Purification and Characterization of a Protein Inhibitor of Adenosine 3',5'-Monophosphate-dependent Protein Kinases*

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SUMMARY

The partial purification and characterization of a factor from skeletal muscle which inhibits the activity of adenosine 3',5'-monophosphate-dependent protein kinases from skeletal muscle, heart, liver, adipose tissue, and brain is described. The inhibitor is stable to heating at 96° and to precipitation with 5%, w/v, of trichloracetic acid, but is assumed to be a protein since it is inactivated by proteolytic enzymes. It has a molecular weight of 26,000 by gel exclusion and an s20,w of 1.5 by sucrose density gradient centrifugation. A kinetic analysis of the effect of the inhibitor on the phosphorylation of casein by skeletal muscle protein kinase indicates that it acts noncompetitively with respect to ATP, the protein substrate, and adenosine 3',5'-monophosphate. The inhibitor promotes a 5-fold increase in the binding constant of adenosine 3',5'-monophosphate to the protein kinase.

Crude skeletal muscle extracts were found to contain a trypsin-labile inhibitor of the phosphorylase kinase activation reaction (1, 2). This inhibitor was shown by Appleson, Birnbaumer, and Torres (3) to inhibit also the conversion of glycogen synthetase from the I to the D form. This paper describes the purification and characterization of this inhibitor which is shown to act by inhibiting a cyclic AMP-dependent protein kinase, the catalyst of both the phosphorylase kinase activation reaction (4), and the conversion of glycogen synthetase I to glycogen synthetase D (5). The protein inhibitor inhibits cyclic AMP-dependent protein kinases from skeletal muscle, heart (6), adipose tissue (7), liver, and brain (8).

MATERIALS AND METHODS

Assay for Cyclic AMP-dependent Protein Kinase

The cyclic AMP-dependent protein kinase was assayed by the method previously described in a preliminary communication (9). In this method the activity of the kinase was determined from the uptake of phosphate by casein in a reaction at 30° utilizing γ-32P-ATP. The complete incubation mixture contained: sodium glycerol-P, 5 μmoles; casein, 0.6 μg; γ-32P-ATP, 0.12 μ mole; magnesium acetate, 0.36 μ mole; sodium fluoride, 2 μmoles; theophylline, 0.2 μ mole; ethylene glycol bis(β-aminoethyl ether) N,N'-tetraacetic acid, 0.03 μ mole; cyclic AMP, 2 μmoles; and the cyclic AMP-dependent protein kinase in a total volume of 0.1 ml with a final pH of 6.0. The reaction was initiated by the addition of magnesium acetate and γ-32P-ATP and terminated after 10 min by the addition of 0.2 ml of bovine serum albumin (6.25 mg per ml) and 1.5 ml of 67%, w/v, trichloroacetic acid. Protein-bound phosphate was determined as described in an accompanying paper (4).

Assay of Protein Inhibitor

Method 1—The initial assay for the inhibitor was based on its ability to inhibit the stimulation of phosphorylase kinase activation by cyclic AMP which occurs using highly purified preparations of phosphorylase kinase (1, 4, 10). The cyclic AMP effect on the activation reaction is now presumed to be due to the presence of contaminating levels of the cyclic AMP dependent protein kinase in the phosphorylase kinase preparation (4), but this was not known at the time this assay was developed. Nonactivated skeletal muscle phosphorylase kinase (0.3 mg), purified as described previously (10), was incubated for 10 min at 30° in a reaction mixture containing: glycerol-P buffer, 7 μmoles; 2-mercaptoethanol, 20 μmoles; EDTA, 0.2 μmole; magnesium acetate, 0.6 μmole; ATP, 0.2 μmole; cyclic AMP, 10 μmoles; and varying aliquots of inhibitor in a total volume of 1.0 ml and a final pH of 6.8. The reaction was initiated by the addition of phosphorylase kinase and terminated by a 21-fold dilution with cold (0°) 0.01 M glycerol-P buffer, pH 6.8, containing 0.045 M 2-mercaptoethanol. Phosphorylase kinase activity was determined at pH 6.8 by the standard procedure (10). The activation of phosphorylase kinase under these conditions is...
highly dependent on cyclic AMP (4), and addition of the inhibitor blocks the reaction almost completely. An example of a typical standard curve is presented in Fig. 1. This assay system has certain major limitations and drawbacks. Because of variations in the rate of activation of different phosphorylase kinase preparations, presumably due to varying degrees of contamination with the cyclic AMP-dependent protein kinase, and due to changes with time of storage for a single preparation, it was difficult to define accurately a unit of inhibitory activity that would remain constant. Nevertheless, it was possible to use this assay method to define amounts of inhibitor within a given set of experiments. A unit of inhibitory activity, utilizing this assay system, was defined as that amount which would inhibit the activation of phosphorylase kinase 50% under these standard conditions (Fig. 1).

