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A CYCLIC AMP BINDING-PROTEIN FROM BARLEY SEEDLINGS

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Abstract—A protein fraction extracted from barley seedlings was shown to bind 3′:5′-cyclic AMP. The binding effect is real and not due to interference with the standard binding-protein assay used. Evidence is presented that this is a specific binding-protein; even at high concentrations other protein fractions from the same source showed no affinity for cyclic AMP. None of a range of cyclic and non-cyclic nucleotides that were examined exhibited a degree of binding with the protein comparable to that with cyclic AMP. The cyclic AMP/binding protein complex has a K_d of 8 nM. This complex eluted at an identical position in the elution sequence from a Sephadex G-150 column as the uncomplexed binding-protein. The barley binding-protein is in a fraction which also exhibits the enzymic activities of glucose 6-phosphatase, ATPase, 5′-nucleotidase, and fructose 1,6-diphosphatase.

INTRODUCTION

The presence of cyclic AMP (adenosine 3′:5′-cyclic monophosphate) in the tissues of various species of higher plants has been reported by several authors, (e.g. refs. [1–6]). Recent work using a specific binding-protein assay and partially purified extracts, from which contaminants which interfere with the assay had been eliminated, has firmly established the presence of cyclic AMP in *Phaseolus vulgaris* and the concentration at which it occurs [6]. The two key enzymes of cyclic AMP metabolism, adenylate cyclase [7] and cyclic AMP phosphodiesterase [8–10] have also been demonstrated in the tissues of this and other species. Thus, the biochemical potential exists in plants for either a secondary messenger role for cyclic AMP analogous to that in animal tissues [11], or for a primary messenger role similar to that in certain bacteria [12].

In the primary messenger system of micro-organisms, there is a central role for a protein which specifically binds cyclic AMP and then initiates a train of metabolic events [12]. In the secondary messenger system in mammalian tissues, there is also a central role for a protein which specifically binds cyclic AMP; in this case the protein is the regulatory unit of a cyclic AMP-dependent protein kinase [13]. Our earlier work with *Phaseolus* [6] and other tissues [14] showed the presence of cyclic AMP-binding activity among proteins extracted from higher plants. We now describe the partial purification and properties of a specific cyclic AMP binding-protein from *Hordeum vulgare*. The main objective was to determine the extent to which the plant binding-protein is similar to that of either the microbial or the mammalian systems. It was hoped that this might give some indication of the role of the cyclic nucleotide in higher plants. *H. vulgare* was chosen for this work in preference to *P. vulgaris* because the cyclic AMP binding-protein from the former had a greater stability during the extraction and purification procedures.

RESULTS

After fractional precipitation with $(NH_4)_2SO_4$, the bulk of the cyclic AMP binding activity in a crude buffered extract of barley seedlings was recovered in the 45–55% saturation fraction. Dialysis of the initial crude extract produced a 50% increase in specific binding activity (Table 1) but the overall purification obtained after the steps shown in Table 1, and culminating in elution from DEAE-cellulose, was 125-fold. Further resolution of this latter fraction by gel-filtration through a column of Sephadex G-150 yielded a single peak of cyclic AMP binding activity at an elution volume of 31 ml. This corresponds to a MW of 1.7×10^5. Incubation of [8-^{3}H]-cyclic AMP with the active fraction obtained from the DEAE-cellulose column produced a radioactive complex which eluted at the same position in the elution sequence from the Sephadex G-150 column as a control sample of the binding-protein that had not been pre-incubated with radioactive cyclic AMP.

The pH optimum for the binding of cyclic AMP by the partially purified protein was found to be at pH 6.5; it was noted that binding activity decreases sharply below pH 6 and above pH 7.5. A plot of data from a time-course study showed the relationship between the amount of cyclic AMP bound and duration of incubation to be that of a rectangular hyperbola. Under the specified assay conditions, maximum binding occurred at 60 min; half-maximum binding was at 15 min.

