Proteins and polypeptides of envelope membranes from spinach chloroplasts

Properties of a membrane-bound ATPase

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Received 5 September 1983

Spinach chloroplasts are known to contain calmodulin and to display an envelope-bound ATPase activity. This activity, stimulated by 0.15 mM Ca^{2+} and 5 mM Mg^{2+}, is further enhanced by calmodulin. The apparent K_m for ATP was 0.55 mM. The enzyme was especially sensitive to NH_4VO_3, $SbCl_3$, $LaCl_3$ and oligomycin. An attempt to isolate the ATPase by calmodulin–Sepharose affinity chromatography was successful. The EGTA-eluted fraction contained 2 proteins out of the 21 proteins separated previously by isoelectric focussing [(1983) Biochim. Biophys. Acta 722, 226–233] and exhibited an ATPase activity.

ATPase *Chloroplast envelope* *Spinach* *Calmodulin* *Cation activation*

1. INTRODUCTION

A Mg^{2+}-dependent ATPase, insensitive to *N,N'*-dicyclohexylcarbodiimide (DCCD), is associated with chloroplast envelopes [1]. This enzyme has a greater affinity for Mn^{2+} than for Mg^{2+} [2]. More recently, oligomycin was found to inhibit partially the envelope ATPase activity [3].

In plant tissues, calmodulin modulates the activity of at least 3 enzymes: NAD^+ kinase, Ca^{2+}-ATPase and quinate: NAD^+ oxidoreductase [4]. Calmodulin is found in spinach [5], pea [6] and wheat [7] leaves and is present mainly in the cytosol (90%) and to a lesser extent in mitochondria (5–9%), chloroplasts (1–2%) and the microsomal fraction (<1%) [7]. In chloroplasts, calmodulin appears to be confined in the stroma [6]. Calmodulin antagonists such as chlorpromazine or phenothiazine inhibit electron transport in photosystem II of spinach chloroplasts [8] and the proton gradients associated with photophosphorylation [9].

The aim of this study was to investigate the properties of the chloroplast envelope-bound ATPase, namely the effect of various ions and calmodulin on its activity. Furthermore, an attempt was made to isolate a protein which has a specific affinity for calmodulin and exhibits an ATPase activity among the 21 chloroplast envelope proteins separated by isoelectric focussing [10].

2. MATERIALS AND METHODS

2.1. *Preparation of envelopes*

Deveined spinach leaves (700–800 g) were homogenized in 1.5 l grinding medium (25 mM Tricine–NaOH, pH 7.8, 300 mM sucrose, 0.1% defatted bovine serum albumin, 1 mM phenylmethanesulfonyl fluoride: PMSF) in a 1 gallon Waring Blendor for 5 × 1 s at 4°C. The homogenate was filtered through 8 layers of cheesecloth and centrifuged at 1500 × *g* for 5 min. The pellets of crude chloro-

This paper is dedicated to Professor Claude Favarger, Director of the Institute of Botany, University of Neuchâtel, Switzerland, in honor of his 70th birthday

This paper is the second of a series (see [10])

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Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

plasts were resuspended in the grinding medium, then layered on a 40% Percoll solution having the same composition as the grinding medium (20 ml suspension/15 ml Percoll 40%) and centrifuged at 2000 × *g* for 5 min [11]. The supernatant was discarded by aspiration and intact chloroplasts were washed once with the grinding medium and spun down at 2000 × *g* for 10 min. The intact and purified chloroplasts were lysed by osmotic shock in 10 mM Tricine–NaOH (pH 7.8), 5 mM $MgCl_2$, 1 mM PMSF. Thylakoids were partially removed by a centrifugation at 12 000 × *g* for 10 min; 18 ml of the resulting yellow-brown supernatant were layered on the top of a two-step sucrose gradient: 1.0 M (10 ml) and 0.6 M (10 ml) sucrose in 10 mM Tricine–NaOH (pH 7.8), 5 mM $MgCl_2$, 1 mM PMSF. A centrifugation at 95 000 × *g* (R_{max}) for 1 h revealed a yellow band (envelope fraction) at the sucrose interface. The envelopes were collected, diluted 4–5-fold with 10 mM Tricine–NaOH (pH 7.8) and sedimented at 122 000 × *g* (R_{max}) for 45 min.

2.2. *ATPase assay*

The ATPase activity was assayed in a 250 μl reaction mixture which contained 50 mM Tris–HCl (pH 7.8), 300 mM sucrose, 10–15 μg envelope protein. Concentrations of ATP, $MgCl_2$ and $CaCl_2$ are indicated in the legends of the figures. When $CaCl_2$ was used, 1 mM EGTA was added to the reaction mixture and free Ca^{2+} concentrations were calculated from the EGTA: Ca^{2+} ratio as in [12]. No correction was made for the ATP-Ca binding. Assays were routinely made in duplicate with adequate controls. After preincubation at 37°C for 10 min, the reaction was started by the addition of ATP. After 15 min at 37°C, the assays were stopped with 50 μl of 60% trichloroacetic acid. Under these conditions, the enzyme kinetics were linear up to 45 min (not shown). Precipitated proteins were removed by centrifugation (1 min in a Beckman Microfuge B) and P_i was measured in the supernatant as in [13]. A P_i calibration curve was made in the presence of 10% trichloroacetic acid.

