PURIFICATION OF THE cAMP RECEPTOR PROTEIN BY AFFINITY CHROMATOGRAPHY

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Summary. 8-(6-aminohexyl)-amino-adenosine 3',5'-monophosphate coupled to agarose has been utilized as affinity chromatography media for separation of the subunits of cAMP-dependent protein kinase and for purification of the regulatory cAMP receptor subunit. Quantitative recovery of highly purified cAMP receptor protein has been achieved using this method. The purified cAMP receptor protein is fully functional in binding cAMP and in reversibly combining with protein kinase catalytic subunits to generate the cAMP-dependent enzyme.

cAMP-dependent protein kinase (E.C.2.7.1.37) consists of two dissimilar subunits: a regulatory cAMP receptor subunit and a catalytic phosphotransferase subunit. Binding of cAMP to the regulatory receptor results in dissociation of the complex with activation of the catalytic kinase subunit. This can be illustrated by the equation:

$R : C + cAMP \rightleftharpoons cAMP : R + C$, where R : C represents the inactive receptor-catalytic complex; cAMP : R, cAMP bound to the receptor subunit; and C, the enzymatically active free kinase subunit (1-12).

The present report describes the successful use of 8-(6-aminohexyl)-amino-adenosine 3',5'-monophosphate (C8-NC_6NH_2-cAMP)-Sepharose[1] as an affinity chromatography medium for separation of the subunits of cAMP-dependent protein kinase and for purification of the cAMP receptor protein. As shown in the equation, specific retention of the receptor protein by immobilized cAMP results in separation of the subunits and flow through of the catalytic kinase subunit; the receptor protein can then be eluted. cAMP receptor protein purified by this method is biologically active in binding cAMP and in reversibly combining with catalytic kinase subunits.

[1]Abbreviations: TMG buffer: 10 mM Tris (pH 7.4)-6 mM 2-mecaptoethanol-10% glycerol. C8-NC_6NH_2-cAMP: 8-(6-aminohexyl)-amino-adenosine 3',5'-monophosphate. BSA: bovine serum albumin.

Materials and Methods. Synthesis of C8-NC_6NH_2-cAMP-Sepharose. The synthesis of C8-NC_6NH_2-cAMP was based on the procedure described by Guilford, Larsson, and Mosbach (13). 8 Bromo-cAMP (500 mg) synthesized as described by Muneyama et al. (14), was refluxed for 6 hr with 1,6 diaminohexane (14 gm) in water (65 ml). The cooled reaction mixture was applied to a Dowex AG1-X8 (acetate) column (2.3 X 15 cm), and washed with water (300 ml) overnight. A linear gradient of 0 to 0.5 M acetic acid was applied until the derivative began to appear; the remainder was then washed from the column with 150 ml of 0.5 M acetic acid, partially dried on a rotary evaporator at less than 30°, and lyophylized (447 mg, 67% yield). The derivative ran as a single spot when chromatographed on Whatman No. 1 paper in ethanol : 0.5 M ammonium acetate (5 : 2) with an Rf of 0.42. The ultraviolet spectrum of the derivative was symmetrical with λ max at 276 nm.

C8-NC_6NH_2-cAMP in solution functions as a competitive inhibitor of cAMP binding to receptor protein (Fig. 1). Approximately 100-fold greater concentrations of the C8 derivative than unmodified cAMP are required for comparable inhibition of [^{3}H]cAMP binding indicating that substitution at the C8 position has decreased the affinity for receptor sites. The C8 derivative also activates cAMP-dependent protein kinase with half maximal activation occurring at approximately 8 X 10^{-8} M.

C8-NC_6NH_2-cAMP was partially solubilized in Na_2CO_3 (0.1 M, pH 8.9; 20 ml) and stirred with CNBr activated Sepharose for 16 hr (15). The affinity gel was then washed extensively with 1 N NaCl followed by TMG buffer[1]. The minimum concentration of the derivative coupled to the gel was determined by synthesizing the derivative from [^{3}H]cAMP and counting aliquots of the beads. Columns containing up to 50 mM derivative were synthesized; the usual concentration used was 10 mM. Stability was determined by synthesizing the derivative from [^{14}C]cAMP. No ^{14}C was eluted from the column which remained active over a 3-month period indicating stability for at least that period of time.

