Studies on the Adenosine 3',5'-Monophosphate-dependent Protein Kinases of Rabbit Skeletal Muscle*

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SUMMARY

The adenosine 3',5'-monophosphate-dependent (cAMP-dependent) protein kinases of rabbit skeletal muscle have been partially characterized in terms of molecular size and subunit composition. Three and possibly four species of protein kinase have been detected. The largest form of the enzyme was estimated by gel filtration and sedimentation to have a molecular weight of 123,000 and to be made up of a catalytic subunit (C) and a regulatory or CAMP-binding subunit (R) with molecular weights of 49,000 and 82,000, respectively. A second muscle protein kinase was estimated to have a molecular weight of 76,000 with a catalytic subunit the same size as that of the larger protein kinase and with an R subunit having a molecular weight of 50,000. The other muscle protein kinases were not purified sufficiently to permit making meaningful estimates of their molecular weights, but it could be concluded that they differed from one another primarily in the size of their R subunits. Experiments were carried out which showed that the R subunits can be readily altered in vitro by aging or by proteolytic attack, so it is possible that some of the forms that were detected may have been formed during enzyme isolation. Resolution of the muscle protein kinases into C and R subunits was achieved by substrate-affinity chromatography after dissociation of the holoenzymes in the presence of [3H]-cAMP. Reconstitution of the original enzyme forms from separated C and R subunits was shown by physical techniques and kinetic measurements.

The finding that a cAMP-dependent protein kinase is involved in the hormonal regulation of glycogen degradation (1, 2) and

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The abbreviations used are: cAMP, adenosine 3',5'-monophosphate; MES, 2-(N-morpholino)ethane sulfonic acid.

synthesis (3-5) suggests that similar protein kinases could catalyze the cAMP-mediated activation or deactivation of other processes. The existence of cAMP-dependent protein kinases in various tissues and species (1, 5-12), the cAMP- and ATP-dependent stimulation of a lipase in adipose tissue by this enzyme (13, 14), and the in vivo phosphorylation of a liver histone by a protein kinase (15) support this view. Recent investigations have focused on the mechanism by which cAMP exerts its stimulatory effect on protein kinase (or kinases). Experiments with the bovine heart enzyme led to the proposal that the kinase is composed of a catalytic or "C" subunit and a CAMP-binding regulatory or "R" subunit, and that the binding of cAMP to the R subunit favors dissociation of the RC complex yielding the free uninhibited C subunit (10). This model has been confirmed in this and other laboratories by the physical separation of R and C subunits of protein kinases from skeletal muscle (16), adrenal cortex (17), reticulocytes (17), liver (18), and heart (19). Kinetic evidence that the separated subunits of the enzyme will recombine is derived from the observation that the R subunit from skeletal muscle inhibits the catalytic activity of the C subunit in the protein kinase reaction and that this inhibition is prevented by cAMP (11, 16-20). In this paper it is shown that physical recombination of the subunits actually occurs in this system as was the case with the subunits of the heart enzyme (19).

Several cAMP-dependent protein kinase activities have been detected in enzyme preparations from skeletal muscle (21), and in a recent communication (16) it was shown that more than one type of R subunit is also present. In the present study the origin and interrelation of the different protein kinases have been investigated by Sephadex chromatography and sucrose density gradient centrifugation of the native enzymes, their separated R and C subunits, and the protein kinases reconstituted from them.

EXPERIMENTAL PROCEDURE

Preparation of Protein Kinases from Rabbit Skeletal Muscle

Method A—The enzymes were purified as described by Reimann et al. (21) and this procedure is simply summarized here. Acid precipitation steps are carried out at pH 6.1 and 5.5. The supernatant fraction from the pH 5.5 precipitation step is centrifuged at 78,000 x g. The resulting supernatant fraction is subjected to calcium phosphate gel adsorption and then the enzyme is chromatographed on a Whatman DE 52 column.
This step resolves two peaks of cAMP-dependent protein kinase activity, referred to as Peaks I and II, with respective purifications of 150- and 400-fold over the crude extract.

