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LIGHT-INDUCED Mg^{2+} ATPase ACTIVITY OF COUPLING FACTOR IN INTACT CHLOROPLASTS

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Summary

Intense illumination of isolated, intact, spinach chloroplasts triggers the well known proton-pumping Mg^{2+} ATPase activity of coupling factor, which can be assayed in subsequently lysed chloroplasts by monitoring ATP-driven quenching of 9-aminoacridine fluorescence. The light-triggered ATPase activity decays slowly in the dark and is inhibited by *N,N'*-dicyclohexylcarbodiimide. After osmotic lysis and washing of the chloroplasts, preillumination no longer triggers maximal proton-pumping ATPase until methylviologen and dithiothreitol are added to the medium. It is suggested that intact organelles contain soluble or loosely bound cofactors necessary for light-triggering of coupling factor ATPase. On osmotic lysis, these endogenous cofactors are diluted or inactivated and must be replaced by addition of a dithiol reagent and an electron acceptor.

Introduction

ATP synthesis in illuminated chloroplasts is catalysed by a membrane-bound enzyme known as coupling factor (CF_1, see Ref. 1 for a review). CF_1 also possesses a latent Mg^{2+} ATPase activity, which can be unmasked by illumination in the presence of a dithiol and an electron acceptor or cofactor [2—4]. Only low, transitory activity results from illumination with cofactor alone [5]. When ATP is present, the fully activated ATPase is stable in the dark and ATP

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; Tricine, *N*-tris(hydroxymethyl)methylglycine; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CF_1, chloroplast coupling factor I.

hydrolysis is accompanied by translocation of protons from the external medium to the intrathylakoid space [6—8]. These results have been much discussed in reference to the chemiosmotic hypothesis of energy coupling [9] and the mechanism of ATP synthesis by CF_1 (Ref. 1 and References therein).

Most experiments outlined above have been performed using broken chloroplast preparations, which lack their outer envelopes and stromal contents [1—8] and hence have lost any endogenous cofactors that are not tightly bound to the membrane. In contrast, intact chloroplasts (which retain the ability to fix CO_2) carry out non-cyclic, cyclic [10], and pseudocyclic [10,11] coupled electron transport in the absence of added acceptors and also reportedly contain several thiol or thiol-reducing compounds [12,13].

It will be shown in this paper that the proton-translocating ATPase is activated by simply illuminating intact chloroplasts. The results suggest that stabilization of ATPase by thiols may be a physiological process.

Materials and Methods

Intact chloroplasts were isolated as previously described [14], using a final resuspension medium of 0.36 M sorbitol, 10 mM Hepes, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, pH 7.55. The preparations contained 70—80% intact chloroplasts as judged by their ability to photoreduce ferricyanide [15]. 9-Aminoacridine fluorescence was measured as previously described [16]. Intact chloroplasts (approx. 30 μg chlorophyll) were preincubated for 3 min in 0.4 ml of a medium containing 0.35 M sorbitol, 0.25 mM Na_2HPO_4 and 50 mM tricine brought to pH 8.1 with KOH/NaOH. Where indicated, the sample was illuminated with red light (Corning CS 2-58 filter) at 2000 W/cm^2. 5 s before the end of the preincubation, chloroplasts were osmotically lysed by adding 1.6 ml of a medium containing 6 mM $MgCl_2$, 10 mM NaCl and 6 μM 9-aminoacridine brought to pH 8 with Tris base. 9-Aminoacridine fluorescence was recorded immediately and all manipulations were carried out in the fluorescence cuvette. When chloroplasts were to be lysed before preincubation, the intact organelles were first suspended in 1.6 ml of the sorbitol-free medium, then 0.4 ml of sorbitol/phosphate/tricine medium was added. In all cases, ATP was injected into the sample using a microsyringe following a standard period of time (usually 40 s) after preincubation.

ATPase activity was also assayed by determining inorganic phosphate release. In this case, the final volume was 2.5 ml, the ATP concentration was 5 mM, and Na_2HPO_4 and 9-aminoacridine were omitted from the assay medium. Preillumination (white light) was provided by two 500 W projectors screened with heat filters. The reaction was initiated by addition of ATP immediately after lysis of the chloroplasts and was terminated by adding 0.5 ml of 20% trichloroacetic acid. Precipitated chloroplasts were removed by centrifugation.

