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MEMBRANE-BOUND ATPase IN CHLOROPLASTS OF *EUGLENA GRACILIS*

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SUMMARY

Membrane-bound ATPase activities in chloroplasts of *Euglena* were examined. Ca^{2+}- and Mg^{2+}-dependent activities were relatively high in membrane preparations and could not be further activated by a number of procedures. The enzyme was found to be highly specific for purine nucleotides and was inhibited by the usual inhibitors of photophosphorylation. K_m values of Ca^{2+} and Mg^{2+} ATPase for ATP were 2.5 and 2.1 mM, respectively. Both activities were competitively inhibited by ADP and inorganic phosphate. A relationship was found between Ca^{2+}- or Mg^{2+}-dependent ATPase activities and chloroplast completeness. The possibilities that these activities result from one enzyme depending on Ca^{2+} or Mg^{2+} or from two different enzymes are discussed.

INTRODUCTION

The coupling factor of chloroplasts from higher plants does not exhibit ATPase activity unless treated by trypsin [1–3], heat [1, 4], or illumination in the presence of dithiothreitol [5]. The interaction of the chloroplast coupling factor 1 with divalent cations was classified as "allotopic" by McCarty and Racker [5]: The enzyme responsible for photophosphorylation and light-induced ATPase activity is membrane bound and Mg^{2+} dependent [6, 7], whereas the purified enzyme was found to be Ca^{2+} dependent [1]. However, more recently it has been shown by Nelson et al. [8] that isolated coupling factor 1 under certain conditions could exhibit Mg^{2+}-dependent ATPase activity and by Bakker-Grunwald [9] that the membrane-bound enzyme shows Ca^{2+} dependency.

In this work we show that the membrane-bound ATPase from *Euglena* chloroplasts differs from that of higher plants. ATPase activity has been detected in chloroplast preparations without any additional treatment. Moreover, in the same preparations both Mg^{2+} and Ca^{2+} dependence was readily demonstrated.

MATERIALS AND METHODS

Cells of *Euglena gracilis* var. *bacillaris* were grown on Hutner's pH 3.3 growing medium [10] in 4-l Erlenmeyer flasks under 200 ft candles at 24–26 °C. The cells were

continuously shaken for about 5 days to the end of the logarithmic growth phase.

Isolation of chloroplasts was performed according to Shneyour and Avron [11] using sucrose/Tris medium (medium composed of 0.2 M sucrose, 0.05 M Tris · HCl buffer (pH 7.8), 0.1 M NaCl and 1 mg per ml albumin). The cells were broken either by transfer through French pressure cell, at 1500 lb/inch^2, or by shaking the cells with glass beads, 1 mm in diameter, in a B. Braun Melsunger cell homogenizer for 45 s. The chloroplasts were isolated from broken cells by differential centrifugation, frozen in liquid nitrogen and stored at −20 °C.

ATPase activity. Ca^{2+}-dependent ATPase activity was measured in a reaction mixture composed of 50 mM Tris · HCl buffer (pH 8.0), 2 mM $CaCl_2$, 5 mM ATP and 0.1 ml chloroplast suspension (containing 1.0–1.2 mg chlorophyll per ml), in a final volume of 1 ml [12]. The reaction mixture for Mg^{2+}-dependent ATPase contained 50 mM Tris/maleate buffer (pH 6.0), 8 mM $MgCl_2$, 5 mM ATP, and 0.1 ml chloroplast suspension (containing 1.0–1.2 mg chlorophyll per ml), in a final volume of 1 ml [12]. After 10 min of incubation at 37 °C in the dark, the reaction was terminated by addition of trichloroacetic acid to a final concentration of 3 %, and the phosphate content of the supernatant was determined according to Fiske and Subba-Row [13].

For determinations of kinetics constants ATPase activity was assayed, using the same reaction mixtures but [γ-^{32}P]ATP was added. The reaction was carried out at 37 °C in the dark for 2 min. The ^{32}P released was determined by the isobutanol-benzene extraction method [14]. Under these conditions about 5 % of the substrate was hydrolyzed during the assay.

