Phytochemistry, 1979, Vol. 18, pp. 1613–1616. © Pergamon Press Ltd. Printed in England. 0031-9422/79/1001-1613 $02.00/0

CYCLIC AMP BINDING PROTEIN FROM JERUSALEM ARTICHOKE RHIZOME TISSUES

MATTEO GIANNATTASIO, GIOVANNA CARRATÙ, GIAN FRANCO TUCCI and ANNA MARIA CARAFA

Istituto Botanico, Facoltà di Agraria, 80055 Portici, Napoli, Italy

(*Revised received* 31 *April* 1979)

Key Word Index—*Helianthus tuberosus*; Compositae; Jerusalem artichoke rhizome tissues; cyclic AMP; adenosine; adenine derivatives; binding protein; protein kinase; chromatin-bound DNA dependent RNA polymerase.

Abstract—A cyclic AMP binding protein has been purified to electrophoretic homogeneity from Jerusalem artichoke rhizome tissues. Its MW is *ca.* 240 000 and the apparent constant of cyclic AMP binding to the protein is 2.3×10^{-7} M. When tested using Millipore filter assay, cyclic AMP binding activity was enhanced by protamine and histone, but not by casein and phosvitin. Of several purine derivatives tested, only 5′-AMP and adenosine inhibited significantly the binding of cyclic AMP by the protein. The protein also binds adenosine and this binding is not affected by cyclic AMP or by other purine derivatives. The apparent binding constant for adenosine is 1.0×10^{-6} M. The binding protein did not show protein kinase activity. In addition, it did not affect the chromatin-bound DNA dependent RNA polymerase of homologous origin, either in the presence or absence of cyclic AMP. The binding protein is devoid of the following activities: cyclic AMP phosphodiesterase, 5′-nucleotidase, adenosine deaminase and ATPase.

INTRODUCTION

We have previously reported the presence of a cyclic AMP binding protein in the soluble fraction isolated from Jerusalem artichoke rhizome tissues [1]. Attempts to show that this protein is involved in the functioning of a cyclic AMP dependent protein kinase failed. Indeed, although protein kinase and cyclic AMP binding protein co-purified, protein kinase activity was unaffected by cyclic AMP. We have further purified the cyclic AMP binding protein and have been able to separate this from the associated protein kinase. In this paper we shall describe the purification procedure adopted, and some properties of the binding protein. The receptor for cyclic AMP appears to bind adenosine as well.

RESULTS

Purification

The results of a typical purification of the binding protein are summarized in Table 1. A purification of *ca* 200-fold with a recovery of 12% of total activity was obtained. Photometric scanning of an electrophoretic disc-gel of the purified fraction (after the Sephadex G-200 chromatography step) stained with Coomassie blue revealed a major protein band and several minor bands. The major band contained 60–70% of total protein and all of the binding activity. Further purification of the binding protein was performed with preparative electrophoresis as reported in Experimental. After this purification step, the binding protein preparation showed a single band component on polyacrylamide gel electrophoresis.

Properties of cyclic AMP receptor

When tested at 30°, the cyclic AMP binding activity was linear as a function of time for 2 min (and reached a

Table 1. Purification of the cyclic AMP binding protein

Step of purification	Specific activity (pmol cyclic AMP bound/mg protein)	Recovery (% of total activity)
105 000 *g* supernatant after dialysis	5	100
40–80% $(NH_4)_2SO_4$ precipitation	8	92
DEAE-cellulose column chromatography	40	44
Hydroxylapatite column chromatography	125	32
Sephadex G-200 column chromatography	630	24
Disc-gel electrophoresis	1020	12

Details of the fractionation procedure are given in Experimental. Cyclic AMP binding activity was tested by Millipore filtration assay.

Table 2. Effect of varying amount of protamine and histone on the binding of cyclic AMP by receptor

Substance added (μg/reaction mixture)		Binding activity measured by Millipore filter assay (pmol of cyclic AMP-[^{3}H])	Binding activity measured by Sephadex G-25 filtration (pmol of cyclic AMP-[^{3}H])
None		0.8	8.2
Protamine	10	4.2	8.3
	20	4.8	8.3
	40	3.6	8.3
Histone	10	4.0	8.2
	20	4.2	8.3
	40	3.4	8.3

Each reaction mixture contained 8 μg of the binding protein.

plateau after 4 min) and as a function of protein concentration (in the range between 1 and 100 μg/reaction mixture). The optimal pH was in the range 6–7 and Mg^{2+} was not required for the binding activity. The apparent binding constant of cyclic AMP to receptor, estimated by determining the amount of bound and free cyclic AMP at various cyclic AMP concentrations and constructing a Scatchard plot [2], was 2.3×10^{-7}. Using Sephadex G-200 chromatography, an apparent MW of 240000 was obtained. The cyclic AMP binding capacity of the receptor was completely destroyed after heat treatment for 5 min at 100°. About 70% of the binding activity was destroyed when the receptor was incubated at 30° in the presence of 10 μg of pronase for 30 min. DNAse and RNAse treatments were ineffective. The above results confirm our previous conclusion on the protein nature of the receptor [1].

