Activation of plant aspartate kinase by calcium and calmodulin-like factor from plants*

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Aspartate kinase partially purified from plants is activated by a protein factor prepared from the same plant material. This activation is further stimulated by calcium and inhibited by anticalmodulin compounds such as trifluoperazine and chlorpromazine. The results suggest that the characteristics of regulation of the enzyme activity by the protein factor are similar to calmodulin regulation of plant and animal enzymes.

Calmodulin *Aspartate kinase* *Spinach* Dolichos lablab

1. INTRODUCTION

The requirement of calcium by plants for several metabolic processes has been well documented [1]. However, there is not much information on the specific calcium requirement by plant enzymes and the precise role of calcium in the enzyme function. In the recent past, studies on many enzymes from animal tissues have shown that calcium regulates the activity of some of the enzymes through a calcium-dependent small molecular mass protein designated as calmodulin [2–4]. In plants also, calmodulin (similar in most respects to animal calmodulin) has been convincingly shown to be involved in the activation of NAD kinase [5,6], calcium ATPase [7] and plant protein kinases [8]. The microsomal calcium uptake in plants has also been shown to be activated by calmodulin [9].

Here, we provide evidence for the requirement of calmodulin-like protein factor for the activity/activation of the enzyme aspartate kinase (ATP:L-aspartate 4-phosphotransferase, EC 2.7.2.4) from plants. The data presented here show that a protein factor which has characteristics of calmodulin can stimulate the activity of aspartate kinase in the presence of calcium (and not other divalent cations) and that the activity of the activated enzyme can be inhibited by chelating out calcium specifically by EGTA or by anticalmodulin compounds such as chlorpromazine and trifluoperazine.

2. MATERIALS AND METHODS

Aspartate kinase was isolated either from seeds of *Dolichos lablab* (soaked for 48 h) or leaves of field-grown spinach and winged beans. The plant material was homogenized in 0.2 M Tris–HCl buffer (pH 7.5), containing dithiothreitol (DTT 1 mM), EDTA (1 mM), threonine (2 mM) and glycerol (30% v/v). The homogenate, after filtration through 8 layers of cheese-cloth, was centrifuged at 10000 × *g* for 30 min. The supernatant was subjected to ammonium sulphate precipitation to obtain a fraction precipitating between 30–60% ammonium sulphate. The pellet was resuspended in potassium phosphate buffer (50 mM, pH 7.5) containing dithiothreitol, EDTA and glycerol as in the extraction buffer. The material was centrifuged at 16000 × *g* for 30 min and the supernatant was desalted on Sephadex G-15 column equilibrated with suspending buffer. The desalted enzyme extract was concentrated against sucrose and used as such or it was loaded on a DEAE cellulose column and eluted with the phosphate buffer containing increasing concentrations of NaCl. The active frac-

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tions were pooled, concentrated, desalted and then used. All the operations were carried out at 4–5°C. Aspartate kinase was assayed as per the procedure described in [10]. The results obtained from any of the plant material used gave similar data.

Calmodulin-like factor was prepared according to the procedure described in [7,9]. Leaves from spinach or winged bean were extracted with 20 mM Tris–HCl buffer, pH 7.5, containing 1 mM EDTA. The extract after filtration and centrifugation was heated for 2 min at 80°C. It was cooled rapidly and centrifuged to obtain supernatant which was mixed with DEAE cellulose and stirred for 1 h before centrifugation to sediment DEAE cellulose. The DEAE cellulose was resuspended in Tris–MES buffer (20 mM, pH 7.5) containing 0.1 mM EGTA and 0.6 M NaCl and eluted with the same buffer. The eluate was dialysed overnight against 20 mM Tris–MES (pH 7.5) to remove NaCl and then used as the factor. The preparation was tested for its calmodulin property by examining the stimulation of NAD kinase activity [6,12]. The activity of NAD kinase partially purified from spinach was stimulated more than 2-fold showing that the preparation contains calmodulin.

3. RESULTS

Aspartate kinase is stimulated over 4-fold by the addition of protein factor (fig.1). The stimulation is dependent on the amount of protein factor added and maximum activation is observed when the protein factor added was 125 μg/1.5 ml of the assay mixture. In several experiments the enzyme preparation that was virtually inactive without the addition of the factor showed good activity on addition of the factor (not shown).

