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ORIGINAL PAPER

The decay of the ATPase activity of light plus thiol-activated thylakoid membranes in the dark

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Abstract Oxidized ATP synthase of spinach thylakoid membranes catalyzes high rates of ATP synthesis in the light, but very low rates of ATP hydrolysis in the dark. Reduction of the disulfide bond in the γ subunit of the ATP synthase in the light enhances the rate of Mg^{2+}-ATP hydrolysis in the dark. The light plus thiol-activated state decays in a few minutes in the dark after illumination in Tris buffer, but not when Tricine was used in place of Tris. In this paper, it is shown that Tris in the assay mixture is an inhibitor of the light plus thiol-activated ATPase activity of thylakoids, but only after the activated membranes had incubated in the dark. Aminopropanediols and diethanolamine, also selectively inhibited ATPase activity of activated membranes after storage in the dark, whereas NH_4Cl and imidazole inhibit the ATPase activity of activated thylakoids almost equally whether they are added directly after the illumination or several minutes later. The fluorescence of 9-amino-6-chloro-2-methoxyacridine (ACMA) is quenched by the establishment of proton gradients by ATP-dependent proton uptake. Addition of ATP to activated membranes results in rapid quenching of ACMA fluorescence. If the activated membranes were incubated in the dark prior to ATP addition, a lag in the ATP-dependent ACMA fluorescence quenching as well as a similar lag in the rate ATP hydrolysis were seen. It is concluded that ADP rebinds to CF1 in the dark following illumination and inhibits the activity of the ATP synthase. Reactivation of the ATP synthase in the dark can occur by the slow generation of proton gradients by ATP hydrolysis in the dark. This reactivation takes place in Tricine buffer, but not in Tris because of its uncoupling action. Whether ADP binding plays a role in the regulation of the activity of the ATP synthase *in situ* remains to be established.

Keywords ATPase activity · Chloroplasts · Thylakoid membranes · ATP synthase

Abbreviations

ACMA	9-amino-6-chloro-2-methoxyacridine
CF1	the catalytic part of the chloroplast ATP synthase
Chl	chlorophyll
DTT	dithiothreitol
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
7-MeG	*N*(7)methylguanosine

Introduction

ATP synthesis by the inner membrane of mitochondria, the plasma membrane of some bacteria, and the thylakoid membrane of chloroplasts is driven by the flow of protons down their electrochemical gradient established by oxidative or photo-electron transport (Pedersen et al., 2000). The enzyme that catalyzes this reaction, ATP synthase, comprises about 10% of the protein of these membranes and is very complex. The chloroplast ATP synthase, for example, is made up of nine different polypeptides and a total of 26 polypeptide chains. ATP synthases are often referred to as F1Fo, an abbreviation that indicates that ATP synthases may be separated into two parts. F1s are extrinsic membrane proteins that contain the nucleotide binding sites of the enzyme. CF1 (chloroplast F1) has a molecular weight of 400,000 and five different polypeptides labeled α–ε in order of decreasing molecular weight. There are three copies of the α and β subunits but just one each of the smaller polypeptides. F1s are highly water-soluble

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after they are removed from the membrane. Fo is what is left of the ATP synthase after dissociation of F1. Fo is an intrinsic membrane protein that translocates protons. CFo (chloroplast Fo) comprises four different polypeptides.

ATP synthases are mechanistically and structurally very similar. The hypothesis advanced by Boyer (1997) of rotary catalysis is now a generally accepted theory. ATP synthases do differ with respect to their modes of regulation of activity. The γ subunit of CF1 contains an insert of about 20 amino acids that is absent in mitochondrial and *Escherichia coli* F1. This insert contains two Cys residues (Cys 199 and Cys 205). Chloroplasts catalyze very rapid ATP synthesis in the light, regardless of whether the Cys199 and Cys205 of the γ subunit of the ATP synthase exists in the dithiol form or the disulfide form (Ketcham et al., 1984; Mills and Mitchell, 1984). In contrast, the rate of hydrolysis of Mg^{2+}-ATP in the dark by thylakoids that contain CF1 in its oxidized state is very low. For spinach thylakoids, for example, the rate of ATP hydrolysis in the dark is 1% or less than that of ATP synthesis in the light (Avron and Jagendorf, 1959).

