\varepsilon)$ binds well to F_0 [63]. Cross-linking yields in the *E. coli* enzyme were dependent on the nucleotide load [10, 23, 26, 27, 35], whereas the chloroplast enzyme behaved much more slackly in this respect (this work).

These differences, however, may represent add-on features of the chloroplast enzyme, which probably do not justify to view chloroplast F_1 as completely different from mitochondrial or *E. coli* F_1. The structural similarity between *E. coli* F_1 and chloroplast F_1 seems warranted by the presence of functional chimeric constructs between them [38, 44, 66]. The accordance of the cross-linking data reported here with those published for the *E. coli* enzyme [21–23, 27, 28] is reassuring in this aspect.

Therefore, given that the two conditions are met, the following conclusion may be drawn: there is no way to place ε underneath γ [even if the α-helical domain of ε changes its relative position to the β-barrel domain upon binding to $CF_1(\text{-}\varepsilon)$]. As a result, the 'stalk' becomes highly asymmetrical. It is tempting to visualize ε and γ serving as connectors between F_0 and F_1, a rotatory element picking up and relaying into the F_1 portion the torque that is generated in F_0 by proton-motive force.

If ATP synthesis is the exact reversal of ATP hydrolysis, the rotation of $\gamma\varepsilon$ (and most likely subunit III of the channel portion) relative to I/II/IV/$(\alpha\beta)_3/\delta$ would be driving it. Then the two roles of ε,inhibitory in hydrolysis and mandatory in synthesis, can be explained on pure mechanical grounds.

In ATP synthesis, the proton-motive force would cause the clockwise rotation (viewed from the membrane) [19] of the c subunits, which are tightly coupled to ε and γ. γ would relay the torque to F_1. Unlike under ATP hydrolysis, there is no steric hindrance since ε and γ act concertedly and with a fixed, time-independent distance between the C-terminal helix-turn-helix domain of ε and the C-terminal α-helical domain of the 'empty' β subunit.

ATP hydrolysis, on the other hand, would be inhibited because the counterclockwise rotation of $\gamma\varepsilon$ [19] would be blocked. The C-terminal helix-turn-helix domain of ε cannot rotate unhindered due to steric hindrance from the C-terminal-α-helical domain of the 'empty' β subunit.

This simple model is supported by findings of Kuki et al. [67] and preliminary data obtained with the chloroplast enzyme (Kirberich, S., Roelevink, M. and Engelbrecht, S., unpublished data). Shortened ε, lacking the two C-terminal α-helices, supported aerobic growth in the respective *E. coli* strains [67]. Similarly, chloroplast ε-(1–89)-peptide together with $F_1(\text{-}\varepsilon)$ reconstituted photophosphorylation to a significant extent in comparison with full-length ε but it lacked the ability to inhibit Ca^{2+}-ATPase activity of soluble F_1 despite apparent binding to $F_1(\text{-}\varepsilon)$. Other than envisaged by Dunn [68], this model assumes a rather rigid structure of at least the two domains of ε.

Subunit ε inhibits Ca^{2+}-ATPase activity of soluble F_1 and is indispensable in photophosphorylation [63]. Both activities were inhibited to some extent after cross-linking of wild-type ε, [Ser6, Cys28]ε and [Ser6,Cys41]ε to subunit γ. This might indicate requirement for some flexibility of γ and ε. On the other hand, Mg^{2+}-ATPase activities of the soluble enzyme were not affected at all by cross-linking, and even reconstitution of photophosphorylation, which is the most demanding activity, in two of three cases was not inhibited to an extent that matched the extent of cross-linking. In view of the rotation of subunit γ relative to $(\alpha\beta)_3$, which is driven by ATP hydrolysis [15–19, 34], γ and ε are probably corotating in ATP synthase. A decisive experiment by polarized absorption recovery after photobleaching, similar to our previous ones on the rotation of γ during ATP hydrolysis [17, 18], is underway in our laboratory.

