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CO-OPERATIVE INTERACTION OF cAMP WITH ITS BINDING PROTEIN FROM *PORTULACA GRANDIFLORA*

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Key Word Index—*Portulaca grandiflora*; Portulacaceae; callus; cyclic AMP; cyclic nucleotides; binding activity.

Abstract—3′:5′-Cyclic AMP (cAMP) binding proteins have been isolated from *Portulaca* callus. The binding activity is increased by cAMP as well as by other nucleotides and kinetin, indicating a co-operative interaction with the binding protein. A cAMP splitting phosphodiesterase as acceptor has been excluded.

INTRODUCTION

The occurrence and physiological role of 3′:5′-cyclic nucleotides in mammals and bacteria are fully documented. Much of the early evidence for the presence of cyclic AMP (adenosine 3′:5′-cyclic monophosphate; cAMP) in higher plants had been presumptive, being based on the observed physiological effects of the exogenously supplied nucleotide [1]. Recently, the occurrence of this nucleotide has been amply confirmed [2] but the physiological role remains unknown. In micro-organisms [3] and in mammalian tissues [4], there is a central role for cAMP and for a protein which specifically binds this nucleotide. In the callus of the red flowering variety of *Portulaca grandiflora* (var. JR), a specific influence of cyclic nucleotides (cAMP, cGMP) on betacyanin biosynthesis at the tyrosine-metabolizing stage was demonstrated by feeding experiments and the cyclic nucleotides identified [5]. The documented existence of a phosphodiesterase with allosteric regulation [6] supports a possible cAMP function in our callus. Nevertheless, the effects could be unphysiological [7]. Only the demonstration of specific cAMP-binding proteins in addition to the enzymes of the cAMP system would be a sound argument for the existence of a cAMP regulator system in plants. Indeed, such specific cAMP binding proteins have been extracted from various plants. None of the wide range of other cyclic and non-cyclic nucleotides is bound to anything like the same extent as cAMP [8]. I now report a specific cAMP binding protein with deviating behaviour from *Portulaca* callus.

RESULTS AND DISCUSSION

cAMP binding protein has been demonstrated in several plants [2]. In contrast to their behaviour, the binding capacity in *Portulaca* callus is increased by cAMP, several other nucleotides and kinetin (Table 3). This increasing effect of unlabelled cAMP on the binding of labelled cAMP demonstrates the high binding capacity of the isolated proteins. The bulk of this capacity was recovered in the 0–40% and the 70–100% saturation fractions of the $(NH_4)_2SO_4$ precipitation (Table 1). A significant inhibition was only reached in the 70–100% $(NH_4)_2SO_4$ fraction by cUMP (30%) and cCMP (32%). cIMP, cGMP, 3′-AMP, 5′-GMP and ATP had no significant effect. All other potential 'competitors' increased the binding capacity in the range of 127% (2′-AMP) to 377% (adenosine) at the optimum temperature (4°) over the original capacity. This suggests an allosteric-regulated co-operativity of the binding protein (perhaps hormone mediated). This high binding of cAMP is not connected with the earlier [6] demonstrated 2′:3′- and 3′:5′-cAMP splitting phosphodiesterase in the 40–70% range of $(NH_4)_2SO_4$ saturation. This enzyme was washed out of the DEAE-cellulose column before elution with the 100 mM KCl buffer (Table 2). Acetone precipitation of the initial crude ethanol extract produced a 200% increase in specific binding capacity but the overall purification obtained after the $(NH_4)_2SO_4$ precipitation was 249-fold

Table 1. Specific binding activity of the binding protein preparation during purification

Step	Specific binding activity (pmol cAMP bound/mg protein)
Crude extract after Amicon concn	0.016
After precipitation	0.049
DEAE-cellulose fraction	0.096
% $(NH_4)_2SO_4$ precipitation of the pooled protein fractions of the DEAE column	
0–20	1.67
20–30	4.0
30–40	2.93
40–50	0.95
50–60	0.43
60–70	0.72
70–80	4.4
80–90	2.87
90–100	1.9

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Table 2. Enzyme activities associated with the binding protein fraction during purification

Step	nkat/mg protein			
	Fructose-1,6-diphosphatase	Glucose-6-phosphatase	5'-Nucleotidase	Phospho-diesterase
Crude extract after Amicon concn	0.1	2.96	0.98	0.76
Acetone precipitation	0.3	0.81	2.15	1.17
DEAE- column eluate after Amicon concn				
(1) Washings	0.25	10.45	1.52	1.79
(2) 100 mM KCl fractions	1.21	8.91	0.97	0.01