Illumination of thylakoids in the presence of sulfhydryl compounds such as DTT causes the rapid reduction of the γ subunit disulfide bond and increases the rate of Mg^{2+}-ATP hydrolysis in the dark by approximately 10-fold (Hoch and Martin, 1963; Marchant and Packer, 1963; Petrack et al., 1965). Light was later determined to have two separate effects on the thiol activation of the ATPase activity of thylakoids. The formation of the electrochemical proton potential causes changes in the structure of CF1 that greatly accelerate the reduction of the γ disulfide bond. In addition, illumination of thylakoids that already contained reduced CF1 stimulated ATPase activity assayed in the dark (Bakker-Grunwald and Van Dam, 1974). The light-stimulated release of ADP bound to CF1 was correlated to the increase in ATPase activity (Shoshan and Selman, 1979).

The Mg^{2+}-ATPase activity of thylakoids that had been illuminated in the presence of DTT was shown by Carmeli and Lipschitz (1972) and McCarty and Racker (1968) to decay in the dark over a period of several minutes. ADP accelerated the dark decay and phosphate retarded it (Carmeli and Lipschitz, 1972). The loss of activity in the dark was not caused by oxidation of the γ disulfide and was correlated to the binding of Mg^{2+}-ADP to CF1 (Shoshan and Selman, 1979). Regulation of the activity of the chloroplast ATP synthase has been reviewed by Hisabori et al. (2002, 2003) and by Richter (2004).

I recently reported that the Mg^{2+}-ATPase activity of light- and DTT-activated spinach thylakoids activated by illumination in the presence of DTT was stable after being stored for at least 20 min in the dark when the buffer used was Tricine (McCarty, 2005). In contrast, in the presence of Tris buffer in both the activation and assay buffers, most of the activity was lost in 2 min. These results prompted me to reinvestigate the dark decay of the ATPase activity of activated thylakoids. The results of these experiments call into question the significance of the binding of ADP in the dark in the regulation of the activity of the chloroplast ATP synthase.

Materials and methods

Thylakoid membrane and chloroplast preparations

Thylakoid membranes were prepared from spinach leaves using a homogenization medium that contained 0.4 M sucrose, 0.02 M Tricine–NaOH (pH 8.0), and 0.01 M NaCl. About 30 g of spinach leaves from which the petioles and larger midribs were removed were ground in a blender with 75 mL of homogenization medium for 15 s and the homogenate filtered though nylon cloth. The filtrate was centrifuged for 5 min at 6000 × *g* and the supernatant discarded. The pellets were resuspended in 40 mL of the buffered sucrose solution and the mixture centrifuged as described previously. The pellet was resuspended in a small volume of the buffered sucrose solution and diluted to 2 mg Chl mL^{-1}. When it was critical to be sure that the γ subunit disulfide bond was fully oxidized, potassium ferricyanide was added to a final concentration of 0.1 mM and the thylakoids were incubated for at least 30 min on ice.

Light and thiol activation

Thylakoids (0.2 mg Chl mL^{-1}) were either illuminated or kept in the dark in an incubation mixture that contained 50 mM Tricine–NaOH or Tris–HCl (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 0.025 mM pyocyanine, and 5 mM DTT. Illumination with white light (2 kW m^{-2}) was for 3–5 min and the temperature was 25°C. Aliquots were taken for ATPase assay either immediately after turning out the light or after the times in the dark indicated in the figures and tables. Thylakoids treated in this manner are referred to as "activated thylakoids."

Assays

Chl was assayed by the method of Arnon (1949). Except where indicated, Mg^{2+}-ATPase activity was determined in 1 mL reaction mixtures that contained 50 mM Tricine–NaOH (pH 8.0) or Tris–HCl (pH 8.0), 5 mM ATP, 2.5 mM $MgCl_2$, and light- and thiol-activated thylakoids equivalent to 10–20 μg of Chl. The incubation time was routinely 5 min and the temperature, 37°C. Mg^{2+}-ATP hydrolysis by activated thylakoids was also monitored continuously using 7-MeG and nucleoside phosphorylase (Banik and Roy, 1990). The stirred 1 mL reaction mixture contained 50 mM Tricine–NaOH (pH 8.0) or

50 mM Tris–HCl (pH 8.0), 1.5 mM $MgCl_2$, 0.2 mM 7-MeG, and about 1 unit of nucleoside phosphorylase from *E. coli*. 7-MeG fluorescence was excited at 300 nm and detected at 417 nm. ATP was added to a final concentration of 3 mM. Two minutes after the addition of ATP activated thylakoids equivalent to 10 μg of chlorophyll were added. The temperature in the sample chamber of the fluorometer was about 30°C. The ATP-dependent quenching of the fluorescence of ACMA was determined as described previously (McCarty, 2005).