Comparison between Millipore filter and Sephadex G-25 assays

When tested using Millipore filter assay, cyclic AMP binding activity was enhanced by the basic proteins, protamine and histone (Table 2), but not by casein and phosvitin (data not shown). Protamine and histone did not themselves bind an appreciable amount of cyclic AMP. They were effective when added either before or after incubation (unpublished). In addition to the Millipore filter assay, we used an alternative method employing Sephadex G-25 filtration of the cyclic AMP-bound protein complex as we have previously reported [1]. When tested with this method (Table 2), cyclic AMP binding activity was unaffected by histone and protamine; in addition, the binding activity was 2–3 times higher than that calculated by Millipore filter assay in the presence of optimal protamine concentration. These results indicate that protamine and histone improve retention of cylcic AMP-bound protein complex on the filter rather than the binding activity itself. Although Sephadex G-25 filtration assay gave higher values of binding activity, we routinely used the Millipore filter assay because of its simplicity and rapidity.

Inhibition effect of purines on cyclic AMP binding

The effect of various purine derivatives (namely guanine and adenine derivatives) on the cyclic AMP binding was tested. As reported in Table 3, among these substances, only some adenine derivatives were inhibitory. At 5×10^{-7} M concentration, the inhibitory compounds were, in order of decreasing effectiveness, cyclic AMP and adenosine to a similar extent (60% of inhibition): 5′-AMP (50%), ADP (20%), ATP (10%). 2′,3′-Cyclic AMP, 3′-AMP, the cytokinin isopentenyladenosine, guanosine, 3′-GMP, 5′-GMP, GDP, 3′,5′-cyclic GMP and 2′,3′-cyclic GMP were ineffective. We also tested the binding capacity of the receptor for adenosine, which was inhibitory towards the cyclic AMP binding activity, by replacing in the reaction mixture the radioactive cyclic AMP-[^{3}H] with an equimolar concentration of adenosine-[^{3}H] (5.25×10^{-7} M). We found that the protein also binds adenosine; when the binding was tested with the Millipore filter assay and in the presence of 8 μg of binding protein, 4.5 pmol of bound adenosine-[^{3}H] were found. The apparent binding constant for adenosine was 1.0×10^{-6} M. Surprisingly, while cold 5×10^{-5} M adenosine almost completely inhibited the binding to adenosine-[^{3}H], cold cyclic AMP as well as 5′-AMP, 3′-AMP, ADP and ATP (all tested at 5×10^{-5} M) was ineffective. In addition, while the binding capacity of the protein preparation to adenosine-[^{3}H] was stable in the long run, the ability to bind cyclic AMP-[^{3}H] decreased quickly and disappeared after storage for one week at −20°. These observations suggest that the protein exhibits two different sites of binding, one for cyclic AMP and the other specific for adenosine

Attempts to show a relationship between cyclic AMP receptor and protein kinase

It is well known that the cyclic AMP dependent protein kinase derived from animal sources is composed of two dissimilar functional subunits: a regulatory subunit which binds cyclic AMP and a catalytic subunit. In the absence of cyclic AMP, protein kinase exhibits a low basal activity because the regulatory subunit inhibits it. Cyclic AMP activates protein kinase *in vitro* by binding to the regulatory subunit, which exhibits maximal activity [3]. We explored the possibility that cyclic AMP binding protein isolated from Jerusalem artichoke was really the regulatory subunit of cyclic AMP dependent protein kinase. The previous observation [1] that cyclic AMP binding activity co-purified with a protein kinase in the initial steps of purification suggested such a possibility. However, when tested with casein, histone and phosvitin as exogenous substrates, the purified protein did not show protein kinase activity, either in the absence or in the presence of cyclic AMP (unpublished). In addition, it did not show an inhibitory effect on both soluble and membrane-bound protein kinase of homologous origin (unpublished).