Activation of ATPase. Chloroplasts were suspended in the reaction mixture without ATP. Dithiothreitol and phenazine methosulfate were added to a final concentration of 5 mM and 50 μM, respectively, and the suspension was illuminated for 10 min at 37 °C. The light was turned off and ATP was added to a final concentration of 5 mM. ATPase activity was assayed as described.

Activation of the membrane-bound ATPase by heat treatment was carried out according to Farron and Racker [4]. Chloroplasts were suspended in a medium containing 20 mM Tricine/NaOH (pH 8.0), 5 mM dithiothreitol and 20 mM ATP. The suspension was heated for 4 min at 64 °C, cooled by tap water and ATPase activity was assayed.

Activation by trypsin was carried out according to Vambutas and Racker [1]. The reaction was started by the addition of trypsin in a final concentration of 400 μg/ml for 5–15 min at 25 °C. The reaction was stopped by addition of 1.2 mg/ml soybean trypsin inhibitor. Aliquots from the suspension were assayed for ATPase activity.

Photophosphorylation. Photophosphorylation was determined in a reaction mixture containing: 18 mM Tricine (pH 7.8), 20 mM KCl, 4 mM $^{32}P_i$ (2.5×10^5 cpm/μmol at pH 7.8), 4 mM MgCl, 1.4 mM ADP, 0.04 mM phenazine methosulfate, 10 mM sodium ascorbate, 6 mM glucose, 4 units hexokinase and chloroplasts containing about 50 μg chlorophyll in a total volume of 3 ml. The reaction was initiated by illumination with four 150-W photoflood lamps at $5 \cdot 10^5$ ergs · cm^{-2} · s^{-1} for 2 min at 22 °C and was stopped by addition of cold trichloroacetic acid to a final concentration of 3 %. [^{32}P]ATP formation was measured according to the method of Avron [14].

The amount of chlorophyll in all preparations used was determined according to Arnon [15] and protein was measured by the method of Lowry et al. [16].

Electron microscopy. Isolated chloroplasts were collected by centrifugation and the pellets were fixed with 5 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at 4 °C for 30 min and post-fixed for 1 h with 2 % OsO_4 in the same buffer.

The samples were dehydrated by a series of graded alcohols and embedded in Epon 812. Thin sections were cut with an LKB ultra-microtome using glass knives, stained on the grids by a saturated solution of uranyl acetate in 30 % ethanol overnight and post-stained by 0.03 % lead citrate for 10 min. The sections were examined with a Joel-100B electron microscope.

Sources. Nucleotide phosphates, phenazine methosulfate, dicyclohexylcarbodimide, hexokinase, trypsin and soybean trypsin inhibitor were purchased from Sigma Chemical Co. St. Louis, Mo. Phlorizin was obtained from Fluka Ag Buchs SG Switzerland, and Dio-9 from Royal Netherland Fermentation Industries Ltd. Delft, Holland. All chemicals employed were analytical grade materials.

RESULTS

The ATPase activity in the chloroplast fraction has been found to be an integral part of the membranes. Chloroplasts were washed twice with sucrose/Tris medium, centrifuged and resuspended. ATPase activity was measured in the supernatants and the resuspended pellets (Table I). About 89 % of Mg^{2+}-dependent ATPase and 81 % of Ca^{2+}-dependent ATPase activities were found in the membrane fraction. The specific activity of Mg^{2+}-dependent ATPase was 2–4 times higher in the membrane fractions than in the supernatants. The Ca^{2+}-dependent ATPase specific activity was also higher in the membrane fraction. The distribution of the enzymic activities between the fractions after second centrifugation of the resuspended membranes was similar. Thus most of the ATPase activities in the chloroplast suspension were membrane bound.

The chloroplast fraction was practically free of other organelles, especially mitochondria. Oligomycin had no effect on the ATPase activities in the preparations.

TABLE I

MEMBRANE-BOUND ATPase ACTIVITY IN *EUGLENA* CHLOROPLASTS

Cells were broken by Braun homogenizer. Chloroplast suspension in sucrose/Tris medium was centrifuged twice at 147 000 × *g* for 60 min at 4 °C. ATPase activity was measured in the supernatants and in the pellets as described under Materials and Methods.

