Dissociation and Activation of Adenosine 3',5'-Monophosphate-dependent and Guanosine 3',5'-Monophosphate-dependent Protein Kinases by Cyclic Nucleotides and by Substrate Proteins*

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SUMMARY

Two adenosine 3',5'-monophosphate (cyclic AMP)-dependent protein kinases, with sedimentation coefficients of 5.4 S (mol wt 84,000) and 7.7 S (mol wt 140,000), respectively, have been purified from bovine brain. Each enzyme migrated as a single band at three different pH values on polyacrylamide disc gel electrophoresis. The enzymes had similar properties.

Catalytic subunits of cyclic AMP-dependent protein kinases from bovine brain, and of cyclic AMP-dependent and guanosine 3',5'-monophosphate (cyclic GMP)-dependent protein kinases from lobster muscle were prepared by means of Enzite CM-cellulose-protamine affinity chromatography, and their properties were studied. The catalytic subunits of the two bovine brain enzymes and of the lobster muscle cyclic GMP-dependent enzyme each had a sedimentation coefficient of 3.6 S (mol wt 40,000), whereas that of the lobster muscle cyclic AMP-dependent enzyme sedimented at 4.5 S (mol wt 60,000). These results agree with kinetic studies, indicating that cyclic GMP-dependent protein kinases of arthropods have certain similarities to cyclic AMP-dependent protein kinases of vertebrates.

Histone and cyclic AMP caused the dissociation of the cyclic AMP-dependent protein kinase from lobster muscle (5.7 S; mol wt 90,000) and those from bovine brain into subunits; histone and cyclic GMP caused the dissociation of the lobster muscle cyclic GMP-dependent protein kinase (7.7 S; mol wt 140,000) into subunits. The dissociation of the enzymes by histone was accompanied by a concomitant increase in cyclic nucleotide-independent activity. Isoelectric points of catalytic and regulatory subunits of the 7.7 S component of the brain enzyme were about pH 7.8 and 3.8, respectively.

Addition of isolated regulatory subunit, derived from bovine brain cyclic AMP-dependent enzyme, inhibited the activity of catalytic subunits obtained from bovine brain enzymes. Interestingly, the regulatory subunit of the cyclic AMP-dependent enzyme from mammalian brain also inhibited the activity of the catalytic subunit prepared from the lobster muscle cyclic GMP-dependent enzyme, converting it into a cyclic AMP-dependent form. Thus, an interaction between catalytic and regulatory subunits occurred, although the subunits were from different tissues, different phyla, and different classes (with respect to cyclic nucleotide specificity) of protein kinase.

Subsequent to the discovery of adenosine 3',5'-monophosphate (cyclic AMP)-dependent protein kinase in skeletal muscle (1) and then in liver (2), brain (3), and a wide variety of other sources (4, 5), it was proposed (4, 5) that the diverse actions of cyclic AMP are mediated through regulation of this class of enzymes. Much recent evidence, obtained in numerous laboratories, is at least consistent with and, in many cases, supports this postulate for the mechanism of action of cyclic AMP in eukaryotic organisms. The discovery (6, 7) of a class of protein kinases activated specifically by guanosine 3',5'-monophosphate (cyclic GMP), rather than by cyclic AMP, has raised the possibility that the effects of cyclic GMP may be mediated through this class of enzymes. However, in contrast to the case of the cyclic AMP-dependent protein kinases, no further studies have been reported on the enzymological properties of cyclic GMP-dependent protein kinases. The fact that cyclic AMP- and cyclic GMP-dependent protein kinases are found in the same tissues raises a number of questions concerning the biochemical and physiological relationship of these two types of enzyme. In view of the importance of these two classes of protein kinase, it

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1The abbreviations used are: cyclic AMP, adenosine 3',5'-monophosphate; cyclic GMP, guanosine 3',5'-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid.
seemed desirable to study and compare their enzymological properties.

In the present investigation, we have compared some of the properties of the holoenzyme and catalytic subunit of cyclic GMP-dependent protein kinase from lobster muscle, cyclic AMP-dependent protein kinase from lobster muscle, and cyclic AMP-dependent protein kinases from mammalian brain. Brain was selected as the source of the mammalian enzymes, since recent evidence suggests that cyclic AMP-dependent protein kinase may play an important role in the functioning of the mammalian nervous system (8-10). The present report includes a description of the preparation of two purified enzymes from mammalian brain.

Studies in several laboratories, using a variety of tissues (11-17), have provided evidence that the mechanism by which cyclic AMP activates cyclic AMP-dependent protein kinases involves dissociation of the holoenzyme into a regulatory subunit, which binds cyclic AMP, and an inhibitory to the enzyme, and a catalytic subunit. In the present study, it has been found that cyclic GMP-dependent protein kinase can be dissociated and activated by cyclic GMP. In a preliminary study (16), certain substrate proteins caused the partial dissociation and activation of cyclic AMP-dependent enzymes. In the present study, both cyclic GMP-dependent and cyclic AMP-dependent protein kinases were completely dissociated by certain proteins capable of acting as substrates. The recombination of subunits has also been studied, including the formation of a "hybrid" enzyme composed of the regulatory subunit of a cyclic AMP-dependent protein kinase and the catalytic subunit of a cyclic GMP-dependent protein kinase.