ATP, ADP, 7 MeG, nucleoside phosphorylase (from *E. coli*), buffers, and amines were obtained from Sigma/Aldrich, St. Louis, MO.

Results

Buffer effects on the decay of ATPase activity

The experiments of Carmeli and Lipschitz (1972) and McCarty and Racker (1968) were carried out in Tris buffer in both the thiol activation medium and in the reaction mixture whereas my recent experiments were carried out in Tricine buffer. A comparison of the two buffers on the stability of the Mg^{2+}-ATPase activity in the dark revealed remarkable differences (Table 1). No ATPase activity was lost in 3 min of incubation in the dark prior to assay when Tricine was used in the assay medium. In contrast, about 80% of the activity was lost in 3 min of darkness when Tris replaced Tricine in the assay medium. The same result was obtained in the presence of either Tris or Tricine in the activation mixture. Note, however, that the rate of ATP hydrolysis was higher in Tris than in Tricine in samples taken from the activation mixtures in the light. This activity increase by Tris is probably a consequence of uncoupling by Tris, a primary amine.

The Mg^{2+}-ATPase activity of CF1 deficient in its ε subunit in Tricine buffer was about twice that in Tris (McCarty, 2005), indicating that Tricine has some ability to overcome inhibition by Mg^{2+} and/or Mg^{2+}-ADP. Tricine does not, however, appear to be an effective activator of the Mg^{2+}-ATPase activity of activated thylakoids. The rate of ATP hydrolysis in the presence of 50 mM Tricine (pH 8.0) was only about 5% higher than that in 5 mM Tricine (pH 8.0), whether the aliquots were taken in the light or after 5 min in the dark. Marked effects were, however, seen when Tris (pH 8.0) added at various concentrations to reaction mixtures that contained Tricine (Fig. 1). Tris up to 50 mM stimulated the ATPase activity in light- and thiol-activated thylakoids that were not incubated in the dark prior to assay. If the activated thylakoids were incubated in the dark for 3 min prior to assay, inhibition of activity was seen at all Tris concentrations greater than 10 mM. Thus, Tris is an inhibitor of the ATPase activity of CF1 in activated thylakoids, but only after the activated thylakoids are incubated in the dark.

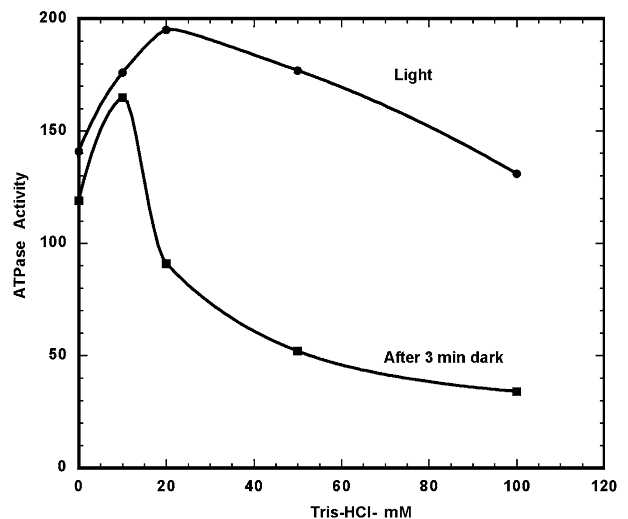

Fig. 1 Tris inhibits Mg^{2+}-ATPase activity of activated thylakoids only after a dark period. Thylakoids (0.2 mg Chl mL^{-1}) were illuminated for 3 min in the presence of Tricine buffer and 5 mM DTT. Aliquots (50 μL) were taken for ATPase assay either just before the light was extinguished or after 3 min in the dark. The reaction mixture contained the concentration of Tris (adjusted to pH 8.0) indicated as well as 50 mM Tricine–NaOH (pH 8.0). ATPase activity is expressed as mol Pi formed h^{-1} mg Chl^{-1}.

