Partial Proteolysis as a Probe of the Conformation of the γ Subunit in Activated Soluble and Membrane-bound Chloroplast Coupling Factor 1*

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Treatments that enhance the latent ATPase activity of the chloroplast coupling factor (CF1) also induce hypersensitivity of the γ subunit toward trypsin. A number of different γ subunit cleavage products are formed (Moroney, J. V., and McCarty, R. E. (1982) J. Biol. Chem. 257, 5910–5914).

We have compared the γ cleavage products of membrane-bound and isolated CF1, activated either by reduction of the γ disulfide bond or by removal of the ε subunit. The γ subunit of isolated CF1, lacking the ε subunit was cleaved to a 27,000-Da species. The same cleavage site became exposed following energy-dependent conformational changes in the membrane-bound enzyme. Activation by reduction of the γ disulfide bond also exposed this site. However, the γ subunit of reduced CF1 was cleaved rapidly at an additional site and trypsin treatment gave rise to a 25,000-Da γ species. The small peptide generated by the second cleavage contains one of the cysteinyl residues of the reduced disulfide bridge of γ. This peptide dissociates from the enzyme and can be isolated by gel filtration. The close proximity of the trypsin cleavage sites to the disulfide bond of γ is discussed with respect to the effects of tryptic cleavage on the ATPase activity of CF1. The data indicate that structural changes in a limited region of the γ subunit strongly influence the catalytic properties of both soluble and membrane-bound CF1.

Chloroplast coupling factor 1 catalyzes the light-dependent formation of ATP from ADP and P_i (1, 2). CF1, is comprised of five different polypeptide subunits with a proposed stoichiometry of αβ2γε (3). The two larger polypeptides, α and β, are thought to contain the catalytic site(s) (4). The γ subunit is involved in regulation of catalysis and may also function in regulating the flow of protons through the hydrophobic proton channel (CF0) portion of the coupling factor complex (1). Both the δ and ε subunits are required for the tight coupling of ATP synthesis to proton translocation (5). The ε subunit is a potent inhibitor of the ATPase activity of the isolated enzyme (6).

Four cysteinyl residues have been identified in the γ subunit (7). One residue reacts with SH-directed probes in membranes kept in the dark and is therefore called the "dark-accessible site." Another residue reacts with probes only under energized conditions (i.e. in the light) and is called the "light-accessible site." The other two cysteinyl residues form a disulfide bond within γ and are only accessible to labeling after reduction, for example by dithiothreitol.

Although illuminated thylakoids catalyze rapid rates of ATP synthesis, the rates of ATP hydrolysis in the dark are very low unless the thylakoids are preilluminated in the presence of thiol reagents (8). Isolated coupling factors of mitochondria and bacteria are active per se in ATP hydrolysis (9), while isolated CF1 is inactive and may be activated by a variety of methods (1, 2).

Activation of the ATPase of CF1, in solution by thiol reagents results from reduction of the disulfide bond in the γ subunit (10), whereas heat (5, 11), detergents (12), and alcohols (6) all cause dissociation of the ε subunit. Activation of the ATPase of oxidized CF1 by proteases was originally thought to result from digestion of ε (13). However, activation appears to correlate well with digestion of the ε subunit (14).

Pretreatment of CF1, with dithiothreitol or with heat both induce a hypersensitivity of the γ subunit toward proteolytic cleavage by trypsin (14). Cleavage of γ under these conditions results in a further increase of the ATPase activity of the enzyme. A similar hypersensitivity of γ toward trypsin was observed with membrane-bound CF1 during illumination of thylakoids (15).

We have investigated the tryptic cleavage products of the γ subunit in further detail. Two trypsin cleavage sites have been identified. One of these sites is exposed by removal of the ε subunit from isolated CF1. The same site also becomes exposed in CF1, attached to the thylakoid membrane during illumination. Reduction of the γ disulfide bond leads to a conformation of CF1, in which both cleavage sites become exposed to trypsin with either the isolated or the membrane-bound enzyme.