	Mg^{2+}-dependent ATPase		Ca^{2+}-dependent ATPase	
	Total activity (μmol P_i/h)	Specific activity (μmol P_i/mg protein per h)	Total activity (μmol P_i/h)	Specific activity (μmol P_i/mg protein per h)
Isolated chloroplasts	665	10	336	5
Chloroplasts after first precipitation	881	13	358	6
First supernatant	127	6	83	3
Chloroplasts after second precipitation	642	13	332	7
Second supernatant	61	5	64	4

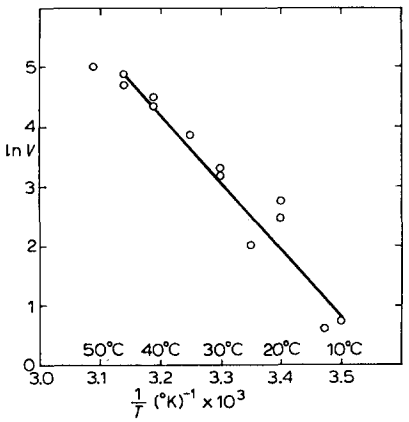

Fig. 1. Activation energy of Ca^{2+}-dependent ATPase of *Euglena* chloroplasts. The data of the effect of temperature on the rate of activity were plotted according to Arrhenius and the activation energy of Ca^{2+}-dependent ATPase was calculated.

Examination of the preparation by electron microscopy did not reveal significant amount of mitochondria.

Effect of temperature on the stability and activity of ATPase. The membrane-bound ATPase was stable in the cold. The same activities were found in preparations isolated either at room temperature or at 4 °C. Stability in the cold was also found in the partially purified enzyme by Chang and Kahn [17]. In this property, the enzyme is different from the cold labile mitochondrial ATPase [18]. The membrane preparations were frozen by liquid nitrogen and were stored at −20 °C for several weeks without any loss of ATPase activity.

TABLE II

SUBSTRATE SPECIFICITY OF THE MEMBRANE-BOUND Ca^{2+}- AND Mg^{2+}-DEPENDENT ATPase OF *EUGLENA* CHLOROPLASTS

Cells were broken by Braun homogenizer. Determination of ATPase activity as described under Materials and Methods.

Substrate	Mg^{2+}-dependent ATPase		Ca^{2+}-dependent ATPase	
	Specific activity (μmol P_i/mg chlorophyll per h)	Activity (%)	Specific activity (μmol P_i/mg chlorophyll per h)	Activity (%)
ATP	68	100	29	100
GTP	48	71	21	75
ITP	47	69	21	74
CTP	4	6	2	8
TTP	10	13	2	9
UTP	29	42	3	11
ADP	17	25	2	8
AMP	1	2	1	5
p-Nitrophenyl phosphate	3	5	0	0
α-Glycerophosphate	0	0	0	0
Glucose 6-phosphate	0	0	0	0

TABLE III

KINETIC CONSTANTS OF ATPase ACTIVITY

Cells were broken by French pressure cell. The effect of ATP concentration on ATPase activity was measured in the concentration range of 0.05 –5 mM ATP. Assays of inhibition constants of P_i and ADP were performed under the same conditions. Concentrations of P_i were 0.5 and 1.0 mM. Those of ADP were 0.2 and 1.0 mM.

Kinetic constants	Mg^{2+}-dependent ATPase	Ca^{2+}-dependent ATPase
K_m	2.1 mM	2.5 mM
K_i (for P_i)	0.8 mM	1.5 mM
K_i (for ADP)	0.4 mM	0.8 mM

The effect of temperature on the reaction rate was measured in the range of 10–50 °C. The data were plotted according to Arrhenius, and the activation energy was found to be 23 kcal/mol for Ca^{2+}-dependent ATPase (Fig. 1). The same value was obtained for Mg^{2+}-dependent ATPase.