**Method B**—The initial steps, starting with 1200 g of fresh skeletal muscle, were performed as described by Reimann et al. (21) through the first acid precipitation at pH 6.1. The supernatant fraction from this step was applied to a histone-Sepharose 4B substrate-affinity column, 6.5 x 30 cm, washed with 2 liters of 1 mM EDTA-10 mM potassium phosphate buffer, pH 6.5, and the protein kinase was eluted with 2 liters of the same buffer containing 1 M NaCl. The protein was then precipitated with (NH₄)₂SO₄ at 70% saturation and was collected by centrifugation for 30 min at 10,000 X g. The precipitate was resuspended in 30 ml of 1 mM EDTA-10 mM potassium phosphate, pH 6.5, and chromatographed on a Sephadex G-100 column, 5 x 150 cm, equilibrated with the same buffer to separate the protein kinase from smaller proteins which entered the gel in contrast to the protein kinases which are excluded. The protein kinase fraction (∼30-fold purified) was concentrated with (NH₄)₂SO₄ and then resuspended in the EDTA-potassium phosphate buffer. A single peak of protein kinase activity (sedimentation constant = 6.8 S) was detected in this preparation by sucrose gradient centrifugation.

**Preparation of R-[3H]cAMP Complex from Protein Kinase Obtained by Method B**

For this preparation [3H]cAMP (1 x 10⁻⁴ M) was added to the protein kinase solution and the mixture was chromatographed at 5° on a Sephadex G-100 column, 5 x 150 cm, equilibrated with 0.5 M NaCl. The R-[3H]cAMP complex and catalytic subunits were separated under these conditions. Fractions containing the former were identified by sedimentation, pooled, and dialyzed against 10 mM MES buffer-1 mM EDTA, pH 6.5. The solution was applied to a Whatman DE 52 column, 1.5 x 10 cm, which had been equilibrated in 10 mM MES, pH 6.5, containing 1 mM EDTA. The column was developed with a 200-ml linear NaCl gradient to 0.2 M salt, and the R-[3H]cAMP complex, which eluted between 0.1 and 0.15 M NaCl, was collected.

**Sucrose Density Gradient Centrifugation**

Sucrose gradients (5 to 20%) were prepared by a slight modification (21) of the procedure of Martin and Ameo (22). The gradients were formed in 10 mM MES buffer, pH 6.5, containing 1 mM EDTA. Human hemoglobin, prepared by the method of Ingram (23), and rabbit muscle phosphorylase b, prepared from a lyophilized powder (24), were used as markers. Centrifugation was carried out at 30,000 rpm in a Beckman SW 40 rotor at 15-20° for 16 hours. Fractions (0.4 ml) were collected and aliquots were assayed for phosphorylase b (25). Hemoglobin was determined by its absorbance at 411 nm. Sedimentation constants of 8.4 S for phosphorylase b (26) and 4.6 S for hemoglobin (27) were used to calculate the sedimentation constants of the proteins studied.

**Protein Kinase Assay**

Assays were performed at pH 6.5 in a mixture containing: potassium phosphate, 0.84 μmole; [3P]ATP, 0.017 μmole; casein or histone (Sigma, type II-A), 0.51 mg; and enzyme in a final volume of 60 μl. Reactions were initiated by the addition of cAMP. After incubation at 30° for 20 min the reactions were terminated by pipetting a 50-μl aliquot of the reaction mixture onto a filter paper disc which was dropped immediately into cold 10% trichloroacetic acid for removal of residual [3P]ATP as described earlier (21). A 15-min wash with slow stirring in this solution (10 ml per disc) was followed by three successive washes with 5% trichloroacetic acid, one in the cold and two at 25°. After 5-min washes in 95% ethanol and then ether, the discs were dried and counted in 10 ml of a toluene-based scintillant. Activity is expressed as units of enzyme activity (picomoles of [3P] incorporated into protein per min) when casein was the substrate or as [3P] incorporated per min when histone was the substrate.

