Regulatory Role of the C-Terminus of the ϵ Subunit from the Chloroplast ATP Synthase†

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ABSTRACT: The ATP synthases from chloroplasts and *Escherichia coli* are regulated by several factors, one of which is the ϵ subunit. This small subunit is also required for ATP synthesis. Thylakoid membranes reconstituted with CF1 lacking the ϵ subunit (CF1-ϵ) exhibit no ATP synthesis and very high ATP hydrolysis. Either native or recombinant ϵ restores ATP synthesis and inhibits ATP hydrolysis. Previously, we showed that truncated ϵ, lacking the last 45 C-terminal amino acids, restored ATP synthesis to membranes reconstituted with CF1-ϵ but was not an efficient inhibitor of ATP hydrolysis. In this paper, we show that this truncated ϵ is unable to inhibit ATP hydrolysis when Mg^{2+} is the divalent cation present, both for the enzyme in solution and on the thylakoid membrane. In addition, the rate of reduction of the disulfide bond of the γ subunit by dithiothreitol is not decreased by truncated ϵ, although full-length ϵ greatly impedes reduction. Thylakoid membranes can synthesize ATP at the expense of proton gradients generated by pH transitions in the dark. Our reconstituted membranes are able to produce a limited amount of ATP under these "acid-bath" conditions, with approximately equal amounts produced by the membranes containing wild-type ϵ and those containing truncated ϵ. However, the membranes containing truncated ϵ exhibit much higher background ATP hydrolysis under the same acid-bath conditions, leading to the conclusion that, without the C-terminus of ϵ, the CF1CFo is unable to check unwanted ATP hydrolysis.

The chloroplast ATP synthase is a member of the F1-ATPase family of protein complexes that utilize an electrochemical proton gradient to produce ATP from ADP and P_i. Other members of the family are found on the inner membrane of *Escherichia coli* and the inner mitochondrial membrane. The F1-ATPases comprise two separate oligomeric domains: the membrane intrinsic Fo[1] and the extrinsic F1. F1 is the site of ATP synthesis and is soluble when removed from Fo. The chloroplast F1, or CF1, comprises five different polypeptides named $\alpha-\epsilon$ in order of decreasing molecular mass in a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$. CFo has four polypeptides, I−IV, all in single copies except for subunit III (or c), which has 14 copies. For reviews, see refs *1* and *2*.

The regulation of the activity of CF1CFo is very complex and is characterized by several different factors. One of these factors is the oxidation/reduction state of the γ disulfide (*3*, *4*). Another aspect of this regulation is the nucleotide occupancy of certain binding sites on CF1. Mg^{2+}-ADP in one of the sites inhibits ATP hydrolysis (*5*). Third, the electrochemical proton gradient impedes ATP hydrolysis. A fourth aspect of regulation is the small ϵ subunit. This subunit is nestled between the larger portions of the CF1 and CFo, in close proximity to the γ subunit (*6*, *7*). CF1 without the ϵ subunit (CF1-ϵ) has high ATPase activity when in solution or when bound to CFo. Either native (*8*) or recombinant (*9*) ϵ inhibits the ATPase activity of CF1-ϵ, restoring it to the low activity characteristic of CF1 or CF1CFo. Membranes containing CF1-ϵ are not able to synthesize ATP at least in part because such membranes are so leaky to protons that they cannot establish a proton gradient. Reconstitution with native or recombinant ϵ allows these membranes to form proton gradients and to synthesize ATP (*10*).

The ϵ subunit of *E. coli* F1 (*11*) is required for the binding of ECF1 to ECFo. This is not the case for the ϵ subunit of CF1, and thus, it is possible to reconstitute thylakoid membranes that have been stripped of CF1 with CF1-ϵ and then add ϵ. Previously, we reported that removal of the last 45 amino acids of the chloroplast ϵ, corresponding to a putative C-terminal helix−turn−helix motif, decreased the inhibition of ATPase activity by this subunit but did not have a significant effect on the initial rates of ATP synthesis by thylakoid membranes (*10*). In this work, we characterize further the aspects of the ϵ subunit, specifically its C-terminus, that allow it to inhibit ATP hydrolysis. It is clear that the C-terminus is involved in the regulation of activity, but its removal is not sufficient to stimulate hydrolysis. Other modes of regulation are still in place to prevent hydrolysis without external stimulation by compounds such as sulfite or detergents or by proton gradients.