MATERIALS AND METHODS

Chloroplast thylakoids were prepared from fresh market spinach (19). CF1 was prepared by a modification (17) of the method of Binder et al. (18), except that chromatography on DEAE-Sephadex A-50 (19) was used instead of sucrose density centrifugation. Procedures for the small scale preparation of CF1 (20) and for preparation of CF1 lacking the ε subunit (6) have been published elsewhere. Enzyme preparations were stored as ammonium sulfate precipitates and were desalted on Sephadex G-50 prior to use. Protein concentrations were determined.
by the method of Lowry et al. (21) or using an extinction coefficient for CF, of 0.483 cm²/mg at 277 nm and a molecular weight of 400,000 (22, 3).

Dithiothreitol activation of the isolated enzyme was carried out by incubation in 50 mM Tris-HCl (pH 8.0), 2 mM EDTA for 2 h at room temperature in the presence of 50 mM dithiothreitol for CF, (10) or 15 min at room temperature in the presence of 20 mM dithiothreitol for \( \epsilon \)-free CF,. Dithiothreitol was removed by gel filtration. For membrane-bound CF, thylakoids (equivalent to 0.1-0.2 mg of chlorophyll/ml) were illuminated for 5 min at room temperature in the illumination medium with trypsin (10-20 pg/ml) either in the dark or in the light. Soybean trypsin inhibitor (60 pg/ml) was added to stop digestion. For trypsin treatment of isolated CF, or \( \epsilon \)-free CF, 2 pg of trypsin were added per 100 pg of enzyme preparation in 50 mM Tris-HCl (pH 8.0), 2 mM EDTA. The reaction was terminated by addition of soybean trypsin inhibitor (50 pg/ml) and gel filtration on a column of Sephadex G-50. Thylakoids were isolated by centrifugation and resuspended in the illumination medium.

For trypsin treatment of membrane-bound CF, thylakoids (equivalent to 0.1-0.2 mg of chlorophyll/ml) were incubated for 5 min at room temperature in the illumination medium with trypsin (10-20 pg/ml) either in the dark or in the light. Soybean trypsin inhibitor (60 pg/ml) was added to stop digestion. For trypsin treatment of isolated CF, or \( \epsilon \)-free CF, 2 pg of trypsin were added per 100 pg of enzyme preparation in 50 mM Tris-HCl (pH 8.0), 2 mM EDTA. The reaction was terminated by addition of soybean trypsin inhibitor (50 pg/ml) and gel filtration on a column of Sephadex G-50. Thylakoids were isolated by centrifugation and resuspended in the illumination medium.

For the identification of cysteine-containing peptides, the SH-reactive probe 6-acyroyl-2-dimethylaminonaphthalene (Acrylodan, Acrylodan, Ref. 23) was used as a fluorescent label. This probe was described as mainly SH-directed (23), and CF, labeled with Acrylodan under various conditions behaves like CF, labeled with fluorescent maleimide with respect to the distribution of label on \( \gamma \) and \( \epsilon \) subunits and a digestive pattern. Pretreatment of CF, with N-ethylmaleimide reduces the amount of incorporated Acrylodan by more than 90%, indicating that labeling of amino groups is low under the conditions employed. The probe was dissolved in dimethyl formamide and was stable for several weeks at 4°C. The final concentration of dimethyl formamide in reaction mixtures was less than 1% (v/v). Acrylodan exhibits a high absorption maximum at 380 nm with an extinction coefficient of 16 \( \times \) 10⁴ cm²/mg at pH 8.0. The fluorescence emission maximum (excitation 380 nm) of the probe reacted with mercaptoethanol in water is at 540 nm. Reaction products of Acrylodan with CF, however, show emission maxima over the range of 475-485 nm, indicative of a hydrophobic environment (23). The disulfide cysteinyl residues of the \( \gamma \) subunit of membrane-bound CF, were labeled as follows. Reactive sulphydryls were blocked by incubation of thylakoids (equivalent to 1 mg of chlorophyll/ml) with N-ethylmaleimide (10 mM) for 15 min at room temperature. Thylakoids were diluted with illumination medium containing 5 mM dithiothreitol and 5 mM MgCl₂, 25 \( \mu \)M procaine (illumination medium), and 5 mM dithiothreitol. Thylakoids were collected by centrifugation and resuspended in the illumination medium.

