Purification and Properties of Rabbit Skeletal Muscle Adenosine 3',5'-Monophosphate-dependent Protein Kinases*

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SUMMARY

A modified procedure for purification of the adenosine 3',5'-monophosphate-dependent (cyclic AMP-dependent) protein kinase from rabbit skeletal muscle is described. This procedure results in the separation of the enzyme into two peaks of activity on diethylaminoethylcellulose. No interconversion of these two peaks was observed. Both peaks exhibit dependence on cyclic AMP, but under certain conditions the dependence of Peak I on cyclic AMP can be reduced. The concentration of cyclic AMP needed for half-maximal stimulation is $3 \times 10^{-5} \text{M}$ for Peak I and $1.5 \times 10^{-4} \text{M}$ for Peak II. The binding of cyclic AMP to the protein kinase can be reversed by a number of mild chemical treatments and by gel filtration on Sephadex G-25. The apparent $K_0$ for ATP in the presence of $10 \text{mM Mg}^{2+}$ is approximately $1.5 \times 10^{-4} \text{M}$ in the presence or absence of cyclic AMP for either Peak I or Peak II. For Peak II the apparent $K_0$ for casein is 0.9 and 0.6 mg per ml in the presence and absence of cyclic AMP, respectively. This apparent $K_0$ is increased to 8 mg per ml when 0.1 M NaCl is present in the reaction mixture. The pH optimum is 6.0 for casein and 6.5 for histone phosphorylation for both enzymes. Peak II contains two components which are dependent on cyclic AMP and separable by sucrose density gradient centrifugation; the sedimentation coefficients of these components are 6.8 S and 4.9 S. Peak II activity sediments as a single peak with a sedimentation coefficient of 4.8 S. In the presence of cyclic AMP the protein kinase activity of Peak I sediments as a single component with a sedimentation coefficient of 3.4 S. The protein kinase activity of Peak II also exhibits a much lower sedimentation coefficient in the presence of cyclic AMP than that shown in the absence of this nucleotide.

Rabbit skeletal muscle phosphorylase kinase was shown by Krebs, Graves, and Fischer (1) to be activated by cyclic adenosine 3',5'-monophosphate by a process requiring ATP and Mg$^{2+}$. This effect of the cyclic nucleotide has since been shown to be mediated by a cyclic AMP-dependent phosphorylase kinase (2, 3), thus identifying the specific site of action of cyclic AMP in the hormonal regulation of glycogenolysis. In the preliminary communication (4) in which the identification of this enzyme was described, it was also shown to catalyze a cyclic AMP-dependent phosphorylation of casein and protamine. Because the enzyme did not appear to be specific for phosphorylase kinase, it was called a "cyclic AMP-dependent protein kinase." It has since been shown to be identical with glycogen synthetase kinase (5, 6). Since the initial report, cyclic AMP-dependent protein kinases have been studied in several other systems. Langan (7) reported that phosphorylation of liver histone in vivo is mediated by a cyclic AMP-dependent protein kinase. Jergil and Dixon (8) isolated an enzyme from trout testis which is involved in the phosphorylation of protamine in that tissue. In addition, cyclic AMP-dependent protein kinases have been demonstrated in adipose tissue (9), brain (10), bacteria (11), heart (12), the adrenal gland (13), the frog bladder (14), and in a number of other tissues (15).

The partial purification and some of the properties of the protein kinase from rabbit skeletal muscle were described in a preliminary communication (4) and in several reports (16, 17). These studies have now been extended and are reported in greater detail in this paper.

EXPERIMENTAL PROCEDURES

Materials

Vitamin-free casein (Nutritional Biochemicals) was suspended in water, heated at 100° for 10 min while maintaining the pH at

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9.5, cooled, and adjusted to a final protein concentration of 30 mg per ml at pH 6.0. Type II-A histone was obtained from Sigma. 3H-Cyclic AMP was obtained from Schwarz BioResearch. Cyclic nucleotides were a gift from Boehringer-Mannheim. Tricalcium phosphate gel (ex sucrose, A grade) and hydroxylapatite (dry powder) were obtained from Calbiochem; DEAE-cellulose (DE-52) was obtained from Whatman.

Methods

Preparation of $\gamma^{32}$P-ATP—Carrier-free $^{32}$P$_1$ (usually 20 mCi) in dilute HCl was obtained from Tracelab for the preparation of labeled ATP. $\gamma^{32}$P-ATP was prepared using a modification (9) of the method described by Glynn and Chappell (18).

