Biochimica et Biophysica Acta, 764 (1984) 138–147
Elsevier

BBA 41442

PARTIAL CHARACTERIZATION OF THE CHLOROPLAST COUPLING FACTOR 1 FROM THE HALOTOLERANT ALGA *DUNALIELLA BARDAWIL*

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(Received July 22nd, 1983)

Key words: Coupling factor; ATPase; Nucleotide-binding site; Chemical modification; (D. bardawil)

The four-subunit chloroplast coupling factor 1 (CF_1) was isolated from the halotolerant alga *Dunaliella bardawil* by a chloroform extraction of membranes prepared from osmotically disrupted cells and purified by ion exchange chromatography on DEAE-Sephadex A-50. The *D. bardawil* CF_1 ATPase activity was completely latent. It could be induced irreversibly as a Ca^{2+}-dependent ATPase by a short heat treatment, or reversibly as a Mg^{2+}-dependent ATPase, by inclusion of selected organic solvents (e.g., methanol and ethanol) in the reaction mixture. Activation of the Ca^{2+}-dependent ATPase required the presence of both dithiothreitol and ATP during the heat treatment. The maximal specific activity of the Mg^{2+}-ATPase was about 4–5-fold higher than that of the Ca^{2+}-ATPase. In contrast to the migration of the subunits of the higher vascular plant spinach CF_1 on sodium dodecyl sulfate polyacrylamide gels, the order of the migration of the *D. bardawil* α- and β-subunits (and presumably their molecular weights) was reversed. This was established by immunocross reactivity experiments, using monospecific antisera directed against the β-subunit of the *Escherichia coli* F_1 and the α-subunit of the *Chlamydomonas reinhardii* CF_1. Fluorescein isothiocyanate (FITC) was a potent inhibitor of the *D. bardawil* CF_1 ATPase. 50% inhibition of the rates of ATP hydrolysis was obtained by 5 and 50 μM FITC for the *D. bardawil* and spinach CF_1, respectively. Inhibition was associated with the binding of FITC to the α-subunit of CF_1. The binding of FITC to the α-subunit of the *D. bardawil* CF_1 and hence the inhibition of the ATPase could be prevented by ATP and ADP, whereas GTP and GDP were much less effective.

Introduction

All photosynthetic organisms couple the light-driven, electron transfer-catalyzed, protonmotive force to the synthesis of ATP by a membrane-bound enzyme known as the reversible energy-transducing ATPase complex. The complex consists of two easily discernable and separable portions: that portion that is embedded in the membrane (F_0) and whose function is to direct the flux of protons to the catalytic portion, and the catalytic portion (F_1), bound to the F_0 portion but peripheral to the membrane, whose function is to convert the energy in the protonmotive force into ATP. Intact coupling factor complexes (F_0-F_1) or the F_1 portions devoid of F_0 have been isolated from photosynthetic bacteria [1], cyanobacteria [2], *Euglena gracilis* [3], green algae [4,5], and a wide variety of higher (vascular) plants [6].

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Abbreviations: CF_1, chloroplast coupling factor 1; CF_0-CF_1, chloroplast DCCD-sensitive ATP synthase complex; FITC, fluorescein isothiocyanate; DCCD, dicyclohexylcarbodiimide; SDS, sodium dodecyl sulphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Ado-P[NH]P, adenylylimidodiphosphate; Tricine, 2-amino-2-hydroxymethylpropane-1,3-diol.

Although there is general agreement that the composition of the various energy-transducing ATPase complexes appears to be quite similar, i.e., the F_0 portions contain three and the F_1 portions five different kinds of polypeptide, there are discrepancies regarding the identification of the individual subunits [4,7], their stoichiometries [6,8–10], and the molecular weight of the F_1 portion [8,10]. There are also some interesting differences in the catalytic properties of some of the isolated coupling factors. For example, whereas the ATPase activity of the spinach CF_1 is latent and must be induced, the activities of the bacterial [1], *E. gracilis* [3], and *Chlamydomonas reinhardii* [5,11] coupling factors are not masked. In addition, whereas the higher (vascular) plant enzyme, which is activated by heat, proteolysis and sulfhydryl reduction, is usually Ca^{2+}-dependent [6], the *C. reinhardii* [5,11] enzyme is Mg^{2+}-dependent.

In this contribution, we report on the properties of a four subunit CF_1 isolated from the halotolerant green alga *Dunaliella bardawil.* As is the case for the higher (vascular) plant enzyme, the *D. bardawil* coupling factor ATPase activity is completely latent. Activation can be achieved either by a short heat treatment, which induces a Ca^{2+}-dependent ATPase, or by selected organic solvents, which induce a Mg^{2+}-dependent ATPase. These two activities are mutually exclusive. In addition, we show by immunological methods that the migration of the two largest subunits of the *Dunaliella* CF_1 on sodium dodecylsulfate (SDS) polyacrylamide gels is reversed with respect to the migration of the spinach CF_1 α- and β-subunits. Finally, we demonstrate that the ATPase inhibitor, fluorescein isothiocyanate (FITC), binds specifically to the *Dunaliella* CF_1 and spinach CF_1 α-subunits, and that they can be protected from FITC by both ATP and ADP.