The specificity of the binding-protein was examined by carrying out the binding assay in the presence of individual potential competitors. The competitors were examined at the same concentration as the radioactive cyclic AMP (165 nm) and also at a concentration (10 μM) *ca* 60-times that of the radioactive cyclic AMP. The validity of the assay was demonstrated by the 50% inhibition of binding of radioactive cyclic AMP produced by an equal concentration of non-radioactive cyclic AMP, and the 100% inhibition of binding produced by a 60-fold

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

Step	Specific binding activity (pmol cyclic AMP bound/mg protein)
Crude extract	0.04
Dialysed crude extract	0.06
Sephadex G-25 fraction	4.06
DEAE-cellulose fraction (100 mM KCl)	5.20

Table 2. Specificity of binding-protein as shown by the effect of potential substrate competitors on the amount of [8-^{3}H]-cyclic AMP bound

Non-radioactive competitor	% [8-^{3}H]-cyclic AMP bound in presence of: 165 nM competitor	10 μM competitor
None	100	100
3′:5′-cyclic AMP	48.9	0
2′:3′-cyclic AMP	97.8	96.7
3′-AMP	77.8	23.8
2′-AMP	100	98.0
5′-AMP	87.8	20
ADP	76.7	24.4
ATP	76.7	32.2
Adenosine	77.8	11.0
Adenine	100	75.5
3′:5′-cyclic dAMP	72.2	17.2
3′:5′-cyclic GMP	85.6	34.4
2′:3′-cyclic GMP	84.4	70.0
N^6,2′-*O*-Dibutyryl 3′:5′-cyclic AMP	100	94.3
3′:5′-cyclic IMP	98	40.0
3′:5′-cyclic XMP	100	96.0
3′:5′-cyclic TMP	100	98.6
3′:5′-cyclic dTMP	100	100
5′-GMP	81	41
6-Benzylaminopurine	100	100
6-Furfurylaminopurine	100	100

excess of non-radioactive cyclic AMP. No significant inhibition of binding was caused by 2′:3′-cyclic AMP, 2′-AMP, N^6,2′-*O*-dibutyryl cyclic AMP, cyclic XMP, cyclic TMP, cyclic dTMP, 6-benzylaminopurine and 6-furfurylaminopurine (Table 2). It is, therefore, concluded that none of these compounds is bound by the binding-protein. From Table 2, it can be seen that the binding-protein does, however, have some affinity for 3′-AMP, 5′-AMP, ADP, adenosine, cyclic 3′:5′-dAMP, 3′:5′-cyclic GMP, 5′-GMP and 2′:3′-cyclic GMP, but these compounds are bound to a lesser extent than cyclic AMP. With 3′:5′-cyclic IMP there was negligible binding at the lower concentration but significant binding at the higher concentration. That the binding of the cyclic nucleotide is attributable to a protein was confirmed by enzymic proteolysis. Pre-incubation of the active fraction with trypsin, resulted in a complete loss of binding activity. If, however, trypsin-inhibitor was also present during the pre-incubation with trypsin, the fraction subsequently showed similar activity to the untreated control in binding [8-^{3}H]-cyclic AMP.

In the current work, the demonstration and investigation of the plant binding-protein is based upon the standard binding-protein assay procedure. In this, unbound cyclic AMP is removed from the incubates by adsorbing it on charcoal and centrifuging. To check that the binding effect with the plant protein was real and not merely due to the protein decreasing the efficiency of the charcoal adsorption step, the following two sets of incubations were set up, both in triplicate. One set contained all the components of the standard binding-protein assay including the plant binding-protein (200 μg of the protein fraction eluted from the DEAE-cellulose column with 100 mM KCl as described in Experimental). The other set contained all the components except the binding-protein. Following incubation of both sets of tubes, charcoal was added to terminate the reaction and the tubes were centrifuged. Samples (100 μl) of the supernatants were taken for determination of radioactivity. Other similar samples were removed into clean tubes, made up to 200 μl with the assay buffer (Tris/cysteine/EDTA/$MgCl_2$), and a second addition of charcoal was made. The charcoal and the amount used were as for the standard assay procedure. After centrifuging, samples (100 μl) were again removed and their radioactivities determined.

