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Zero-length crosslinking between subunits δ and I of the H^+-translocating ATPase of chloroplasts

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Treatment of spinach thylakoids with 1-ethyl-3-(dimethylaminopropyl)-carbodiimide (EDC)/*N*-hydroxysulfosuccinimide (sulfo-NHS) induced formation of a zero-length crosslink of an apparent molecular mass of 38 kDa. This product was shown, by immunodetection, to consist of subunit δ of CF_1 and subunit I of CF_0. The crosslink was isolated by preparative SDS gel electrophoresis and subjected to cyanogen bromide cleavage. Electrophoretic and immunological analysis of the resulting peptides suggested that the crosslink was formed between a glutamyl or aspartyl residue at the C-terminal end of subunit I and a basic amino acid of subunit δ in the range between Val-1 to Met-165. Treatment of thylakoids with EDC/Sulfo-NHS resulted in inhibition of photophosphorylation and CF_0CF_1-catalyzed ATP hydrolysis without affecting formation of a proton gradient related to phenazine methosulfate-mediated cyclic electron transport. Inhibition of H^+ transport-coupled ATP hydrolysis was more pronounced than non-coupled methanol-stimulated ATP hydrolysis. The results suggest that subunits δ and I form a connection between the partial complexes CF_1 and CF_0 in situ. Crosslinking of the two subunits may impede the translocation of protons through CF_0CF_1.

Introduction

The proton translocating ATPase of chloroplasts consists of the membrane-integral sector CF_0, which forms a transmembrane proton channel, and the catalytic peripheral sector CF_1. CF_0 is composed of four different subunits I to IV, CF_1 consists of five subunits α to ε. The probable subunit stoichiometry is α_3, β_3, γ, δ, ε, I, II, III_{6-12}, IV [1]. CF_1 is easily released from the thylakoid membrane by treatment with EDTA [2] or the chaotropic salt NaBr [3]. The high proton conductance of CF_1-stripped membranes, indicating open CF_0 channels, is blocked by dicyclohexyl carbodiimide, which binds to Glu-61 of subunit III, or by reassociation of CF_1. Re-binding of CF_1 reconstitutes the energy-conserving function of the thylakoid membrane [1]. Several experimental results suggest an important role of the δ subunit in functional connection between CF_1 and CF_0 [4]. It was found that a δ-deficient isolated CF_1 was unable to reconstitute phosphorylation [5–9]. Although the protein was bound to the membrane at almost stoichiometric amounts to the number of open CF_0 channels, the proton leaks were not resealed. It was, therefore, concluded that subunit δ controls the productive proton flux through the CF_0 channel [8,9]. This view was supported by the finding that isolated δ can reconstitute photophosphorylation of partially CF_1-depleted thylakoids by plugging the open CF_0 pores [9].

Immunological and proteolytic studies revealed that a large part of the δ subunit is hidden within the CF_1 head [10]. The strong in vitro interaction between subunits δ and α [11] suggests a connection between these two polypeptides also in situ. On the other hand, the subunit(s) of CF_0 which anchor δ to the membrane sector of the ATPase, are actually unknown. In this paper we report on crosslinking of subunit δ to the CF_0 subunit I induced by treatment of thylakoid membranes with 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC).

EDC induces zero-length crosslinks between vicinal proteins by the formation of secondary amido bonds

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Abbreviations: CF_0, membrane integral moiety of chloroplast H^+-ATPase; CF_1, peripheral sector of chloroplast H^+-ATPase; DCCD, *N,N'*-dicyclohexyl-carbodiimide; DTT, dithiothreitol; EDC, 1-ethyl-3-(dimethylaminopropyl)-carbodiimide; Sulfo-NHS, *N*-hydroxysulfosuccinimide; tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl] glycine.

between adjacent carboxylic and amino groups, respectively. This crosslinking reaction is greatly enhanced by the catalytic reagent *N*-hydroxysulfosuccinimide (Sulfo-NHS), which reacts with the *O*-acylurea intermediate of EDC [12]. The resulting activated NHS-ester is less sensitive to hydrolysis and shifts the pH optimum of the EDC-mediated crosslinking reaction from 4–5 to the range of 7–8. The crosslinks formed at these physiological pH values give information about the natural vicinities between polypeptide chains in protein complexes.

