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The activation and inactivation of the *Dunaliella salina* chloroplast coupling factor 1 (CF_1) in vivo and in situ

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Preillumination of intact cells of the eukaryotic, halotolerant, cell-wall-less green alga *Dunaliella salina* induces a dark ATPase activity the magnitude of which is about 3–5-fold higher than the ATPase activity observed in dark-adapted cells. The light-induced activity arises from the activation and stabilization in vivo of chloroplast coupling factor 1 (CF_1). This activity, ~ 150–300 μmol ATP hydrolyzed/mg Chl per h, rapidly decays (with a half-time of about 6 min at room temperature) in intact cells but only slowly decays (with a half-time of about 45 min at room temperature) if the cells are lysed by osmotic shock immediately after illumination. The activated form of the ATPase in lysed cells is inhibited if the membranes are treated with ferri- but not ferrocyanide, suggesting that the stabilization of the activated form of CF_1 is due to the reduction of the enzyme in vivo in the light.

Chloroplast coupling factor 1; ATPase; (*Dunaliella salina*)

1. INTRODUCTION

The catalytic activity of the chloroplast coupling factor 1 (CF_1) both in vitro and in situ is latent. A wide variety of treatments induces the ATPase activity of CF_1 in vitro, including but not limited to proteolytic digestion, incubation at high temperature for a short time, and incubation in the presence of high concentrations of reductant, some detergents, and some organic solvents [1]. Similarly the ATP synthase activity of CF_1 in situ can be induced by the formation of a protonmotive force across the chloroplast thylakoid membrane, and the ATPase activity by some of the treatments used to induce the activity of the soluble enzyme [1]. Undoubtedly all of the methods used to activate the catalytic activity of the CF_1 result in some conformational changes in the enzyme structure, some of which are reversible.

In the absence of a protonmotive force, a disulfide bridge on the thylakoid-bound spinach CF_1 γ-subunit reacts very slowly with aqueous reductants although it can be reduced by dithiothreitol upon prolonged incubation [2,3]. A protonmotive force across the chloroplast thylakoid membranes alters the reactivity of the γ-disulfide bridge with respect to polar reductants [2,3]. Reduction of the disulfide bridge, though not a prerequisite for catalytic activity, has the effect of decreasing the magnitude of the protonmotive force needed to drive phosphorylation [3–5], and, in addition, stabilizes the ATPase activity of the enzyme after the dissipation of the protonmotive force [3], presumably by stabilizing the ATPase active conformation of the CF_1. For studies with washed thylakoid membranes, the reductant most often chosen is dithiothreitol; however, in vivo, for both spinach and pea chloroplasts, strong arguments have been made for the reductant being thioredoxin [5–8]. Although

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Abbreviations: CF_1, chloroplast coupling factor 1; DTT, dithiothreitol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea

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studies with other plant materials are not nearly as exhaustive as they have been for spinach and pea, it appears, nevertheless, that a similar reduction-stabilization mechanism exists for the active conformation of the CF_1 in situ in algae [9] as well.

In this communication we confirm the existence of redox activation/deactivation of the in vivo ATPase activity of CF_1 in the eukaryotic, cell-wall-less, green alga *Dunaliella*, recently demonstrated by Noctor and Mills [9]. We show that the activated enzyme is rapidly inactivated in vivo but is only slowly inactivated when the cells are lysed after illumination. The activity of the activated enzyme associated with the lysed cells is inhibited by chemical oxidants such as ferricyanide, but not by glutathione. In this manuscript we demonstrate the utility of *D. salina* as a test system for purifying the putative in vivo inhibitory factor.

2. MATERIALS AND METHODS

D. salina was cultured in the laboratory in 1.5 M NaCl in growth medium as previously described [10] and maintained in log phase. Cells were harvested by centrifugation (1600 × *g*, 3.0 min) and resuspended in 20 mM Tricine-NaOH (pH 8.0) (plus 1.0 M NaCl) at a density equivalent to 0.2 mg Chl/ml. Intact cells were illuminated at room temperature with heat-filtered white light for 5 min. Thereafter, the cells were either maintained intact in the dark for variable periods or lysed in the dark by the addition of 10 μl to the hypotonic ATPase assay mixture.

