Molecular Forms and Subunit Composition of a Cyclic Adenosine 3', 5'-Monophosphate-dependent Protein Kinase Purified from Bovine Heart Muscle*

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SUMMARY

A cyclic adenosine 3', 5'-monophosphate (cyclic AMP)-dependent protein kinase has been purified from bovine heart muscle. Its molecular weight was estimated to be 280,000 by gel filtration chromatography, and it was composed of cyclic AMP-independent protein kinase and cyclic AMP-binding subunits with molecular weights of 42,000 and 55,000, respectively. When the purified protein kinase was subjected to polyacrylamide gel electrophoresis, ultracentrifugation, or storage there appeared smaller forms of cyclic AMP-dependent kinase with molecular weights of approximately 140,000 and 90,000. A close structural relationship between all of these forms of protein kinase was suggested by the observation that each was composed of the same two kinds of subunits.

During the past few years, investigations designed to elucidate the mechanisms by which cyclic adenosine 3', 5'-monophosphate exerts its diverse biological effects have concentrated on the ability of this nucleotide to stimulate protein kinase activity (1-10). Cyclic AMP-dependent protein kinases appear to serve as proximate receptors of cyclic AMP linking the membrane-bound, hormone-sensitive adenylyl cyclase system and the physiological responses of the cell. Protein kinases alter the activity of specific cellular proteins by catalyzing the transfer of the γ-phosphate of ATP to specific serine and threonine residues (1, 8, 11, 12). Optimal concentrations of cyclic AMP have been shown to stimulate protein kinase activity 2- to 10-fold in vitro (3) and in vivo (1). Proteins which serve as substrates for the protein kinases derived from mammalian tissues include histones (1, 3), protamine (12), phosphorylase kinase (4, 13), glycogen synthetase (4), hormone-sensitive lipase (5, 6), ribosome-associated proteins (10), microtubular protein (7), and the 7 factor of the RNA-polymerase from Escherichia coli (9). Thus, protein kinases may transform a change in the intracellular concentration of cyclic AMP into an alteration in glycogen metabolism, lipid metabolism, protein synthesis, or DNA transcription, depending upon the type of cell examined.

Previous work from this (14) and other laboratories (15-18) has shown that cyclic AMP-dependent protein kinases are composed of two functionally distinct components, a cyclic AMP-binding moiety and a cyclic AMP-independent catalytic moiety. It has been postulated that cyclic AMP activates protein kinase by releasing the catalytic moiety from the inhibition exerted by its association with a cyclic AMP-binding protein (14-18). There is, however, little detailed information about the molecular structure of cyclic AMP-dependent protein kinases.

The present report is concerned with the purification of the cyclic AMP-dependent protein kinase from beef heart and an analysis of its molecular forms and subunit composition.

EXPERIMENTAL PROCEDURE

Materials

Protamine sulfate was purchased from Eli Lilly, ATP and cyclic AMP from Sigma. [γ-32P]ATP (10 Ci per mmole) was obtained from New England Nuclear; cyclic [3H]AMP (16.3 Ci per mmole) from Schwan-Mann. DEAE-cellulose (Whatman DE-52) was secured from Reeve-Angel; DEAE-Sephadex A50 from Pharmacia; Bio-Gel P-300 from Bio-Rad; hydroxylapatite from Clarkson Chemical; and alumina C₃ from Sigma. Coomassie brilliant blue and Procion brilliant blue M-RS were obtained from Colab; the magnesium salt of 1-anilino-8-naphthalene sulfonic acid, from Eastman.

Glyceraldehyde 3-phosphate dehydrogenase, liver alcohol dehydrogenase and fumarase were from Boehringer-Mannheim; pyruvate kinase, xanthine oxidase, aldolase, urease, catalase, and rabbit γ-globulin from Sigma; alkaline phosphatase from Worthington; and bovine serum albumin from Pentex. All proteins were dialyzed against 0.05 M potassium phosphate, pH 7.0, prior to use.
Methods

**Enzyme Assays**—Protein kinase was assayed by measuring 32P incorporation from [γ-32P]ATP into protamine with a modification (14) of the method of De Lange et al. (13). The reaction mixture (0.2 ml) contained 0.05 M potassium phosphate buffer, pH 7.0, 0.01 M MgSO4, 50 μM [γ-32P]ATP (15,000 cpm per nmole), 1.0 μM cyclic AMP, 0.01 M dithiothreitol, 0.25 mg of protamine, and 0.5 mg of bovine serum albumin. Incubations were performed at 30° for 5 min unless otherwise noted. One unit of kinase activity was defined as that amount of enzyme necessary to catalyze the transfer of 1 n mole of 32P from [γ-32P]ATP to protamine per min at 30°. Protein kinase assays were carried out under conditions in which the reaction velocity was constant with respect to time and proportional to enzyme concentration (0.1 to 2.5 μg). The activity in assays of crude fractions (1 to 2.5 μg of protein per assay) was constant for more than 10 min, whereas the activity in assays of highly purified preparations (0.1 to 0.2 μg of protein per assay) was constant for only 2 to 3 min. This may have been due to instability of the kinase in dilute solution or to a destabilizing effect of cyclic AMP at low enzyme concentration (19). The 32P incorporated into protamine was stable in hot trichloroacetic acid but was released by boiling in 1 N HCl.

