ATP Synthase of Chloroplast Thylakoid Membranes: A More in Depth Characterization of Its ATPase Activity

Richard E. McCarty[1]

In contrast to everted mitochondrial inner membrane vesicles and eubacterial plasma membrane vesicles, the ATPase activity of chloroplast ATP synthase in thylakoid membranes is extremely low. Several treatments of thylakoids that unmask ATPase activity are known. Illumination of thylakoids that contain reduced ATP synthase (reduced thylakoids) promotes the hydrolysis of ATP in the dark. Incubation of thylakoids with trypsin can also elicit higher rates of ATPase activity. In this paper the properties of the ATPase activity of the ATP synthase in thylakoids treated with trypsin are compared with those of the ATPase activity in reduced thylakoids. The trypsin-treated membranes have significant ATPase activity in the presence of Ca^{2+}, whereas the Ca^{2+}-ATPase activity of reduced thylakoids is very low. The Mg^{2+}-ATPase activity of the trypsinized thylakoids was only partially inhibited by the uncouplers, at concentrations that fully inhibit the ATPase activity of reduced membranes. Incubation of reduced thylakoids with ADP in Tris buffer prior to assay abolishes Mg^{2+}-ATPase activity. The Mg^{2+}-ATPase activity of trypsin-treated thylakoids was unaffected by incubation with ADP. Trypsin-treated membranes can make ATP at rates that are 75–80% of those of untreated thylakoids. The Mg^{2+}-ATPase activity of trypsin-treated thylakoids is coupled to inward proton translocation and 10 mM sulfite stimulates both proton uptake and ATP hydrolysis. It is concluded that cleavage of the γ subunit of the ATP synthase by trypsin prevents inhibition of ATPase activity by the ε subunit, but only partially overcomes inhibition by Mg^{2+} and ADP during assay.

KEY WORDS: ATPase activity; ATP synthase; chloroplasts; thylakoid membrane; trypsin.

INTRODUCTION

The chloroplast ATP synthase (reviewed by McCarty *et al.*, 2000), a member of the F-ATPase family of enzymes (reviewed by Pedersen *et al.*, 2000), consists of a catalytic portion, CF1, and a thylakoid membrane-embedded part, CFo, that transports protons across the membrane. Remarkably, the ATPase activity of oxidized thylakoid membranes in the dark is 1% or less of the rate of ATP synthesis in the light (Avron and Jagendorf, 1959). Among the factors that prevent the wasteful hydrolysis of ATP by the chloroplast ATP synthase in the dark are inhibition by the ε subunit of CF1 (Richter *et al.*, 1984), inhibition by the binding of Mg^{2+}-ADP (Du and Boyer, 1990; Malyan, 1994; Digel *et al.*, 1996), lack of a substantial electrochemical proton gradient and oxidation of the γ subunit vicinal dithiol groups to a disulfide (Nalin and McCarty, 1984; Ort and Oxborough, 1992).

Several treatments elicit the ATPase activity of CF1 in thylakoids. Alcohols (Sakurai *et al.*, 1981; Anthon and Jagendorf, 1983) and some detergents (Pick and Bassilian, 1982; Yu and McCarty, 1985) cause the reversible dissociation of the inhibitory ε subunit and prevent Mg^{2+}-ADP inhibition. Heating CF1 can selectively denature the ε subunit (Patrie and McCarty, 1984; Wang *et al.*, 1993). Although reduction of the γ disulfide is not in itself sufficient to activate the ATPase of CF1 in thylakoids, reduced thylakoids can hydrolyze ATP in the dark after a brief period of illumination (Bakker-Grunwald and Van Dam,

[1] Department of Biology, Johns Hopkins University, Baltimore, Maryland 21218; e-mail: rem1@jhu.edu.

Key to abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; CF1, the catalytic portion of the chloroplast ATP synthase; CF1-ε, CF1 lacking its ε subunit; Chl, chlorophyll; DTT, dithiothreitol.

1974). Illumination of thylakoids in the presence of proteases also activates ATP hydrolysis (Lynn and Straub, 1969). Activation by trypsin in the light has been correlated to the specific cleavage of the γ subunit (Moroney and McCarty, 1982). In thylakoids in which the disulfide bond of the γ subunit is reduced, trypsin can cleave the γ subunit in the dark as well as in the light (Schumann et al., 1985). Trypsin-treated, reduced thylakoids have very high rates of Mg^{2+}-ATP hydrolysis, especially in the presence of sulfite, which prevents inhibition by free Mg^{2+} and Mg^{2+}-ADP (Larson and Jagendorf, 1989). The ATPase activity of CF1 with its γ subunit cleaved by trypsin is not inhibited by the ε subunit (Soteropoulos et al., 1992; Hightower and McCarty, 1996) and the ε subunit fails to bind to trypsin-treated CF1.

A study of the effects of trypsin on the ATPase activity of thylakoids that contain either reduced or oxidized CF1 is presented. It is concluded that in trypsin-treated thylakoids, ΔpH is not required for the reversal of ε inhibition, but for overcoming inhibition by Mg^{2+}-ADP produced by Mg^{2+}-ATP hydrolysis during assay.