Table 1 Effects of tris and tricine buffers on the dark decay of Mg^{2+}-ATPase activity

	Mg^{2+}-ATPase activity[a]			
	Tris in assay[b]		Tricine in assay[b]	
Buffer in activation mix[a]	Light	3 min dark	Light	3 min dark
Tris	273[c]	57	163	176
Tricine	281	55	148	163

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

[b] Tris–HCl or Tricine–NaOH (both pH 8.0) were present at 50 mM.

[c] Aliquots of the activation mixture (50 μL) were taken either with the light still on or after 3 min in the dark.

Table 2 Buffer effects on the decay of Mg^{2+}-ATPase activity in the dark[a]

	Mg^{2+}-ATPase activity[b]	
Buffer in assay mix	No dark period	After 4 min dark
Tris	230	29
Trizma grade Tris	204	16
Tes	177	169
Tricine	152	182
Bistrispropane	177	148

[a] Activation was carried out in 50 mM Tricine–NaOH (pH 8.0) and 5 mM DTT for 3 min in the light.

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

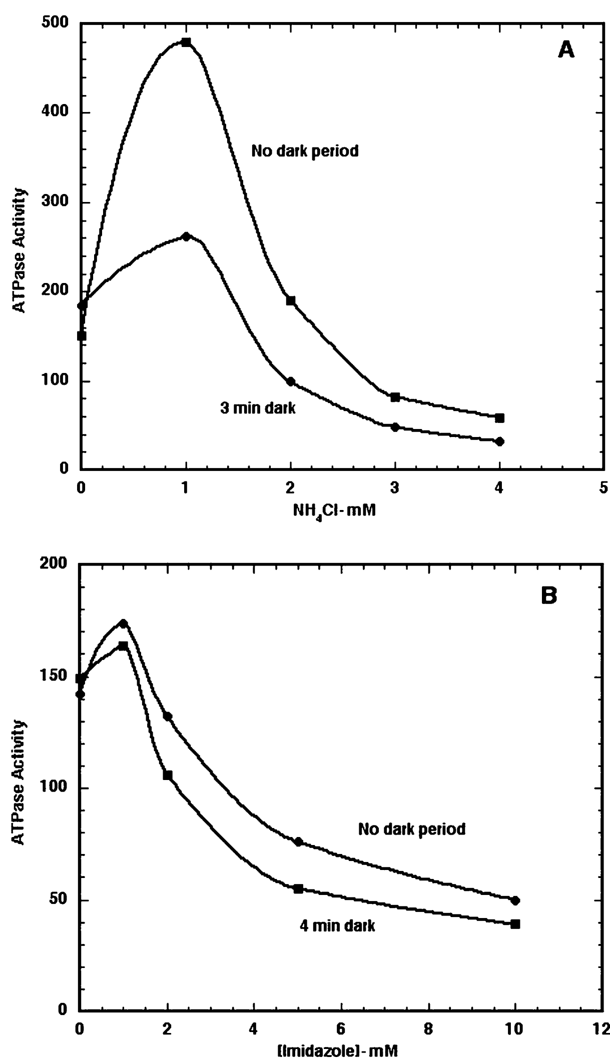

Fig. 2 NH_4Cl and imidazole have similar effects on the Mg^{2+}-ATPase activity of activated thylakoids whether the aliquots were taken just before the end of the light period or after 3 min in the dark. Activation and assay were carried out as described in the caption to Fig. 1. (A) Effects of NH_4Cl. (B) Effects of imidazole.

The effects of several other buffers on the loss of the ATPase activity of light- and thiol-activated thylakoids in the dark were tested (Table 2). Highly purified Tris (Sigma Ultra Trizma base) was as effective as reagent grade Tris, indicating that the effects of Tris are probably not caused by a contaminant. In the presence of either TES (*N*-tris(hydroxymethyl-methyl-2-aminoethanesulfonic acid) or bistris propane (1,3-bis[tris(hydroxymethyl)amino]propane (both adjusted to pH 8.0) in the assay mixtures at 50 mM, there was little loss of Mg^{2+}-ATPase activity after 4 min in the dark.