The results of this experiment (Table 3) show that after the first addition of charcoal in the absence of binding-protein, 5700 dpm of the original 393 600 dpm remain in solution; the charcoal adsorption is, therefore, 98.5% efficient. If the radioactivity determined in the supernatants of those incubations containing binding-protein is corrected for this, there are still 19 300 dpm remaining in solution. Addition of the second portion of charcoal to these tubes gave a supernatant with a radioactive count of 20 000 dpm, i.e. no further adsorption of radioactive cyclic AMP occurred. Since the binding-protein concentration was not increased between the first and second additions of charcoal, the only explanation for 93.6% of the radioactivity being removed in the first step and none being removed in the second, is that all the [8-^{3}H]-cyclic AMP remaining in solution is bound in a form unavailable for adsorption.

By a Scatchard plot [15] of equilibrium binding data, which gave a good linear relationship between $[S_b]$ and $[S_b]/[S_f]$, the dissociation constant for the cyclic AMP

Table 3. Charcoal adsorption of [8-^{3}H]-cyclic AMP in presence and absence of the binding-protein from barley

Stage	Radioactivity in supernatant (dpm/100 μl) Binding-protein present	Binding-protein absent
Initial incubation (0 min)	393 600	393 600
After 1st addition of charcoal	25 000	5700
After 2nd addition of charcoal	20 000	105

Incubation conditions were as described in the text for the standard binding-protein assay of cyclic AMP.

Table 4. Cyclic AMP phosphodiesterase activity of binding-protein fraction during purification

Step	Cyclic AMP phosphodiesterase activity (pkat/mg protein)
Crude extract	9.3
Dialysed crude extract	9.5
$(NH_4)_2SO_4$ ppt. (45–55% satn)	4.0
Sephadex G-25 fraction	4.0
DEAE-cellulose column washings	15.5
DEAE-cellulose fraction (100 mM KCl)	1.5
DEAE-cellulose fraction (200 mM KCl)	1.0

binding-protein complex was calculated to be 8 nM. Comparison of the results in Table 4 with those in Table 1 shows that the binding of cyclic AMP described in this work is not attributable to binding onto a catalytically active or inactive cyclic AMP phosphodiesterase. The binding activity and the phosphodiesterase activity are demonstrably separable on DEAE-cellulose by which the binding activity is retained and has to be eluted whereas the phosphodiesterase is not retained (Tables 1 and 4). The results presented in Table 5 show that the binding-protein is present in a fraction containing glucose 6-phosphatase, ATPase, fructose 1,6-diphosphatase and 5′-nucleotidase.

DISCUSSION

That the binding of cyclic AMP described in this work is attributable to protein was demonstrated by the gel-filtration behaviour of the active fraction, by its precipitation with $(NH_4)_2SO_4$, its behaviour upon dialysis, abolition of activity following incubation with trypsin and by dependence of optimal activity upon pH. The results show that the binding effect is real and that the activity is specific and not due to non-specific binding by protein. This is substantiated by the finding that only one fraction is active and that the others, even at very high concentrations, showed no affinity for cyclic AMP. Further, Table 2 shows that none of a wide range of other cyclic and non-cyclic nucleotides is bound to anything like the same extent as cyclic AMP.

It has been previously reported [16] that cyclic AMP binding-proteins obtained from higher plants do not modulate protein kinase activity. The presence of nucleotidase, ATPase, glucose 6-phosphatase and fructose 1,6-diphosphatase activity in the same fraction as the cyclic AMP binding activity is similar to the cyclic AMP-binding 5′-nucleotidase from wheat seedlings [17] but the latter also binds cyclic GMP very tightly. The barley binding-protein has, however, a much lower affinity for cyclic GMP and as shown by the K_d values, binds cyclic AMP much more tightly than was reported for the proteins from other plant tissues [16, 17]. The K_d of 8 nM for the barley binding-protein is of the same order as that (2–3 nM) of a typical mammalian cyclic AMP binding-protein, i.e. that of bovine skeletal muscle [18].

The properties of the binding-protein that we have studied do not readily categorize it as either an 'animal-type' protein kinase, or, a 'microbial-type' cyclic nucleotide receptor-protein. Both possibilities remain, as does the possibility that a completely different function is served by the cyclic AMP binding-protein of higher plants. In this connexion, it may be of significance that the latter is associated with nucleotidase, ATPase, glucose phosphatase, and fructose 1,6-diphosphatase, especially as it is suggested that this last enzyme may be sensitive to cyclic AMP concentration [19].

