Proteolytic Cleavage within a Regulatory Region of the γ Subunit of Chloroplast Coupling Factor 1[†]

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Received December 11, 1995; Revised Manuscript Received February 12, 1996[⊗]

ABSTRACT: The γ subunit of chloroplast coupling factor 1 (CF_1) is susceptible to selective proteolysis when the enzyme is in solution and the ϵ subunit is removed [$CF_1(-\epsilon)$]. In spinach thylakoid membranes, rapid cleavage of γ is dependent on the generation of an electrochemical proton potential. The tryptic cleavage sites within the γ of oxidized CF_1 in illuminated thylakoids as well as of reduced $CF_1(-\epsilon)$ in solution were determined by N-terminal amino acid sequencing. Two large γ fragments of 27 000 (γ27) and 10 000 (γ10) molecular weight were generated by trypsin treatment of membrane-bound CF_1. The N-terminal γ27 contains amino acids 1−215, and the C-terminal γ10 contains 232−323. These polypeptides were tightly associated with the trypsin-resistant core of the enzyme. In contrast, three large γ fragments were produced by trypsinolysis of reduced $CF_1(-\epsilon)$. These polypeptides, which were also tightly associated with the trypsin-resistant core, have molecular weights of 7900 (γ8), 14 850 (γ15), and 10 000 (γ10). These fragments contain residues 1−70, 71−204, and 232−323, respectively. The C-terminal γ10 fragment generated by trypsin treatment of membrane-bound and soluble CF_1 are identical. These results suggest that the γ subunit of CF_1 in illuminated thylakoids resembles that of $CF_1(-\epsilon)$ with respect to accessibility to proteolytic cleavage. Cleavage of γ between residues 215 and 232 is sufficient to fully activate the ATPase activity of the enzyme without reduction of the γ disulfide. In addition, cutting within this region is responsible for loss of affinity for the inhibitory ϵ subunit.

The chloroplast ATP synthase, CF_1CF_o, catalyzes the formation of ATP from ADP and P_i at the expense of the thylakoid membrane proton gradient. The catalytic portion, CF_1,[1] is composed of five subunits, α through ϵ in order of decreasing molecular weight, with a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$. The transmembrane region, CF_o, is involved in proton translocation and attachment of CF_1 (McCarty & Moroney, 1985).

The proteolytic enzyme trypsin, which cleaves peptides on the carboxyl side of lysine and arginine residues, has been an important tool for probing the structure and function of the F_1-ATPases. Trypsin has been used to determine the sites of solvent accessibility on *Escherichia coli* F_1 (Dunn et al., 1980; Bragg & Hou, 1987; Gavilanes-Ruiz et al., 1988; Mendel-Hartvig & Capaldi, 1991; Tang et al., 1994), mitochondrial F_1 (Hundal & Ernster, 1981; Todd & Douglas, 1981; Walker et al., 1985), and chloroplast F_1 (Moroney & McCarty, 1982a,b; Schumann et al., 1985; Berzborn & Finke, 1989a,b). Trypsin activation of ATPase activity has been reported for F_1-ATPases from *E. coli* (Bragg & Hou, 1987; Gavilanes-Ruiz et al., 1988; Tang et al., 1994), plants (Deters et al., 1975; Moroney & McCarty, 1982a; Schumann et al., 1985; Cohen, 1989), and cyanobacteria (Binder & Bachofen, 1979; Werner-Grüne et al., 1994).

The γ subunit of spinach CF_1 is a 323 amino acid polypeptide with a molecular weight of 35 800. Four cysteine residues are found in the subunit at positions 89, 199, 205, and 322 (Miki et al., 1988). Cys-89 is referred to as the "light site" since it reacts with *N*-ethylmaleimide in illuminated thylakoids. Cys-322 is called the "dark site" because it is accessible to *N*-ethylmaleimide in the dark (Moroney & McCarty, 1984). Cys-89 is strictly conserved among the chloroplast, bacterial, and mitochondrial F_1-ATPases with the exception of the γ from a thermophilic bacterium. Cys-199 and -205 form a disulfide bond that is found in an extra domain, approximately Ser-193 to Phe-237, that is only present in photosynthetic organisms (Miki et al., 1988).

For membrane-bound CF_1, trypsin cleavage of oxidized γ under energized conditions results in the formation of two fragments, of 27 and 10 kDa, which remain tightly associated with the $\alpha_3\beta_3$ core (Schumann et al., 1985; Hightower & McCarty, 1996a). Mg^{2+}-ATPase activity is activated and photophosphorylation is uncoupled when thylakoid membranes are treated with trypsin in the light (Moroney & McCarty, 1982b). CF_1 isolated from these thylakoids has a decreased affinity for the ϵ subunit (Soteropoulos et al., 1992).