**Cyclic AMP Binding**—Binding assays were carried out according to the method of Gilman (20). The only modification involved the substitution of 0.05 M potassium phosphate buffer, pH 7.0, for 0.05 M acetic buffer, pH 4.0.

**Protein Determination**—Protein was assayed by the method of Lowry et al. (21) with bovine serum albumin as the standard.

**Polyacrylamide Gel Electrophoresis**—Standard 7.5% gels (containing 0.13 M Tris buffer, pH 8.1) were prepared and run according to the method of Davis (22). The sample and stacking gels were omitted. Samples containing 10 to 60 μg of protein in 50 to 150 μl of 20% sucrose, pH 7.0, were layered on 55-mm gel columns. Electrophoresis was carried out at 2.5 ma per gel until the bromphenol blue marker approached the bottom of the gels. Gels were then simultaneously fixed and stained in 0.2% Coomassie brilliant blue containing 45% methanol and 10% glacial acetic acid for 1 hour. Destaining was carried out in 5% methanol containing 7% glacial acetic acid. Staining with Procion brilliant blue M-RS was performed in the same manner.

Molecular weights were determined by electrophoresis on polyacrylamide gels of varying acrylamide concentration as described by Hedrick and Smith (23). The acrylamide to bisacrylamide ratio was 30:1.

Molecular weight determinations in sodium dodecyl sulfate-polyacrylamide gels were carried out according to the procedure of Shapiro et al. (24) with the conditions described by Weber and Osborn (25). Each gel received 5 to 20 μg of protein. Enzymatic activity on standard 7.5% gels was assayed in two ways. (a) Gels were cut into 1-mm slices and assayed directly in the standard assay mixture (see above). (b) Slices (1 mm) were eluted overnight at 4° in 200 μl of 0.05 M potassium phosphate buffer, pH 7.0, containing 0.004 M 2-mercaptoethanol. Aliquots were then withdrawn and assayed.

Cyclic AMP-binding activity on acrylamide gels was also determined by two procedures. (a) Slices (1 mm) were eluted as described above and then assayed by the modified Gilman method (20). (b) The whole gel was incubated for 2 hours at 4° in the presence of 6 × 10^-8 M cyclic AMP·[4 Ci per mmole], rinsed with two 500-ml changes of cold (4°) deionized water for 16 hours and sliced into 1-mm segments. The slices were placed in scintillation vials with 1 ml of 30% H2O2 and the polyacrylamide was solubilized by heating for 5 hours at 55° (26). Ten milliliters of Triton-toluene (3:7) scintillation fluid (containing 4 g of Omnifluor per liter) were added to each vial and the radioactivity determined in a liquid scintillation spectrometer. A similar binding assay has recently been reported (27).

Stained gels were scanned at a wave length of 510 nm with a Beckman DU spectrophotometer equipped with a Gilford model 2410 linear transport and a Gilford model 2000 recorder.

**Ultracentrifugation**—Sucrose density gradient centrifugation was performed according to the method of Martin and Ames (28) with rabbit γ-globulin and bovine serum albumin as markers.

Sedimentation velocity experiments were performed with a Beckman model E ultracentrifuge equipped with schlieren optics. Protein kinase (4 mg per ml in 0.05 M potassium phosphate buffer, pH 7.0, containing 0.004 M 2-mercaptoethanol) was subjected to centrifugation at 20° in an An D rotor at 59,780 rpm. Pictures were taken at 5, 15, 30, 55, 70, 90, and 120 min after the rotor reached constant speed.

Sedimentation equilibrium analysis was performed according to the meniscus depletion method of Yphantis (29) with a difference sector interference cell at 4°. The protein concentration was 0.3 mg per ml in potassium phosphate buffer, pH 7.0.

Results

**Purification of Protein Kinase**—Beef hearts were purchased from a local slaughterhouse and delivered to the laboratory immediately after the animals were killed. After removal of the pericardium and fat tissue, the heart muscle was minced and stored at -20°.