Methods and Materials

Coupling factor preparations

Log-phase cultures of *D. bardawil* (5-10L) were harvested by centrifugation ($600 \times g$, 5 min) and washed twice in 500 ml buffer comprising 40 mM Na-Tricine (pH 7.4)/1.5 M NaCl/5 mM $MgCl_2$. After the second wash, the cells were osmotically shocked by suspension in 250 ml hypotonic buffer comprising 50 mM Na-Hepes (pH 7.6)/10 mM NaCl/1 mM EDTA and incubated at 4°C for 5 min. Membrane fragments were collected by centrifugation ($3000 \times g$, 10 min) and washed four times (250 ml/wash) with 10 mM sodium pyrophosphate (pH 7.8) buffer.

CF_1 was extracted by chloroform and purified on a DEAE-Sephadex column essentially according to Selman-Reimer et al. [5], with the exception that the dialysis prior to the DEAE-Sephadex purification was omitted. The four- [12] and five- [13] subunit spinach coupling factors were prepared as previously described. Prior to use, aliquots of the ammonium sulphate precipitated proteins were centrifuged ($12\,000 \times g$, 2 min), and the proteins were redissolved in buffer comprising 40 mM Na-Tricine (pH 8.0)/1 mM EDTA.

ATPase assays

(a) Ca^{2+}-dependent ATPase. Samples containing 0.5 to 2.5 mg protein/ml in a total volume of 50 μl were heated at 60°C for 3.0 min in a medium comprising 40 mM Na-Tricine (pH 8.0)/1.0 mM EDTA/5.0 mM ATP/5.0 mM dithiothreitol. The samples were then rapidly cooled to room temperature. Unless otherwise stated, reaction mixtures contained, in a total volume of 0.1 ml, 40 mM Na-Tricine (pH 8.0), 1.0 mM EDTA, 6.0 mM $CaCl_2$, 5.0 mM [γ-^{32}P]ATP (approx. $2 \cdot 10^5$ cpm) and 0.5 μg protein. Reaction mixtures were incubated at 37°C for 2.0 min. Reactions were stopped by the addition of 2.0 ml of a solution containing 0.8 M $HClO_4$ and 1% (w/v) ammonium molybdate. Complexed inorganic [^{32}P]phosphate was determined by liquid scintillation as previously described [14]. One unit of activity is defined as the amount of protein required to hydrolyze 1 μmol ATP/min.

(b) Mg^{2+}-dependent ATPase. Unless otherwise stated, standard reaction mixtures for the *D. bardawil* CF_1 contained, in a total volume of 0.1 ml, 40 mM Na-Tricine (pH 8.0), 1.0 mM EDTA, 6.0 mM $MgCl_2$, 5.0 mM [γ-^{32}P]ATP (approx. $2 \cdot 10^5$ cpm), 25% (v/v) ethanol and 0.5 μg protein. The assay mixture for the spinach CF_1 Mg^{2+}-ATPase was similar, with the exception that 30 mM octylglucoside was added instead of ethanol. All other conditions were as described above for the Ca^{2+}-dependent ATPase assay.

Transblotting. The subunits of the *D. bardawil* and spinach coupling factors were separated by SDS-polyacrylamide slab gel electrophoresis (7.5% acrylamide, 25 μg protein/lane) [15]. The electrotransfer of the polypeptides to cellulose nitrate paper was performed essentially according to Rott and Nelson [16] with slight modifications. The buffer comprised 25 mM Tris/192 mM glycine/0.02% (w/v) SDS/20% (v/v) methanol, at a final pH of 8.3. Electrotransfer was performed at 0.4 A for 2 h. Complete transfer was confirmed by the lack of Coomassie blue staining material remaining in the polyacrylamide gel. The cellulose nitrate paper was incubated for 2 h at 37°C in buffer comprising 2% bovine serum albumin/140 mM NaCl/25 mM Tris-HCl (pH 7.4). It was cut into strips, and each strip was incubated with the primary monospecific antiserum in the same buffer. The incubation was performed in a total volume of 3.0 ml at 37°C for 20 h using 10 μl antiserum/lane. The strips were washed and then incubated for 10 h at 4°C with the secondary antiserum, ^{125}I-labelled goat anti-rabbit IgG. The strips were washed again [16] to remove unbound, labelled antiserum and exposed to X-ray film for autoradiography [17].

Chemical modification with FITC. FITC chemical modification of spinach CF_1 and *Dunaliella* CF_1 was performed essentially as previously described [18]. Prior to the separation of the subunits by SDS-polyacrylamide gel electrophoresis, the enzymes were centrifuged through Sephadex G-50 columns, preequilibrated with 40 mM Na-Tricine (pH 8.0)/0.1 mM EDTA [19]. This removed free FITC.

Localization of covalently bound FITC. After treating the coupling factors with FITC and removing the excess reagent by gel filtration, the subunits were separated by slab SDS-polyacrylamide gel electrophoresis. The gels were illuminated with an ultraviolet lamp (Chromato-Vue transilluminator model C-62, Ultra-Violet Products, San-Gabriel, CA, U.S.A.). The fluorescence was filtered through a Leitz GGr filter to block the ultraviolet and photographed with Kodak Plus X film. Finally, for comparison, the slab gels were stained with Coomassie Blue G [20] to visualize the polypeptide bands.

