Adenylate cyclase activity in a higher plant, alfalfa (*Medicago sativa*)

Valentina C. CARRICARTE,* Graciela M. BIANCHINI,* Jorge P. MUSCHIETTI,*
María Teresa TÉLLEZ-INÓN,* Alejandro PERTICARI,† N. TORRES*
and Mirtha M. FLAWIÁ*

*Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (INGEBI-CONICET),
Facultad de Ciencias Exactas y Naturales, UBA, Obligado 2490, (1428) Buenos Aires, Argentina, and

†Instituto Nacional de Tecnología Agropecuaria, Castelar, Argentina

An adenylate cyclase activity in *Medicago sativa* L. (alfalfa) roots was partially characterized. The enzyme activity remains in the supernatant fluid after centrifugation at 105 000 *g* and shows in crude extracts an apparent M_r of about 84 000. The enzyme is active with Mg^{2+} and Ca^{2+} as bivalent cations, and is inhibited by EGTA and by chlorpromazine. Calmodulin from bovine brain or spinach leaves activates this adenylate cyclase.

INTRODUCTION

The control of metabolism, cell growth and differentiation by cyclic AMP in some eukaryotic organisms has been well established. In these organisms, which belong to the Animalia, Protocista and Fungi kingdoms, the cyclic nucleotide mediates cell response to different environmental signals, acting as intracellular second messenger. In higher plants, however, the role of cyclic AMP is unknown. Several reports indicate the existence of this compound in plant tissues, as well as the presence of cyclic AMP phosphodiesterases, adenylate cyclase and cyclic AMP-binding protein activities (Brown & Newton, 1981). These studies, however, do not give a detailed characterization of some of these enzyme activities and their regulation, or provide major evidence on the involvement of the cyclic nucleotide in the control of some important responses of plant cells to environmental stimuli.

Ca^{2+} and calmodulin constitute another alternative for the transduction of environmental signals, modulation of cell metabolism and regulation of development in higher plants (Vanderhoef & Kosuge, 1984; Hepler & Wayne, 1985). In this regard, the involvement of Ca^{2+} in geotropic and phototropic responses has been indicated (Slocum & Roux, 1983; Hale & Roux, 1980). Similarly, the cation seems to be related to the actions of gibberelic acid; auxin and cytokinins on senescence, germination and growth, as well as in the secretion of indolylacetic acid (Poovaiah & Leopold, 1973; Moll & Jones, 1981; Saunders & Hepler, 1983; de la Fuente, 1984; de Guzmán & de la Fuente, 1984). Some of these actions are inhibited by phenothiazine derivatives (Elliot *et al.*, 1983; Raghothama *et al.*, 1985), which may indicate the involvement of the Ca^{2+} -calmodulin complex in such regulatory phenomena.

It is also known that calmodulin activates some enzyme activities in higher plants, such as NAD kinase (Anderson & Cormier, 1978), Ca^{2+} -ATPase (Dieter & Marmé, 1981) and quinate:NAD⁺ 3-oxidoreductase (Ranjeva *et al.*, 1983). The involvement of the Ca^{2+} -calmodulin complex in protein phosphorylation in plants has also been reported (Veluthambi & Poovaiah,

1984, 1986). The involvement of phosphoinositides in controlling cytosolic Ca^{2+} concentrations in plants was also proposed (Drobak & Ferguson, 1985; Poovaiah *et al.*, 1987).

The present paper reports studies on the characterization and partial purification of a soluble adenylate cyclase activity from alfalfa (*Medicago sativa* L.) roots. This enzyme was found to be activated by calmodulin.

EXPERIMENTAL

Materials

Rabbit muscle creatine kinase and lactate dehydrogenase were from Boehringer (Mannheim, Germany), and ATP, cyclic AMP, phosphocreatine, EDTA, EGTA, horse heart cytochrome *c*, phenylmethanesulphonyl fluoride, pigeon breast muscle malate dehydrogenase, ox liver catalase, ^2H_2O , Tris and Pipes were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sucrose was from Schwartz-Mann (Orangeburg, NY, U.S.A.), forskolin from Calbiochem (San Diego, CA, U.S.A.), DEAE-cellulose (DE-52) from Whatman (Clifton, NJ, U.S.A.), 3-isobutyl-1-methylxanthine from Aldrich (Milwaukee, WI, U.S.A.), Trasylol from Bayer (Leverkusen, Germany) and Ultrogel AC-34 from LKB-Produkter (Bromma, Sweden). Neutral alumina was purchased from Merck (Darmstadt, Germany), AG50 W-X4 (200–400 mesh) from Bio-Rad (Richmond, CA, U.S.A.), and [α - ^{32}P]ATP and cyclic [^3H]AMP were from New England Nuclear. All other chemicals were reagent grade.