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[1] Abbreviations: F1, the catalytic portion of ATP synthases; Fo, the proton-conducting part of ATP synthases; CF1, chloroplast F1; CFo, chloroplast Fo; ECF1, F1 from *Escherichia coli*; CF1-ϵ, CF1 lacking its ϵ subunit; Δ45c, recombinant ϵ subunit from which 45 amino acids were deleted from the C-terminus; ACMA, 9-amino-6-chloro-2-methoxyacridine; Tricine, *N*-tris(hydroxymethyl)methylglycine; PMS, *N*-methylphenazonium methosulfate; STN, 0.4 M sucrose, 0.02 M Tricine−NaOH (pH 8.0), and 0.01 M NaCl.

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MATERIALS AND METHODS

CF1 (*4*) and CF1-ϵ (*8*) were prepared from market spinach and stored at 4 °C as an $(NH_4)_2SO_4$ precipitate. For use, aliquots of the suspensions were centrifuged at 16000*g* for 5 min and the pellets dissolved in either 20 mM Tris-HCl (pH 8.0) and 0.1 mM $CuCl_2$ (for experiments in solution) or 5 mM Tricine-NaOH (pH 8.0) and 2 mM EDTA (for membrane reconstitutions). Remaining $(NH_4)_2SO_4$ was removed by passage through Sephadex G-50 spin columns equilibrated with the same buffer (replacing $CuCl_2$ with 2 mM EDTA when used).

Recombinant ϵ subunits were expressed as inclusion bodies in *E. coli* (*9*). The washed inclusion bodies were dissolved in a buffer containing 8 M urea. When used for reconstitutions of CF1-ϵ in solution, the urea-solubilized ϵ was diluted with an ethanol/glycerol buffer first. When used for membrane reconstitutions, the urea-solubilized ϵ solutions were either diluted directly into the diluted thylakoid membrane suspension or first diluted with the ethanol/glycerol buffer. This dilution of urea-solubilized recombinant ϵ into the ethanol/glycerol mixture generates full-length ϵ subunit that is nearly as effective as native ϵ in inhibition of Ca^{2+}-ATPase activity of CF1-ϵ (*9*). The recombinant ϵ subunits used for these experiments all contained Ser at residue 6, replacing a Cys (C6S). Cys6 is the only Cys residue in ϵ, and this replacement has negligible effect on activity but makes the ϵ inclusion bodies easier to work with because it eliminates the possibility of dimer formation by oxidation.

For membrane reconstitutions with CF1-ϵ, it is necessary to add excess δ subunit to replace the amount lost during removal of ϵ. This was achieved by adding recombinant spinach CF1 δ subunit, expressed in *E. coli* from a clone provided by Dr. Mark L. Richter of the University of Kansas. The resulting inclusion bodies were collected and washed as those of recombinant ϵ. Folding of δ was performed by dissolving the inclusion bodies in 0.2% SDS, followed by removal of the detergent by passage through a 3 mL column of AG11 A8 resin (Bio-Rad). Native δ treated with SDS regained full function after treatment by this protocol (*12*).

NaBr-treated thylakoids were prepared and reconstituted with CF1-ϵ and recombinant ϵ as described (*10*). The final concentration of thylakoids reconstituted with recombinant ϵ was either 5 or 10 μg of chlorophyll mL^{-1} for Mg^{2+}-ATPase activity assays, 10 μg mL^{-1} for ATP-dependent proton pumping, and 20 μg of chlorophyll mL^{-1} for the assays of ΔpH-stimulated ATP synthesis or hydrolysis.

The Mg^{2+}-ATPase activity of CF1-ϵ in solution was assayed at 37 °C in a reaction mixture that contained 50 mM Tris-HCl (pH 8.0), 5 mM ATP, 5 mM $MgCl_2$, and 50 mM Na_2SO_3. P_i was determined spectrophotometrically (*13*). Reconstituted NaBr thylakoids (5 μg of chlorophyll) were used directly in a 1 mL reaction containing 50 mM Tricine−NaOH (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 50 mM Na_2SO_3, and 0.05 mM PMS. The mixtures were illuminated for 10 s, and the reaction was initiated by the addition of 0.05 mL of 0.1 M ATP and then incubated at 37 °C for 5 min. P_i was determined spectrophotometrically.

For reductive activation experiments, CF1 or CF1-ϵ was oxidized by incubation with 0.1 mM $CuCl_2$ for 30 min. After removal of the $CuCl_2$ by centrifugal gel filtration, CF1 was used without further treatment, and CF1-ϵ was either assayed directly or reconstituted with ϵ. The preparations (0.1 mg mL^{-1}) were incubated at 25 °C with 10 mM DTT in 20 or 50 mM Tris-HCl (pH 8.0) and 2 mM EDTA. Ca^{2+}-ATPase activity was measured at 37 °C at various times after addition of DTT in a reaction mixture that contained 50 mM Tris-HCl (pH 8.0), 5 mM ATP, and 5 mM $CaCl_2$. P_i was determined spectrophotometrically.