Other methods. [γ-^{32}P]ATP was prepared as described [21]. Protein was determined by the Coomassie Blue G dye binding method [22] using bovine serum albumin as the standard, and chlorophyll was measured as described by Arnon [23]. Non-dissociating [24] and SDS-dissociating [15] polyacrylamide gel electrophoresis were performed as previously described.

Materials

Monospecific rabbit antisera directed against the native spinach CF_1 and native *C. reinhardii* CF_1, the SDS-denatured *C. reinhardii* CF_1 and the SDS-denatured *C. reinhardii* α- and β-subunits were generous gifts from Dr. Sabeeha Merchant (Department of Biochemistry, University of Wisconsin, Madison, WI). Rabbit antiserum directed against the *Escherichia coli* β-subunit was generously supplied by Prof. Nathan Nelson (Department of Biology, Technion, Technological Institute of Israel, Haifa). ^{125}I-labelled goat anti-rabbit IgG was a gift from Dr. A. Zilberstein at the Weizmann Institute of Science, Rehovot, Israel. All other reagents used were of the best analytical quality available.

Results

Preliminary characterization of the D. bardawil CF_1 ATPase. The *Dunaliella* CF_1 was removed from membrane fragments of osmotically shocked cultures of *D. bardawil* by chloroform extraction [12] and purified by anion exchange chromatography on DEAE-Sephadex A-50 [14]. Under the conditions used (20 mM Tris-SO_4 (pH 8.0)/1.0 mM EDTA/0.1 mM ATP), it eluted from the column between 170–200 mM ammonium sulphate. The preparation ran as a single major band on non-dissociating polyacrylamide gels but did contain a very small amount of an apparently much higher molecular weight species (not shown). On SDS-polyacrylamide gels, only four bands were observed (see, for example, Fig. 2), consistent with other chloroform-released coupling factors [12].

In contrast to the CF_1 isolated from the green alga *C. reinhardii* [5], the *Dunaliella* CF_1 ATPase activity was completely latent. Attempts to release this latency by proteolytic digestion of the ATPase with trypsin [13] were unsuccessful; however, the activity could be induced by either a short heat

treatment or by the inclusion of selected organic solvents in the assay mixture.

Heat-induced Ca^{2+}-dependent ATPase. Heat activation has been used to induce or stimulate the activity of coupling factors isolated from a wide variety of sources [8]. In the case of the *Dunaliella* CF_1, heat induces a Ca^{2+}-dependent ATPase activity, the ratio of the ATPase rate with Ca-ATP compared to Mg-ATP being about 10–15. Optimal activation was obtained after a 3.0 min incubation at 60°C and was independent of the protein concentration over the range 0.5–2.5 mg/ml. The activation absolutely required the presence of both ATP and a thiol reducing agent (e.g., dithiothreitol) during the heating process.

The requirement for ATP is probably to protect the protein against irreversible heat denaturation, which occurs rapidly at 60°C in the absence of ATP (data not shown). The requirement for dithiothreitol during the heating process is not understood, but may reflect the reduction of vicinal dithiols that are only exposed at elevated temperatures. The addition of dithiothreitol after the heat treatment was without effect on the *Dunaliella* CF_1 ATPase activity. These observations are similar to those recently reported by Pick [25] for the heat-activation of the spinach CF_1, although the magnitude of the effect of dithiothreitol on the *Dunaliella* CF_1 compared to the spinach CF_1 is much larger (2-fold for the spinach CF_1 compared to about 5--7-fold for the *Dunaliella* CF_1).

Double-reciprocal plots for the activity of the Ca^{2+}-ATPase are linear over the substrate range from 0.1–5.0 mM Ca-ATP. The apparent K_m for Ca-ATP is about 0.8 mM and the apparent V_m is about 14 units/mg protein. The Ca^{2+}-ATPase was inhibited by ADP, phloridzin, dicyclohexylcarbodiimide (DCCD), and (rabbit) antisera directed against both native spinach and native *C. reinhardii* CF_1, and SDS-denatured *C. reinhardii* CF_1. The activity was not inhibited by (rabbit) antisera directed against the isolated SDS-denatured subunits of the *C. reinhardii* CF_1 (or combinations thereof), nor was it inhibited by tentoxin. ADP caused the double-reciprocal plots of activity vs. substrate concentration to become non-linear, as has been observed for other coupling factors [26–28], suggesting that the interaction of the *Dunaliella* CF_1 with ADp is probably quite complex (data not shown).

Solvent-induced Mg^{2+}-dependent ATPase. Selective organic solvents have been reported to activate a number of coupling factors [29]. Most notably, because of the magnitude of the stimulation of the ATPase rates, are the CF_1's isolated from spinach [30,31] and *C. reinhardii* [5,11]. As shown in Fig. 1A, the *Dunaliella* CF_1 ATPase activity was dramatically affected by ethanol, the optimal Mg^{2+}-ATPase activity being obtained at about 25% (v/v) ethanol. Methanol (35%, v/v), as well as other solvents [11,31], were also effective in inducing similar activities. The ATPase activity induced by solvents was reversible and Mg^{2+}-dependent. The ratio of the rates obtained for Mg-ATP compared to Ca-ATP was about 50 (see, for example, Fig. 1A).