RESULTS

Cleavage of Membrane-bound CF, by Trypsin—CF, was isolated from thylakoid membranes treated with trypsin in the dark or in the light. The thylakoids were either illuminated in the presence of dithiothreitol or kept in the dark in the absence of dithiothreitol prior to trypsin treatment. After isolation, ATPase activities were measured (Table I) and samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1). Consistent with a previous report (15), CF, from membranes treated with trypsin in the dark had only a slightly higher Ca²⁺-ATPase activity than control CF, (Table I). The amount of \( \gamma \) in this preparation was not reduced and no degradation products were seen (Fig. 1, lane B). Trypsin treatment in the light, however, resulted in higher ATPase activities and most of the \( \gamma \) subunit was degraded to a smaller polypeptide of about 27 kDa (Fig. 1, lane C).

Pretreatment of thylakoids with dithiothreitol in the light, which causes reduction of the disulfide bond in the \( \gamma \) subunit (27), and subsequent incubation with trypsin either in the dark or in the light resulted in CF, preparations with Ca²⁺-ATPase activities similar to that of CF, isolated from thylakoids trypsin-treated in the light (Table I). Again, the \( \gamma \) subunit of these preparations was clipped, but this time the major cleavage product was a polypeptide of approximately 28 kDa (lanes D and E). The amount of \( \gamma \) remaining was much higher in the sample from thylakoids treated with trypsin in the dark (lane D) than in that from thylakoids exposed to trypsin in the light (lane E). It should be pointed out that all samples were exposed to a high concentration of dithiothreitol prior to electrophoresis. This eliminated the possibility that the disulfide bond in oxidized CF, altered the migration of the 27-kDa species. Trypsin cleavage of \( \gamma \) either the reduction or 27-kDa species also resulted in a small peptide visible at the gel front. This species, as judged by electrophoresis on higher percentage gels, is about 6 to 8 kDa and is probably the same fragment previously shown to contain the dark-accessible cysteinyl residue of \( \gamma \) (28).

Cleavage of Isolated CF, by Trypsin—The \( \gamma \) subunit of oxidized CF, is relatively stable during short-term incubation (<5 min) with low concentrations of trypsin (14). Longer incubations with trypsin result in cleavage of all five subunits with a concomitant increase in the Ca²⁺-dependent ATPase activity of the enzyme to a maximum value after about 60-}

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Both dithiothreitol-treated CF₁ and ε-free CF₁ after only 5 min of incubation with trypsin. No further activation was observed in either case after longer incubation with trypsin (not shown). Consistent with earlier reports, the rapid further activation of reduced CF₁ and ε-free CF₁ by trypsin coincided with a rapid cleavage of the γ subunit (Fig. 2, lane C). The major γ cleavage product of reduced CF₁ was the 25-kDa species also produced by tryptic cleavage of reduced CF₁ on the membrane (Fig. 1). Interestingly, the γ subunit of CF₁(−ε) was rapidly cleaved to a 27-kDa species similar to that obtained from trypsin cleavage of oxidized CF₁ bound to immobilized thylakoid membranes (Fig. 2, lane E; Fig. 1, lane C). Trypsin rapidly cleaved the γ subunit of reduced CF₁(−ε) to the 25-kDa species (Fig. 2, lane F). Treatment of reduced CF₁(−ε) with trypsin had no effect on the ATPase activity since the enzyme was already maximally activated (Table II). Regardless of whether a 27-kDa or 25-kDa species was formed, the 6-8-kDa γ fragment appeared at the front.

