A proenzyme of the protein kinase described in the accompanying paper (Takai, Y., Kishimoto, A., Inoue, M., and Nishizuka, Y. (1977) J. Biol. Chem. 252, 7603-7609) was found in the soluble fraction of rat brain. Upon limited proteolysis by calcium-dependent protease occurring in the same tissue, the proenzyme was converted to an active protein kinase which could phosphorylate five species of histone fractions. Trypsin also catalyzed the conversion. The proenzyme and protease were separated by DEAE-cellulose (DE52) column chromatography. By this procedure the proenzyme was resolved into two components; each was purified further by gel filtration followed by isoelectrofocusing electrophoresis. Both components showed a sedimentation coefficient of about 5.1 with a molecular weight of 7.7 × 10^4 and a Stokes radius of about 42 Å. Upon isoelectrofocusing electrophoresis the proenzyme exhibited heterogeneity with isoelectric points of 4.0 to 5.6. The proenzyme showed no glycogen phosphorylase kinase activity but was always associated with activity to phosphorylate protamine. Ca^{2+}-dependent protease was also purified further by gel filtration followed by DE52 column chromatography. The protease showed a sedimentation coefficient of about 5.8 with a molecular weight of 9.3 × 10^4 and a Stokes radius of about 47 Å, and an isoelectric point of 4.8. The protease required a divalent cation almost absolutely; Ca^{2+} was most active and the maximum activity was obtained at 3 mM. Se^{2+} and Mn^{2+} were 24% and 11% as active as Ca^{2+}, respectively, but other cations including Mg^{2+}, Ba^{2+}, Zn^{2+}, and Cu^{2+} were inactive. The optimum pH of the protease was 7.5 to 8.5. The active protein kinase thus produced from the proenzyme in vitro showed a sedimentation coefficient of about 3.9 with a molecular weight of 5.1 × 10^4 and a Stokes radius of about 38 Å. Although these values were slightly different from those of the protein kinase which was obtained from bovine cerebellum stored frozen (see the accompanying paper), the active protein kinases were indistinguishable from each other in their kinetic and catalytic properties. A preliminary survey revealed that such proenzyme and protease were distributed in many other tissues including lung, liver, kidney, cerebellum, heart, skeletal muscle, and adipose tissue.

In the accompanying paper (1) a new species of protein kinase has been described which is partially purified from bovine cerebellum. The enzyme is independent of cyclic nucleotides, and is separable from both cyclic AMP-dependent and cyclic GMP-dependent protein kinases in the same tissue. The enzyme is not the catalytic subunit of cyclic AMP-dependent protein kinase, and distinguishable from the catalytic fragment of cyclic GMP-dependent protein kinase which may be produced by trypsin in vitro (2). Nevertheless, the enzyme phosphorylates histone and protamine. In this paper evidence will be presented indicating that the enzyme is produced from its proenzyme by a proteolytic reaction which is catalyzed by calcium-dependent protease occurring in the same tissue. Partial purification and properties of the proenzyme and protease will be described. For the present studies rat whole brain was employed instead of bovine cerebellum, since fresh tissues can be obtained immediately after sacrifice of the animals. The protein kinase previously obtained from bovine cerebellum (1) is tentatively referred to in this paper as protein kinase M, and cyclic AMP-dependent and cyclic GMP-dependent protein kinases as protein kinase A and protein kinase G, respectively.

**EXPERIMENTAL PROCEDURES**

**Materials**—Male Sprague-Dawley rats, weighing 200 to 250 g, maintained ad libitum on CLEA laboratory chows were employed. The animals were decapitated, tissues and organs were quickly removed, and homogenized in a Potter Elvehjem Teflon glass homogenizer with 3 volumes of 20 mM Tris/HCl at pH 7.5 containing 0.25 mM sucrose, 2 mM EDTA, and 10 mM EGTA. The homogenate was centrifuged for 90 min at 80,000 × g. The supernatant was employed as soluble fraction. All operations were performed at 0-4°C. Bovine cerebellar protein kinase M was prepared by the method described in the accompanying paper (1). Glycogen phosphorylase and glycogen phosphorylase kinase were purified from rabbit skeletal muscle by the method of Fischer and Krebs (3) and Cohen (4).

* The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N′,N′,N′-tetraacetic acid.
respectively. Protein inhibitor was purified from bovine cerebellum by the method of Donnelly et al. (5), and the preparation of the step of DEAE-cellulose (DE52) column chromatography was employed for the present study. The regulatory subunits of rat liver protein kinase A was purified by the method described previously (6). Calf thymus whole histone was prepared by the method of Johns (1971, H1, H2A, and H2B histones were prepared by the method of Oliver et al. (8). H3 and H4 histones were prepared by the method of Johns (9) and further purified as described by Hnilica and Bess (10) and Starbuck et al. (11). Trypsin (type III), soybean trypsin inhibitor (type 1S, lyophilized), salmon sperm protein (grade I, histone-free), bovine serum albumin, heparin, yeast cytochrome c (9), and various cyclic nucleotides were obtained from Sigma. Bovine serum albumin (Hammarsten) and egg yolk phosvitin were obtained from Merck AG-Darmstadt and Mann, respectively. Human γ-globulin (Fraction II) and ovalbumin (twice recrystallized) were from Nutritional Biochemicals. γ-32P-ATP was prepared by the method of Glynn and Chappell (12). Other materials were obtained from commercial sources.

Enzyme Assay—The protein kinase produced from the proenzyme was routinely assayed at 75 mM Mg2+ with 100 μg of whole histone as substrate under the conditions employed for protein kinase M (1) except that incubation was made for 5 min. Proteolysis was assayed with 100 μg of protease as substrate under the same conditions except that 5 mM Mg2+ was used and that the reaction was stopped by ice cold 10% trichloroacetic acid and the precipitate was collected on a Toyo Roshi membrane filter (pore size, 0.45 μ). Protein kinases A and G were assayed at 5 mM and 100 mM Mg2+, respectively, with 100 pg of whole histone as substrate as described previously (13).