Double-reciprocal plots of the velocity vs. the substrate concentration over the concentration range from 0.1 to 5.0 mM Mg-ATP are linear. The apparent K_m for Mg-ATP is about 1.2 mM and the apparent V_m is about 50–80 units/mg protein. The V_m for the solvent-induced Mg^{2+}-ATPase was about 5–6-fold higher than the V_m for the Ca^{2+}-ATPase. This difference in V_m cannot be explained

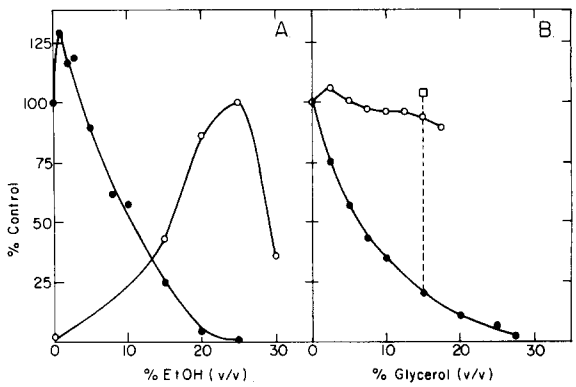

Fig. 1. (A) Contrasting effects of ethanol on the ATPase activities of the *Dunaliella* CF_1. The heat-induced Ca^{2+}-dependent (●——●) and solvent-induced Mg^{2+}-dependent (○——○) ATPase activities of the *Dulaliella* CF_1 were assayed as described in Methods and Materials, except that the final concentration of ethanol (v/v) was varied as indicated. (B) Glycerol sensitivity of the Ca^{2+}-dependent and Mg^{2+}-dependent ATPase activities of the *Dunaliella* CF_1. Ca^{2+}-dependent ATPase (●——●); Mg^{2+}-dependent ATPase (○——○).

by a partial heat denaturation of the protein during the heat treatment used to induce the Ca^{2+}-dependent ATPase because it remained even after the protein had been heat-treated (see, for example, Fig. 1B, dashed line).

In a manner similar to the heat-induced Ca^{2+}-ATPase, the Mg^{2+}-ATPase activity was inhibited by ADP, phloridzin and the various antisera directed against the spinach and *C. reinhardii* coupling factors, but was not inhibited by antisera directed against the SDS-denatured *C. reinhardii* CF_1 subunits, nor was it inhibited by tentoxin. ADP had the same effects on the kinetics of the Mg^{2+}-dependent ATPase activity that were observed for the Ca^{2+}-dependent ATPase activity (not shown).

Contrasts between the Dunaliella CF_1 Ca-ATPase and Mg-ATPase activities. Although both the Ca^{2+}-ATPase and Mg^{2+}-ATPase activities of the *Dunaliella* CF_1 showed similar responses to the various antisera and inhibitors noted above, the two activities are distinct and mutually exclusive. This is clearly demonstrated in the ethanol titration curves for the two activities shown in Fig. 1A. Whereas the Ca^{2+}-ATPase was inhibited 50% at about 10–12% (v/v) ethanol, 50% of the maximal ethanol-induced, Mg^{2+}-dependent ATPase activity occurred at approx. 13–15% (v/v) ethanol.

As shown in Fig. 1B, both the Ca^{2+}-dependent and Mg^{2+}-dependent ATPase activities also displayed differential responses when titrated against glycerol, the osmoticum accumulated by *D. bardawil* when cultured in high salt [32]. The heat-induced, Ca^{2+}-dependent ATPase activity was markedly inhibited by glycerol, 50% inhibition occurring at about 7% (v/v) glycerol, whereas the solvent-induced, Mg^{2+}-dependent ATPase activity was largely unaffected by concentrations of glycerol up to 20% (v/v) (10% inhibition of activity). In addition, even when the heat-treated *Dunaliella* CF_1 Ca^{2+}-ATPase activity was inhibited 80% by 15% glycerol, complete Mg^{2+}-ATPase activity was recovered by the addition of ethanol (25%) to the reaction mixture (Fig. 1B; dashed line). The differential effects of glycerol on the ATPase activities of the *Dunaliella* CF_1 were not specific for glycerol. Other polyols (e.g., ethylene glycol, sucrose, ficols) as well as NaCl displayed the same differential inhibition phenomenon.

The above results demonstrate that the reversible, solvent-induced activation of a dependent Mg^{2+}-ATPase in *Dunaliella* CF_1 is associated with structural modifications of the enzyme.