EXPERIMENTAL PROCEDURE

Materials

Frozen bovine brains were obtained from Pel-Freez. They were stored at 20° until the enzyme purification was started. Histone mixture (sulfur thymus) obtained from Schwarz-Mann was the histone preparation used throughout this study, unless otherwise specified. Slightly lysine-rich histone, arginine-rich histone, lysine-rich histone, protamine (salmon sperm), and bovine serum albumin (fraction V) were purchased from Sigma. Casein was obtained from Nutritional Biochemicals. Cyclic AMP and cyclic GMP were purchased from Boehringer Mannheim. Cyclic [γ-32P]AMP (16.3 Ci per millimole) and cyclic [γ-3H]GMP (4.5 Ci per millimole) were purchased from Schwarz-Mann. DEAE-cellulose (medium mesh, 0.85 meq per g) was obtained from Sigma; hydroxylapatite (dry powder) was from BioRad; Enzite CM-cellulose hydrazide was from Miles-Seravac; and Sepharose 6B was from Pharmacia. Catalase, horse liver alcohol dehydrogenase, acetylcholinesterase, l-arginine decarboxylase, avidin, and d-fructose-phosphate kinase were obtained from Sigma; glyceraldehyde 3-phosphate dehydrogenase was from Boehringer Mannheim; lysozyme, lactate dehydrogenase, and β-galactosidase were from Worthington; poly-L-arginine was from Miles.

Methods

Assay for Protein Kinase—The activity of cyclic AMP-dependent and cyclic GMP-dependent protein kinases and of their catalytic subunits was assayed in a standard incubation system by a procedure described previously (18). The incubation mixture contained, in a final volume of 0.2 mL, sodium phosphate buffer (pH 6.0), 10 μmoles; histone mixture, 40 μg; [γ-32P]ATP, 1.0 μmole, containing about 1.8 x 10^6 cpm; magnesium acetate, 2 μmoles; and cyclic nucleotide as indicated. One unit of enzyme activity was defined as that amount of enzyme which transferred 1 pmole of ^3P from [γ-32P]ATP to recovered protein in 5 min at 30° in the standard assay system.

Analytical Gel Electrophoresis—Analytical gel electrophoresis was performed at pH 8.1, pH 0.5, and pH 12.0 in accordance with the procedures of Ornstein (19) and Davis (20), with a CanaL probe model 66 electrophoresis apparatus without stacking gel. Gels were stained with aniline blue-black and destained electrophoretically.

Assay for Cyclic AMP-binding Activity—The cyclic AMP-binding activity of protein was determined by the method of Gilmour (21), except for omission of the heat-stable inhibitor. The incubation mixture contained, in a final volume of 0.2 ml, 10 μmoles of sodium acetate buffer (pH 4.0) and 3 μmoles of cyclic [γ-3H]AMP (16.3 Ci per millimole). The reaction was initiated by the addition of binding protein.

Sucrose Density Gradient Centrifugation—Ultracentrifugation of protein kinases was carried out at 37,500 rpm in a Beckman SW 39 L rotor for 16 hours at 4°. In addition to 5 to 20% sucrose gradient, the centrifuge tube contained 50 μM acetate buffer (pH 6.0), 0.3 μM EGTA, and 2.5 μM 2-mercaptoethanol. At the termination of the centrifugation, the bottom of the tube was punctured and 7-drop fractions, containing about 0.14 ml, were collected. Sedimentation coefficients and molecular weights were determined by the method of Martin and Ames (22), with catalase (11.6 S, mol wt 232,000) (23) which was assayed by the method of Beers and Sizer (24), glyceraldehyde 3-phosphate dehydrogenase (7.7 S, mol wt 140,000) (25) which was assayed by the method of Velick (26), and horse liver alcohol dehydrogenase (5.4 S, mol wt 84,000) (27) which was assayed by the method of Vallee and Hoch (28), as internal markers in each experiment.

Isoelectric Focusing—Isoelectric focusing was carried out by a slight modification of the procedure described previously (29), by using a 110-ml column (LKB Instrument). The amount of carrier ampholyte used, with a pH range of 3 to 10, was 2% (w/v) in a 0 to 47% (w/v) sucrose gradient. Electrophoresis was initiated at 400 volts; as the current decreased, the voltage was increased to and maintained at 500 volts. The duration of electrophoresis was 24 hours, and the current was constant (0.9 ma) for the last 4 hours. Upon completion of the isoelectric focusing, 3-ml fractions were collected.

Other Methods—Cyclic GMP-dependent and cyclic AMP-dependent protein kinases from lobster muscle were prepared by a method described earlier (6). Enzite CM-cellulose-protamine was prepared by the method of Mitz and Summaria (30). [γ-32P]ATP was prepared by the procedure of Post and Sen (31). Protein was measured by the method of Lowry et al. (32), with bovine serum albumin as the protein standard.

RESULTS AND DISCUSSION

Preparation of Holoenzyme and Catalytic Subunit of Cyclic AMP-dependent and Cyclic GMP-dependent Protein Kinases

Preparation of Brain Holoenzyme—Four frozen bovine brains, with a combined weight of 1,450 g, were thawed, cut into small pieces, homogenized with 3 volumes of 5 mM potassium phosphate buffer (pH 7.0), containing 2 mM EDTA, and the homogenate was centrifuged at 13,000 x g for 20 min. This and all succeeding steps of purification were performed at 4°, and all buffers used contained 2 mM EDTA. Protamine sulfate (15
mg per 100 ml) was added to the crude extract; after 10 min of gentle stirring, the resultant nucleic acid precipitate was removed by centrifugation at 13,000 \( \times g \) for 20 min. Solid ammonium sulfate (27.7 g per 100 ml) was added to the supernatant solution. After stirring for 30 min, the solution was centrifuged; the protein precipitate was dissolved in about 450 ml of 5 mm potassium phosphate buffer (pH 7.0); and the enzyme solution was dialyzed overnight against 10 volumes of the same buffer, with two changes of buffer. Precipitate formed during the dialysis was removed by centrifugation.

The enzyme solution was then applied to a DEAE-cellulose column (4 x 30 cm) which had been previously equilibrated with 5 mm potassium phosphate buffer (pH 7.0), and the column was washed with 2 volumes of the same buffer. The enzyme was then eluted from the column with a linear gradient of phosphate buffer (0.005 to 0.2 ml), pH 7.0, in a total volume of 4.0 liters. Two active enzyme peaks, eluted at 0.03 and 0.2 ml phosphate, respectively, were obtained. The enzyme activity eluted at 0.03 ml phosphate was less than 5% of that eluted at 0.2 ml phosphate and was discarded. The active fractions eluted at 0.2 ml phosphate were pooled and dialyzed extensively against 5 mm potassium phosphate buffer.