**Trypsin Cleavage of Dithiothreitol-activated CF₁**—A fluorescent maleimide (anilinonaphthylemaleimide) was previously used to investigate the trypsin digestion pattern of isolated CF₁ labeled either on the α-accessible or light-accessible γ sulfhydryls (28). A similar approach was carried out to examine the fate of the sulfhydryls that participate in the γ disulfide using 6-acryloyl-2-dimethylaminonaphthalene (Acrylodan), a new fluorescent probe with sulfhydryl, but little amine reactivity (23). The accessible sulfhydryls of the γ subunit (Fig. 2, lane F) were blocked by incubation with N-ethylmaleimide. After reduction of the γ disulfide...
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bond with dithiothreitol, the resulting free sulphydryls were reacted with Acrylodan and the modified enzyme was treated with trypsin. A time course of the digestion by trypsin is shown in Fig. 3. Within the first minute after addition of trypsin, the fluorescence of γ decreased while several new fluorescent bands appeared. The most prominent bands were the 27-kDa and 25-kDa species described above. A diffuse band was also formed with a molecular mass of about 11–11.5 kDa; at longer incubation times, these fragments formed a sharp band of 11 kDa which is probably the 11-kDa species found as a cleavage product of the 25-kDa fragment (14). The 14-kDa species formed 3–5 min after trypsin addition exhibited a low fluorescence, presumably due to a small amount of label incorporated into the “light”-accessible γ sulphydryl. Another diffuse band running below the free dye band appeared after 1–2 min. The amount of fluorescence in this band was difficult to estimate. However, it seemed to increase until γ and the 27-kDa species were nearly completely missing. In addition, the amount of fluorescence in the 25-kDa species after digestion for 5 min appeared to be lower than that of the original γ band. Essentially the same digestion pattern was obtained with CF₁ labeled with fluorescein maleimide on the disulfide cysteines; a 27-kDa species was formed which was further cleaved into the 25-kDa and 2-kDa fluorescent species. Therefore, the two labeled cysteines of the disulfide bond are both likely to be present in the 27-kDa species. An additional clip creates a 25-kDa fragment, as well as a small peptide with a molecular mass of about 2000 Da. Both the 25-kDa and 2-kDa fragments contain a disulfide cysteine. The faint fluorescent band above the free dye band probably originated from a slight unspecific labeling of the α and β subunits.

Isolation of the Small Fluorescent Peptide by Gel Filtration—
A fluorescent band near the front was detected when disulfide-labeled, trypsin-treated CF, was subjected to acrylamide gel electrophoresis in the absence of sodium dodecyl sulfate. This result suggests that the 2-kDa fragment dissociates from the remainder of the enzyme. To isolate this species, CF₁ was treated with trypsin for 4 min and was passed through a Sephadex G-75 column.

Two peaks of Acrylodan fluorescence and of A277 and A380 were observed (Fig. 4). The first peak eluted with the void volume (fractions 8–12); its fluorescence emission maximum was at 475 nm which is identical with that of labeled undigested CF₁. The second peak eluted in fractions 18–30; the relationship between the absorption at 380 nm (the absorption maximum of Acrylodan) and 277 nm varied throughout the peak indicating that several peptides were present. The absorption at 380 nm matched well with the fluorescence. The emission maximum was at 525 nm in the second peak samples, indicating that the probe was in a more polar environment than in peak I. The molecular mass of peptides eluted in peak II (deduced from the separation range of Sephadex G-75) is 5 kDa or less. As judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, peak II contained only fluorescent and Coomassie blue staining material that migrated near the front (not shown). Samples of the first peak contained the slightly modified α and β subunits, together with ε, and the 25-kDa and 6–8 kDa fragments of the γ subunit. δ was completely digested in accordance with its high sensitivity to trypsin (14). Fractions 18 to 30 were combined and applied to a small DEAE-cellulose column (0.5 x 5 cm). Free label and other fluorescent material eluted with the void volume. The small fluorescent peptide was eluted with 0.2–0.3 M NaCl. CF₁ labeled on the dark site with Acrylodan, reduced by dithiothreitol, and treated with trypsin in the same way as described above showed no second fluorescent peak upon Sephadex G-75 separation (data not shown).

Identification of Acrylodan-labeled Tryptic Peptides—A detailed analysis of cysteine-containing tryptic peptides of the γ subunit was carried out previously by modification of sulphydryl groups with 4-vinylpyridine followed by total tryptic digestion and separation of peptides by HPLC (7). The two cysteines forming the disulfide bond and the light- and dark-accessible cysteines eluted on four different peptides, S1 to S4 (7). A similar analysis was carried out using Acrylodan-labeled CF₁ to identify the γ peptide released from the enzyme by trypsin. The peptides were detected by their fluorescence since the sensitivity of this method is much higher than detection by UV absorption (7).