Table 3. Effect of different potential substrate competitors on the amount of [8-^{3}H]cAMP bound

Non-radioactive competitors (10 μM)	% [8-^{3}H]cAMP bound by	
	% $(NH_4)_2SO_4$ fraction	
	0–40	70–100
None	100	100
3':5'-cAMP	+300	+364
3':5'-cUMP	0	−30
3':5'-cCMP	0	−32
2':3'-cAMP	+208	+178
2':3'-cGMP	+225	+217
2'-AMP	+38	+127
5'-AMP	0	−21
Adenosine	+337	+377
Adenine	+27	0
ADP	+139	+225
Kinetin	+306	+283

for the 20–30% fraction and 274-fold for the 70–80% fraction. This precipitation with $(NH_4)_2SO_4$, the dependence of optimal activity upon pH (7.4) for both fractions, characterized by a sharp peak, and the activity loss by boiling for 30 min demonstrates the protein character of the binding substances.

The presence of nucleotidase, glucose-6-phosphatase and fructose-1,6-diphosphatase activity in one fraction with binding activity is in accordance with the cyclic AMP-binding 5'-nucleotidase from wheat [9] and barley [8] seedlings. As previously reported, cAMP binding proteins from higher plants do not modulate protein kinase activities. Therefore, other possibilities must be taken into consideration. Despite the suggested sensitivity of fructose-1,6-diphosphate to cAMP [10], a possible activation of polyphenoloxidases [11] or, comparable to the situation in animals, activation of tyrosinase or DOPA decarboxylase must be taken into account in our betalain-synthesizing callus of *P. grandiflora*.

EXPERIMENTAL

Callus of *Portulaca grandiflora* Hook var. JR [12] was induced and cultivated as described [13]. After lyophilization, the homogenized callus was used for the tests.

Extraction and purification of cyclic nucleotide binding protein. Callus (3 g) was homogenized at 4° in aluminium–silicon oxide (white clay, Merck) (9 g) and polyclar AT (water-insoluble PVP, Serva) (3 g) containing EtOH–Tris–HCl (8:2; 100 mM, pH 7.4) buffer soln with cysteine (0.2 M), EDTA (1 mM) and $MgCl_2$ (2 mM). After filtration, the solid was washed with Me_2CO–Tris–HCl (99:1) (25 mM, pH 7.4) soln, complemented as above. The colourless Me_2CO dry powder was dissolved in the same Tris–HCl buffer, stirred for 30 min and centrifuged at 10000 *g* for 15 min. The soln was concd by Amicon filtration (YM 10). Protein was precipitated by Me_2CO (1.5-fold) and stirred for 30 min in a ice bath, centrifuged (15 min, 5000 *g*) and again suspended in the same buffer (50 mM, pH 7.4), complemented as described, concentrated, and the protein separated on a DEAE-cellulose (Whatman DE 52) column, equilibrated with the 25 mM Tris–HCl buffer. The cAMP binding protein was eluted by including KCl (100 mM) in the buffer [8]. The protein-containing fractions were collected and subjected to fractional precipitation with $(NH_4)_2SO_4$ at 4°. The separated fractions were dialysed overnight against the Tris–HCl buffer (50 mM).

Measurement of cyclic AMP binding activity. The cAMP binding activity was routinely determined using the charcoal method [8] with 0.5 μCi [8-^{3}H]cAMP (26 Ci/mmol, 250 μCi; Amersham Buchler).

Enzyme tests. 5'-Nucleotidase [14], fructose-1,6-diphosphatase [15], phosphodiesterase and glucose-6-phosphatase [6] were tested as described. Protein concn was determined [16] using bovine serum albumin as standard.

pH dependence. The conditions for incubation were as described for the binding assay except that the buffers were Na-succinate (0.2 M) (pH 3.8–6.0), Tris–Na-maleate (0.2 M) (pH 5.2–8.6) and Tris–HCl (0.2 M) (pH 7.2–9.1).

Specificity of the binding activity. The specificity of the binding process was examined using the assay procedure for [8-^{3}H]cAMP binding activity in the presence of various non-radioactive potential competitors. For each incubation, radioactive cAMP was present at a concn of 165 nM; the potential competitors were examined at 10 μM.

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