Substrate specificity. Both Ca^{2+}-dependent and Mg^{2+}-dependent ATPase activities have shown high specificity for purine trinucleotides. A low rate of hydro-

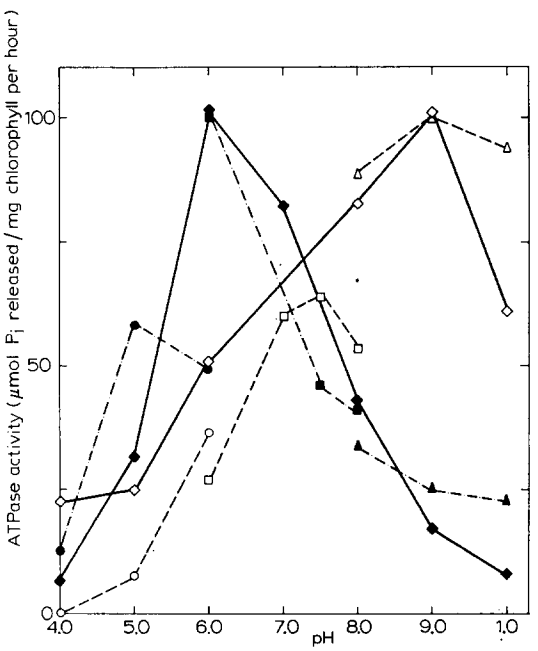

Fig. 2. Effect of H^+ concentration and the nature of buffer on the membrane-bound ATPase activity of *Euglena* chloroplasts. The reaction conditions are as described under Materials and Methods except that different buffers were used. In one set of experiments a buffer containing acetate, maleate and Tris (50 mM of each) was used and the pH was adjusted by NaOH. ◆, Mg^{2+}-; ◇, Ca^{2+}-dependent ATPase activity. In another set of experiments different buffers were used: ●, ○, Tris/acetate; ■, □, Tris/maleate; ▲, △, Tris · HCl. Closed symbols (●, ■, ▲) for Mg^{2+}- and open symbols (○, □, △) for Ca^{2+}-dependent ATPase activity.

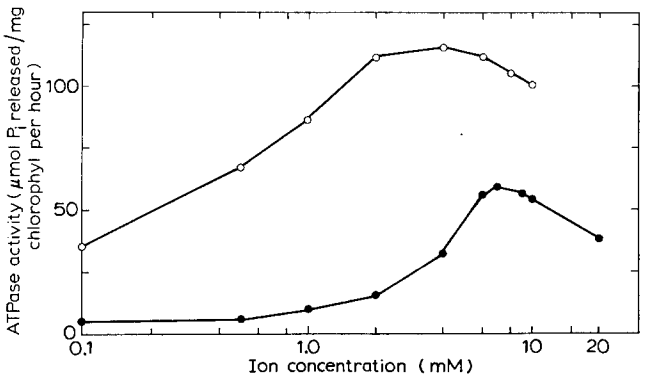

Fig. 3. Effect of Mg^{2+} and Ca^{2+} concentrations on the membrane-bound ATPase activity of *Euglena* chloroplasts. The reaction conditions are described under Materials and Methods except that the ion concentrations were varied. Mg^{2+}-dependent ATPase (●–●) and Ca^{2+}-dependent ATPase (○–○) were assayed in the chloroplast preparations.

lysis was obtained with pyrimidine-nucleotides, ADP and AMP, whereas other phosphate esters, such as substrates, were hydrolized very slowly (Table II).

Kinetic constants. Using ATP as a substrate, the Michaelis constant of Mg^{2+}-dependent ATPase was found to be 2.1 mM, and of Ca^{2+}-dependent ATPase 2.5 mM. ADP and inorganic phosphate competitively inhibit the enzymic activities and the values of the inhibition constants are represented in Table III. ADP was found to be a competitive inhibitor also in a partially purified Ca^{2+}-dependent ATPase of *Euglena* chloroplasts [17].

Effect of H^+ concentration. The ATPase activity was markedly affected by H^+ concentration. Mg^{2+}-ATPase activity has a maximum at pH 6.0, whereas Ca^{2+}-ATPase activity reaches its maximum at pH 9.0 (Fig. 2).

Effect of Mg^{2+}, Ca^{2+} and Na^+ concentrations. Either Mg^{2+} or Ca^{2+} are essential for the ATPase activity. Using different ion concentrations it was found that

TABLE IV

EFFECT OF ACTIVATION PROCEDURES ON ATPase ACTIVITY

Cells were broken by Braun homogenizer for the first two experiments and by French pressure cell for the third one. ATPase activity measurements and activation procedures as described under Materials and Methods.

