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STUDY OF A CYCLIC AMP-DEPENDENT PROTEIN KINASE FROM CALF THYMUS

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SUMMARY

A cyclic AMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37), which appears to consist of catalytic and regulatory subunits, has been isolated from calf thymus microsomal supernatant. Two regulatory subunits were found. The heavy subunit (mol. wt 85 000–90 000) is probably a dimer of the light subunit (mol. wt 40 000–45 000). Cyclic AMP did not affect the apparent K_m for ATP and histones but increased V. The study of the affinity of the receptor for cyclic AMP showed two types of binding sites.

INTRODUCTION

Cyclic AMP-dependent protein kinases (ATP: protein phosphotransferase, EC 2.7.1.37) have been demonstrated in various animal tissues[1–5]. These enzymes modify the activity of cellular proteins by phosphorylating serine and threonine residues[6,7].

Epinephrine, growth hormone, parathyroid hormone and vasopressin stimulate adenyl cyclase activity and increase the cyclic AMP level in the thymocyte population. These hormones and cyclic AMP show, *in vivo* and in *vitro*, a mitogenic activity on thymocytes[8]. At the molecular level, the mechanism of action of cyclic AMP is unknown.

As recent works performed on other tissues[9–17] have shown the existence of receptors for cyclic AMP, which have a high affinity and appear to be the regulatory moiety of these protein kinases, we searched for a cyclic AMP receptor and a cyclic AMP-dependent protein kinase in thymocytes.

MATERIAL AND METHODS

[γ-^{32}P]ATP was obtained from C.E.A. (France), ^{3}H-labeled cyclic AMP (spec. act., 20.6 Ci/mmole) was obtained from Schwarz Bioresearch. Filters of cellulose ester

Abbreviation: MES 2-(*N*-morpholino)ethanesulfonic acid.

(HAWP, 0.45 μm) were obtained from Millipore, paper discs No. 3 MM from Whatman. Total calf thymus histones were given by Choay Laboratories, Paris. Fresh calf thymus from two different animals was obtained from a slaughtherhouse, transported in ice to the laboratory and eventually stored at −20 °C.

Binding of cyclic AMP was measured by the method of Walton and Garren[18], in a total volume of 0.3 ml containing 20 mM Tris, pH 7.5, or 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6, (as indicated in the text), 8 mM $MgCl_2$, 4 mM theophylline, 0.2 μM ^{3}H-labeled cyclic AMP and up to 200 μg of protein. After 120 min incubation at 4 °C, bound and free cyclic AMP were separated by filtration through a Millipore filter. Filters were dissolved in 10 ml of Bray's solution.

Protein kinase activity was measured by a method adopted from Reiman *et al.*[19]. The reaction mixture contained, in a total volume of 0.5 ml: 20 mM Tris (pH 7.5) or 20 mM MES (pH 6) (as indicated in the text), 8 mM $MgCl_2$, 4 mM theophylline, 2 mM dithiothreitol, 0.5 mg total histones, 5 μM cyclic AMP (when indicated) and 1–50 μg of protein-enzyme. The reaction was initiated by adding 0.1 mM [γ-^{32}P]-ATP. After incubation for 10 min at 37 °C, an aliquot was spotted on a Whatman paper disc and washed for 45 min in a small volume of trichloroacetic acid 10% containing 20 mM ATP. The discs were washed three times for 30 min in 5% trichloroacetic acid containing 10 mM $Na_4P_2O_7$ and 10 mM Na_3PO_4. Finally, the discs were washed for 15 min in a mixture of alcohol–acetone (1:1, v/v) and acetone, and dried before counting in a scintillator.

RESULTS

Purification of the cyclic AMP-binding protein and the cyclic AMP-dependent protein kinase

The same techniques as those described previously for rat liver[14] were utilized. Calf thymus was homogenized in 4 vol. of 250 mM sucrose containing 1 mM $MgCl_2$. The homogenization and all subsequent steps were carried out at 4 °C. Proteins from the microsomal supernatant which precipitate at pH 5 were removed and the enzyme was precipitated by $(NH_4)_2SO_4$ (45% saturation). The preparation was applied to a Sephadex G-200 column. Chromatography gave a single peak (molecular weight about 160 000). The elution profile of the cyclic AMP-binding activity followed the activity profile of the protein kinase (not shown).