To perform the ΔpH-stimulated ATP synthesis and hydrolysis (*14*−*16*), the following protocol was used: Thylakoids totaling 20 μg of chlorophyll in 100 μL of STN-Mg^{2+} [0.4 M sucrose, 0.02 M Tricine−NaOH (pH 8.0), 0.05 M NaCl, 5 mM $MgCl_2$] were diluted with 100 μL of a pH-adjusted 30 mM succinate, 5 mM $MgCl_2$ buffer for 30 s on ice to generate the acid stage. To effect catalysis, 800 μL of the base stage [100 mM Hepes−KOH (pH 8.0), 1 mM ADP, 2 mM K_2HPO_4, 5 mM $MgCl_2$, 10 μM diadenosine pentaphosphate] was added, and the reaction was quenched after 10 s by addition of 30 μL of 50% TCA. Membranes were removed by centrifugation, and 50 μL of supernatant was diluted into 450 μL of 50 mM Tris−acetate (pH 7.75). This dilution was used in a luciferin/luciferase determination of ATP as prescribed by the manufacturer (Roche Molecular Biosystems). For hydrolysis, thylakoids totaling 10 or 20 μg of chlorophyll in 100 μL of STN-Mg^{2+} were equilibrated in the same fashion, except the base stage contained 5 mM ATP instead of the ADP and K_2HPO_4, and the $MgCl_2$ concentration was reduced to 1.25 mM. Immediately after addition of the base stage the reaction was placed at 37 °C for 10 min. The reaction was quenched by 1 mL of 0.5 N TCA and placed on ice, and P_i was determined spectrophotometrically. The tubes were centrifuged for 5 min to remove suspended membranes before absorbance was determined.

ATP-dependent proton pumping was assayed by quenching of the fluorescence of ACMA. ACMA fluorescence was excited at 410 nm and emission measured at 475 nm. The 1 mL incubation mixture contained 50 mM Tricine−NaOH (pH 8.0), 2 mM $MgCl_2$, and 1 μM ACMA. In some runs, Na_2SO_3 was present at 5 mM. The reaction was initiated by the addition of ATP to 4 mM. After the ACMA fluorescence reached a steady state, NH_4Cl was added to 4 mM to collapse the ΔpH. Quenching is given as $\Delta F/F$, were ΔF is the change in ACMA fluorescence after addition of the NH_4Cl and F is the the steady-state fluorescence after NH_4Cl addition. Both sulfite and ATP quench ACMA fluorescence to some extent and the reporting of energy-dependent ACMA fluorescence as $\Delta F/F$ obviates these effects.

RESULTS

Previously, we showed that a truncated form of the ϵ subunit, Δ45c, is a poor inhibitor of the ATPase activity of either CF1-ϵCFo in thylakoids or isolated CF1-ϵ (*10*). However, this reduced inhibition was not merely due to decreased binding of the Δ45c to CF1-ϵ, as shown by competition between Δ45c and full-length ϵ and by the fact that Δ45c is as effective as full-length ϵ in restoring ATP synthesis to thylakoids reconstituted with CF1-ϵ. Questions still remain as to how the C-terminus of ϵ is related to the other mechanisms of regulation of the activity of CF1CFo.

Previously, we measured the Ca^{2+}-ATPase activity of both membrane-bound CF1 and CF1 in solution containing Δ45c. However, the relevant divalent cation in vivo is Mg^{2+}. Thus,