Treatment	Mg^{2+}-dependent ATPase (μmol P_i/mg chlorophyll per h)		Ca^{2+}-dependent ATPase (μmol P_i/mg chlorophyll per h)	
	Before treatment	After treatment	Before treatment	After treatment
Heat (64 °C; 4 min)	40	0	24	0
Light; dithiothreitol; phenazine methosulfate	65	85	34	40
Trypsin (200 μg; 5 min)	43	69	57	46

8 mM Mg^{2+} or 2 mM Ca^{2+} are the optimal concentrations for ATPase activity (Fig. 3). When the ions are omitted from the reaction mixture, ATPase activity is about one-tenth of the activity in the presence of the ions. Concentrations of 0–50 mM Na^{+} had no effect on the ATPase activities.

Influence of ATPase activators. ATPases from different sources, such as mitochondria [19], higher plant chloroplasts [7, 20] and bacterial chromatophores [21] are latent enzymes and different treatments are needed for their activation. The activity of the membrane-bound enzymes increased markedly by heat treatment [12], trypsin digestion [2, 3], or illumination in the presence of dithiothreitol [5]. When these procedures were applied to chloroplast membranes of *Euglena*, the effects were quite different (Table IV). Heat treatment, by incubation at 64 °C for 4 min [4], caused total inactivation of both Ca^{2+}- and Mg^{2+}-dependent activities. A mild increase in the enzymic activity was found after illumination in the presence of dithiothreitol and phenazine methosulfate, but the effect was low in comparison to the effect on ATPase from other sources [5, 12]. Digestion by trypsin resulted in a rapid decrease of the Ca^{2+}-dependent ATPase activity, which disappeared after 15 min incubation with trypsin (Fig. 4). The effect of trypsin on Mg^{2+}-ATPase was different: During the first 10 min of incubation with trypsin the activity increased, about 2-fold, but from then on, a rapid decrease of the activity was noted (Fig. 4). Both Mg^{2+}- and

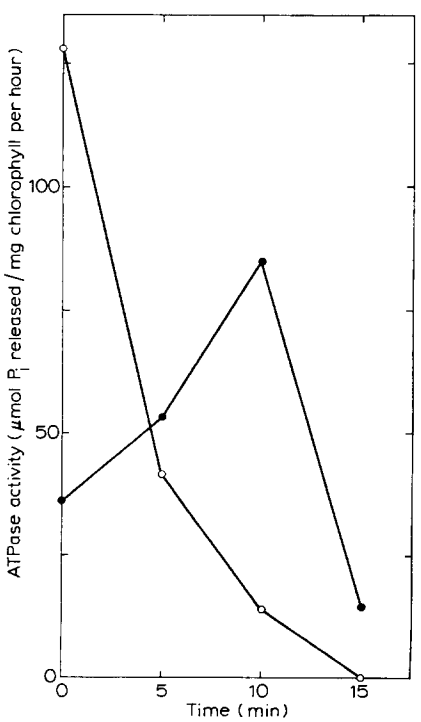

Fig. 4. Effect of trypsin on the membrane-bound ATPase activity of *Euglena* chloroplasts. Chloroplasts were activated by trypsin (400 μg/ml) and at various time intervals samples were assayed for Mg^{2+}-dependent ATPase (●–●) and Ca^{2+}-dependent ATPase (○–○) as described under Materials and Methods.

TABLE V

THE EFFECT OF INHIBITORS ON PHOTOPHOSPHORYLATION AND ATPase ACTIVITIES

Cells were broken by either "Yeda Press" (at 800 lb/inch^2 under argon [22]) for photophosphorylation measurements, or by Braun homogenizer for ATPase (as described under Materials and Methods). The rate of activity without any addition was taken at 100 %. The activity of photophosphorylation was 101 μmol ATP formed/mg chlorophyll per h. The activities of Mg^{2+}-dependent and Ca^{2+}-dependent ATPase were 108 and 40.5 μmol P_i/mg chlorophyll per h, respectively.