Method 2—An alternate and more reproducible method for the assay of the protein inhibitor became available upon the identification of the effect of the inhibitor on the phosphorylation of casein catalyzed by purified cyclic AMP-dependent protein kinase (9). This method of assay is an adaptation of the assay for the latter enzyme described above. Reaction mixtures contained all of the components as listed, except that the amount of protein kinase was fixed (see below) and varying amounts of inhibitor were included. The amount of cyclic AMP used was 0.4 mM instead of 2 mM, aminophylline was used instead of theophylline, and the total volume was 0.13 ml. Reactions were run for a period of 40 min rather than 10 min, since it was found that the sensitivity of the assay was enhanced by the use of low concentrations of cyclic AMP-dependent protein kinase and compensating for the associated decrease in phosphate incorporation by extending the time of reaction. In a typical assay using a protein kinase solution that would catalyze the incorporation of 65 μmoles of 32P per min into casein under the standard assay conditions, 0 to 0.45 unit of inhibitor (as defined by assay Method 1) caused a linear decrease in incorporation from 0 to 60% with a standard deviation between 16 independent assays of less than ±5%. A standard assay curve, typical of that obtained, is presented in Fig. 7 under "Results." All activities of inhibitor presented in this paper have been calculated by comparison to the units defined as in Method 1.

Materials Used

Proteolytic enzymes and standard proteins were obtained from the following commercial sources: trypsin, twice crystallized; chymotrypsin, crystallized three times; ribonuclease, crystallized once, Worthington; lysozyme, crystallized once, Pentex; phospholipase C, myoglobin, crystallized once, Nutritional Biochemicals; soy bean trypsin inhibitor; pepsinogen, ovalbumin, Sigma; and cytochrome c, Calbiochem. Histone, Fraction Fb, was prepared by the method of Johns (11). DEAE cellulose (Sigma) was washed with 0.5 N sodium hydroxide, 0.5 M sodium chloride before equilibration in the appropriate buffer. The method for purifying the cyclic AMP-dependent protein kinase was in the course of development at the same time that the present study was being carried out, and some of the experiments described herein were performed utilizing fractions from intermediate stages of the purification procedure or minor modifications thereof, instead of the most extensively purified fraction (8, 12). The experiments of Figs. 9 and 10 utilized material that had not been subjected to the chromatography on DEAE-cellulose (DE32) or Sephadex G-200. The experiment of Fig. 11 utilized the protein that had been purified to the stage of elution from calcium phosphate gel of the modified purification procedure (12). The experiments of Figs. 6, 7, 8, 12, 13, and Table II utilized the latter enzyme that had been further purified by chromatography on a column of Sephadex G-200 equilibrated in 10 mM Tris buffer, pH 7.5, 1 mM EDTA. The specific activity of the latter was 1 × 10^6 μmoles per min per mg of protein (12). All other chemicals and methods used in this study are as described elsewhere (4, 9).