Results

The light plus dithiothreitol activated ATPase is a proton pump that generates a pH gradient across the thylakoid. Its activity can therefore be specifically monitored by the quenching of 9-aminoacridine fluorescence [7,8] which

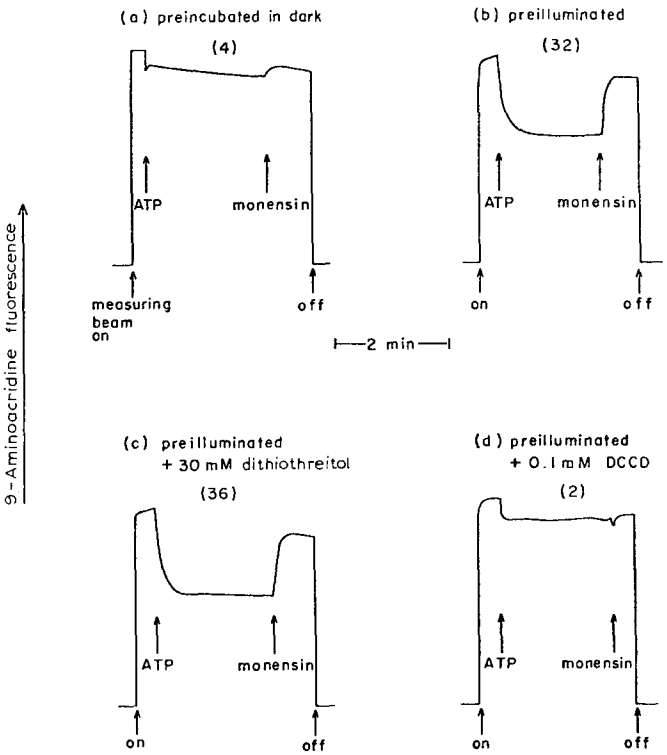

Fig. 1. ATP-driven quenching of 9-aminoacridine fluorescence in lysed chloroplasts. Chloroplasts (33 μg chlorophyll) were maintained intact during the preincubation (see Methods) either in darkness (a), or illumination (b), (c) and (d). Concentrations of dithiothreitol and DCCD (where indicated) are those during preincubation and were thus 5 times lower in the final assay medium after osmotic lysis of the chloroplasts. ATP and monensin were added to a final concentration of 0.3 mM and 3 μM respectively. The number in parentheses is the percentage of ATP-driven quenching that was reversed by monensin.

occurs when the amine distributes itself across the membrane in response to the proton gradient [18]. Fig. 1 shows the quenching of 9-aminoacridine fluorescence observed when ATP is added to chloroplasts that have been preincubated intact and then osmotically lysed immediately before switching on the measuring beam. In all cases a rapid initial decrease in fluorescence is observed which does not require the presence of chloroplasts and thus is not related to any ATPase activity. When chloroplasts are preincubated in the dark, little further quenching is observed (Fig. 1a). However, if chloroplasts are preilluminated, addition of ATP induces considerable slow quenching of fluorescence that is readily reversed on adding monensin (which dissipates any pH gradient by facilitating Na^+/H^+ exchange across the thylakoid). Addition of 30 mM dithiothreitol to the preincubation mixture only slightly increases ATP-driven fluorescence quenching. Addition of 0.1 mM DCCD which at this concentration inhibits Mg^{2+}-dependent ATPase activity of CF_1 [6], also inhibits 9-aminoacridine quenching. The results show that considerable proton-pumping ATPase activity can be triggered on simply illuminating intact chloroplasts. These preparations apparently contain endogenous 'activation cofactors' obviating the