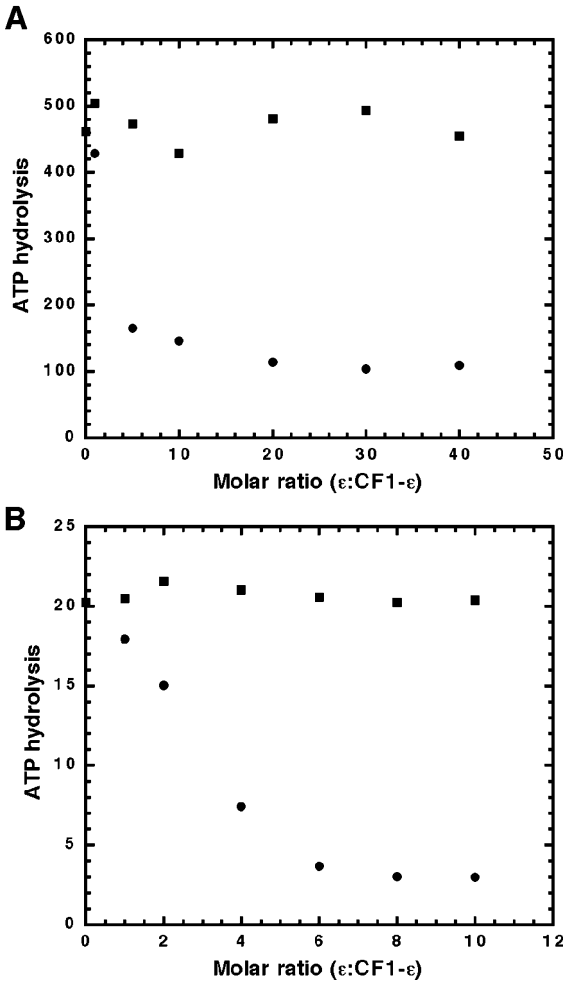

FIGURE 1: Hydrolysis of Mg^{2+}-ATP by reconstituted CF1-ϵ in solution and on the thylakoid membrane. (A) 5 μg of CF1-ϵ was incubated for 5 min with the indicated amount of either full-length ϵ or Δ45c. ATP hydrolysis is shown in units of μmol of P_i min^{-1} (mg of CF1-ϵ)$^{-1}$. The reaction mix was as stated under Materials and Methods, with 50 mM Na_2SO_3. (B) NaBr-treated thylakoids were reconstituted with CF1-ϵ and then washed before dilution with recombinant ϵ in TBU buffer [8 M urea, 25 mM Tris-HCl (pH 8.0)] at various molar ratios. ATP hydrolysis is shown in units of μmol of P_i formed h^{-1} (mg of chlorophyll)$^{-1}$. Key: circles, ϵ; squares, Δ45c.

we performed analogous experiments with reconstituted NaBr-treated membranes and reconstituted CF1-ϵ in solution; these data are presented in Figure 1. In contrast to the results with Ca^{2+}-ATP in which partial inhibition of ATP hydrolysis was obtained, Δ45c did not inhibit the Mg^{2+}-ATPase activity of thylakoid membranes. Full-length ϵ is an effective inhibitor of the Mg^{2+}-ATPase activity of both soluble and bound CF1-ϵ. Why Δ45c inhibits Ca^{2+}-ATPase activity partially, but Mg^{2+}-ATPase activity not at all, remains elusive. It is, however, evident that the truncated ϵ is much less effective as an inhibitor of the ATPase activity of CF1-ϵ regardless of the divalent cation in the reaction mixture. The same is also true for other truncations of ϵ (Table 1). Although the deletion of three residues from the C-terminus of ϵ had no effect on inhibition of the Mg^{2+}-ATPase activity of CF1-ϵ bound to thylakoid membranes, inhibition was abolished when 10 or more residues were deleted.

Removal of ϵ increases susceptibility of γ to proteolysis (*17*) and to reduction by either DTT (*18*) or thioredoxin (*19*). Reduction of the γ disulfide, the only disulfide bond in the enzyme, increases the ATPase activity of CF1 by a factor of 10−15 and that of CF1-ϵ by a factor of about 2. The extent of reduction has been correlated to ATPase activity (*3*) and may be determined by the ATPase activity of the enzyme. The rates of activation of the ATPase activity of CF1, CF1-ϵ, and CF1-ϵ reconstituted with Δ45c or native ϵ, purified directly from CF1, were determined. The data were fit with the equation for a single exponential rise, allowing the calculation of the half-time of the activation. Table 2 shows the data from these experiments, and Figure 2 shows two examples of the data sets for CF1-ϵ and CF1-ϵ reconstituted with a 3:1 molar ratio of native ϵ. As expected, removal of ϵ greatly enhances the rate of activation by DTT. Native ϵ reverses this effect, showing that it is the absence of ϵ, not damage by the procedure used to remove ϵ, which causes the exposure of the γ disulfide to reduction. Reconstitution of CF1-ϵ with full-length recombinant ϵ also slowed the rate of reductive activation (R. E. McCarty, unpublished data). Reconstitution of CF1-ϵ with Δ45c, in contrast, did not retard the rate of activation by DTT. By tryptophan fluorescence and SDS−PAGE, Δ45c remains bound after removal of excess by gel filtration on Sephadex G-100 resin (K. F. Nowak, thesis dissertation), and in this case Δ45c has no effect on the rate of reductive activation (Table 2). Even when present at 10 times (Table 2) and 50 times (data not shown) the concentration of CF1-ϵ, Δ45c had no effect. In combination with previous data showing that Δ45c competes with full-length ϵ for inhibition (*10*), these data