Effect of cyclic AMP binding protein on chromatin-bound dependent RNA polymerase isolated from Jerusalem artichoke rhizome tissues

It has been shown that in a bacterial cell-free system cyclic AMP binds to specific receptor protein and stimulates the transcription of *m*RNA [4]. Furthermore, it has been found that in mammalian systems [5] cyclic AMP stimulates RNA synthesis. We tested the effect of the cyclic AMP binding protein from Jerusalem artichoke on the chromatin-bound DNA dependent RNA polymerase of homologous origin. In the experimental conditions used, and in the absence of the binding protein, 4000–4500 cpm of UTP-[^{3}H] were incorporated; this activity was unaffected by the cyclic AMP binding protein (tested in the range 1–20 μg) either in the presence or absence of cyclic AMP (tested in the range 10^{-7}–10^{-5} M) as well as by cyclic AMP alone (at 10^{-5} M concentration).

Determination of some enzymatic activities

The purified cyclic AMP binding protein was devoid of the following activities: cyclic AMP phosphodiesterase, 5′-nucleotidase, adenosine deaminase and ATPase. In order to determine if cyclic AMP-[^{3}H] and adenosine-[^{3}H] were altered upon binding, the cyclic AMP-[^{3}H]-bound protein complex or the adenosine-[^{3}H]-bound protein complex were separated by Sephadex G-25, boiled for 1 min and subjected to chromatography on PEI-cellulose thin layer plates. More than 90% of radioactivity moved with authentic cyclic AMP and adenosine, respectively, indicating that no alteration occurred after binding.

DISCUSSION

Following our previous observations [1] concerning the presence of a cyclic AMP binding protein in the soluble fraction isolated from Jerusalem artichoke rhizome tissues, we have purified to electrophoretic homogeneity and characterized this binding protein. Although protein kinase activity and cyclic AMP binding co-purified in the initial steps of purification, we succeeded in resolving the two activities by means of hydroxylapatite column chromatography. The highly purified preparation of cyclic AMP binding protein does not exhibit protein kinase activity either in the absence or presence of cyclic AMP. Furthermore, from the various experiments performed to examine for any possible relationship between the binding protein and protein kinases of homologous origin, it appears that the binding protein is not the regulatory subunit of a cyclic AMP dependent protein kinase (unpublished). Moreover, no catalytic activity can yet be assigned to it.

A protein has been recently purified from wheat germ that binds cyclic AMP [6]. Although similar in many respects, namely MW, pH optimum, chromatographic behaviour on DEAE-cellulose, absence of protein kinase activity, inhibitory response to adenosine and other adenine derivatives, Jerusalem artichoke and wheat germ cyclic AMP binding proteins appear different as far as the response to 2',3'-cyclic AMP and 3'-AMP is concerned; in fact, these compounds were reported to be inhibitory for wheat germ binding protein, whereas they were ineffective for Jerusalem artichoke binding protein.

The inhibitory effect of 5'-AMP on the binding of cyclic AMP to the highly purified protein receptor is surprising, since this nucleotide had been found ineffective when tested on an almost crude preparation from the same tissue source [1]. The explanation for this discrepancy could be that crude preparation contains factor(s) interfering with the inhibitory action of 5'-AMP on the binding of cyclic AMP to the receptor (for instance, protein that specifically binds 5'-AMP or 5'-AMP destroying enzyme as 5'-AMP deaminase) and that the interfering factor is removed by the purification procedure adopted.

The binding protein from Jerusalem artichoke binds adenosine in addition to cyclic AMP. Our preliminary observations (the binding of adenosine may exclude the binding of cyclic AMP but not vice versa and further the binding capacity of adenosine is stable whereas that of cyclic AMP is not) suggest that the protein exhibits two different sites of binding, one for cyclic AMP and adenosine, and the other specific for adenosine.

Cyclic AMP–adenosine binding protein has also been found in animal tissues as rabbit erythrocytes [7], mouse liver [8], bovine and rat liver [9]. Since in mammalian tissues cyclic AMP and adenosine metabolism appears to be interrelated [10, 11] and adenosine plays a role of metabolic and physiological regulator [12–15], it has been proposed that cyclic AMP–adenosine binding protein is involved in these processes [7–10].