If the effects of Tris were caused by its weak uncoupling, uncouplers should behave similarly. As shown in Fig. 2, however, neither NH_4Cl (Fig. 2A) nor imidazole (Fig. 2B) showed the dramatic selective inhibition of the ATPase activity of activated thylakoids after incubation in the dark that was observed with Tris. Even after 20 min of incubation of activated thylakoids in the dark, the ATPase activity assayed in the presence of 0.5 mM NH_4Cl was 63% of that of activated thylakoids not incubated in the dark. Imidazole and NH_4Cl inhibit the ATPase activity of activated thylakoids assayed right after illumination almost equally to that in thylakoids incubated in the dark prior to assay.

Table 3 Effect of polar amines on the Mg^{2+}-ATPase activity of activated thylakoids[a]

Amine in assay mix[b]	Mg^{2+}-ATPase activity[c] No dark period	4 min dark
3-Amino-1,2-propanediol (20 mM)	295	52
2-Amino-1,3-propanediol (20 mM)	313	93
Diethanolamine (5 mM)	239	102
Triethanolamine (10 mM)	76	83
Glucosamine (50 mM)	182	177

[a] Thylakoids were illuminated for 3 min in the presence of 5 mM DTT and 50 mM Tricine–NaOH (pH 8.0). Aliquots were taken for assay either right after activation or after 4 min in the dark. The experiment with the propanediols was done on the same day. Each of the other amines was tested in separate experiments.

[b] Amines were adjusted to pH 8.0 with HCl. Tricine–NaOH (pH 8.0) was also present in the assay mixtures at 50 mM.

[c] μmol Pi formed h^{-1} mg Chl^{-1}.

Several other polar amines affected the ATPase activity of activated thylakoids in a manner very similar to Tris. Among them are 3-amino-1,2-propane diol and 2-amino-1,3-propane diol (Table 3). Diethanolamine was somewhat effective, but triethanolamine inhibited the ATPase activity of the activated thylakoids to similar extents both with no dark incubation and after 4 min in the dark. The difference in the effects of triethanolamine and diethanolamine may be in part a consequence of the difference in the pK_a values for the two compounds (8.88 for diethanolamine and 7.76 for triethanolamine). At pH 8.0, the concentration of triethanolamine at 50 mM total amine concentration would be almost 32 mM, whereas the concentration of diethanolamine would be just 6 mM. For amines to uncouple, the neutral form of the amine must cross the thylakoid membrane. The concentration of Tris base at a total Tris concentration of 50 mM in the assay medium is about 25 mM, close to that of triethanolamine. Yet, Tris is a less effective uncoupler than triethanolamine. The octanol/water partition coefficient of ethanol (0.182) is significantly lower than that of methanol (0.50). Thus, Tris base would be predicted to be more polar than triethanolamine. The concentration of amines required to uncouple photophosphorylation decreases sharply as the octanol/water partition coefficient of the basic form of the amine increases (McCarty and Coleman, 1970). Very polar, bulky amines should be poor uncouplers. In agreement

with this notion, 50 mM D-glucosamine (pK_a 7.8) had no effect on the ATPase activity of light- and thiol-activated thylakoids assayed either directly after illumination or after 4 min in the dark. Tricine and TES do not uncouple because the amine forms of these buffers are negatively charged and will not cross the thylakoid membrane rapidly.

Buffer effects on ATP-dependent ACMA fluorescence quenching

The quenching of the fluorescence of ACMA in thylakoid suspensions upon addition of ATP is a convenient way to estimate ATP-dependent proton uptake. When ATP is added to activated thylakoids, there is a rapid quenching of ACMA fluorescence, followed by a slower phase (Fig. 3). The rapid phase is a result of direct interaction between ATP and ACMA and is independent of thylakoids. The slower phase results from ATP-dependent proton pumping by the ATP synthase as shown by reversal of the quenching by addition of NH_4Cl to 4 mM. Proton uptake starts quickly after ATP addition to activated thylakoids that had incubated in the dark for 1 min prior to ATP addition to a reaction mixture that contains Tricine buffer (Fig. 3A). If the thylakoids were incubated for 10 min in the dark prior to ATP addition, a pronounced lag in the rate of ACMA fluorescence quenching in Tricine assay mix was apparent (Fig. 3B). The extent of the quenching was, however, less affected by the incubation in the dark. In the presence of 50 mM Tris–HCl in the assay medium, ATP addition caused very little NH_4Cl-reversible ACMA fluorescence quenching after activated thylakoids had incubated for 10 min in the dark (Fig. 3C).