MATERIALS AND METHODS

Thylakoid Membrane and CF1 Preparations

Thylakoid membranes were prepared from market spinach as described by McCarty and Racker (1968). For convenience, thylakoids are denoted as "oxidized" when they contain CF1 in which Cys199 and Cys205 of the γ subunit in the disulfide form and "reduced" when these residues are in the dithiol form. To prepare oxidized thylakoids, the membranes were incubated with 0.1 mM ferricyanide for at least 30 min on ice. Thylakoids were reduced by incubation with dithiothreitol and the SH groups alkylated by N-ethylmaleimide as described (Evron and McCarty, 2000). In some experiments, thylakoids were incubated with 5 mM dithiothreitol in the light to reduce the γ subunit disulfide bond. CF1 (Soteropoulos et al., 1992) and CF1-ε (Richter et al., 1984) were prepared as described.

Treatment With Trypsin

Trypsin solutions were prepared fresh daily in 1 mN H_2SO_4 and its concentration determined spectrophotometrically using an extinction coefficient of 1.43 A mg ml^{-1} at 280 nm (Worthington Catalog). Oxidized or reduced thylakoids (0.2 mg Chl ml^{-1}) were incubated in the light (about 2 kW m^{-2}) or dark at 25°C

with the amounts of trypsin and times indicated in the figure and table legends in an incubation mixture that contained 50 mM Tricine-NaOH (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, and 0.025 mM pyocyanine. Tris-HCl (pH 8.0) was sometimes used in place of Tricine. An amount of soybean trypsin inhibitor six times that of the trypsin (by weight) was added after the incubation with trypsin. This amount of the inhibitor effectively blocked further proteolysis.

Assays

Chlorophyll was determined by the method of Arnon (1949). ATPase activity was routinely assayed at 37°C in a reaction mixture that contained 50 mM Tricine-NaOH (pH 8.0), 5 mM ATP, 2.5 mM $MgCl_2$ and thylakoids equivalent to 5–20 μg of Chl. On occasion, Tris buffer was used instead of Tricine. After 1 to 5 min, Pi was determined spectrophotometrically (Taussky and Shorr, 1953). Photophosphorylation was assayed in a reaction mixture that contained 2 mM KPi buffer (pH 8.0) and 3 mM ADP in addition to the components given for the trypsin incubation mixture. After illumination with white light (2 kW m^{-2}) at 25°C, Pi was determined.

Ca^{2+}-ATPase activity of trypsin-treated thylakoid membranes was determined after the membranes had been incubated with EDTA to remove Mg^{2+} that strongly inhibits Ca^{2+}-ATPase activity. Membranes were collected by centrifugation at $6000 \times g$ for 5 min and resuspended in 0.4 M sucrose, 0.02 M Tricine-NaOH (pH 8.0), 0.01 M NaCl and 0.01 M EDTA at a Chl concentration of 0.5 mg ml^{-1}. After at least 30 min on ice, Ca^{2+}-ATPase activity was determined at 37°C in a reaction mixture (1 ml) that contained 50 mM Tris-HCl (pH 8.0), 5 mM ATP, 5 mM $CaCl_2$, and thylakoids equivalent to 20 μg of Chl. The Mg^{2+}- and Ca^{2+}-ATPase activities of CF1-ε were routinely determined at 37°C in reaction mixtures of the same composition as those used for the determination of these activities in thylakoids.

The ATP-dependent quenching of the fluorescence of ACMA (excitation, 410 nm; emission, 475 nm) was taken as an indication of proton pumping linked to the hydrolysis of ATP by the chloroplast ATP synthase. The stirred incubation mixture at room temperature contained in 1 ml, 50 mM Tricine-NaOH (pH 8.0), 2.5 mM $MgCl_2$, thylakoids equivalent to 20 μg of Chl and 1 μM ACMA. In some experiments, sodium sulfite was present at 10 mM. Higher sulfite concentrations could not be used because sulfite directly quenches ACMA fluorescence and has some uncoupling activity. Quenching was initiated by the addition of ATP to 2 mM. When the fluorescence reached a steady state, NH_4Cl was added to 4 mM. Results are

reported as $\Delta F/F$, where ΔF is the change in fluorescence after addition of the NH_4Cl and F, the steady state fluorescence after NH_4Cl addition. Light-dependent quenching of ACMA fluorescence was determined at room temperature using an incubation mixture identical to that used for the incubation of thylakoids with trypsin in the light except that the mixture also contained 1 μM ACMA.