EXPERIMENTAL

Materials. Dormant rhizomes of Jerusalem artichoke (*Helianthus tuberosus* L. cv Violet de Rennes) were harvested in November and stored at 4° for 4–6 months before experimental use. All unlabelled nucleotides were obtained from Bohringer. Histone (type IIa) and phosvitin were purchased from Sigma; BsA, protamine sulfate and casein from BDH; DEAE-cellulose (DE 52) from Whatman; cellulose–ester membrane filters (HA 0.45 μ) from Millipore. ATP-[γ-^{32}P] (sp. act. 17 Ci/mmol), cyclic AMP [8-^{3}H] (sp. act. 27 Ci/mmol), ATP-[2-^{3}H] (sp. act. 27 Ci/mmol) and adenosine-[2-^{3}H] (sp. act. 20 Ci/mmol) were obtained from Amersham. Radioactive purine derivatives were purified by TLC using the solvent systems previously reported [16].

Purification of cyclic AMP binding protein. All operations were carried out at 4°. The initial purification steps were essentially similar to those previously reported [1] except that $(NH_4)_2SO_4$ precipitation was performed at 40–80% satn. The 40–80% $(NH_4)_2SO_4$ ppt., containing both protein kinase and cyclic AMP binding activity, was collected by centrifugation and dissolved in 25 mM Tris–HCl buffer, pH 7.6, containing 1 mM 2-mercaptoethanol (buffer A) and dialysed overnight with several changes of buffer A. 25 ml of the above fraction (containing 232 mg of protein) were applied to a 2.6 × 15.4 cm DEAE-cellulose column which had previously been equilibrated with buffer A. Following adsorption of the protein, the column was washed with 250 ml buffer A and then eluted with a 500 ml linear gradient of 0 to 0.2 M KCl in the buffer A. Fractions (*ca* 5 ml) were collected and assayed for cyclic AMP binding capacity and kinase activity. When the protein kinase activity was tested using casein as exogenous substrate, two peaks of activity were resolved from DEAE-cellulose column, one eluting at about 0.06 M and the other at 0.1 M KCl. One peak of cyclic AMP binding activity was found and it was coincident with the protein kinase peak eluting at 0.1 M KCl. The cyclic AMP binding fractions were pooled and $(NH_4)_2SO_4$ was added to 80% satn. The resulting ppt. was centrifuged, dissolved in 5 mM K-Pi buffer, pH 7, containing 1 mM 2-mercaptoethanol (buffer B) and dialysed against the same buffer. The enzyme soln was subsequently applied to a hydroxylapatite column (0.9 × 12.6 cm) which had been previously equilibrated with buffer B. This step separated the binding protein from the protein kinase. In fact, the binding protein was not adsorbed to the gel and emerged at the breakthrough, while protein kinase remained adsorbed to the gel and could be eluted in two peaks by application of a linear gradient of 5 to 300 mM KCl. The binding fractions were pooled, concd by Diaflo ultrafiltration and dialysed overnight against buffer A. The protein soln was chromatographed on a Sephadex G-200 column (1.6 × 84 cm) previously equilibrated with buffer A and elution was performed with the same buffer at an upward flow rate of *ca* 5 ml/hr. The binding protein emerged as a single peak eluting at a vol. of 66 ml. The active fractions were pooled, concd by Diaflo filtration and kept at −20° in 20% glycerol until required. All the expts concerning binding protein characterization were performed with this prepn except for the MW determination and the tests for inhibitory action of purine derivatives on the binding activity either to cyclic AMP or to adenosine. In these latter cases, a prepn further purified by electrophoresis was used. Preparative electrophoresis was performed essentially as described by Yuh and Tao [7]. The buffer system used was Tris (3 g/l.)–glycine (14.4 g/l.), pH 8.3. A separating gel of 7.5% and a stacking gel were polymerized in a 5 mm × 6 cm glass column. About 150 μl of protein binding fraction after Sephadex G-200 containing 36 μg of protein in 20% sucrose were applied to each of the gels. Electrophoresis was performed at 4° at 2 mA/gel for *ca* 2 hr until the dye (bromophenol blue) front was 5.2 cm. Some gels were stained with Coomassie blue; the others were sliced laterally in 2 mm thick sections by a mechanical slicer and each section was eluted with 150 μl of buffer A for 3 hr at 4°. The elution buffer was separated from the gel section by aspiration and tested for binding activity. Binding activity was found as a single peak

coincident with a protein band migrating at $R_f = 0.18$–0.21. When subjected again to electrophoresis according to the procedure described above, a single band was visible at $R_f = 0.18$ and this was coincident with the binding activity.