RESULTS

Purification of Inhibitor from Rabbit Skeletal Muscle—The purification of the inhibitor was greatly aided by its heat stability. The procedure that has been utilized for its partial purification is as follows. The supernatant solution from the pH 6.1 acid precipitation step of a standard phosphorylase kinase preparation (10) served as the source of the protein. This solution, obtained from a preparation utilizing 2.4 kg of rabbit skeletal muscle, was adjusted to pH 7.0 by the addition of 6 M ammonium hydroxide. The neutral solution (5,500 ml) was heated in a stainless steel bucket with vigorous stirring and brought to 95° over a 20-min period. The resultant suspension was cooled to 10° in an ice bath and then filtered through cheesecloth and filter paper (Eaton Dikeman No. 615). By the addition of a cold solution of 100%, w/v, of trichloracetic acid the heat filtrate was adjusted to 15%, w/v, with respect to the acid, and the precipitate was separated by centrifugation at 10,000 × g for 20 min. The precipitate, which contained the inhibitor protein, was resuspended in 25 ml of 0.05 M glyceral-P buffer, pH 6.8, containing 0.002 M EDTA, by homogenization in a Potter-Elvehjem homogenizer. During this procedure the pH was maintained at 6.8 by the addition of 6 M ammonium hydroxide. The resultant turbid solution was dialyzed extensively against three changes (12 liters each) of 0.005 M potassium phosphate buffer, pH 7.0, containing 0.001 M EDTA. The suspension obtained by this procedure was centrifuged at 34,000 × g for 20 min. The supernatant solution containing the inhibitor could be stored at −15°.
Fig. 2 (left). Elution profile of the chromatography of the protein inhibitor on Sephadex G-75. The elute from a column of DEAE-cellulose of the protein inhibitor (Fraction 4) prepared from 4.8 kg of rabbit skeletal muscle as described in the text was subjected to electrophoresis on polyacrylamide gel (5%). The column was monitored for absorbance at 235 nm (●), and for inhibitor activity (○) by Method 1.

DF indicates the dye front marked by India ink. An equivalent gel was sliced, the fractions were eluted with 5 mM glycerol–P buffer, pH 6.8, containing 0.003 M EDTA and were then assayed for inhibitor activity (Method 1). Only the fraction marked by an arrow contained activity.

For the final steps in the purification, the supernatant solution from two separate preparations utilizing 2.4 kg of muscle each were pooled. The combined solutions were adjusted to pH 5.0 with 0.1 M acetic acid and the resultant suspension was clarified by centrifugation at 34,000 × g for 10 min. The clarified solution was then chromatographed on a column of DEAE-cellulose (1.25 × 24 cm) equilibrated with 0.005 M sodium acetate buffer, pH 5.0, containing 0.001 M EDTA, and the column was then developed with a linear gradient (1,000 ml) of sodium acetate buffer, pH 5.0, between 0.005 M and 0.3 M. The eluate was monitored by absorption at 235 nm. The fraction containing the inhibitor was eluted at 0.14 M sodium acetate buffer, well separated from the bulk of ultraviolet absorbing material. The pooled fractions of inhibitor protein were dialyzed against 5 × 10⁻⁴ M glycerol–P buffer, pH 7.0, containing 2 × 10⁻⁴ M EDTA. This solution was concentrated by lyophilization, the resultant powder was suspended in a minimal volume of water (approximately 2 ml), and the solution was clarified by centrifugation. The inhibitor solution was chromatographed on a column of Sephadex G-75 (1.25 × 26 cm) equilibrated in 0.005 M Tris chloride buffer, pH 7.5, containing 0.001 M EDTA, the elution profile of which is shown in Fig. 2. The fractions contained in the inhibitor were pooled and stored at −15°C with little loss in activity. A summary of the purification procedure is given in Table I. The purified protein had a specific activity of approximately 2 × 10⁴ units of inhibitor activity per mg of protein determined by the method of Lowry et al. (13). The inhibitor was purified 760-fold with respect to units of activity per mg of protein with a 34% yield. The final purification step of gel filtration did not increase the specific activity (Table I) but did separate contaminating ultraviolet absorbing material (Fig. 2). The purified inhibitor or fractions from earlier stages in the preparation was used for the studies of this report. The final preparation exhibited two major bands of protein on disc gel electrophoresis, the minor of which was demonstrated to be the inhibitor (Fig. 3).

Characterization of Inhibitor—The activity of the inhibitor was destroyed by treatment with low concentrations of either trypsin or chymotrypsin (Table II) but was not affected significantly by RNase, DNase, lysozyme, or phospholipase, even after incubation for 24 hours at 30°C at high concentrations of the latter hydrolytic enzymes. From this behavior, together with its precipitability by trichloroacetic acid and its staining by Amido Schwarz following disc gel electrophoresis, the inhibitor has been tentatively identified as a protein. The protein inhibitor was found to have a molecular weight of 26,000 as determined by the gel filtration method of Whitaker (15) (Fig. 4). Sucrose gradient sedimentation of the protein inhibitor according to the method of
Effect of hydrolytic enzymes on activity of protein inhibitor