**Step 1: Homogenization**—Frozen heart muscle was thawed overnight at 4°. Unless otherwise indicated, all further handling of protein kinase was carried out at 4° and all buffer systems contained 0.004 M 2-mercaptoethanol. Heart muscle (2 kg, wet weight) was mixed with 4 liters of 0.04 M potassium phosphate buffer, pH 6.1, containing 0.002 M EDTA and homogenized in small batches at high speed for 1 min in a Waring blender. The homogenate was then centrifuged at 10,000 × g for 10 min and the supernatant fluid collected and filtered through Whatman No. 54 paper. The pellets were extracted two more times with 1 liter of buffer and the extracts combined (Step 1, Table I).

**Step 2: Ammonium Sulfate Fractionation**—The pooled extracts were brought to 55% saturation by the addition of solid (NH4)2SO4 (320 g of (NH4)2SO4 per liter) and the pH was maintained between pH 7 and 8 by the addition of NH4OH (5.0 ml of concentrated NH4OH per liter). Protein was allowed to precipitate for 24 hours and then collected by centrifugation at 10,000 × g for 10 min. The supernatant fluid was discarded and the precipitate dissolved in 500 ml of 0.05 M Tris buffer, pH 7.6, containing 0.01 M NaCl. This solution was dialyzed against two 4-liter volumes of the same buffer.

**Step 3: DEAE-Sephadex Batch Elution**—The dialyzed enzyme preparation, containing 18 g of protein, was stirred for 1 hour with 800 ml of DEAE-Sephadex that had been equilibrated with 0.05 M Tris buffer, pH 7.6, containing 0.01 M NaCl. Under these conditions, the kinase was adsorbed by the resin and could be

*Tris buffers were adjusted to pH 7.6 by the addition of concentrated HCl.*
formed after 1 hour was collected by centrifugation at 10,000 g. The precipitate which was then brought to 75% saturation by the addition of solid (NH₄)₂SO₄ (258 g per liter). The precipitate was then centrifuged at 10,000 × g for 10 min and discarded. The supernatant fluid was then brought to 75% saturation by the addition of solid (NH₄)₂SO₄ (258 g per liter). The precipitate which formed after 1 hour was collected by centrifugation at 10,000 × g for 10 min. The pellet was suspended in 100 ml of 0.05 M Tris buffer, pH 7.6, containing 0.30 M NaCl. The pooled filtrates (1800 ml) containing the protein kinase activity were brought to 35% saturation by the addition of solid (NH₄)₂SO₄ to 85% saturation (569 g per liter). The precipitate which formed after 1 hour was collected by centrifugation at 10,000 × g for 10 min and discarded. The supernatant fluid was then brought to 75% saturation by the addition of solid (NH₄)₂SO₄ (258 g per liter). The precipitate which formed after 1 hour was collected by centrifugation at 10,000 × g for 10 min. The pellet was suspended in a minimal volume of 0.05 M potassium phosphate buffer, pH 7.0, and dialyzed against 2 liters of the same buffer overnight. The combination of the DEAE batch elution and a second (NH₄)₂SO₄ fractionation served to clarify the enzyme solution. Five individual preparations were purified through Step 3 and were pooled prior to Step 4.

Step 4: Alumina C₅ Elution—The dialyzed (NH₄)₂SO₄ fraction (10.6 g of protein in 500 ml) was adsorbed to alumina C₅ gel by mixing the protein solution with gel (2.2 g of alumina C₅ per g of protein) that had been previously washed with two 500-ml portions of 0.05 M potassium phosphate buffer, pH 7.0. After stirring for 45 min the gel was collected by centrifugation at 3000 × g for 5 min and the supernatant fluid discarded. The gel was then resuspended and washed two additional times with 250 ml of the same buffer. Protein kinase was eluted by suspending the alumina C₅ gel in 0.15 M potassium phosphate buffer, pH 7.0, stirring for 15 min and removing the gel by centrifugation. Elution was carried out once with 200 ml of buffer and twice with 125 ml of buffer. The supernatant fluids were pooled and concentrated by the addition of solid (NH₄)₂SO₄ to 85% saturation (569 g per liter). After centrifugation the pellet was suspended in 100 ml of 0.05 M Tris buffer, pH 7.6, containing 0.01 M NaCl and dialyzed overnight against two 1-liter changes of the same buffer.