**Histone and Casein-Sepharose 4B Affinity Columns**

The protein-Sepharose 4B affinity columns were prepared essentially as described by Cuatrecasas (28). Twenty grams of solid cyanogen bromide were added to 400 ml of a suspension of Sepharose 4B in H₂O (200 ml of H₂O plus 200 ml of settled Sepharose). The solution was maintained at pH 11 with 4 N NaOH and ice was added periodically to keep the temperature at 25°. The reaction was complete in 30 to 45 min. After adding more ice the suspension was transferred to a Buchner funnel and washed with cold 0.1 M NaHCO₃ for 5 min. Casein or histone (600 mg) in 200 ml of the same buffer was added to the treated Sepharose and the mixture was incubated 12 hours at 4° with slow stirring and then washed with 20 volumes of 10 mM MES buffer, pH 6.5, containing 1 mM EDTA.

**Gel Filtration**

A Sephadex G-150 column, 1.5 x 90 cm, was equilibrated at 4° in 10 mM MES buffer, pH 6.5, containing 1 mM EDTA. Samples (0.2 to 5 ml) were placed on the column and 2- to 4-ml fractions were collected. The buffer flow was 12 to 14 ml per hour. The column was calibrated with globular proteins having known physical constants. The void volume was taken as the elution volume of blue dextran. Cytochrome c was detected by its absorbance at 411 nm. Other standards were detected by their absorbance at 280 nm. Stokes radii for cytochrome c and the bovine serum albumin dimer were obtained from data of Siegel and Monty (29). Stokes radii for other marker proteins were calculated (29) from molecular weights, sedimentation coefficients, and partial specific volumes derived from several literature sources (30-33).

**Treatment of Protein Kinase or Its Subunits with Trypsin**

Samples (0.2 to 1.0 ml) of protein or a subunit fraction were incubated with 1:100 (g/g) trypsin (10 to 20 μl) for 5 min at 30°. The reactions were terminated by addition of a 3-fold excess (g/g) of soybean trypsin inhibitor (10 to 50 μl) and placing on an ice bath.

**Chemicals**

Sephadex G-150 and Sepharose 4B were obtained from Pharmacia and [8-3H]cAMP from New England Nuclear. Casein was purchased from Nutritional Biochemicals Co. and histone (type II A) from Sigma. Trypsin was obtained from Worthington Biochemical Corp. Analytical reagent grade sucrose was from Mallinckrodt Chemical Works.

**RESULTS**

**Separation of Rabbit Muscle Protein Kinases into Catalytic and Regulatoty Subunit Fractions,—** Cyclic AMP-dependent protein kinases can be dissociated by cAMP into regulatory or
FRACTION NUMBER

The protein kinases of Peaks I and II after dissociation of the enzymes with cAMP were used to study the substrate-affinity chromatography. To 7.2 ml of Peak I (7 mg of protein) or Peak II (4 mg of protein), shown in A and B, respectively, was added 0.1 ml of 7.5 × 10⁻⁴ M [³H]cAMP. Following a 10-min incubation at 0°C, the solutions were applied to a column, 0.9 × 7 cm, equilibrated with 10 mM Mes buffer, pH 6.0, containing 1 mM EDTA. This addition was followed by 7.2 ml of the same buffer containing 0.1 ml of 7.5 × 10⁻⁴ M [³H]cAMP and then by 3 ml of buffer without cAMP.

The column was then eluted with a linear gradient of NaCl in the same buffer with no added cAMP. Fractions (3.5 ml) were assayed for protein kinase activity in the presence (○—○) and absence (●—●) of cAMP; 0.05-ml aliquots were analyzed for [³H]cAMP (A—A) by counting. The absorbance at 280 nm was determined for each fraction. The recovery of enzyme activity was greater than 60% for both Peaks I and II.