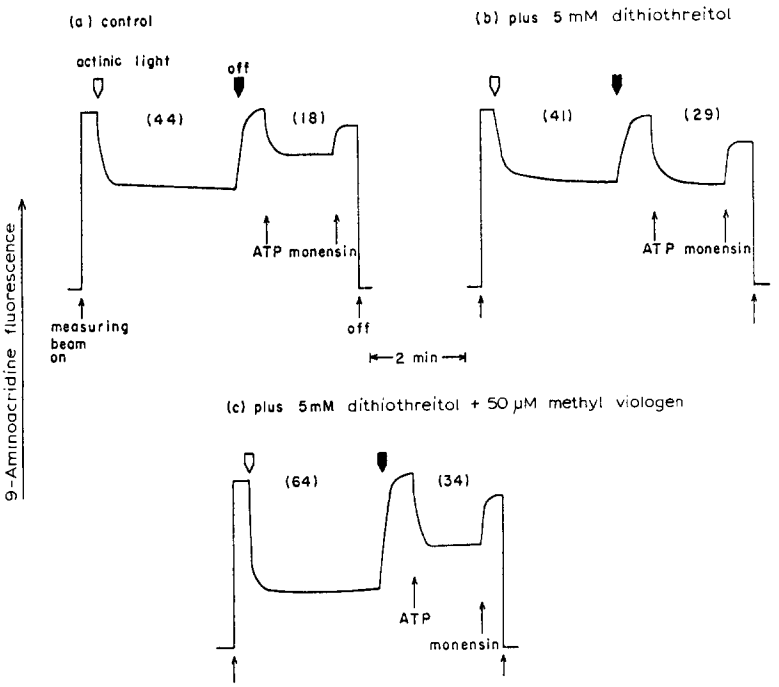

Fig. 2. Light and ATP-driven quenching of 9-aminoacridine fluorescence in lysed chloroplasts. In this case, intact chloroplasts that had not been preilluminated were lysed immediately before turning on the measuring beam. Concentrations of dithiothreitol and methyl viologen are those during final assay. Other details as given in Fig. 1.

need to add exogenous dithiols or electron acceptors.

When intact chloroplasts are osmotically lysed immediately before the preillumination with actinic light, subsequent ATP-driven fluorescence quenching is 44% inhibited (18 units in Fig. 2a against 32 units in Fig. 1b). Addition of 5 mM dithiothreitol now stimulates the quenching (Fig. 2b) whilst further addition of an electron acceptor such as methyl viologen before preillumination (Fig. 2c) restores ATP-driven quenching to that observed when chloroplasts

TABLE I

QUENCHING OF 9-AMINOACRIDINE FLUORESCENCE INDUCED BY ILLUMINATION AND ADDITION OF ATP TO LYSED, WASHED CHLOROPLASTS

Intact chloroplasts were lysed in 1/25-strength resuspension medium containing 5 mM $MgCl_2$, centrifuged after 5 min and finally resuspended in full strength medium. Each sample contained 32 μg chlorophyll. Experiments were performed exactly as shown in Fig. 2.

Additions (final concentration)	9-Aminoacridine fluorescence quenched (%)	
	light-driven	ATP-driven
None	47	10
50 μM methyl viologen	68	9
5 mM dithiothreitol	47	17
50 μM methyl viologen + 5 mM dithiothreitol	65	27

TABLE II

LIGHT-TRIGGERING OF ATPase AS A FUNCTION OF CHLOROPLAST INTACTNESS

Intact chloroplasts were prepared as previously described [14], but the isolation medium contained 20 mM morpholinoethanesulfonic (Mes) acid in place of pyrophosphate buffer and the resuspension medium contained 10 mM rather than 50 mM Hepes. One preparation of intact chloroplasts was shocked in 1/25-strength resuspension medium (40 ml), centrifuged, washed once by resuspension in dilute medium and finally resuspended as for the intact chloroplasts. Chloroplasts (176 μg chlorophyll) were illuminated or dark incubated for 3 min in 0.5 ml 0.35 M sorbitol plus 25 mM tricine (pH 8.1); then 2 ml 6 mM $MgCl_2$, 10 mM NaCl plus either 10 mM tricine or Mes to give a final pH of 8.1 or 5.5, were added to shock the (intact) chloroplasts (from the ability to reduce ferricyanide in the light it was shown that this regime produced approx. 95% breakage of intact chloroplasts within 15 s). 50 μl 0.25 M ATP was added 15 s later and ATPase activity was stopped after 5 min (dark, 20°C).