ATPase assay mixtures contained in a total volume of 0.1 ml, lysed cells, equivalent to 2 μg Chl, 20 mM Tricine-NaOH (pH 8.0), 20% ethanol (v/v), 6.0 mM $MgCl_2$, and 1.0 mM EDTA. Additions, when made, were to these suspensions, and the suspensions were further incubated at room temperature in the dark for various periods prior to starting the assay for ATP hydrolysis. ATPase assays were initiated by the addition of 5.0 mM [γ-^{32}P]ATP. Reaction mixtures were incubated at 37°C for 5.0 min. Reactions were terminated by the addition of 2.0 ml of 0.8 N $HClO_4$ in 1% (w/v) ammonium molybdate, and inorganic [^{32}P]phosphate was determined as previously described [11]. One unit of activity is defined as 1.0 μmol phosphate released/min. The ATPase activity from dark-adapted, non-illuminated cells ranged from 0.5 to 1.5 units/mg Chl. This activity has been shown not to arise from CF_1 [9] and was therefore subtracted from all subsequent measurements. Net light-stimulated rates ranged from 2 to 6 units/mg Chl.

3. RESULTS

When dark-adapted cells of *D. salina* are lysed directly in a hypotonic reaction mixture, a basal rate of ATPase activity, varying from 0.5 to 1.5 units/mg Chl (dependent upon the culture), is

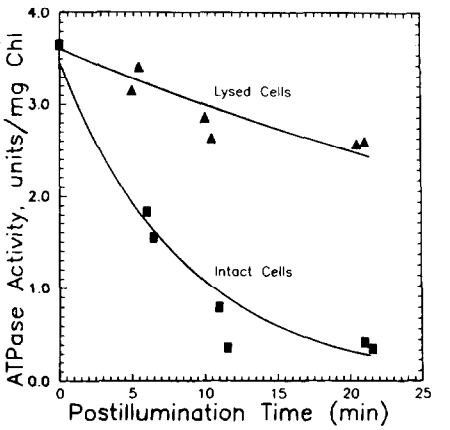

Fig.1. Time course for the decay of the in vivo light-induced ATPase activity in *D. salina*. Intact cells of *D. salina* were illuminated with white light for 5.0 min at room temperature as described in section 2. Upon extinguishing the light (at $t = 0$) samples were taken and either maintained intact or lysed in ATPase reaction mixtures (minus ATP) in the dark at room temperature. At the indicated times, either ATP was added to the lysed cells (△) or intact cells were lysed by addition to ATPase reaction mixtures containing ATP (□). ATP hydrolysis was measured at 37°C as described in section 2.

observed. Noctor and Mills [9] have obtained similar results with the alga *Dunaliella tertiolecta* and have demonstrated that this basal activity is not due to the thylakoid-bound CF_1. Illumination of the culture with white light for 5 min prior to the assay, however, results in a marked increase in the ATPase activity of 3–5-fold over the basal rate. As reported by Noctor and Mills [9], the light-induced increase in activity, but not the basal activity, is inhibited by methylviologen and DCMU (when present during illumination) and a polyclonal, monospecific chicken antiserum directed against the *D. salina* CF_1 (when present in the dark phase; not shown). Therefore, the light-induced increase in ATPase activity most likely arises from an activation of the catalytic activity of the CF_1.

When illuminated cells are returned to the dark, the light-induced increased ATPase activity rapidly decays. The half-time for the decay of the light-induced increased activity varies with the temperature of the culture ranging from about 10 to 15 min at 4°C (not shown) to 5 to 10 min at 20°C (fig.1; squares). However, if the cells are lysed immediately following the 5 min illumination period, the half-time for the decay of the light-

induced increased ATPase activity is markedly increased, from greater than 50 min at 4°C (not shown) to ~ 45 min at 20°C (fig.1; triangles). These data are reminiscent of those obtained for the in organello activation/deactivation of the thylakoid-bound CF_1 for both spinach and pea chloroplasts [7,8,12]. In the latter organisms the rapid in organello deactivation of the light-induced increased ATPase activity has been attributed to the rapid re-oxidation of CF_1 (γ-subunit) vicinal dithiols [3], a process that is greatly retarded when the organelles are lysed and the soluble stroma factors (oxidants) diluted.