Dissociation into subunits of the calf thymus cyclic AMP-dependent protein kinase

Previous work[9–17] has shown that cyclic AMP promotes the dissociation of the cyclic AMP-dependent protein kinase of some tissues into a regulatory moiety (the receptor for cyclic AMP) and a catalytic moiety. We have used this property for studying the structure of the calf thymocyte protein kinase. Fig. 1 shows the results of an experiment where the $(NH_4)_2SO_4$ fraction was chromatographed on a calibrated Sephadex G-150 column, after preincubation with cyclic AMP. Two regulatory subunits were found. The heavy subunit was estimated to be about 85 000–90 000 and the light subunit about 40 000–45 000. In this experiment, the catalytic moiety seemed to form aggregates because it was found in the void volume. ^{3}H radioactivity found in the void volume may indicate that the regulatory subunit form ag-

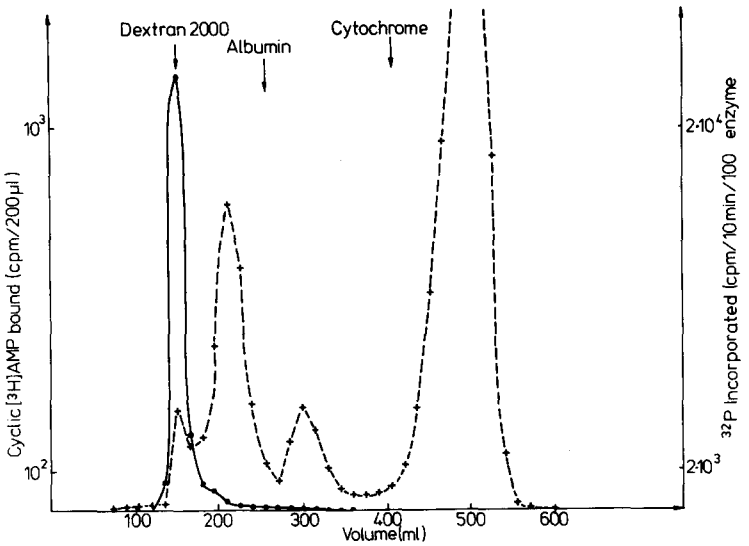

Fig. 1. Chromatography of protein kinase on a Sephadex G-150 column after incubation with cyclic AMP. 50 mg of protein from $(NH_4)_2SO_4$ fraction were applied, after incubation with ^3H-labeled cyclic AMP (2 μM), on a Sephadex G-150 column equilibrated with 10 mM MES (pH 6) and 1 mM dithiothreitol. Elution volumes of Dextran 2000 albumin and cytochrome *c* are indicated. ———, protein kinase activity was measured in the presence of 5 μM cyclic AMP as described under Material and Methods. Specific radioactivity of [γ^{32}P]ATP was $2 \cdot 10^4$ cpm/nmole. — — —, binding of cyclic AMP was measured by ^3H radioactivity of 0.2 ml aliquots in 10 ml of Bray's solution.

gregates or that the binding of cyclic AMP to the regulatory moiety does not promote the dissociation of all molecules of protein kinase.

Effect of cyclic AMP on the apparent K_m for substrates

The activity of the enzyme was measured in the presence or absence of cyclic AMP at different concentrations of substrates. The apparent K_m for ATP (Fig. 2a) ($1.5 \cdot 10^{-5}$ M) was not modified by cyclic AMP. As observed with ATP, the histone concentration needed for half-maximum activity (0.15 mg/ml) was not modified by cyclic AMP, but an inhibition of the activity in the absence and in the presence of cyclic AMP was found for histone concentrations higher than 1 mg/ml (Fig. 2b). For this particular preparation the enzyme was activated 2-fold by cyclic AMP (5 μM).

Affinity for cyclic AMP

Cyclic AMP binding to a $(NH_4)_2SO_4$ fraction was studied at cyclic AMP concentrations ranging from 0.3–150 nM .When the amount of cyclic AMP bound was plotted against the ratio of bound to free cyclic AMP (Fig. 3), two slopes were obtained. The measured dissociation constants K_d are: 2–3 nM and 0.1–0.2 nM.