The loss of Mg^{2+}-ATPase activity of activated thylakoids that occurs in the dark when the assay medium contained Tris is completely reversed by sulfite in the assay medium (McCarty, 2005). This result makes it likely that Mg^{2+}-ADP released during illumination binds to CF1 during the incubation of thylakoids in the dark. Sulfite promotes exchange bound ADP for medium ATP and overcomes Mg^{2+} and Mg^{2+}-ADP inhibition of ATP hydrolysis (Digel et al., 1996; Larson and Jagendorf, 1989).

As shown in Table 4, ATP addition to a reaction mixture that contained 50 mM Tris (pH 8.0), sulfite at 10 mM and activated thylakoids that had been incubated in the dark for 8 min caused ACMA fluorescence quenching of a similar extent as that when the buffer used was Tricine.

Illumination of activated thylakoids with red light for 10 s in the presence of 15 μM pyocyanine after dark adaptation for 15–20 min also restored ATP-dependent ACMA fluorescence quenching to thylakoids assayed in the presence of Tris. The ATP was added 30 s after turning off the light. The lag in the quenching by ATP in the presence of Tricine was eliminated by illumination and the quenching

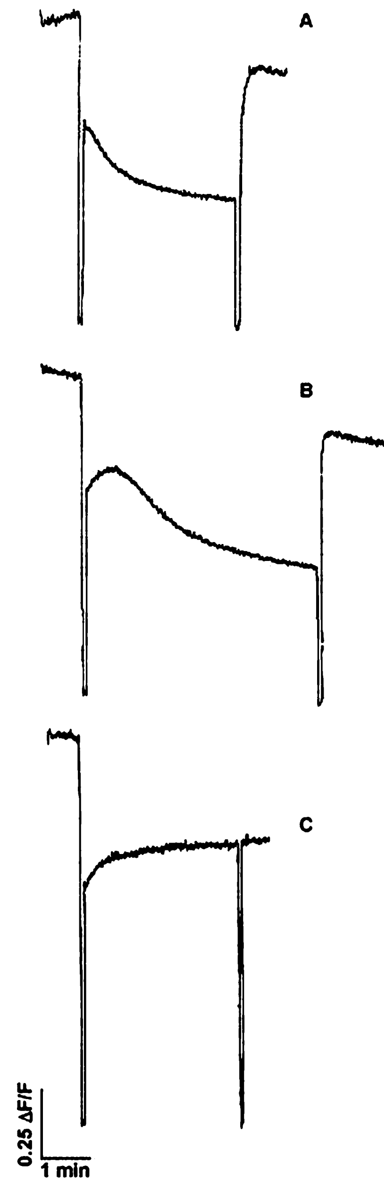

Fig. 3 Buffer effects on ATP-dependent ACMA fluorescence quenching. Thylakoids were activated as described in the caption to Fig. 1 and 50 μL aliquots taken for assay of ATP-dependent quenching of ACMA fluorescence. (A) The buffer used was 50 mM Tricine–NaOH (pH 8.0) and ATP was added 1 min after the end of the activation illumination. (B) As in (A), except that the activated thylakoids were incubated for 10 min in the dark prior to ATP addition. (C) The buffer in the ACMA fluorescence quenching assay mix contained both 50 mM Tricine–NaOH (pH 8.0) and 50 mM Tris–HCl (pH 8.0). ATP was added 10 min after the activation was completed. ATP (3 mM final concentration) was added at the first downward deflection of the traces (caused by the shutter closing upon opening the sample compartment) and NH_4Cl (4 mM final concentration), at the second.

was complete in just 30 s after addition of the ATP. These effects of light raised concern that the slow ACMA fluorescence quenching by ATP addition may be assisted by the 410 nm measuring beam. However, the slow quenching after ATP addition was seen when the reaction mixture

Table 4 Effects of sulfite and light on the ATP-dependent quenching of the fluorescence of ACMA

Buffer in assay mix[a]	Treatment of activated thylakoids	ACMA fluorescence quenching[b]	Lag[c]-s
Tris	2.5 min dark before ATP addition	0.08	nd[d]
Tris	23 min dark before ATP addition	0.06	nd
Tris + 10 mM Na_2SO_3	8 min dark before ATP addition	0.48	15
Tris + pyocyanine	16 min dark then 30 s light before ATP addition	0.25	None
Tricine	28 min dark before ATP addition	0.43	45
Tricine + pyocyanine	38 min dark then 30 s light before ATP addition	0.36	None

[a]Thylakoids were illuminated in the presence of 50 mM Tricine buffer and 5 mM DTT for 3 min and kept in the dark for the times shown. The pyocyanine concentration in the assay mix was 15 μM and the samples were illuminated within the fluorometer with red light (660 nm) from an array of light-emitting diodes.