Assay of Protein Kinase Activity—Protein kinase activity was measured at pH 6.0 in a 0.1-ml reaction mixture incubated at 30°C and containing the following: 50 mM glycerol-P, 1 mM potassium phosphate, 20 mM NaF, 0.3 mM EGTA, 2 mM theophylline, 6 mg per ml of casein, 0.2 mM $\gamma^{32}$P-ATP, 10 mM magnesium acetate, 2 mM cyclic AMP (when added), and enzyme. Reactions were started by addition of the $\gamma^{32}$P-ATP and initial reaction velocities were determined over a 20-min period. Two methods were used for terminating the reaction and determining the amount of $^{32}$P incorporated into the protein substrate. Method A is described in an accompanying paper (3) and in Method B the protein-bound $^{32}$P was determined in a manner similar to that described by Thomas, Schlender, and Larner (19) for $^{14}$C-glycogen in the assay of glycogen synthetase. Adaptation of this method to the determination of protein-bound $^{32}$P was kindly suggested by Dr. Keith Schlender and also by Dr. Steven Mayer. Reactions were terminated by pipetting 50 μl of reaction mixture onto squares (2 cm × 2 cm) of Whatman No. 31ET chromatography paper. These papers were then washed in cold 10% trichloroacetic acid for 30 min, cold 5% trichloroacetic acid for 30 min, and twice in 5% trichloroacetic acid for 30 min at room temperature, using 5 to 10 ml of trichloroacetic acid per paper in each wash. The papers were washed in a stainless steel wire basket placed in a beaker of trichloroacetic acid stirred gently with a magnetic stirring bar. The papers were washed briefly in ethanol, rinsed with ether, dried, and transferred to liquid scintillation counting vials to which had been added 5 ml of toluene-based scintillation fluid containing 6 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(5-phenyloxazoyl)]benzene per liter. In the experiments of Figs. 11, 12, and 13, the protein kinase activity was determined in a slightly modified reaction mixture containing 60 mM glycerol-P, 0.36 mM EGTA, 24 mM NaF, 2.4 mM theophylline, 7.2 mg per ml of casein, 0.24 mM $\gamma^{32}$P-ATP, 12 mM magnesium acetate, and enzyme in a volume of 70 μl and with a pH of 6.0; protein-bound $^{32}$P was determined by Method B.

Sucrose Density Gradient Centrifugation Studies—Sucrose density gradient studies were conducted by the method of Martin and Ames (20) using a Beckman SW 40 rotor at 39,000 rpm at 2°C for 16 hours. Linear sucrose gradients from 5 to 20% sucrose in 50 mM Tris Cl, 1 mM EDTA, pH 7.5, were employed. From 0.01 mg to 2.75 mg of protein were applied to the gradients in a volume of 0.1 to 0.5 ml. The gradient tubes were emptied by washing the contents of the tube from the bottom with a long hypodermic needle connected to a peristaltic pump. Fractions were collected and assayed for protein kinase activity and for the markers phosphorylase a and human hemoglobin.

Lyophilized phosphorylase b was prepared as described by Brostrom and Krebs (21), and assayed in the presence of AMP as described for phosphorylase a (22). Hemoglobin was prepared as described by Ingram (23) and measured by optical density at 405 μg.

Other Methods—Protein determinations were done by the biuret test or the method of Lowry et al. (24) using bovine serum albumin as a standard.

RESULTS

Purification of Cyclic AMP-dependent Protein Kinase

Six New Zealand white female rabbits, weighing 7 to 10 pounds, were anesthetized by intravenous injection of Nembutal and exsanguinated. The muscle was removed from the hind legs and back, ground, and homogenized in 4 mM EDTA at pH 7 for 1 min in a Waring Blendor. The grinding, homogenization, and all subsequent steps were conducted at 4°C. The homogenate was centrifuged at 10,000 × g for 30 min and the supernatant (extract) was adjusted to pH 6.1 by adding 1 N acetic acid. After removal of the precipitate, which contained phosphorylase kinase and glycogen synthetase, by centrifuging at 10,000 × g for 30 min, the supernatant was adjusted to pH 5.5 by adding 1 N acetic acid and centrifuged at 10,000 × g for 30 min. The pH 5.5 supernatant with a volume of 10 liters was brought to near
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Disc gel electrophoresis was performed using 30 μg of Peak I and 30 μg of Peak II per gel. A Tris asparagine buffer system (26) with 6% polyacrylamide gel and bromophenol blue to mark the buffer front was used. The gels were stained with Coomassie blue and destained as described previously (5).

Neutralization by adding a mixture of 400 ml of 1 M K₂HPO₄, 400 ml of 1 M KH₂PO₄, and 400 ml of 0.2 M neutral EDTA. The enzyme was precipitated by adding 3,250 g of powdered ammonium sulfate followed by centrifugation for 40 min at 10,000 × g. The precipitate was suspended in 5 mm potassium phosphate-1 mm EDTA, pH 6.5 (final volume of 250 ml), and dialyzed against several changes of the same buffer for 16 hours. The enzyme was centrifuged at 78,000 × g for 1 hour and the precipitate was discarded. To 200 ml of the supernatant containing approximately 5 g of protein were added 125 ml of hydroxylapatite (200 mg per ml) equilibrated in the same buffer, to adsorb unwanted proteins.