DISCUSSION

As in other eukaryotic tissues[9–17], we have found a receptor for cyclic AMP

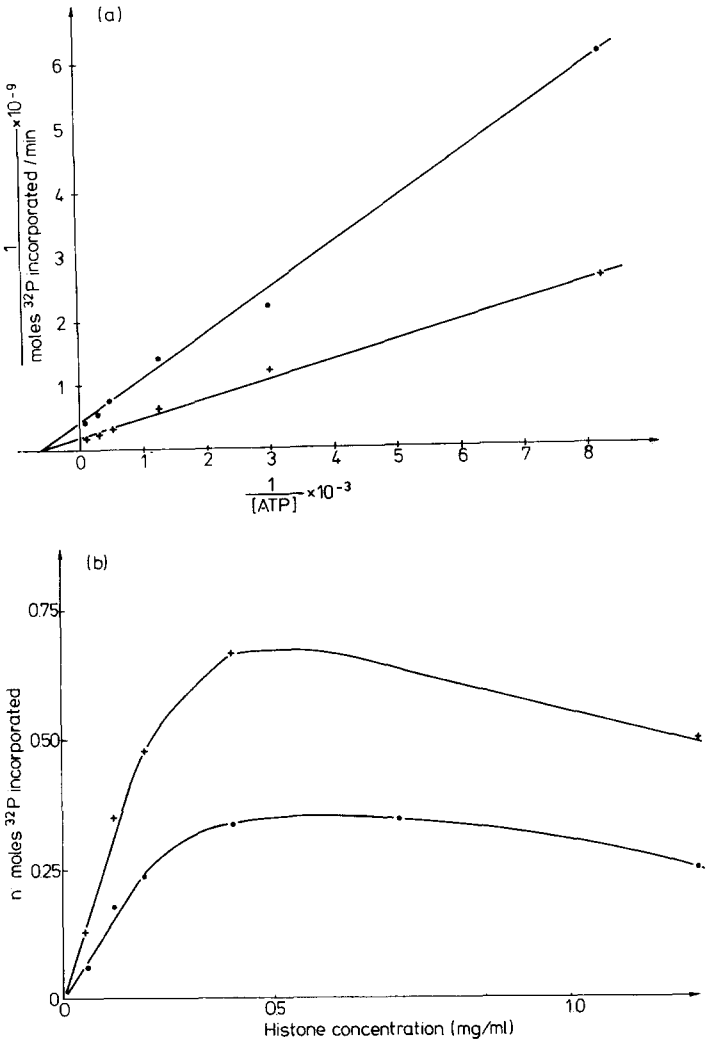

Fig. 2. (a) Double reciprocal plots illustrating the effect of ATP concentration on protein kinase activity. $(NH_4)_2SO_4$ fraction (40 μg) was assayed in the absence (●) or presence (+) of 5 μM cyclic AMP, as described under Material and Methods. (b) Effect of histone concentration on protein kinase activity. $(NH_4)_2SO_4$ fraction (40 μg) was assayed in the absence (●) or presence (+) of 5 μM cyclic AMP as described under Material and Methods.

which seems to be the regulatory moiety of a cyclic AMP-dependent protein kinase. Cyclic AMP does not modify the affinity of the enzyme for its substrates. We find, in the absence or presence of cyclic AMP, the same apparent K_m for ATP and histones but we find that V is increased. Reiman *et al.*[19] obtained a similar result for the rabbit muscle enzyme.

Two regulatory subunits were found. The heavy subunit (mol. wt 85 000–90 000) is probably a dimer of the light subunit (mol. wt 40 000–45 000). Gill and Garren[20] obtained a value of 90 000 for the molecular weight of the regulatory moiety

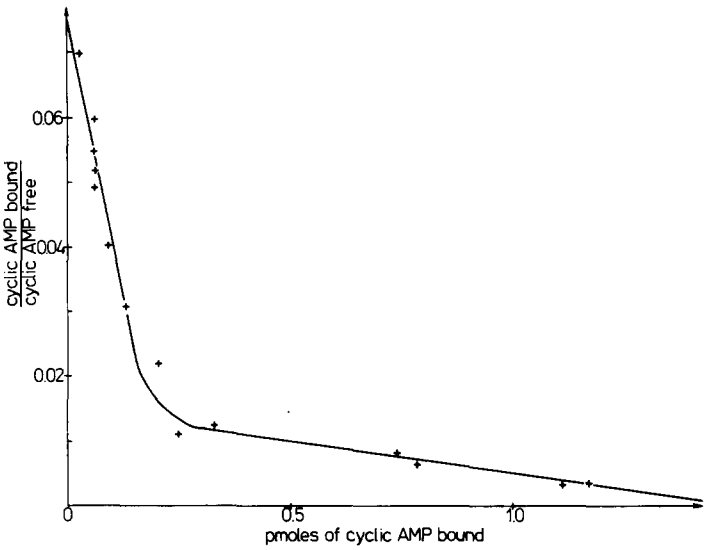

Fig. 3. Evidence for sites with different affinities for cyclic AMP. $(NH_4)_2SO_4$ fraction (150 μg) was incubated 90 min at 4 °C in 1 ml 10 mM MES (pH 6), 5 mM $MgCl_2$ with ^{3}H-labeled cyclic AMP 0.3 nM–150 nM. The incubate was filtered through a Millipore filter as described under Material and Methods. The maximum binding was 16 255 cpm. The amount of cyclic AMP bound for each concentration of cyclic AMP was plotted against the ratio of bound to free cyclic AMP.

of bovine adrenal protein kinase. Reiman *et al.*[10] also found two regulatory subunits for the enzyme from rabbit muscle. The catalytic moiety of the calf thymocyte enzyme formed aggregates. Gill and Garren[20] for the bovine adrenal enzyme also found that the catalytic moiety forms aggregates. However, we found about 70 000 for the molecular weight of the catalytic subunit with a fraction obtained after DEAE-chromatography (unpublished observations).