Step 5: Chromatography on DEAE-cellulose—Concentrated protein kinase from the previous step (2 g of protein in 125 ml) was injected onto a column, 2.5 × 95 cm, of DEAE-cellulose previously equilibrated with 0.05 M Tris buffer, pH 7.6, containing 0.01 M NaCl. The column was then washed with 100 ml of 0.05 M Tris buffer, pH 7.6, containing 0.06 M NaCl. Protein kinase activity was eluted with a linear gradient of Cl⁻ generated by a reservoir containing 1 liter 0.05 M Tris buffer, pH 7.6, and 0.30 M NaCl (0.34 M Cl⁻), and a mixing chamber containing 1 liter 0.05 M Tris buffer, pH 7.6, and 0.06 M NaCl (0.10 M Cl⁻). Ascending chromatography was carried out at a flow rate of 30 ml per hour and 15-ml fractions were collected. Protein kinase was eluted as a single peak of enzymic activity between 0.13 and 0.15 M Cl⁻. The peak fractions were combined and concentrated by precipitation with (NH₄)₂SO₄ as described above and the protein was redissolved and dialyzed against 0.05 M potassium phosphate buffer, pH 7.0.

Step 6: Gel Filtration on Bio-Gel P-300—The concentrated protein kinase solution (510 mg in 12 ml) was next injected onto a column, 2.5 × 95 cm, of Bio-Gel P-300 that had been previously washed with 0.05 M potassium phosphate buffer, pH 7.0. Ascending gel filtration was performed with the same buffer at a flow rate of 5 ml per hour and 3-ml fractions were collected. Protein kinase emerged from the column as a single peak of enzymic activity which was coincident with the major peak of protein (Fig. 1). Peak fractions were combined, concentrated with (NH₄)₂SO₄ and dialyzed against 0.05 M potassium phosphate buffer, pH 7.0, as described earlier.

<table>
<thead>
<tr>
<th>TABLE I Purification of protein kinase</th>
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<tbody>
<tr>
<td>Stepa</td>
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<td>-------</td>
</tr>
<tr>
<td>1. Homogenate (10,000 × g supernatant)</td>
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<tr>
<td>2. Ammonium sulfate</td>
</tr>
<tr>
<td>3. DEAE-Sephadex</td>
</tr>
<tr>
<td>4. Alumina C₅</td>
</tr>
<tr>
<td>5. DEAE-cellulose</td>
</tr>
<tr>
<td>6. Bio-Gel P-300</td>
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<tr>
<td>7. Hydroxylapatite</td>
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</table>

a The data given in Steps 1 to 3 represent the combined and averaged (where appropriate) values obtained from five small scale preparations (as described under “Results”). Subsequent purification was carried out with the pooled protein kinase preparation.

b A unit of catalytic activity is defined as that amount of enzyme necessary to catalyze the transfer of 1 nmole of [γ-32P]ATP to protamine per min. A unit of binding activity is equivalent to the binding of 1 nmole of cyclic AMP under the conditions of the binding assay employed (20).

c Specific activity is defined as units per mg of protein.
**Step 7: Chromatography on Hydroxylapatite**—The protein kinase preparation (111 mg in 5 ml) was injected onto a column, 2.5 × 20 cm, of hydroxylapatite that had previously been equilibrated with 0.05 M potassium phosphate buffer, pH 7.0. The column was washed in stepwise fashion with 200-ml aliquots of 0.05, 0.10, and 0.15 M potassium phosphate buffer, pH 7.0. Protein kinase was then eluted with 0.25 M potassium phosphate buffer, pH 7.0.

**Comments on Puri$cation Procedure**—A summary of the purification procedure is given in Table I. Approximately 55% of the total units of soluble protein kinase were found in the initial extract (Step 1). The second and third extractions yielded 30 and 15% of the units, respectively. Additional extractions or extractions in the presence of 0.2% Triton X-100 did not significantly increase the yield of kinase units. Extraction of fresh heart muscle that had never been frozen yielded an equivalent number of kinase units per kg, wet weight. All of the fractions in the peak of enzyme activity eluted from DEAE (Step 5) were stimulated by 10^{-6} M cyclic AMP. Half-maximal stimulation occurred at 6 × 10^{-8} M cyclic AMP. The apparent $K_m$ values for ATP, and Mg$^{2+}$ determined at a proteinate concentration of 1.25 mg per ml were $1.3 	imes 10^{-5}$ and $1.7 	imes 10^{-4}$ M, respectively. These values are in agreement with those reported by Brostrom et al. (19).