RESULTS

Trypsin Activation of the ATPase Activity of Oxidized and Reduced Thylakoids

Trypsin was shown (Lynn and Straub, 1969; Moroney and McCarty, 1982) to activate markedly the Mg^{2+}-ATPase activity of oxidized thylakoids only when thylakoids were illuminated in the presence of the protease. Schumann *et al.* (1985) and Larson and Jagendorf (1989) showed, however, that light is not required for the activation of the Mg^{2+}-ATPase activity of reduced thylakoids by trypsin. The effects of trypsin treatment on the Ca^{2+}-dependent ATPase activity of the ATP synthase in thylakoids were not explored. A comparison of the Ca^{2+}- and Mg^{2+}-ATPase activities of oxidized and reduced thylakoids with and without trypsin treatment is shown in Table I. In agreement with previous results, light is not required for the activation of Mg^{2+}-ATPase activity of reduced membranes. The Ca^{2+}-ATPase activity of either oxidized or reduced membranes was very low after incubation in the light or dark without treatment with trypsin (Table I). Incubation of oxidized thylakoids in the dark with trypsin caused very little Ca^{2+}-ATPase activation, whereas incubation with trypsin in the light resulted in substantial activation. Trypsin treatment in the dark caused a large activation of the Ca^{2+}-ATPase activity of reduced thylakoids. CF1-ε has high Ca^{2+}-ATPase activity

both in solution (Richter *et al.*, 1984) and when bound to thylakoid membranes (Nowak *et al.*, 2002) and this activity is fully sensitive to inhibition by the ε subunit. Thus, the activation of Ca^{2+}-ATPase activity by trypsin is an indication that inhibition by the ε subunit has been at least partially lost in the trypsin-treated thylakoids.

Effects of Trypsin on ATP Synthesis

Time courses of the activation of Mg^{2+}-ATPase activity and loss of ATP synthesis activity by trypsin were determined in reduced thylakoids in the dark and oxidized thylakoids in the light (Fig. 1). The incubation of oxidized
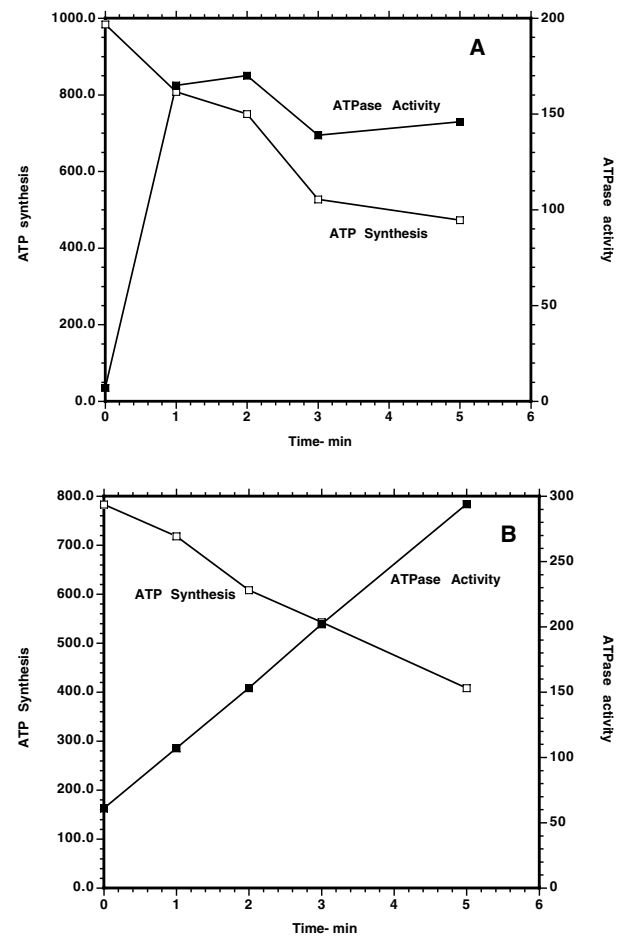
Fig. 1. Effect of trypsin treatment of thylakoids on ATP synthesis. Oxidized (Panel A) or reduced thylakoids (Panel B) were incubated with trypsin (5 and 200 μg ml^{-1} Chl for oxidized thylakoids and 10 and 400 μg ml^{-1} Chl for reduced thylakoids) and Mg^{2+}-ATPase and photophosphorylation measured. The experiments were performed on different days. Rates are expressed as μmol Pi formed (ATPase activity) or Pi consumed (ATP synthesis) h^{-1} mg Chl^{-1}.

Table I. Activation of the ATPase Activity of Thylakoids by Trypsin

| | | Oxidized | | Reduced | |
| | | ATPase activity[b] | | ATPase activity[b] | |
Trypsin[a]	Light/dark	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}
−	Dark	10	13	10	45
−	Light	10	41	10	81
+	Dark	13	65	127	201
+	Light	144	222	182	197

[a]Trypsin was added to 20 μg ml^{-1} and incubated for 3 min with oxidized or reduced thylakoids equivalent to 0.2 mg ml^{-1} of Chl in the dark or the light.

[b]The units of the ATPase rates are μmol Pi formed h^{-1} mg Chl^{-1}.

thylakoids with trypsin in the dark does not inhibit ATP synthesis (not shown). Although the Mg^{2+}-ATPase activity of reduced thylakoids had not peaked after 5 min of incubation in the dark (Fig. 1a), the Mg^{2+}-ATPase of the oxidized thylakoids reached its maximal activity after 1 min of illumination in the presence of trypsin (Fig. 1b). The rates and extents of loss of ATP synthesis were, however, similar in the two preparations, indicating that the decrease in photophosphorylation is not directly related to ATPase activation. It is now clear that substantial activation of the ATPase activity of the chloroplast ATP synthase by trypsin can take place with little loss in ATP synthesis activity.

