PURIFICATION OF RABBIT SKELETAL MUSCLE PROTEIN KINASE REGULATORY SUBUNIT USING CYCLIC ADENOSINE-3':5'-MONOPHOSPHATE AFFINITY CHROMATOGRAPHY*

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SUMMARY

A method for the preparation of homogeneous protein kinase regulatory subunit has been developed using adenosine-3',5'-monophosphate (cAMP) affinity chromatography. N^6-(2-Aminoethyl)-cAMP was synthesized and covalently linked to CNBr-activated Sepharose 4B. When a crude rabbit skeletal muscle protein kinase preparation was applied to the gel, cAMP independent activity was recovered in the effluent. The bound regulatory subunit was not removed with 2 M NaCl but could be eluted with 30 mM cAMP. This regulatory subunit was identical to regulatory subunit prepared by other methods as judged by electrophoretic migration in the presence of sodium dodecylsulfate, cAMP binding, and inhibition of protein kinase catalytic subunit activity in the absence of cAMP.

Cyclic AMP (cAMP)[1] dependent protein kinase [EC. 2.7.1.37] has been implicated as a mediator of many hormonal actions. This enzyme has been shown to consist of catalytic subunits (C) which carry out the phosphorylation reaction and cAMP binding regulatory subunits (R) which inhibit catalytic activity in the absence of cAMP (see reviews 2,3). Studies on the enzyme have been aided by the recent

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[1] The abbreviations used are: cAMP, adenosine-3',5'-monophosphate; N^6-(2-aminoethyl)-cAMP, N^6-(2-aminoethyl)-adenosine-3',5'-monophosphate; 6-Cl-cPRMP, 6-chloropurine-riboside-3',5'-monophosphate; MES, morpholinoethanesulfonic acid; C, cAMP dependent protein kinase catalytic subunit; and R, cAMP dependent protein kinase regulatory subunit (isolated by this method as a protein kinase regulatory subunit·cAMP complex).

availability of homogeneous preparations from heart (4) and skeletal muscle (5). However, isolation of large quantities of R from the purified enzyme by these techniques is difficult. In order to provide an easier method for obtaining R its purification was undertaken by affinity chromatography using N^6-(2-aminoethyl)-cAMP covalently linked to Sepharose 4B. Other laboratories have investigated the problem of isolating R using cAMP affinity columns but have encountered difficulty in the elution of the bound protein (6,7). Ramseyer et al. (8) recently reported the successful elution of R from an affinity column using urea; their product recovered at least part of its activity following removal of the urea. In this communication we describe a convenient method for the preparation of cAMP affinity gels and the elution of R using cAMP. The protein is not subjected to denaturing conditions and the product is fully active.

MATERIALS AND METHODS

Polyox was obtained from Union Carbide, 6-Cl-cPRMP from Boehringer-Mannheim, 8-[^{3}H]-cAMP from Schwarz-Mann and CNBr-activated Sepharose 4B from Pharmacia. Casein was prepared by the method of Reimann et al. (9). [γ-^{32}P]-ATP was prepared by the method of Glynn and Chappel (10) as modified by Walsh et al. (11). Rabbit skeletal muscle protein kinase was purified one step through the first DEAE column (5). Peak I from this preparation, which contains the majority of the activity in muscle and is about 10 to 30 fold purified over the initial extract, was used for further purification on affinity columns and for kinetic studies. Homogeneous cAMP dependent protein kinase and C were prepared as described (5). Molecular weight values of 38,000 and 48,000 for C and R, respectively (12), were used for the stoichiometry calculations.

Thin layer chromatography was performed on Brinkman Cellulose F plates using either isopropanol-concentrated NH_4OH-H_2O, 7:1:2, or n-butanol-acetic acid-H_2O, 5:2:3. Nucleotides were visualized with UV light and primary amines with ninhydrin. Evaporations were carried out with a Buchler rotary evaporator under reduced pressure with a water bath temperature of 40°. Ultraviolet spectra were determined on a Gilford Model 2400 spectrophotometer. Spectra

of Sepharose-bound N^6-(2-aminoethyl)-cAMP were obtained from 0.5% Polyox gel suspensions (13). Ribose was determined by the orcinol method (14) and primary amines by the reaction with trinitrobenzene sulfonic acid (15). Phosphate was determined by the Malachite green method (16) as modified by Stull[2] using the ashing procedure of Ames (17). Protein concentrations were determined by the method of Lowry et al. (18) using bovine serum albumin as a standard. Sodium dodecylsulfate electrophoresis was conducted as described elsewhere (19). Protein kinase assays were performed by the filter paper method of Reimann et al. (9) using casein as a substrate with the modified buffer (5).