When isolated oxidized CF₁ is treated with Acrylodan, most of the label was incorporated into the γ subunit (about 80%) and a small amount into the ε subunit. After total tryptic digestion of the labeled protein, two major fluorescent peaks were separated by HPLC (Fig. 5A). The second peak with a retention time of 34 min corresponds to the peptide containing the dark-accessible site (S4) and the first peak (retention time 31 min) to the cysteine-containing peptide of ε. However, the tryptic peptide of S3 (containing the light-accessible γ sulphydryl) eluted at nearly the same position. It was found that
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**FIG. 5.** HPLC elution profile of the total trypsin digest of Acrylodan-modified CF$_1$. Acrylodan-modified CF$_1$ (0.5–1.5 mg/ml) was treated with trypsin (2 $\mu$g/100 $\mu$g of CF$_1$) in the presence of CaCl$_2$ (3 mM) for 1–3 h at 37 °C. The samples were then heated in boiling water for 10–15 s. After a second addition of trypsin (2 $\mu$g/100 $\mu$g of CF$_1$), the samples were incubated at 37 °C overnight. The solutions were centrifuged in a Beckman Airfuge for 5–10 min to remove small amounts of precipitate and 20-$\mu$l aliquots were applied to HPLC. Peptides were eluted using gradients formed by mixing 0.1% phosphoric acid in H$_2$O (solvent A) and phosphoric acid in acetonitrile (solvent B). The elution program was composed of two linear gradients from 20–34% solvent B for 15 min followed by a 34–45% solvent B linear gradient for 40 min at a constant flow rate of 2 ml/min. A, CF$_1$; B, dithiothreitol-activated CF$_1$.

This peak varies in height in different preparations and may contain S3 as well as the $\epsilon$ peptide. CF$_1$ labeled with Acrylodan after pretreatment of the enzyme with dithiothreitol showed two additional peaks with retention times of 18 and 25 min (Fig. 5B), corresponding to the two disulfide sulfhydryls of $\gamma$. Therefore, the four peptides S1 to S4 appear to elute in the same sequence as the peptides labeled with 4-vinylpyridine (7).

Treatment of isolated CF$_1$ with N-ethylmaleimide prior to dithiothreitol incubation, and subsequent labeling with Acrylodan, decreased the amount of label incorporated into $\epsilon$ and the accessible $\gamma$ sulfhydryl. Consequently, HPLC peaks $\epsilon$/S3 and S4 were reduced (Fig. 6A). CF$_1$ specifically labeled with Acrylodan on the disulfide sulfhydryls was subjected to a brief exposure to trypsin and the fluorescent products separated by Sephadex G-75 chromatography (cf. Fig. 4). Polypeptides in the two fluorescent peaks were digested with trypsin overnight and examined by HPLC. The first peak (void volume fractions) (Fig. 6B) contained only S1, while S2 was nearly exclusively present in the second G-75 peak (Fig. 6C). The overnight trypsin digestion of the S2-containing fractions did not affect the retention time of S2. This result indicates that the S2 fragment is released from the enzyme in its final form rather than as a larger precursor.

Modification of the disulfide sulfhydryls with Acrylodan is not a prerequisite for trypsin cleavage and release of S2 because trypsin treatment of the reduced, unmodified CF$_1$ gave the same cysteine-containing peptide. In this case, released S2 could be identified by addition of Acrylodan to the late fractions of Sephadex G-75 chromatography and subsequent HPLC separation (data not shown).

**FIG. 6.** HPLC elution profiles of Acrylodan-modified CF$_1$ fractions separated by gel filtration after a short trypsin treatment. Total trypsin digest of fractions from the Sephadex G-75 column described in the legend to Fig. 4 were analyzed by HPLC as in Fig. 5. A, CF$_1$ labeled with Acrylodan on the $\gamma$ disulfide cysteines (accessible SH-groups were blocked with N-ethylmaleimide before reduction of the disulfide bond); B, Fraction 10 (Fig. 4); C, Fraction 23 (Fig. 4).