Table 1 shows that a similar process of re-oxidation might be responsible for the decay of the light-induced increased ATPase activity in *D. salina*. In this experiment, after illumination, the cells were lysed and incubated in the dark for 5 min in various reaction mixtures. The addition of dithiothreitol to the lysis mixture results in a slight stimulation of the ATPase activity, ranging from 5 to 20% (row 2). Ferricyanide (1.0 mM), however, completely inhibits the light-induced increased ATPase activity (but not the basal activity; data not shown) (row 3). The ferricyanide-dependent inhibition of the light-induced activity is completely prevented by dithiothreitol (2.0 mM), probably because of the reduction of ferricyanide to ferrocyanide (row 4). Notably, glutathione (at 1.0 mM) does not inhibit the light-induced activity (rows 5 and 6). Though these results, of course, do not demonstrate that the stabilization of the light-induced active conformation of the CF_1 in situ is due to the reduction of a disulfide bridge and the decay of the active form due to its re-oxidation, the parallels of the behavior of the *D. salina* CF_1 to those of spinach and pea are striking. It seems reasonable, therefore, to conclude that the activity of the *D. salina* CF_1 in vivo is probably modulated by the redox state of the enzyme, though it remains to be shown exactly which group(s) is (are) involved.

Table 1

Oxidant-induced inhibition of the light-triggered CF_1 ATPase activity

Additions to the reaction mixture prior to the assay		ATPase activity (units/mg Chl)
I	II	
None	none	2.05
None	DTT	2.13
Ferricyanide	none	0.11
Ferricyanide	DTT	1.95
Glutathione	none	1.71
Glutathione	DTT	2.52

ATPase reaction mixtures (minus ATP) containing either ferricyanide or glutathione (1.0 mM each) or no additions (as indicated in column I) were preincubated with 2.0 mM dithiothreitol (as indicated in column II) at room temperature in a total volume of 80 μl. 10 μl of a preilluminated cell suspension of *D. salina* were added and further incubated in the dark for 5 min at room temperature. The ATPase assay was initiated by the addition of 10 μl ATP (final concentration 5 mM) and the assay performed at 37°C as described in section 2

4. DISCUSSION

Preillumination of *D. salina* activates an ATPase activity that can be measured when intact cells are lysed in a subsequent dark phase. The rate of decay of the light-activated ATPase activity is greatly diminished if the cells are broken. And, the rate of decay of the light-activated ATPase can be accelerated in lysed cells in the presence of a chemical oxidant such as ferricyanide. It appears, therefore, that the catalytic activity of the *D. salina* thylakoid-bound CF_1 is modulated in a manner analogous to that of the in organello pea and spinach CF_1. Namely, light induces an active conformation of the enzyme which can then be trapped upon reduction, whereas in the dark the enzyme is oxidized, allowing it to reassume its inactive conformation. For both the pea and spinach CF_1 the nature of the reductant has been argued to be thioredoxin [5–8] and the target site a disulfide bridge on the CF_1 γ-subunit [2,3]. From our studies, of course, we cannot address either the nature of the reductant or the target site for that reductant in *D. salina*, though it seems unlikely that they will be different from those found in the vascular plants.

In this work we have been mainly concerned with designing a convenient and reliable test system to be able to rapidly screen possible factors that might be involved in the in vivo regulation of CF_1, vis-à-vis the inactivation of the light-activated, stabilized form of the CF_1. These initial studies could have been performed with higher plant chloroplasts; however, the cell-wall-less,

halotolerant alga provides a much better test system than either intact spinach or pea chloroplasts. Firstly, though intact vascular plant chloroplasts can be readily prepared on a relatively small scale, the technical difficulties in preparing the large quantities (~100 mg Chl) needed for the isolation of a factor responsible for the inactivation of the CF_1 are enormous. In contrast, it is relatively easy to culture large quantities of algae, whose internal volume is approx. 60% or higher stroma [13].

Secondly, because *Dunaliella* is halotolerant and cell-wall-less, it can be cultured in the presence of high salt which makes it susceptible to osmotic shock when resuspended in or added to a hypotonic medium. Lysis is facilitated by the fact that it does not possess a cell wall. Thus, the assays for the light-activation and dark inactivation of the ATPase are far easier than similar assays with the vascular plant chloroplasts.

Finally, it has been our experience with intact pea chloroplasts that, although the activated state of the pea CF_1 is somewhat stable in the dark after the intact chloroplasts have been lysed, the activity of that enzyme decays fairly rapidly, when compared to comparable experiments with *D. salina* (see also [14]). That rate of decay would complicate the use of intact pea chloroplasts as a convenient and rapid test system for the putative oxidant. On the other hand, although the activated state of the *D. salina* CF_1 decays very rapidly in intact cells, once the cells have been lysed, the half-time for the decay is at least 45 min. As we show in the subsequent communication, this test system can be readily adapted for use as a routine screen for possible regulatory factors.

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