After dialysis, the enzyme solution was applied to a column (2.6 x 12 cm) of hydroxylapatite which had been previously equilibrated with 5 mm potassium phosphate buffer at pH 7.0. The enzyme was then eluted from the column with a linear gradient of potassium phosphate buffer (0.005 to 0.2 ml), pH 7.0, in a total volume of 2.0 liters. Two enzyme peaks, designated Peak I and Peak II, respectively, were obtained (Fig. 1). The active fractions from each peak were pooled, dialyzed overnight against 5 mm potassium phosphate buffer at pH 7.0, and then concentrated separately by means of ultrafiltration on Diaflo membranes (PM-10, Amicon Corp.) under pressure of nitrogen gas. The resultant enzyme preparations were designated protein kinase I and protein kinase II, respectively.

About 5 mg of protein kinase I, in a 2.0 ml volume, containing 50 mm potassium phosphate buffer (pH 7.0), 1.2 mM cysteine, 5% sucrose, and 0.0015% bromphenol blue (as tracking dye), was applied to a column (2.1 x 8.0 cm) of 7% acrylamide gel, and preparative gel electrophoresis was carried out at 20 ma and 4° for 11 hours. The gel column had been subjected to a "blank" electrophoresis run at 20 ma and 4° for 3 hours before the application of the enzyme sample; this prerun was found to increase recovery of enzyme activity in the gel purification step. The upper buffer was 0.05 M Tris-glycine (pH 8.9) containing 1.2 mm cysteine, and the lower buffer was 0.1 M Tris Cl (pH 8.1). After completion of electrophoresis, the gel was washed from the glass tube and soaked in 5-anilino-naphthalene-sulfonate (magnesium salt); the protein bands were visualized under ultraviolet light, according to the method of Hartman and Udenfriend (33). Five protein bands were observed. The gel was cut into 2-mm slices, each slice was homogenized in a Teflon homogenizer with 10 ml of 10 mm potassium phosphate buffer at pH 7.0, and the homogenate was centrifuged at 27,000 \( \times g \) for 5 min. The gel was then resuspended in 5 ml of the same buffer and again removed by centrifugation. The supernatant fluids (containing enzyme eluted from the gel) were combined and dialyzed overnight against 5 mm potassium phosphate buffer at pH 7.0. The protein kinase activity stimulated by cyclic AMP was found to be associated with two of the five protein bands. The slower moving of the two active bands was designated protein kinase IA, and the faster moving was designated protein kinase IB.

About 4 ml (4 to 5 mg) of protein kinase II, the enzyme from Peak II of the hydroxylapatite chromatography, was applied to a column (1.3 x 90.0 cm) of Sepharose 6B, which had been previously equilibrated with 5 mm potassium phosphate buffer at pH 7.0. The enzyme was then eluted from the column with about 100 ml of the same buffer. The active fractions were pooled and concentrated by means of Diaflo membrane ultrafiltration. Two milliliters of this concentrated enzyme solution, containing about 4 mg of protein, was then subjected to preparative gel electrophoresis, and the protein was eluted from the gel by the procedure described above for protein kinase I. After exposure to 5-anilino-naphthalene-sulfonate, three bands (and in some preparations, a fourth band) were visualized. The cyclic AMP-dependent enzyme activity was found to be associated with only one band on the gel. This activity is designated protein kinase IIA. The procedure used for the preparation of the various brain protein kinases, together with their S values, is given in Scheme 1. The results of the enzyme purification are summarized in Table 1. The low recovery of the purified enzyme was because only the chromatographic fractions with highest activity were used for subsequent purification steps.

Protein kinases IA, IB, and IIA were subjected to analytical gel electrophoresis. Protein kinases IB and IIA each migrated as a single band at each of three pH values, namely pH 8.1, 9.5, and 12.0 (Fig. 2); in each case, enzyme activity was found only in the region of the gel corresponding to the protein band. Protein kinase IA contained two protein bands. The one staining more intensely contained all of the enzyme activity.

**Preparation of Regulatory and Catalytic Subunits from Bovine Brain Cyclic AMP-dependent Protein Kinases—Subunits were prepared from bovine brain cyclic AMP-dependent protein kinases by affinity chromatography on columns containing covalently bound protamine. Protein kinase II (6.3 mg) from bovine brain was preincubated at 0° for 10 min with 5 \( \times 10^{-6} \) M cyclic [G-3H]AMP (25 \( \mu \)Ci) in 7.0 ml of 10 mm 2-(N-morpholino)-ethane sulfonic acid buffer (pH 6.0), containing 1 mm EDTA. The solution was then applied to a column (0.9 x 7.0 cm) of Enzite CM-cellulose-protamine which had been previously equilibrated with the same buffer, and the column was washed with 7 ml of the buffer containing 5 \( \times 10^{-4} \) M of the radioactive cyclic AMP. The subunits were eluted from the column with 170 ml of a linear gradient of sodium chloride (0 to 1 M), present
**Scheme 1.** Procedure used for preparing cyclic AMP-dependent protein kinases from bovine brain.