EXPERIMENTAL

Seeds of *Hordeum vulgare* L. cv Astrix were washed in running H_2O for 24 hr and germinated in moist vermiculite at 24°. Seedlings were grown for 7–8 days in a light cycle of 18 hr light (5.5 klx) and 6 hr dark.

Extraction and purification of cyclic nucleotide binding-protein. Leaves (250 g) were homogenized at 4° in 500 ml of 100 mM Tris–HCl buffer (pH 7.4) containing 0.2 mM cysteine, 1 mM EDTA and 2 mM $MgCl_2$. After filtering the homogenate through cotton-gauze, the filtrate was centrifuged at 10000 *g* for 20 min at 4°. The supernatant was decanted and subjected to fractional precipitation with $(NH_4)_2SO_4$ at 4°. The ppt. obtained between 45 and 55% satn with $(NH_4)_2SO_4$ was collected by centrifugation, resuspended in a minimal vol. of 25 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA, 0.2 mM cysteine and 2 mM $MgCl_2$, and desalted on a Sephadex G-25 (fine) column (2.2 cm × 14 cm) previously equilibrated with the same buffer. The desalted extract was loaded onto a DEAE-cellulose (Whatman DE 52) column (1.5 cm × 13.5 cm) that had previously been equilibrated with 25 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA and 2 mM $MgCl_2$. After washing the column with the same buffer, the cyclic AMP binding-protein was eluted by including KCl (100 mM) in the buffer. The fractions containing binding-protein activity were pooled and dialysed for 16 hr against 50 mM Tris–HCl buffer (pH 7.4), containing 0.2 mM cysteine, 1 mM EDTA and 2 mM

Table 5. Enzymic activities associated with the binding-protein fraction during purification

Step	nkat/mg protein			
	Glucose 6-phosphatase	ATPase	Fructose 1,6-diphosphatase	5′-Nucleotidase
Crude extract	1.98	5.43	4.04	4.03
Dialysed crude extract	2.61	5.30	2.42	4.13
Sephadex G-25 fraction	7.81	7.68	9.82	9.60
DEAE-cellulose washings	12.95	30.50	2.22	15.00
DEAE-cellulose fraction (100 mM KCl)	10.41	4.75	4.00	13.88

$MgCl_2$. The dialysed protein fraction was concd to 6 ml by ultrafiltration through an Amicon UM 10 membrane.

Measurement of cyclic AMP binding activity. The cyclic AMP binding activity of plant extracts containing up to 1 mg of protein was routinely determined in triplicate in a medium (200 μl) containing 50 mM Tris–HCl buffer (pH 7.4), 0.2 mM cysteine, 1 mM EDTA, 2 mM $MgCl_2$, 0.5 μCi [8-^{3}H]-cyclic AMP (sp. act. 30 Ci/mmol) to a final concn of 83 nM. Following incubation at 0° for 1 hr, 100 μl of a charcoal suspension were added. This contained 10 mg of charcoal in a 2% (w/v) soln of bovine serum albumin (BSA) in the assay buffer. After shaking, the tubes were centrifuged at 4000 *g* for 20 min. The radioactivity of aliquots of the supernatant was determined by liquid scintillation counting as previously described [6]. Adsorption of unbound cyclic nucleotide by the added charcoal was not 100% efficient; results were therefore corrected by subtracting the radioactivity determined for the supernatant of a control incubation from which the protein had been omitted.

Measurement of enzyme activities and determination of protein concentration. Cyclic nucleotide phosphodiesterase activity and that of the enzymes ATPase, fructose 1,6-diphosphatase, glucose 6-phosphatase and 5′-nucleotidase were determined as previously described [9, 10]. Protein concn was determined by the method of ref. [20] using BSA to construct a standard curve over the range 50 μg/ml.

Estimation of MW of binding-protein. The fraction containing the cyclic AMP binding-activity (60 mg protein) was eluted from the DEAE-cellulose column and passed through a column of Sephadex G-150 (fine; 2.2 cm × 20 cm) in 50 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA, 0.2 mM cysteine and 2 mM $MgCl_2$. The Sephadex column had been previously equilibrated with the same buffer. Fractions (1.5 ml) were collected and assayed for cyclic AMP binding activity. Using catalase, aldolase, BSA, chymotrypsinogen A and cytochrome *c* as standards, the MW of the binding-protein was calculated from the elution vol. as described in ref. [9].