The hydroxylapatite was removed by centrifugation and the supernatant was treated with 125 ml of calcium phosphate (20 mg per ml) equilibrated in the same buffer, to adsorb the enzyme. The suspension was centrifuged and the gel was washed with a series of phosphate buffers composed of dibasic and monobasic potassium phosphate (9:1) containing 1 mM EDTA. The volume and phosphate concentration of the washes were as follows: 100 ml of 0.05 M, 20 ml of 0.05 M, 20 ml of 0.1 M, 20 ml of 0.2 M, and 20 ml of 0.3 M. The enzyme typically was eluted in the two final washes. These were combined and equilibrated with 5 mM Tris Cl-1 mM EDTA at pH 7.5 on Sephadex G-25. The equilibrated enzyme was concentrated by ultrafiltration with a Diaflo membrane (XM-50, Amicon Corporation) to approximately 25 ml and applied to a column (0.9 × 15 cm) of Whatman DEAE-cellulose equilibrated in the same buffer. The enzyme was eluted at pH 7.5 with a 0.005 M to 0.5 M linear Tris Cl gradient containing 1 mM EDTA with a total volume of 200 ml. This column resolved the enzyme into two peaks of activity as shown by the activity profile presented in Fig. 1. The specific activities and yields at several stages of purification are shown in Table I. For the particular preparation described the specific activity of Peak I was 150-fold and that of Peak II was 350-fold greater than that of the initial extract. However, the specific activity and yield of Peak I were often greater than those of Peak II. This procedure is a modification of that described by Walsh, Perkins, and Krebs (4). It differs primarily in that the calcium phosphate step replaces one of the DEAE-cellulose columns and the DEAE-cellulose column that is used is much smaller than that in the original procedure. These modifications result in the resolution of the enzyme activity into the two major peaks. Part I migrates as a single broad band on disc gel electrophoresis, but Peak II shows the presence of multiple protein bands by this technique (Fig. 2).

No interconversion of Peaks I and II was observed upon rechromatography of each fraction on DEAE-cellulose. In addition, the two fractions were identified in a crude muscle extract that was subjected to chromatography on DEAE-cellulose either within 3 hours postmortem or after storage for 1 week at 4°C. Storage for 1 week did not alter the relative yields of Peak I and Peak II.

Properties of Protein Kinases

Time Course of Casein Phosphorylation and Effect of Varying Enzyme Concentration—The skeletal muscle protein kinases catalyzed the phosphorylation of casein or histone. This is illustrated in Fig. 3 for the enzyme from Peak II with casein as the substrate; similar data were obtained using Peak I. In utilizing

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Fig. 2. Disc gel electrophoresis of Peak I and Peak II. Disc gel electrophoresis was performed using 30 μg of Peak I and 30 μg of Peak II per gel. A Tris asparagine buffer system (26) with 6% polyacrylamide gel and bromophenol blue to mark the buffer front was used. The gels were stained with Coomassie blue and destained as described previously (5).

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3 Recently by the use of very shallow gradients it has sometimes been possible to demonstrate a third peak (Peak III) on the DEAE-cellulose column. When present, this peak is eluted immediately after Peak II. Peak III is a minor component, and even when incompletely separated from Peak II, it has only small influence on the total activity of this fraction. Nevertheless, the existence of this enzyme is of interest and it has been partially characterized. It appears to differ from Peak I and Peak II in that it is not stimulated by cyclic AMP, and in contrast to Peak I and Peak II, it phosphorylates casein more readily than histone. Preliminary data indicate that this enzyme has an apparent K₅₀ for ATP greater than 10⁻⁴ M. The heat-stable protein inhibitor of protein kinases (25) does not inhibit this enzyme whereas it inhibits both Peak I and Peak II.
FIG. 3. Effect of incubation time on casein phosphorylation by the protein kinase. The reaction mixture, with a composition as described under "Methods," contained 26 μg of Peak II in a total volume of 0.5 ml; 50-μl aliquots were withdrawn at the indicated times for measurement of 32P incorporation by Method B. The values have not been corrected for 32P incorporation in the absence of added enzyme. The values shown for incubation times of zero are indicative of the values typically observed for such blanks.

FIG. 4. Effect of protein kinase (Peak II) concentration on the rate of casein phosphorylation. Reaction mixtures were identical with those described in the legend of Fig. 3 except for enzyme concentrations. 32P incorporation was measured by Method B. Initial reaction velocities were determined from measurements made at 5, 10, and 20 min.