The data presented in table 1 show that the enzyme activity in the absence of factor can be considerably inhibited by EGTA which is known to chelate Ca^{2+} preferentially. The factor-stimulated enzyme activity is also inhibited by EGTA (table 1). Thus, to obtain full enzyme activity not only the factor but also Ca^{2+} is essential. This is further supported by the data presented in table 2. In the absence of the factor the enzyme activity inhibited by EGTA can be reversed by the addition of Ca^{2+} in a dose-dependent manner. At 0.5 mM Ca^{2+} the

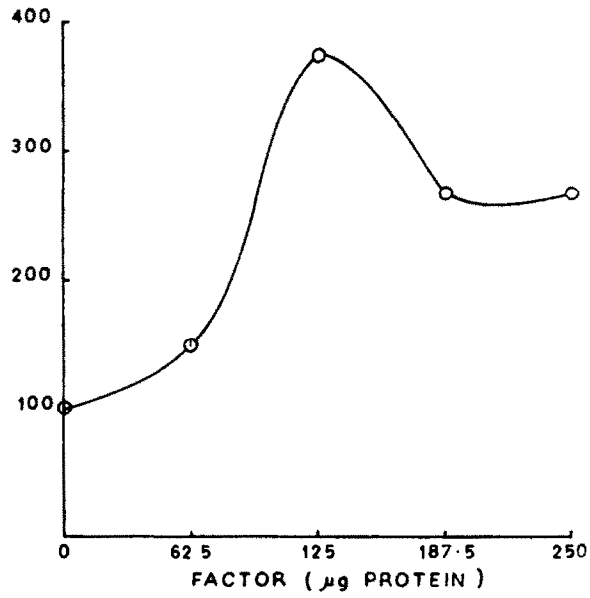

Fig.1. Effect of varying concentrations of the protein factor on aspartate kinase activity.

enzyme activity is more than the control. This activity is further stimulated by the factor and maximum activity is observed when both Ca^{2+} and protein factor are present. The requirement of Ca^{2+} cannot be substituted by other divalent cations such as Mn^{2+}, Mg^{2+} or Zn^{2+} which could only marginally (10–20%) improve the EGTA-inhibited activity (not shown) as against 4-fold stimulation by $Ca_2{}^{+}$.

These data suggest that the activation of the enzyme by the protein factor has characteristics similar to those of calmodulin stimulation of enzyme activity observed for many of the animal enzymes and two plant enzymes, viz., NAD-kinase and Ca-ATPase. The protein factor under study therefore seems similar to the calmodulin. It can be argued that if the factor is equivalent to calmodulin then its stimulatory activity should be inhibited by anticalmodulin compounds such as trifluoperazine and chlorpromazine. These compounds have been shown to block activities of calmodulin-stimulated enzymes from animal sources. Data presented in table 3 show that trifluoperazine inhibits the aspartate kinase activity in the presence of the factor. The chlorpromazine similarly inhibits the activity (not

Table 1

Inhibition of aspartate kinase by EGTA in the presence and absence of the protein factor

Treatment	Percent activity	
	− Factor	+ Factor
1. Control (Enz)	100	378
2. Enz + 0.1 mM EGTA	100	271
3. Enz + 0.4 mM EGTA	100	247
4. Enz + 0.5 mM EGTA	100	165
5. Enz + 0.8 mM EGTA	48	111
6. Enz + 1.0 mM EGTA	9	70

shown). These data, therefore, show that the protein factor responsible for stimulation of aspartate kinase must be calmodulin. To the best of our knowledge this is the first demonstration of calmodulin regulation of aspartate kinase isolated from any source.

The calmodulin-regulation enzymes can be depleted of calmodulin by passing through the DEAE cellulose column where the enzyme is less tightly bound and hence eluted at low salt concentration. The calmodulin binds more tightly to the column and can be eluted with high salt concentration. The depleted enzyme then can be reconstituted by addition of calmodulin [5,6,11–13]. Such an experiment was carried out to

Table 2

Reversal of EGTA inhibition of aspartate kinase by Ca^{2+} in the presence and absence of protein factor

Treatment	Percent activity	
	− Factor	+ Factor
1. Enzyme alone	100	378
2. Enz + EGTA (1 mM)	9	70
3. Enz + EGTA (1 mM) + Ca^{2+} (0.1 mM)	39	154
4. Enz + EGTA (1 mM) + Ca^{2+} (0.2 mM)	100	211
5. Enz + EGTA (1 mM) + Ca^{2+} (0.3 mM)	111	259
6. Enz + EGTA (1 mM) + Ca^{2+} (0.5 mM)	133	483
7. Enz + EGTA (1 mM) + Ca^{2+} (1.0 mM)	111	336