### TABLE I
**Summary of purification of cyclic AMP-dependent protein kinases from bovine brain**

Four whole bovine brains (1450 g) were used as the starting material. Protein kinase activity was determined in the presence of 5 μM cyclic AMP. The purification procedure used is described in the text.

| Fraction                     | Specific activity (units/mg protein) | Purification fold | Recovery of activity (%) |
|------------------------------|-------------------------------------|-------------------|-------------------------|
| Crude extract                | 1,749                               | 1.0               | 100                     |
| Protamine sulfate supernatant| 1,694                               | 1.0               | 78                      |
| Ammonium sulfate precipitate | 5,252                               | 3.0               | 44                      |
| DEAE-cellulose eluate        | 16,526                              | 9.4               | 9.4                     |
| Hydroxylapatite, protein kinase (Peak) I | 31,282                        | 17.9              | 1.2                     |
| Preparative gel, protein kinase (Band) I | 210,302                         | 125.4             | 0.2                     |
| Preparative gel, protein kinase (Band) II | 175,481                         | 100.3             | 0.2                     |
| Hydroxylapatite, protein kinase (Peak) II | 72,245                          | 41.3              | 2.0                     |
| Sepharose 6B eluate          | 135,290                             | 77.4              | 0.9                     |
| Preparative gel, protein kinase (Band) II | 271,449                         | 155.2             | 0.5                     |

in the same buffer, in the absence of cyclic AMP (Fig. 3). The protein peak containing bound radioactive cyclic AMP was not retained by the column, and there was no catalytic activity associated with this peak. The catalytic activity retained by the column was eluted at approximately 0.2 M sodium chloride. This catalytic activity was not associated with protein-bound radioactive cyclic AMP. Protein kinase IC (3.5 mg), prepared from protein kinase I, as described in the next section, was also chromatographed on Enzite CM-cellulose-protamine and gave results similar to those shown in Fig. 3.

Regulatory subunit free of bound cyclic AMP was prepared from brain protein kinase by taking advantage of the ability (16) of histone to cause the dissociation of the enzyme into subunits. For this purpose, 20 μg of the enzyme preparation from the DEAE-cellulose column was preincubated at 30°C for 10 min in the presence of 100 μg of histone per ml. After preincubation, the solution was applied to a column (2.1 × 1.5 cm) of hydroxylapatite, which had been previously equilibrated with 5 mM potassium phosphate buffer (pH 7.0), containing 2 mM EDTA. The column was then washed with 10 ml of the same buffer, and the protein was eluted with 0.02 M potassium phosphate buffer.
buffer (pH 7.0), containing 2 mM EDTA. Cyclic AMP-binding activity was assayed on an aliquot of each fraction. The peak of cyclic AMP-binding protein appeared in the 7th through the 12th ml. Active fractions were combined and used as cyclic AMP-binding protein in the recombination experiments described below. Protein kinase catalytic activity of this preparation was negligible. Previous methods of preparing regulatory subunits of protein kinase have used cyclic AMP to dissociate the holoenzyme and have suffered from the disadvantage that it is extremely difficult to free the regulatory subunit from bound cyclic nucleotide. Dissociation of holoenzymes by protein substrates provides an effective means of preparing regulatory subunits without cyclic nucleotides being attached.

Preparation of Catalytic Subunits from Lobster Muscle Cyclic GMP-dependent and Cyclic AMP-dependent Protein Kinases—For the preparation of catalytic subunit from lobster muscle cyclic GMP-dependent protein kinase, lobster muscle cyclic GMP-dependent holoenzyme (40 mg) was preincubated at 0°C for 10 min with 5 × 10^{-6} M cyclic GMP (25 μCi) in 8.0 ml of 10 mM 2-(N-morpholino)ethane sulfonic acid buffer (pH 6.0), containing 1 mM EDTA. The solution was then applied to a column (0.9 × 5.0 cm) of Enzite CM-cellulose-protamine, which had been previously equilibrated with the same buffer, and the column was then washed with 5 ml of the buffer containing 5 × 10^{-6} M of the radioactive cyclic GMP. The elution of protein from the column was carried out with 170 ml of a linear gradient of sodium chloride (0 to 1 M) present in the same buffer, in the absence of cyclic GMP. About 73% of the total enzyme activity, and all of the protein-bound radioactive cyclic GMP and the free radioactive cyclic GMP, passed straight through the column. Attempts to purify further the cyclic GMP-binding protein from this fraction have not been successful. The remaining 27% of the enzyme activity retained by the column was eluted at approximately 0.2 M sodium chloride; this activity was independent of added cyclic GMP. The results suggested that only about one-fourth of the holoenzyme had been dissociated into subunits by this procedure.

The catalytic subunit from lobster muscle cyclic AMP-dependent enzyme was prepared by the procedure described for the cyclic GMP-dependent enzyme, except that the enzyme was preincubated with 5 × 10^{-6} M cyclic GMP (15 μCi) instead of cyclic GMP. The yield of the catalytic subunit was 32%. Its activity was found to be independent of added cyclic AMP, and it was free from protein-bound radioactive cyclic AMP. The cyclic nucleotide-independent catalytic subunits of the two classes of protein kinase from lobster muscle were separately concentrated on PM-10 Diaflo membranes.

Sucrose Density Gradient Centrifugation of Holoenzyme and Catalytic Subunit of Cyclic AMP-dependent and Cyclic GMP-dependent Protein Kinases

Protein kinase I, the enzyme from Peak I of the hydroxylapatite column, was found on sucrose density gradient centrifugation to consist of two distinct components, with sedimentation coefficients of 7.7 S (mol wt 140,000) and 5.4 S (mol wt 84,000) (Fig. 4). Both components of the enzyme were activated by cyclic AMP. The 5.4 S component of protein kinase I was designated protein kinase IC, and the 7.7 S component was designated protein kinase ID. Protein kinase II consisted of only one active component, with a sedimentation coefficient of 7.7 S (Fig. 4). The two active enzyme bands, protein kinase IA and IB, eluted from the gel following preparative gel electrophoresis of protein kinase I, were found to have sedimentation coefficients of 7.7 S and 5.4 S, respectively. Protein kinase II, prepared from protein kinase IP by Sepharose 6B chromatography and preparative gel electrophoresis, as described above, sedimented at a position corresponding to 7.7 S.