Purified inhibitor (0.37 mg, 2 × 10^4 units per mg, Fraction 5) was incubated at 30º in 0.05 M glycerol-P buffer, pH 7.5, containing 0.002 M EDTA with the various hydrolytic enzymes. In addition, the incubation mixture with phospholipase C and chymotrypsin contained 0.05 M CaCl₂ and that with DNase contained 0.05 M magnesium acetate. The incubation with trypsin and chymotrypsin were terminated by the addition of a 300-fold excess of soy bean trypsin inhibitor and 1 × 10^-4 M phenylmethylsulfonylfluoride, respectively. Inhibitor activity was determined by Method 1.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Ratio of inhibitor to enzyme</th>
<th>Time of incubation (hrs)</th>
<th>Activity% remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td>Trypsin</td>
<td>100:1</td>
<td>3/5</td>
<td>0</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>100:1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>RNase</td>
<td>1:1</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td>DNase</td>
<td>1:1</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>1:1</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td>Phospholipase C</td>
<td>10:1</td>
<td>24</td>
<td>80</td>
</tr>
</tbody>
</table>

*Activity expressed as a percentage of that at zero incubation time.

FIG. 5. The determination of the sedimentation coefficient of the protein inhibitor by sucrose gradient ultracentrifugation. The sedimentation coefficient was determined essentially as described by Martin and Ames (21). Sedimentation was performed at 3º in a Beckman SW 65 rotor for 18 hours in a continuous sucrose gradient between 5% and 20% sucrose in 0.05 M Tris hydrochloride buffer, pH 7.5, containing 0.2 mM EDTA. Standard proteins were detected by absorption at 280 nm and the inhibitor was assayed by Method 2. The sedimentation coefficient (s_{20,w}) of standard proteins were: myoglobin, 1.97 (17); papain, 2.42 (22); pepsin, 2.88 (19).

Martin and Ames (21) gave an s_{20,w} = 1.5 (Fig. 5). This sedimentation value is significantly lower than that characteristic of a simple globular protein with a molecular weight of 26,000 and a partial specific volume of 0.74 (20). This divergence might be explained if the inhibitor has a bound carbohydrate or lipid moiety.

Inhibition of Skeletal Muscle Cyclic AMP-dependent Protein Kinase—The initial studies showed that the protein inhibitor blocked the cyclic AMP-stimulated activation of phosphorylase kinase (2, 4) and this effect was originally used in the purification study as the basis for an assay (Method 1, Fig. 1). In concurrent studies it became established that the cyclic AMP-dependent activation of phosphorylase kinase was catalyzed by an enzyme that was present in skeletal muscle and could be obtained free of phosphorylase kinase (9). This new enzyme was referred to as a cyclic AMP-dependent protein kinase on the basis of its fairly broad substrate specificity in the phosphorylation of proteins by γ-32P-ATP (9). The experiment of Fig. 6 shows that the inhibi-
CASEIN CONCENTRATION [mg/ml]^{-1}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig8.png}
\caption{Double reciprocal plots of initial velocity of the phosphorylation of casein by cyclic AMP-dependent kinase concentration versus casein concentration at constant levels of the inhibitor protein. The initial velocities of reaction were determined utilizing the standard assay system for cyclic AMP-dependent protein kinase described in the text with the exception of varying the concentration of casein as shown. The latter assay utilizes a protein kinase activity that was approximately 4-fold higher than that of the experiments presented in Figs. 6, 7, 9, and 10. The concentrations of inhibitor (Fraction 5) used in the reaction were: \(\Delta\rightarrow\Delta\), none; \(\circ\rightarrow\circ\), 13.2 units per ml; and \(\bullet\rightarrow\bullet\), 26.4 units per ml.}
\end{figure}

A kinetic analysis of the effect of the protein inhibitor on the catalysis of casein phosphorylation by cyclic AMP-dependent protein kinase indicates the reaction to be noncompetitive with respect to the two substrates, casein (Fig. 8) and ATP (Fig. 9), and to the activator molecule, cyclic AMP (Fig. 10). The protein inhibitor does not act by the destruction of cyclic AMP. This is demonstrated in the experiment presented in Table III in which it is shown, that in the presence of sufficient inhibitor to block greater than 95% of the protein kinase-catalyzed phosphorylation of casein, cyclic AMP can be completely recovered at the termination of the incubation period. Under the conditions of the experiment presented in Table III, theophylline, an inhibitor of phosphodiesterase, was present as a standard component of the protein kinase assay reaction. Additional experiments, performed in the absence of the methylated xanthine derivative, failed to show the presence of any phosphodiesterase activity in the inhibitor protein preparation.