**Molecular Forms of Cyclic AMP-dependent Protein Kinase**—A single protein band was observed after the purified protein kinase was subjected to electrophoresis in 7.5% acrylamide gels (Fig. 2). The molecular weight of the cyclic AMP-stimulated protein kinase from bovine heart was determined by gel filtration according to the method of Andrews (31) and estimated to be 280,000. Following storage for 2 weeks at 4°C, the protein kinase was again examined by electrophoresis. One major and two minor components were observed (Fig. 3). Parallel, unstained gels were cut into 1-mm slices and assayed for catalytic and cyclic AMP-binding activities. The major band possessed >90% of the total enzymic and cyclic AMP-binding activities, but both minor components also exhibited catalytic and cyclic AMP-binding activities (Fig. 3). On further storage, the major component decreased to 70% of the total protein kinase activity with a concomitant increase in the concentration of the two other forms.

The over-all purification of cyclic AMP-binding activity was 1154-fold with a recovery of 14%. Both activities were stable to storage at 4°C for 30 days.

**Properties**—At all stages of purification, protein kinase activity was stimulated approximately 5-fold by the addition of 10^{-6} M cyclic AMP. Half-maximal stimulation occurred at 6 × 10^{-8} M cyclic AMP. The apparent $K_m$ values for ATP, and Mg$^{2+}$ determined at a proteinate concentration of 1.25 mg per ml were $1.3 	imes 10^{-5}$ and $1.7 	imes 10^{-4}$ M, respectively. These values are in agreement with those reported by Brostrom et al. (19).
Protein Kinase from Bovine Heart Muscle

FIG. 4 Chromatography of stored protein kinase on Bio-Gel P-300. The column and conditions were identical with those described under “Step 6” of the purification procedure. The column was calibrated with the following pure proteins: bovine serum albumin (molecular weight (MW) = 66,000), alkaline phosphatase (molecular weight = 80,000), glyceraldehyde 3-phosphate dehydrogenase (molecular weight = 140,000), pyruvate kinase (molecular weight = 237,000), xanthine oxidase (molecular weight = 280,000), and urease (molecular weight = 483,000). A plot of log molecular weight versus elution volume (Ve) is shown in the inset. Also indicated, are the molecular weights of three peaks of kinase activity. Samples, 20 μg, from each kinase peak were subjected to electrophoresis in 7.5% polyacrylamide gels. The electrophoretic patterns developed with Coomassie Blue are shown below the three protein peaks. The gel at the extreme left shows the pattern exhibited by the stored kinase prior to chromatography on Bio-Gel P-300.

(Fig. 4). The nature of the molecular heterogeneity of the protein kinase was further investigated by rechromatography of the stored enzyme on Bio-Gel P-300. Three distinct protein peaks (Fig. 4) corresponding to molecular weights of 280,000, 140,000, and 90,000 were resolved: Protein Kinases 1, 2, and 3, respectively (inset, Fig. 4). Protein Kinases 1, 2, and 3 had similar specific catalytic (990, 1080, 806 units per mg, respectively), and specific binding (9.3, 12, 7.0 units per mg, respectively) activities. Protein Kinases 1 and 3 migrated as single bands on electrophoresis in standard 7.5% acrylamide gels while Protein Kinase 2 exhibited a unique band as well as a band that corresponded to that of Protein Kinase 1 (Fig. 4). Each form of the enzyme had a unique mobility that corresponded to one of the three bands seen after electrophoresis of the original stored enzyme (Figs. 3 and 4). All proteins observed were characterized as cyclic AMP-dependent protein kinases. These results suggested that, although all of the protein components resolved by these techniques contained protein kinase activity, the preparation was heterogeneous with respect to the molecular weight and perhaps net charge of these species.

The molecular weight of Protein Kinase 1 was determined by electrophoresis in six different concentrations of acrylamide in accordance with the method of Hedrick and Smith (23, see also Reference 32). A value of 140,000 daltons was obtained (Fig. 5A), one-half the value determined by gel filtration. The two minor bands were too diffuse to measure accurately by this technique.

