SEPARATION OF REGULATORY AND CATALYTIC SUBUNITS OF THE CYCLIC 3',5'-ADENOSINE MONOPHOSPHATE-DEPENDENT PROTEIN KINASE(S) OF RABBIT SKELETAL MUSCLE*

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SUMMARY

The cyclic 3',5'-adenosine monophosphate-dependent (cAMP-dependent) protein kinase(s) from rabbit skeletal muscle has been separated into catalytic and regulatory subunits by affinity chromatography utilizing a casein-Sepharose column in the presence of cAMP. The isolated catalytic subunit manifests full activity in the absence of cAMP but its requirement for this nucleotide is regained when the enzyme is reconstituted by addition of the regulatory subunit. Evidence is presented for the existence of more than a single type of regulatory or cAMP-binding subunit in muscle.

The finding of a cAMP-dependent protein kinase which catalyzes the phosphorylation and activation of phosphorylase kinase has helped in our understanding of how cAMP serves as a regulatory agent (1). This protein kinase also catalyzes the phosphorylation and inactivation of glycogen synthetase (2,3) and has recently been shown to activate the hormone-sensitive lipase of adipose tissue (4,5). A similar enzyme catalyzes the phosphorylation of histones in liver (6) and protamine in trout testis (7). The presence of cAMP-dependent protein kinases has been demonstrated in a variety of tissues (8-12) and in E. coli (13).

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A study of the kinetic and physicochemical behavior of the cAMP-dependent protein kinase from bovine heart (9) provided evidence to support the theory that this enzyme is made up of a catalytic or "C" subunit and a regulatory or "R" subunit which binds cAMP. The following model was proposed:

$$\underset{\text{(inactive complex)}}{C \cdot R} \rightleftharpoons \underset{\text{(active form of the enzyme)}}{C} + \underset{\text{(cAMP binding subunit)}}{R} \qquad (1)$$

In this model it was assumed that the binding of cAMP to R would favor dissociation of C·R yielding more of the active form, C. Gill and Garren (11) found that adrenal cortical extracts contained a cAMP-binding protein or proteins which could be partially separated from the protein kinase activity in their preparations. They also found that addition of the cAMP-binding protein to the protein kinase fraction caused a decrease in its activity when measured in the absence of cAMP but was without effect in its presence. These workers postulated independently that cAMP activation of the protein kinase might result from dissociation of a binding moiety from the enzyme (11). A very recent study on the effects of cAMP on the quaternary structure of a protein kinase is that of Tao, et al. (14) who found that in the presence of cAMP the protein kinase from reticulocytes separates into a subunit binding the nucleotide and a catalytic subunit.

In the present work the cAMP-binding regulatory and cAMP-independent catalytic subunits of the cAMP-dependent protein kinase(s) of rabbit skeletal muscle have been completely separated chromatographically on a casein-Sepharose column in the presence of cAMP. Recombination of the subunit fractions results in reconstitution of the cAMP-stimulated enzyme.

MATERIALS AND METHODS

The protein kinase used in the experiments to be described was the first peak of rabbit skeletal muscle cAMP-stimulated protein kinase activity eluted from a Whatman DEAE cellulose (DE 52) column in the purification procedure of Walsh et al. (1) as modified by Reimann et al. (15,16). By density gradient centrifugation this fraction has been shown to contain two cAMP-dependent protein kinases, one of which has a sedimentation coefficient of 6.8S and the other a coefficient of 4.9S (16).

Assays of protein kinase activity were carried out by a modification of a previously described method (1). Assay reaction mixtures at pH 6.5 contained: potassium phosphate, 0.84 μmole; γ^{32}P-ATP, 0.017 μmole; magnesium acetate, 0.17 μmole; cAMP (where added), 1 X 10^{-4} μmole; casein, 0.51 mg; enzyme (≃ 1 μg); and binding fraction (where added) in a final volume of 80 μl. Reactions were initiated by the addition of 10 μl of binding fraction (where added) and 10 μl of enzyme in rapid succession, and terminated by pipetting a 50 μl aliquot of the reaction mixture onto a filter paper disc. The disc was then dropped immediately into cold 10% trichloroacetic acid for washing and subsequent counting as described previously (16). Casein-Sepharose 4B affinity gels were prepared by the method of Cuatrecasas (17). cAMP was identified by descending chromatography using Whatman No. 3 chromatography paper and a solvent consisting of n-butanol, acetone, glacial acetic acid, ammonia (28-30%), and water (35:25:15:2.5:22.5 v/v). γ^{32}P-ATP (Sp. act.: 20 c/m mole) was prepared by a modification of the method of Glynn et al. (18). [8-^{3}H]-adenosine 3',5'-monophosphate (Sp. act.: 15.7 c/m mole) was purchased from Schwartz Bioresearch, Orangeburg, N. Y.