Experimental procedures

Thylakoids were isolated from spinach leaves as described in Ref. 13, washed and resuspended in 0.3 M sucrose, 50 mM NaCl, 1 mM $MgCl_2$ and 10 mM tricine buffer (pH 8.0). Crosslinking reactions were carried out in the same medium containing additional 10 mM EDC, 5 mM sulfo-NHS and thylakoids corresponding to 1 mg chlorophyll/ml. After 30 min stirring at 20°C the reaction was stopped by addition of a 5-fold volume of a solution consisting of 0.3 M sucrose, 50 mM NaCl, 1 mM $MgCl_2$, 0.1 M glycine and 10 mM tricine buffer (pH 8.0). After centrifugation and washing in the same medium, the pellet was resuspended in 0.2 M sucrose, 10 mM $MgCl_2$, 0.2 M $(NH_4)_2SO_4$, 0.5% sodium cholate, 30 mM *n*-octyl β-D-glucopyranoside and 10 mM Tricine buffer (pH 8.0), and stirred for 1 h at 4°C. This treatment solubilizes CF_0CF_1 [14]. After centrifugation to remove non-solubilized membrane material, the supernatant was fractionated by ammonium sulfate precipitation. The 35% to 50% $(NH_4)_2SO_4$ precipitate was collected by centrifugation, re-dissolved in 10 ml 0.2% Triton X-100, 0.5 mM EDTA, 0.1 mM ATP and 30 mM Tris-succinate (pH 6.5), and subjected to sucrose gradient (15–50%) centrifugation (20 h at $220000 \times g$). The single fractions were analyzed by SDS gel electrophoresis and subsequent silver staining [15]. Protein contents were measured by the method of Bensadoun and Weinstein [16].

The fractions containing pure CF_0CF_1 were pooled, subjected to preparative SDS-PAGE [17] and stained by Coomassie blue [18]. The bands containing subunits δ, I and a δ-I crosslink (38 kDa) were excised, washed three times with a 5-fold volume of 70% formic acid and incubated for 4 h in a 5-fold volume of a solution containing 70% formic acid and 1% cyanogen bromide. After washing three times in a 10-fold volume of 0.1% SDS and 60 mM Tris-phosphate (pH 6.7), the gel pieces were placed into the slots of another SDS-polyacryalamide gel for analysis of the fragmentation products.

Antisera were raised in rabbits [19] with about 100 μg of the respective CF_0CF_1 subunits. The pure polypeptides were obtained by electroelution [20] after preparative SDS gel electrophoresis of the enriched CF_0CF_1 complex.

For Western blot analysis, the proteins and peptides, respectively, were transferred to nitrocellulose membranes, incubated with polyclonal antisera and assayed by the Enhanced Chemoluminescence System of Amersham [21]. The blotted protein standards were detected by ink stain (Pelikan Brillant Black 4001) [22].

For measurements of thylakoid activities, the EDC reaction was stopped by 1:1 dilution with 50 mM NaCl, 1 mM $MgCl_2$, 1 M glycine and 2 mM Tricine buffer (pH 8.0). After centrifugation and washing twice in the same medium, but without glycine, the membranes were resuspended in this medium and stored at a chlorophyll concentration of 1 mg/ml.

Time courses of transmembrane proton gradient formation were followed by monitoring the fluorescence quench of 9-aminoacridine, as reported in Ref. 23. The basal medium contained 50 mM NaCl, 5 mM $MgCl_2$, 10 mM dithiothreitol and 50 μM phenazine methosulfate. The chlorophyll concentration was 25 μg/ml and the 9-aminoacridine concentration was 5 μM.

Non-coupled ATPase activity was assayed at 37°C in a medium containing 35% methanol (v/v), 25 mM NaCl, 25 mM $MgCl_2$, 5 mM [γ-^{32}P]ATP, 25 mM Tris buffer (pH 8.8), and thylakoids corresponding to 25 μg chlorophyll/ml [24]. Samples were taken after 30, 60, 90 s, deproteinized by $HClO_4$ (0.5 mM) and analyzed for [^{32}P]P_i [25].