Determination of cyclic AMP binding activity. Cyclic AMP binding activity was determined by modification of the procedure previously reported [1]. Each reaction mixture contained, in a total vol. of 200 μl, 20 mM Tris–HCl, pH 7, 1 mM 2-mercaptoethanol, 105 pmol of cyclic AMP-[^{3}H] (1900 cpm/pmol), 15 μg of protamine and the binding protein. After incubation at 30° for 3 min, the reaction was arrested by addition of 2 ml of cold 25 mM Tris–HCl (pH 7.6) containing 10 mM $MgCl_2$, and the mixture filtered through a Millipore filter which had been presoaked in the same buffer. The filter was washed × 4 with 5 ml portions of the same buffer, placed in a counting vial, dried and finally counted in toluene–0.5% diphenyloxazole–0.005% 1,4-bis-2-(5-phenyloxazole)benzene in a liquid scintillation spectrometer. Protamine was added to the reaction mixture because it was found to improve the filter assay.

Protein kinase assay. This was determined as previously reported [1] using ATP-[γ-^{32}P] as a phosphate donor. Incubation was carried out at 30° for 15 min.

Estimation of MW by gel filtration. A Sephadex G-200 column (1.6 × 84 cm) was equilibrated with 25 mM Tris–HCl (pH 7.5) at 4°. About 0.5 ml of each of the protein soln was applied to the column and the elution was carried out with the same buffer at an upward flow rate of 12 ml. Blue dextran T-2000 was used to determine the void vol. The protein standards used were phosphorylase a (MW 370000), catalase (MW 244000), phosphorylase b (MW 185000), lipoxidase (MW 97000), creatin kinase (MW 80000), peroxidase (MW 49000) and ovalbumin (MW 43000).

Isolation of chromatin and RNA polymerase assay. Chromatin was extracted from Jerusalem artichoke rhizome tissue according to the method reported in ref. [20]. RNA polymerase assay was performed according to ref. [20].

Determination of enzymatic activities. Cyclic AMP phosphodiesterase was determined as previously reported [16]. 5'-Nucleotidase and adenosine triphosphatase were determined as described in ref. [17]; adenosine deaminase according to ref. [18]. Protein concn was determined by the method of ref. [19] using BSA as standard.

Acknowledgements—The authors thank Prof. E. Brown, Department of Biochemistry, Swansea, U.K., for critical reading of the manuscript and Sig. Gennarino Scotto for the excellent technical assistance. This work was supported by a grant from Consiglio Nazionale delle Ricerche, Italy, to M.G.

REFERENCES

1. Giannattasio, M., Carratù, G. and Tucci, G. F. (1974) *FEBS Letters* **49**, 249.
2. Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **57**, 660.
3. Langan, T. A. (1973) *Adv. Cyclic Nucleotide Res.* **3**, 99.
4. Pastan, I. and Perlan, R. L. (1972) *Adv. Cyclic Nucleotide Res.* **1**, 11.
5. Wicks, W. D. (1974) *Adv. Cyclic Nucleotide Res.* **4** 335.
6. Polya, G. and Sia, J. P. H. (1976) *Plant Sci. Letters* **7**, 43.
7. Yuh, K. C. and Tao, M. (1974) *J. Biol. Chem.* **13**, 5220.
8. Ueland, P. M. (1978) *Eur. J. Biochem.* **86**, 27.
9. Sugden, P. H. and Corbin, J. D. (1976) *Biochem. J.* **159**, 423.
10. Fain, J. N., Pointer, R. H. and Ward, W. F. (1972) *J. Biol. Chem.* **247**, 6866.
11. Huang, M. and Daly, J. W. (1974) *Life Sci.* **14**, 489.
12. Schwabe, Y., Scnonhofer, P. S. and Ebert, R. (1974) *Eur. J. Biochem.* **46**, 537.
13. Berne, R. M., Rubio, R., Dobson, J. G. and Curnish, R. R. (1974) *Circ. Res.* **28**, 115.
14. Fredholm, B. B. (1976) *Acta Physiol. Scand.* **96**, 422.
15. Manohar, S. V., Lerner, M. H. and Rubistein, D. (1968) *Can. J. Biochem.* **46**, 445.
16. Giannattasio, M., Sica, G. and Macchia, V. (1974) *Phytochemistry* **13**, 2729.
17. Polya, G. M., Brownlee, A. G. and Hynes, M. J. (1975) *J. Bacteriol.* **124**, 693.
18. Sharpless, T. K. and Wolfenden, R. (1967) *Methods Enzymol.* **12**, 126.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
20. Cullis, C. A. (1976) *Planta* **131**, 293.