We gratefully acknowledge the excellent technical assistance of Martina Blomeier. We are particularly thankful to Stephan Wilkens and Roderick A. Capaldi (University of Oregon, Eugene OR, USA) for the coordinates of *E. coli* F_1 ε and to Andrew G. W. Leslie and John E. Walker (Medical Research Council, Cambridge, UK) for the mitochondrial F_1 coordinates. This work was supported by a grant of the Deutsche Forschungsgemeinschaft (SFB 171-B3), by the Fonds der chemischen Industrie and the Land Niedersachsen.

REFERENCES

1. Senior, A. E. (1988) ATP synthesis by oxidative phosphorylation, *Physiol. Rev. 68*, 177–231.
2. Boyer, P. D. (1989) A perspective of the binding change mechanism for ATP synthesis, *FASEB J. 3*, 2164–2178.
3. Futai, M., Noumi, T. & Maeda, M. (1989) ATP synthase (H^+-ATPase): results by combined biochemical and molecular biological approaches, *Annu. Rev. Biochem. 58*, 111–136.

4. Junge, W. (1989) Protons, the thylakoid membrane, and the chloroplast ATP synthase, *Ann. N.Y. Acad. Sci. 574*, 268–286.
5. Senior, A. E. (1990) The proton-translocating ATPase of *Escherichia coli*, *Annu. Rev. Biophys. Biophys. Chem. 19*, 7–41.
6. Boyer, P. D. (1993) The binding change mechanism for ATP synthase – some probabilities and possibilities, *Biochim. Biophys. Acta 1140*, 215–250.
7. Capaldi, R. A., Aggeler, R., Turina, P. & Wilkens, S. (1994) Coupling between catalytic sites and the proton channel in F_1F_0-type ATPases, *Trends Biochem. Sci. 19*, 284–289.
8. Fillingame, R. H. (1996) Membrane sectors of F- and V-type H^+-transporting ATPases, *Curr. Opin. Struct. Biol. 6*, 491–498.
9. Cross, R. L. & Duncan, T. M. (1996) Subunit rotation in F_0F_1-ATP syntheses as a means of coupling proton transport through F_0 to the binding changes in F_1, *J. Bioenerg. Biomembr. 28*, 403–408.
10. Capaldi, R. A., Aggeler, R., Wilkens, S. & Grüber, G. (1996) Structural changes in γ and ε subunits of the *Escherichia* coli F_1F_0-type ATPase during energy coupling, *J. Bioenerg. Biomembr. 28*, 397–401.
11. Dimroth, P. (1987) Sodium ion transport decarboxylases and other aspects of sodium ion cycling in bacteria, *Microbiol. Rev. 51*, 320–340.
12. Dimroth, P. (1992) Mechanisms of sodium transport in bacteria, *Philos. Trans. R. Soc. Lond. Biol. Sci. 326*, 465–477.
13. Abrahams, J. P., Leslie, A. G. W., Lutter, R. & Walker, J. E. (1994) Structure at 2.8 Å resolution of F_1-ATPase from bovine heart mitochondria, *Nature 370*, 621–628.
14. Gogol, E. P., Johnston, E., Aggeler, R. & Capaldi, R. A. (1990) Ligand-dependent structural variations in *Escherichia coli* F_1 ATPase revealed by cryoelectron microscopy, *Proc. Natl Acad. Sci. USA 87*, 9585–9589.
15. Duncan, T. M., Bulygin, V. V., Zhou, Y., Hutcheon, M. L. & Cross, R. L. (1995) Rotation of subunits during catalysis by *Escherichia coli* F_1-ATPase, *Proc. Natl Acad. Sci. USA 92*, 10964–10968.
16. Zhou, Y., Duncan, T. M., Bulygin, V. V., Hutcheon, M. L. & Cross, R. L. (1996) ATP hydrolysis by membrane-bound *Escherichia* coli F_0F_1 causes rotation of the γ subunit relative to the β subunits, *Biochim. Biophys. Acta 1275*, 96–100.
