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The partial purification of a factor from *Dunaliella salina* that causes the rapid in situ inactivation of light-activated chloroplast coupling factor 1 (CF_1)

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A factor having the expected properties of the in vivo oxidant responsible for inactivating the in vivo light-activated chloroplast coupling factor 1 (CF_1) has been partially purified from cell-free extracts of *Dunaliella salina*. This factor is highly polar, weakly acidic, and relatively temperature stable. The ability of this factor to inactivate light-activated CF_1 is prevented if it is pretreated with reductants such as dithiothreitol. The factor has virtually no effect on the ethanol-induced, Mg^{2+}-dependent ATPase activity of the isolated CF_1.

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

1. INTRODUCTION

Conformational changes in the structure of the chloroplast CF_1 have been inferred by many studies [1]. Perhaps the most detailed studies are those showing the correlation of changes in the accessibility of both sulfhydryls and disulfides on the γ-subunit of the spinach CF_1 with changes in the activity of the enzyme as demonstrated by McCarty and co-workers [2–5]. Upon the formation of a protonmotive force across the chloroplast thylakoid membranes, the reactivity of the γ-disulfide with polar reductants greatly increases as it presumably becomes more exposed to the solvent [4,6]. Though reduction of this disulfide bridge is not a prerequisite for catalytic activity, its reduction has the effect of stabilizing the ATPase activity of the enzyme after the dissipation of the protonmotive force [6], presumably by stabilizing the ATPase active conformation of the CF_1.

The reoxidation of the resultant γ-subunit vicinal dithiols results in the rapid, reversible decay of the ATPase activity [7–9]. This is usually achieved either by the prolonged incubation of the intact (spinach and pea) chloroplasts in the dark or by the addition of a chemical oxidant [8]. Although several candidates for the in vivo oxidant have been suggested, e.g. H_2O_2 [8], convincing evidence for any substance has not yet been forthcoming.

In this contribution we take advantage of the properties of the in vivo light-activated *Dunaliella salina* CF_1 to partially purify a small molecular mass, polar, heat-stable, factor from *Dunaliella*. This factor rapidly inactivates the ATPase activity of the in vivo, light-activated, but not the in vitro ethanol-induced, Mg^{2+}-dependent, enzyme. The ability of this factor to inhibit the ATPase activity is a function of the redox state of the factor.

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Abbreviations: CF_1, chloroplast coupling factor 1; DTT, dithiothreitol

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2. MATERIALS AND METHODS

2.1. *Preparation of materials*

A crude cell-free extract was prepared from cell cultures in the following manner. Cells from a ~4 l culture (equivalent to ~80–100 mg Chl total), grown in the presence of 1.5 M NaCl, were collected by centrifugation (600 × *g*, 5 min) and washed once in 100 ml Tricine-NaOH (pH 8.0) (plus 1.0 M NaCl). In order to lyse the cells, the pellet was resuspended in 40 ml of 2 mM Tricine buffer (pH 8.0). The suspension was centrifuged (3900 × *g*, 30 min) to remove the cell debris. The supernatant was heated to 95°C for 5.0 min, and the flocculent removed by centrifugation (3900 × *g*, 30 min). Absolute ethanol was added to a final concentration of 75% (v/v) and the resulting flocculent again removed by centrifugation (3900 × *g*, 30 min). The solution was evaporated to dryness at 65°C under vacuum, and the yellow residue redissolved in distilled water (~5 ml per l of the original culture). The solution was applied to a Dowex 1 (Dowex 1X2-400; Sigma) column (2.6 × 22 cm) equilibrated in distilled water. The column was developed with a linear HCl gradient (0–0.1 M; 100 ml total volume), and 1.0 ml samples were collected. Aliquots were assayed for inhibitory activity. The most active fractions, usually eluting in a total volume of 4 ml were pooled, evaporated to dryness, and redissolved in distilled water. The active fraction usually eluted from the column at pH ~4. Using this procedure about 80–90% of the total activity in the original extract could be recovered in a sample containing less than 5% of the original UV absorbing color. The active fraction is colorless and has no characteristic absorption in the near UV.

The *D. salina* CF_1 was purified to homogeneity as described [10].

2.2. *Analytical methods*

All analytical methods were as described in the accompanying paper.

3. RESULTS AND DISCUSSION

The light-induced ATPase activity of the *D. salina* CF_1 rapidly decays when intact, but not lysed, cells are returned to the dark (see fig.1 of the accompanying paper). If the rapid decay of the ATPase active form of thylakoid-bound CF_1 in vivo is indeed due to the re-oxidation of the light-dependent reduction of CF_1 (by thioredoxin?), then it should be possible to identify the in vivo oxidant. Before attempting to isolate the putative oxidant, we set the following minimal criteria: (i) because the rate of decay of the active form of CF_1 is greatly diminished upon lysis of the cells and dilution of the soluble stroma phase, the factor should be hydrophilic and not thylakoid membrane-bound; (ii) any ATPase inhibitory factor must itself be a redox substance and, therefore, ineffective when reduced; (iii) any ATPase inhibitory factor must be effective at relatively low concentrations; and (iv) the factor, regardless of its redox state, should not inhibit the solvent-induced, Mg^{2+}-dependent, ATPase activity of the CF_1 in vitro. We recognize, of course, that these criteria are by necessity rather general and that it is quite possible to isolate any number of factors whose properties might fit these criteria.

