A CYCLIC-3',5'-ADENOSINE MONOPHOSPHATE DEPENDENT PROTEIN KINASE FROM THE ADRENAL CORTEX: COMPARISON WITH A CYCLIC AMP BINDING PROTEIN+

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SUMMARY

A cyclic AMP activated protein kinase has been identified in adrenal cortical tissue. The K_m and nucleotide specificity of this enzyme resemble the K_d and nucleotide specificity of the adrenal cortical protein which specifically binds cyclic AMP. Initial purification procedures which result in a 200-fold purification of the binding protein and a 50-fold purification of the protein kinase are presented. Though a partial separation of these two activities is achieved, a functional interaction of these two proteins is suggested.

Current evidence indicates that cyclic-3',5'-adenosine monophosphate (cyclic AMP) is the intracellular mediator of the action of adrenocorticotropin (ACTH) on the adrenal cortex (1,2). In addition to stimulating corticosteroid production (3), cyclic AMP can substitute for ACTH in maintaining adrenal cortical weight and protein content in hypophysectomized animals (4). Despite these striking effects of the cyclic nucleotide the mechanism of action of cyclic AMP at the molecular level remains to be elucidated.

Recent investigations have disclosed that the nucleotide binds specifically to a protein (5) and seems to stimulate directly the activity of a protein kinase (6). We isolated a protein from the adrenal cortex which specifically and tightly binds cyclic AMP and postulated that the protein is involved in the initial action of the cyclic nucleotide (5). Walsh, et al., isolated from skeletal muscle a protein kinase that catalyzed the activation of phosphorylase kinase and suggested that cyclic AMP acted directly upon the kinase in stimulating its activity. In addition to phosphorylase kinase, the protein kinase phosphory-

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lated casein, protamine, and histone (6). A similar protein kinase (or kinases) have been identified in mammalian adipose tissue (7), liver (8) and brain (9) and have been described more recently in trout testis (10) and in bacteria (11). To further study the action of cyclic AMP within the adrenal cortex a cyclic AMP activated protein kinase has been identified and its relationship to the cyclic AMP binding protein investigated.

The method for determining the binding of ^{3}H cyclic AMP to protein will be described elsewhere (12). ^{3}H cyclic AMP (Schwartz 2.35 C/mM) was added to a final reaction volume of 0.3 ml containing 8 mM theophylline, 10 mM $MgCl_2$, 50 mM Tris pH 7.4, and protein at various stages of purification. After incubation for 30 minutes at 4° C the bound cyclic AMP was separated from the free by passage through a Millipore filter, washed with 25 mM Tris, 10 mM $MgCl_2$ buffer, the filters placed in 15 ml of Bray's solution (13), and the radioactivity determined in a liquid scintillation counter. This method yields a K_a of 1.25×10^{-8}*. Specific activity measurements were performed at saturating concentrations of cyclic AMP and expressed as amount of cyclic AMP bound per mg protein.

Kinase activity was assayed as described by Walsh, Perkins, and Krebs (6) in a reaction volume of 0.1 ml. ATP^{32} (γ labeled) was prepared by the method of Glynn and Chappel (15). Purity was estimated by chromatography on paper using 0.1 M phosphate pH 6.8, ammonium sulfate, n propanol (100/60/2). Specific activity measurements were performed at saturating concentrations of ATP, histone (240 μg) and cyclic AMP and expressed as the amount of ATP^{32} incorporated into TCA insoluble material per mg of enzyme protein during a 10 min. incubation at 30° C. During purification procedures assays were performed both with and without the addition of cyclic AMP. Activity was linearly responsive to increasing amounts of enzyme protein in all experiments. Enzyme activity was linear throughout a 10 min. incubation at 30° C.

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*The previously reported K_a (average intrinsic equilibrium constant (14)) of cyclic AMP binding was 5×10^{-8} determined by equilibrium dialysis. The presented method (utilizing Millipore filters) for cyclic AMP binding appears to be more accurate.