FIG. 5. a, determination of the molecular weight of Protein Kinase 1 by polyacrylamide electrophoresis. Electrophoresis and molecular weight determinations were carried out according to the method of Hedrick and Smith (23). 1, Alkaline phosphatase (molecular weight = 80,000); 2, bovine serum albumin (dimer, molecular weight = 136,000); 3, aldolase (molecular weight = 160,000); 4, fumarase (molecular weight = 194,000); bovine serum albumin (trimer, molecular weight = 204,000); 6, catalase (molecular weight = 240,000); and 7, urease (molecular weight = 480,000) were employed as standards for the calibration curve. Protein Kinase 1 and the standard proteins were subjected to electrophoresis in gels containing six different concentrations of polyacrylamide (5 to 12%). Plots of the log of the relative mobility X 100 versus the percentage of gel concentration (not shown) were linear and the slope was determined for each protein. The secondary plot of the slope versus molecular weight was also linear and was used to estimate the molecular weight of Protein Kinase 1. b, determination of the molecular weights of the catalytic and cyclic AMP-binding subunits of protein kinase by SDS-polyacrylamide gel electrophoresis. Electrophoresis and the molecular weight estimation were carried out according to the method of Shapiro et al. (24) with the conditions described by Weber and Osborn (25). Molecular weights were determined from a calibration curve obtained with six pure protein subunits: 1, bovine serum albumin (molecular weight = 66,000); 2, catalase (molecular weight = 237,000); 3, fumarase (molecular weight = 280,000); 4, alcohol dehydrogenase (molecular weight = 41,000); 5, glyceraldehyde 3-phosphate dehydrogenase (molecular weight = 36,000); and 6, IgG light chains (molecular weight = 24,000).
Sedimentation velocity studies on Protein Kinase 1.
The pictures were taken (from right to left) at 15, 55, and 90 min with bar angles of 65°, 55°, and 55°*, respectively. The sedimentation coefficients of the two peaks were 6.9 and 4.9 S.

**Table II**

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Molecular weight</th>
<th>Sedimentation coefficient</th>
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<tbody>
<tr>
<td></td>
<td>Gel filtration</td>
<td>Electrophoresis*</td>
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<tr>
<td>Freshly purified protein kinase (Step 7, Table I)</td>
<td>280,000</td>
<td>140,000</td>
</tr>
<tr>
<td>Protein Kinase 1</td>
<td>280,000</td>
<td>140,000</td>
</tr>
<tr>
<td>Protein Kinase 2</td>
<td>140,000</td>
<td>100,000–120,000</td>
</tr>
<tr>
<td>Protein Kinase 3</td>
<td>90,000</td>
<td>4.9</td>
</tr>
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</table>

*Method of Hedrick and Smith (23) (see "Methods").

**Fig. 8.** SDS gel electrophoresis of protein kinase and its components. The purified protein kinase was resolved into its major and minor components by polyacrylamide gel electrophoresis and detected by staining with the magnesium salt of anilinoazophenol-sulfonic acid (33) (not shown). The major band was excised, eluted, and treated with SDS and mercaptoethanol (25). The catalytic and cyclic AMP-binding moieties obtained by chromatography on DEAE were also exposed to SDS and mercaptoethanol. These preparations were then subjected to electrophoresis in 0.1% SDS (24, 25). A, 10 μg of catalytic protein; B, 15 μg of intact protein kinase; C, 10 μg of cyclic AMP-binding protein.

Preliminary sedimentation equilibrium studies on dilute solutions (see "Methods") of Protein Kinases 1 and 3 disclosed that they both behaved as multicomponent systems. The maximal molecular weight of Protein Kinase 3 approached 90,000, whereas

**Fig. 7.** Polyacrylamide gel electrophoresis of the protein kinase catalytic (A) and cyclic AMP-binding (B) components. Catalytic protein (25 μg) and cyclic AMP-binding protein (25 μg) were subjected to electrophoresis in 7.5% gels, stained, and assayed as described in Fig. 3 and under "Methods." (- - -) absorbance at 510 nm; ( ) protein kinase activity (units per 20 μl of gel eluate); (O--O) cyclic AMP-binding activity (units per 20 μl of gel eluate).
the maximal molecular weight of Protein Kinase 1 approached 140,000.

Catalytic and Cyclic AMP-binding Components—Purified protein kinase (5 mg in 1 ml 0.05 M potassium phosphate buffer, pH 7.0) was applied to a column, 1 x 3 cm, of DEAE-cellulose that had been previously equilibrated with the same buffer. After washing with 20 ml of buffer, a cyclic AMP-independent protein kinase (catalytic protein) was eluted with 10 ml of buffer containing 10^{-6} M cyclic AMP. Approximately 85 to 90% of the original protein kinase activity was recovered in this fraction. Residual cyclic AMP was then washed off the resin with 20 ml of 0.12 M potassium phosphate buffer, pH 7.0, and a cyclic AMP-binding protein was eluted with 10 ml of 0.25 M potassium phosphate buffer, pH 7.0. Catalytic and binding components were then separately dialyzed against 0.05 M potassium phosphate buffer, pH 7.0, before further use. The catalytic moiety did not bind cyclic AMP and was not stimulated by the addition of this nucleotide. In fact, the addition of cyclic AMP (10^{-6} M) inhibited its activity. The protein which bound cyclic [PH]AMP did not possess any catalytic activity.