The test system we chose for the purification of the putative oxidant was the ability of a test sample to inhibit the light-induced increased ATPase activity 5 min after the lysis of intact, illuminated cells (analogous to ferricyanide; see table 1, row 3 of the accompanying paper). In this period of time the control light-induced activity decays (in the absence of any added factor) about 5%.

Initially cell-free extracts were prepared from concentrated suspensions of cells that were disrupted either by osmotic shock or sonication, the supernatant having been clarified by centrifugation. When tested for inhibitory activity (the assay is limited to ~30 μl of sample), fresh extracts yielded a maximal inhibition of only 20–25%. The ability of the extract to inhibit the ATPase activity increased markedly upon storage at 4°C for 24 h, or by freezing and thawing. In all cases the extract became cloudy and could be clarified by centrifugation without loss of in-

Table 1

The ability of dithiothreitol to prevent the factor-dependent inhibition of the light-induced CF_1 ATPase activity

Additions to the reaction mixture prior to the assay			ATPase activity	
I	II	III	Units/mg Chl	Percent control
None	none	cells	2.19	100
None	DTT	cells	2.41	110
None	factor	cells	0.39	18
Factor	DTT	cells	2.28	104
Factor	cells	DTT	1.34	61

The following additions were made to ATPase reaction mixtures (final total volume 100 μl) in the order indicated above: 10 μl partially purified factor, dithiothreitol (2.0 mM final concentration), or *D. salina* cells that had been preilluminated for 5.0 min at room temperature. After each addition (except in line 5) the mixture or suspension was incubated in the dark for 5.0 min at room temperature. After the third addition, or concomitant with the third addition (line 5), the assay was initiated by the addition of ATP, and ATP hydrolysis was measured at 37°C as described in section 2

hibitory activity. Heating the fresh extract to 95°C for 5 min resulted in the precipitation of almost all of the soluble protein, and after removal of the precipitate by centrifugation, the clear yellow supernatant contained a very potent ATPase inhibitor, 5 μl of a 16 ml extract from 4 l of log phase cultured cells inhibiting the ATPase activity about 90%.

The inhibitory activity could not be removed by passage of the extract through either a Chelex or Dowex 50 column, nor could it be extracted into ether, butanol, or propanol. When evaporated to dryness, the activity could be recovered in water, methanol, and 95% (v/v) ethanol/H_2O, but not acetone or absolute ethanol. Inhibitory activity could be removed by passage of the extract through either a Dowex 1 or silica gel column, but could not be recovered from the latter. When bound to a Dowex 1 column, the activity could be eluted in weak acid (~0.1–1 mM HCl). These observations led to the partial purification protocol outlined in section 2. The factor appears to be rather polar, weakly anionic, and very stable (see table 2) (criterion (i) above).

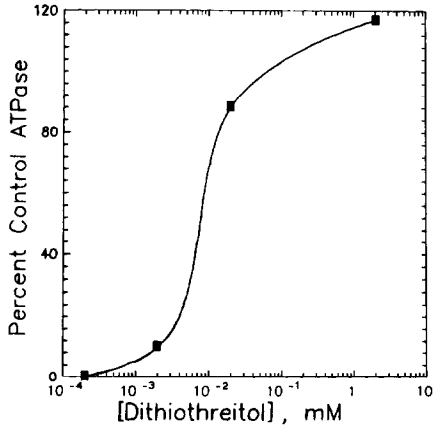

Fig.1. Concentration curve for the dithiothreitol prevention of the factor-dependent inhibition of the in vivo light-induced ATPase activity. Aliquots (10 μl) of partially purified factor were preincubated with the indicated concentrations of dithiothreitol for 5.0 min at room temperature in 80 μl ATPase reaction mixtures (minus ATP). *D. salina* cells were preilluminated for 5.0 min following which 10 μl samples were added to the reaction mixtures containing both the partially purified factor and dithiothreitol. The suspensions were incubated for an additional 5.0 min at room temperature prior to the addition of 10 μl ATP. ATP hydrolysis was measured at 37°C as described in section 2. The control rate of ATP hydrolysis for samples added to reaction mixtures containing neither partially purified factor nor dithiothreitol was 3.84 units/mg Chl.

Table 1 demonstrates that the factor is only active as an ATPase inhibitor in its oxidized form. In the experiment shown, preincubation of activated membranes with factor for 5 min prior to the ATPase assay caused a complete inhibition of activity (row 3). (Note that the level of inhibition is proportional to the amount of factor added to the reaction mixture; see fig.1, triangles.) When, however, the factor is first preincubated with dithiothreitol prior to being incubated with activated membranes, the factor is no longer inhibitory (row 4). Finally, if the factor is first allowed to incubate with the activated membranes prior to the addition of dithiothreitol, dithiothreitol is no longer capable of preventing the factor from inhibiting the ATPase activity (row 5). These data seem to conform to criterion (ii) as stated above.