Sulfite greatly stimulates the Mg^{2+}-ATPase activity of trypsin-treated thylakoids (Larson and Jagendorf, 1989), likely by preventing inhibition by Mg^{2+} and Mg^{2+}-ADP. The time courses for trypsin activation of Mg^{2+}-ATPase activity of thylakoids were repeated with sulfite in the assay mixtures to realize the full catalytic potential of the ATP synthase (Fig. 2). The Mg^{2+}-ATPase activity of oxidized thylakoids reached its maximal value within just 1 min of illumination in the presence of trypsin. In contrast, the Mg^{2+}-ATPase activity of reduced thylakoids was still increasing after 10 min of incubation with trypsin
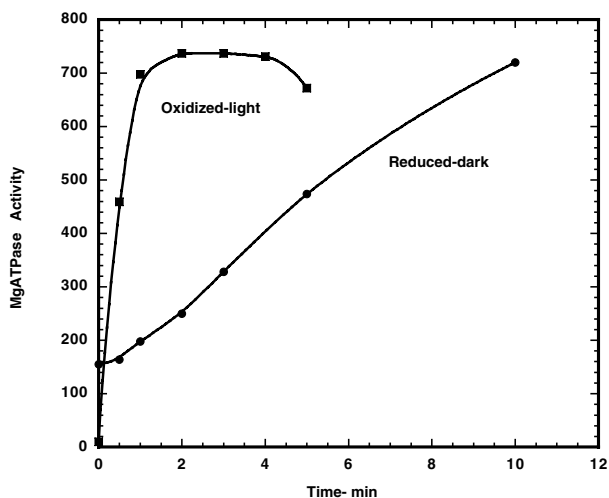
Fig. 2. Time courses of the activation of the Mg^{2+}-ATPase activity of oxidized and reduced thylakoids by trypsin. Oxidized thylakoids (0.2 mg of Chl ml^{-1}) were incubated in the light with trypsin at 5 μg ml^{-1}. At the times shown 0.2 ml aliquots were added to tubes in the dark that contained 30 μg of trypsin inhibitor. Mg^{2+}-ATPase activity, expressed as μmol Pi formed h^{-1} mg Chl^{-1}, was measured in the presence of 10 mM sulfite. Reduced thylakoids were incubated with trypsin under the conditions described for oxidized thylakoids except that the incubation was carried out in the dark. Mg^{2+}-ATPase activity was determined in the presence of 20 mM sulfite.

in the dark. Thus, the region of the γ subunit that is attacked by trypsin is more accessible to trypsin in illuminated thylakoids than in reduced thylakoids in the dark.

Uncoupler Effects

The Mg^{2+}-ATPase activity of trypsinized, oxidized, or reduced thylakoids is only partially inhibited by uncouplers. NH_4Cl (5 mM) in the assay mixture decreased the Mg^{2+}-ATPase activity by an average of about 40% (range, 17–70% in eight experiments). Inhibition was incomplete even when gramicidin D (3 μM) was present in the assay medium together with the NH_4Cl. In contrast, 4 mM NH_4Cl in the assay medium fully inhibited the Mg^{2+}-ATPase activity of light-activated, reduced thylakoids (McCarty and Racker, 1968). Larson and Jagendorf (1989) showed that when sulfite was present in the assay mixture uncouplers had very little effect on the Mg^{2+}-ATPase activity of trypsin-treated thylakoids.

Effects of Incubation With Mg^{2+}-ADP

Light triggers the Mg^{2+}-ATPase activity of reduced thylakoids. The capacity of reduced thylakoids to hydrolyze ATP after illumination was lost in the dark over a period of several minutes. Mg^{2+}-ADP addition caused a rapid loss of light-triggered Mg^{2+}-ATPase activity (Carmeli and Lipschitz, 1972). The experiments of Carmeli and Lipschitz (1972) were carried out in Tris buffer. As shown in Fig. 3, however, the Mg^{2+}-ATPase activity of light-activated reduced thylakoids is stable in the dark in the presence of Tricine, but most of the activity is lost within 2 min in the presence of Tris. The loss of activity in the presence of Tris in the dark cannot be attributed to the weak uncoupling action of Tris since uncouplers do not accelerate dark decay of activity (Carmeli and Lipschitz, 1972).

The loss of Mg^{2+}-ATPase activity in the dark in the absence of added ADP is probably the result of the binding of ADP that had been released from CF1 in the light. In agreement with this notion, the rate at which Mg^{2+}-ATPase activity is lost in the dark in Tris is dependent on the thylakoid membrane concentration. At 200 μg Chl ml^{-1}, the time for 50% loss of activity was 75 s, whereas at 20 μg Chl ml^{-1}, half of the activity was lost in 105 s.