Table 1: Mg^{2+}-ATPase Activity of NaBr Thylakoids[a]

NaBr thylakoids + CF1-ϵ + δ +	n[b]	ATP hydrolysis [μmol of P_i h^{-1} (mg of chlorophyll)$^{-1}$] range[c]	mean ± SD[d]
no ϵ	9	447−827	614 ± 136
full-length ϵ	9	75−390	180 ± 122
$\epsilon\Delta$3c	8	57−353	125 ± 102
$\epsilon\Delta$6c	8	181−658	373 ± 169
$\epsilon\Delta$10c	8	348−791	544 ± 132
$\epsilon\Delta$32c	5	384−893	644 ± 194
$\epsilon\Delta$45c	9	377−870	577 ± 142

[a] Experiments were performed as in Figure 1 with at least a 40:1 molar ratio of ϵ:CF1-ϵ. [b] n = number of independent experiments. [c] Range = minimum and maximum values observed. [d] SD = standard deviation.

Table 2: Reductive Activation of CF1[a]

	ϵ:CF1-ϵ[c]	n[d]	$t_{1/2}$ (min)[b] range[e]	$t_{1/2}$ (min)[b] mean ± SD[f]
CF1		4	19.5−30.1	25.5 ± 4.4
CF1-ϵ		4	3.0−5.6	4.1 ± 1.1
CF1-ϵ + native ϵ	5:1, 3:1	2	36.5−40.0	38.3
CF1-ϵ + Δ45c	10:1	1	5.1	
CF1-ϵ + Δ45c[g]	>1:1	3	2.7−4.1	3.4 ± 0.7

[a] CF1 or CF1-ϵ was oxidized by incubation with 0.1 mM $CuCl_2$ for 30 min. CF1 was used without further treatment, and CF1-ϵ was either assayed directly or reconstituted with ϵ. Ca^{2+}-ATPase activity was measured at 37 °C at various times after addition of DTT. Data were fit with a single exponential rise using the program Kaleidagraph (Synergy Software); R values for all fits were >0.98. [b] $t_{1/2}$ = half-time of exponential. [c] ϵ:CF1-ϵ = molar ratio of ϵ added. [d] n = number of independent experiments. [e] Range = minimum and maximum values observed. [f] SD = standard deviation. [g] The CF1-ϵ + Δ45c sample was reconstituted with an excess of Δ45c and then purified on Sephadex G-100.

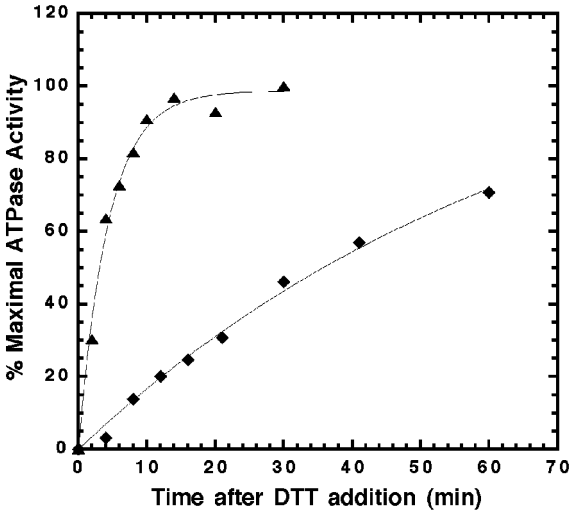

FIGURE 2: Reductive activation of CF1 vs CF1-ϵ. Data were generated as for Table 2. Data were fit with a single exponential rise using the program Kaleidagraph (Synergy Software); *R* values for both fits were >0.99. Key: triangles, activity of CF_1-ϵ; diamonds, activity of CF_1-ϵ reconstituted with a 3:1 molar ratio of native ϵ. The maximal ATPase activities were 33 μmol min^{-1} (mg of protein)$^{-1}$ for CF1-ϵ and 8 μmol min^{-1} (mg of protein)$^{-1}$ for the reconstituted CF1.

show that the truncated ϵ can bind to CF1-ϵ in solution. These results indicate that the C-terminus of ϵ interacts with the γ subunit in such a way as to hinder the accessibility of the regulatory region of γ to DTT.

Some reductive activation experiments were also carried out with CF1-ϵ bound to thylakoid membranes. Interactions between CFo and CF1-ϵ appear to slow the rate of reduction somewhat. In one experiment the half-time of the activation of CF1-ϵ bound to CFo in thylakoids by 10 mM DTT was 10 min, and that of the same preparation reconstituted with Δ45c was 17 min. The half-time of the activation of CF1 in native thylakoids that had been extensively washed to remove residual thioredoxin was 35 min (data not shown). Again, these data are consistent with the idea that the ϵ C-terminus contacts, or partially occludes, that part of the γ subunit that contains the regulatory disulfide/dithiol.