The inhibitor does not function by reversal of the phosphorylation reaction. This is shown by the experiment presented in

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig9.png}
\caption{Double reciprocal plots of the initial velocity of the phosphorylation of casein by cyclic AMP-dependent protein kinase concentration versus ATP concentration at constant levels of the inhibitor protein. The initial velocities of reaction were determined utilizing the standard assay for the protein inhibitor (Method 2) with the exception that Mg\(^{2+}\) concentration was 10 mM. The concentrations of inhibitor (Fraction 5) used in the reaction were: \(\circ\rightarrow\circ\), none; \(\bullet\rightarrow\bullet\), 2.1 units per ml; \(\circ\rightarrow\circ\), 4.2 units per ml, respectively.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig10.png}
\caption{Double reciprocal plots of the initial velocity of the phosphorylation of casein by cyclic AMP-dependent protein kinase concentration versus cyclic AMP concentration at constant levels of the inhibitor protein. The initial reaction velocity was determined utilizing the standard assay for the protein inhibitor (Method 2). The concentrations of inhibitor used were: \(\circ\rightarrow\circ\), none; \(\bullet\rightarrow\bullet\), 2.1 units per ml; \(\circ\rightarrow\circ\), 4.2 units per ml, respectively.}
\end{figure}
TABLE III
Recovery of cyclic AMP in reaction of protein kinase blocked by protein inhibitor

The activity of the protein kinase was determined in the standard assay system described under "Materials and Methods" utilizing "H-cyclic AMP in the presence or absence of inhibitor (Fraction 5). At the termination of the reaction (20 min), the solution was deproteinized and the content of "H-cyclic AMP determined following separation of the nucleotides by paper chromatography on Whatman No. 1 paper, utilizing the solvent system of Levey and Epstein (23).

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Amount of protein added</th>
<th>3H-AMP incorporated into casein</th>
<th>Cyclic AMP</th>
<th>AMP formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein kinase</td>
<td>Inhibitor</td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>1</td>
<td>3.3 g</td>
<td>0</td>
<td>489</td>
<td>200</td>
</tr>
<tr>
<td>2</td>
<td>3.3 g</td>
<td>1</td>
<td>200</td>
<td>208</td>
</tr>
</tbody>
</table>

Effect of Inhibitor on Binding of Cyclic AMP to Skeletal Muscle Protein Kinase—The binding of cyclic AMP to the partially purified preparation of cyclic AMP-dependent protein kinase was examined by the gel filtration method of Hummel and Dryer (24) (Fig. 12). At a nucleotide concentration of $1 \times 10^{-7} M$, the 200-fold purified preparation of cyclic AMP-dependent protein kinase bound 1 mole of cyclic AMP per $2 \times 10^6$ g of protein. No binding of the cyclic nucleotide to the purified inhibitor could be demonstrated. In the presence of a level of inhibitor sufficient to completely inhibit the activity of the amount of cyclic AMP-dependent protein kinase used, the binding of cyclic AMP to the latter enzyme was increased to a level of 1 mole of cyclic AMP per $1.6 \times 10^6$ g of protein. The apparent effect of the inhibitor on cyclic AMP binding was examined in more detail over a range of cyclic AMP concentrations of $1 \times 10^{-7}$ to $5 \times 10^{-4} M$. In this experiment the amount of protein kinase used at each of the

Fig. 12. Sephadex G-50 elution profiles used for the measurement of binding of "H-cyclic AMP to the protein kinase in the presence of the protein inhibitor. The experiments were performed essentially by the method of Hummel and Dryer (24). The column of Sephadex G-50 (24.1 cm) was equilibrated with 0.001 m glycerol phosphate buffer, pH 6.0, containing 0.001 m EDTA and $1 \times 10^{-7} M ^{3}H$-cyclic AMP (specific activity, 1.31 $\times 10^8$ cpm per pmole). To separate columns were applied the following protein solutions, equilibrated in the column buffer: O---O, 1 mg of protein kinase in 2 ml; O---O, 0.20 mg of inhibitor (Fraction 5) in 1 ml, 0.20 mg of inhibitor, and 1 mg of protein kinase in 2 ml, 0.20 mg of inhibitor in 1 ml in successive additions; B---B, 0.6 mg of inhibitor in 4 ml. The amount of inhibitor (0.20 mg) applied concomitantly with 1 mg of protein kinase was sufficient to inhibit greater than 95% of the activity of the enzyme, as determined by the standard protein kinase assay.