Tricine buffer also prevented rapid inactivation of Mg^{2+}-ATPase activity of light-activated reduced thylakoids by Mg^{2+}-ADP in the dark. For example, the Mg^{2+}-ATPase activity of thylakoids that had been illuminated in the presence of 5 mM DTT with Tricine as the buffer and stored in the dark in Tricine for 3 min prior to
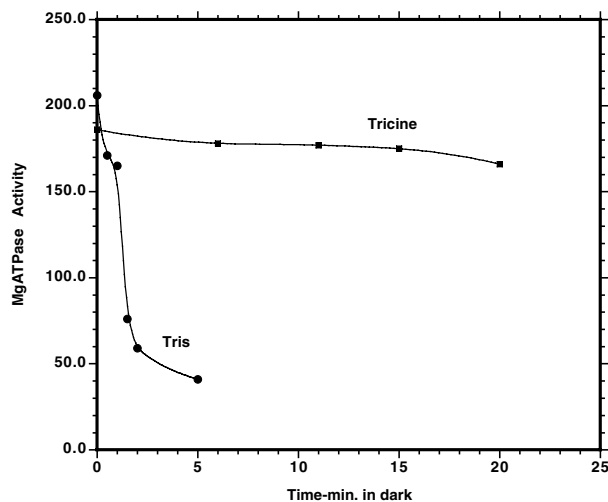
Fig. 3. Loss of the capacity of light-triggered, reduced thylakoids to hydrolyze Mg^{2+}-ATP upon storage in the dark. Thylakoids (0.2 mg Chl ml^{-1}) were incubated for 3 min in the light in the presence of 5 mM DTT in incubation mixtures that contained either 50 mM Tris-HCl (pH 8.0) or 50 mM Tricine-NaOH (pH 8.0). The mixtures were transferred to the dark and aliquots taken at the time indicated for the assay of Mg^{2+}-ATPase activity in the absence of sulfite. The assay mixtures contained the same buffer (also 50 mM) as that present in the incubation media. ATPase rates are expressed as μmol Pi formed h^{-1} mg Chl^{-1}.

assay was 138 μmol Pi formed h^{-1} mg Chl^{-1}. The activity of a sample incubated in the dark under otherwise identical conditions in the presence of 0.5 mM Mg^{2+}-ADP was 147. Tricine was used in the reaction mixtures at 50 mM. In Tris buffer, in contrast, the activity is nearly totally lost when 0.5 mM Mg^{2+}-ADP was present in just 15 s in the dark (Table II). Note, however, that sulfite (40 mM) in

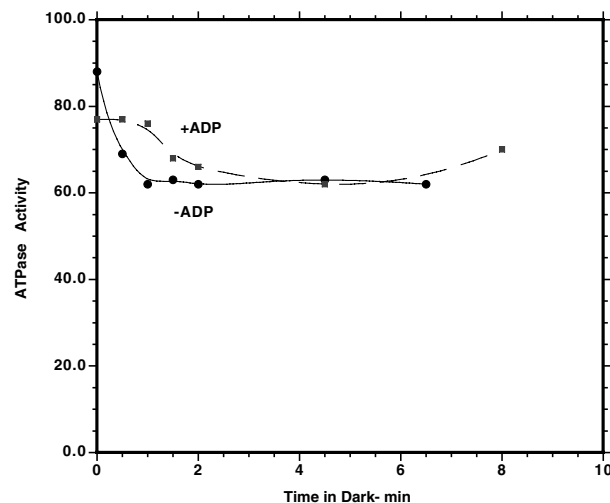**Table II.** Dark Decay of the Mg^{2+}-ATPase Activity Light-Triggered, Reduced Thylakoids[a]

Dark interval	Mg^{2+}-ATPase activity	
	($-$) Sulfite	($+$) 40 mM sulfite
None	268	477
15 s	131	425
15 s $+$ 0.5 mM ADP	50	451

[a]Thylakoids equivalent to 0.2 mg of Chl were suspended in an incubation mixture that contained 1.0 ml, 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM MgCl$_2$, 0.025 mM pyocyanine and 5 mM DTT. After 3 min of illumination aliquots were added to the Mg^{2+}-ATPase assay mixture that also contained 50 mM Tris-HCl (pH 8.0) with and without 40 mM sodium sulfite either immediately after illumination and or after 15 s in the dark. ATPase activity is expressed as μmol Pi formed h^{-1} mg Chl^{-1}.

the assay mixture restores Mg^{2+}-ATPase activity in samples incubated in the dark with or without ADP to control levels. Because sulfite prevents Mg^{2+}-ADP inhibition (Larson and Jagendorf, 1989) and promotes exchange of bound ADP for nucleotide in the medium (Larson et al., 1989; Digel et al., 1996), loss of activity in the dark is likely a consequence of the binding of Mg^{2+}-ADP to an inhibitory site.

Some Mg^{2+}-ATPase activity of oxidized thylakoids treated with trypsin in the light was lost in the first minute or so of incubation in the dark in the presence of Tris, but most of the activity was stable for at least 6.5 min of incubation in the dark. The addition of 0.5 mM ADP did not accelerate the loss of activity in the dark (Fig. 4). Incubation of thylakoid membranes treated with trypsin after reduction with 0.5 mM Mg^{2+}-ADP for 5 min in the dark prior to assay also had no effect on the Mg^{2+}-ATPase activity (not shown). These results suggest that cleavage of the γ subunit either prevents the binding of inhibitory Mg^{2+}-ADP or promotes its rapid exchange with ATP in the medium. In view of the fact that sulfite gives such strong stimulation of steady-state Mg^{2+}-ATP hydrolysis in trypsin-treated thylakoids, it is clear that cleavage of the γ subunit does not completely overcome the Mg^{2+}-ADP inhibition that occurs during catalysis.