For soluble CF_1, the ATPase activity can be activated by removal of the ϵ subunit, reduction of the γ disulfide, or trypsin cleavage. The combination of any two of these treatments maximally activates the enzyme (Schumann et al., 1985). Removal of the ϵ subunit and reduction of the γ disulfide make the trypsin cleavage sites on γ more accessible (Moroney & McCarty, 1982a; Schumann et al., 1985). It was previously reported that trypsinolysis of reduced $CF_1(-\epsilon)$ results in the formation of four γ polypeptides. The

[†] This work was supported by National Science Foundation Grants MCB 94 05713 and DMB 91-04742.

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[⊗] Abstract published in *Advance ACS Abstracts,* April 1, 1996.

[1] Abbreviations: CF_1, chloroplast coupling factor 1; $CF_1(-\epsilon)$, CF_1 lacking the ϵ subunit; DTT, dithiothreitol; FM, fluorescein maleimide; TPCK-trypsin, trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone; PVDF, polyvinylidene difluoride; S2, the small γ tryptic peptide released from reduced CF_1; ECF_1, *E. coli* coupling factor 1.

0006-2960/96/0435-4846$12.00/0

fragments, with molecular weights of approximately 14 000, 11 000, 1300, and 6000−8000 (N-terminus to C-terminus) were proposed to contain one cysteine residue each (89, 199, 205, and 322, respectively) (Schumann et al., 1985).

We have further investigated the trypsin cleavage of the γ subunit of spinach CF_1. The large polypeptides formed during cleavage on the membrane or in solution remain tightly associated with the enzyme core. The exact cleavage sites were determined by N-terminal amino acid sequencing and the orientation of the fragments within the γ subunit was determined. The region of γ involved in ϵ binding and activation of ATPase activity was determined to be within the extra domain of CF_1, previously implicated in regulation.

MATERIALS AND METHODS

CF_1 was isolated from market spinach by modifications of the procedure of Shapiro and McCarty (1990) (Soteropoulos et al., 1994; Digel & McCarty, 1995), and the contaminating ribulose bisphosphate carboxylase/oxygenase was removed as described previously (Soteropoulos et al., 1992). $CF_1(-\epsilon)$ was prepared by published procedures (Richter et al., 1984, 1985; Soteropoulos et al., 1994).

For trypsin treatment of thylakoid membrane bound CF_1, the cleavage was performed in the dark or during illumination for 3 min as described elsewhere (Schumann et al., 1985; Soteropoulos et al., 1992).

For trypsin treatment of soluble $CF_1(-\epsilon)$, the γ disulfide was first reduced by incubation with 20 mM dithiothreitol (DTT) in 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5 mM EDTA, for 15 min. The DTT was removed by passing the protein through a 3 mL Sephadex G-50 centrifuge column (Penefsky, 1977) and Cys-199, -205, and -322 were alkylated with either iodoacetic acid (Soteropoulos et al., 1994) or fluorescein maleimide (FM). The protein was labeled for 10 min in the dark with 5 mol of FM:1 mol of $CF_1(-\epsilon)$. The unreacted FM was removed by addition of *N*-acetylcysteine to 2 mM and passage through another 3 mL Sephadex G-50 centrifuge column. The reduced and alkylated $CF_1(-\epsilon)$ (1−50 mg) was treated with TPCK-trypsin for 20 min at 1 μg of trypsin per 100 μg of $CF_1(-\epsilon)$. The trypsin was removed by passing the protein through a 1 mL column of soybean trypsin inhibitor bound to 4% beaded agarose.

All forms of CF_1 were stored, in pH 8.0 buffers, at 4 °C as 50% ammonium sulfate precipitates in the presence of 1 mM ATP and 2 mM EDTA. The protein was desalted prior to use by passage through a 3 mL Sephadex G-50 centrifuge column.

The γ15 fragment was isolated from trypsinized, reduced $CF_1(-\epsilon)$ by a modification of the procedure of Gao et al. (1995) in which the 200 mM NaH_2PO_4 wash was omitted and the isolated fragment was dialyzed against a buffer containing 50 mM Tris-HCl (pH 8.0), 1 M guanidine-HCl, 2 mM EDTA, 20% glycerol. DTT was added to 5 mM before storage at −80 °C.