Although the factor is somewhat heat stable, prolonged exposure of the factor to high temperatures gradually diminishes both its ability to inhibit the light-induced ATPase activity and the extent of the dithiothreitol prevention of inhibition (table 2).

Table 2

Heat lability of the inhibitory factor in the crude extract

Conditions of heat treatment	Pretreatment of factor prior to assay		
	None	+DTT	Δ (extent reversible)
95°C, 5 min	0.81	2.46	1.65
110°C, 24 h	1.59	2.34	0.75
180°C, 3 h	1.81	2.28	0.47
180°C, 24 h	1.66	1.63	0

Samples of *D. salina* cell-free extract, prepared as described in section 2, were heated in sealed containers at the temperatures and times shown in column 1. Aliquots (10 μl) of treated sample were added to 70 μl of ATPase reaction mixture (minus ATP) without (column 2) and with (column 3) dithiothreitol (2.0 mM final concentration) and incubated for 5.0 min at room temperature. To these mixtures were added 10 μl of *D. salina* cells that had been preilluminated for 5.0 min at room temperature. The suspensions were incubated for a further 5.0 min in the dark at room temperature. The ATPase assay was initiated by the addition of 10 μl ATP (5 mM final concentration) and measured at 37°C as described in section 2. The control rates without added factor were 2.45 in the absence and 2.86 in the presence of dithiothreitol. Results are expressed as units/mg Chl

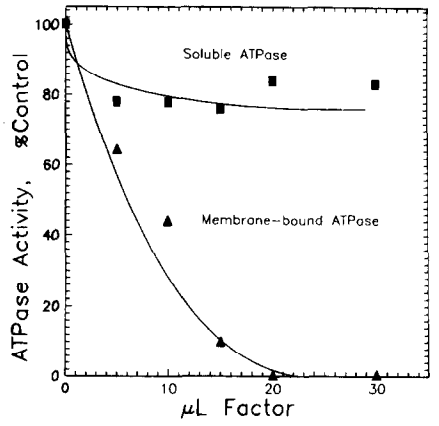

Fig.2. The ability of partially purified factor to inhibit either the in vivo light-induced or in vitro ethanol-induced *D. salina* CF_1 ATPase activity. (△) Intact *D. salina* cells were preilluminated for 5.0 min and then lysed in ATPase reaction mixtures (minus ATP) containing the indicated amounts of partially purified factor in a total volume of 90 μl. The suspensions were incubated in the dark at room temperature for 5.0 min prior to the initiation of the ATPase assays by the addition of 10 μl ATP. ATP hydrolysis was measured at 37°C as described in section 2. (□) Purified *D. salina* CF_1 was preincubated in ATPase reaction mixtures containing 20% ethanol (v/v) and the indicated amounts of partially purified factor at room temperature for 5.0 min in a total volume of 90 μl. ATP hydrolysis was initiated as above and determined as described [10]. The control rates of ATP hydrolysis for samples incubated in the absence of the partially purified factor were 5 units/mg Chl for the lysed cells (membrane-bound ATPase) and 25 μmol ATP hydrolyzed/mg protein per min for the purified CF_1 (soluble ATPase).

Fig.1 shows a titration curve for the dithiothreitol prevention of the factor-dependent inhibition of the light-induced ATPase activity. Approx. 5 μM dithiothreitol is needed to prevent 50% inhibition by 10 μl of the partially purified extract. Assuming that the dithiothreitol is completely reduced and that the only oxidant in the partially purified preparation is the factor, the upper limit for the concentration of the partially purified factor in the extract can be estimated to be about 0.2 mM equivalents. Since 4 ml of extract are obtained from ~25 mg Chl, this would correspond to about 25 Chl/oxidizing equivalent. Although this calculation would seem to conform to criterion (iii) as stated above, it must be considered cautiously.

Fig.2 compares the ability of the partially purified factor to inhibit either the light-induced ATPase activity of lysed cells or the ethanol-induced, Mg^{2+}-dependent ATPase activity of the isolated *D. salina* CF_1. Whereas 15 μl of this preparation inhibited the light-induced, membrane-bound ATPase activity about 90% (the triangles), the maximal inhibition of the soluble CF_1 ATPase activity was about 20% and was independent of the amount of factor added in the range of 5–30 μl (the squares). These results are consistent with criterion (iv) as stated above.

4. CONCLUDING REMARKS

In our search for the putative factor responsible for the inactivation of the in vivo light-activated *D. salina* CF_1, we defined four criteria that we believed this factor should fulfill. (i) It should be water soluble and easily removed from membrane preparations; (ii) it should be a redox agent, capable of inhibiting the activated form of the enzyme only in its oxidized form (table 1); (iii) it should be in relatively low abundance; and (iv) it should not inhibit the ATPase activity of the soluble enzyme when that activity is induced in a manner independent from the reduction of the enzyme (fig.2). Obviously these criteria are quite general, and once we identify the chemical nature of the active component of our fraction, we will have to establish its biological function.

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