Plant material

Medicago sativa L. was grown in fields fertilized with ammonium superphosphate (30 kg/hectare) for 1–2 years. After removal of soil, roots were gently washed with water and stems were cut at about 1 cm above the root-stem confluence. Thereafter these trimmed plants were transferred to pots containing a nitrogen-rich sterile soil and further cultured at 25 °C, 60–70 % humidity and 16 h/day illumination (1000 lm; 40 W Sylvania Gro-lux fluorescent tubes) with daily watering (Vincent, 1970). These plants did not contain any nodule in the roots.

Enzyme preparation

Crude extract. Except otherwise indicated, all operations were performed at 2–5 °C. Roots were gently washed with tap and distilled water. After excision from stems (about 1 cm below the junction), roots were cut into small pieces with scissors and frozen with liquid N₂. The material was ground in a mortar in the presence of liquid N₂ and further homogenized with 1 ml of 50 mM-Tris/HCl buffer, pH 7.4, containing 1 mM- β -mercaptoethanol (Buffer A)/g of the powder with an all-glass Dounce-type homogenizer. The homogenate was centrifuged at 10000 *g* for 10 min, and the supernatant fluid was further centrifuged at 105000 *g* for 120 min. The supernatant thus obtained was termed 'crude extract'.

DEAE-cellulose column chromatography. A suspension of the cellulosic exchanger was first adjusted to pH 7.5 with dil. HCl. The column (16 cm $\times$ 1.7 cm), equilibrated with Buffer A, was loaded with 25 ml of the 'crude extract' (adenylate cyclase activity 10 pmol/min per mg of protein; 7 mg of protein/ml). After washing with 110 ml of Buffer A, the column was eluted with 200 ml of a 0–1.0 M-NaCl linear gradient in Buffer A. Fractions (5 ml) were collected at 0.5 ml/min. Adenylate cyclase activity was eluted as a single peak at about 0.3 M-NaCl. Pooled fractions corresponding to this peak, termed 'DEAE fraction', were pooled and stored at 0–2 °C.

Estimation of molecular and hydrodynamic parameters

Gel filtration. A sample of 'crude extract' or 'DEAE fraction' (1 ml) was loaded on an Ultrogel AC-34 column (40 cm $\times$ 1.5 cm) equilibrated with Buffer A containing 0.15 M-NaCl. Fractions (1 ml) were collected at 0.2 ml/min. The fractions with the highest specific activity, termed 'Ultrogel fractions', were combined, concentrated and stored as indicated above.

Sucrose-gradient centrifugation. A sample of 'crude extract' or 'DEAE fraction' (0.5 ml) was overlaid on 5–20% sucrose linear gradients made in Buffer A, containing 0.15 M-NaCl. Some of the gradients were also made in solutions made in ²H₂O. Centrifugations were carried out at 45000 rev./min for 16 h in a Beckman SW40 rotor. Fractions (0.2 ml) were collected by pumping from the bottom at a rate of 0.5 ml/min.

Calibrating proteins and calculations. Samples of calibrating proteins were filtered through an Ultrogel AC-34 column or subjected to sucrose gradient centrifugation under conditions described above. Calibrating proteins were added at the following concentrations: catalase (ox liver), 100 μ g/ml; lactate dehydrogenase (rabbit muscle), 30 μ g/ml; malate dehydrogenase (pigeon breast muscle), 10 μ g/ml; cytochrome *c* (horse heart), 2 mg/ml. Calculations of sedimentation coefficients, partial specific volumes, Stokes radius, *M_r*, and frictional ratio were performed as previously described (Kornblihtt *et al.*, 1981; Reig *et al.*, 1982).

Purification of calmodulin from *Spinacea oleracea* L. (spinach) leaves

Fresh spinach leaves (200 g) were washed with distilled water and homogenized with a Waring Blendor in 200 ml of 50 mM-Tris/HCl buffer, pH 7.4, containing 3 mM-

EDTA. The homogenate was filtered through two layers of gauze and the filtrate centrifuged at 10000 *g* for 20 min. The supernatant (120 ml) was loaded on a DEAE-cellulose column (17 cm $\times$ 1.7 cm) equilibrated with 50 mM-Tris/HCl buffer, pH 7.4, and washed with 100 ml of this solution. Elution was performed with a linear gradient of 0–0.6 M-NaCl (300 ml) in the same solution; 6 ml fractions were collected at a rate of 1 ml/min and assayed for calmodulin activity after the activation of bovine brain cyclic AMP phosphodiesterase as described by Téllez-Iñón *et al.* (1985).

Purification of bovine calmodulin

The modulator was purified by the method of Bazari & Clarke (1981) with the modifications of Téllez-Iñón *et al.* (1985).