N-terminal amino acid sequencing was performed on γ27 and γ10 from thylakoid membrane trypsinized CF_1 and γ8, γ15, and γ10 from trypsinized, reduced $CF_1(-\epsilon)$ by the Analytical Chemistry and Peptide/DNA Synthesis Facility at Cornell University. The γ27, γ10, and γ8 fragments were transferred from polyacrylamide gels onto polyvinylidene difluoride (PVDF) membranes according to manufacturer specifications (Immobilon Tech Protocol TP006). The γ15 was sequenced from the isolated fragment.

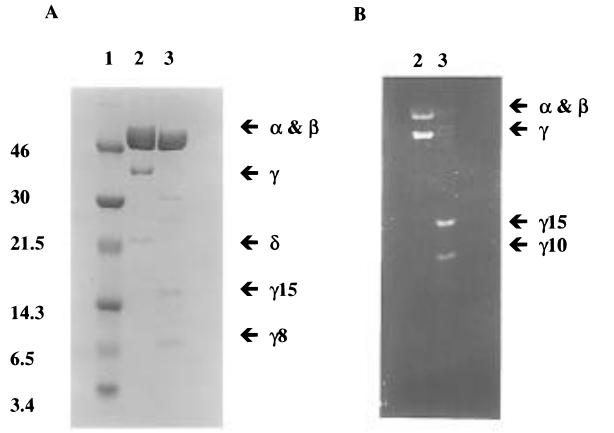

FIGURE 1: Trypsin treatment of reduced and alkylated $CF_1(-\epsilon)$ in solution. Lane 1, molecular mass standards (kDa); lane 2, control $CF_1(-\epsilon)$; lane 3, trypsinized, reduced $CF_1(-\epsilon)$. A. Coomassie-stained gel. The γ disulfide had been alkylated with iodoacetic acid. Each lane contained 30 μg of protein. B. Fluorescent gel. Cys-322 was labeled with FM for both samples. Cys-199 and Cys-205 had also been alkylated with FM for the trypsinized, reduced $CF_1(-\epsilon)$. Each lane contained 25 μg of protein.

To determine if cleavage of γ at Lys-70 affects ATPase activity, $CF_1(-\epsilon)$ from thylakoids incubated with trypsin in the light was trypsin treated without reduction of the disulfide. The protein was incubated with 10 μM $CuCl_2$ for 10 min at room temperature to ensure that the dithiol was oxidized to the disulfide. The $CuCl_2$ was removed with a 3 mL Sephadex G-50 centrifuge column. The enzyme was cleaved with 1 μg of trypsin:100 μg of $CF_1(-\epsilon)$ for 20 min before removal of the trypsin with the soybean trypsin inhibitor column.

CF_1 concentrations were determined by absorbance at 277 nm [ϵ = 0.483 cm^2/mg, Bruist and Hammes (1981)] or by published procedures (Lowry et al., 1951). The molecular mass of CF_1 is 400 kDa (Moroney et al., 1983). Ca^{2+}-ATPase assays were carried out in 50 mM Tris-HCl (pH 8.0), 5 mM ATP, 5 mM $CaCl_2$, and Mg^{2+}(sulfite)-ATPase assays were done in 50 mM Tris-HCl (pH 8.0), 100 mM Na_2SO_3, 4 mM ATP, 2 mM $MgCl_2$. The assays were performed at 37 °C for 2−10 min. The P_i formed during hydrolysis was measured colorimetrically (Taussky & Schorr, 1953). SDS−polyacrylamide gel electrophoresis was performed on 18% polyacrylamide gels with a Tris/Tricine buffer system (Schägger & von Jagow, 1987).

FM was purchased from Molecular Probes (Eugene, OR), and PVDF membranes were purchased from Millipore (Bedford, MA).

RESULTS

γ Fragments of Trypsin-Treated $CF_1(-\epsilon)$. Trypsin treatment of reduced $CF_1(-\epsilon)$ results in the formation of three large γ polypeptides. From 18% gels and N-terminal sequencing, we have determined that the γ tryptic fragments have apparent molecular weights of 7900 (γ8), 14 850 (γ15), and 10 000 (γ10) (Figure 1A,B). Two of the fragments, γ8 and γ15, can be visualized by Coomassie staining (Figure 1A). The third polypeptide, γ10, is not stained well by Coomassie Blue R-250. By labeling Cys-199, -205, and -322 with FM, it is possible to identify γ10 and γ15 by their fluorescence (Figure 1B). As described later, γ8 does not