Gel Filtration Using Sephadex G-150—In order to achieve further purification of the subunit fractions and to obtain information concerning the molecular sizes of individual protein components, gel filtration experiments were carried out with a calibrated Sephadex G-150 column. Fig. 2, A and B, shows the elution profiles of the C subunit fractions, C₁ and C₂, obtained from Peaks I and II, respectively (Fig. 3 presents a scheme explaining the nomenclature applied to subunit fractions derived from the protein kinases of Peaks I and II). In each instance a single symmetrical activity peak was present at the same elution volume. The elution profile for the first peak of [³H]cAMP from the experiment of Fig. 1A is shown in Fig. 4A. Two [³H]cAMP-binding protein peaks, R₁a·[³H]cAMP and R₁b·[³H]cAMP, were observed, as well as a large peak of free [³H]cAMP. The second peak of [³H]cAMP from the experiment of Fig. 1A showed an elution pattern (not illustrated) essentially identical with that of Fig. 4A except that much less free [³H]cAMP was present. The first peak of [³H]cAMP from the experiment of Fig. 1B, i.e. from the casein-Sepharose 4B column separation of Peak II subunits, consisted entirely of free [³H]cAMP. The second peak of [³H]cAMP from this experiment gave the elution pattern shown in Fig. 5A. One major peak of protein bound [³H]cAMP, R₁, [³H]cAMP, was present together with a small shoulder which was not further characterized. The residual undissociated protein kinase activity which overlapped

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All peaks of radioactivity were shown chromatographically to consist solely of free [³H]cAMP or protein-bound [³H]cAMP, the latter form being separable from free [³H]cAMP by gel filtration. Free [³H]cAMP was released by heating samples of protein-bound [³H]cAMP.
The second [3H]cAMP peak of Fig. 1B eluted ahead of the binding protein (not shown in Fig. 5A). Stokes radii for the subunit components were estimated by comparing their elution volumes with those of known standards as shown in Fig. 6 (29). No estimation was made for the first [3H]cAMP-binding protein peak eluted in Fig. 4A, since as will be shown, this fraction R4A-[3H]cAMP, consists of more than a single component.

Stokes radii were also determined (Fig. 6) for two intact protein kinases or holoenzymes which were deemed of sufficient enzymatic purity to warrant this determination. One of these was the protein kinase of Peak II (Method A), and the other was the protein kinase obtained by Method B as described under "Experimental Procedure." In the latter method fresh muscle extract was chromatographed on a histone-Sepharose 4B substrate-affinity column and no calcium phosphate gel step is included in contrast to Method A. This point may have a bearing on the nature of the product obtained as will be discussed later. The R-[3H]cAMP complex derived from the protein kinase prepared by Method B was also characterized by gel filtration. This complex, assumed to be identical with R-[3H]cAMP of the scheme shown in Fig. 3, is designated as R-[3H]cAMP (Method B) in Fig. 6. The C subunit derived from the protein kinase prepared by Method B behaved identically with C4 and C11 on gel filtration.

**Sucrose Density Gradient Centrifugation and Estimations of Molecular Weights**—Sucrose density gradient centrifugations were carried out to characterize further the skeletal muscle protein kinase subunits. Fig. 2, C and D, shows the sedimentation patterns obtained for the catalytic subunit fractions from Peaks I and II after gel filtration, i.e. C4 and C11. In each case a sedimentation constant of 4.1 S was obtained.9 In Fig. 4, B and C, shows the sedimentation patterns obtained for the two [3H]cAMP-binding protein fractions, R4A-[3H]cAMP and R11-[3H]cAMP, derived by gel filtration of the binding proteins of Peak I. It will be noted (Fig. 4B) that R4A-[3H]cAMP consists of two species, one with a sedimentation constant of approximately 4.9 S and the other with a constant of 3.9 S. The complex R11-[3H]cAMP consists of a single species with a sedimentation constant of 3.4 S (Fig. 4C). The [3H]cAMP-binding protein fraction from Peak II, R11-[3H]cAMP, sedimented as a single species with a sedimentation constant of 3.9 S (Fig. 5B). In these determinations it is apparent that a considerable amount of free [3H]cAMP was present in the samples tested. This was not an unusual finding in working with the R-[3H]cAMP complexes which appeared to be relatively unstable. Denaturation of the proteins in these fractions often occurred on aging and was accompanied by release of the bound [3H]cAMP. Another R-[3H]cAMP complex which was characterized by gel filtration was the complex derived from the protein kinase prepared by Method B. This subunit fraction, R-[3H]cAMP (Method B), gave a symmetrical peak with a sedimentation constant of 4.9 S identical with that of R4A-[3H]cAMP. The catalytic subunit fraction derived from the protein kinase prepared by Method B had a sedimentation constant of 4.1 S, identical with that of C4 and C11.