When the catalytic moiety was subjected to electrophoresis in 7.5% acrylamide gels, two distinct protein bands of low mobility were detected (Fig. 7A). These corresponded to two peaks of cyclic AMP-independent protein kinase activity. Since only one kind of subunit was apparent after this preparation was fully dissociated by treatment with SDS and mercaptoethanol (see Fig. 6), these two bands may have resulted from aggregation or limited proteolytic degradation.

Polyacrylamide gel electrophoresis of the cyclic AMP-binding moiety revealed that >90% of the binding activity was associated with the major protein band (Fig. 7B). A minor peak of cyclic AMP-binding activity with a greater mobility was also detected. It would appear, therefore, that, as previously suggested (14), bovine heart muscle protein kinase consists of two kinds of functionally distinct components: a cyclic AMP-binding protein and a cyclic AMP-independent catalytic protein.

The molecular weights of the cyclic AMP-binding and catalytic subunits were found to be 55,000 and 42,000, respectively, by SDS-acrylamide gel electrophoresis (Fig. 5B). The purified cyclic AMP-dependent protein kinase consisted of two dissimilar subunits, one of which corresponded to the subunit obtained from the catalytic moiety and another which corresponded to the subunit obtained from the cyclic AMP-binding moiety (Fig. 8). Protein Kinases 2 and 3 were composed of the same two kinds of subunits as Protein Kinase 1 (Fig. 9).

The relative proportions of binding and catalytic subunits in the major component of protein kinase were estimated by scanning the optical density along the SDS gels after staining with either Coomassie brilliant blue or Procion blue (34) (representing reversible and covalently bound stains, respectively). Scans of gels after both staining procedures indicated a 2:1 molar ratio of catalytic to binding subunits suggesting a minimal molecular weight of 140,000.

**DISCUSSION**

A cyclic AMP-stimulated protein kinase was isolated from bovine heart and purified to apparent homogeneity by the procedure summarized in Table I. Although kinase and cyclic AMP-binding activities were both purified approximately 1200-fold, the enrichment of these activities did not parallel each other at each step in the procedure (Table I). This may have been due to the removal of cyclic nucleotide phosphodiesterases or to enrichment of phosphatases, ATPases, or a protein kinase inhibitor (35, 36) during early stages in the purification. Another possibility is that a significant amount of endogenous cyclic AMP remained bound to the enzyme during homogenization and was released only at a later stage in the purification procedure.

Although catalytic activity was enhanced approximately 5-fold by the presence of an optimal concentration of cyclic AMP, activity was never totally dependent upon the addition of this cyclic nucleotide. The cyclic AMP-stimulated protein kinases which have been isolated from other tissues also exhibited activity in the absence of cyclic AMP (1, 2, 15, 16). This basal activity may have been due to the presence of tightly bound, endogenous cyclic AMP which was not dissociated during purification. Gill and Garren (37) and Gilman (20) have demonstrated that protein kinases have a high affinity for cyclic AMP and that the binding reaction is not readily reversible.

* The term “subunit” is used to denote the polypeptide chains derived from the isolated cyclic AMP-independent protein kinase and cyclic AMP-binding moieties.

![Image](http://www.jbc.org/)

Fig. 9. Subunit composition of protein kinases. Purified protein kinase was resolved into its major and minor components by polyacrylamide gel electrophoresis (B, see Fig. 4). The bands were detected, excised, and subjected to SDS-polyacrylamide gel electrophoresis (A and C) as described in Fig. 8. A, Protein Kinase 1; C, Protein Kinase 2. Similar experiments have shown that Protein Kinase 3 is also composed of the same two subunits.
The penultimate step in the purification procedure involved the isolation of protein kinase in an elution volume that corresponded to a molecular weight of 280,000. Upon storage at 4°C, however, two additional protein kinases appeared. All three forms were resolved by gel filtration; the major component (Protein Kinase 1) had a molecular weight of 280,000 and the two minor components derived from it were found to have molecular weights of 140,000 and 90,000. When the molecular weight of Protein Kinase 1 was determined electrophoretically, however, a value of 140,000 was obtained, suggesting that Protein Kinase 1 had dissociated under the conditions of electrophoresis.

Similarly, in sedimentation velocity experiments with Protein Kinase 1, two peaks of protein having molecular weights of approximately 140,000 and 90,000 were observed (Fig. 6). Sedimentation equilibrium studies performed on Protein Kinases 1 and 3 disclosed the presence of a polydisperse system in each case implying that dissociation into even smaller species was occurring.

The data obtained by electrophoresis, ultracentrifugation, and gel filtration led us to conclude that the purified protein kinase was functionally homogeneous, but heterogeneous in composition. This heterogeneity could have been the result of dissociation or limited digestion by trace amounts of a contaminating proteolytic enzyme. The increase in concentration of the smaller protein kinase species which occurred during storage of the purified protein kinase as well as the ultracentrifugal studies strongly suggested a common origin for all of the protein kinases observed. This suggestion was further supported by the demonstration that Protein Kinases 1, 2, and 3 were composed of the same kinds of subunits.