Identification of the Dunaliella CF_1 α- and β-subunits on SDS-polyacrylamide gels. Fig. 2 shows an SDS-polyacrylamide slab gel (12.5%) of the chloroform-released *Dunaliella* CF_1 (lane 2) and spinach CF_1 (lane 3). Four major bands from the *Dunaliella* CF_1, having apparent M_r values of 52 000, 49 000, 35 000 and 16 500, can be seen. The two smaller M_r bands probably correspond to the γ- and ε-subunits. The identification of the two larger *Dunaliella* CF_1 subunits was, however, questionable, particularly in light of the suggestion

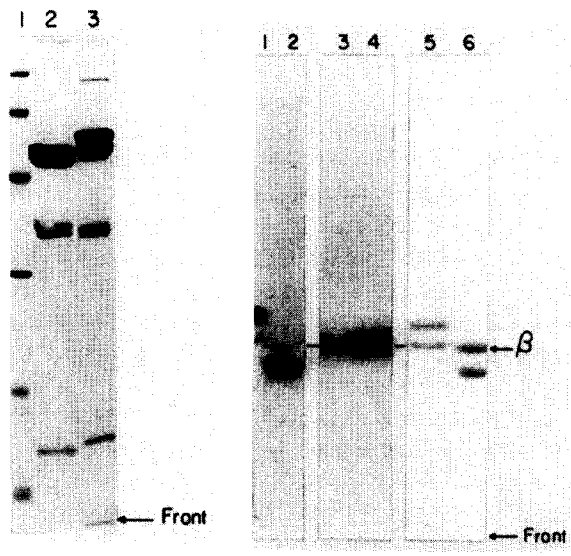

Fig. 2. SDS-polyacrylamide gel of the purified *Dunaliella* CF_1. SDS-polyacrylamide gel electrophoresis (12.5%) was performed as described [5]. Lane 1, molecular weight standards; lane 2, *Dunaliella* CF_1; lane 3, chloroform-released spinach CF_1.

Fig. 3. Absolute identification of the *Dunaliella* CF_1 α- and β-subunits by immunological cross-reactivity with monospecific antisera. The transblot experiment was performed as described in Methods and Materials. Lanes 1 and 2, the primary antiserum was a rabbit anti *C. reinhardii* α-subunit antiserum; Lanes 3 and 4, the primary antiserum was a rabbit anti *E. coli* β-subunit antiserum; Lanes 5 and 6, Coomassie blue stained SDS-polyacrylamide gel electrophoresis before transblotting. Lanes 1, 3 and 5 are spinach CF_1. Lanes 2, 4 and 6 are *Dunaliella* CF_1.

by Piccioni et al. [4] that the order of migration of the *C. reinhardii* CF_1 α- and β-subunits on SDS-polyacrylamide gels reversed when compared to other coupling factors (although under our SDS-polyacrylamide gel electrophoresis conditions, this has now been shown not to be the case [7,33]).

In order to establish the absolute identification of the two larger subunits of the *Dunaliella* CF_1, the following experiment was performed. After separation of both the spinach and *Dunaliella* CF_1 subunits by SDS-polyacrylamide gel electrophoresis, the polypeptides were transblotted to nitrocellulose paper [16], and their crossreactivities with various monospecific antisera were determined. These results are depicted in Fig. 3. An antiserum directed against the *C. reinhardii* CF_1 α-subunit (lanes 1 and 2) crossreacted with the slower-running α-subunit band from the spinach CF_1 (compare lanes 1 and 5), but with the faster-running band (M_r 49000) from the *Dunaliella* CF_1 (compare lanes 2 and 6). An antiserum directed against the *E. coli* β-subunit (lanes 3 and 4) crossreacted with the faster-running β-subunit band from spinach CF_1 (compare lanes 3 and 5), but with the slower-running polypeptide (M_r 52000) from the *Dunaliella* CF_1 (compare lanes 4 and 5). Clearly, these data strongly support the conclusion that the order of migration of the Dunaliella CF_1 subunits on SDS-polyacrylamide gel electrophoresis is indeed reversed from that of spinach CF_1. The order in apparent decreasing molecular weight on SDS-polyacrylamide gels of the *Dunaliella* CF_1 subunits is therefore: β, α, γ, ε. (These results were confirmed by an analysis of the Coomassie R-250 fluorescence associated with various dye-polypeptide complexes and the specific binding of DCCD to the β-subunit of the *Dunaliella* CF_1, Finel, M., Pick, U.,, Selman-Reimer, S. and Selman, B.R., unpublished data.)

The interaction of fluorescein isothiocyanate (FITC) with the Dunaliella CF_1

FITC has been shown to be a very potent inhibitor of a number of ATP-utilizing enzymes [34,35], and Pick [18] has recently characterized the interaction of lettuce CF_1 with this covalent modifier. It was not surprising, therefore, to find that chemical modification of the *Dunaliella* CF_1 by FITC led to a loss in the ethanol-induced, Mg^{2+}-dependent ATPase activity of the enzyme. A comparison of the FITC concentration curves for the inhibition of the *Dunaliella* CF_1 and spinach CF_1 Mg^{2+}-ATPase activities showed that the *Dunaliella* CF_1 is somewhat more sensitive to FITC than the spinach CF_1. 50% inhibition of the Mg-ATPase activities was obtained at about 5 and 50 μM FITC for the *Dunaliella* CF_1 and spinach CF_1, respectively (protein concentration 10 μg/ml, pH 8.0, and a 30 min preincubation at 37°C). The kinetics for the interaction of FITC with the *Dunaliella* CF_1 were quite complex and, as has previously been observed for the red-blood-cell Ca^{2+}-pump [36], multiphasic (data not shown).