This reaction as the basis for an assay for the kinases, enzyme concentrations were kept sufficiently low so that reaction rates in the presence of cyclic AMP were linear for at least 20 min. It was shown that reaction rates were proportional to enzyme concentration under these conditions (Fig. 4).

pH Optima—Fig. 5A illustrates the effect of pH on casein phosphorylation by Peak I. Maximal activity in the presence of cyclic AMP was observed at pH 6, whereas in the absence of cyclic AMP, maximal activity was observed at pH 5.6. This was the lowest pH tested, because casein precipitated when the pH was lowered further. Similar results were obtained with Peak II except that the activity in the absence of cyclic AMP was relatively unaffected by pH. When histone (6 mg per ml) was used as a substrate, both peaks exhibited a rather broad pH curve with an optimum at pH 6.5.

Variable Requirement for Cyclic AMP—It was found that the relatively high activity of Peak I at low pH in the absence of cyclic AMP (Fig. 5A) occurred only when the reaction was initiated by addition of ATP as the final component. If the reaction was started by the addition of enzyme, or under conditions in which essentially no time was allowed for preliminary incubation of enzyme and casein, then the activity in the absence of cyclic AMP remained low even below pH 6. Enzyme activity in the presence of cyclic AMP was unaffected by the order of addition of components. Similar observations were made when histone (6 mg per ml) was used as a substrate for Peak I. In this case essentially no requirement for cyclic AMP was seen at any of the pH values tested if the enzyme was previously incubated with histone prior to addition of ATP. Again, if the reaction was initiated by the addition of enzyme, the activity in the absence of cyclic AMP was markedly reduced, whereas activity in the presence of cyclic AMP was unaffected. The mechanism or mechanisms by which casein at low pH or histone alleviates the requirement for cyclic AMP is not clear. However, investigation to elucidate this mechanism is in process, since it may reveal additional information about the mechanism of action of cyclic AMP. It is particularly interesting that this effect is seen only with Peak I and not with Peak II.

Apparent Kₐ for Cyclic AMP—In experiments with varying concentration of cyclic AMP it was found that this nucleotide
Fig. 6. Effect of cyclic AMP concentration on casein phosphorylation by Peak II. Assay conditions were as described in the text except that 10 mM MES buffer at pH 6.0 was used instead of glycerol-P. Enzyme concentration was 4 µg per ml of Peak II. Protein-bound ³²P was determined by Method A. In the absence of added cyclic AMP the activity was 0.2 µmole ³²P incorporated per min. This basal activity was subtracted from the activity observed in the presence of cyclic AMP.

Table II

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>10⁻⁶ M nucleotide</th>
<th>10⁻⁴ M nucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak I</td>
<td>Peak II</td>
</tr>
<tr>
<td>Cyclic AMP</td>
<td>5.85</td>
<td>6.25</td>
</tr>
<tr>
<td>Cyclic IMP</td>
<td>4.50</td>
<td>5.42</td>
</tr>
<tr>
<td>Dibutyryl cyclic AMP</td>
<td>1.95</td>
<td>0.54</td>
</tr>
<tr>
<td>Cyclic UMP</td>
<td>-0.40</td>
<td>0.51</td>
</tr>
<tr>
<td>Cyclic TMP</td>
<td>0</td>
<td>-0.14</td>
</tr>
<tr>
<td>Cyclic GMP</td>
<td>0.35</td>
<td>0.78</td>
</tr>
<tr>
<td>Cyclic dAMP</td>
<td>0.15</td>
<td>-0.01</td>
</tr>
<tr>
<td>Cyclic CMP</td>
<td>-0.25</td>
<td>0.82</td>
</tr>
</tbody>
</table>

exerted a slight cooperative effect on the protein kinase from either Peak I or Peak II. This is illustrated for Peak II in Fig. 6. As can be seen the reciprocal plot was nonlinear. From the Hill plot a coefficient of 1.26 was obtained. For Peak I the Hill coefficient was 1.27. The concentration of cyclic AMP needed for half-maximal stimulation was 3 X 10⁻⁴ M for Peak I and 1.5 X 10⁻⁴ M for Peak II.

Nucleotide Specificity of Peak I and Peak II—It was of interest to test the effect of cyclic GMP on these two enzymes because cyclic GMP has been reported as a naturally occurring cyclic nucleotide (28, 29) and because a protein kinase stimulated by cyclic GMP has recently been reported (30). The effect of di-butyryl cyclic AMP was also of interest since it has been found to reproduce the effects of cyclic AMP in many systems (31). These and a number of other cyclic nucleotides were tested at 10⁻⁶ and 10⁻⁴ M as shown in Table II. At 10⁻⁶ M cyclic IMP was nearly as effective as cyclic AMP in stimulating either enzyme. The dibutyryl derivative of cyclic AMP was somewhat effective in stimulating Peak I, but had little effect on Peak II activity. None of the other nucleotides stimulated either enzyme to a significant degree at 10⁻⁶ M. At 10⁻⁴ M, however, nearly all the nucleotides were as effective as cyclic AMP. Cyclic TMP at this concentration was somewhat less effective than the other nucleotides tested, particularly in stimulating Peak I activity. Aside from these minor differences it appears that these two enzymes have the same nucleotide specificity. The stimulation of both enzymes by cyclic GMP at 10⁻⁴ M was very low compared to the stimulation by cyclic AMP at 10⁻⁶ M suggesting that cyclic GMP is not important for activating either of these enzymes in vivo.