A protein kinase activated by cyclic AMP was identified in the high speed supernatant fraction of bovine adrenal cortical tissue. The adrenal cyclic AMP activated enzyme utilizes histone, protamine, phosphorylase kinase* and casein as substrate. The endogenous adrenal substrate or substrates were not identified and histone was used in the following experiments. Enzyme activity which was stimulated by increasing concentrations of cyclic AMP revealed a half maximum stimulation (K_m) at 1.4×10^{-8} M and a maximum stimulation at a concentration of approximately 1×10^{-7} M (Fig. 1); the enzyme was activated approximately fourfold by the high concentrations of the nucleotide.

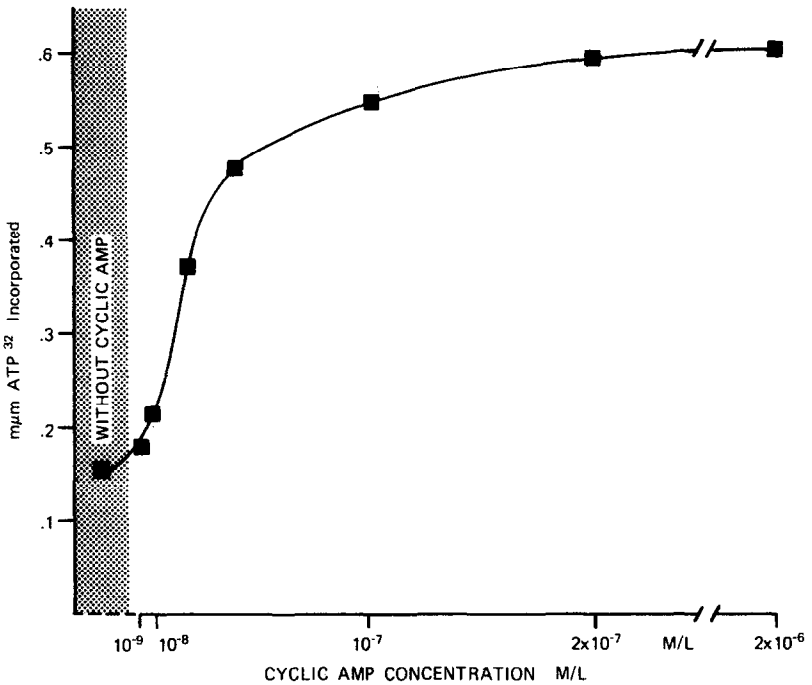

Fig. 1. Effect of cyclic AMP concentration on histone phosphorylation. The incubation mixture was as described in the text and contained 20 μg of enzyme protein (15-fold purified and without self phosphorylation), 240 μg histone (saturating concentration) and cyclic AMP added to give the final concentrations as indicated. Incubations were for 10 min. at 30° C.

In comparing various nucleotides with cyclic AMP in stimulating the enzyme the same specificity was obtained as that demonstrated for the cyclic AMP binding protein (5).

*A purified preparation obtained from rabbit skeletal muscle generously supplied by Dr. Steven Mayer.

As shown in Table I, only nucleotides with the 3',5' cyclic ring intact are able to substitute for cyclic AMP in activating the protein kinase; the efficiency of substitution appears related to the structural similarity of the entire molecule to cyclic AMP, cyclic-3',5'-inosine monophosphate being most efficient.

TABLE I

Nucleotide specificity. The reaction mixtures were as described in Fig. 1 with nucleotide substitutions for cyclic AMP as indicated.