2.3. *Other methods*

The isolation of the partially purified ATPase from chloroplast envelopes was carried out essentially as in [14], using calmodulin–Sepharose affinity chromatography. Protein was determined as in [15]. Calmodulin was purified from bovine brain [16].

3. RESULTS AND DISCUSSION

3.1. *Effect of ATP*

Fig.1 shows that the envelope-bound ATPase activity as a function of ATP concentrations followed Michaelis–Menten kinetics with an apparent K_m-value for ATP of 0.55 mM as determined by the double-reciprocal plot of $1/V$ *vs* $1/S$ (inset of fig.1). Compared to the value reported earlier [2], our enzyme had a greater affinity for ATP by a factor of 1.4. A basal ATPase activity, varying between 60–80 nmol P_i released · mg protein^{-1} · min^{-1} was associated with chloroplast envelopes (see control in fig.2). This activity was much higher than that (about 3 nmol $P_i \cdot mg^{-1} \cdot min^{-1}$) found in [2]. This basal activity apparently did not depend on divalent cations which could be externally bound to envelope vesicles since it was EDTA-insensitive.

3.2. *Effect of ions, buffers and inhibitors*

The ATPase activity was stimulated by divalent cations with maximal rates at 0.15 mM $CaCl_2$ and 5–10 mM $MgCl_2$ (fig.2). It is noteworthy that

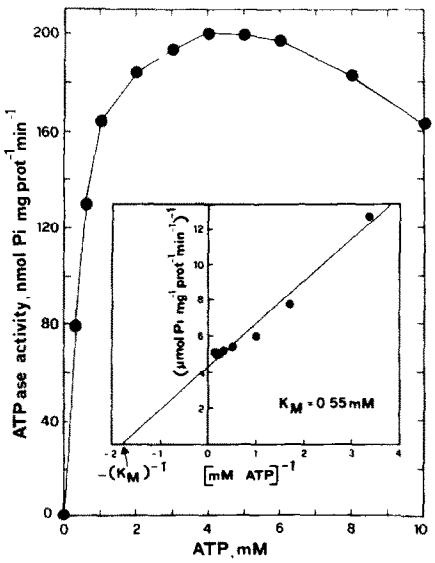

Fig.1. ATP concentration dependence of Mg^{2+}-stimulated envelope-bound ATPase activity. The reaction mixture contained 5 mM $MgCl_2$ and ATP as indicated. A double-reciprocal plot of $1/V$ *vs* $1/S$ is shown in the inset ($r = 0.98$).

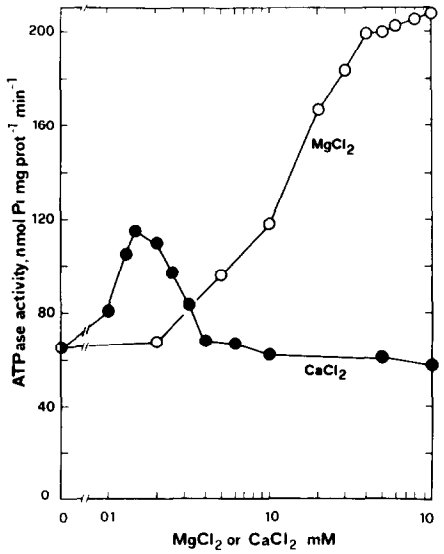

Fig.2. Stimulation of envelope-bound ATPase activity by $MgCl_2$ and $CaCl_2$. The reaction mixture contained 4 mM ATP and salts at the concentrations indicated.

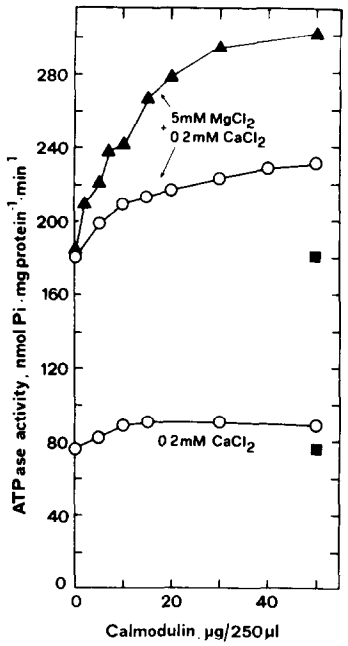

Fig.3. Stimulation of envelope-bound ATPase activity by calmodulin in the presence of $CaCl_2$ alone or $MgCl_2$ + $CaCl_2$. The reaction mixture contained 4 mM ATP and calmodulin at the concentrations indicated. (▲) spring spinach; (○) summer spinach; (■) effect of 80 μM chlorpromazine.