Table 1: N-Terminal Sequences of the γ Tryptic Fragments

trypsin treatment	γ fragment	sequence	amino acids
on the membrane			
	27	ANLRELRDRI	1−215[a]
	10	TETPAF(T)PIL[b]	232−323
in solution			
	8	ANLRELRDRI	1−70[a]
	15	IRTVKKVALMVVTGDRG	71−204
	10	TETPAFSPIL	232−323

[a] The C-terminal amino acid of these fragments has not been strictly determined. [b] The actual γ sequence is TETPAFSPIL. The serine residue was read as a threonine during sequencing.

contain a cysteine residue that can react with FM. A small fourth fragment (S2), amino acids 205−215, is released from the enzyme after cleavage (Schumann et al., 1985; Cozens & Walker, 1988). The α and β subunits, which are slightly modified by trypsin treatment, run closer together. The δ subunit is fully cleaved and cannot be detected by electrophoresis (Moroney & McCarty, 1982a). Trypsin cleavage for up to 3 h did not significantly affect the appearance of the three large γ fragments (data not shown).

The γ fragments are tightly associated with the $\alpha_3\beta_3$ core. It is significant that greater than 90% of the mass of the γ subunit remains firmly attached to the core. The structure remains intact during precipitation with ammonium sulfate, gel filtration, ion exchange chromatography, and molecular sieving high-performance liquid chromatography. Dissociation methods, such as incubation of the protein in high concentrations of urea in the cold (Gao et al., 1995), have proven successful for removing the fragments.

N-Terminal Amino Acid Sequencing. Even though trypsin cleavage of the γ subunit of spinach CF_1 has been studied for many years, the exact cut sites, with the exception of Lys-204 and Arg-215, were not known. The identities of the tryptic fragments and their positions within the γ subunit could only be surmised by the specific reaction of fluorescent reagents with the four cysteine residues followed by proteolysis. The sites of trypsin cleavage were determined for the fragments generated both on the membrane and in solution by N-terminal amino acid sequencing of either isolated material (γ15) or polypeptides transferred to PVDF membranes (γ8, γ10, and γ27). Ten amino acids were sequenced for all fragments with the exception of γ15 for which 17 residues were determined. Comparison of these partial sequences to the published γ sequence (Miki et al., 1988) made it possible to identify each of the γ tryptic fragments (Table 1).

Trypsin treatment of membrane-bound, oxidized CF_1 during illumination results in the formation of two polypeptides. The γ27 fragment begins with the first amino acid and probably ends at Arg-215. When the γ disulfide is reduced, γ27 is further cleaved to γ25 and the S2 peptide, which ends at Arg-215 (Schumann et al., 1985; Cozens & Walker, 1988), is released. Three of the four cysteine residues, 89, 199, and 205, are present on γ27. The tryptic peptide γ10 extends from Thr-232 to the last amino acid, Val-323. It was previously determined that Cys-322, the penultimate amino acid, is not lost after trypsin cleavage (Hightower & McCarty, 1996a).

The three γ fragments generated by trypsin treatment of reduced $CF_1(-\epsilon)$ in solution encompass almost the entire subunit. The N-terminal polypeptide, γ8, begins at the first amino acid and continues to probably Lys-70, giving the fragment a molecular weight of 7900. On SDS gels, the γ8 fragment migrates slightly farther than the γ10 fragment but not as far as the 6.5 kDa molecular mass standard (Figure 1A). Even though the C-terminal amino acid in γ8 has not been determined, the next closest cut site would result in a much smaller polypeptide (4.7 kDa). The 14.85 kDa fragment (γ15), Ile-71 to Lys-204, contains both the light site cysteine (Cys-89) and one of the disulfide cysteines (Cys-199). The 10 kDa C-terminal polypeptide (γ10), Thr-232 through Val-323, has the dark site cysteine at position 322. The S2 peptide, Cys-205−Arg-215, is released from the enzyme (Schumann et al., 1985; Cozens & Walker, 1988). Amino acids 216−231, which are in the extra domain, could not be identified.

With the exception of the sequence around Lys-70, the same regions of γ are accessible to trypsin both on the membranes during illumination and in solution if the ϵ subunit has been removed. Limited trypsinolysis of oxidized $CF_1(-\epsilon)$ in solution results in the formation of γ27 and γ10, the same fragments that are produced by trypsin treatment on the membranes (Schumann et al., 1985). With reduction, γ27 is cleaved to a 25 kDa polypeptide which, after cutting at position 70, produces the γ8 and γ15 fragments. The C-terminal γ10 fragments generated from membrane treated and soluble treated γ are identical. Regardless of whether the enzyme is cleaved on the membranes or in solution, the N- and C-termini of γ remain intact.