Adenylate cyclase assay

The standard incubation mixture contained 50 mM-Tris/HCl buffer, pH 7.4, 0.2 mM-3-isobutyl-1-methyl-xanthine, 1 mM-cyclic AMP, 2.5 mM-MgCl₂, 0.25 mM-CaCl₂, 0.5 mM-[α -³²P]ATP (sp. radioactivity 100–300 c.p.m./pmol), 2 mM-phosphocreatine, 0.2 mg of creatine kinase and enzyme fractions. The volume was 0.1 ml. Incubations were performed at 37 °C for 2.5–10 min, and reactions were stopped by addition of a solution containing 12.5 mM-cyclic [³H]AMP (sp. radioactivity 3800 c.p.m./ μ mol) plus 40 mM-ATP and heating in a boiling-water bath for 3 min (Rodbell, 1967). Each sample was adjusted to 1 ml with water, and cyclic AMP was purified by the sequential column procedure (Dowex 50 and alumina) described by Salomon *et al.* (1974). According to the criteria previously described (Flawiá & Torres, 1972), by this assay procedure only cyclic AMP was detected as reaction product.

Other analytical procedures

Enzyme activities of *M_r* standards were measured as previously described (Kornblihtt *et al.*, 1981). Protein was measured by the procedure of Lowry *et al.* (1951).

RESULTS

Evidence of a 'non'-sedimentable' form of adenylate cyclase in root tissues

Table 1 shows the distribution of enzyme activity in different fractions obtained after three successive centrifugations of a root 'homogenate'; most of the enzyme activity was found in the 105000 *g* supernatant fluid. The possibility that this 'non-sedimentable' adenylate cyclase may be generated by the proteolytic breakdown of a membrane-bound enzyme could be discounted, since the presence of two different proteinase inhibitors in the extracting buffer did not modify the enzyme distribution. As a soluble adenylate cyclase, the alfalfa root enzyme could be subjected to purification procedures, in the absence of detergents, often employed with other soluble proteins. These procedures included ultracentrifugation, DEAE-cellulose chromatography (Fig. 1), gel filtration (Fig. 2) and centrifugation through sucrose gradients (Fig. 3). The two last methods was employed for the determination of hydrodynamic and molecular characteristics of the enzyme. The values for these parameters were as follows: sedimentation coefficient, 4.1 S; Stokes

Table 1. Distribution of adenylate cyclase activity after centrifugation of a homogenate of alfalfa roots

The homogenate (5 ml) was centrifuged at 10000 *g* for 20 min, and the supernatant fluid thus obtained was centrifuged for 120 min at 105000 *g*. Other conditions were given in the text and in the Experimental section.

Additions to Buffer A	Fraction	Total activity (pmol/min)	Specific activity (pmol/min per mg of protein)
None	Homogenate	8783	68.6
	10000 <i>g</i> sediment	46	14.0
	105000 <i>g</i> sediment	52	7.7
	105000 <i>g</i> supernatant	10480	630.0
5 mM-Phenylmethanesulphonyl-fluoride	Homogenate	8750	70
	10000 <i>g</i> sediment	50	15
	105000 <i>g</i> sediment	58	8
	105000 <i>g</i> supernatant	9960	650
Trasyolol (25 units/ml)	Homogenate	8690	71
	10000 <i>g</i> sediment	52	14.9
	105000 <i>g</i> sediment	59	8.3
	105000 <i>g</i> supernatant	11000	642

Fig. 1. DEAE-cellulose column chromatography of a 'crude extract' from alfalfa roots

○, Adenylate cyclase activity; ■, protein; —, [NaCl]. Other conditions were as described in the Experimental section.

radius, 4.4 nm; partial specific volume, 0.74 ml/g; M_r , 84000; and axial ratio, 1.54.

It is important to point out that amounts of adenylate cyclase activity in alfalfa roots can be affected by some undefined factors. Enzyme specific activities in the same plant increased from negligible values and decreased in periods of about 3–5 weeks.

Requirements of alfalfa root adenylate cyclase activity

Requirements for enzyme activity were studied in a partially purified enzyme preparation obtained after chromatography in DEAE-cellulose. As shown in Table 2, adenylate cyclase activity, measured with MgATP as substrate, was stimulated by Ca^{2+} ions. This stimulation was blocked by EDTA. In addition, the enzyme activity was stimulated by calmodulin from two different sources: bovine brain and spinach leaves. Such

stimulation was also blocked by EDTA and by the neuroleptic phenothiazine derivatives chlorpromazine and flufenazine.