Nucleotide used	mμm ATP^{32} incorporated Nucleotide concentration	
	2×10^{-7} molar	2×10^{-4} molar
None	.069	
Cyclic 3',5' AMP	.342	.351
5'AMP	.068	.105
ADP	.069	.099
Cyclic 3',5' IMP	.261	.360
Cyclic 3',5' CMP	.120	.374
Cyclic 3',5' GMP	.111	.357

The cyclic AMP activated protein kinase was compared with the cyclic AMP binding protein through several purification procedures. All were carried out at 4° C. Subcellular fractionation was performed as previously described (5). The 100,000 x g supernatant was brought to 45% saturation by the addition of solid ammonium sulfate; the precipitate was dissolved in 0.01 M Tris pH 7.4, 0.006 2-mercaptoethanol buffer (TM) and dialyzed against the same for 16 hours. Calcium phosphate gel (3%, aged) was added to the protein solution (10-12 mg/ml) at a dry gel to protein ratio of 1 : 1. After stirring for 45 min. the gel was collected, washed once with TM buffer, and the protein eluted with 0.25 M KPO_4 buffer pH 8.1. The eluate was dialyzed for 16 hours against TM buffer containing 10% glycerol (TMG), and then approximately 900 mg applied to a 33 x 3 cm DEAE 52 column previously equilibrated with the same buffer. The column was washed with 2 volumes of TMG and then eluted with a 0.04 to 0.4 M linear NaCl gradient in TMG. The binding and kinase activities were parallel through the initial purification steps (Table II) but a partial separation was

TABLE II

Purification procedure. Binding and kinase assays were performed as described in the text and in the legend of Fig. 1. The kinase reaction mixture contained histone (240 μg) and 2×10^{-7} M cyclic AMP. Peak numbers refer to the DEAE cellulose chromatography shown in Fig. 3A.

Procedure	μμm Cyclic AMP bound/mg protein	mμm ATP^{32} incorporated/ mg protein/10 minutes
Cortical homogenate	1.69	1.19
100,000 x g supernatant	3.17	2.1
Ammonium sulfate	8.31	3.6
Calcium phosphate gel	15.99	8.09
DEAE-cellulose chromatography - Peak II	90.0	9.2
- Peak III	51.0	25.0
7s Sucrose gradient peak (from III)	49.8	56.6
4s Sucrose gradient peak (from II)	326.6	4.4

obtained on DEAE chromatography (Fig. 2A). The amount of Peak I obtained was variable but the binding and kinase activities in the fraction remained consistently parallel. In Peak II the cyclic AMP binding protein was relatively free of kinase activity. Sedimentation of Peak II on 5-20% linear sucrose gradients yielded highly purified cyclic AMP binding protein which sedimented at 4s as previously described (5) (Table II, last line); a small peak of protein kinase, not stimulable by cyclic AMP and sedimenting at 7s was also seen in this fraction. Peak III, the cyclic AMP activated protein kinase peak, contained a significant shoulder of binding activity. The sedimentation of Peak III on sucrose gradients is shown in Fig. 2B. Cyclic AMP activated protein kinase sediments at 7s. There is a parallel peak of binding activity at 7s as well as the expected peak of binding activity at 4s. The isoelectric point of both the binding and kinase activities of Peak III was determined by electrofocusing to be 4.5. This is identical to that obtained for a brain cyclic AMP dependent protein kinase studied by Miyamoto, *et al.* (17). Thus the partially purified adrenal cyclic AMP dependent protein kinase studied here retains significant cyclic AMP binding activity even after chromatography on DEAE and electrofocusing.

The relation of the cyclic AMP binding activity associated with the protein kinase (co-sedimenting on sucrose gradients at 7s) to the binding fraction separated from kinase