Conditions	Rate of ATP hydrolysis (μmol phosphate released/mg chlorophyll per h)			
	Intact chloroplasts		Shocked, washed chloroplasts	
	pH 8.1	pH 5.5	pH 8.1	pH 5.5
Preilluminated	33	22	13	11
Preincubated in dark	14	19	5	11
Difference due to light	19	3	8	0

are preilluminated intact (Fig. 1b). It appears that osmotic lysis causes partial loss (or inactivation) of endogenous cofactors necessary for light-triggering of the ATPase. Indeed, when lysed chloroplasts are washed (by centrifugation and resuspension), ATP-driven quenching is further diminished (Table I) but not totally inhibited. Addition of dithiothreitol to washed chloroplasts restores some of the quenching but methyl viologen plus dithiothreitol are needed for optimal recovery. The data indicate that the endogenous cofactor replaceable by dithiothreitol may be more easily lost than cofactors of coupled electron flow.

For probes such as 9-aminoacridine, the relation of percentage quenching to ΔpH is only qualitative at low quenching values, as was shown by Schuldiner et al. [18]; accordingly, ATPase activity was also measured directly by phosphate release. Table II shows the importance of chloroplast intactness in retaining the ability to trigger ATPase activity by light, without addition of dithiol or cofactor. Shocking the chloroplasts, followed by washing, removes 58% of the light-triggered ATPase activity assayed at pH 8.1; failure to remove all activity by washing shows that some component(s) needed for triggering are loosely bound to the membranes. As originally shown by Kraayenhof et al. [19], significant ATPase activity was observed in chloroplasts preincubated in the dark. It should be noted however that this may not be due entirely to activity associated with CF_1 since the stroma contains several soluble ATPases and phosphatases. Indeed, when the assay (but not the preincubation) medium was lowered to pH 5.5, considerable dark ATPase activity was found, which was not stimulated by preillumination. This activity too, is loosely associated with the membrane as evidenced by its incomplete (42%) removal upon shocking and washing the chloroplasts.

The effects of dithiothreitol on light-triggering in intact chloroplasts is small (Table III), as expected from Fig. 1, but 0.1 mM DCCD inhibits all activity

TABLE III

LIGHT-TRIGGERING OF ATPase IN INTACT CHLOROPLASTS

ATPase activity of subsequently lysed chloroplasts was assayed by inorganic phosphate release. Each sample contained 180 μg chlorophyll. Concentrations of dithiothreitol and DCCD refer to those during preincubation. Where indicated, chloroplasts assayed intact were diluted with 0.3 M sorbitol, 50 mM tricine (pH 8.0) in place of the sorbitol-free medium.

Conditions	Rate of ATP hydrolysis (μmol phosphate released/mg chlorophyll per h)			
	Preilluminated		Preincubated in dark	
	pH 8.1	pH 5.5	pH 8.1	pH 5.5
Control	42	31	13	45
Control + 50 mM dithiothreitol	50	45	—	—
Control + 0.1 mM DCCD	0	26	5	31
Assayed intact	10	—	—	—

when presented before illumination. The activity assayed at pH 5.5 is only moderately sensitive to DCCD and interestingly, is partially inhibited by preillumination (see also Table II, the extent of this effect is variable). Further effort is required to characterize and identify the dark ATPase activity.

Table III also shows that light-triggered ATPase activity corresponding to the activity of the 20—30% broken chloroplasts in the preparation could be detected if the chloroplasts were not osmotically lysed. This result would be expected in view of the reported low rate of entry of ATP into the intact chloroplast (either by passive diffusion or via the adenylate translocator [20]).