Assays. cAMP receptor activity was determined as described by Walton and Garren (16). Aliquots for assay were diluted in TMG containing BSA[1] (5 mg/ml) so that each assay contained

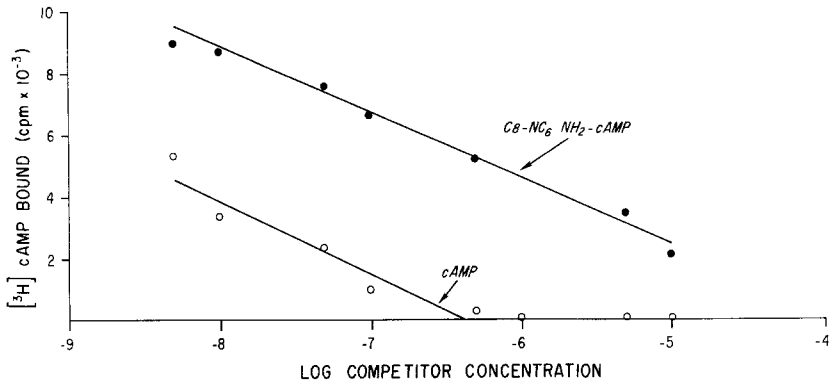

Figure 1. C8-NC_6NH_2-cAMP as a competitive inhibitor of the binding of cAMP to receptor protein. The indicated concentrations of unlabeled cAMP or C8-NC_6NH_2-cAMP were mixed with 2 X 10^{-8} M [^{3}H]cAMP prior to the addition of 16 µg of pig muscle protein kinase purified through the calcium phosphate step. Receptor assay was carried out as described under Methods.

100 to 200 µg of protein. BSA was necessary for successful use of the cellulose ester membrane filter assay with the small quantities of receptor protein present in purified fractions. This assay was verified by comparison to equilibrium dialysis (17). Protein kinase activity was measured by the method of Walsh et al. (18) using protamine as substrate.

Procedures. Pig muscle was purified through the first DEAE batch step described by Rubin et al. or through the subsequent calcium phosphate fractionation (8). Beef adrenal cortices were processed through the calcium phosphate step of Gill and Garren (4). Electrophoresis in polyacrylamide gels of varying cross-linkage was performed as previously described (4). Gels were either fixed with acetic acid and the protein stained with Coomassie blue or assayed for receptor activity as described (4). Protein was determined by the method of Lowry et al. (19) after dialysis overnight against 0.05 M sodium phosphate buffer pH 7.4.

Results. Denaturation of the cAMP receptor protein. The primary elution procedure utilized for recovery of the retained receptor protein was reversible denaturation in 7 M urea. The inhibition of binding in urea is observed with the cellulose ester membrane filter assay, with equilibrium dialysis, and when Sephadex G-25 chromatography is used to

separate bound from free cAMP. The presumed unfolding of the cAMP receptor protein in 7 M urea is completely reversible upon removal of urea by either dialysis or dilution resulting in regeneration of full receptor activity. Parallel studies on protein kinase activity indicated full inhibition of activity in 5 M urea but restitution of only 40-60% of the initial kinase activity following urea removal.