17. Sabbert, D., Engelbrecht, S. & Junge, W. (1996) Intersubunit rotation in active F-ATPase, *Nature 381*, 623–625.
18. Sabbert, D., Engelbrecht, S. & Junge, W. (1997) Functional and idling rotatory motion within F_1-ATPase, *Proc. Natl Acad. Sci. USA 94*, 4401–4405.
19. Noji, H., Yasuda, R., Yoshida, M. & Kinosita K. Jr (1997) Direct observation of the rotation of F_1-ATPase, *Nature 386*, 299–302.
20. Sabbert, D. & Junge, W. (1997) Stepped versus continuous rotatory motors at the molecular scale, *Proc. Natl Acad. Sci. USA 24*, 2312–2317.
21. Zhang, Y., Oldenburg, M. & Fillingame, R. H. (1994) Suppressor mutations in F_1 subunit ε recouple ATP-driven H^+-translocation in uncoupled Q42E subunit *c* mutant of the *Escherichia coli* F_0F_1 ATP synthase, *J. Biol. Chem. 269*, 10221–10224.
22. Zhang, Y. & Fillingame, R. H. (1995) Subunits coupling H^+ transport and ATP synthesis in the *Escherichia coli* ATP synthase, *J. Biol. Chem. 270*, 24509–24614.
23. Haughton, M. A. & Capaldi, R. A. (1995) Asymmetry of *Escherichia coli* F_1-ATPase as a function of the interaction of α–β subunit pairs with the γ and ε subunits, *J. Biol. Chem. 270*, 20568–20574.
24. Grüber, G. & Capaldi, R. A. (1996) Differentiation of catalytic sites on *Escherichia coli* F_1ATPase by laser photoactivated labeling with [^{3}H]-2-azido-ATP using the mutant βGlu381Cys:εSer108Cys to identify different β subunits by their interactions with γ and ε subunits, *Biochemistry 35*, 3875–3879.
25. Grüber, G. & Capaldi, R. A. (1996) The trapping of different conformations of the *Escherichia coli* F_1 ATPase by disulfide bond formation, *J. Biol. Chem. 271*, 32623–32628.
26. Feng, Z., Aggeler, R., Haughton, M. A. & Capaldi, R. A. (1996) Conformational changes in the *Escherichia coli* ATP synthase (ECF_0F_1) monitored by nucleotide-dependent differences in the reactivity of Cys-87 of the γ subunit in the mutant βGlu-381 →Ala, *J. Biol. Chem. 271*, 17986–17989.
27. Aggeler, R., Haughton, M. A. & Capaldi, R. A. (1995) Disulfide bond formation between the COOH-terminal domain of the β subunits and the γ and ε subunits of the *Escherichia coli* F_1-ATPase, *J. Biol. Chem. 270*, 9185–9191.
28. Tang, C. & Capaldi, R. A. (1996) Characterization of the interface between γ and ε subunits of *Escherichia coli* F_1-ATPase, *J. Biol. Chem. 271*, 3018–3024.
29. Aggeler, R. & Capaldi, R. A. (1996) Nucleotide-dependent movement of the ε subunit between α and β subunits in the *Escherichia coli*, F_0F_1-type ATPase, *J. Biol. Chem. 271*, 13888–13891.
30. Watts, S. D., Tang, C. & Capaldi, R. A. (1996) The stalk region of the *Escherichia coli* ATP synthase – tyrosine 205 of the γ subunit is in the interface between the F_1 and F_0 parts and can interact with both the ε and *c* oligomer, *J. Biol. Chem. 271*, 28341–28347.
31. Engelbrecht, S. & Junge, W. (1990) Subunit δ of H^+-ATPases: at the interface between proton flow and ATP synthesis, *Biochim. Biophys. Acta 1015*, 379–390.
32. Lill, H., Hensel, F., Junge, W. & Engelbrecht, S. (1996) Cross-linking of engineered subunit δ to $(\alpha\beta)_3$ in chloroplast F-ATPase, *J. Biol. Chem. 271*, 32737–32742.