Sedimentation coefficients were also determined for the intact protein kinases or holoenzymes. In an earlier report (21) it was shown that Peak I (Method A) contains two sedimenting species of the cAMP-dependent enzyme with sedimentation coefficients of 6.8 and 5.2 S. These values were reconfirmed in the present study. The protein kinase of Peak II was shown (21) to sediment as a single component with a sedimentation coefficient of 5.9 S, a value which was also reconfirmed in the present study. The protein kinase prepared by Method B was found to sediment as a single species with a sedimentation coefficient of 6.8 S identical with that of the heavier component of Peak I (Method A).

Utilizing the Stokes radii as determined by gel filtration and the sedimentation coefficients as determined above, it was possible to estimate the molecular weights of several of the muscle protein kinases and their component subunits as described by Siegel and Monty (29). In making the calculation a value of 0.725 cm2 per g was assumed as the partial specific volume. The results are given in Table I.

**Recombination of Regulatory and Catalytic Subunits of Protein Kinases**—In an earlier communication giving a preliminary report of this work (16) it was shown that the R-cAMP complex derived from Peak I inhibited the C subunit fraction from Peak I when tests were carried out in the absence of cAMP, i.e. in the absence of any cAMP other than the trace amount carried over into the reaction mixture as a part of the complex. No inhibition was present when cAMP was added. Kinetic evidence for recombination of R and C subunits derived from different muscle

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9 In a previous publication (21) an incorrect sedimentation constant for the hemoglobin marker was used, and 4.1 and 5.2 S components were erroneously reported as having sedimentation constants in the range of 3.4 to 3.8 S and 4.9 S, respectively.
Protein kinase fractions:  

**Fig. 3.** Scheme for designating the various protein kinases and their subunits as derived from rabbit skeletal muscle. The two cAMP-dependent protein kinase fractions obtained by chromatography with DE 52 (21) are referred to as Peaks I and II. The derivation of particular subunit fractions from Peaks I or II are indicated by arrows. In addition to subunits stemming from Peaks I and II, these fractions were also derived from the protein kinase prepared by Method B as described in the text.

![Diagram of Protein Kinase Fractions](image)

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**Fig. 4.** Gel filtration and sucrose density gradient centrifugation of R-[3H]cAMP complexes from the protein kinase of Peak I. Curve A: Five milliliters of pooled Fractions 1 to 7 containing [3H]cAMP from the casein-Sepharose 4B chromatography of Peak I (Fig. 1A) were chromatographed (A) on a standardized Sephadex G-150 column. Fractions (0.1 ml) were analyzed for [3H]cAMP by liquid scintillation counting. This figure is reproduced from a previous communication (16) by permission of Academic Press. Curves B and C: An aliquot (0.2 ml) was taken from each peak of protein-bound [3H]cAMP in Curve A and examined independently by sucrose density gradient centrifugation. Tritium analyses were performed on aliquots as in Fig. 1. Curve B shows the sedimentation profile obtained for the first peak and Curve C the pattern for the second peak. Sedimentation constants were calculated with phosphorylase b and hemoglobin as markers (profiles not shown in figure).