The binding of cAMP was assayed by a modification of the Millipore filter method of Gill and Garren (20). One to two µg of R were incubated with purified C (0.1-2.5 µg) and 10 µg of bovine serum albumin in 2 ml of 20 mM phosphate buffer pH 6.9 containing 4 mM magnesium acetate, 1 mM ATP and 2 x 10^{-6} M [8-^{3}H]-cAMP (300-700 cpm/pmole). After 30 min at 23° the filtration was performed as described (21). These conditions allowed exchange of the bound non-labeled cAMP to occur without significant loss of cAMP binding capacity and agreed with gel filtration and equilibrium dialysis methods (22) for the measurement of the total cAMP binding capacity of Peak I protein kinase.

RESULTS AND DISCUSSION

Preparation and coupling of N^6-(2-aminoethyl)-cAMP. The synthesis of N^6-(2-aminoethyl)-cAMP and its coupling to Sepharose 4B were adapted from the methods of Guilford et al. (23). The structures of the derivatives are depicted in Figure 1. Twenty-five mg (0.067 mmoles) of 6-Cl-cPRMP were added to a solution of 0.26 g (4.35 mmoles) of ethylenediamine free base in 5 ml of water. After refluxing for 6 hours the reaction was cooled and applied to a Dowex-1-X2 column (acetate form, 1.5 x 20 cm) which was sequentially washed with 25 ml of distilled water, 25 ml of 0.01 M NH_4Cl and again with 25 ml of distilled water. The

[2] Stull, J. T., personal communication.

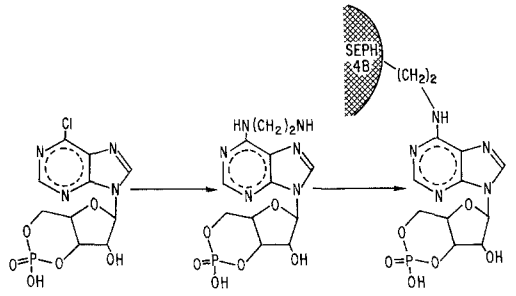

Figure 1. Synthesis of N^6-(2-aminoethyl)-cAMP covalently linked to Sepharose-4B. 6-Cl-cPRMP was converted to the desired derivative which was then coupled to CNBr-activated Sepharose 4B. Conditions for these reactions are described in the text.

nucleotide was eluted with a linear gradient of 0 to 0.5 N acetic acid (4 column volumes). Those fractions containing ninhydrin positive material which also absorbed at 265 nm were pooled and concentrated resulting in an 80% yield of N^6-(2-aminoethyl)-cAMP (20 mg, 0.054 mmoles). The compound was free from other nucleotides and ninhydrin positive material, as judged by thin layer chromatography in two solvent systems, and contained ribose, phosphate and primary amine in a ratio of 1:1:1. The N^6-(2-Aminoethyl)-cAMP showed a λmax of 262 nm at pH 1 and of 267 nm at pH 11 with a molar extinction coefficient of about 16,000 at both pH's. The apparent Ka for the activation of protein kinase by the derivative was about 2×10^{-7} M which was similar to that for cAMP itself (data not shown).

Before coupling of the cAMP derivative to CNBr-activated Sepharose 4B, the latter was washed with 5 volumes of 1 mM HCl. The washed gel was suspended in an equal volume of 100 mM sodium bicarbonate buffer, pH 9.5, containing enough N^6-(2-aminoethyl)-cAMP to give a final concentration of 2 µmoles per ml of packed gel. Although the coupling was essentially complete in a matter of minutes, the reaction was allowed to continue for 2 hours at room temperature at which time an equal volume of buffer containing 1 M ethanolamine (pH 9.5) was added. The gel was resuspended and allowed to react with the ethanolamine for 16 hours at 4°. Then the gel was filtered, washed with 5-10 volumes of the bicarbonate buffer followed by several volumes of distilled water. Phosphate analysis indicated

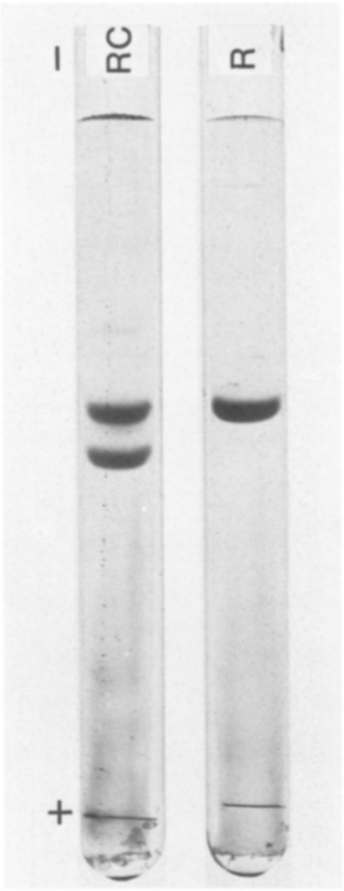

Figure 2. Electrophoretic patterns of rabbit skeletal muscle cyclic AMP dependent protein kinase (RC) and its regulatory subunit (R) purified by cAMP affinity chromatography. Electrophoresis on the 7.5% polyacrylamide gels in the presence of sodium dodecylsulfate was carried out as described (19) and the gels stained in Coomassie brilliant blue. Four µg protein were applied to the gel containing R and five µg to that containing RC.