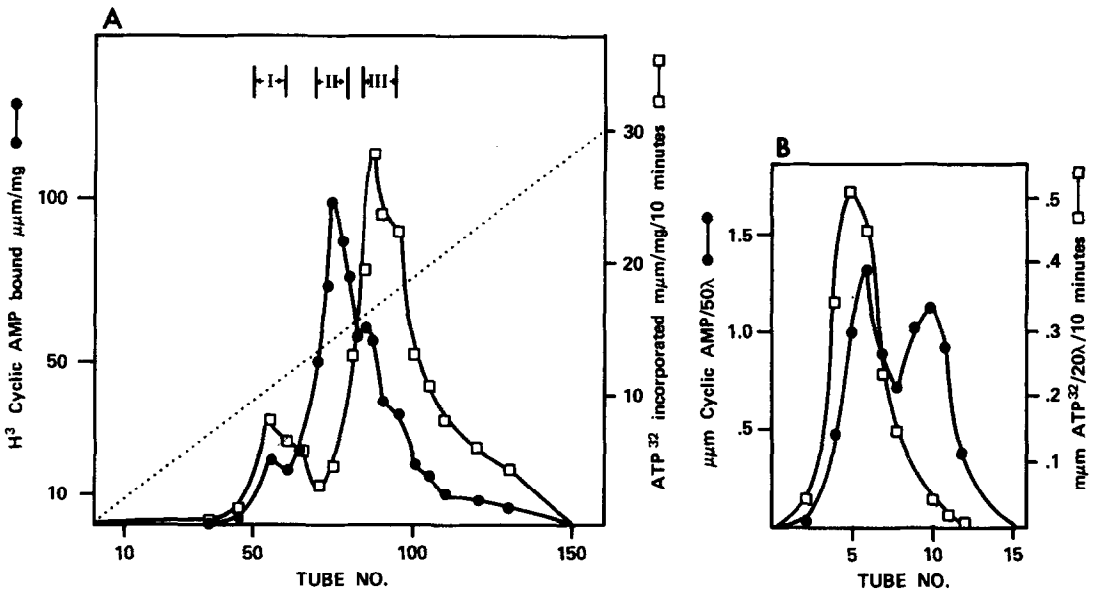

Fig. 2A. DEAE 52 cellulose chromatography of the cyclic AMP binding ●—● and cyclic AMP activated □—□ protein kinase activities. Peak activities were pooled as indicated by brackets.

Fig. 2B. Sucrose gradient sedimentation of Peak III from DEAE column. Peak III was concentrated by ultrafiltration and aliquots of protein (0.2 ml) were layered onto 4.6 ml gradients of 5-20% sucrose in TM and centrifuged for 18 hours at 39,000 rpm and 3° C as described by Martin and Ames (16). Gamma globulin and ovalbumin were run as markers in parallel tubes and OD at 280 mμ determined. Equal aliquots from each fraction were assayed for binding and kinase activities. The peak kinase activity was stimulated 3-fold by cyclic AMP.

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activity by DEAE chromatography (sedimenting at 4s) remains unknown. Studies of the effect of the addition of binding protein to the partially purified protein kinase are presented in Table III. Binding protein (Peak II after sucrose gradient sedimentation, 4s) almost entirely free of protein kinase activity suppressed the basal protein kinase reaction 20–45% in various experiments. The cyclic AMP stimulated reaction was unaffected suggesting that neither a nonspecific protein inhibitor nor a phosphatase was responsible for this suppression. Heating of the protein kinase preparation to 43° C at pH 6.0 for 15-30 min. is associated with a 25% decrease in cyclic AMP binding activity and a 10-25% increase in basal protein kinase activity; the total activity in the presence of cyclic AMP was unchanged. Addition of

TABLE III

Effect of binding protein on kinase activity. Binding protein isolated from sucrose gradient sedimentation of the DEAE column Peak II was incubated for 15 min. at 32° C with kinase protein prepared from the sucrose gradient illustrated in Fig. 2B. The assay mixture was then added and reactions incubated at 30° C for 10 min. Boiled binding protein was without effect.