The capacity of the reconstituted membranes to synthesize ATP at the expense of a proton gradient generated in the dark was determined. The membranes were acidified with succinate buffers at various pH values and then quickly diluted with a basic reaction mix containing ADP and P_i. The ΔpH generated in this way can drive ATP synthesis (*16*) and activate ATP hydrolysis (*20*). The reconstituted membranes have similar extents of ATP synthesis at similar pH jumps (Figure 3A). However, as seen in Figure 3B, membranes reconstituted with Δ45c catalyze significantly more ATP hydrolysis after the pH jump than those reconstituted with full-length ϵ. This result also suggests that the C-terminus is required for ATPase inhibition even in the absence of sulfite.

Mills and Mitchell (*15*) showed that reduction of the γ subunit disulfide shifted the curve that relates the amount of ATP formed to the size of the ΔpH to lower ΔpH values. There was, however, little or no change in the amount of ATP synthesized when the reconstituted membranes were reduced with DTT prior to assay (data not shown). Δ45c does not inhibit Mg^{2+}-ATP hydrolysis, as shown in Figure

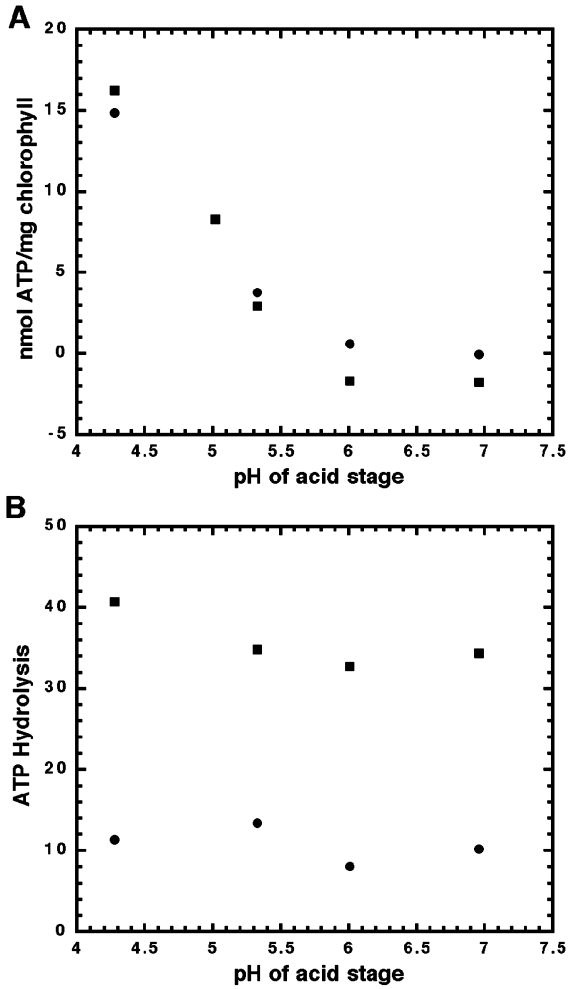

FIGURE 3: ΔpH-stimulated ATP synthesis and hydrolysis. (A) Reconstituted thylakoids equivalent to 20 μg of chlorophyll were used in the assay, with a total incubation of 10 s in the base stage. ATP was measured by the luciferin/luciferase method. (B) ΔpH was generated as above, replacing ADP and P_i with ATP. ATP hydrolysis is shown in units of μmol of P_i h^{-1} (mg of chlorophyll)$^{-1}$. Key: circles, full-length ϵ; squares, Δ45c.

1, and thus is unable to prevent ATP hydrolysis under conditions when hydrolysis would normally be in check. Exactly how much of the ATP generated undergoes hydrolysis cannot be determined by the results of these two experiments. Because the ATP synthesis was performed at 4 °C and the hydrolysis at 37 °C, and with differing initial nucleotide conditions, the results cannot be compared directly.