that an essentially quantitative yield of 2 µmoles ligand per ml of packed gel was obtained. The UV spectrum of the washed gel suspended in 0.5% Polyox showed a peak at 265 nm which was absent in the case of CNBr-activated Sepharose 4B treated in the same manner in the absence of nucleotide. Before use the Sepharose 4B containing covalently linked N^6-(2-aminoethyl)cAMP was equilibrated with 5 mM MES buffer (pH 6.5) containing 0.1 mM EDTA, 15 mM β-mercaptoethanol and 100 mM NaCl (Buffer A).

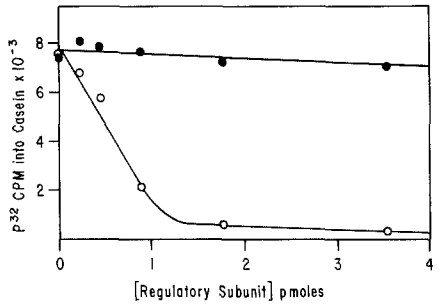

Figure 3. Inhibition of kinase activity by R purified by affinity chromatography. The reaction mixture contained 0.0425 µg pure C (1.1 pmoles) in a final volume of 0.10 ml and appropriate amounts of R in the presence (●) or absence (○) of 10^{-6} M cAMP.

Purification of R on N^6-(2-aminoethyl)-cAMP columns. The effectiveness of the N^6-(2-aminoethyl)-cAMP containing gel was tested by using it for isolation of R from a relatively crude preparation of protein kinase. Cyclic AMP dependent protein kinase from the muscle of four rabbits was purified through the first DEAE column step (5). Peak I from this preparation was passed slowly through a 1.2 x 2.0 cm column of packed gel at room temperature. In typical preparations greater than 80% of the cAMP binding capacity was retained on the column and nearly all of the kinase activity was recovered in the effluent in a cAMP independent form.

After adsorption of R the gel was washed batchwise four times for 30 minutes at 23° with 25 ml of Buffer A containing 2 M NaCl. In some cases these washes were performed by slow passage of the NaCl solution through the column. These washes removed essentially all remaining protein from the gel except for the bound R. Lack of thorough washing generally resulted in the elution of impure enzyme.

R was eluted by incubating the gel for 60 minutes at 30° in three times its volume of Buffer A containing 30 mM cAMP (pH 6.5). The gel was then removed by suction filtration and the filtrate containing the R was dialyzed exhaustively against six 1:100 v/v changes of Buffer A to remove unbound cAMP. Table I shows the results of a typical purification using this method. Yields of R generally ranged from 30 to 50% of that contained in the Peak I protein

kinase fraction. The R was purified over 500 fold to homogeneity by the affinity chromatography step.

Characterization of R. As shown in Figure 2, R prepared in this manner migrated as a single species on electrophoresis in the presenceof sodium dodecylsulfate and had a molecular weight identical to that of R component of pure cAMP dependent protein kinase (5). Since pure preparations of R from rabbit skeletal muscle have been shown to bind 1 mole of cAMP per mole of protein and to recombine with C in a 1 to 1 molar ratio (12,24) these parameters were tested with R isolated by cAMP-affinity chromatography. As shown in Table I, the eluted R bound 19,200 pmoles cAMP per mg protein when

Table I

Affinity chromatography purification of the cyclic AMP dependent protein kinase regulatory subunit

Skeletal muscle protein kinase from four rabbits was purified through the first DEAE column step (5). Four hundred ml of this preparation was applied to a 1.2 x 2.0 cm column of gel containing N^6-(2-aminoethyl)-cAMP. The gel was then washed with 2 M NaCl. The bound R was eluted by washing with 3 volumes of neutralized 30 mM cAMP for 60 minutes at 30°.

Fraction	Total protein	Cyclic AMP binding[a]		
	mg	pmoles/mg	total pmoles bound	% recovery
Peak I	1790	35	63,000	100
Effluent	1750	3.9	6,800	11
Cyclic AMP Eluate	1.6	19,200	30,700	49

[a]Assayed as described in Materials and Methods.

measured by the cAMP exchange binding assay. This corresponds to a value of 0.92 moles cAMP bound per R monomer. As seen in Figure 3, R also inhibited C activity in the absence of cAMP. Greater than 90% inhibition was obtained after the addition of 1.1 to 1.2 pmoles of R to 1.1 pmoles of C corresponding to a molar ratio of 1:1. From these data it would appear that R eluted from affinity columns with cAMP is a fully active protein.

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