Pick [18] has shown that the rate of FITC inhibition of the lettuce CF_1 can be markedly decreased by the inclusion of various nucleotides in the incubation mixture, presumably reflecting a competition or masking of exclusive or mutual binding sites. Table I demonstrates that the FITC inhibition of the *Dunaliella* CF_1 ATPase activity is greatly suppressed by ATP and ADP but only partially by Ado-P[NH]P, GTP, GDP or inorganic phosphate, and not at all by AMP. In the presence of 20 μM FITC (all other conditions as above) half inhibition is prevented by about either 50 μM ATP or ADP (not shown).

In order to identify the subunit(s) of the *Dunaliella* CF_1 and spinach CF_1 that bind FITC, the subunits were separated by SDS-polyacryla-

TABLE I

PROTECTION OF THE *D. BARDAWIL* CF_1 AGAINST INHIBITION BY FITC

The *D. bardawil* CF_1 was treated with 20 μM FITC for 30 min at 37°C and the ethanol-induced, Mg^{2+}-dependent ATPase assayed as described in Methods and Materials. The final concentration of all nucleotides and phosphate, added prior to the addition of FITC, was 1.0 mM. The control rate was 25 units/mg protein.

Addition	ATPase activity (% control)
None	15
ATP	86
ADP	98
AMP	9
Ado-P[NH]P	36
GTP	27
GDP	44
P_i	25

mide gel electrophoresis (7.5%) after treating the enzymes with various concentrations of the chemical modifier in the presence and absence of ATP. The FITC fluorescence, induced by ultraviolet illumination of the gel, was photographed. These results are depicted in Fig. 4. Lanes 7–9 demonstrate that the covalent binding of FITC to the *Dunaliella* CF_1 occurs almost exclusively by a modification of the α-subunit. Similar results were also obtained for the spinach CF_1 (lanes 4–6) but there was, in addition, a very narrow fluorescent band that migrated close to the spinach β-subunit. Fig. 4 also demonstrates that (i) the FITC fluorescence intensity of the α-subunit is greater with the *Dunaliella* CF_1 than with the spinach CF_1 when both are treated with equal concentrations of FITC, consistent with the observation that the *Dunaliella* CF_1 is more sensitive to FITC than is spinach CF_1, (ii) the amount of FITC bound to the α-subunit of both *Dunaliella* CF_1 and spinach CF_1, but not the narrow band associated with the spinach CF_1, increases with increasing FITC concentrations, and (iii) ATP partially inhibits the covalent incorporation of FITC into the α-subunit of both enzymes and into the narrow band (lanes 3 and 10).

The inhibition by FITC was dependent on the pH of the reaction mixture during the chemical modification, as demonstrated in Fig. 5. Both enzymes showed maximal inhibition at pH 8. (It should be noted that the controls, enzymes incubated in the absence of FITC (pH 7–9) or in the presence of FITC and (1 mM) ATP (pH 7 and 8), retained most of their ATPase activity (over 85%) while at pH 9, there was a partial decrease in the protective effect of ATP (data not shown)).

There is also a partial correlation between the FITC incorporation into the α-subunits of both enzymes and the inhibition of ATPase activity, as demonstrated in Fig. 6. At pH 7, there was only a

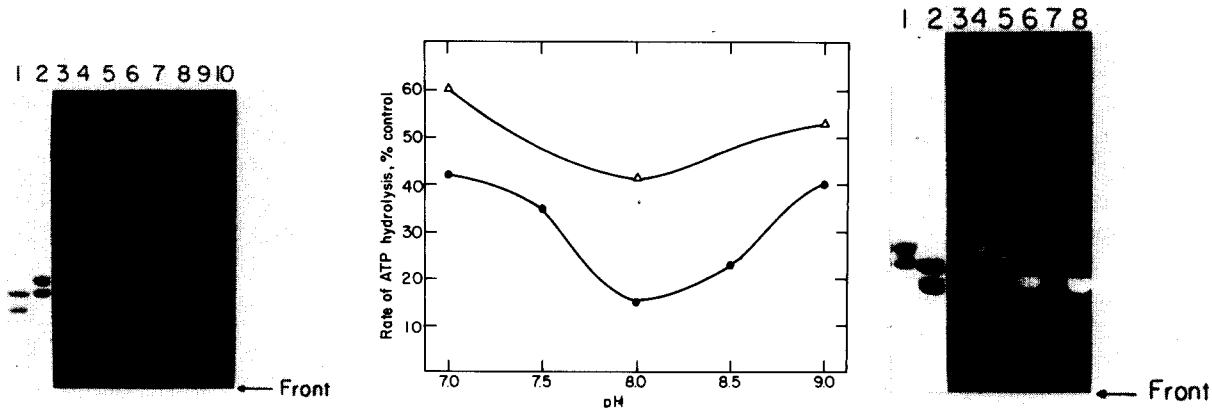

Fig. 4. Identification of the *Dunaliella* CF_1 subunit that covalently binds FITC. Spinach CF_1 (lanes 2–6) and *Dunaliella* CF_1 (lanes 1, 7–10) were incubated with FITC (10 μM, lanes 4 and 9; 20 μM, lanes 5, 8 and 10; 50 μM lanes 3, 6 and 7). ATP (1 mM), when present (lanes 3 and 10), was added before FITC. Incubation conditions and separation on 7.5% SDS-polyacrylamide gel electrophoresis were as described in Methods and Materials.