Addition	Concentration	Percent of control rate of activity		
		Photophosphorylation	Ca^{2+}-ATPase	Mg^{2+}-ATPase
Dicyclohexylcarbodimide	0.1 mM	5	66	73
	0.2 mM	0	53	82
	0.3 mM	0	25	65
Phlorizin	3.0 mM	0	69	96
	9.0 mM	–	15	43
Dio-9	10.0 μg/ml	7	65	72
	30.0 μg/ml	3	–	–
	90.0 μg/ml	0	–	–
Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone	1.0 μM	24	98	100
	2.0 μM	11	–	–
	5.0 μM	2	97	103

Ca^{2+}-dependent activities were relatively high in the membrane preparations and the activation procedures applied did not have significant effect.

Effect of inhibitors on ATPase activity. Phlorizin, dicyclohexylcarbodimide and Dio-9 which are known to inhibit photophosphorylation also inhibited ATPase activity in the chloroplast preparations. Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone, although inhibiting photophosphorylation, did not affect ATPase activity (Table V). The effect of phlorizin was more pronounced than that of the other drugs used. Both Ca^{2+}- and Mg^{2+}-dependent ATPase activities were inhibited but that of Ca^{2+} dependent was inhibited to a higher degree.

TABLE VI

EFFECT OF CHLOROPLAST ISOLATION PROCEDURE ON Mg^{2+}- AND Ca^{2+}-DEPENDENT ATPase ACTIVITIES

Details of the procedures as described under Materials and Methods.

	Mg^{2+}-dependent ATPase (μmol P_i/mg chlorophyll per h)	Ca^{2+}-dependent ATPase (μmol P_i/mg chlorophyll per h)
Chloroplasts from cells broken by French pressure cell	50	129
Chloroplasts from cells broken by Braun homogenizer	61	61.5

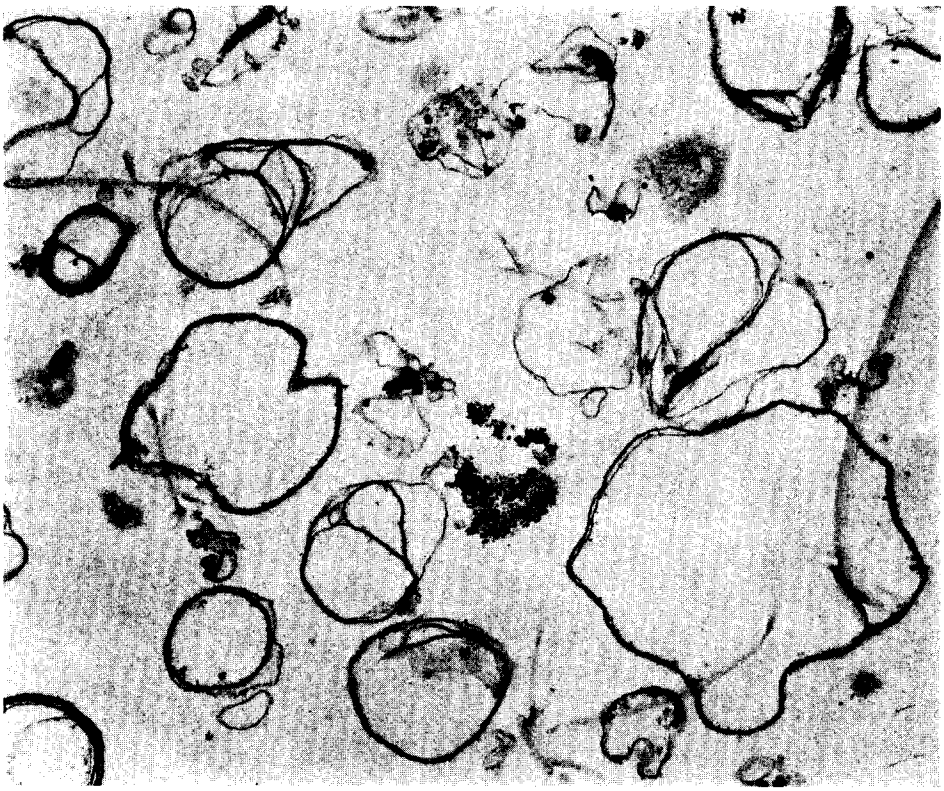

Fig. 5. Chloroplast fraction isolated from cells broken with a French pressure cell, ×11 000.