Use of C8-NC_6NH_2-cAMP-Sepharose for affinity chromatography. C8-NC_6NH_2-cAMP-Sepharose removed cAMP receptor activity from solutions of partially purified cAMP-dependent protein kinase (Table I). Protein kinase appearing in the column eluate was largely independent of cAMP for activity. After extensive washing, 7 M urea in TMG was applied to the column; the 7 M urea fraction contained more than 80% of the applied receptor activity and less than 1% of the applied protein kinase. Specific activities of the purified receptor protein varied from 1,000 to 5,000 pm cAMP bound/mg. It can be estimated that a 94,000 to 98,000 MW receptor protein (4,8) binding one molecule of cAMP per molecule of protein would contain 11,000 pm of cAMP bound per mg of protein suggesting that the protein obtained is not homogeneous (Fig. 2, left). Receptor specific activities have been higher when more purified material is applied to the column and when larger amounts of starting protein are utilized. Attempts to further purify the receptor by a second passage through the affinity column have resulted in recoveries of less than 10%. Receptor protein can be further purified by standard protein separation procedures. Sedimentation in 5-20% glycerol gradients yields a highly purified receptor preparation sedimenting at 4.6 S (Fig. 2, right). Aging in 7 M urea generated a faster migrating (Rf = 0.58) cAMP binding protein of 24,000 MW (Fig. 2, left); addition of methylamine to the 7 M urea prevented formation of the smaller form. Methylamine is now routinely added to urea solutions immediately prior to use.

Characteristics of the cAMP receptor protein purified by affinity chromatography. The interaction of cAMP with the purified receptor protein was identical to that observed in

TABLE I. Chromatography of Partially Purified cAMP-Dependent Protein Kinase on C8-NC_6NH_2-cAMP-Sepharose

	Protein	Receptor		Protein Kinase			
		pm cAMP bound		pm ^{32}P incorporated/μg/min		Stimulation	Total
Fraction	mg	Total	mg^{-1}	cAMP (-)	cAMP (+)	X	nm/min
Crude (100 ml)	2,300	44,000	19	0.89	2.40	2.70	5,520
Not retained (200 ml)	1,720	7,000	4	1.75	1.85	1.06	3,010
7 M urea (20 ml)	10	37,000	3,700	<0.20	0.68	--	6.8

Pig muscle (1.0 kg) was purified through the DEAE step described by Rubin et al. (8). A 70% ammonium sulfate cut of this fraction was dialyzed against TMG and vortexed with 10 ml of C8-NC_6NH_2-cAMP-Sepharose in 200 ml of TMG. The non-absorbed protein was removed by centrifugation. Nonspecifically bound protein was removed by washing the gel with the following in TMG: 2 liters of 0.1 M NaCl; 20 ml of 0.4 M NaCl; 20 ml of 1 M urea containing 1.5 mM methylamine. The receptor protein was eluted with 20 ml of 7 M urea containing 12 mM methylamine in TMG.

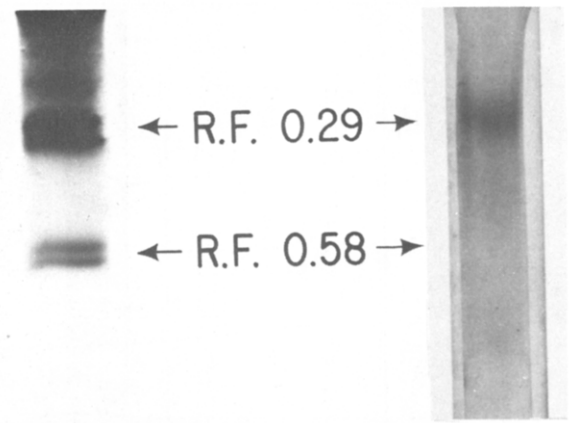

Figure 2. Polyacrylamide gel electrophoresis of receptor protein purified by affinity chromatography. 7.5% polyacrylamide gels were run at 4° in system B described by Rodbard and Chrambach (20). [^{3}H]cAMP was incubated with receptor protein prior to electrophoresis and run on parallel gels to determine the position of cAMP binding activity (4). cAMP binding activity corresponded to the major protein bands migrating at Rf = 0.29 and Rf = 0.58. Binding activity encompassed both doublets shown in the gel on the left. Left: Receptor protein obtained by elution from the affinity column with 7 M urea. Right: Receptor protein eluted from the affinity column with 7 M urea containing methylamine and subsequently purified by sedimentation in a 5-20% linear glycerol gradient.

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the starting material. Scatchard plots for the binding of [^{3}H]cAMP to the receptor were linear yielding KD = 2.0 X 10^{-8} M.