Fig. 5. pH profile for the FITC inhibition of the Mg-ATPase activities of the spinach (△) and *D. bardawil* CF_1 (●). Spinach CF_1 and *Dunaliella* CF_1 (0.5 mg/ml) were incubated for 30 min at 37°C with 50 and 20 μM FITC, respectively, in a medium comprising 50 mM Tris-Hepes (pH 7.0–8.0) or Tris-Cl (pH 8.5 and 9.0). Mg^{2+}-ATPase activity was measured in the presence of 25% ethanol (*Dunaliella* CF_1) or 30 mM octylglucoside (spinach CF_1) as described in Methods and Materials. The control (100%) activities were 21 and 12 units/mg protein for *Dunaliella* CF_1 and spinach CF_1, respectively.

Fig. 6. pH effect on the incorporation of FITC into subunits of spinach and *D. bardawil* CF_1. Spinach CF_1 (lanes 1 and 3–5) and *Dunaliella* CF_1 (lanes 2 and 6–8) were incubated with FITC at pH 7.0 (lanes 5 and 7), 8.0 (lanes 4 and 8) and 9.0 (lanes 3 and 6). Incubation conditions were as described in Fig. 5. Enzymes samples (20 μg) were separated on 7.5% SDS-polyacrylamide gels. FITC fluorescence photography (lanes 3–8) and Coomassie blue staining (lanes 1 and 2, which correspond to lanes 5 and 6, respectively) were performed as described in Methods and Materials.

partial labeling of the α-subunit of *Dunaliella* CF_1 (lane 7) or of spinach CF_1 (lane 5), although the α-subunit of *Dunaliella* CF_1 as well as the narrow fluorescent band in spinach CF_1 were labeled more intensely than at pH 8. In contrast at pH 9, there was a significant incorporation of FITC into the α-subunit of both enzymes (lanes 6 and 3) in spite of the reduced inhibition (Fig. 4), possibly indicating nonspecific modification of other lysine residues. These results suggest that FITC inhibition probably results from a modification of the α-subunit in both *Dunaliella* CF_1 and spinach CF_1.

The narrow fluorescein-labeled polypeptide found in spinach CF_1 seems to be unrelated to the inhibition of the ATPase and different from the β-subunit. This is supported by the following observations: (i) it was most intensely labeled at pH 7 while the optimal pH for ATPase inhibition was 8; (ii) maximal fluorescein binding to the narrow band was obtained at 10 μM FITC (Fig. 4) which only partially inhibited the ATPase activity; (iii) after FITC-labeling at pH 7.0, spinach CF_1 was passed through a high-performance liquid chromatography column. A pure β-subunit fraction containing no fluorescein label and another fraction, highly enriched in the narrow band polypeptide and in fluorescein label were obtained (data not shown). The nature of this protein is not known and is currently under investigation.

Discussion

In this report we have presented the initial characterization of the CF_1 associated with the thylakoid membranes of the halotolerant green alga *D. bardawil.* It is interesting to compare the similarities and dissimilarities of this enzyme to coupling factors previously isolated from other sources.

When released from osmotically shocked membranes by a chloroform extraction, the enzyme contains only four subunits, apparently missing the δ-subunit. This is also the case for both the higher (vascular) plant CF_1 [12] and the eukaryotic unicellular green algal *C. reinhardii* CF_1 [37].

The ATPase activity of the *Dunaliella* CF_1 is completely latent but the latency is relived either by a heat treatment, which induces a Ca^{2+}-dependent ATPase, or by selective organic solvents, which induce an Mg^{2+}-dependent ATPase. Qualitatively, these results are similar to those obtained with the higher (vascular) plant CF_1, although the V_m for the solvent-induced, Mg^{2+}-dependent, ATPase activity of the *Dunaliella* CF_1 (50–80 units/mg protein) is substantially higher than the solvent-induced rates that have been obtained for the spinach enzyme (10–15 units/mg protein) [31]. We have, as yet, been unsuccessful in inducing either a Ca^{2+}-dependent or Mg^{2+}-dependent ATPase activity with the *Dunaliella* CF_1 by proteolytic digestion.

The heat-induced Ca-ATPase and the solvent-induced Mg^{2+}-ATPase activities of the *Dunaliella* CF_1 are mutually exclusive in the sense that solvents inhibit the Ca^{2+}-ATPase but concomitantly activate the Mg^{2+}-ATPase. In this respect, the effect of solvents on the *Dunaliella* CF_1 resembles the effect of octylglucoside micelles on the higher plants CF_1 [38]. Although both the Ca^{2+}- and the Mg^{2+}-dependent ATPase activities of *Dunaliella* CF_1 show a similar sensitivity to most inhibitors, there is a notable difference between them seen in the higher sensitivity of the former to osmoticums (e.g., glycerol and NaCl). The fact that the membrane-bound enzyme is a Mg^{2+}-dependent ATPase (data not shown) and is capable of functioning in the presence of the high glycerol concentrations accumulated in the alga in vivo [32] suggests that the solvent-induced Mg^{2+}-ATPase activity of *Dunaliella* CF_1 represents a conformation of the enzyme that is more physiologically important than the conformation of the enzyme that has Ca^{+}-dependent ATPase activity.