Thylakoids reconstituted with CF1-ϵ and then with either no ϵ, full-length ϵ, or truncated ϵ were tested for their ability to hydrolyze Mg^{2+}-ATP in the dark and to carry out ATP-dependent quenching of ACMA fluorescence, an indicator of proton pumping. All membranes had low Mg^{2+}-ATPase activity in the absence of sulfite (Table 3). In the presence of 5 mM sulfite, however, the membranes that contained no ϵ and Δ45c showed high ATPase activity, whereas that of membranes containing full-length ϵ remained low. No preparation showed significant ATP-dependent ACMA fluorescence quenching in the absence of sulfite. In the presence of sulfite, only the membranes reconstituted with Δ45c showed extensive quenching. These results show that the Mg^{2+}-ATPase activity of CF1CFo containing the truncated

Table 3: Mg^{2+}-ATPase Activity and Associated Quenching of the Fluorescence of ACMA in Reconstituted Membranes[a]

	Mg^{2+}-ATPase[b]		ACMA fluorescence[d]	
ϵ added	$-HSO_3^-$	$+HSO_3^-$ [c]	$-HSO_3^-$	$+HSO_3^-$ [c]
none	5	113	0.01	0.02
full-length ϵ	7	3	0.02	0.03
Δ45c	13	164	0.00	0.43

[a] NaBr-treated thylakoids were reconstituted with CF1-ϵ and with no ϵ or with recombinant full-length ϵ or Δ45c. [b] Determined at 30 °C in the presence of 50 mM Tricine−NaOH (pH 8.0), 2 mM ATP, and 2 mM $MgCl_2$. The same conditions were used for ACMA fluorescence quenching experiments. Rates are given as μmol h^{-1} (mg of chlorophyll)$^{-1}$. [c] HSO_3^- was present at 5 mM. [d] Expressed as $\Delta F/F$.

ϵ is linked to inward proton translocation. In the absence of sulfite, both ATPase activity and proton pumping are inhibited, likely by bound Mg^{2+}-ADP. Membranes that contain full-length ϵ are inactive because of inhibition by the C-terminal domain of the subunit, even when sulfite is present.

DISCUSSION

The ϵ C-terminus is absolutely required for full inhibition of ATP hydrolysis by CF1 in solution. There is an as yet unexplained difference between Ca^{2+}- and Mg^{2+}-ATP hydrolysis by the ATP synthase, both in solution and on the membrane. Ca^{2+}-ATP hydrolysis by the membrane-bound holoenzyme or CF1-ϵ in solution is partially inhibited by truncated ϵ, whereas Mg^{2+}-ATP hydrolysis is unaffected. The Mg^{2+}-ATPase activity is more relevant to the in vivo action of the enzyme, since this is the physiologically relevant divalent cation present in the chloroplast. Ca^{2+} cannot support ATP synthesis, for example, and Ca^{2+}-ATPase activity is inhibited by NH_4^+ (*21*), but Mg^{2+}-ATPase activity is not. ATP hydrolysis itself is not physiologically relevant because several regulatory mechanisms combine to switch off the synthase in the dark. What could be causing these differences?

Hochman et al. (*22*) postulated that, in the presence of Mg^{2+}, inhibition by bound Mg^{2+}-ADP helps to slow hydrolysis of the newly generated ATP when the chloroplast is no longer illuminated, thus serving a physiologically relevant role. In this way, Mg^{2+} regulation complements ϵ inhibition to prevent wasteful hydrolysis of ATP. Mg^{2+} is a more effective in vitro activator of ATP hydrolysis by CF1 in solution than Ca^{2+}. Also, Ca^{2+} fails to activate catalysis by CF1CFo in thylakoids, but activation by Mg^{2+} remains the same as in the soluble enzyme (*22*). These differences could partially account for the results shown here, where Ca^{2+} allows C-terminally truncated ϵ to inhibit ATP hydrolysis to a greater extent than Mg^{2+} does. Another notable difference is that Ca^{2+} has a larger atomic radius than Mg^{2+} and, because of shorter coordination bond length, is bound more weakly to the ATP synthase (*22*). Mg^{2+}-ADP and Mg^{2+}-ATP differentially affect the structure of *E. coli* F1, as shown through specific cross-link formations (*23*), and thus may affect orientation of and inhibition by the ϵ subunit. The structure of CF1 may also be affected differently by the binding of Ca^{2+}-ATP than by that of Mg^{2+}-ATP, producing the varied results seen in the in vitro hydrolysis experiments. These differences between the two cations may help to explain the results presented here.