above 0.4 mM, $CaCl_2$ did not stimulate the enzyme activity. In the presence of 5 mM $MgCl_2$, 0.01 mM $CaCl_2$ caused a 30% increase in the activity (not shown). $MnCl_2$ (5 mM, pH 7.8) also stimulated the ATPase activity but to a lesser extent than $MgCl_2$ (about 2/3 of the stimulation observed with $MgCl_2$). A survey of the effect of other salts showed that $CuSO_4$ (5 mM) and $ZnSO_4$ (5 mM) in the absence of $MgCl_2$, KCl (50 mM), NaCl (50 mM), NH_4Cl (50 mM) in the presence of 5 mM $MgCl_2$, only slightly activated the ATPase. However, $SbCl_3$ (1 mM) and $LaCl_3$ (1 mM) in the presence of 5 mM $MgCl_2$ inhibited 50% of the activity. Among 3 different Mg salts ($MgCl_2$, $MgSO_4$, $Mg(NO_3)_2$) tested, the chloride salt was the most efficient on the enzyme activity. Comparison of ATPase activity at pH 7.8 showed that the specific activity with the standard buffer (50 mM Tris–HCl) was up to 25% higher than that in 50 mM Tricine–NaOH. Among several inhibitors tested (NaN_3, oligomycin, ouabain, NH_4VO_3, DCCD, EGTA) only oligomycin (5 μg/250 μl) and NH_4VO_3 (0.2 mM) inhibited the activity by 25 and 98%, respectively.

Thus, the present envelope-bound ATPase exhibited several differences with that described in [2]: (i) The ATPase activity did not depend strictly on divalent cations; (ii) $MgCl_2$ (≥5 mM) and to a lesser extent $MnCl_2$ stimulated the activity; (iii) $CaCl_2$ was found to be an activator of the enzyme at low concentrations (0.15 mM). In addition to the inhibitory effect of oligomycin, which was already described [3], we found that NH_4VO_3, $LaCl_3$ and $SbCl_3$ were potent inhibitors of the envelope-bound ATPase.

3.3. *Effect of calmodulin*

In the presence of both $MgCl_2$ and $CaCl_2$, calmodulin further stimulated the activity (fig.3). The extent of the stimulation was 63% with spring and 28% with summer spinach. This stimulation was not only $CaCl_2$- but also $MgCl_2$-dependent. An antagonist of calmodulin such as chlorpromazine, suppressed completely the effect of calmodulin, as shown in fig.3. It is the first time that a chloroplast envelope-bound enzyme is found to be stimulated by calmodulin.

3.4. *Partial purification of an envelope ATPase*

Since calmodulin was found to enhance the activity of the envelope-bound ATPase, we postulated

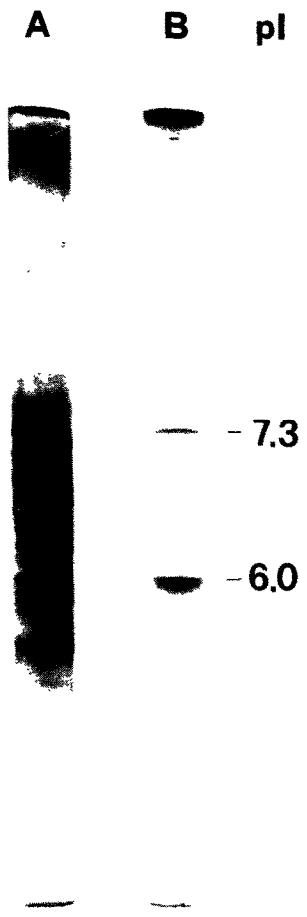

Fig.4. Separation of proteins by isoelectric focussing. (A) chloroplast envelope proteins. (B) EGTA-fraction eluted from the calmodulin–Sepharose column. Conditions for isoelectric focusing were as in [10].

that the enzyme might have a specific affinity for calmodulin. We attempted therefore to retain specifically the ATPase on a calmodulin–Sepharose affinity column in the presence of calcium. Fig.4 shows the separation by isoelectric focusing of the total envelope proteins and of the EGTA-eluted fraction which contained only two proteins. This latter fraction displayed an ATPase activity which was also stimulated by Ca^{2+}, Mg^{2+} and calmodulin (not shown).

In conclusion, the fact that the envelope-bound ATPase is sensitive to Ca^{2+} at low concentrations, to Mg^{2+} at much higher concentrations and to calmodulin opens interesting perspectives in the understanding of the regulation of photosynthesis. Due to its special properties this enzyme might well modulate ion, metabolite and/or protein exchanges between the chloroplast and the cytosol.

ACKNOWLEDGEMENTS

This research was supported by the Swiss National Science Foundation grant 3.661.0.80 to P.A.S. We thank Dr John Golbeck for critical reading of the manuscript and Françoise Bohren for technical assistance. This work is part of a doctoral program which is carried out by T.D.N. in the Laboratoire de Physiologie Végétale, Université de Neuchâtel.

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