Treatment of binding-protein with trypsin. A 1 ml sample (2.5 mg protein) of the binding-protein fraction was incubated with 100 μl of trypsin (0.5 mg/ml) for 60 min at 30°. The reaction was terminated by adding trypsin-inhibitor (Boehringer) at a concn of 80 mg/ml of incubate. Two controls were included; in one, the plant protein was incubated alone, and in the other, it was incubated together with trypsin and trypsin-inhibitor. After incubation, aliquots (100 μl) from each treatment were assayed for cyclic AMP binding activity.

Gel-filtration of binding-protein/cyclic AMP complex. A sample (1.2 ml) of the fraction containing the binding-activity was incubated with [8-^{3}H]-cyclic AMP as described for the assay of binding-protein. Following addition of the charcoal, the incubate was centrifuged and a portion of the supernatant, containing 3 × 10^5 dpm, was loaded onto the Sephadex G-150 column described above for MW determination. Elution was with the same buffer as before; 1 ml fractions were collected and their radioactivity measured.

pH-Dependence of cyclic AMP binding. Incubation conditions were as described for the assay of binding-protein except that the buffers were 50 mM K succinate (pH 4–6) or 50 mM Tris–HCl (pH 7–9). Binding-activity was determined at pH 4.2, 5.4, 6.6, 7.4, 8 and 8.8.

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

Dissociation constant of cyclic AMP/binding protein complex. Cyclic AMP binding-activity was determined at the following concns of cyclic AMP: 8.5, 16.7, 33.3, 66.7, 100, 133 and 167 nM. The procedure was that described above. The binding constant was derived from a Scatchard plot [15] of S_b against S_b/S_f, where S_b and S_f are substrate bound and free, respectively.

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REFERENCES

1. Brown, E. G. and Newton, R. P. (1973) *Phytochemistry* **12,** 263.
2. Raymond, P., Narayanan, A. and Pradet, A. (1973) *Biochem. Biophys. Res. Commun.* **53,** 1115.
3. Giannattasio, M. and Macchia, V. (1973) *Plant Sci. Letters* **1,** 259.
4. Brewin, N. J. and Northcote, D. M. (1973) *J. Exp. Botany* **24,** 881.
5. Ashton, K. and Polya, G. M. (1977) *Biochem. J.* **165,** 27.
6. Brown, E. G., Al-Najafi, T. and Newton, R. P. (1979) *Phytochemistry* **18,** 9.
7. Smith, C. J., Brown, E. G., Newton, R. P., Al-Najafi, T. and Edwards, M. J. (1979) *Biochem. Soc. Trans.* **5,** 1351.
8. Brown, E. G., Al-Najafi, T. and Newton, R. P. (1977) *Phytochemistry* **16,** 1333.
9. Brown, E. G., Edwards, M. J., Newton, R. P. and Smith, C. J. (1979) *Phytochemistry* **18,** 1943.
10. Brown, E. G., Edwards, M. J., Newton, R. P. and Smith, C. J. (1980) *Phytochemistry* **19,** 23.
11. Robison, G. A., Butcher, R. W. and Sutherland, E. W. (1971) *Cyclic AMP.* Academic Press, New York.
12. Pastan, I. R. and Perlman, R. L. (1972) *Adv. Cyclic. Nucleotide Res.* **1,** 11.
13. Langan, T. A. (1973) *Adv. Cyclic Nucleotide Res.* **6,** 99.
14. Smith, C. J., Brown, E. G., Newton, R. P. and Edwards, M. J. (1979) *Biochem. Soc. Trans.* **6,** 1268.
15. Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51,** 660.
16. Giannattasio, M., Carratu, G. and Tucci, G. F. (1974) *FEBS Letters* **49,** 249.
17. Polya, G. M. and Sia, J. P. H. (1976) *Plant Sci. Letters* **7,** 43.
18. Gilman, A. G. and Murad, F. (1974) in *Methods in Enzymology* (Hardman, J. G. and O'Malley, B. W., eds.) Vol. 38, p. 56. Academic Press, New York.
19. Clark, M. G., Kneer, N. M., Bosch, A. L. and Lardy, M. A. (1974) *J. Biol. Chem.* **249,** 5695.
20. Lowry, O. H., Roseborough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265.