H^+-coupled ATP hydrolysis at clamped ΔpH was measured at 20°C with an instrument described in Ref. 26. The medium consisted of 50 mM KCl, 5 mM $MgCl_2$, 10 mM DTT, 50 μM PMS, 2 mM phosphoenolpyruvate, 80 μg/ml pyruvate kinase, 0.1 μM valinomycin, 5 μM 9-aminoacridine and 25 mM tricine buffer (pH 8.0); the chlorophyll concentration was 25 μg/ml. After pre-illumination for 2 min to activate the ATPase, a pre-chosen pH of 2.5 was provided by the instrumental device by modulation of photosynthetic light intensity. After 2 min, 0.5 mM [γ-^{32}P]ATP plus 80 nM nigericin was injected. ΔpH was maintained at 2.5 by automatic regulation of light intensity [26]. After 30, 60 and 90 s samples were taken deproteinized by $HClO_4$ (0.5 mM) and analyzed for [^{32}P]P_i [25].

Results

EDC-mediated crosslinking between CF_0CF_1 subunits

After EDC/Sulfo-NHS treatment of thylakoids, the CF_0CF_1 complex was solubilized by octylglucoside and isolated by sucrose gradient centrifugation [14]. SDS-PAGE analysis revealed a variety of crosslinks of molecular weights above 50 kDa (Fig. 1). By immunodetection, using antisera against isolated CF_0CF_1 subunits, crosslinking between nearly all subunits could be

observed (data not shown) in accordance with published results [27,28]. Only subunit γ was not linked to any other subunit by EDC treatment. Furthermore, derivatives of α and β subunits were detected by their slightly modified migrations in SDS-PAGE. As those do not contain a crosslinking partner, they are most probably intramolecular crosslinks of these subunits.

One crosslink formed at high yield was detected in the range of an apparent molecular weight of 38 kDa (Fig. 1). Immunoblot analysis revealed that this protein band contains the subunits CF_1-δ and CF_0-I (Fig. 2).

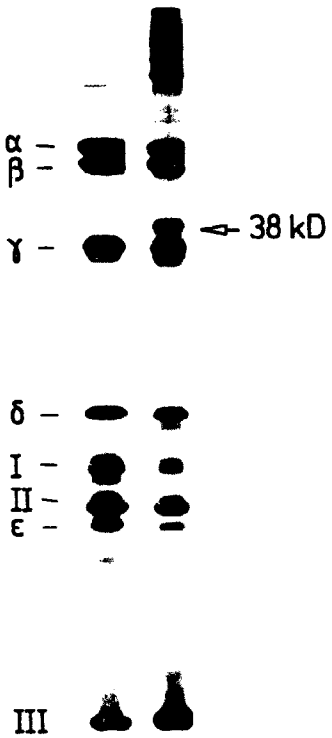


Fig. 1. SDS gel electrophoretic patterns of isolated CF_0CF_1 from non-treated (A) and EDC-treated (B) thylakoid membranes. Electrophoresis was carried out on a 15% polyacrylamide-gel with 20 μg protein in each lane; the gel was stained with silver [15]; subunit CF_0-IV does not stain under these conditions. The arrow marks the 38 kDa δ-I-crosslink.