A clue as to why such different results are obtained when Tricine is used from those obtained in the presence

Fig. 4. Dark decay of the Mg^{2+}-ATPase activity of light- and trypsin-treated thylakoids. Oxidized thylakoids were incubated with trypsin in the light under the conditions given in the caption to Fig. 1, except that the buffer used was 50 mM Tris-HCl (pH 8.0). Trypsin inhibitor was added and the sample transferred to the dark. Mg^{2+}-ATPase, expressed as μmol Pi formed h^{-1} mg Chl^{-1} was assayed in the absence of sulfite. The buffer used was 50 mM Tris-HCl (pH 8.0).

Table III. Buffer Effects on the ATPase Activity of CF1-ε in Solution

Treatment of CF1-ε	Buffer	Ca^{2+}-ATPase[a]	Mg^{2+}-ATPase
None	Tris	25.1	2.4
None	Tricine	25.5	5.0
Incubated with Mg^{2+}	Tris	nd[c]	0.6
Incubated with Mg^{2+}	Tricine	nd	1.6

[a] ATPase rates are expressed as μmol Pi formed min^{-1} mg protein^{-1}.
[b] Incubated for 5 min at room temperature in the presence of 1 mM MgCl$_2$ prior to assay.
[c] Not determined.

of Tris was obtained in experiments using soluble CF1 deficient in its ε subunit (CF1-ε). The steady-state rate of ATP hydrolysis with 5 mM ATP and 2.5 mM Mg^{2+} in the presence of 50 mM Tricine was more than twice as high as that assayed in the presence of 50 mM Tris (Table III). The rate of hydrolysis of Ca^{2+}-ATP was the same in Tricine and Tris buffers. Also, the Mg^{2+}-ATPase activity of CF1-ε incubated for 5 min with 1 mM Mg^{2+} in Tricine prior to assay was more than double that of the enzyme incubated in Tris. Thus, Tricine has some ability to offset Mg^{2+}-ADP inhibition, an observation in line with the findings of Nelson *et al.* (1972) that some carboxylates can enhance the Mg^{2+}-ATPase activity of heat-treated CF1.

Effects of Light on the Mg^{2+}-ATPase Activity of Trypsin-Treated, Oxidized Thylakoids

The Mg^{2+}-ATPase activity of thylakoids may be conveniently monitored by following proton release during ATP hydrolysis using the fluorescent pH indicator, pyranine. The reaction mixture may be illuminated with red light and ATP hydrolysis followed continuously by the decrease in pyranine fluorescence. Oxidized thylakoids (20 μg Chl ml^{-1}) were added to a stirred cuvette within the fluorometer in an incubation mixture (1 ml) that contained pyocyanine, a mediator of cyclic electron flow, 2 mM ATP and 5 mM Mg^{2+} and pyranine. Trypsin (5 μg) was then added and the cuvette was illuminated with red light. After about 20 s of illumination, the pyranine fluorescence began to decrease rapidly (Fig. 5). When the light was switched off, the fluorescence change rapidly decreased to a low rate similar to that observed before illumination. The rapid fluorescence change in the light was dependent on the addition of trypsin, required ATP and was abolished by tentoxin or 4 mM NH$_4$Cl (not shown). These observations are consistent with the notion that trypsin treatment can induce a light-dependent Mg^{2+}-ATPase activity. To confirm this notion, oxidized thylakoids were illuminated
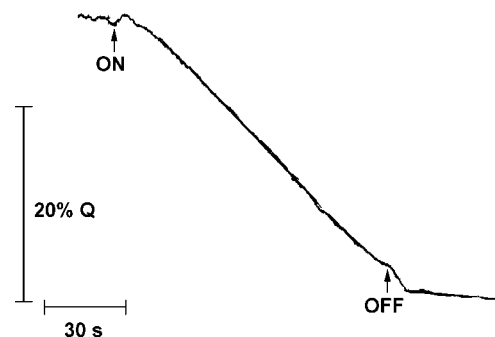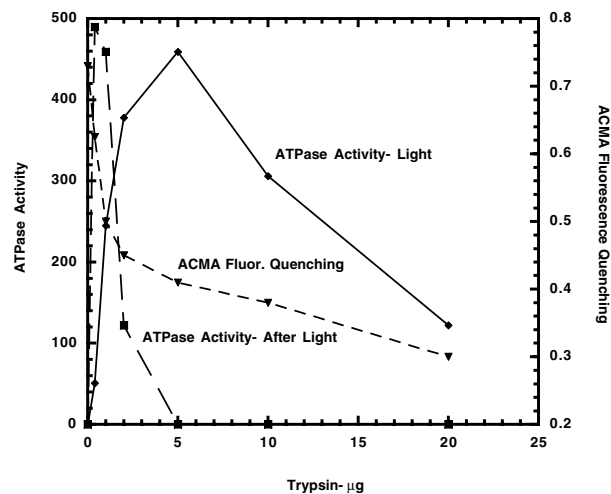