The γ subunit of spinach CF_1 (Miki et al., 1988) and of *E. coli* F_1 (ECF_1) (Saraste et al., 1981) have 38.9% amino acid identity in an overlap of 285 residues (Figure 2). The N- and C-termini, as well as the region around Cys-89, are reasonably well conserved. However, there is little similarity in the rest of the sequence. Limited proteolysis of ECF_1 results in cleavage of the γ subunit at Lys-201. With longer incubations, the ECF_1 γ subunit can also be cut at positions 70, 199, and 212 (Gavilanes-Ruiz et al., 1988; Tang et al., 1994). Two of the cleavage sites in spinach CF_1 γ, Lys-70 and Lys-231, are analogous to trypsin sensitive sites in ECF_1 γ, Lys-70 and Lys-199 or -201. The other cleavage sites on spinach CF_1 γ, Lys-204 and Arg-215, are present in the extra domain and do not correspond to the ECF_1 sequence. There is no site in the spinach γ sequence comparable to the Lys-212 cut in *E. coli*.

ATPase Activity. Trypsinized, reduced $CF_1(-\epsilon)$ is an active Ca^{2+}- and sulfite-stimulated Mg^{2+}-ATPase (Table 2). As previously reported (Schumann et al., 1985), cleavage of reduced CF_1 lacking the ϵ subunit does not significantly affect the ATPase activity of the enzyme. Both the Ca^{2+}- and Mg^{2+}-ATPase activities are comparable for reduced $CF_1(-\epsilon)$ and trypsinized, reduced $CF_1(-\epsilon)$. Storage of the trypsin-treated protein as an ammonium sulfate precipitate for several weeks did not cause appreciable loss of activity (data not shown).

Activation of ATPase Activity by Trypsin Treatment. Trypsin treatment of membrane-bound CF_1 during illumination results in cleavage of γ within the extra domain between residues 215 and 232. CF_1 loses its affinity for the ϵ subunit as a result of this cleavage and is, therefore, more active (Soteropoulos et al., 1992). However, the enzyme is almost fully active without the addition of reducing agents (Table 3). In contrast, CF_1 isolated from membranes treated with trypsin in the dark with intact γ shows the normal response