The binding constant for cyclic AMP of the regulatory moiety of protein kinases has been found to be about $1 \cdot 10^{-8}$ M[9–14]. However, for the calf thymus protein kinase, by working over a large range of cyclic AMP concentrations, we found binding sites with different affinities for cyclic AMP ($K_d = 2 \cdot 10^{-9}$–$3 \cdot 10^{-9}$ M and $1 \cdot 10^{-8}$–$2 \cdot 10^{-8}$ M). Otherwise Walsh *et al.*[21] have shown that a protein inhibitor of cyclic AMP-dependent protein kinases promotes a 5-fold increase in the binding affinity for cyclic AMP (K_d $1.4 \cdot 10^{-8}$ M and $2.8 \cdot 10^{-9}$ M). However, it is difficult to present a structural interpretation for these binding constants before one is able to measure directly the affinity of the isolated regulatory subunits.

The observation that cyclic AMP can induce mitosis of cultured thymocytes[8] has suggested that it could play an important role in the induction of lymphocyte differentiation. Smith *et al.*[22] have shown that phytohemaglutinin increases the activity of adenyl cyclase in homogenates of human lymphocytes and increases the level of endogenous cyclic AMP 2–4-fold. The existence of a receptor for cyclic AMP associated to a protein kinase supports the hypothesis previously suggested[8] that the mechanism by which cyclic AMP induces mitosis in thymocytes involves the activation of a protein kinase.

REFERENCES

1 D. A. Walsh J. P. Perkins and E. G. Krebs, *J. Biol. Chem.* 243 (1968) 3763.
2 T. A. Langan, *Science*, 162 (1968) 579.
3 J. D. Corbin and E. G. Krebs, *Biochem. Biophys. Res. Commun.*, 36 (1969) 328.
4 S. Jard and F. Bastide, *Biochem. Biophys. Res. Commun.*, 139 (1970) 539.
5 J. F. Kuo B. K. Krueger, J. R. Sanes and P. Greengard, *Biochim. Biophys. Acta* 211 (1970) 79.
6 T. A. Langan, *J. Biol. Chem.*, 244 (1969) 5763.
7 B. Jergil and G. H. Dixon *J. Biol. Chem.*, 245 (1970) 425.
8 J. P. Mac Manus and J. F. Whitfield *Exp. Cell Res.*, 58 (1970) 188.
9 G. N. Gill and L. D. Garren, *Biochem. Biophys. Res. Commun.*, 39 (1970) 335.
10 E. M. Reimann C. O. Brostrom J. D. Corbin, C. A. King and E. G. Krebs *Biochem. Biophys. Res. Commun.*, 42 (1971) 187.
11 M. Tao M. L. Salas and F. Lipmann, *Proc. Natl. Acad. Sci. U.S.*, 67 (1970) 408.
12 A. Kumon H. Yamamura and Y. Nishizuka, *Biochem. Biophys. Res. Commun.*, 41 (1970) 1290.
13 L. J. Chen and D. A. Walsh *Biochemistry*, 10 (1971) 3614.
14 M. Pierre and J. E. Loeb, *Biochimie*, 53 (1971) 727.
15 J. Erlichman A. L. Hirsch and O. M. Rosen, *Proc. Natl. Acad. Sci. U.S.*, 68 (1971) 731.
16 A. M. Chambaut F. Leray and J. Hanoune, *FEBS Lett.*, 15 (1971) 328.
17 L. Rappaport J. F. Leterrier and J. Nunez, *Biochimie*, 53 (1971) 721.
18 G. M. Walton and L. D. Garren, *Biochemistry*, 9 (1970) 4227.
19 E. M. Reimann D. A. Walsh and E. G. Krebs *J. Biol. Chem.* 246 (1971) 1986.
20 G. N. Gill and L. D. Garren, *Proc. Natl. Acad. Sci. U.S.* 68 (1971) 786.
21 D. A. Walsh, C. D. Ashby, C. Gonzales, D. Calkins, E. H. Fischer and E. G. Krebs *J. Biol. Chem.* 246 (1971) 197.
22 J. W. Smith, A. L. Steiner and C. W. Parker, *J. Clin. Invest.*, 50 (1971) 432.