Lysine-sensitive plant aspartate kinase is not regulated by calcium or calmodulin

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Received 18 September 1985; revised version received 11 November 1985

Lysine-sensitive plant aspartate kinase purified from *Daucus carota* to a specific activity 20-fold higher than previously reported was not activated by calcium or calmodulin. The calmodulin antagonists calmodazolium and compound 48/80 did not inhibit activity. However, trifluoperazine exhibited an inhibitory effect, but only at high concentrations. The results do not agree with a previous proposal that calcium and calmodulin play a key role in regulating higher plant lysine-sensitive aspartate kinase.

Ca^{2+} *Calmodulin* *Lysine sensitivity* *Aspartate kinase* (Daucus carota)

1. INTRODUCTION

Calmodulin extractable from plant tissue bears a number of striking similarities to that from animal tissue, these similarities include: molecular mass, amino acid composition and sequence, the affinity towards calcium and the potency in activating animal and plant calmodulin-dependent enzymes. These enzymes include protein kinases, NAD kinase and calcium ATPase [1]. Recently, it has been reported that the enzyme aspartate kinase (ATP:aspartate 4-phosphotransferase, EC 2.7.2.4) is activated by calcium and a calmodulin-like factor from plants [2].

Aspartate kinase is present in both plants and microbes and catalyses the phosphorylation of aspartate to aspartyl phosphate. The reaction is the first step in the biosynthesis of the amino acids isoleucine, lysine, threonine and methionine. Interest in these pathways stems from the fact that all the previously mentioned amino acids are essential for simple-stomached animals and that plant proteins are low in lysine (cereals) or methionine plus cysteine (legumes) [3].

In barley leaves, there are 3 isoenzymes of aspartate kinase: one enzyme is inhibited by threonine, the other 2 have their inhibition by lysine potentiated by *S*-adenosylmethionine [4]. We are presently purifying the lysine-sensitive forms of aspartate kinase from a *Daucus carota* cell suspension culture and its potential regulation by calcium and calmodulin has been investigated with enzyme preparations whose specific activities are over 20-fold higher than previously reported [5].

2. MATERIALS AND METHODS

Aspartate kinase was purified from carrot (*Daucus carota* L. D.C.3) cell suspension culture; 100 g of material (stored in liquid N_2) was homogenised in 100 ml of 50 mM K_2PO_4/NaOH buffer (pH 7.4) containing 1 mM DTT, 50 mM KCl and 20% (v/v) ethanediol. After centrifugation at 20000 × *g* for 30 min the supernatant was brought to 60% saturation with ammonium sulphate and centrifuged at 20000 × *g* for 30 min. The precipitate was dissolved in the extraction buffer, applied to a Sephacryl S-200 (Pharmacia) column (2.6 × 65.0 cm) and equilibrated with 25 mM Tris/HCl (pH 7.1) containing 50 mM NaCl, 1 mM DTT and 10% (v/v) ethanediol. Active fractions were pooled, applied to a DEAE-Sephacel (Pharmacia) column (2.5 × 8.0 cm) and equilibrated with 25 mM Tris/HCl (pH 7.1) containing 50 mM

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

NaCl, 1 mM DTT and 10% (v/v) ethanediol. Bound material was eluted with a linear gradient of 50–300 mM NaCl, and active fractions pooled and used in the experiments. The activity was measured by incubating 0.1 ml enzyme at 30°C with 0.1 ml of 250 mM L-aspartate (pH 7.4), 0.1 ml of 50 mM K_2PO_4/NaOH (pH 7.4) containing 1 mM DTT, 50 mM KCl and 10% (v/v) ethanediol, 0.05 ml of 4 M hydroxylamine (pH 7.4), 0.05 ml of 20 mM ATP (pH 7.4), 0.05 ml of 250 mM $MgSO_4$ and 0.05 ml H_2O. The reaction was terminated by the addition of 0.5 ml ferric chloride reagent (0.37 M $FeCl_3$, 0.67 M HCl, 0.2 M trichloroacetic acid) and the absorbance at 505 nm measured. Additional plant material from barley and pea seedling leaves was purified to the ammonium sulphate stage and desalted on Sephadex G-25 (Pharmacia). One unit of activity is defined as nmol aspartyl hydroxyamate formed $min^{-1} \cdot mg^{-1}$ protein.

Calmodulin was partially purified from spinach leaves as described in [6] to the DEAE-cellulose step, with ammonium sulphate precipitation replacing heat denaturation. Using bovine calmodulin and Ca^{2+}-dependent phosphodiesterase (PDE) outlined in [7], 2% of the spinach extracted was estimated to be calmodulin. One unit of PDE hydrolysed 1 μmol cyclic AMP $\cdot min^{-1}$ at 25°C.

All chemicals were purchased from Sigma.

3. RESULTS

Table 1 shows the effect of calcium and inhibitors of calmodulin-dependent enzymes on the highly purified preparations of lysine-sensitive aspartate kinase isolated from *D. carota* cells. There was no significant inhibition of enzyme activity by calmodazolium, EGTA or compound 48/80 [8]. The only detectable inhibition of enzyme activity was observed with trifluoperazine at the same concentrations used by Sane et al. [2]. Essentially similar results were obtained with the

Table 1

The effect of inhibitors of calmodulin on the activity of aspartate kinase purified from *D. carota* cells