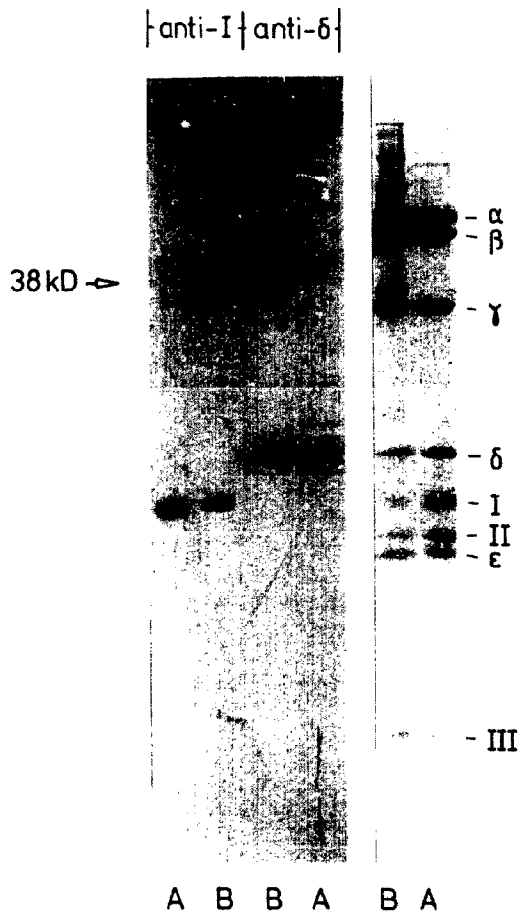


Fig. 2. Immunoblot of CF_0CF_1 isolated from non-treated (A) and EDC-treated (B) thylakoid membranes, with antisera against subunits I (no. 392) and δ (no. 306). Left side: immuno blot with 20 μg of protein per lane. Right side: 30 μg of CF_0CF_1 standards electroblotted on nitrocellulose and stained with ink. SDS-gel-electrophoresis was carried out on a 15% polyacrylamide gel.

The formation of the 38 kDa crosslink is related to a significant loss of intensities in the bands of subunit δ and subunit I (Figs. 1 and 2).

Cyanogen bromide cleavage of the δ-I crosslink

The 38 kDa band, as well as the non-crosslinked CF_1-δ and CF_0-I bands, were taken from Coomassie-stained preparative SDS gels and subjected to cyanogen bromide treatment, which provides cleavage of peptide bonds at the carboxy-side of methionine residues. Since electrophoretic elution of the proteins from the gels resulted in considerable loss of protein and possibly oxidation of methionines, the cleavage reaction was carried out by in-gel treatment of the excised bands for 4 h at 20°C. Afterwards the gel pieces were put on a second gel to separate the fragments by another SDS-

PAGE, as described in Materials and Methods, blotted on nitrocellulose membranes and analyzed by antisera against CF_1-δ and CF_0-I. Fig. 3A shows, schematically, the cyanogen bromide cleavage sites of CF_0-I and CF_1-δ, as well as the molecular weights of the expected cyanogen cleavage products. According to the translated DNA sequence, spinach CF_0-I contains four methionines in addition to the N-terminal methionine (Met-90, Met-108, Met-175, Met-179) [29]. By amino acid sequencing, however, the protein was found to start with Gly-18 [30,31], suggesting a post-translational processing of 17 N-terminal amino acids [29]. Accordingly complete cyanogen bromide cleavage should yield five peptides designated by the letters a (7.8 kDa), b (2.2 kDa), c (7.8 kDa), d (0.4 kDa) and e (0.6 kDa). CF_1-δ has only two methionines close to the C-terminal end (Met-165, Met-180) [32] yielding three cleavage products, which were designated A (18 kDa), B (1.7