The peak of catalytic activity (catalytic subunit) and that of cyclic AMP-binding protein (regulatory subunit), derived from protein kinase II by chromatography on Enzite CM-cellulose-protamine (Fig. 3), were centrifuged in a sucrose density gradient; protein kinase II holoenzyme was included for purposes of comparison. The 2 subunits were found to sediment in positions corresponding to 3.6 S (mol wt 40,000) and 6.1 S (mol wt 100,000), respectively (Fig. 5). The cyclic AMP-binding activity peak of the holoenzyme sedimented in a position corresponding to 7.7 S (mol wt 140,000) (Fig. 5) as did its catalytic activity peak (Fig. 4).

The peak of catalytic activity (catalytic subunit) and that of cyclic AMP-binding protein (regulatory subunit), derived from protein kinase IC by chromatography on Enzite CM-cellulose-protamine, were also analyzed by sucrose density gradient centrifugation, along with the holoenzyme (5.4 S) from which they were obtained. The catalytic subunit sedimented in a position corresponding to 3.6 S (mol wt 40,000), whereas the protein-bound radioactive cyclic AMP sedimented in positions corresponding to 6.1 S (mol wt 100,000) and 2.0 S (mol wt 20,000), respectively. The 6.1 S component of the regulatory subunit may be an aggregate form of the 2.0 S component.

The isolated catalytic subunit of lobster muscle cyclic GMP-dependent enzyme sedimented in a sucrose density gradient at a position corresponding to 3.6 S (mol wt 40,000), and the holoenzyme from which it was derived sedimented at a position of 7.7 S (mol wt 140,000) (Fig. 6A). The isolated catalytic subunit and the holoenzyme of the lobster muscle cyclic AMP-dependent protein kinase, on the other hand, sedimented at positions corresponding to 4.5 S (mol wt 60,000) and 5.7 S (mol wt...
In the case of these two enzyme preparations, the catalytic activation of protein kinase IR (Fig. 8) and protein kinase IC, experiment of Fig. 7, histone also caused the dissociation and activity of this enzyme preparation, which had a peak of 90,000, respectively (Fig. 6B). With both classes of enzyme, the catalytic activity of the holoenzyme was dependent upon added cyclic nucleotide, whereas the catalytic activity of the subunit was independent of added cyclic nucleotide.

It is interesting that the catalytic subunits derived from lobster muscle cyclic GMP-dependent protein kinase and from bovine brain cyclic AMP-dependent protein kinases had the same sedimentation coefficient (3.6 S) which differed from that for the catalytic subunit (4.5 S) from lobster muscle cyclic AMP-dependent protein kinase. These findings correlate with our earlier observations (6, 7) that, with respect to some kinetic properties, cyclic GMP-dependent enzymes from arthropods resembled mammalian cyclic AMP-dependent enzymes and were dissimilar to arthropod cyclic AMP-dependent enzymes.

**Dissociation and Activation of Protein Kinases by Histone and by Cyclic Nucleotides**

**Bovine Brain Cyclic AMP-dependent Protein Kinases**—When protein kinase II was preincubated with histone (1 mg per ml) and then centrifuged in the presence of the same concentration of histone, the enzyme became dissociated. Thus, the catalytic activity of this enzyme preparation, which had a peak of 7.7 S (mol wt 140,000) and had been cyclic AMP-dependent, shifted in the presence of histone to a position of 3.6 S (mol wt 40,000) and was now cyclic AMP-independent (Fig. 7). Studies of the effect of histone on protein kinase IIA which had been further purified, through the stages of Sepharose 6B chromatography and preparative gel electrophoresis, gave results similar to those observed with protein kinase II.

Under the same experimental conditions as were used in the experiments of Fig. 7, histone also caused the dissociation and activation of protein kinase IB (Fig. 8) and protein kinase IC. In the case of these two enzyme preparations, the catalytic activity shifted, in the presence of 1 mg of histone per ml, from a cyclic AMP-dependent peak at 5.4 S (mol wt 84,000) to a cyclic AMP-independent peak at 3.6 S.

The effect of cyclic AMP, alone and in combination with histone, was studied on the dissociation of protein kinase IC and protein kinase II from bovine brain. In these experiments, preincubation and centrifugation in the presence of cyclic AMP, with or without histone, were carried out in a manner analogous to that used for the studies of histone alone (Fig. 7). In high concentrations (5 × 10^{-4} M), cyclic AMP caused complete dissociation of each of these enzymes. In some experiments, the effects of suboptimal concentrations of histone and of cyclic AMP were examined. A low concentration of histone (200 μg per ml) caused the partial dissociation of protein kinase IC, so that two peaks of activity were observed, one at 5.4 S which was cyclic AMP-dependent, and one at 3.6 S which was cyclic AMP-independent. A low concentration of cyclic AMP (5 × 10^{-4} M) also caused the partial dissociation of protein kinase IC: two peaks of catalytic activity appeared, one at 5.4 S and one at 3.6 S. In the combined presence of these low concentrations of histone and cyclic AMP, protein kinase IC was completely dissociated: all catalytic activity appeared in a position correspond-
as described in the legend to Fig. 7. The enzyme (100 μg) was preincubated at 30° for 5 min in a volume of 0.22 ml containing 50 mM acetate buffer (pH 6.0), 0.3 mM EGTA, and 2.5 mM 2-mercaptoethanol, in the absence (△, ▲) or presence (Δ, □) of histone (1 mg per ml). At the end of the preincubation, the entire volume of the solution was layered onto 4.8 ml of a 5 to 20% sucrose density gradient containing the same concentrations of acetate buffer, EGTA, and 2-mercaptoethanol, with or without histone, as were present in the preincubation solution. After centrifugation, the fractions obtained from each tube were assayed for enzyme activity under standard conditions in the absence (——) or presence (—) of 5 μM cyclic AMP.

In the absence of histone, a two-peak pattern of the enzyme activity appeared, one at 5.4 S and one at 3.6 S. In the combined presence of these low concentrations of histone and cyclic AMP, protein kinase II was completely dissociated, all catalytic activity appearing in a position corresponding to 3.6 S. Thus, the effects of histone and of cyclic AMP were additive in bringing about the dissociation of each of the bovine brain protein kinase preparations studied. The value of 3.6 S for the catalytic subunit of protein kinase IC and of protein kinase II agrees with the corresponding data, obtained by sucrose density gradient centrifugation, for the catalytic subunits of these enzymes isolated by column chromatography on Enzite CM-cellulose protamine.