Effect of Inhibitor on Binding of Cyclic AMP to Skeletal Muscle Protein Kinase—The binding of cyclic AMP to the partially purified preparation of cyclic AM-dependent protein kinase was examined by the gel filtration method of Hummel and Dryer (24) (Fig. 12). At a nucleotide concentration of $1 \times 10^{-7} M$, the 200-fold purified preparation of cyclic AMP-dependent protein kinase bound 1 mole of cyclic AMP per $2 \times 10^6$ g of protein. No binding of the cyclic nucleotide to the purified inhibitor could be demonstrated. In the presence of a level of inhibitor sufficient to completely inhibit the activity of the amount of cyclic AMP-dependent protein kinase used, the binding of cyclic AMP to the latter enzyme was increased to a level of 1 mole of cyclic AMP per $1.6 \times 10^6$ g of enzyme. The apparent effect of the inhibitor on cyclic AMP binding was examined in more detail over a range of cyclic AMP concentrations of $1 \times 10^{-7}$ to $5 \times 10^{-4} M$. In this experiment the amount of protein kinase used at each of the

* The binding of cyclic AMP to the protein kinase is noncovalent (12).
sufficiently sensitive to detect any small deviation from linearity in the double reciprocal plots. It is questionable whether the method used in the direct binding studies (Fig. 13) would be adequate for the amount of inhibitor applied concomitantly with the protein kinase in the absence of the inhibitor determined from a least square evaluation of the reciprocal plot was 1.4 \times 10^{-8} \text{ M}, the intercept on the ordinate indicates a maximum binding of 1 mole of cyclic AMP per 1.9 \times 10^8 g of the partially purified protein kinase. In the presence of the inhibitor the binding of cyclic AMP to the protein kinase was increased at all concentrations of nucleotide examined, the maximum effect observed being a 3-fold stimulation at a nucleotide concentration of 5 \times 10^{-4} \text{ M}. The slight difference in maximum binding observed in Fig. 13 is not considered significant. In the presence of the inhibitor the binding constant of cyclic AMP for the protein kinase is increased j-fold to 2.8 \times 10^{-10} \text{ M}, and 5 \times 10^{-9} \text{ M}, respectively. In the absence of cyclic AMP the quantity of protein kinase was preserved in Fig. 13 is not considered significant. In the presence of the inhibitor the binding constant of cyclic AMP for the protein kinase was increased j-fold to 2.8 \times 10^{-10} \text{ M}, and 5 \times 10^{-9} \text{ M}, respectively. In the absence of inhibitor the amounts of inhibitor used were 0.2, 0.14, 0.048, and 0.048 mg, respectively. In control experiments no binding of cyclic AMP to the inhibitor could be detected over the entire nucleotide concentration used. The preparations of inhibitor and protein kinase were identical with those used in the experiment given in Fig. 12.

Inhibitor

<table>
<thead>
<tr>
<th>Inhibitor Units</th>
<th>Activity in the Absence of Cyclic AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>80</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
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<tr>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

Effect of Inhibitor on Cyclic AMP-dependent Protein Kinases from Different Tissues—The effect of the inhibitor on cyclic AMP-dependent protein kinase from a number of different tissues was examined. Tissues from the rabbit were excised and homogenized in 5 volumes of 0.01 Tris chloride buffer, pH 8.0, containing 2 \text{ mM EDTA}. The homogenate was centrifuged for 30 min at 14,000 \times g and the resultant supernatant, after filtration through glass wool, was used as a source of the cyclic AMP-dependent protein kinases. The effect of the protein inhibitor on the activities of these preparations is presented in Fig. 14. These were examined under conditions in which the initial rates of reactions were linear for at least 15 min. This necessitated dilutions for
the assay of 1:40, 1:40, 1:40, 1:50, and 1:16, respectively, for the extracts of brain, heart, muscle, liver, and adipose tissue. These differing dilutions reflect to some extent the differing cyclic AMP-dependent protein kinase activities of these tissues, which were determined in this experiment to be 1.70, 1.76, 0.75, 1.81, and 0.25 amoles of $^{32}$P incorporated per g of tissue, respectively, under the assay conditions described in the legend of Fig. 14. Cyclic AMP stimulated the reactions 2.7-, 3.0-, 2.0-, 3.1-, and 2.5-fold, respectively, for these tissues under these conditions. The protein inhibitor inhibited the activities of all five enzymes examined (Fig. 14). In all cases the inhibitor completely blocked the cyclic AMP stimulation. A slight effect of the inhibitor was detected on the protein kinase activities occurring in the absence of the cyclic AMP. The purified inhibitor was essentially equally effective for all the enzymes tested. As a corollary to this experiment, it was shown that the inhibitor was present in all the tissues utilized in the experiment of Fig. 14 and in a wide range of other tissues. The highest levels were detected in muscle and brain.