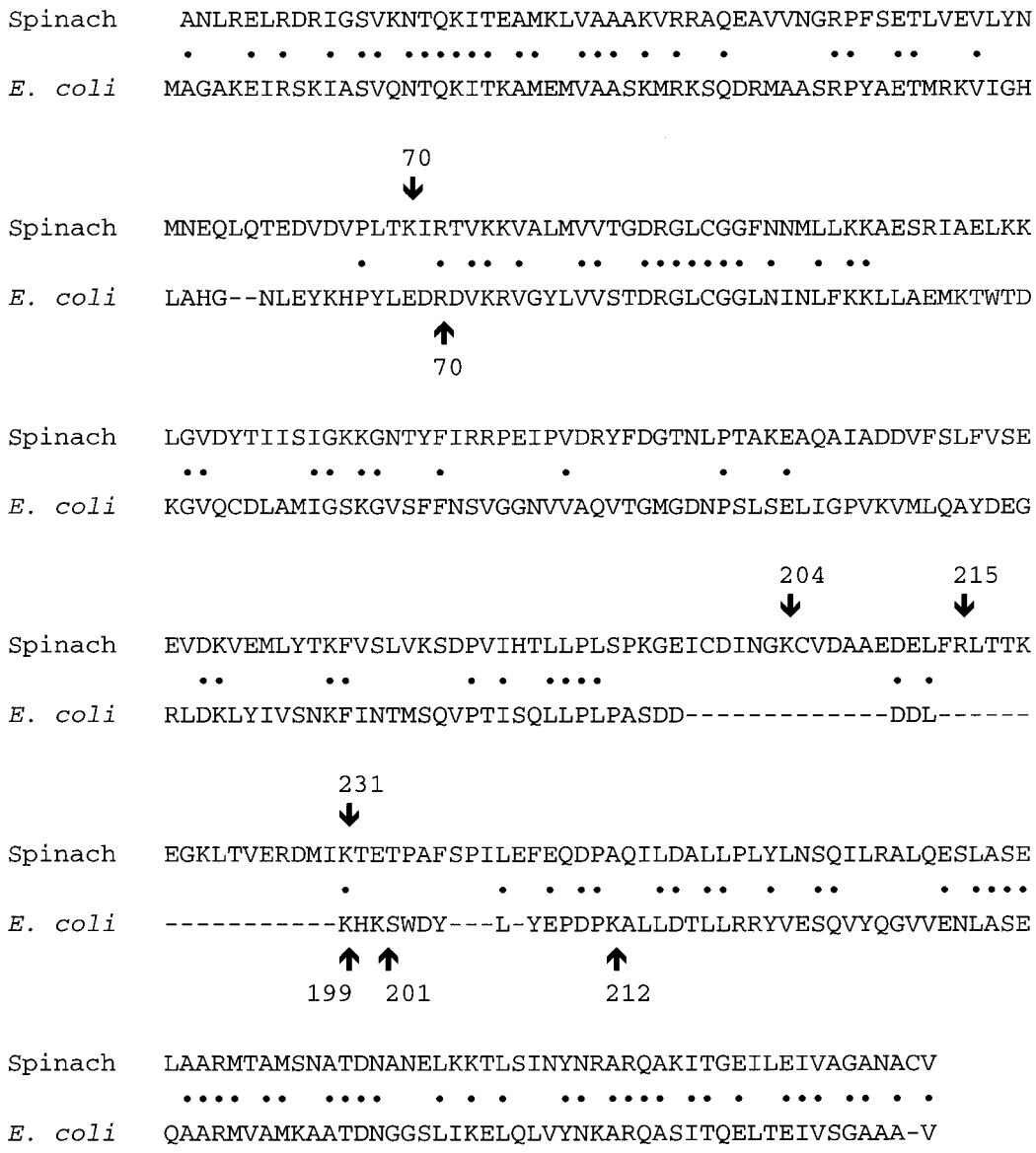

FIGURE 2: Alignment of the γ sequences from spinach CF_1 (Miki et al., 1988) and *E. coli* F_1 (Saraste et al., 1981). Gaps in the sequences are marked by dashes. Identical residues are denoted by ·. Arrows indicate the trypsin cleavage sites in both sequences. The residue number for the cut site is above the arrow. Sequence alignment was performed using the Dayhoff matrix on MULTALIN (Corpet, 1988).

Table 2: Ca^{2+}- and Mg^{2+} (Sulfite)-ATPase Activities of Different Forms of $CF_1(-\epsilon)$

form of CF_1	ATPase activity (μmol of P_i/min/mg of protein)	
	Ca^{2+}	Mg^{2+} (sulfite)[a]
$CF_1(-\epsilon)$	15.7	34.2
reduced $CF_1(-\epsilon)$[b]	31.6	56.3
trypsinized, reduced $CF_1(-\epsilon)$	26.7	51.6

[a] 100 mM sodium sulfite was used to stimulate the Mg^{2+}-ATPase activity. [b] The disulfide cysteines were alkylated with iodoacetic acid after reduction.

Table 3: Activation of the ATPase Activity of CF_1 Isolated from Trypsin-Treated Membranes

condition of trypsin treatment	form of CF_1	Ca^{2+}-ATPase activity (μmol of P_i/min/mg of protein)
dark[a]	CF_1	2.4
	$-\epsilon$	17.0
	$-\epsilon$, + DTT[b]	29.7
illumination[c]	CF_1	14.7
	$-\epsilon$	22.9
	$-\epsilon$, + DTT[b]	27.0

[a] The γ subunit of CF_1 from thylakoids treated with trypsin in the dark was intact. [b] The protein was incubated with 20 mM DTT for 20 min prior to assaying for ATPase activity. [c] The γ subunit of CF_1 from thylakoids incubated with trypsin in the light was cleaved into γ27 and γ10. The high activity of this preparation was partially due to a loss of affinity for the ϵ subunit.

(Schumann et al., 1985) to removal of the ϵ subunit (~8−10-fold increase) followed by reduction of the γ disulfide (additional ~2-fold increase). The slight increase in the activity of $CF_1(-\epsilon)$ from thylakoids treated with trypsin during illumination upon reduction is probably due to incomplete cleavage of the γ subunit during illumination. Under the conditions used for trypsin treatment in the light, approximately 70%−80% of the γ is cleaved (Soteropoulos et al., 1992).