Fig. 5. Apparent light-dependent ATP hydrolysis by trypsin-treated thylakoids. Acidification of the incubation mixture by the hydrolysis of ATP was followed by monitoring the fluorescence of the pH indicator, pyranine as described under "Materials and Methods" section. Trypsin was present at 5 μg ml^{-1} and oxidized thylakoids equivalent to 20 μg Chl ml^{-1} were used. On and off refer to light on and off, respectively. The rate of acidification in the light was 314 μeq. H$^+$ h^{-1} mg Chl^{-1}.

in the presence of ATP and trypsin in the fluorometer and aliquots of the reaction mixture analyzed for Pi. The rate of Pi release in the light was very similar to the rate of ATP hydrolysis determined by acidification (not shown).

The observation of light-dependent Mg^{2+}-ATPase activity in trypsin-treated thylakoids was perplexing in that it is clear that trypsin can elicit Mg^{2+}-ATPase activity that persists in the dark for at least 10 min. A plausible explanation for light-dependent Mg^{2+}-ATPase was derived from experiments on the effects of a broad range of trypsin concentrations on the Mg^{2+}-ATPase activity of oxidized thylakoids and on the proton gradient, as reported by ACMA fluorescence quenching (Fig. 6). Oxidized thylakoids were incubated in the fluorometer with the amounts of trypsin shown in the figure. The mixtures were illuminated for 90 s within the fluorometer and 30 μg of trypsin inhibitor were added. Pyranine fluorescence was followed in the light and after turning off the light. In a separate experiment, thylakoids were treated in the same manner with trypsin in the light and the light-dependent quenching of the fluorescence of ACMA was measured. The lowest amount of trypsin used (0.4 μg) elicited little Mg^{2+}-ATPase activity in the light. However, when the light was turned off, rapid Mg^{2+}-ATPase hydrolysis was seen. As the amount of trypsin was increased to 5 μg, the rate of Mg^{2+}-ATPase activity in the light increased and that in the dark decreased. The light-dependent quenching of ACMA fluorescence decreased as the trypsin concentration increased.

These findings may be understood in light of the effects of the proton gradient on Mg^{2+}-ATPase activity. The Mg^{2+}-ATPase activity of reduced thylakoids is inhibited

Fig. 6. Effect of trypsin concentration on light-induced and light-dependent ATP hydrolysis. The quenching of pyranine fluorescence was used to determine the rates (expressed as μeq. H$^+$ released h^{-1} mg Chl^{-1}) of Mg^{2+}-ATP hydrolysis by oxidized thylakoids both during illumination in the presence of several concentrations of trypsin and after illumination. In separate runs the effect of trypsin treatment on ACMA fluorescence quenching in the light was determined as an indication of the magnitude of the ΔpH.

The hydrolysis of Mg^{2+}-ATP by the trypsinized membranes in the presence of 10 mM sulfite is coupled to the inward translocation of protons as indicated by the quenching of the fluorescence of ACMA (Table IV). Because sulfite quenches ACMA fluorescence directly and would be predicted to have some uncoupling action at high concentrations, 10 mM sulfite was the highest concentration used for the assays of the quenching of ACMA fluorescence by addition of ATP. The ΔpH formed under these conditions is rapidly collapsed by the addition of 5 mM NH$_4$Cl. The resistance of the Mg^{2+}-ATPase activity of these membranes to inhibition by NH$_4$Cl is, thus, not a consequence of a residual ΔpH in the presence of the uncoupler.

DISCUSSION

The properties of the ATPase activity elicited by treatment of thylakoids with trypsin differ significantly from those of the ATPase activity of reduced thylakoids brought on by illumination in the absence of trypsin. The ability of reduced membranes to hydrolyze Mg^{2+}-ATP after illumination is lost in several minutes of storage of the membranes in the dark in an incubation mixture that contains Tris. If, however, 0.5 mM ADP were added, the ATPase activity was lost in a manner of seconds. In contrast, the Mg^{2+}-ATPase activity of reduced membranes treated with trypsin in the dark or oxidized membranes incubated with trypsin in the light is not inhibited by incubation with 0.5 mM ADP (in the presence of Mg^{2+}) for 5 min prior to assay. Also, trypsin-treated thylakoids exhibit significant Ca^{2+}-ATPase activity, whereas the Ca^{2+}-ATPase activity of the reduced, membrane bound ATP synthase is low and is not enhanced by illumination prior to assay. After its release from thylakoid membranes, reduced CF1 has significant Ca^{2+}-ATPase activity (McCarty and Racker,

by ΔpH (Davenport and McCarty, 1986). When the ΔpH reaches a critical value, however, Mg^{2+}-ATPase activity is lost, unless sulfite is present. At low trypsin levels the ΔpH is high enough in the light to inhibit ATP hydrolysis and to maintain the enzyme in an active form. As the trypsin concentration increases and the ΔpH decreases, activity in the dark decreases because the membranes are leaky to protons and the proton gradient cannot be maintained in the dark at the critical level needed to keep the synthase active. In the light, however, proton transport linked to electron flow can generate a ΔpH sufficient to keep the enzyme active (Table IV).