	Specific activity (units)	% inhibition
Enzyme	329	0
Enzyme + 5 mM lysine	29	91
Enzyme + 5 mM threonine	316	4
Enzyme − ATP	0	100
Enzyme + 0.1 mM EGTA	334	0
Enzyme + 1 mM EGTA	329	0
Enzyme + 0.05 mM Ca^{2+}	334	0
Enzyme + 0.5 mM Ca^{2+}	310	6
Enzyme + 0.4 μM calmodazolium	329	0
Enzyme + 2 μM calmodazolium	329	0
Enzyme + 25 $\mu g \cdot ml^{-1}$ compound 48/80	278	16
Enzyme + 50 $\mu g \cdot ml^{-1}$ compound 48/80	291	12
Enzyme + 100 μM trifluoperazine	214	35
Enzyme + 500 μM trifluoperazine	94	72

Table 2

The effect of bovine and spinach calmodulin on the activity of *D. carota* lysine-sensitive aspartate kinase and Ca^{2+}-bovine heart phosphodiesterase

	Specific activity (units)
D. carota aspartate kinase	330
+ 0.5 mM Ca^{2+} + 5 $\mu g \cdot ml^{-1}$ spinach calmodulin	321
+ 0.5 mM Ca^{2+} + 10 $\mu g \cdot ml^{-1}$ spinach calmodulin	326
+ 0.5 mM Ca^{2+} + 12 $\mu g \cdot ml^{-1}$ bovine calmodulin	308
+ 0.5 mM Ca^{2+} + 12 $\mu g \cdot ml^{-1}$ bovine calmodulin + 25 $\mu g \cdot ml^{-1}$ compound 48/80	278
	Activity (units × 10^{-3})
Bovine Ca^{2+}-phosphodiesterase	0.52
+ 0.2 $\mu g \cdot ml^{-1}$ bovine calmodulin	2.26
+ 5 $\mu g \cdot ml^{-1}$ spinach calmodulin	1.11

D. carota enzyme at all stages of purification. Cruder preparations of aspartate kinase isolated from *Hordeum vulgare* and *Pisum sativum* leaves were tested with 2 mM EGTA and 25 $\mu g \cdot ml^{-1}$ compound 48/80, no inhibition of activity was observed.

The results in table 2 show that both spinach and bovine calmodulin activated bovine heart Ca^{2+}-dependent phosphodiesterase but were unable to activate *D. carota* lysine-sensitive aspartate kinase. In addition the lysine-sensitive aspartate kinase did not bind to calmodulin agarose (Sigma) under conditions which normally bind calmodulin-dependent enzymes.

4. DISCUSSION

Work on plant aspartate kinase in the past has been limited due to loss of activity during purification. Sane et al. [2] have attributed this loss in activity to a removal of calmodulin. However, we have recently obtained enzyme preparations in good yield purified >300-fold from *D. carota* cell suspension cultures.

The inhibition of activity by trifluoperazine is almost identical to that observed by Sane et al. [2] at the same concentrations. However, trifluoperazine has been cited as a non-specific antagonist of calmodulin and Roufogalis [9] has questioned the relevance of studies at concentrations above 100 μM.

The majority of the enzymes (including aspartate kinase) of the biosynthetic pathway which leads to lysine, threonine, isoleucine and methionine are totally located within the chloroplasts [10]. Over 90% of plant calmodulin is located in the cytosol with only 1–2% associated with the chloroplasts [1]. Recent estimates put the concentration of calmodulin inside the chloroplasts as low as 1–3 $\mu g \cdot ml^{-1}$ [11,12], thus the concentration within the chloroplasts would be lower than the concentration of bovine calmodulin tested in table 2. Bearing this in mind we can find no evidence at this present time to support a role for calcium and calmodulin in regulating lysine-sensitive plant aspartate kinase.

ACKNOWLEDGEMENTS

We are indebted to ICI Plant Protection Division for financial support and to our collaborators Drs S.W.J. Bright, B.J. Miflin, J. Relton and R.M. Wallsgrove for stimulating discussions. The facilities at the Food Research Institute, Norwich supplied by Dr M. Hilton for growing the *D. carota* tissue cultures are also gratefully acknowledged.

REFERENCES

[1] Dieter, P. (1984) Plant Cell Environ. 7, 371–380.
[2] Sane, P.V., Kochhar, S., Kumar, N. and Kochhar, V.K. (1984) FEBS Lett. 175, 238–242.
[3] Bright, S.W.J. and Shewry, P.R. (1984) CRC Crit. Rev. Plant Sci. 1, 49–93.
[4] Bright, S.W.J., Lea, P.J., Arruda, P., Hall, N.P., Kendall, A.C., Keys, A.J., Kueh, J.S.H., Parker, M.L., Rognes, S.E., Turner, J.C., Wallsgrove, R.M. and Miflin, B.J. (1984) in: The Genetic Manipulation of Plants and its Application to Agriculture (Lea, P.J. and Stewart, G.R. eds) pp.141–169, Oxford University Press, Oxford.
[5] Davies, H.M. and Miflin, B.J. (1978) Plant Physiol. 62, 536–554.
[6] Anderson, J.M. (1983) Methods Enzymol. 102, 9–17.
[7] Murray, A. and Rogers, A. (1978) Biochem. J. 17, 120–126.
[8] Gretzen, K., Sanchez-Delgado, E. and Bader, H. (1983) IRCS Med. Sci. 11, 12–13.
[9] Roufogalis, B.D. (1982) in: Calcium and Cell Function vol.3 (Cheung, W.Y. ed.) pp.130–159, Academic Press, New York.
[10] Wallsgrove, R.M., Lea, P.J. and Miflin, B.J. (1983) Plant Physiol. 71, 780–784.
[11] Muto, S. (1982) FEBS Lett. 147, 161–164.
[12] Jarret, H.W., Brown, C.J., Black, C.C. and Cormier, M.J. (1982) J. Biol. Chem. 257, 13795–13804.