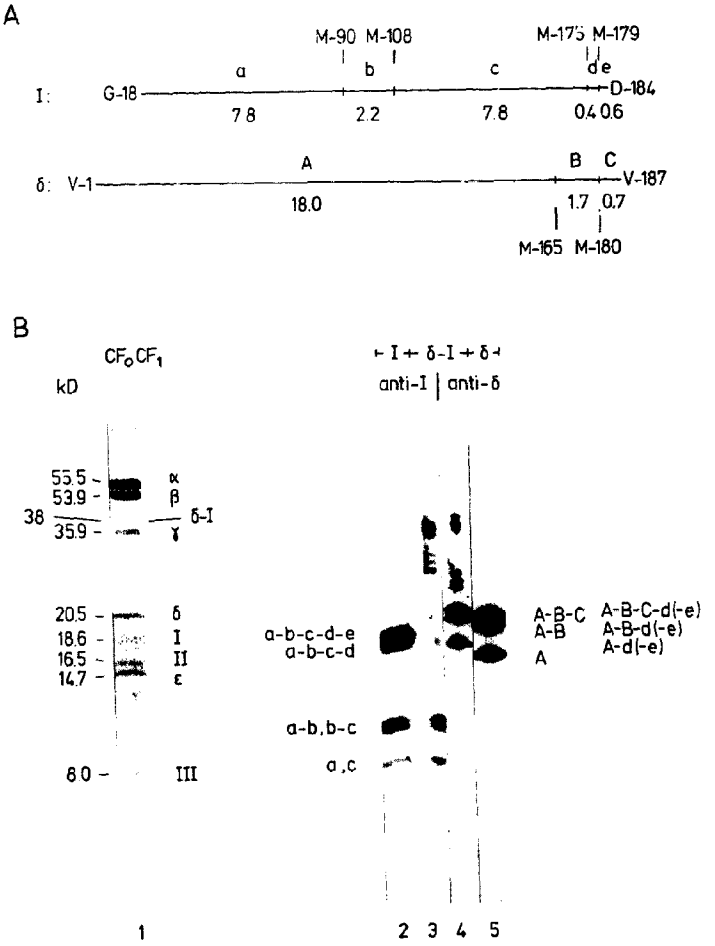


Fig. 3. (a) Cyanogen bromide cleavage sites of spinach CF_1-δ and CF_0-I and molecular weights of the fragments in kDa. The letters a to e and A to C designate the cleavage products of subunits I and δ, respectively. (B) Lane 1, 30 μg of ink-stained CF_0CF_1 isolated from EDC-treated thylakoid membranes separated by SDS PAGE and blotted on nitrocellulose. Real molecular weights deduced from the amino acid sequences are shown. Lane 2, immuno blot of cyanogen bromide-cleaved isolated subunit CF_0-I, decorated with anti-I serum no. 392. Lanes 3, 4, immunoblot of the isolated δ-I crosslink, cleaved, separated and decorated with anti-I serum no. 392 and anti-δ serum no. 306, respectively. Lane 5, immunoblot of the cyanogen bromide-cleaved isolated subunit δ decorated with anti-δ serum no. 306. The bands identified by molecular weight are assigned according to the designations given in A. SDS-gelelectrophoresis was carried out on a 17% polyacrylamide gel. After electroblotting the gel was silver-stained. Due to incomplete transfer to the nitrocellulose membrane, the immunoreactive peptide bands could be identified on the gel. Comparison of the two tracks corresponding to lanes 4 and 5, for example, clearly confirmed the shift to slightly higher molecular masses of the cleavage products of the crosslink compared with the δ subunit.

kDa) and C (0.7 kDa). After incomplete cyanogen bromide cleavage, a number of further fragments can be expected.

Fig. 3B shows the experimentally obtained cleavage patterns of the isolated subunits CF_0-I (lane 2), CF_1-δ (lane 5) and of the δ-I crosslink (lanes 3, 4) detected by immunoblot analysis. In lanes 2 and 3 anti-CF_0-I, in lanes 4 and 5 anti-CF_1-δ was employed.

Separation of the fragments of isolated CF_0-I shows three bands of apparent molecular weights of 18–19 kDa, about 10 kDa and about 8 kDa. The shapes of the bands suggest that each of them may be composed of more than one single peptide. According to the molecular weight, the upper broad band may contain the uncleaved subunit (18.8 kDa) and the a-b-c-d fragment, which is smaller by only 1 kDa. The middle band may be assigned to the a-b fragment and the b-c fragment, both having a real molecular weight of 10 kDa. The lower band, most likely, contains the fragments a and c (7.8 kDa). The absence of fragments c-d (8.2 kDa) and c-d-e (8.8 kDa) – which should be clearly distinguished from the a and c fragments – suggests that the cleavage site at M-175 may be very sensitive to cyanogen bromide treatment. The small peptides ≤ 2.2 kDa (b, d–e, d and e) are not detected in the employed gel system. Cyanogen bromide treatment of isolated CF_1-δ yields three bands of 20, 19 and 17.5 kDa apparent molecular weight. They may be assigned to uncleaved δ (20.4 kDa), fragment A-B (19.7 kDa) and fragment A (18 kDa), respectively. The small B, C and B-C peptides (≤ 2.4 kDa) are again undetectable.