The order of migration of the *Dunaliella* CF_1 subunits, when separated by SDS-polyacrylamide gel electrophoresis, becomes an important question when one wants to identify the subunits and attempt to determine their function. In this regard, it is interesting to note that Piccioni et al. [4] have suggested that, under their electrophoresis conditions, the *Chlamydomonas* CF_1 α-subunit runs faster than the *Chlamydomonas* CF_1 β-subunit. Under our experimental conditions, this is clearly not the case for the *Chlamydomonas* CF_1 subunits [7,33]. However, it does seem to be the case for the migration of the *Dunaliella* CF_1 subunits. This conclusion is based on the immunological cross-reactivity studies presented here and the studies

performed on the comparative fluorescence yields eminating from Coomassie R-250-polypeptide complexes and the specific binding of DCCD to the β-subunit (to be presented elsewhere).

Thus, we can now suggest with certainty that FITC inhibition of the *D. bardawil* CF_1 and spinach CF_1 ATPase activities is due to the specific binding of FITC to the α-subunit of the enzymes. We have previously shown that inhibition of the lettuce CF_1 ATPase by FITC is associated with the incorporation of fluorescein into both the α- and β-subunits [18]. The reasons for the difference between the lettuce and spinach CF_1 with respect to the fluorescein incorporation into the β-subunit are not clear, but it should be noted that, in lettuce CF_1, the ATP protection against fluorescein incorporation was more pronounced for the α- rather than the β-subunit (see Table II, Ref. 18) consistent with the correlation between ATPase inhibition and modification of the α-subunit.

Although the results presented here do not provide direct evidence that FITC modifies an ε-amino group of a lysine residue in CF_1, the following four facts strongly suggest that FITC indeed modifies a lysine residue in the α-subunit of CF_1 which is associated with the binding of ATP: (i) other lysine reagents protect CF_1 against FITC modification (data not shown; see also Table II, Ref. 18); (ii) similar inhibition by other lysine reagents are also protected by ATP [39,40]; (iii) the α-subunit in CF_1 contains no manifest -SH groups (reviewed in Ref. 43), -SH groups being the major alternative target for FITC; and (iv) the site of FITC modification of the sarcoplasmic reticulum Ca^{2+}-ATPase has been identified as a lysine ε-amino group [42].

The location as well as the function of the adenine nucleotide binding site that, when filled, protects the enzyme from both covalent chemical modification and concomitant inhibition of activity, is still uncertain. Adenine nucleotide binding sites on both the α- and the β-subunits of spinach CF_1 have been observed by the use of photoaffinity adenine nucleotide analogues [43–45], and directly determined by measuring the binding of ATP to the isolated, non-denatured *E. coli* α-subunit [46,47]. Yet, the active site is generally assumed to be located on the β-subunit of CF_1 [6,48,49]. From the data presented here, it is difficult to conclude whether FITC reacts with catalytic sites, with the tight sites or with another type of adenine nucleotide binding site. However, there are three observations which suggest that FITC does not block the tight sites. (i) Depleting of spinach CF_1 of bound nucleotides [50] did not affect the sensitivity of the enzyme to FITC (data not shown). (ii) The apparent affinity for ATP protection appears to be too low for the tight sites. (iii) Modification of spinach CF_1 by FITC, which caused 90% inhibition of ATPase activity, does not inhibit but rather stimulates binding of ADP to the tight sites (unpublished data). Therefore, it is possible that the FITC binding site is at the catalytic site of the *D. bardawil* and spinach CF_1. This has been previously suggested for a number of different FITC-sensitive cation-transporting ATPases [32–34]. If this were the case, it would have to be assumed that either the active site is made up of both the α and β-subunits, as has been suggested for the mitochondrial and bacterial F_1 [51]. Alternatively, the nucleotide binding site is on the CF_1 and *Dunaliella* CF_1 β-subunit, and, when occupied by either ATP or ADP, the conformation of the enzyme is such that the reactivity of the group(s) that bind FITC on the α-subunit is (are) greatly diminished.

Acknowledgements

This research was supported in part by grants from the Weizmann Institute of Science (B.R.S., Meyerhoff Fellow), the College of Agricultural and Life Sciences, University of Wisconsin (B.R.S.), the U.S.-Israel Binational Research Foundation (BSF 2891/82 U.P. and B.R.S.), the United States Department of Agriculture/SEA CRGO (82-CRCR-1-1151; B.R.S.), and the National Institutes of Health (GM 31384-01; B.R.S.). In addition, we are indebted to Dr. Sabeeha Merchant and Prof. Nathan Nelson for the generous supply of monospecific antisera, to Dr. A. Zilberstein for her help with the transblot experiments, and to Prof. M. Avron for his suggestion to purify and study CF_1 from *Dunaliella* which gave us the initiative for this work.

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