The molecular weights of some of the forms of beef heart protein kinase are similar to those reported for protein kinases from other tissues. Reimann et al. (38) have described protein kinases from skeletal muscle with sedimentation coefficients of 6.8 and 4.9 S, essentially the same values obtained when the purified Protein Kinase 1 of heart muscle was subjected to ultracentrifugation. Gill and Garren (27) have also reported that the major form of protein kinase from bovine adrenals has a molecular weight of 140,000 to 150,000 (S = 7), whereas a cyclic AMP-binding protein from this source was found to have a molecular weight of 92,000. Tan et al. (15) estimated that the molecular weight of rabbit reticulocyte protein kinase was 140,000 on the basis of sucrose gradient centrifugation. They also found a cyclic AMP-independent protein kinase of molecular weight = 60,000. The latter protein may be a dissociated catalytic subunit, similar in size to the catalytic subunit isolated from bovine heart (see "Results"). Thus, protein kinases from four different tissues have been reported to have molecular weights of approximately 140,000. In the case of bovine heart muscle, this species may have been generated by dissociation of a species of molecular weight = 280,000.

Other complex oligomeric proteins are known to undergo dissociation-association reactions. Glutamate dehydrogenase, for example, can be isolated as an octamer of molecular weight 2.2 X 10^6 daltons. It undergoes reversible dissociation, however, when the protein concentration is reduced below 6 mg per ml (39, 40). Following dissociation, an active form of molecular weight 300,000 was isolated. In a situation analogous to that reported here, the molecular weight of tryptophan synthetase from E. coli could not be determined by sedimentation equilibrium techniques because of continuous aggregation and disaggregation of the subunits (41). The complete enzyme was most stable to gel filtration and its molecular weight was determined by filtration on Sephadex G-200 (41).

The major form of beef heart protein kinase also appeared to be stable to gel filtration. The possibility exists, however, that the high molecular weight assigned to this form may be in error. Covalently bound carbohydrate, for example, is often responsible for aberrant behavior on gel filtration (31). It is of interest in this regard that after SDS-acrylamide gel electrophoresis, beef heart protein kinase failed to stain with the periodic acid-Schiff test (42).

During the purification procedure, only one form of protein kinase was observed. Neither cyclic AMP-independent protein kinases nor free cyclic AMP-binding proteins were seen at any stage of purification. These findings are in contrast to the observations reported from a number of laboratories in which multiple forms of cyclic AMP-dependent protein kinase (15, 38), cyclic AMP-independent kinase (11, 27, 38), and free cyclic AMP-binding protein have been found (27, 43). This may be due in part to the relative cellular homogeneity of heart muscle. Alternatively, the different forms of protein kinase seen in other laboratories may be the result of dissociation of a high molecular weight aggregate into smaller protein kinases or even into cyclic AMP-independent catalytic and cyclic AMP-binding subunits in vitro. As reported here, spontaneous dissociation can occur slowly on storage or rapidly with changes in the physical environment. Thus, the nature of the buffers used during enzyme preparation, as well as the pH and the ionic strength to which the enzyme is exposed, may determine its molecular form. Another factor may be the concentration of cyclic AMP in the tissue immediately before homogenization. Since cyclic AMP can induce the dissociation of protein kinase into cyclic AMP-binding and catalytic moieties in vitro (14-18), it may also do so in vivo. Partial degradation due to proteolysis may also explain some of these observations since most of the available data on protein kinases have been obtained with partially purified preparations.

Protein kinase was shown to consist of two dissimilar subunits; the cyclic AMP-binding subunit had a molecular weight of 55,000, whereas the smaller catalytic subunit had a molecular weight of 42,000. All of the different forms of protein kinases observed appear to represent various combinations of these subunits although the number of catalytic and cyclic AMP-binding subunits in each form of protein kinase has not yet been accurately determined. The biological significance of the different forms of protein kinase is not known. It is possible that some or all of these forms may occur in vivo, depending upon the nature of the microenvironment. Under certain in vitro conditions, free catalytic and cyclic AMP-binding moieties can be demonstrated (see Fig. 7), but it is not known whether they exist in vivo. It will be important to determine whether the different forms of protein kinase have different substrate specificities, or whether they are subject to different kinds of regulation. Another possible role for protein kinase or cyclic AMP-binding protein might be to function as a storage form of "bound" or physiologically inactive cyclic AMP (44).

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