Fig. 3 depicts the stability of the light-triggered ATPase in a dark period in the presence of 1 mM Na_2HPO_4 (which helps to stabilize the activity [7]). The

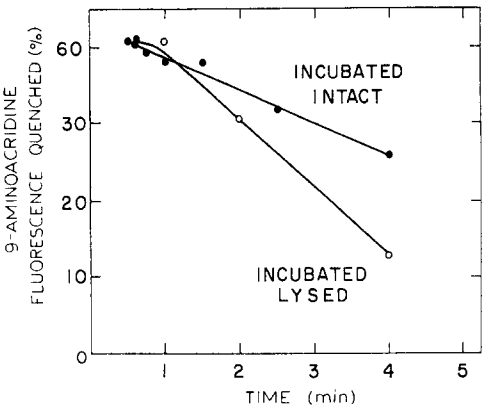

Fig. 3. Inactivation of the light-triggered ATPase in the dark, monitored as ATP-driven quenching of 9-aminoacridine fluorescence. The abscissa gives the total time (in the dark) between terminating preillumination and adding ATP (0.3 mM). During this period, chloroplasts were maintained either intact (i.e. lysed 5 s before adding ATP) or lysed (i.e. lysed immediately after termination of preincubation). Otherwise, experiments were performed as shown in Fig. 1.

abscissa represents the time between terminating preillumination and addition of ATP. When chloroplasts are lysed immediately at the end of the preillumination, ATPase activity declines over a period of several minutes, as shown by others [2—5,7]. If the chloroplasts are maintained intact during the dark incubation period, activity also declines but at a slower rate. Apparently therefore, light-triggered ATPase is both more easily activated and more stable in intact than in lysed chloroplasts.

Discussion

It has been unequivocally shown in this paper that illumination alone is sufficiently to trigger Mg^{2+} ATPase and proton pumping activity of CF_1 in intact chloroplasts. Some years ago, Kraayenhof et al. [19] concluded from studies of inorganic phosphate release that considerable ATPase activity of CF_1 is present both in dark and preilluminated 'intact' chloroplasts. It should be noted however that their chloroplast preparations were permeable to ATP, and may not therefore have been intact (no biochemical evidence of the intactness was given). Although we also detect dark ATPase activity by the inorganic phosphate assay, there is little quenching of 9-aminoacridine fluorescence. The dark ATPase activity is incapable of maintaining a very large proton gradient and may therefore be due mostly to other ATPases released on osmotically lysing the organelles.

The light-triggered ATPase in the intact chloroplast appears to be analogous to that observed in lysed chloroplasts in the presence of an electron acceptor and dithiothreitol. In the intact organelle, ferredoxin-catalysed cyclic [10] and non-cyclic [11] electron transport activity is high. Osmotic lysis and washing of the thylakoid probably removes most of the soluble and loosely bound ferredoxin thereby creating the need for an exogenous electron acceptor. In a similar and more dramatic way, lysing the chloroplasts also causes the loss of another soluble activation cofactor replaceable by dithiothreitol. The identity of the latter cofactor is not presently known, though one possible candidate is thioredoxin, a low molecular weight protein with a redox-active disulfide bridge [21]. Chloroplast thioredoxin, following its reduction by thylakoids in the light, has recently been proposed to activate several stromal enzymes involved in CO_2 fixation [12,13]. It is possible that this dithiol protein also activates Mg^{2+} ATPase of CF_1 in vivo — a process that may have important implications in the mechanism kinetics of ATP synthesis.

Finally, it is pertinent that the ATPase activity of CF_1 decays relatively slowly in the dark. Recently, a report by Inoue et al. [22] has appeared, describing activation of the proton-translocating ATPase in intact chloroplasts under flash illumination. Although these authors also found that activation of ATPase required no external cofactors, they concluded the ATPase activity was unstable in the dark and decayed with a half-time of about 2 s, after terminating illumination. Measurements of its activity, described here, show that ATPase is relatively stable in the dark, therefore the apparent activation described by Inoue et al. [22] must arise from some other cause. A possible explanation is that the ADP formed, inhibits membrane-bound ATPase allosterically as is the case with solubilised CF_1 [1]. Allosteric inhibition by ADP would become

rapidly apparant in studies with intact chloroplasts where the endogenous pool of ATP is relatively small (approx. 15 nmol/mg chlorophyll; Ref. 22). However, such an inhibition may not be seen, or may be reversed on adding large amounts of ATP (20 μmol/mg chlorophyll) to lysed organelles as in the present study. Thus, the two sets of results can be reconciled.

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