Table 3

Inhibition of aspartate kinase by anticalmodulin compound trifluoperazine (TFP) in the presence of protein factor

Treatment	Percent activity	
	− Factor	+ Factor
1. Enzyme (control)	100	248
2. Enzyme + TFP (100 μM)	69	127
3. Enzyme + TFP (500 μM)	27	69

The enzyme activity is similarly inhibited by chlorpromazine

provide final proof for calmodulin requirement of aspartate kinase. The data shown in table 4 show that the non-depleted enzyme activity is reduced to 41% on depletion by DEAE cellulose column chromatography. The factor (calmodulin) eluted from the column at high concentration of NaCl can reconstitute enzyme activity to 82% which can be further stimulated by Ca^{2+} to 101%. The stimulated activity is inhibited by trifluoperazine to 20% as expected. These data show that the factor which has properties of calmodulin can be

Table 4

Depletion of aspartate kinase of activating factor and its reconstitution by the addition of the factor (calmodulin) eluted from the same enzyme extract

Treatment	Percent activity
1. Non-depleted enzyme	100
2. Depleted enzyme	41
3. Depleted enzyme + factor (eluted from the column)	82
4. Depleted enzyme + factor + Ca (1 mM)	101
5. Depleted enzyme + factor + Ca (1 mM) + trifluoperazine (5 mM)	30

The enzyme extract (20 ml) used as non-depleted enzyme was loaded on a DEAE cellulose column as described in section 2. The column was eluted with the same buffer containing 200 mM NaCl in a total volume of 20 ml. The bound factor was eluted by 2 M NaCl in 20 ml and concentrated 10-fold before use. Per reaction mixture of 1.5 ml, factor added was 0.2 ml (164 μg) protein

dissociated from the enzyme and reassociated to reconstitute the activity.

A final confirmation for the activation of aspartate kinase by calmodulin came from the experiment in which calmodulin obtained from Sigma was used. This authentic calmodulin (30 μg/ml of reaction mixture) isolated from bovine brain stimulated the activity of aspartate kinase (which was depleted of the factor) over 2-fold in the presence of 500 μM calcium. This suggests that not only is the factor that stimulates aspartate kinase calmodulin but also that calmodulin from the animal tissue can substitute for plant calmodulin – at least in the case of aspartate kinase.

4. DISCUSSION

The protein factor was isolated using the procedure for isolation of calmodulin. The properties of protein factor such as stimulation of NAD-kinase depleted of calmodulin, the requirement of calcium for its stimulation, inhibition of stimulated activity by anticalmodulin compounds, and activation of depleted aspartate kinase by the authentic sample of calmodulin obtained from Sigma lead us to conclude that the protein factor responsible for aspartate kinase stimulation is calmodulin. Considering the fact that several kinases from animal sources [14–22] are regulated by calmodulin it is not surprising that aspartate kinase is also regulated by calmodulin. To the best of our knowledge this is the first demonstration of calmodulin requirement for aspartate kinase.

The observations made here have several interesting implications. In plants the work on aspartate kinase has been restricted due to loss of enzyme activity during purification. We believe that calmodulin dissociates and separates away from the enzyme during purification procedure and hence the activity is lost. It should now be possible to 'reconstitute' aspartate kinase activity by calmodulin. This should enable detailed studies of this important regulatory enzyme. Aspartate kinase is under control by feedback inhibition and one would like to know how this regulation is affected in the presence of calmodulin. Are the kinetic properties changed? We are presently studying these aspects.

The enzyme aspartate kinase can be used as a test system for assay of calmodulin as this enzyme can be easily depleted of calmodulin and the stimulation is quite considerable. The enzyme assay is also much simpler. The physiological significance of this regulation needs to be investigated; however, a change in calcium fluxes postulated to be controlled by phytochrome could easily subject the enzyme under regulatory control of calmodulin in vivo.

The results reported here, thus show that:

(a) Aspartate kinase activity is considerably stimulated by a protein factor which was isolated by the procedure for calmodulin isolation.

(b) This stimulation is inhibited by EGTA which preferentially chelates Ca^{2+} and the inhibition is relieved by addition of Ca^{2+}.

(c) The maximum stimulation requires both Ca^{2+} and the protein factor.

(d) The enzyme activity is inhibited by anticalmodulin compounds such as trifluoperazine and chlorpromazine.

(e) The enzyme depleted of the factor can be activated over 2-fold by low concentrations of commercially available calmodulin isolated from the animal tissue.

These data are consistent with the conclusion that the protein factor under study is akin to calmodulin and that aspartate kinase is regulated by calmodulin in plants.

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