RESULTS

Since it was known that casein is a good substrate for cAMP-

dependent protein kinases, it was reasoned that an affinity column (17) consisting of casein linked to Sepharose might be used in separating the catalytic and regulatory subunits of this enzyme on a preparative scale. An experiment in which this objective was achieved is shown in Fig. 1. The protein kinase was first pre-incubated with 1 X 10^{-5} M ^{3}H-cAMP to dissociate the C and R subunits in accordance with Equation 1 (see above) and was then chromatographed. As can be seen, there was a large breakthrough peak of ^{3}H followed by a low secondary peak of ^{3}H. All of the radioactivity in these peaks was shown to be ^{3}H-cAMP by chromatographic analysis of boiled samples. The ^{3}H-cAMP peaks were followed by a peak of protein kinase activity which showed essentially no requirement

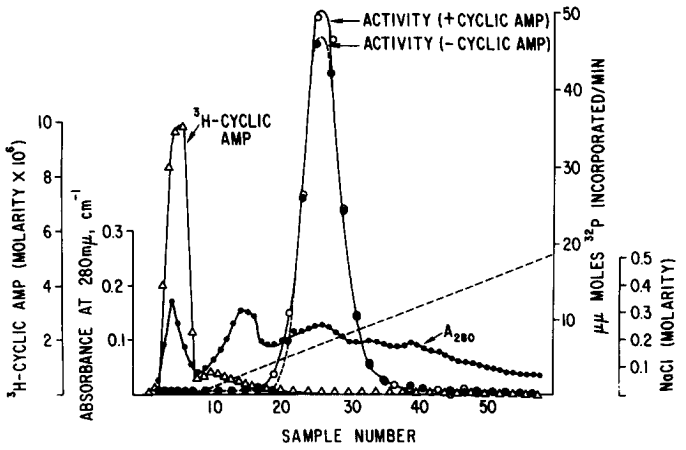

Fig. 1. Chromatography of skeletal muscle cAMP-dependent protein kinase using casein-Sepharose in the presence of cAMP. To 7.2 ml (≃ 7 mg) of rabbit skeletal muscle cAMP-dependent protein kinase (Peak 1) (16), 0.1 ml of 7.5 X 10^{-4} M ^{3}H-cAMP was added. Following a 10 min incubation at 0°, this solution was applied to a 0.9 X 7 cm casein-Sepharose 4B column equilibrated with 10 mM 2[N-morpholino] ethane sulfonic acid (MES) buffer, pH 6.0, containing 1 mM EDTA. This addition was followed by 7.2 ml of the buffer containing 0.1 ml of 7.5 X 10^{-4} M ^{3}H-cAMP and then by 3 ml of buffer with no cAMP added. The column was eluted with 170 ml of a linear gradient of NaCl in the same buffer with no added cAMP. Samples (3.5 ml) were assayed for protein kinase in the presence (O —— O) and absence (● —— ●) of cAMP; 0.05 ml aliquots were analyzed for ^{3}H (Δ —— Δ) in 10 ml dioxane-based scintillant; and the absorbance at 280 mμ was determined for each sample. The recovery of enzyme activity was greater than 60%.

for cAMP. This was in contrast to the enzyme originally applied to the column which was stimulated 50-fold by cAMP.

Samples consisting of the original breakthrough peak of ^{3}H-cAMP and the secondary ^{3}H-cAMP peak from the chromatographic experiment of Fig. 1 were pooled separately and portions were rechromatographed on Sephadex G-150 (Figs. 2A and 2B). Both fractions showed the presence of two ^{3}H-cAMP-binding protein peaks as well as having a peak of free ^{3}H-cAMP. Although the ^{3}H-cAMP-binding peaks from the two fractions were eluted in the same position, the relative proportions of the two peaks differed. The cAMP-independent protein kinase activity peak from the experiment of Fig. 1 was also rechromatographed on Sephadex G-150 (not illustrated). This fraction gave a single symmetrical peak with a maximum at an elution volume of 122 ml. Ovalbumin peaked at an elution volume of 89 ml with the Sephadex column used in these experiments.