33. Aggeler, R., Weinreich, F. & Capaldi, R. A. (1995) Arrangement of the ε subunit in the *Escherichia coli* ATP synthase from the reactivity of cysteine residues introduced at different positions in this subunit, *Biochim. Biophys. Acta 1230*, 62–68.
34. Junge, W., Sabbert, D. & Engelbrecht, S. (1996) Rotatory catalysis by F-ATPase: real-time recording of intersubunit rotation, *Ber. Bunsen-Ges. Phys. Chem. 100*, 2014–2019.
35. Aggeler, R., Chicas-Cruz, K., Cai, S.-X., Keana, J. F. W. & Capaldi, R. A. (1992) Introduction of reactive cysteine residues in the ε subunit of *Escherichia coli* F_1 ATPase, modification of these sites with tetrafluorophenyl azide-maleimides, and examination of changes in the binding of the ε subunit when different nucleotides are in catalytic sites, *Biochemistry 31*, 2956–2961.
36. Weber, T. & Brunner, J. (1995) 2-(Tributylstannyl)-4-[3-(trifluoromethyl)-3H-diazirin-3-yl]benzyl alcohol: a building block for photolabeling and cross-linking reagents of very high specific radioactivity, *J. Am. Chem. Soc. 117*, 3084–3095
37. Rosenberg, A. H., Lade, B. N., Chui, D.-S., Lin, S.-W., Dunn, J. J. & Studier, F. W. (1987) Vectors for selective expression of cloned DNAs by T7 RNA polymerase, *Gene (Amst.) 56*, 125–135.
38. Steinemann, D., Lill, H., Junge, W. & Engelbrecht, S. (1994) Overproduction, renaturation and reconstitution of δ and ε subunits from chloroplast and cyanobacterial F_1, *Biochim. Biophys. Acta 1187*, 354–359.
39. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular cloning: a laboratory manual*, 2nd edn, Cold Spring Harbor Laboratory, Cold Spring Harbor NY.
40. Sanger, F., Nicklen, S. & Coulsen, A. R. (1977) DNA sequencing with chain terminating inhibitors, *Proc. Natl Acad. Sci. USA 74*, 5463–5467.
41. Cruz, J. A., Harfe, B., Radkowski, C. A., Dann, M. S. & McCarty, R. E. (1995) Molecular dissection of the ε subunit of the chloroplast ATP synthase of spinach, *Plant Physiol. (Bethesda) 109* 1379–1388.
42. Engelbrecht, S., Schürmann, K. & Junge, W. (1989) Chloroplast ATP synthase contains one single copy of subunit δ that is indispensable for photophosphorylation, *Eur. J. Biochem. 179*, 117–122.
43. Krause, I. & Elbertzhagen, H. (1987) *Eine 5-Minuten schnelle Silberfärbung für getrocknete Polyacrylamidgele*, in *Elektrophoreseforum 1987* (Radola, B. J., ed.) pp. 382–384, TU München.
44. Lill, H., Burkovski, A., Altendorf, K., Junge, W. & Engelbrecht, S. (1993) Complementation of *Escherichia coli unc* mutant strains by chloroplast and cyanobacterial F_1-ATPase subunits, *Biochim. Biophys. Acta 1144*, 278–284.
45. Polle, A. & Junge, W. (1986) The slow rise of the flash-light-induced alkalization by photosystem II of the suspending medium of thylakoids is reversibly related to thylakoid stacking, *Biochim. Biophys. Acta 848*, 257–264.
46. Shoshan, V. & Shavit, N. (1973) On the reconstitution of photophosphorylation in chloroplast membranes, *Eur. J. Biochem. 37*, 355–360.
47. Nelson, N. & Eytan, E. (1979) Approach to the membrane sector of the chloroplast coupling device, in *Cation fluxes across biomem-*