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In another experiment, the lightest (3.4 S) form of R subunit derived from Peak I, R1B-[3H]cAMP, was recombined with C1. This produced one peak of cAMP-dependent protein kinase having a sedimentation coefficient of 5.0 S (Fig. 8C). It is presumed that if both the 5.4 and 5.0 S protein kinases produced by recombination (Fig. 8B and C) were present in the original enzyme, they might well have shown up as one peak at 5.2 S as is found in Peak I (Fig. 8A). The sucrose density profile of the original Peak I enzyme could thus reflect a mixture of at least three protein kinases, i.e. those which can be produced by recombination of three R subunits (R1A, R1A2 and R1B) with the C subunit. Further support for this concept was obtained in other experiments in which physical reconstitution of the protein kinases of Peak I was carried out with the complete R-[3H]-cAMP complex, R1-[3H]-cAMP, obtained prior to fractionation by gel filtration, plus either C1 or C17 (Fig. 8D). In these experiments three distinct peaks were discernible on density gradient centrifugation of the reconstituted enzyme. The reason for the lack of cAMP dependence of the lighter peak is not clear.

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**Modification of Muscle Protein Kinases and Their Subunits—**Preliminary experiments have been carried out which show that the complex that is seen in regard to multiple forms of rabbit skeletal muscle protein kinase may be due to modifica-
Fig. 5. Gel filtration and sucrose density gradient centrifugation of the R-[3H]cAMP complex from the protein kinase of Peak II. A 2-ml sample of pooled Fractions 13 to 29 containing [3H]-cAMP from the casein-Sepharose 4B chromatography of Peak II (Fig. 1B) was chromatographed (A) on a Sephadex G-150 column and fractions were analyzed for tritium on 0.1-ml aliquots. Sucrose density gradient centrifugation (B) was done on another sample (0.2 ml) of the same pooled fractions above. Aliquots were analyzed for [3H]cAMP. Sedimentation constants were calculated with phosphorylase b and hemoglobin as markers (profiles not shown in figure).

Table I

<table>
<thead>
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<th>Component</th>
<th>Stokes radius</th>
<th>Sedimentation coefficient</th>
<th>Molecular weight</th>
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<td>Protein kinase prepared by Method B</td>
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<td>6.8</td>
<td>123,000</td>
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<tr>
<td>Protein kinase of Peak II</td>
<td>36.0</td>
<td>5.2</td>
<td>76,000</td>
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<tr>
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<td>3.4</td>
<td>37,000</td>
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<tr>
<td>C1, CII, and C (Method B)</td>
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<td>4.1</td>
<td>49,000</td>
</tr>
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</table>

* Assumed to be identical with the 6.8 S protein kinase of Peak I.

* Assumed to be identical with the 4.9 S component derived from Peak I, i.e. RIA-[3H]cAMP.

Fig. 6. Estimation of Stokes radii of rabbit skeletal muscle protein kinases and their subunits by gel filtration on Sephadex G-150. The marker proteins are indicated by open circles. Data are plotted according to the method of Siegel and Monty (29). The nomenclature used for designating protein kinase fractions (closed circles) is given in Fig. 3. BSA, bovine serum albumin.

Fig. 7. Inhibition of the C subunit fraction of Peak II protein kinase (Cn) by the R-[3H]cAMP complex from Peak I. R-[3H]cAMP was Fraction 10 from the experiment of Fig. 1A. The source of Cn was Fraction 45 from the experiment of Fig. 1B; this fraction was diluted 3-fold prior to use. Casein served as the substrate in this experiment.
The protein kinase activity eluted from the column following Both of these trypsin-treated proteins chromatographed on the protein kinase of Peak II and of RI1. [3H]cAMP has also been done.

Fig. 8. Physical recombination of C and R subunits of cAMP-dependent protein kinases. A, density gradient centrifugation of Peak I protein kinase, 0.05 ml, prepared according to Method A. B, density gradient centrifugation of reconstituted protein kinase made by incubating 0.2 ml of RII-[3H]cAMP with 0.02 ml of CI (Fraction 20 of Fig. 1A) for 3 hours at 25°. The source of RII-[3H]-cAMP was the peak tube of the heavier complex obtained by gel filtration of RII-[3H]cAMP (see Fig. 3A). C, density gradient centrifugation of reconstituted protein kinase made by incubating 0.2 ml of RII-[3H]cAMP with 0.2 ml of CI as above. Protein kinase activities in A to C were measured with (O-O) and without (●-●) CAMP. Hemoglobin and phosphorylase b markers (not shown in the figure) were included for calculation of the sedimentation constants.