Several proteins, other than histone, were examined for their effectiveness in dissociating the brain enzymes, protein kinase II and protein kinase IC. Protamine (500 μg per ml) caused the dissociation of both protein kinases with formation of the 3.6 S catalytic component, whose activity was no longer stimulated by cyclic AMP. Casein (3 mg per ml) caused protein kinase II to dissociate partially into the 5.4 S and 3.6 S components, and caused partial dissociation of protein kinase IC into the 3.6 S component. Neither protein kinase was dissociated when preincubated with a variety of other proteins, such as bovine serum albumin (500 μg per ml), fructose 6-phosphate kinase (200 μg per ml), poly-L-serine (200 μg per ml), catalase (200 μg per ml), glyceraldehyde-3-phosphate dehydrogenase (200 μg per ml), or horse liver alcohol dehydrogenase (200 μg per ml). Tao (34), using a cyclic AMP-dependent protein kinase from rabbit erythrocytes, was able to confirm our earlier observations (16) on the dissociation of brain cyclic AMP-dependent protein kinases by protamine, but could not demonstrate dissociation of the erythrocyte enzyme by histone. His somewhat different results may be a reflection of tissue or species differences in the properties of protein kinases.

The conversion of protein kinase in the presence of histone to a cyclic AMP-independent form, observed in the sucrose density gradient experiments (Figs. 7 and 8), could also be demonstrated simply by preincubation of the enzyme with histone, followed by assay of protein kinase activity in the absence of cyclic AMP. Thus, preincubation of protein kinases IB, IC, II, or IIA in the presence of histone (5 to 200 μg per ml) caused a substantial increase in the activity of the enzyme in the absence of cyclic AMP, with a corresponding decrease in the stimulatory effect of cyclic AMP. The effect of the activity of protein kinase II of preincubation in the presence of various concentrations of histone is shown in Fig. 9. It is clearly possible that substrate-
Protein Kinase

Cyclic AMP Independent Protein Kinase Activity—Cyclic AMP-independent protein kinase activity has been found in a number of systems. For example, in lobster muscle, cyclic AMP-independent activity of hepatic lysyltactin) appeared at a pH value of 7.8, and the peak of cyclic AMP-independent protein kinase activity (catalytic and regulatory subunits) had a pI of pH 7.6 to 8.9. Chen and Walsh (35) reported protein-bound radioactive cyclic AMP (regulatory subunit) appeared at a pH value of 3.8. Preincubation of protein kinase II in the presence of ATP plus Mg" did not cause dissociation of the brain enzymes. In contrast to the ability of some substrate proteins to cause dissociation and activation of the kinases, preincubation of protein kinase IC or II in the presence of ATP plus Mg" did not cause dissociation or activation of these enzymes.

Isoelectric Points of Catalytic and Regulatory Subunits from Protein Kinase II—Protein kinase II was dissociated into its catalytic and regulatory subunits by preincubation in the presence of histone and cyclic AMP; it was then applied to a 110-ml isoelectric focusing column which did not contain added histone or cyclic AMP. By using these experimental conditions, it was possible to measure the pI for cyclic AMP-dependent enzyme and for the separate catalytic and regulatory subunits in the same experiment. A typical isoelectric focusing pattern of this enzyme and its subunits is illustrated in Fig. 10 A pI of pH 4.6 was observed for the cyclic AMP-dependent activity. The peak of cyclic AMP-independent protein kinase activity (catalytic subunit) appeared at a pH value of 7.8, and the peak of protein-bound radioactive cyclic AMP (regulatory subunit) appeared at a pH value of 3.8. Chen and Walsh (35) reported earlier that the cyclic AMP-independent activity of hepatic protein kinases had pI values of pH 7.6 to 8.9.

Lobster Muscle Cyclic GMP-dependent and Cyclic AMP-dependent Protein Kinases—Lobster muscle cyclic GMP-dependent enzyme, either not preincubated or preincubated in the absence of histone and cyclic GMP, was found to have a sedimentation coefficient of 7.7 S (Fig. 11A). When this enzyme was preincubated and then centrifuged in the presence of histone (1 mg per ml), a partial dissociation of the enzyme was observed (Fig. 11A). The peak of catalytic activity at 7.7 S which had been cyclic GMP-dependent decreased in size, and a new peak of catalytic activity which was cyclic GMP-independent appeared in a position of 3.6 S. When this enzyme was preincubated and then centrifuged in the presence of 5 x 10^-6 M cyclic GMP (Fig. 11B), the enzyme was only partially dissociated. Two peaks of catalytic activity again appeared at positions corresponding to 7.7 S and 3.6 S. Cyclic GMP was more effective than cyclic AMP in dissociating and in activating the cyclic GMP-dependent enzyme. When the cyclic GMP-dependent enzyme was preincubated with both histone (1 mg per ml) and cyclic GMP (5 x 10^-6 M), an almost complete dissociation of the enzyme into the 3.6 S component was observed (Fig. 11B).

Histone and cyclic nucleotides also caused dissociation of the cyclic AMP-dependent protein kinase from lobster muscle. Thus, the holoenzyme, which sedimented at a position of 5.7 S (mol wt 90,000), dissociated about 50% to a cyclic AMP-independent 4.5 S component (mol wt 50,000). When the enzyme was preincubated and centrifuged in the presence of 200 mg of histone per ml, complete dissociation of the enzyme into the 3.6 S component was observed (Fig. 11B).
effective in causing dissociation of lobster muscle cyclic GMP-dependent and cyclic AMP-dependent protein kinases into the corresponding cyclic nucleotide-independent catalytic subunits. These proteins which were ineffective in causing the dissociation of the brain enzymes were also ineffective against the two lobster enzymes.