[b]Expressed as $\Delta F/F$ where ΔF is the change in fluorescence that occurred after the addition of NH_4Cl to 4 mM and F is the steady-state fluorescence after NH_4Cl addition.

[c]The time before rapid quenching started after ATP addition.

[d]Not detectable.

was supplemented with 10 μM DCMU, a photosystem II inhibitor. This concentration of DCMU strongly inhibited the red-light-induced acceleration of ACMA quenching by ATP (not shown).

Time course of ATP hydrolysis

The lag in the quenching of the fluorescence of ACMA after the addition of ATP to activated thylakoids that had been incubated for several minutes in the dark suggested there should be a similar lag in the onset of ATP hydrolysis. A convenient way to monitor ATP hydrolysis continuously is to follow Pi release using a fluorescent nucleoside and the enzyme, nucleoside phosphorylase (Banik and Roy, 1990). 7-*N*-Methylguanosine (7-MeG) is a suitable fluorescent substrate for bacterial nucleoside phosphorylase and 7-*N*-methylguanine, a product of the phosphorolytic cleavage of 7MeG, is much less fluorescent. Activated thylakoids were added to the reaction mixture that contained 7-MeG, ATP, $MgCl_2$, Tricine buffer, and nucleoside phosphorylase either 15 s or 20 min after the end of the illumination. Little or no lag in the decrease in fluorescence of 7-MeG was seen when the activated thylakoids had incubated just 15 s in the dark prior to their addition to the reaction mixture (Fig. 4). In contrast, a lag of approximately 60 s was seen when activated thylakoids were aged for 20 min in the dark prior to assay. After the lag period was completed, the rate of decrease in 7-MeG fluorescence in the sample that contained thylakoids aged for 20 min in the dark was about 80% of that incubated in the dark for 15 s.

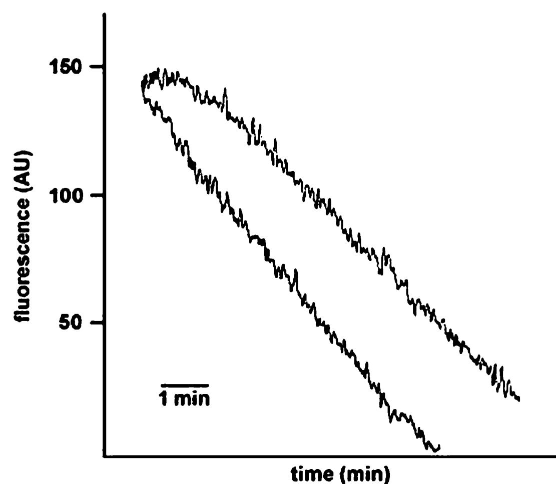

Fig. 4 Time courses of Mg^{2+}-ATPase activity in activated thylakoids. Thylakoids were activated as described in the caption to Fig. 1, except that the illumination time was 3.5 min. Aliquots (50 μL) were added to reaction mixtures that contained 7-MeG, nucleoside phosphorylase, Tricine buffer, and $MgCl_2$, either 15 s after the end of the activating illumination (lower trace) or after 20 min in the dark (upper trace). The temperature was 30°C.

Discussion

It is remarkable that the incubation of activated thylakoids in the dark results in little loss of Mg^{2+}-ATPase activity when the buffer present in the assay mixture is Tricine, but quite significant loss when the buffer is Tris. Tris and Tricine gave similar results when used in light activation and dark incubation mixtures. There was little loss of ATPase activity in the dark when the ATPase assay mixtures contained another zwitterionic buffer, TES. Tris was not unique in its apparent induction of decay of ATPase activity in the dark. Other polar amines, including aminopropanediols and diethanolamine had quite similar effects. Imidazole and NH_4Cl are stronger uncouplers of photophosphorylation than Tris and the other polar amines. Both imidazole and NH_4Cl affected the ATPase activity of activated thylakoids almost equally whether the aliquots were taken immediately at the end of illumination or after 4 min in the dark. The inhibition of the Mg^{2+}-ATPase activity of activated

thylakoids by uncouplers is likely the result of the decrease in ΔpH to below a threshold level required to keep the reduced ATP synthase in active form.