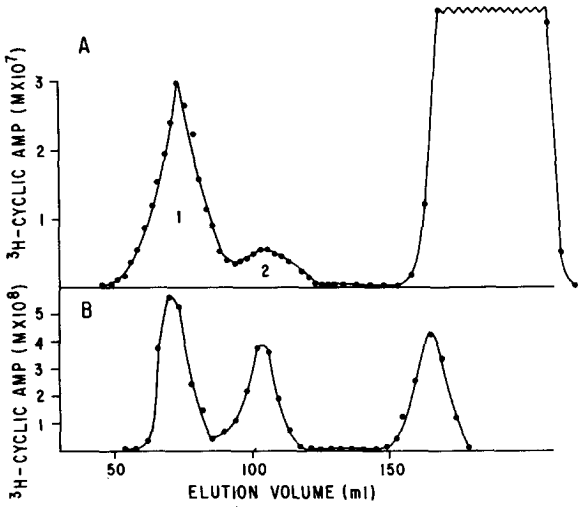

Fig. 2. Chromatography of cAMP binding fractions on Sephadex G-150. Five ml of pooled Samples 1-7 and 2.3 ml of Samples 8-15 described in Fig. 1 were chromatographed (A and B, respectively) on a 1.5 X 90 cm Sephadex G-150 column which had been equilibrated with 10 mM MES buffer, pH 6.5, containing 1 mM EDTA. Aliquots (0.1 ml) of each sample were analyzed for ^{3}H-cAMP by liquid scintillation counting.

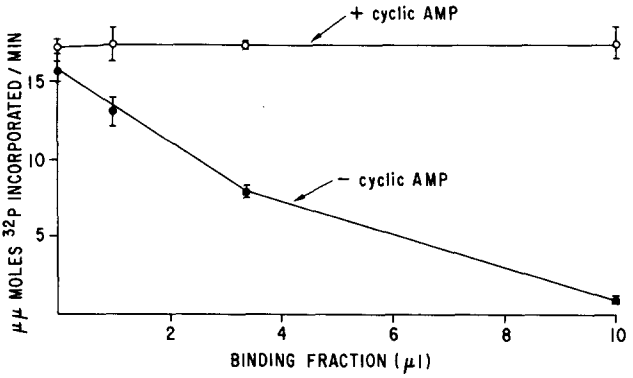

Fig. 3. Effect of increasing concentrations of the cAMP-binding fractions on the catalytic activity of the protein kinase fraction. The binding and catalytic fractions used were Samples 10 (diluted appropriately) and 26 (diluted 10-fold) respectively, from the casein-Sepharose 4B column described in Fig. 1. The length of the bars represents the range of duplicates in one experiment carried out.

Material from any of the ^{3}H-cAMP-binding peaks was found to inhibit the cAMP-independent protein kinase fraction of Fig. 1 when tests were carried out in the absence of added cAMP. No inhibition was obtained in the presence of added cAMP. This is illustrated in Fig. 3. Since the extent of inhibition was roughly

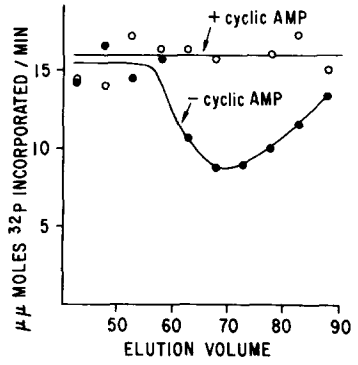

Fig. 4. Inhibition of the protein kinase by individual fractions from the first ^{3}H-cAMP binding peak of Fig. 2B. Samples from the individual tubes making up Peak 1 of Fig. 2B were tested for their ability to inhibit the cAMP-independent protein kinase obtained in the experiment of Fig. 1. For these tests each sample was combined undiluted with a 1 to 5 dilution of Sample 26 of Fig. 1 and protein kinase activity (ordinate) was determined in the presence or absence of 1 X 10^{-6} M cAMP.

proportional to the amount of ^{3}H-cAMP-binding fraction added (Fig. 3), it was possible to utilize this property as the basis for an assay to determine whether ^{3}H-cAMP binding and inhibitory potency coincide in the various chromatographic separations. This was found to occur in all instances and is illustrated in Fig. 4 for the first ^{3}H-cAMP-binding peak from Fig. 3B. The profile for protein kinase activity reaches a minimum at the same elution volume at which ^{3}H-cAMP binding was maximal. None of the ^{3}H-cAMP binding fractions were inhibitory after being boiled for 1 min.

DISCUSSION

The experiments reported in this paper provide further evidence supporting the concept (9,11,14) that the mechanism of action of cAMP in activating protein kinases involves the dissociation of an enzyme-inhibitor complex designated as R·C in Equation 1. By using an affinity-chromatographic column, R and C were effectively separated in the presence of ^{3}H-cAMP. The R subunits were located in the chromatographic fractions by virtue of their retention of ^{3}H-cAMP, but the amount of nucleotide present was not sufficient to block their ability to inhibit the protein kinase when assays were carried out in the absence of additional cAMP. The purified protein kinase used in this study was known to consist of two cAMP-dependent enzymes separable by density gradient centrifugation (16) (Also see Materials and Methods). It is of interest that on dissociation it appears to yield two types of R subunits and one type of catalytic unit as evidenced by chromatography on Sephadex G-150. In preliminary experiments it has been shown that the original sucrose density gradient pattern of the protein kinase can be reconstituted when the C and R subunits are recombined.

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