It remains to be determined whether the dissociation and activation of protein kinases by substrate proteins may represent a physiological mechanism for regulating protein kinase activity in response to extracellular signals; conceivably, availability of substrate proteins might constitute an intracellular mechanism for regulating the activity of this class of enzyme.

Recombination of Regulatory and Catalytic Subunits—Recombination of the regulatory and catalytic subunits derived from cyclic AMP-dependent protein kinases has been reported for a number of mammalian tissues (11, 14, 15, 17, 35-37). It was found in the present study that regulatory subunit prepared from bovine brain cyclic AMP-dependent protein kinase was able to inhibit the enzymatic activity of catalytic subunits derived from bovine brain cyclic AMP-dependent protein kinase, or, interestingly, from lobster muscle cyclic GMP-dependent protein kinase, accompanied in each case by a concomitant restoration of cyclic nucleotide dependence of the resultant holoenzyme (Fig. 12). For each enzyme, the activity of the free catalytic subunit, measured in the presence or absence of added cyclic AMP, and that observed with the reconstituted holoenzyme in the presence of added cyclic AMP were comparable. The relatively high amount of regulatory subunit required to inhibit catalytic activity may have been due either to partial denaturation of the isolated regulatory subunit, or to an incomplete association of regulatory and catalytic subunits under the experimental conditions used. The reconstituted "homologous" holoenzyme, obtained by combining regulatory and catalytic subunits both derived from the cyclic AMP-dependent class of protein kinase, was found to be activated preferentially by cyclic AMP rather than by cyclic GMP (Fig. 13A), as seen for the brain kinases before dissociation. Cyclic AMP was also more effective than cyclic GMP in activating the reconstituted "hybrid" holoenzyme consisting of the regulatory subunit from a cyclic AMP-dependent protein kinase and the catalytic subunit from a cyclic GMP-dependent protein kinase (Fig. 13B). The latter results indicate that the cyclic nucleotide specificity of the regulatory subunit was not affected by its combination with the "heterologous" catalytic subunit. It would be interesting to study the effect of the regulatory

![Figure 11](http://www.jbc.org/)

**FIG. 11.** Dissociation of lobster muscle cyclic GMP-dependent protein kinase by histone and cyclic GMP. The enzyme (2.0 mg) was preincubated at 30° for 5 min in a volume of 0.22 ml containing 50 mM sodium acetate buffer (pH 6.0), 0.3 mM EGTA, 2.5 mM 2-mercaptoethanol, and the following additions: (A) none (O--O), 1 mg of histone per ml (△, △); (B) 50 μM cyclic GMP (■), or 1 mg of histone per ml plus 50 μM cyclic GMP (■). At the end of the preincubation, the entire volume of the solution was layered onto 4.8 ml of a 5 to 20% sucrose density gradient containing the same concentrations of acetate buffer, EGTA, and 2-mercaptoethanol, and the same additions as were present in the preincubation solution. After centrifugation, the fractions obtained from each tube were assayed for protein kinase activity under the standard conditions in the absence ( - - - ) or presence (□□□□□) of 5 μM cyclic AMP.

![Figure 12](http://www.jbc.org/)

**FIG. 12.** Inhibition of the activity of isolated catalytic subunits by the addition of regulatory subunit and restoration of cyclic AMP dependence in the reconstituted holoenzyme. The catalytic subunits were prepared by column chromatography on Enzite CM-cellulose-protamine, as described in the text. Various amounts of the isolated regulatory subunit prepared from bovine brain cyclic AMP-dependent protein kinase were added to (A) catalytic subunit (1.6 μg) from protein kinase II, (B) catalytic subunit (2.4 μg) from protein kinase IC, or (C) catalytic subunit (1.6 μg) from lobster muscle cyclic GMP-dependent enzyme. Enzyme activity was assayed under the standard conditions in the absence (O--O) and presence (●●●●) of 5 μM cyclic AMP.
Some Characteristics of Catalytic Subunits from Cyclic AMP-dependent and Cyclic GMP-dependent Protein Kinases

Protein Substrate Specificity—The relative abilities of several proteins to serve as phosphate acceptor for the holoenzymes and the catalytic subunits of bovine brain and lobster muscle protein kinases are compared in Table II. All histone preparations were more effective than other proteins, including protamine, tested as substrates for both the holoenzyme and the catalytic subunit of brain cyclic AMP-dependent protein kinase. The order of effectiveness of the various substrates was identical for the holoenzymes of protein kinases II, II A, II B, and IC, as well as for the catalytic subunits from protein kinases II and IC.

The order of effectiveness of substrates for the brain enzyme and for the lobster muscle cyclic AMP-dependent and cyclic GMP-dependent enzymes differed from one another, the largest differences being observed between the two cyclic AMP-dependent enzymes. For example, protamine was one of the poorest substrates for the brain enzyme and one of the best for the lobster cyclic AMP-dependent protein kinase. It was found in several experiments, including that shown in Table II, that the efficacy of casein relative to histones and protamine to serve as substrate for cyclic AMP-dependent and cyclic GMP-dependent protein kinases from lobster muscle was much poorer in the presence of holoenzyme plus cyclic nucleotide than in the presence of the isolated catalytic subunit. The explanation of these observations may lie in the greater ability of histones and protamine to