To test if cleavage within the extra domain is sufficient for maximum ATPase activity, the γ27 fragment of $CF_1(-\epsilon)$ from thylakoids treated with trypsin in the light was further cleaved by trypsin without reduction of the γ disulfide. The resulting fragments are probably γ8 and a 16 kDa fragment consisting of Ile-71 to Arg-215 (data not shown). No further

Table 4: Effect of Cleavage at γ Lys-70 on the ATPase Activity of $CF_1(-\epsilon)$ from Thylakoids Incubated with Trypsin during Illumination

treatment	Ca^{2+}-ATPase activity (μmol of P_i/min/mg of protein)
none	34.3
DTT[a]	38.3
trypsin	35.8
trypsin then DTT[a]	34.2

[a] The protein was incubated with 20 mM DTT for 20 min prior to assay.

increase in ATPase activity is observed following this cleavage and the enzyme is not activated by DTT (Table 4).

DISCUSSION

Cleavage of the γ subunit of spinach CF_1 by trypsin is site specific. Even though there are 43 potential cleavage sites, trypsinolysis of the membrane-bound or soluble enzyme results in the formation of several large polypeptides that remain tightly associated with the $\alpha_3\beta_3$ core. It was previously determined that cleavage of the γ subunit of either membrane-bound or soluble spinach CF_1 at positions 204 and 215 releases the small S2 peptide. The cut at Lys-204 is only possible when the γ disulfide is reduced (Schumann et al., 1985; Cozens & Walker, 1988). Two additional trypsin sensitive sites, at positions 70 and 231, have been identified. Lys-231 is accessible to trypsin in illuminated membranes and in solution after removal of the ϵ subunit or reduction of the γ disulfide. Arg-215 is probably also susceptible to trypsin treatment under these conditions. Cleavage at Lys-70 has only been observed for the soluble protein following disruption of the extra domain. The fate of residues 216−231 has not been established. This region, which is part of the extra domain found in photosynthetic organisms, appears to be protease sensitive. It is possible that trypsin cuts this sequence into several small peptides that may, like the S2 peptide, be lost from the enzyme.

The specificity of the cleavage of the γ subunit by trypsin is a direct consequence of the structure of the enzyme. At least part of the γ subunit is located in the central cavity of the $\alpha_3\beta_3$ heterohexamer (Gogol et al., 1989; Boekema et al., 1990; Boekema & Böttcher, 1992; Wilkens & Capaldi, 1992; Abrahams et al., 1994). It is likely that the hexamer structure protects this region of γ, presumably the N- and C-termini, from proteolytic cleavage. However, most of the γ subunit, including the extra domain, is probably located outside of the hexamer. The presence of the ϵ subunit protects this region of γ from proteolytic cleavage. Trypsin will not cut the γ subunit of CF_1 when ϵ is bound to the enzyme (Richter et al., 1985). None of the trypsin sensitive sites in the extra domain is accessible. The binding of the ϵ subunit and the position of the γ within the hexamer cannot fully explain the trypsin resistance of the three fragments. Even though the extra domain is protease sensitive, the rest of γ is not. The fragments remain intact after prolonged cleavage of the $CF_1(-\epsilon)$ with trypsin. The tertiary structure of the γ subunit must shield most of the lysine and arginine residues from the trypsin.

The cleavage patterns of the γ subunits of spinach CF_1 and ECF_1 are very similar. Initial trypsin studies indicated that the γ subunit of ECF_1 is cleaved into a 20 kDa fragment consisting of the N-terminus of the subunit (Bragg & Hou, 1987). Gavilanes-Ruiz et al. (1988) found that, with limited proteolysis, trypsin will cleave the ECF_1 γ at position 201 to form fragments with molecular weights of 22 000 (N-terminal) and 9000 (C-terminal). With longer incubation times, the 22 000 molecular weight fragment is cut at residue 70 (Tang et al., 1994). In CF_1, the first trypsin cut produces a 10 kDa C-terminal fragment (γ10) beginning at residue 232 that, like its ECF_1 counterpart, does not stain well with Coomassie Blue. As with ECF_1, prolonged trypsinolysis of the N-terminal polypeptide results in cleavage of CF_1 γ at position 70. Unfortunately, none of the γ trypsin cleavage sites in CF_1 or ECF_1 is resolved in the partial crystal structure of the bovine heart mitochondrial F_1 (Abrahams et al., 1994). The similarity of the trypsin cleavage patterns of CF_1 γ and ECF_1 γ suggests that these subunits, with the exception of the CF_1 extra domain, are structurally similar.