Apparent Kₘ for ATP—Double reciprocal plots of ATP concentration against casein phosphorylation are depicted in Fig. 7 for Peak I and in Fig. 8 for Peak II. In these studies magnesium acetate was maintained at 10 mM. It is clear from the experiment presented in Fig. 7 that cyclic AMP did not significantly change the apparent Kₘ for ATP for Peak I. Values of 1.0 and 1.2 X 10⁻⁴ M were obtained in the absence and presence of cyclic AMP, respectively. On the other hand, maximal velocity was increased 7-fold by cyclic AMP in this experiment. Fig. 8 illustrates typical double reciprocal plots obtained with Peak II. In the presence of cyclic AMP, an apparent Kₘ for 1.1 X 10⁻⁴ M was obtained in agreement with that observed for Peak I. In the absence of cyclic AMP a biphasic reciprocal plot was obtained. This plot is arbitrarily interpreted as being due to a mixture of two enzymes differing in their apparent Michaelis constants for ATP. Support for this interpretation comes from the fact that another protein kinase not stimulated by cyclic AMP and exhibiting an apparent Kₘ for ATP greater than 10⁻⁴ M has been detected as a minor contaminant of Peak II.³
FIG. 8. Double reciprocal plots illustrating the effect of ATP concentration on casein phosphorylation by Peak II. Peak II was assayed in the presence (■-■) and absence (○-○) of 10 mM cyclic AMP. Magnesium acetate was held constant at 10 mM. Protein-bound 32P was determined by Method A.

TABLE III
Effect of magnesium on apparent Km values for ATP
Experimental conditions were as described for Fig. 7, except that apparent Michaelis constants were determined at three different magnesium concentrations. Peak I was used at a final concentration of 18 μg per ml in this experiment. No incubations were done in the absence of cyclic AMP. Protein-bound 32P was determined by Method B.

<table>
<thead>
<tr>
<th>Magnesium (mM)</th>
<th>Maximal velocity (μmols 32P incorporated/min)</th>
<th>Apparent Km for ATP (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>3</td>
<td>12.6</td>
<td>2.8</td>
</tr>
<tr>
<td>10</td>
<td>12.7</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Effect of Varying Magnesium—Data demonstrating the effect of varying the concentration of Mg2+ on the maximal velocity and Km for ATP are given in Table III. As can be seen, maximal velocities are the same between 1 mM and 10 mM Mg2+, while Km values decrease significantly in this range. A complete interpretation of these data is not possible at this time, but several observations can be made relative to the Mg2+ effect. It is possible that Mg2+ may bind at more than one site on the enzyme and serve in a regulatory capacity in addition to its substrate role with ATP. On the other hand, although the true stability constant of Mg-ATP is approximately 75,000, the apparent stability constant of the magnesium-ATP complex at the low pH used in the enzyme assay is much lower (32). Furthermore, the reaction mixture contains F-, glycerol-P, and EDTA which would reduce the availability of Mg2+ for ATP. Under these conditions it is possible that an increase in Mg2+ between 1 mM and 10 mM may simply be affecting the extent of magnesium-ATP complex formation even though Mg2+ is in great molar excess over ATP. It was found that 10 mM Ca2+ in place of Mg2+ gave approximately 75% of the activity found with Mg2+. Mn2+, Zn2+, and Ca2+ at this concentration did not fulfill the requirement for a divalent cation. Concentrations of Mg2+ up to 10 mM did not reduce the dependence of this enzyme on cyclic AMP for casein phosphorylation. This is in contrast to the report of Huijing and Lamer (33) using a relatively impure glycogen synthetase preparation containing glycogen synthetase kinase now believed to be identical with the cyclic AMP-stimulated protein kinase (5).

Apparent Km for Casein—The effect of varying casein concentration on enzyme activity in the presence and absence of cyclic AMP was studied for Peak II. As was observed when ATP was varied, the concentration needed for half-maximal activity was not affected significantly by the addition of cyclic AMP, whereas maximal velocity was greatly increased. These data are illustrated in Fig. 9. The apparent Km for casein was 0.6 mg per ml in the absence and 0.9 mg per ml in the presence of cyclic AMP. The addition of cyclic AMP increased maximal velocity from 79 to 526 μmoles of 32P incorporated per 10 min.