Table II

| Substrate Specificity of Holoenzymes and Catalytic Subunits of Cyclic Nucleotide-dependent Protein Kinases from Bovine Brain and Lobster Muscle |
|---|
| The concentration of cyclic AMP or cyclic GMP, where present, was 5 μM. The amounts of holoenzyme and catalytic subunit used were: 1.8 and 3.6 μg, respectively, for cyclic AMP-dependent enzyme (protein kinase II) from bovine brain; 145 and 9.2 μg, respectively, for cyclic AMP-dependent enzyme from lobster muscle; 50 and 16.3 μg, respectively, for cyclic GMP-dependent protein kinase from lobster muscle. Enzyme activity was assayed under standard conditions except for the variation in kind and amount of substrate proteins used. |

| Substrate | Bovine brain cyclic AMP-dependent enzyme | Lobster muscle cyclic AMP-dependent enzyme | Lobster muscle cyclic GMP-dependent enzyme |
|---|---|---|---|
| Holoenzyme | Catalytic subunit | Holoenzyme | Catalytic subunit | Holoenzyme | Catalytic subunit |
| Cyclic AMP | +Cyclic AMP | −Cyclic AMP | Cyclic AMP | +Cyclic AMP | −Cyclic AMP | cyclic GMP | +Cyclic GMP | −Cyclic GMP |
| None | 1.2 | 1.5 | 0.9 | 3.2 | 4.2 | 1.4 | 2.5 | 3.7 | 2.3 |
| Histone mixture | | | | | | | | | |
| 10 μg | 10.6 | 83.0 | 82.6 | 8.9 | 14.7 | 3.7 | 9.0 | 25.4 | 10.8 |
| 40 μg | 8.4 | 39.0 | 36.6 | 8.7 | 14.3 | 5.3 | 6.7 | 23.9 | 11.4 |
| Arginine-rich histone, 40 μg | 6.2 | 39.6 | 18.7 | 10.9 | 19.0 | 5.6 | 7.0 | 23.0 | 11.2 |
| Slightly lysine-rich histone, 40 μg | 6.1 | 25.1 | 15.3 | 13.1 | 25.7 | 8.2 | 3.9 | 15.5 | 6.9 |
| Casein, 600 μg | 3.1 | 11.1 | 7.7 | 8.6 | 10.9 | 7.4 | 5.4 | 7.8 | 9.8 |
| Protamine, 100 μg | 4.6 | 7.6 | 5.6 | 20.4 | 35.9 | 8.4 | 10.8 | 18.0 | 12.0 |
| Bovine serum albumin, 100 μg | 1.7 | 1.9 | 1.2 | 3.7 | 5.0 | 2.5 | 2.0 | 3.3 | 2.8 |

The incorporation of 32P into protein from catalytic subunits from cyclic AMP-dependent and cyclic GMP-dependent enzymes was 17 and 7.9 pmol, respectively.
promote dissociation of the holoenzyme in the absence or in the presence of low concentrations of cyclic AMP.

Effect of ATP and Histone Concentration—From double reciprocal plots of enzyme activity versus ATP concentration, which obeyed classical kinetics, the apparent $K_m$ values for ATP of the catalytic subunits from lobster muscle cyclic GMP-dependent and cyclic AMP-dependent protein kinases were calculated to be $4.2 \times 10^{-4}$ M and $8.3 \times 10^{-6}$ M, respectively. The apparent $K_m$ values for ATP of the holoenzymes of protein kinases IB, IC, II, and IIA (in the presence of $5 \times 10^{-6}$ M cyclic AMP) and of isolated catalytic subunits of protein kinases IC and II were all in the range from $3.8 \times 10^{-4}$ to $5.6 \times 10^{-4}$ M.

Double reciprocal plots of enzyme activity versus histone concentration showed a biphasic response for all protein kinase preparations studied. The concentration of histone required to give half-maximal activity of the catalytic subunits of lobster muscle cyclic AMP-dependent and cyclic GMP-dependent protein kinases were 95 pg per ml and 105 pg per ml, respectively, and the activity reached a plateau at a histone concentration of about 500 pg per ml with both enzymes. The relationship between enzyme activity and histone concentration was also studied using the catalytic subunits and holoenzymes of protein kinases IB, IC, II, and IIA and the catalytic subunits derived from protein kinases IC and II. In all six cases, the results were similar to those found with the catalytic subunits of the two lobster enzymes.

Metal Ion Requirement—A comparison of the effects of divalent metal ions was carried out with the holoenzymes of protein kinases IB, IC, II, and IIA, the catalytic subunits derived from protein kinases IC and II, and the catalytic subunits derived from the lobster muscle cyclic AMP-dependent and cyclic GMP-dependent protein kinases. All eight enzyme preparations had an absolute requirement for a divalent ion: Ca$^{2+}$, Mg$^{2+}$, and Mn$^{2+}$ supported protein kinase activity, but Ca$^{2+}$ did not. In fact, Ca$^{2+}$ antagonized the stimulatory effect of the other metal ions. For all eight enzyme preparations, enzyme activity was highest in the presence of $10 \text{ mm} \text{Ca}^{2+}$.

Inhibition by ADP and Related Compounds—It was observed in earlier studies that ADP, adenosine, and 2'-deoxyadenosine inhibited the activity of cyclic AMP-dependent protein kinases from brain, liver, and a variety of other tissues (5, 29, 38). In the present study, it was found that the activity of the holoenzymes of protein kinases IB, IC, II, and IIA, as well as of the catalytic subunits derived from protein kinases IC and II, were each inhibited 50 to 60% by 50 $\mu$M ADP. Adenosine and 2'-deoxyadenosine were about half as potent as ADP. These results are similar to those reported earlier with holoenzymes and indicate that the inhibitory action of these substances occurs on the catalytic subunits. The inhibition by ADP, adenosine, and 2'-deoxyadenosine of the catalytic subunits of the brain enzymes was readily overcome by increasing the ATP concentration, suggesting that these inhibitors compete with ATP for a common site on the catalytic subunit. Results similar to those obtained with the brain enzymes, i.e. inhibition by the three compounds mentioned above and removal of the inhibition by ATP, were also obtained with the catalytic subunits of lobster muscle cyclic AMP-dependent and cyclic GMP-dependent protein kinases.

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Dissociation and Activation of Adenosine 3',5'-Monophosphate-dependent and Guanosine 3',5'-Monophosphate-dependent Protein Kinases by Cyclic Nucleotides and by Substrate Proteins

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