As shown in Fig. 4(a), activation of alfalfa adenylate cyclase had a saturable dependence on bovine brain or spinach calmodulin, with a half-maximal stimulation at about 1.0 μg of the factor/ml. On the other hand, half-maximal inhibition by chlorpromazine was observed at 150 μM (Fig. 4b). This extent of inhibition was achieved at concentrations of chlorpromazine which were similar to those required to block the stimulatory action of brain calmodulin in *Neurospora crassa* cyclic AMP phosphodiesterase (Téllez-Iñón *et al.*, 1985).

In addition, the effects of some well-known modulators of animal, membrane-bound, adenylate cyclase activities were also studied. In this regard, GTP, guanosine 5'-[$\beta\gamma$ -imido]triphosphate, forskolin, fluoride or cholera toxin

Fig. 2. Gel filtration of a 'crude extract' from alfalfa roots

The inset show the relationship between K_{el} (elution-volume/exclusion-volume ratio) and Stokes radii for markers (●) and adenylate cyclase activity (○). Other conditions were described in the Experimental section. The standard proteins, shown at the top (Stokes radii in parentheses), were: G, β -galactosidase (6.84 nm); C, catalase (5.21 nm); M, malate dehydrogenase (3.69 nm); Cc, cytochrome *c* (1.87 nm). V_0 , exclusion volume.

(preactivated with dithiothreitol) had no effect on enzyme activity (results not shown).

DISCUSSION

The results indicate the existence in alfalfa roots of an adenylate cyclase activity with two important properties: (1) it is a 'non-sedimentable' enzyme activity, which is amenable to purification by procedures commonly used for other soluble, globular, proteins; and (2) it is activated by Ca^{2+} ions and calmodulin.

From an evolutive point of view, these characteristics of solubility and dependence on Ca^{2+} are not new facts. In the Fungi kingdom, *Neurospora* adenylate

Fig. 3. Sucrose gradient centrifugation of a 'crude extract' from alfalfa roots

Gradients were made in H_2O or $^2\text{H}_2\text{O}$. The inset shows the relationship between the position in the gradient (r) and sedimentation coefficients for markers (●) and adenylate cyclase activity (○). Other conditions were described in the Experimental section. The standard proteins, shown at the top, were: Cc, cytochrome *c* (1.7 S); M, malate dehydrogenase (4.3 S); L, lactate dehydrogenase (7.3 S); C, catalase (11.3 S).

Table 2. Effect of Ca^{2+} , EGTA, calmodulin and neuroleptic drugs on the activity of alfalfa root adenylate cyclase

A 'DEAE fraction' (40 μg of protein) was used as source of enzyme. Other conditions were described in the Experimental section.

Additions to the assay mixture	Specific activity (pmol/min per mg of protein)
None (Ca^{2+} omitted)	68
0.25 mM- CaCl_2	204
0.25 mM- CaCl_2 plus 1 mM-EGTA	74
0.25 mM- CaCl_2 plus brain calmodulin (0.3 $\mu\text{g}/\text{ml}$)	2856
0.25 mM- CaCl_2 plus brain calmodulin (0.3 $\mu\text{g}/\text{ml}$) plus 1 mM-EGTA	74
0.25 mM- CaCl_2 plus brain calmodulin (0.3 $\mu\text{g}/\text{ml}$) plus 100 μM -chlorpromazine	1529
0.25 mM- CaCl_2 plus brain calmodulin (0.3 $\mu\text{g}/\text{ml}$) plus 100 μM -flufenazine	1400
0.25 mM- CaCl_2 plus spinach calmodulin (0.3 $\mu\text{g}/\text{ml}$)	1680

Fig. 4. Relationship between adenylate cyclase activity and (a) concentration of bovine brain (●) or spinach (○) calmodulin, and (b) chlorpromazine concentration

Mixtures contained peak fractions corresponding to the sucrose-gradient centrifugation of a 'crude extract' made in H_2O (10 μg of protein). In (b), mixtures contained 0.5 μg of bovine brain calmodulin. Other conditions were described in the Experimental section.

cyclase activity has these two properties (Reig *et al.*, 1982, 1984). Moreover, in the Animalia kingdom, a soluble adenylate cyclase was found in mammalian testicular tissues, which might also be under the control of Ca^{2+} (Morton *et al.*, 1974; Braun & Dods, 1975; Kornblihtt *et al.*, 1981). From a regulatory point of view, it seems that eukaryotic cells have two different types of control mechanisms for cyclic AMP synthesis. One of them involves GTP-binding proteins (Gilman, 1984); the other may implicate the interaction with the Ca^{2+} -calmodulin system. In the particular case of alfalfa adenylate cyclase, the limited evidence available might suggest that this Ca^{2+} -dependent adenylate cyclase activity could be associated with proliferative responses in root tissues.

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