Inhibition by High Salt—Casein phosphorylation was inhibited approximately 75% by the addition of NaCl, KCl, or potassium phosphate to an ionic strength of 0.25 M. It was postulated that the inhibitory effect of high salt might be mediated by a decreased affinity of the enzyme for the protein substrate and that this phenomenon might be reflected by an increase in apparent Km for that substrate. Fig. 10 depicts a study of the effect of 0.1 M NaCl on the initial reaction velocities with varying casein concentration. A double reciprocal plot (inset) indicates that the NaCl did, indeed, increase the Km for casein by a factor of 6. The deviation that was observed between the value of Vmax found by extrapolating the double reciprocal plots in Fig. 10 and that determined experimentally would appear to indicate some degree of protein substrate inhibition at high salt.

Reversibility of Binding of Cyclic AMP to Protein Kinase—It is reported in an accompanying paper (25) that the cyclic AMP-
interacted with phosphorylase kinase (40). In consideration of this possibility, it seemed advisable to carry out further experiments relating to the question of reversibility of cyclic AMP binding to the protein kinase. Enzyme from Peak I, a 0.25-mg sample which was stimulated 50-fold by cyclic AMP, was incubated for 10 min at 30° with a large excess of 3H-cyclic AMP (5 × 10⁻⁸ M) and 0.5% casein, pH 6.0, in a final volume of 0.6 ml. (The casein was present as a protective protein to help prevent loss of enzyme activity.) A portion of the mixture, 0.2 ml, was then passed through a Sephadex G-25 column (1 × 30 cm) equilibrated with 5 mM potassium phosphate, 1 mM EDTA buffer, pH 6.9, to separate the protein kinase from the 3H-cyclic AMP. The pooled enzyme peak, with a volume of 2 ml, still contained 30 μmoles of 3H-cyclic AMP, as determined by measuring radioactivity, and essentially all of the protein kinase activity (assayed in the presence of cyclic AMP) that had been applied to the column. The enzyme from this fraction, assayed in the absence of any cyclic AMP other than that which would be carried over into the reaction mixture with the enzyme, had only 14% of its maximal activity. This assay was performed at a 1 to 5 dilution of the enzyme fraction, and the concentration of 3H-cyclic AMP carried over under these conditions was 3 × 10⁻⁸ M in the reaction mixture. The activity that was found is what was anticipated at that concentration of cyclic AMP (see Fig. 6). It is clear that the Sephadex treatment resulted in a major separation of 3H-cyclic AMP, which would not have occurred if the nucleotide had been bound covalently. In other experiments, it was shown that all of the cyclic AMP bound to protein kinase fractions could be released and recovered as cyclic AMP when the enzyme was denatured by heat, urea, 0.1 M NaOH, 5% trichloracetic acid, or 32% ethanol. No labeled peptides were found after subjecting mixtures of the protein kinase plus 3H-cyclic AMP to tryptic attack. It has also been demonstrated that during the course of casein phosphorylation cyclic AMP is not destroyed (25).

Sucrose Density Gradient Centrifugation of Peak I and Peak II—Sucrose density gradient centrifugation was employed in order to study the sedimentation behavior of the protein kinase and to gain further information regarding its purity. Fig. 11 illustrates activity and protein profiles for Peak I. As can be seen, the major protein peak was not associated with activity, indicating that this enzyme fraction is still quite impure despite the finding of a single band on electrophoresis (Fig. 4). It is also apparent that Peak I has two active components differing in their sedimentation coefficients. In separate experiments, using phosphorylase b (8.4 S) and hemoglobin (4.1 S) as markers, the sedimentation coefficients of these two components were found to be 6.8 S and 4.9 S. Both of the components were highly dependent on cyclic AMP for activity. To determine if interconversion of the 6.8 S and 4.9 S components occurred, they were separated by sucrose density gradient centrifugation, dialyzed, and reconstituted. The sedimentation pattern of the separated components and the markers used are illustrated in Fig. 12, A and B. It is clear that each retained the same sedimentation coefficient observed initially.

The effect of cyclic AMP on the sedimentation of each of the two protein kinase components from Peak I was studied by adding 10⁻⁴ M cyclic AMP to the sucrose gradients. The enzymes were not equilibrated with cyclic AMP prior to their application to the gradients. In the presence of cyclic AMP each component now sedimented at 3.4 S, as can be seen from the activity and marker profiles illustrated in Fig. 12, C and D. In a
Fig. 12. Sucrose density gradient centrifugation of the separated components from Peak I. Sucrose density gradient centrifugations were performed as described under Fig. 11. A and C, approximately 30 μg of the 6.8 S component from the gradient in Fig. 11 was resedimented. B and D, approximately 15 μg of the 4.9 S component from the gradient in Fig. 11 was resedimented. C and D, the gradients contained 10^{-6} M cyclic AMP. Phosphorylase b and hemoglobin were used as markers in the calculation of the sedimentation constants given in the figure. Fraction volume was 0.3 ml.