The purified receptor protein can be functionally recombined with the catalytic protein kinase to regenerate the cAMP-dependent enzyme (Fig. 3). Increasing amounts of receptor protein were added to protein kinase which has been largely freed of receptor subunits by passage through the affinity column twice. The cAMP-dependent protein kinase was progressively inhibited by added receptor protein; this inhibition was completely reversed by addition of cAMP.

The interaction of the small molecular weight receptor with cAMP is similar to the larger form with KD = 1.6 X 10^{-8} M; it is, however, inefficient in suppressing activated protein kinase subunits.

Discussion. The present studies indicate that 8-(6-aminohexyl)-amino-cAMP coupled to Sepharose functions as an efficient affinity chromatography medium for use with cAMP-

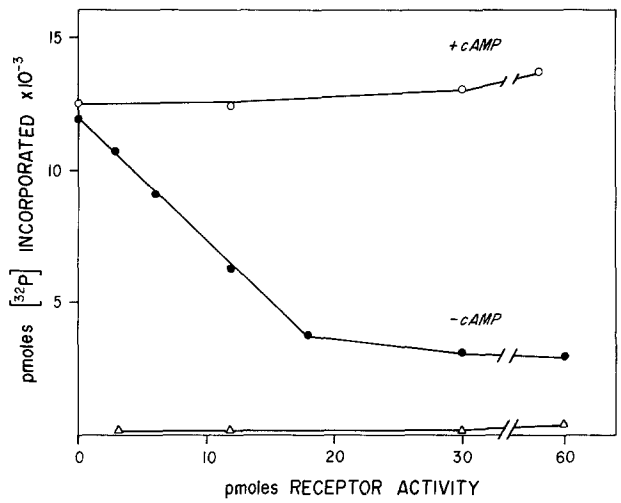

Figure 3. Effect of isolated cAMP receptor on protein kinase activity. cAMP-dependent protein kinase from porcine muscle purified through the calcium phosphate gel step of Rubin et al. (8) was passed successively through two affinity columns to remove receptor activity. The final protein kinase preparation contained 1.98 pm of [^{3}H]cAMP binding activity per mg of protein; protein kinase activity was largely cAMP independent. cAMP receptor, purified from the affinity column and completely in the 94,000 MW form, was added in increasing amounts to the cAMP independent protein kinase (52 μg) and activity assayed using protamine (160 μg) as substrate. Receptor activity is expressed as pmoles of [^{3}H]cAMP which could be bound to the protein fraction. Incubations were at 30° for 8 min. ●——●, without cAMP; O——O, with 200 pm cAMP added; and Δ——Δ, receptor alone.

dependent protein kinase. In the initial description of the synthesis of this derivative, Guilford, Larsson, and Mosbach predicted its potential usefulness in affinity chromatography (13). Wilchek et al. reported the use of N^6-caproyl-cAMP-Sepharose as an affinity column which activated protein kinase, but from which recovery of the cAMP receptor protein was not accomplished (21). Recovery of the cAMP receptor protein from the N^6-caproyl-cAMP-Sepharose column using urea has also been unsuccessful in our laboratory.

The cAMP receptor protein purified by this method is active in its two major known functions: binding of cAMP and reversible inhibition of the catalytic activity of protein kinase subunits. Material purified from bovine adrenal cortices and porcine skeletal muscle were identical in all properties studied. A smaller molecular weight form was obtained from both which contained the cAMP binding site. Generation of the smaller form could be

prevented by addition of methylamine to the urea solutions used. Though the 24,000 MW form could represent a smaller subunit as indicated by the intersection of the Rf values at 0% acrylamide (22) (data not shown), it may represent an acid cleavage product which contains the cAMP binding site. The failure of the smaller form to inhibit catalytic kinase subunits suggests alteration of the site involved in interaction with kinase subunits.

Because the catalytic protein kinase subunit differs from the receptor-kinase complex in both size and charge, retention of the receptor protein on the affinity column allows for subsequent purification of the catalytic subunit as well.

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