Procedures for preparation of chloroplasts membranes and their effect on the ATPase activities. To obtain chloroplasts preparations, cells were broken in two ways: By a French pressure cell or by shaking the cells with glass beads in a Braun homogenizer. The Ca^{2+}-dependent ATPase activity in "French press" preparations was 2.5 times higher that that of Mg^{2+}-dependent activity, whereas in the "Braun homogenizer" preparations the activities were either similar or the Mg^{2+}-dependent activity was higher (Table VI). Electron microscopy of the two preparations has revealed distinct morphological differences. Samples from French press contained broken chloroplasts and free thylakoids (Fig. 5), whereas those from the Braun homogenizer contained relatively undisrupted chloroplasts with densely packed thylakoids (Fig. 6). A correlation between the degree of chloroplast completeness and the level of Ca^{2+}- and Mg^{2+}-dependent ATPase activities could be noted.

DISCUSSION

As a part of our work on the localization of enzymes in chloroplast membranes of *Euglena*, we have examined some properties of membrane-bound ATPase. The activity of the enzyme in untreated different chloroplast preparations, was 40–130 units (μmol P_i released/mg chlorophyll per h). The activity in untreated preparations

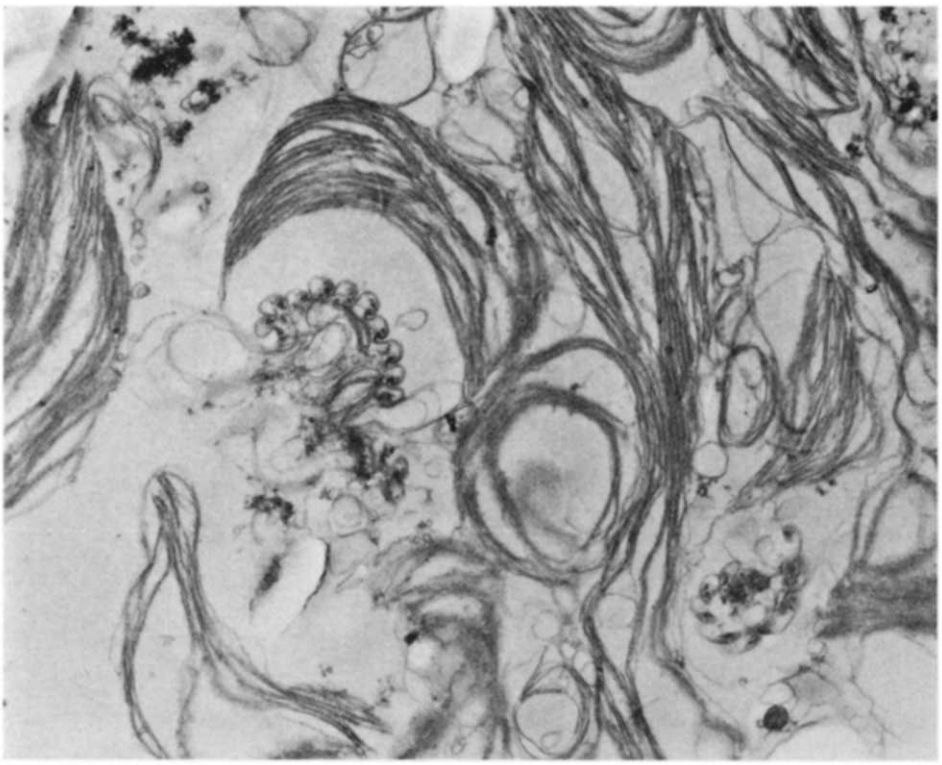

Fig. 6. Chloroplast fraction isolated from cells broken with a Braun homogenizer, ×11 000.

from other organisms is only 6–30 units for Mg^{2+}-dependent ATPase [12]. A higher activity could be obtained only if the preparations were pretreated by various means. Known procedures for ATPase activation, such as digestion with trypsin [2, 3] or illumination in the presence of dithiothreitol [5, 12] have only little effect on the enzyme from *Euglena*. Heat activation caused total loss of activity. It seems that the inability to increase enzyme activity by the various activation procedures is due to the fact that its potential activity is expressed without additional treatments. This is in agreement with the fact that carbonylcyanide *p*-trifluoromethoxyphenylhydrazone uncoupled photophosphorylation but did not inhibit ATPase activity. Thus, the high energy state of the membrane is not essential for the hydrolytic activity of ATPase.

