

Proteins and polypeptides of envelope membranes from spinach chloroplasts

Properties of a membrane-bound ATPase

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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–Sephacryl 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 $1500 \times g$ 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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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 $2000 \times g$ for 5 min [11]. The supernatant was discarded by aspiration and intact chloroplasts were washed once with the grinding medium and spun down at $2000 \times g$ 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 $12000 \times g$ 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 $95000 \times g$ (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 $122000 \times g$ (R_{max}) for 45 min.

2.2. ATPase assay

The ATPase activity was assayed in a 250 μ l reaction mixture which contained 50 mM Tris-HCl (pH 7.8), 300 mM sucrose, 10–15 μ g 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 μ l 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 $\cdot$ mg protein $^{-1} \cdot$ 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

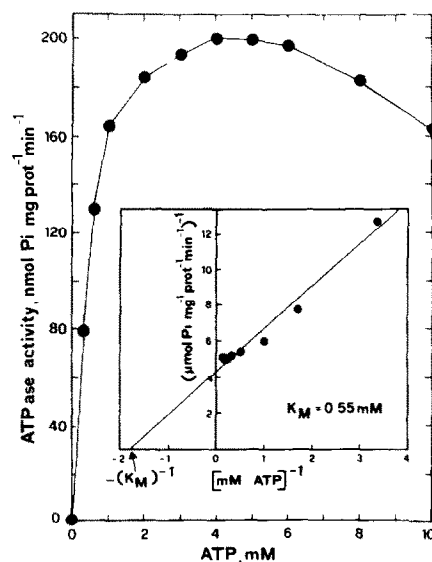


Fig.1. ATP concentration dependence of Mg^{2+} -stimulated envelope-bound ATPase activity. The reaction mixture contained 5 mM $MgCl_2$ and ATP as indicated. A double-reciprocal plot of $1/V$ vs $1/S$ is shown in the inset ($r = 0.98$).

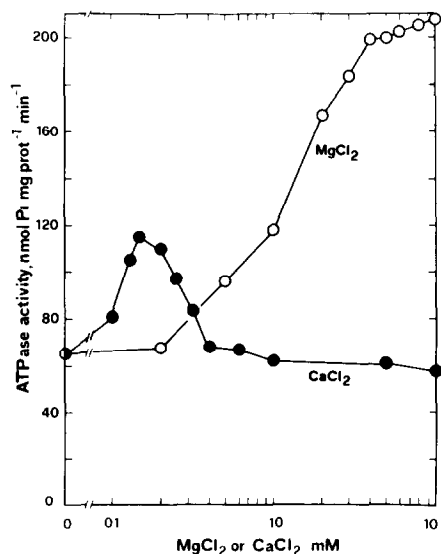


Fig. 2. Stimulation of envelope-bound ATPase activity by MgCl_2 and CaCl_2 . The reaction mixture contained 4 mM ATP and salts at the concentrations indicated.

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 , $\text{Mg}(\text{NO}_3)_2$) 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 ($5 \mu\text{g}/250 \mu\text{l}$) 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)

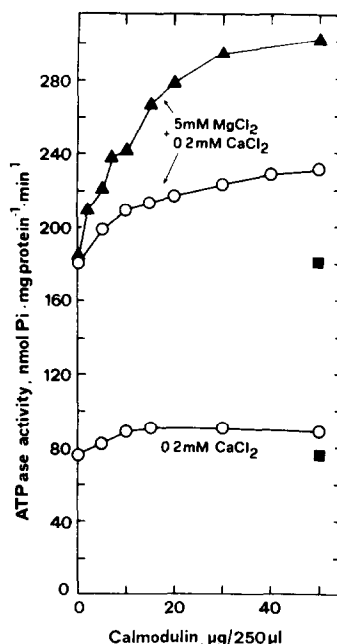


Fig. 3. Stimulation of envelope-bound ATPase activity by calmodulin in the presence of CaCl_2 alone or $\text{MgCl}_2 + \text{CaCl}_2$. The reaction mixture contained 4 mM ATP and calmodulin at the concentrations indicated. ($\blacktriangle$) spring spinach; ($\circ$) summer spinach; ($\blacksquare$) effect of $80 \mu\text{M}$ chlorpromazine.

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

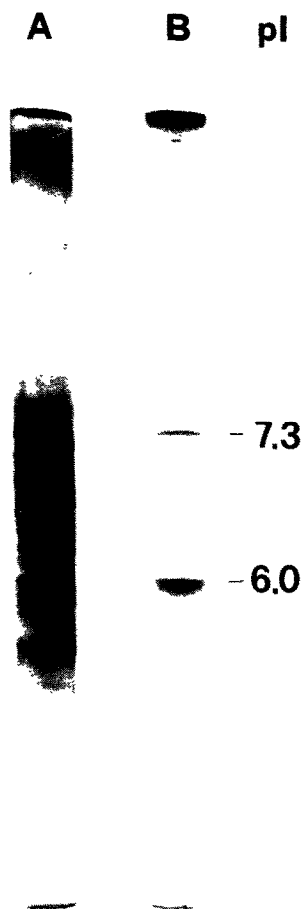


Fig.4. Separation of proteins by isoelectric focussing. (A) chloroplast envelope proteins. (B) EGTA-fraction eluted from the calmodulin-Sephrose column. Conditions for isoelectric focusing were as in [10].

that the enzyme might have a specific affinity for calmodulin. We attempted therefore to retain specifically the ATPase on a calmodulin-Sephrose 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 cal-

modulin 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.

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