branes (Mukohata, Y. & Packer, L., eds) pp. 409–415, Academic Press, New York.
48. Engelbrecht, S. & Junge, W. (1987) Fragmentation of chloroplast coupling factor in dependence of bound nucleotides, *FEBS Lett. 219*, 321–325.
49. Engelbrecht, S. & Junge, W. (1988) Purified subunit δ of chloroplast coupling factor CF_1 reconstitutes photophosphorylation in partially CF_1-depleted membranes, *Eur. J. Biochem. 172*, 213–218.
50. Engelbrecht, S., Althoff, G. & Junge, W. (1990) Reconstitution of photophosphorylation in EDTA-treated thylakoids by added chloroplast coupling factor 1 (ATPase) and chloroplast coupling factor 1 lacking the δ subunit, *Eur. J. Biochem. 189*, 193–197.
51. Pick, U. & Racker, E. (1979) Purification and reconstitution of the *N,N'*-dicyclohexylcarbodiimide-sensitive ATPase complex from spinach chloroplasts, *J. Biol. Chem. 254*, 2793–2799.
52. Fromme, P., Boekema, E. J. & Gräber, P. (1987) Isolation and characterization of a supramolecular complex of subunit III of the ATP synthase from chloroplasts, *Z. Naturforsch. Sect. C Biosci. 42*, 1239–1245.
53. LeBel, D., Poirier, G. G. & Beaudoin, A. R. (1978) A convenient method for the ATPase assay, *Anal. Biochem. 85*, 86–89.
54. Sedmak, J. J. & Grossberg, S. E. (1977) A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G 250, *Anal. Biochem. 79*, 544–552.
55. Vriend, G. (1990) WHAT IF: a molecular modeling and drug design program, *J. Mol. Graph. 8*, 52–56.
56. Jones, T. A., Zou, J.-Y., Cowan, S. W. & Kjeldgaard, M. (1991) Improved methods for building protein models in electron density maps and the location of errors in these models, *Acta Crystallogr. A47*, 110–119.
57. Wilkens, S., Dahlquist, F. W., McIntosh, L. P., Donaldson, L. W. & Capaldi, R. A. (1995) Structural features of the ε subunit of the *Escherichia coli* ATP synthase determined by NMR spectroscopy, *Nat. Struct. Biol. 2*, 961–967.
58. Sayle, R. A. & Milner-White, E. J. (1995) RASMOL: biomolecular graphics for all, *Trends Biochem. Sci. 20*, 374–376.
59. Richter, M. L., Snyder, B., McCarty, R. E. & Hammes, G. G. (1985) Binding stoichiometry and structural mapping of the ε polypeptide of chloroplast coupling factor 1, *Biochemistry 24*, 5755–5763.
60. Nalin, C. M. & McCarty, R. E. (1984) Role of a disulfide bond in the γ subunit in activation of the ATPase of chloroplast coupling factor 1, *J. Biol. Chem. 259*, 7275–7280.
61. Smith, J. B. & Sternweis, P. C. (1977) Purification of membrane attachment and inhibitory subunits of the proton translocating adenosine triphosphatase from *Escherichia coli*, *Biochemistry 16*, 306–311.
62. Tuttas-Dörschug, R. & Hanstein, W. G. (1989) Coupling factor 1 from *Escherichia coli* lacking subunits δ and ε: preparation and specific binding to depleted membranes, mediated by subunits δ or ε, *Biochemistry 28*, 5107–5113.
63. Patrie, W. J. & McCarty, R. E. (1984) Specific binding of coupling factor 1 lacking the δ and ε subunits to thylakoids, *J. Biol. Chem. 259*, 11121–11128.
64. Riddles, P. W., Blakeley, R. L. & Zerner, B. (1983) Reassessment of Ellman's reagent, *Methods Enzymol. 91*, 49–60.
65. Kabsch, W. & Sander, C. (1983) Dictionary of protein secondary structure: pattern recognition of hydrogen bonded and geometrical features, *Biopolymers 22*, 2577–2637.
66. Burkovski, A., Lill, H. & Engelbrecht, S. (1994) Complementation of *Escherichia coli uncD* mutant strains by a chimeric F_1-β subunit constructed from *E. coli* and spinach chloroplast F_1-β, *Biochim. Biophys. Acta 1186*, 243–246.
67. Kuki, M., Noumi, T., Maeda, M., Amemura, A. & Futai, M. (1988) Functional domains of ε subunit of *Escherichia coli* H^+-ATPase (F_0F_1), *J. Biol. Chem. 263*, 17437–17442.
68. Dunn, S. D. (1995) A barrel in the stalk, *Nat. Struct. Biol. 2*, 915–918.