ATPase from *Euglena* is highly specific for purine nucleotides as substrates. The highest rate of hydrolysis was obtained with ATP but GTP and ITP were also suitable substrates (Table II). Isolated unbound ATPase from *Euglena* [17] and higher plants [4] hydrolyses GTP at a rate of about 20 % that of ATP, whereas for the membrane-bound enzyme in higher plants the degree of specificity was found to be dependent on the energetic state of the membrane [23].

The K_m values for ATP of *Euglena* chloroplasts ATPase were found to be higher than those measured in higher plants [23]. The K_m value for ATP reported for isolated Ca^{2+}-dependent ATPase from *Euglena* is 0.097 mM [17]. For the membrane-bound enzyme we have measured a value of 2.5 mM. The reported K_i value for ADP

from isolated enzyme from *Euglena* is 0.39 mM [17] and that of the membrane-bound measured by us is 0.8 mM. These differences in kinetic constants can be explained by conformational changes which are the result of the removal of the enzyme from the membrane.

Maximal enzymatic activity was obtained with 8 mM Mg^{2+} and 2 mM Ca^{2+}. Higher or lower concentrations reduced the activity, possibly due to inhibition of ATPase by excess of either ATP or the divalent cations. Similar results were obtained in the same system but from a different organism [24].

In most chloroplasts ATPase systems it was found that Mg^{2+} stimulates the activity of the bound enzyme, whereas Ca^{2+} stimulates the activity of the isolated one. This effect was explained as an allotopic effect by McCarty and Racker [5]. Recently, it was found that under certain conditions, Mg^{2+}-dependent ATPase activity could be expressed by the isolated enzyme [8] and Ca^{2+}-dependent ATPase activity by the membrane-bound ATPase [9]. In *Euglena* chloroplasts, we have found both Ca^{2+}- and Mg^{2+}-stimulated ATPase activity of the membrane-bound enzyme. A correlation was found between the rate of the two activities and the procedure of chloroplasts isolation. A higher rate of Ca^{2+}-dependent ATPase activity was measured in "French press" preparations containing broken chloroplasts and free thylakoids. It can be speculated that this activity is "embedded" in the inner parts of the chloroplast and exposed only when the chloroplast is disintegrated. On the other hand the Mg^{2+}-ATPase is "external" in comparison, and could be expressed in more "complete" chloroplasts obtained by the Braun homogenizer. If the Mg^{2+}-dependent ATPase is "external" in relation to the "embedded" Ca^{2+}-ATPase it is expected that trypsinization will especially increase the activity of the latter. However, that was not the case. The Ca^{2+}-ATPase activity decreased rapidly. The increase in Mg^{2+} activity in the "trypsinates" can be rationalized assuming that the native ATPase inhibitor affects the Mg^{2+}-dependent enzyme and thus, the trypsin "digests" the inhibitor while releasing the enzyme activity.

The data presented substantiate the assumption that one enzyme has both the activities of Ca^{2+}- and Mg^{2+}-dependent ATPase. The kinetic constants: K_m values for ATP and K_i values for ADP and P_i were found to be similar in the presence of either Mg^{2+} or Ca^{2+}. The effect of inhibitors was also similar, and activation energy was the same for the two activities. The ATPase inhibitors affected both ATP hydrolysis and ATP synthesis by photophosphorylation. On the other hand, Douce et al. [25] have reported for spinach chloroplasts that Mg^{2+}-ATPase activity is located in the chloroplast envelope, while Ca^{2+}-ATPase activity is in the thylakoids. Therefore, the possibility of two different enzymes cannot be excluded. Some of our data could be related to activities of two enzymes: Ca^{2+}-dependent ATPase activity was better expressed in a relatively disintegrated chloroplasts, whereas Mg^{2+}-dependent activity was similar in both broken or more complete preparations. The different effects of trypsin on Mg^{2+}- and Ca^{2+}-dependent ATPase activities may support the assumption of two enzymes. More research has to be carried out in order to clarify these possibilities, such work is under way.

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