Table IV. ATP-Dependent Proton Transport in Trypsin-Treated Thylakoids[a]

Thylakoid preparation	Mg^{2+}-ATPase activity[b] (+10 mM sulfite)	ACMA fluorescence ($\Delta F/F$)	
		(No sulfite)	(10 mM sulfite)
Control, no trypsin	49	0.03	0.19[c]
Trypsin-treated in light	1401	0.10	0.45

[a] Oxidized thylakoids (0.2 mg Chl ml^{-1}) were incubated for 1 min in the light with trypsin at 5 μg ml^{-1}. Control thylakoids were illuminated under the same conditions, but without trypsin. Soybean trypsin inhibitor (30 μg ml^{-1}) was added to both preparations and the thylakoids collected by centrifugation.

[b] Expressed as μmol Pi formed h^{-1} mg Chl^{-1}.

[c] The rate of quenching after ATP addition was much slower in this sample than in the trypsin-treated sample in the presence of sulfite.

1968). Reduced CF1 has a decreased affinity for the inhibitory ε subunit (Duhe and Selman, 1990; Soteropoulos *et al.*, 1992). At the concentration at which soluble CF1 is assayed, some ε dissociates from the enzyme. The high Ca^{2+}-ATPase activity of the trypsin-treated thylakoids is, however, not a consequence of solubilization of CF1 by the protease since all of the CF1 remains attached to thylakoids after trypsin treatment.

CF1 purified from trypsin-treated thylakoids is deficient in the ε subunit (Soteropoulos *et al.*, 1992) and CF1 that contains cleaved γ subunit cannot bind ε. The extent of dissociation of the ε subunit from trypsin-treated membranes is unlikely to be large. ATP synthesis would be strongly inhibited by trypsin if much of the ε subunit were lost from the ATP synthase since dissociation of ε renders the membranes very leaky to protons (Richter *et al.*, 1984). Thus, although the cleavage of the γ subunit clearly weakens the interaction between ε and CF1, ε interactions with CFo subunits, likely subunit III (Wetzel and McCarty, 1993) stabilize the binding of ε to the ATP synthase of trypsin-treated thylakoids.

The properties of the ATPase activity of trypsin-treated, reduced thylakoids both resemble and differ from those of CF1-ε bound to thylakoid membranes. Both preparations have high Ca^{2+}-ATPase activity. The Ca^{2+}-ATPase activity of the trypsin-treated membranes is, however, not inhibited by the ε subunit of CF1, whereas that of membrane-bound or soluble CF1-ε is. The ATPase activity of CF1 purified from trypsin-treated membranes is also not inhibited by the ε subunit (Hightower and McCarty, 1996). CF1-ε, either in solution or thylakoid membrane bound, has significant steady-state Mg^{2+}-ATPase activity only in the presence of alcohols, some detergents, or oxyanions. These reagents overcome the inhibition by free Mg^{2+} and Mg^{2+}-ADP. Reduced, trypsin-treated CF1 in thylakoids, however, has significant rates of Mg^{2+}-ATPase in the absence of these reagents. Thus, trypsin cleavage appears to desensitize the membrane-bound enzyme to Mg^{2+}-ADP. To reach the highest rates of ATPase activity, however, sulfite must be present in the assay medium.

Free Mg^{2+} and Mg^{2+}-ADP are potent inhibitors of the ATPase activity of both soluble and membrane-bound CF1. In contrast, Mg^{2+} is required for ATP synthesis and excess Mg^{2+} or Mg^{2+}-ADP does not inhibit ATP formation. Thus, the ΔpH must reverse inhibition by these reagents. Inhibition by azide is also prevented by ΔpH (Wei *et al.*, 1988). Energy-dependent release of bound ADP and exchange of bound ADP for ADP in the medium have been observed and it is very likely that ΔpH greatly reduces the affinity for ADP binding to an inhibitory site (or sites). Sulfite markedly enhances the rate of exchange under the same conditions that it stimulates Mg^{2+}-ATPase activity of CF1 in solution (Digel and McCarty, 1995) or bound to thylakoid membranes (Larson *et al.*, 1989).

ATP hydrolysis by the trypsin-treated thylakoids is linked to the inward translocation of protons as shown in this paper by the ATP-dependent quenching of the fluorescence of ACMA. Trypsin-treated thylakoids resemble thylakoid membranes reconstituted with CF1 containing recombinant ε subunit from which the C-terminal putative helix–loop–helix domain had been deleted (Nowak and McCarty, 2004). The helical domain of ε is necessary for ATPase inhibition, but not for ATP synthesis. Thus, trypsin cleavage of the γ subunit of CF1 nullifies inhibition by the ε subunit. Reduction of the γ disulfide weakens, but does not eliminate, inhibition by the ε subunit. This fact may explain why the Mg^{2+}-ATPase activity of reduced CF1 in thylakoids is lower than that of the trypsin-treated membranes, especially in the presence of lower concentrations of sulfite.

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