*Enzyme used			mμm ATP^{32} incorporated	
Binding protein (4s peak)		Kinase protein (7s peak)	-cyclic AMP	+cyclic AMP
9.0 μg	+	0	0	.05
0	+	8 μg	.158	.455
4.5 μg	+	8 μg	.125	---
9.0 μg	+	8 μg	.109	.555
13.5 μg	+	8 μg	.093	---

binding protein to protein kinase previously heated demonstrates suppression of the elevated basal kinase activity. In addition to the partial separation of binding and kinase activities achieved by DEAE cellulose chromatography and heating, incubation of the fraction obtained prior to DEAE chromatography (calcium phosphate step, Table II, line 4) with cyclic AMP results in a partial separation of binding activity from protein kinase. As shown in Fig. 3 the fraction eluted from calcium phosphate gel on sucrose gradient sedimentation displays a homogeneous peak at 7s with parallel activities of protein kinase and cyclic AMP binding. Incubation of cyclic AMP with the fraction prior to sedimentation results in a shift of the peak of binding protein from 7s to 4s; significant binding activity, however, still remains with the protein kinase which still sediments predominantly at 7s. As previously demonstrated (5), cyclic AMP remains tightly bound to the protein even after extensive dialysis. Therefore it is difficult to ascertain the effect of the removal of binding protein on protein kinase activity without the presence of cyclic AMP.

The present investigation demonstrates that protein kinase and cyclic AMP binding protein are enriched in parallel throughout the enzyme purification procedures. Cyclic AMP binding protein is partially separated from the protein kinase by prior incubation with

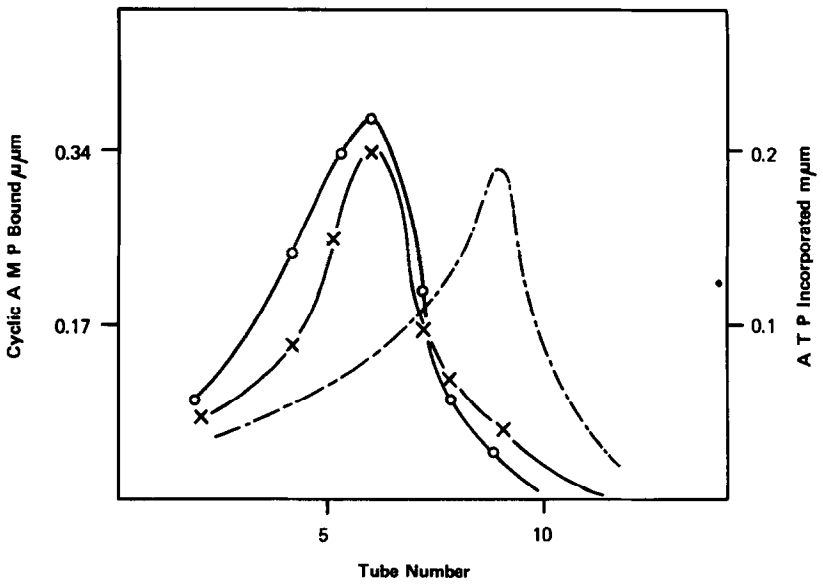

Fig. 3. Effect of cyclic AMP on sedimentation of binding and kinase activities. Sucrose density gradients were prepared and run as described in Fig. 2. Aliquots of 0.02 ml from each fraction were assayed for binding and kinase activities in the presence of saturating quantities of cyclic AMP. Protein kinase O——O and binding X——X activities demonstrate parallel sedimentation. Incubation of an equal aliquot of protein with ^{3}H cyclic AMP (10^{-6} M) prior to sedimentation resulted in a shift in the peak of binding protein activity (broken line) without a marked change in the kinase sedimentation (not shown).

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the nucleotide and by DEAE chromatography. The binding protein is also more labile to heat than the protein kinase. Also the addition of binding protein to the protein kinase fraction decreases the activity of protein kinase which is still entirely responsive to activation by cyclic AMP.

Complete understanding of the association of the cyclic AMP binding protein and cyclic AMP dependent protein kinase must await complete purification. The possibility has not been excluded that the cyclic AMP binding activity that remains associated with the protein kinase and that separated from the enzyme are different. Nevertheless, a tentative model for this association is suggested by the presented data. The cyclic AMP binding moiety in association with the protein kinase suppresses its activity; the binding of cyclic AMP to its receptor relieves the suppression. The cyclic AMP activation of the protein kinase may result by favoring the dissociation of the binding moiety from the protein kinase (Fig. 3).

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