Although sulfite or detergents are necessary to allow sustained Mg^{2+}-ATPase activity of oxidized CF1 and CF1CFo, reduced thylakoids have no such requirement for ATP hydrolysis. This is counterintuitive, because the sulfite is not merely necessary to activate the complex but to prevent binding of Mg^{2+}-ADP to the ATP synthase and, thus, inhibition of hydrolysis. Hydrolysis of ATP by thylakoid-bound ATP synthase can pump protons into the thylakoid lumen and create substantial ΔpH values (*24*). It seems that only the C-terminally truncated ϵ allows continued hydrolysis, and thus generation of ΔpH, in the presence of sulfite. This must be because of differential regulation that occurs when the C-terminal helix−turn−helix of ϵ is missing. Alterations in subunit a of *E. coli* (equivalent to subunit IV in chloroplasts) can perturb ATP-dependent proton pumping by ECF1ECFo (*25*). Mutation of the nucleotide binding sites in β is also capable of altering ATP-dependent proton pumping in *E. coli* (*26*). In chloroplasts, the ϵ subunit and Mg^{2+}-ADP binding can inhibit ATPase activity and associated proton pumping. Removal of the C-terminus of ϵ and the presence of sulfite allow the reconstituted membranes to perform ATP-dependent proton pumping, although membranes containing full-length ϵ are not able to generate such proton gradients even in the presence of sulfite. Thus, the ϵ C-terminus and Mg^{2+}-ADP binding are parts of a highly redundant regulatory mechanism.

The properties of the reconstituted membranes in the presence of an artificial pH gradient are also very interesting. We see a much higher activation of Mg^{2+}-ATP hydrolysis with membranes that contain Δ45c than those that contain full-length ϵ. At the same time, we see approximately equal extents of ATP synthesis under the same conditions. We cannot correct the synthesis for background hydrolysis because (1) the two experiments were carried out at different temperatures and (2) the initial buffer conditions for hydrolysis and synthesis contained ATP and ADP + P_i, respectively. The curve that relates rates of ATP synthesis in the light to ΔpH is shifted toward lower ΔpH values by reduction of the γ disulfide bond (*27*). A similar shift was seen for the extent of ATP synthesis induced by acid to base transitions in the dark (*15*). Membranes containing Δ45c might mimic reduced thylakoids in that reduction weakens ϵ inhibition. Thus, these considerations suggest that there may be a lower threshold ΔpH required for ATP synthesis by thylakoids containing Δ45c. ϵ is an inhibitor of the ATPase activity of the synthase and must also be an inhibitor of ATP synthesis. The conversion of the synthase to its active form requires energy; further analysis of the properties of membranes reconstituted with truncated ϵ may yield information as to the extent of this requirement.

There is movement of the ϵ C-terminus upon energization of the membrane, seen both from the accessibility of ϵLys109 to pyridoxal phosphate (*28*) and from the interaction of the C-terminus with specific antibodies (*29*). Cross-linking of the C-terminus to the β-sandwich of ϵ activates ATP hydrolysis of *E. coli* F1, though it has little effect on proton pumping-coupled ATP hydrolysis, DCCD sensitivity, or ATP synthesis (*30*). This fits the model put forth by Hara et al. (*31*) that the C-terminus wraps around the helices of γ when the complex is in the inhibited state, and then the ϵ helices move down to a position similar to that seen in both the cross-links formed by Schulenberg and Capaldi (*30*) and the

structure of the ϵ alone in solution (*32*). The conclusion by Schulenberg and Capaldi that the ϵ structure in the whole ATP synthase must be that observed of ϵ alone in solution, because of the few effects seen when cross-linked in that position, may be erroneous. It may very well be that this is the position of ϵ during catalytic activity, and there is no observable activity when the ϵ C-terminus is positioned otherwise.

Deletion of as few as 10 amino acids from the C-terminus of the ϵ subunit is sufficient to prevent its inhibition of the Mg^{2+}-ATPase activity of membrane-bound CF1-ϵ. Antibodies directed toward residues 125−134 (the last 10 residues) of the ϵ subunit were shown to interact with the ϵ subunit of CF1 in thylakoids only when thylakoid membranes were illuminated in the presence of the antibodies (*29*). Thus, the electrochemical proton potential formed in the light causes the C-terminus of the ϵ subunit to be exposed. This conformational change may be sufficient to overcome the inhibition of photophosphorylation by ϵ. Our results are consistent with this conclusion.

A scheme for the activation of the chloroplast ATP synthase during dark to light transitions is emerging. In the dark, Mg^{2+}-ADP is bound to an inhibitory site and the C-terminal domain of ϵ is in its inhibitory conformation. The disulfide bond of the γ subunit is shielded from reduction by part of the C-terminus of ϵ. Changes in the structure of the enzyme are elicited by the formation of the ΔpH. These changes include movement of the C-terminus of ϵ, exposure of the γ disulfide and γ Cys85 to the medium, and changes that result in the release of bound Mg^{2+}-ADP. Further modulation of activity is achieved by reduction of the γ disulfide by reduced thioredoxin.

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