Release of S2 from Membrane-bound CF$_1$—Membrane-bound CF$_1$ was labeled with Acrylodan on the disulfide sulfhydryls and the release of fluorescent peptides into the medium during incubation with trypsin in the light or the dark was followed. After trypsin treatment, the thylakoids were precipitated by centrifugation and the resulting supernatants were passed through small DEAE-cellulose columns. Fluorescent peptides were eluted and identified by HPLC without further trypsin digestion. A single, small peak was present in the supernatants derived from membranes treated with trypsin either in the dark or in the light (Fig. 7, A and B). The identity of this peak as S2 was confirmed by co-chromatography with authentic S2 derived from isolated CF$_1$ (Fig. 7C). The higher yield of the S2 fragment in the light is again indicative of a light-induced increase in the sensitivity of $\gamma$ toward proteolytic attack.
Two major sites of trypsin cleavage of the \( y \) subunit were identified. Although the \( y \) subunit of oxidized thylakoids treated with trypsin in the dark or in the light. Soybean trypsin inhibitor EDTA buffer and were subjected directly to HPLC without further trypsin treatment.

The results are summarized in the degradation scheme shown in Fig. 8. Both sulfhydryls are present on a precursor of the 25-kDa fragment having an apparent molecular mass of 27 kDa. This fragment also contains the light site sulfhydryl residue. The dark site is found on a 6-8-kDa polypeptide which appears without any apparent precursor. The scheme of Fig. 8 assumes that no fragments other than the 27- and 6-8-kDa species are formed by the first trypsin clip.

Upon formation of the 25-kDa species from the 27-kDa fragment, one of the labeled cysteines is lost and appears on a 2-kDa peptide. This labeled molecule freely dissociates from the CF\(_1\) complex and can be isolated by gel filtration. HPLC showed that this peptide probably is the tryptic peptide S2 (7) containing 11 amino acid residues, including one of the \( y \) sulfhydryl sulfhydryls.

The 25-kDa species is further cleaved to two fragments of 14 and 11 kDa, containing the light site and the other sulfhydryl residue, respectively. Although the arrangement of these two fragments within the \( y \) subunit was not confirmed, we assume that the 14-kDa species, containing the light site cysteine, is the N-terminal portion of \( y \) because in the oxidized enzyme the 27-kDa species is sometimes clipped at trypsin sites I and III only giving a slightly larger precursor of the 11-kDa species (see Fig. 3). Cleave at the second trypsin site is very slow with the oxidized enzyme indicating that the sulfhydryl bridge keeps this region of \( y \) in a conformation inaccessible to trypsin. Removal of \( \epsilon \) from oxidized CF\(_1\) exposes only one of the tryptic sites (I in Fig. 8). This might indicate either that \( \epsilon \) is bound to this region of \( y \) or that \( \epsilon \) binds to an “allosteric” site and keeps the coupling factor in a conformation in which the tryptic sites are inaccessible.

The latent ATPase of CF\(_1\) is activated by reduction of the \( y \) disulfide bond or further activated by trypsin cleavage at a site or sites which appear to be close to the disulfide bond. All of these effects appear, therefore, to be related in that they involve changes in a limited region of the \( y \) polypeptide. The \( y \) disulfide bond may be involved in keeping CF\(_1\) in a constrained, inactive conformation. Reduction, or trypsin cleavage of \( y \), could result in a higher flexibility of the modified complex in which rapid ATP hydrolysis is possible. Removal of \( \epsilon \) from CF\(_1\) may invoke similar changes in the \( y \) subunit. Although the effect of \( \epsilon \) removal is only partial, reduction of the disulfide bond in \( \epsilon \)-free CF\(_1\) results in complete activation. Together with the effect of \( \epsilon \) removal on accessibility of trypsin or dithiothreitol to \( y \), such results have been taken to indicate a close physical and functional relationship between \( \epsilon \) and \( y \). A critical region of the \( y \) subunit...
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**FIG. 8.** Schematic presentation of the trypsin cleavage sites of the $\gamma$ subunit of CF, and of fragments generated by trypsin cleavage. Cleavage of $\gamma$ at the first trypsin site (I) generates a 27-kDa and a 6-8-kDa fragment (with the dark site cysteine). When the disulfide bond in $\gamma$ (S-S) is reduced, a second site (II) is exposed and trypsin cleavage leads to a 25-kDa fragment and the 1.3-kDa peptide containing a disulfide cysteine (S2 cysteine). The 25-kDa fragment is slowly cleaved at a third site (III) giving a 14-kDa and an 11-kDa fragment.

may, therefore, play a central role in the process of activation of CF, both on and off the thylakoid membrane.

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