Cleavage of the δ-I crosslink yields three main bands in the range from 18 to 21 kDa, which are positive with anti-δ. The pattern of the bands is similar to the one of isolated δ after cleavage (cf. lane 5), but all three bands are shifted to ≤ 1 kDa higher apparent molecular weights. This result permits the conclusion that it is the A peptide of CF_1-δ, which is enlarged by 1 kDa or less. The three peptides are not reactive with anti-I, indicating that the attached CF_0-I fragment has no epitope that is recognized by this antiserum. The CF_0-I fragment that is contained in the three different bands, must be either the d-e or e peptide. This is consistent with the fact that the fragments a-b-c, a-b, b-c and a,c are found unchanged in the cleavage pattern of the crosslink (cf. lanes 2 and 3). Additional larger (> 20 kDa) cleavage products of the crosslink, which are partly identical in the anti-I- and anti-δ-treated lanes, were not further identified.

Effect of EDC on the function of the ATPase

Treatment of thylakoids with EDC under the experimental conditions which lead to formation of the δ-I crosslink, did not inhibit the generation of a transmembrane proton gradient coupled to PMS-mediated cyclic electron transport. This result demonstrates that neither the employed electron transport system is significantly impaired nor that EDC acts as an uncoupler (Fig. 4). If compared to the signal of untreated thylakoids, EDC treatment even slightly increases the extent of pH. Phenomenologically EDC acts like the chemically-related carbodiimide derivative dicyclohexylcarbodiimide (DCCD). It was shown, however, that the hydrophilic EDC does not bind to the DCCD binding site (Glu-61) in subunit III of CF_0 [33]. Starting photophosphorylation by addition of phosphate to untreated thylakoids in the light, results in a significant decrease in the proton gradient due to acceleration of H^+ efflux, coupled with ATP formation (Fig. 4a). In an EDC-treated thylakoid preparation, however, the gra-

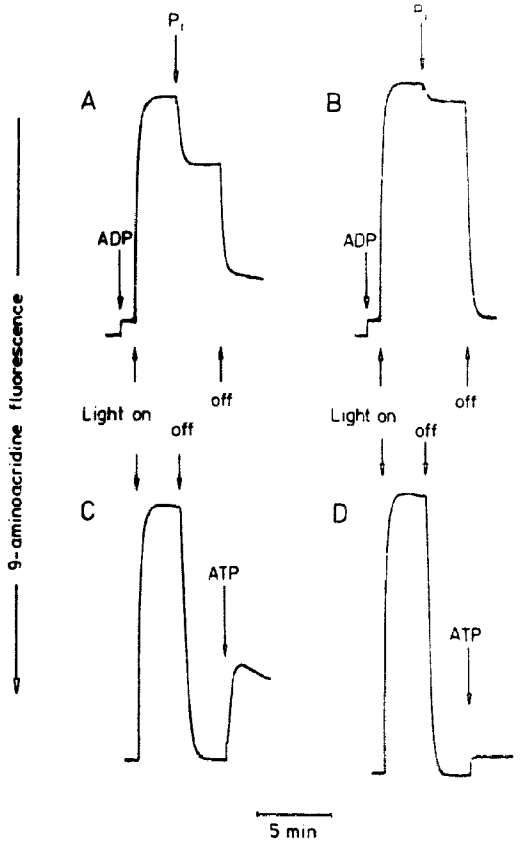


Fig. 4. Effect of EDC treatment on light-dependent formation of a proton gradient as measured by 9-aminoacridine fluorescence quenching. (A), Change of the fluorescence signal due to ADP phosphorylation in control thylakoids and (B), in EDC-treated thylakoids. (C), Formation of a proton gradient upon addition of ATP after pre-illumination in the presence of DTT in control thylakoids and (D), in EDC-treated thylakoids. Assay conditions as described in Experimental procedures. Pre-treatment with EDC was carried out for 20 min. The substrates were added at final concentrations of 200 μM (ADP and ATP) and 1 mM (P_i). The instantaneous small fluorescence quench observed on ADP or ATP addition is an artefact due to interaction of the nucleotides with 9-aminoacridine [26].