Fig. 13. Sucrose density gradient centrifugation of Peak II. Sucrose density gradient centrifugations of Peak II (200 μg) were performed as described in Fig. 11 in the absence (A) and in the presence (B) of 10^{-6} M cyclic AMP. Phosphorylase b and hemoglobin were used as markers. Fraction volume was 0.3 ml.

DISCUSSION

The cyclic AMP-dependent protein kinase activity of rabbit skeletal muscle extract has been shown to be separable into two major peaks by DEAE-cellulose chromatography. In this paper the properties of the protein kinases in these two peaks are compared. Toward the end of this study it was realized that Peak I can be further resolved into two components by density gradient centrifugation, and it was also determined that Peak II is often contaminated with traces of a cyclic AMP-independent protein kinase. Under these circumstances it is somewhat arbitrary to treat Peaks I and II as entities, but it was deemed desirable and useful, nevertheless, to report the results at this stage in the development of the problem. It is obvious that many properties will have to be redetermined when each of the protein kinases is available in the pure form.
In general, the protein kinases of Peaks I and II were remarkably similar. The pH dependence of casein and histone phosphorylation did not differ greatly for the two enzyme fractions, except that preliminary incubation of Peak I with casein at low pH or with histone decreased the dependence of this enzyme on cyclic AMP as was discussed above. The concentration of cyclic AMP needed for half-maximal stimulation and the degree of cooperativity exhibited by this activator were nearly identical for the two enzymes. Likewise the apparent \( K_m \) for ATP was the same for both enzymes in the presence or absence of cyclic AMP. The two peaks exhibited very similar behavior toward a number of different cyclic nucleotides except for a greater response of Peak I to dibutyryl cyclic AMP and a slightly lower response to cyclic TMP. Although only two concentrations of the cyclic nucleotides were used in this study, the similar response of the two peaks at each concentration suggests that the two enzymes have the same Michaelis constants for any given cyclic nucleotide tested. Furthermore, the degree of stimulation by different cyclic nucleotides and the apparent \( K_m \) for ATP and cyclic AMP of Peak I and Peak II are in good agreement with values published previously for the enzyme prior to its resolution into two peaks (16). As is reported in detail elsewhere (5), Peaks I and II both phosphorylated casein, histone, glycogen synthetase, and phosphorylase kinase. Furthermore, the relative rates of phosphorylation of these four substrates at the concentrations tested were the same for the two enzymes. Although the possibility remains that under different conditions, e.g. protein substrate concentrations, a difference between the two enzymes may be observed, it appears that they do not differ in substrate specificity. No evidence was obtained that the protein kinases in Peaks I and II were interconvertible in vitro.

The properties of cyclic AMP-stimulated protein kinases from a number of tissues have now been reported. Although identical assay conditions were not used in studying these enzymes, it is of interest to compare some of their reported properties. In general, the enzymes from heart (12), adipose tissue (9), liver (30), brain (10), and trout testis (8), as well as the one from rabbit skeletal muscle (5), phosphorylate histones more readily than casein. This is true despite the fact that in some cases mixtures of histones and in others specific histone fractions were used. An exception to this generality is the observation that the cyclic AMP-stimulated protein kinases from some lower species phosphorylate casein more readily than histone (15). The ability of these protein kinases to phosphorylate phosphorylase kinase and glycogen synthetase has not been widely tested. However, in those instances in which such studies have been made, namely the enzymes from rabbit skeletal muscle (5), bovine heart (12), and rat epididymal fat pad, phosphofructokinase of rabbit skeletal muscle phosphorylase kinase and glycogen synthetase did occur. It appears, therefore, that these enzymes in general exhibit a rather broad specificity toward protein substrates. Only limited kinetic data concerning apparent \( K_m \) values for the protein substrates of the protein kinases are available. Miyamoto, Kuo, and Greengard (10) have reported that half-maximal activity of the brain protein kinase was achieved at approximately 100 \( \mu \)g of histone per ml. They concluded that cyclic AMP had no significant effect on the apparent \( K_m \) for histone. Brostrom et al. (12) likewise found no effect of cyclic AMP on the apparent \( K_m \) for casein, using the heart protein kinase. No effect of cyclic AMP on the apparent \( K_m \) for casein was also observed for the muscle protein kinase (16) as is reported here in more detail.

The effect of ATP concentration on protein kinase activity has been reported for the protein kinases from bovine heart (12) and brain (10). The heart enzyme exhibited an apparent \( K_m \) of 5.0 \( \times 10^{-5} \) M in the presence or absence of cyclic AMP. The brain enzyme exhibited an apparent \( K_m \) of 1.3 \( \times 10^{-4} \) M in the presence of cyclic AMP, but in the absence of cyclic AMP Michaelis-Menten kinetics were not followed. The value of 1.3 \( \times 10^{-4} \) M agrees well with the value of 1.1 \( \times 10^{-4} \) M reported in this publication for Peak II in the presence of cyclic AMP and for Peak I in the presence or absence of cyclic AMP. The results presented here are at variance with those from some other workers (33) in two respects. They reported that the apparent \( K_m \) values for ATP were much higher and that cyclic AMP reduced the \( K_m \) for ATP. The reason for these differences is not clear.