Sulfite in the assay medium overcomes the inhibition of Mg^{2+}-ATPase activity in thylakoids by uncouplers (Larson and Jagendorf, 1989) and stimulates the exchange of bound ADP for medium ATP (Larson et al., 1989). The loss of ATPase activity with time in the dark detected when Tris is the buffer used in the assay medium is completely offset by the presence of sulfite in the assay mixture (McCarty, 2005). These results and the fact that the addition of ADP accelerates the loss of ATPase activity in the dark (Carmeli and Lipschitz, 1972) strongly suggest that the binding of ADP to CF1 in activated thylakoids takes place in the dark after the illumination in the presence of DTT. Illumination promotes an exchange of medium nucleotide with ADP bound to CF1 (Harris and Slater, 1975; Strotmann and Bickel-Sandkoetter, 1977) and illumination of thylakoids permits the binding of ADP to CF1 in the post-illumination dark (Magnusson and McCarty, 1976).

The binding of Mg^{2+}-ADP to CF1 in activated thylakoids inhibits ATPase activity, whether the assay mixture contains Tris or Tricine. In Tris-buffered assay mixtures, the inhibition is permanent whereas in Tricine buffer, the inhibition is partially overcome over a period of about 1 min at about 30°C. The lag in the ATP hydrolysis and in the ATP-dependent ACMA fluorescence quenching observed in activated thylakoids that had incubated in the dark for 20 min prior to assay in Tricine buffer confirms this notion. The lag likely is a reflection of the slow initial build up of ΔpH after ATP addition in the presence of a nonpermeant buffer such as Tricine. A few ATP synthase molecules seem to be active even after 20 min in the dark following activation and ATP hydrolysis by these molecules causes inward directed proton translocation. The ΔpH generated results in the activation of more ATP synthase molecules (likely by release of bound ADP) and the ΔpH increases further. The switching on of the ATPase activity continues until the steady-state rate is achieved. The buildup of ΔpH in this manner cannot occur in Tris buffer because of the uncoupling action of the Tris. The rate of ATP-dependent proton uptake in activated thylakoids that had been incubated in the dark before ATP addition is initially quite low and even a relatively weak uncoupler like Tris would be expected to—and does—prevent the ATP-dependent rise in ΔpH. Therefore, the enzyme remains inactive in Tris buffer after the addition of ATP.

The ATP-dependent activation of the ATPase activity of dark adapted, activated thylakoids resembles the activation by DTT of the Mg^{2+}-ATPase and Pi-ATP exchange activities of thylakoids in the dark (McCarty and Racker, 1968). Both activations are prevented by Tris in the assay medium and are sensitive to uncouplers. As shown by Davenport and McCarty (1981) the activation required DTT and ATP and took place during assay. At 31°C and with 9 mM DTT and 50 mM Tricine–NaOH (pH 8.0) the rate of ATP-Pi exchange did not reach the steady-state until 10 min of incubation in the dark. In contrast, in the experiments reported in this paper the lag was of the order of 1 min. The much slower activation in the experiments of Davenport very likely reflects the fact that both reduction of the γ subunit disulfide bond and ADP release were required for activation. In my experiments, the γ disulfide was reduced during the illumination of the thylakoids in the presence of 5 mM DTT. The γ subunit disulfide can be reduced by incubation of thylakoids for 2 h with 50 mM DTT in the dark and the dithiol generated by reduction alkylated with *N*-ethylmaleimide to prevent reoxidation. DTT was not required for the ATP-dependent activation of the ATPase activity of thylakoid membranes that had been illuminated and then stored in the dark (R. E. McCarty, unpublished observations).

Ort and Oxborough (1992) questioned whether nucleotide binding to CF1 in situ was involved in regulation of the activity of the ATP synthase in part on the basis of the fact that Pi prevents inactivation in the dark. The results presented in this paper also call this possibility into question. It must be stressed, however, that the experiments reported here were carried out under distinctly nonphysiological conditions. An examination of the regulation of the activity of the ATP synthase at physiologically relevant concentrations of ADP, ATP, and Pi (Giersch et al., 1990) seems to be in order, as does regulation in intact chloroplasts capable of high rates of CO2 fixation in the light.

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