Fig. 9. Density gradient centrifugation of reconstituted protein kinase prepared by incubation of the total R-[3H]cAMP complex of Peak I (RI-[3H]cAMP) with catalytic subunit fractions. In the upper part of the figure (A) the catalytic subunit fraction, CI, was used. This was prepared from Peak I protein kinase as in the experiment of Fig. 1A. In the lower part of the figure (B) the catalytic subunit fraction, CI, was used. This was prepared from Peak II as in Fig. 1B. RII-[3H]cAMP was from Peak I prepared as in Fig. 1A. The hemoglobin profile (Δ——Δ) is shown for reference.

The modifications observed in the experiments of Figs. 10 and 11 might well be explained on the basis of limited proteolysis. Such an explanation seemed particularly pertinent with respect to the experiment with added calcium, since it is known that muscle extract contains a Ca2+-activated protease (34). It was of interest, therefore, to examine the effect of a known protease on the protein kinases and their subunits. Fig. 12, A and B, shows the elution patterns of Peak I protein kinases obtained by gel filtration before and after trypsin treatment. It is clear that the enzyme, which remained almost completely dependent on CAMP, was converted to a form or forms with a much greater elution volume than those present originally. The effect of trypsin on the RII-[3H]cAMP complexes derived from Peak I (RII-[3H]cAMP) is shown in Fig. 12, C and D. Two peaks were present originally, i.e. RIIA-[3H]cAMP and RIB-[3H]cAMP, but after the trypsic attack essentially all of the complex eluted in the position of RII-[3H]cAMP. Trypsin treatment of the protein kinase of Peak II and of RII-[3H]cAMP has also been done. Both of these trypsin-treated proteins chromatographed on the Sephadex G-150 column almost exactly the same as the trypsin-treated protein kinase and RII-[3H]cAMP complex from Peak I. The protein kinase activity eluted from the column following trypsin action also remained cAMP-dependent.

Fig. 10. Conversion of R-[3H]cAMP (Method B) to a lower molecular weight form (or forms) on storage at 10°. The R-[3H]-cAMP complex was derived from the protein kinase prepared by Method B. The protein was in MES buffer, pH 6.5, containing 1 mm EDTA. A 200-μl sample was placed on sucrose density gradients after 0 (A), 10 (B), 38 (C), and 60 (D) days of storage at 10°. After centrifugation, 0.5-ml fractions were collected and 100-μl aliquots were counted for tritium (O). The sedimentation pattern (4.6 S) for hemoglobin (Δ) is shown for reference.

Fig. 11. Effect of incubation of muscle extracts on the sucrose density gradient centrifugation pattern of cAMP-dependent protein kinase. A homogenate (2 ml per g) of rabbit skeletal muscle was prepared in 10 mm MES, pH 6.5, containing 4 mm EDTA. The homogenates were centrifuged at 8,000 X g for 30 min and the supernatant fractions were collected. Aliquots (200 μl) of this fraction were either placed on ice (A) or incubated at 37° for 2 hours without (B) or with (O) 400 μm CaCl2 before applying to sucrose density gradients. Protein kinase activity (O) was assayed with casein as substrate in the presence of 2 mm CAMP. The hemoglobin profile (Δ) represents the endogenous hemoglobin (4.6 S). Phosphorylase b (profile not shown) was added to the gradients for calculation of the sedimentation constants shown in the figure.