### DISCUSSION

Initial observations (1, 2) on the protein inhibitor isolated from skeletal muscle indicated that it inhibited the activation of phosphorylase kinase. These preliminary studies were then extended by Appleman et al. (3) who showed that it also effectively blocked the conversion of glycogen synthetase I to the D form. An understanding of these observations has since come from the identification of the cata
ylases of the phosphorylase kinase activation and glycogen synthetase inactivation reactions. The former reaction is comprised of at least two elements (4). One is a autophosphorylation reaction catalyzed by phosphorylase kinase itself, the second is catalyzed by a cyclic AMP-dependent protein kinase. Only the latter activation process requires cyclic AMP and is also the only reaction that is blocked by the inhibitor. The conversion of glycogen synthetase I to the D form is catalyzed by the same protein kinase (5) which as a consequence explains the effect of the protein inhibitor on this reaction. Use of the protein inhibitor was, in fact, instrumental in the initial elucidation of these processes (4, 5).

The biochemical mechanism of action of the protein kinase inhibitor remains to be established. Interaction between it and cyclic AMP has been ruled out as a possible mode of action. The inhibitor possesses no cyclic AMP phosphodiesterase activity, and cyclic AMP can be recovered from a protein kinase reaction mixture that has been completely inhibited by its addition. The inhibitor does not bind cyclic AMP. The inhibitor is equally effective in the protein kinase reaction mixture independent of the use of histone, casein, phosphorylase kinase, or glycogen synthetase as substrate and does not act as an alternate protein substrate. The inhibitor does not inhibit pyruvate kinase, phosphofructokinase, or phosphorylase kinase (3), nor does it interfere with the phosphorylation of phosphorylase kinase that occurs concurrently with autoactivation (4); thus the protein inhibitor does not function in the destruction of ATP. The remaining possible site of action of the protein inhibitor is by a direct interaction with the cyclic AMP-dependent protein kinase. Indirect evidence for such an interaction was obtained from the observation that in the presence of the inhibitor, the affinity of cyclic AMP to the protein kinase is increased 5-fold. No evidence is currently available to elucidate this apparent anomalous behavior; comparison can be drawn, however, to the work of Gerhart and Schachman (25) with aspartyltranscarbamylase. These workers have shown that upon dissociation of the catalytic and regulatory subunits of this enzyme, the maximum velocity of the enzyme per unit of active site is enhanced. By analogy, therefore, the inhibitor may function to dissociate the potential subunits of the protein kinase resulting in an increase in affinity of cyclic AMP; however, the dissociated form of the enzyme would be inactive in the presence of the inhibitor.

The physiological significance of the protein inhibitor remains to be elucidated. The inhibitor, which is present in many tissues, blocks the activity of cyclic AMP-dependent protein kinases from all tissues so far examined. It can be calculated from the data presented in the accompanying paper (4) that the level of inhibitor in resting skeletal muscle would be sufficient to inactive approximately one-sixth of the total cyclic AMP-dependent protein kinase present in the tissue. Fluctuations in either the cyclic AMP-dependent protein kinase or the protein inhibitor could thus have a marked effect on the over-all metabolism of this tissue. Future work is, however, needed to determine that it actually functions in this role in vivo. Even in the absence of an in vivo function it exists as a potentially powerful tool for the elucidation of cyclic AMP functions. Its utilization (4) led to the discovery of cyclic AMP-dependent protein kinase in skeletal muscle which is currently serving as the model for cyclic AMP function in other tissues.

### REFERENCES


*The existence of catalytic and regulatory subunits of protein kinase is discussed in the accompanying manuscript (12).*


Purification and Characterization of a Protein Inhibitor of Adenosine
3',5'-Monophosphate-dependent Protein Kinases
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