Treatment of membrane-bound, oxidized CF_1 with trypsin during illumination results in formation of the γ27 and γ10 fragments. Under these circumstances, the only area of γ that is accessible to trypsin is the region between residues 215 and 232. CF_1 isolated from these membranes has a greatly reduced affinity for the ϵ subunit (Soteropoulos et al., 1992). Tang et al. (1994) concluded that in trypsinized ECF_1 the loss of ϵ inhibition of ATPase activity is due to γ cleavage at position 70. Cleavage of this region of CF_1 γ is not required for the loss of high-affinity ϵ binding. For membrane-bound, oxidized CF_1, trypsin does not cleave at positions 70 or 204. Therefore, cleavage between or loss of residues 216−231 is sufficient to significantly decrease the affinity of CF_1 for the ϵ subunit. The addition of up to 50 mol of ϵ:1 mol of $CF_1(-\epsilon)$ (from thylakoids treated with trypsin in the light) results in only 40% inhibition of ATPase activity, whereas in control $CF_1(-\epsilon)$ (from thylakoids treated with trypsin in the dark), 50% inhibition is observed at a 2:1 molar ratio of ϵ to CF_1 (Hightower & McCarty, 1996a). At least part of this inhibition may be due to incomplete cleavage of γ during illumination (Soteropoulos et al., 1992). It is not known if the loss of ϵ binding is due to a localized effect of trypsin cleavage or to a larger conformational change in the structure of the enzyme.

The γ subunit is considered to be involved in regulation of the complex. Photosynthetic organisms have an extra domain in their γ subunits, of approximately 30−40 amino acids, that is not present in nonphotosynthetic organisms. The photosynthetic eukaryotes have an additional disulfide bond within this region. Reduction of this disulfide in membrane-bound CF_1 allows for the production of ATP at a lower electrochemical proton potential (Ketcham et al., 1984; Junesch & Gräber, 1987). Reduction or trypsin treatment on the membranes results in increased levels of ATP hydrolysis (Moroney & McCarty, 1982b; Ketcham et al., 1984). The disulfide has also been implicated in activation of ATPase activity for the soluble enzyme by both DTT and the natural reductant thioredoxin (Arana & Vallejos, 1982; Nalin & McCarty, 1984; Dann & McCarty, 1992). Recently, the disulfide was inserted into the γ subunit of the cyanobacterium *Synechocystis* 6803. The enzyme became activated by DTT only after insertion of the disulfide bond (Werner-Grüne et al., 1994). We have shown that maximal levels of ATPase activity are possible without reducing the disulfide. Cleavage within the extra domain is sufficient to activate the enzyme as well as abolish binding of the ϵ subunit. Relaxation of the structure of the γ subunit,

either by trypsin cleavage or reduction of the disulfide, may be responsible for the increase in activity.

It was previously shown that illumination of membrane bound CF_1 or removal of the ϵ subunit from soluble CF_1 makes the γ subunit more sensitive to reduction by DTT and thioredoxin (Richter et al., 1985; Dann & McCarty, 1992) and to cleavage by trypsin (Moroney & McCarty, 1982b; Schumann et al., 1985). We have determined that the C-terminal γ fragments from both forms of CF_1 are identical. The evidence seems to indicate that there are changes in the conformation of the enzyme during illumination that mimic the effect of removal of the ϵ subunit. As previously suggested (McCarty et al., 1988), energization of the thylakoids may result in changes in the conformation of the γ and ϵ subunits that are important for regulation of the complex. In particular, changes in the interactions between the γ and ϵ subunits induced by the electrochemical proton potential could overcome inhibition by ϵ and allow synthesis of ATP.

Our selective proteolysis studies clearly show that the γ subunit of soluble CF_1 may be cleaved into several peptides, three of which remain tightly bound to the trypsin-resistant $\alpha_3\beta_3$ core. This preparation is very active as an ATPase, and, as shown in the accompanying paper (Hightower & McCarty, 1996b), the stability of the enzyme is not markedly affected by proteolysis of γ. In addition, reconstitution of two cloned pieces of the γ subunit, residues 1−196 and 206−323, with α and β results in an almost fully active complex (M. L. Richter, personal communication). These results should be kept in mind when mechanisms involved in overcoming the structural asymmetry of the enzyme during catalysis are considered.

ACKNOWLEDGMENT

We wish to thank Ted Thannhauser at the Analytical Chemistry and Peptide/DNA Synthesis Facility at Cornell University for performing the N-terminal sequencing and Dale Seburn for advice on the soybean trypsin inhibitor column.

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BI952913P