Cyclic nucleotides other than cyclic AMP have previously been reported to stimulate several protein kinases (9,12-15). In addition, less purified systems presumably containing protein kinases have been tested for their response to different cyclic nucleotides (37,38). Stimulation of the protein kinase from rabbit skeletal muscle by cyclic nucleotides has been reported by Schlender, Wei, and Villar-Palasi (39) using glycogen synthetase as a substrate and by Soderling et al. (5) using both phosphorylase kinase and glycogen synthetase as substrates. Except for an apparently greater sensitivity to low concentrations of the cyclic nucleotides other than cyclic AMP in the latter report, the results are similar to those reported here. In general, studies with cyclic nucleotides other than cyclic AMP have shown that cyclic AMP is the most effective at low concentrations. This is not surprising considering the high degree of structural similarity between cyclic AMP and cyclic AMP. Cyclic GMP has also been found to stimulate protein kinases, but in general much higher concentrations are needed than for cyclic AMP. An example is the cyclic GMP-stimulated protein kinase recently reported by Kuo and Greengard (30). This enzyme has a lower apparent \( K_m \) for cyclic GMP than for cyclic AMP.

Since cyclic AMP mediates the effects of many different hormones (31) it is possible that these effects are mediated in turn by protein phosphorylation. There is now evidence that in addition to stimulating the phosphorylation of phosphorylase kinase (40) and glycogen synthetase (41) in muscle, cyclic AMP may also stimulate phosphorylation of a number of other potentially physiological protein substrates such as a hormone sensitive lipase in adipose tissue (42,43), histone in liver (7), pyruvate dehydrogenase in heart (44), proteinase in trout testis (8), phosphorylase phosphatase in muscle (45), and RNA polymerase in Escherichia coli. It would not be unreasonable to expect that different protein kinases are responsible for phosphorylating different protein substrates, but the apparent lack of specificity of protein kinases suggests that this may not be the case. Furthermore, in skeletal muscle it seems quite clear that there are not different enzymes responsible for activating phosphorylase kinase and inactivating glycogen synthetase (5).

Activation of this enzyme by cyclic AMP can be classified as a "V" system activation as defined by Monod, Wyman, and Changeux (48), since cyclic AMP increased the maximum veloc-
ity without affecting the $K_a$ for the substrates. According to the model, cooperativity in these systems is expected only for the activator and not for the substrates. These are exactly the observations we have made, i.e. cooperativity was observed for cyclic AMP, whereas casein and ATP obeyed Michaelis-Menten kinetics. The mechanism by which cyclic AMP affects the change in catalytic efficiency of the enzyme appears to involve dissociation of the enzyme, since a decrease in sedimentation coefficient was observed in the presence of cyclic AMP. Either the dissociation of a regulatory subunit, which binds cyclic AMP, from a catalytic subunit, or the dissociation of the enzyme into identical subunits, could be involved in activation of the enzyme. A model for the mechanism of action of cyclic AMP on the protein kinase from bovine heart has been proposed (12). This model involves dissociation of the protein kinase into a regulatory subunit and a catalytic subunit in the presence of cyclic AMP. In the absence of cyclic AMP, the regulatory, i.e. cyclic AMP-binding, subunit binds to the catalytic subunit thereby reducing its activity. The fact that both components of Peak I as well as Peak II sediment at essentially the same rate in the presence of cyclic AMP suggests that they may have a common kinase subunit and that they differ primarily in the number or type of regulatory subunits. Recently Gill and Garren (13) have reported a partial resolution of the catalytic and cyclic AMP binding activities of the protein kinase from adrenal cortical tissue. They have also interpreted their data as fitting the model described above. In an even more recent report, Tao, Salas and Lipmann (47) have shown that the protein kinases from erythrocytes separate into different catalytic and cyclic AMP-binding subunits in the presence of cyclic AMP.4

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REFERENCES


4 In recent work from this laboratory (E. M. Reimann, C. O. Brostrom, J. D. Corbin, C. A. King, and E. G. Krebs, unpublished results) the skeletal muscle protein kinase has been separated into distinct regulatory and catalytic subunits. Recombination of the isolated cyclic AMP-binding subunit with the catalytic subunit restores the dependence of the enzyme on cyclic AMP.

Purification and Properties of Rabbit Skeletal Muscle Adenosine 3',5'-Monophosphate-dependent Protein Kinases
Erwin M. Reimann, Donal A. Walsh and Edwin G. Krebs


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