DISCUSSION

The present study shows that at least three and probably four forms of cAMP-dependent protein kinase can be obtained from rabbit skeletal muscle. This conclusion is based on the number of holoenzyme forms, i.e. RC complexes, detected during fractionation plus information gained by recombining isolated R and C subunit fractions. The largest of the protein kinases, i.e. the enzyme prepared by Method B, was estimated to have a molecular weight of 123,000. The R-[3H]cAMP complex derived from this protein kinase was estimated to have a molecular weight of 82,000, and the catalytic subunit molecular weight was estimated as 49,000. The protein kinase of Peak II was estimated to have a molecular weight of 76,000. Its component parts, i.e. RII-[3H]cAMP and CI, were found to have molecular weights of 50,000 and 49,000, respectively. These subunit molecular weights add up to more than 76,000 which could be accounted for by various possibilities. One of these would simply be the error inherent in molecular weight determinations by the methods used. Another would be the possibility that the complex formed between cyclic AMP and the regulatory subunit is a dimer. Peak I would appear to contain a total of three species of protein kinase. One of these is the 6.8 S component.
believed to be identical with the protein kinase prepared by Method B. The other two would be the 5.4 and 5.0 S components which were first believed to consist of a single 5.2 S species. These were not obtained as separate entities so that it was not possible to estimate their molecular weights.

The cAMP-dependent protein kinases can be resolved into catalytic and regulatory subunits with substrate-affinity chromatography after treatment of the holoenzyme with \([\text{HI}]\text{cAMP}. The regulatory subunits are obtained in the form of complexes with \([\text{HI}]\text{cAMP} which is tightly bound and serves as a convenient marker during characterization studies. Recombination of catalytic and regulatory subunits is readily achieved as indicated by kinetic experiments or by density gradient centrifugation. During recombination the \([\text{HI}]\text{cAMP} which is bound to the R subunits is released as free \([\text{HI}]\text{cAMP} (35).

From a study of the molecular weights and sedimentation coefficients of the separated catalytic and regulatory subunits of the muscle protein kinases, it was found that whereas only a single size of catalytic subunit appears to be present, a number of different R subunits are found. In other words, insofar as size is concerned, the protein kinases seem to differ according to the type of regulatory subunit which they contain. Preliminary studies (4) have not revealed any striking differences, however, in the specificity or kinetic properties of the various protein kinases that result from combination of different R's with C. Inasmuch as the activity of these protein kinases in the presence of cAMP is believed to be a function of free catalytic subunit, which arises from dissociation of the RC complex (18), one would not expect kinetic differences unless they related to differences in the ease of dissociation of the RC complex.

Further work is required to determine whether the various protein kinases in skeletal muscle are actually present in the intact cell or whether they arise during the isolation procedure of Method A. By Method B only a single species of protein kinase, presumably identical with the largest species obtained by Method A, is isolated (36). Although it is possible that Method A results in an enrichment of lower molecular weight forms discarded during fractionation by Method B, it is also possible that the largest protein kinase is degraded during steps employed in the first procedure. Several observations suggest that the regulatory subunit is readily altered in vitro. This was seen in the experiment in which a smaller sedimenting species appeared when a R-[\text{HI}]\text{cAMP complex was aged. Changes were also noted when crude muscle extracts were incubated. Finally, it was shown that the protein kinase could be degraded by trypsin in a reaction characterized by breakdown of the R subunit although its ability to bind cAMP was not affected. The smaller forms of protein kinase found by Rubin et al. (37) after storage of the enzyme could have been the result of a proteolytic process.

The molecular weights of the various skeletal muscle protein kinases and their subunits are similar to those reported for some forms of the proteins derived from other tissues. Rubin et al. (37) resolved three forms of holoenzyme from heart muscle with molecular weights of 250,000, 140,000, and 90,000. The catalytic and regulatory subunits derived from all three of the kinases had molecular weights of 42,000 and 55,000, respectively. Gill and Garren (20) reported the major form of protein kinase from adrenals with a molecular weight of 110,000 to 150,000 and a regulatory subunit had a molecular weight of 92,000. Tao et al. (17) estimated the molecular weight of the reticulocyte protein kinase to be 140,000. The cAMP-independent kinase or catalytic subunit from this source had a molecular weight of 60,000. The differences in the molecular sizes of some of the protein kinases and subunits from the four tissue sources could be explained by either aggregation, dissociation, proteolysis, or tissue specificity of the proteins, or a combination of any of these factors.

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![Graph](https://example.com/graph.png)
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Studies on the Adenosine 3', 5'-Monophosphate-dependent Protein Kinases of Rabbit Skeletal Muscle

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