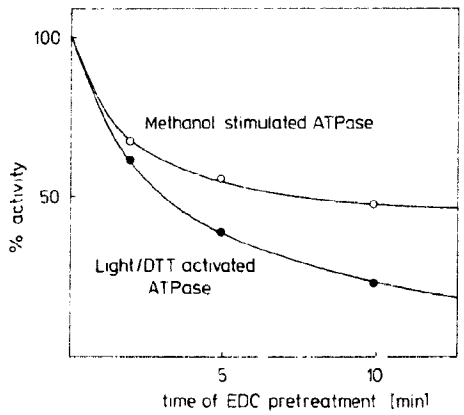


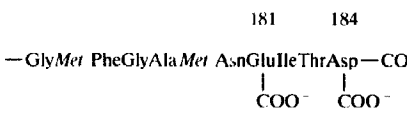
Fig. 5. Effect of EDC treatment on the activity of H^+-coupled (light/DTT-triggered) and non-coupled (methanol-stimulated) ATPase of isolated thylakoid membranes. Experimental details are given in the Experimental procedures. Control activities: 108.5 μmol P_i mg chlorophyll^{-1} h^{-1} (coupled ATPase) and 245.8 μmol P_i mg chlorophyll^{-1} h^{-1} (non-coupled ATPase).

dient is only slightly decreased upon phosphate addition (Fig. 4b), indicating inhibition of photophosphorylation at the level of the H^+-translocating ATPase. Control thylakoids, which were pre-illuminated in the presence of DTT to activate the ATPase ("light-triggered ATPase"), show generation of a proton gradient on addition of ATP in the subsequent dark (Fig. 4c). However, no significant gradient formation is detected with EDC-treated thylakoids (Fig. 4d). The results of Fig. 4 indicate that EDC is a rather specific inhibitor of CF_0CF_1.

EDC treatment might effect inhibition of either the scalar ATPase reaction or the vectorial proton translocation step. To test this, two different ATPase activities of thylakoids were measured as functions of EDC incubation time, the non-coupled methanol-stimulated ATPase [24] and the H^+ transport-coupled light-triggered ATPase [1]. In order to obtain energetically-controlled and comparable conditions, the proton gradient was clamped at $\Delta pH = 2.5$ in the latter system. This was achieved by feed-back modulation of the intensity of simultaneously employed photosynthetic light [26]. Fig. 5 shows that both ATPase activities are inhibited by EDC treatment. Particularly at prolonged incubation times, however, the coupled reaction is more sensitive to EDC. Inhibition of the uncoupled ATPase may be due to the formation of intermolecular and intramolecular crosslinks of CF_1 subunits, including α and/or β (cf. Fig. 1). The additional inhibition of coupled ATPase as a function of EDC incubation time is related with the loss of staining intensities of subunits δ and I and the increase of staining intensity of the 38 kDa δ-I aggregate (not shown). We admit, however, that this semi-quantitative estimation does not really prove a correlation between the two parameters.

Discussion

The cyanogen bromide cleavage pattern of the EDC-induced δ-I crosslink permits the conclusion that the link is formed between a segment of subunit δ including the amino acids Val-1 to Met-166 and the C-terminal end of subunit I. Inspection of the C-terminal sequence of CF_0-I,



shows that only two acid amino acids are suitable candidates for EDC-mediated crosslinking, Glu-181 and the C-terminal Asp-184, comprising, altogether, three carboxylic groups as possible reaction sites. The predicted secondary structure of CF_0-I shows five helical segments separated by short β-turns [29]. The hydrophobic N-terminal helix, comprising the amino acids Ser-19 to Phe-43, is supposed to span the membrane, whereas the remaining part of the polypeptide – including the C-terminal crosslinking site – is exposed to the CF_1-facing membrane side and is accessible to antibodies [34] and proteases [35]. Accordingly, the co-reacting amino group must be in the δ subunit. From our results its location cannot be further specified within the large segment of CF_1-δ, designated 'peptide A'. In addition to the N-terminal amino group there are 16 arginines or lysines, respectively [32], as possible linking sites.

Proteolytic and immunological studies have shown that in isolated thylakoids subunit δ is virtually inaccessible, except for about 15 C-terminal amino acids which are clipped by V8-protease [10]. In isolated CF_1, however, δ is rapidly degraded by trypsin and recognized by monoclonal antibodies against different epitopes [36]. These results suggest that in situ most of subunit δ is hidden within the CF_1 head. Since the V8-protease-accessible C-terminal segment of δ is not involved in crosslinking to CF_0-I, the contact faces of the two subunits seem to be screened by other subunits. Nevertheless, the interacting surfaces seem to be accessible by the smaller EDC molecule.

The formation of a zero-length crosslink presumes close vicinity of the reacting acidic and basic amino acid, respectively. As the two oppositely charged amino acids can undergo ionic binding, it is likely, although unproven, that they play a role in natural binding between the two subunits I and δ.

The role of δ as a structural link between CF_0 and CF_1 was proposed by Nelson and Karny [37]. Later on, however, several workers found that δ is not required

for CF_1 binding, but is necessary for the functional integrity of the ATPase complex. With the exception of Patrie and McCarty [38], who reported some restoration of photophosphorylation of CF_1-stripped thylakoids with δ-deficient CF_1 at higher Mg^{2+} concentrations, most authors found no reconstitution unless isolated δ was also added [6,39,40]. Chemical crosslinking studies with dimethyl suberimidate and hexamethylene diisocyanate revealed structural relationships between δ and all other CF_1 subunits, as well as between δ and the CF_0 subunits I and II [27], which, in part, is in accordance with our results on EDC-catalyzed crosslinking.

The CF_0-I homologous subunit b is the probable target for binding of F_1 in *E. coli*. Trypsin or chymotrypsin treatment of EF_1-stripped membranes or EF_0-containing liposomes, which affected only subunit b, abolished re-binding of EF_1 and the cleavage of b was protected by the presence of EF_1 [41–44]. Treatment of EF_1-stripped membranes or EF_0-liposomes with the arginine-modifying reagent, phenylglyoxal, which resulted in predominant incorporation into subunit b likewise inhibited EF_1 binding [41,45]. Similar results were obtained with 2,3-butanedione [46]. The conclusion that arginine residues on subunit b are involved in binding of F_1, is not exactly in agreement with the results reported here on CF_0-I-CF_1-δ crosslinking, where the reaction partner in subunit I is a glutamic or aspartic acid, respectively. Comparison of the primary structures shows large variations in both, subunits δ (or OSCP in the mitochondrial enzyme) and subunit I/b from different sources, but quite homologous secondary structures [29,32,47]. The possible amino acids of CF_0-I that form the crosslink with subunit δ in spinach, are not conserved, suggesting that in other species the interaction between the respective subunits may be through other amino acid residues of the same region of the polypeptide chain. This is also supported by the finding that EF_1-δ can replace CF_1-δ in reconstitution of photophosphorylation [40]. It is also likely that more sites than the one identified here are involved in the interaction of the two polypeptides.

The higher sensitivity of the coupled, compared to the uncoupled, ATPase reaction towards EDC treatment suggests inhibition of the proton translocation step in addition to some inhibition by crosslink formation between CF_1 subunits. Since δ plays an important role in the control of proton conductance through the ATPase complex [7,48], it is tempting to trace back the additional inhibition of the coupled reaction to the observed formation of a δ-I crosslink. Covalent fixation of δ to CF_0 may either block the transfer of protons at the interface between the two subcomplexes or act indirectly by preventing structural changes or motions of δ and I which may be related with proton coupling of the ATPase reaction.

In addition to its function as a driving force in ATP formation, a proton gradient is also necessary for ATPase activation [1]. It is unknown if activation requires only enzyme protonation from the intra-thylakoidal side (possibly related with deprotonation at the external side) [23,49] or proton flow through the enzyme complex. From the results of Fig. 5 we cannot decide whether EDC treatment inhibits enzyme activation or the proton-coupled catalytic reaction or both. Since the proton gradient was clamped in this experiment, i.e., the internal proton concentration was constant, it is clear, however, that the pre-requisites for protonation and deprotonation, respectively, at the two sides of the thylakoid membrane were the same in the non-treated and EDC treated samples.

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