The proenzyme was quantitated by measuring the formation of an active protein kinase after preincubation with either trypsin or Ca2+-dependent protease. The preincubation mixture (0.1 ml) contained 1 μmol of Tris/HCl at pH 8.0, 0.5 μmol of 2-mercaptoethanol, a fixed amount of either trypsin or Ca2+-dependent protease plus 0.2 μmol of CaCl2, and a proenzyme preparation to be assayed. After preincubation for 30 min for the period indicated in each experiment, 10-μl aliquot of the mixture was taken and immediately assayed for protein kinase in the presence of 75 mM Mg2+ as described above. When trypsin was used for the limited proteolysis, the second incubation mixture contained 2 μg of soybean trypsin inhibitor as an additional ingredient. In the control experiment either trypsin or CaCl2 was omitted during the preincubation, but the protein kinase assay was made under the same conditions as each comparable experiment.

Ca2+-dependent protease was assayed under analogous conditions with an excess amount of the proenzyme and a protease preparation to be assayed. The proteolytic activity was also assayed with casein as substrate under the conditions described by Guroff (14) by measuring the increase of trichloroacetic acid-soluble tyrosine-reacting materials by the method of Waalkes and Udenfriend (15). When trypsin was used for the limited proteolysis, the second incubation mixture contained 2 μg of soybean trypsin inhibitor as an additional ingredient. In the control experiment either trypsin or CaCl2 was omitted during the preincubation, but the protein kinase assay was made under the same conditions as each comparable experiment.

Results

Formation of Protein Kinase in Crude Extract—The soluble fraction of rat brain was preincubated at 30° in the presence of 5 mM Ca2+ under the conditions specified in Fig. 1. After various periods of time an aliquot was taken and protein kinase activity was assayed with whole histone as phosphate acceptor. As shown in this figure, the enzymatic activity measurable at 75 mM Mg2+ was increased linearly but remained unchanged when Ca2+ was omitted from the preincubation mixture. In contrast, the enzymatic activity measurable at 5 mM Mg2+, which was attributed mostly to protein kinase A (1), was not affected significantly by such preincubation with Ca2+. In brain extracts the level of protein kinase G, which was also measurable at 75 mM Mg2+, was extremely low and a similar profile was obtained in the presence of cyclic GMP (1 mM) in the assay mixture (data not shown).

The increase in enzymatic activity at 75 mM Mg2+ was also observed when Ca2+ was replaced by a small quantity of trypsin, and the results of such experiments are shown in Table I. It is likely, therefore, that a protein kinase which is active at 75 mM Mg2+ may be produced from some other precursor molecule by a proteolytic reaction.

Resolution of Proenzyme and Protease—All operations were carried out at 0-4 °C. The brain soluble fraction obtained from 20 g of wet tissue was applied to a DE52 column under the conditions given in the legend to Fig. 2. When each fraction was directly assayed for protein kinase in the presence of 75 mM Mg2+, practically no enzymatic activity was found as shown in Fig. 2A. If, however, each fraction was preincubated with a small amount of trypsin, two peaks appeared. The first and second peaks are tentatively referred to hereafter as proenzyme I and proenzyme II, respectively. Under these conditions protein kinase A was not measurable since higher concentrations of Mg2+ were highly inhibitory (1). Proenzymes I and II were purified further as described below. Next, the protease activity in each fraction was assayed by measuring the increase in protein kinase activity in the presence of Ca2+ and an excess amount of purified proenzyme I. As shown in Fig. 2B, two peaks of the protease appeared; both peaks were markedly stimulated by Ca2+. When the protease was assayed with purified proenzyme II, an essentially similar profile was obtained (data not shown). The first minor peak was not studied here, and the second peak was purified further as described below. Finally, the proenzyme in each fraction was again quantitated using the purified preparation of Ca2+-dependent protease. The results plotted together in Fig. 2A show that the peak obtained in this way exactly coincides with those obtained with trypsin, and that the proenzyme and Ca2+-dependent protease may be separable from each other.

Purification of Proenzyme I—Fractions 9 through 17 in Fig. 2 were pooled and concentrated to 5 ml by an Amicon ultrafiltration cell equipped with PM 10 filter membrane. After centrifugation for 10 min at 20,000 g to remove insoluble materials, the proenzyme (30 mg of protein) was subjected to gel filtration on a Sephadex G-100 column under the conditions described in the legend to Fig. 2A. When each fraction was assayed for proenzyme, a single peak appeared (Fig. 3A). The active fractions were pooled and concentrated to 10 ml by ultrafiltration. The proenzyme (7.1 mg of protein) was then subjected to isoelectrofocusing electrophoresis under the conditions described previously (17) except that the electrophoresis was run at 500 V for 38 h in the presence of carrier ampholytes (pH 5 to 7, 1.5%) and 1 × 10-3 M cyclic AMP. After electrophoresis, fractions of 2 ml each were collected. The proenzyme appeared as a single peak in Fractions 14 through 21 with an isoelectric point of around 5.6. These fractions were pooled, dialyzed overnight against a
Then, the solution was mixed with 2 pg of trypsin inhibitor and protein kinase M except that the incubation was made for 10 min. Assayed for protein kinase at 75 mM Mg+ under the conditions for contained 10 mM Tris/HCl at pH 8.0 and 5 mM 2-mercaptoethanol.

Fractions of 15 ml each were collected. A, lo-p1 aliquot of each fraction was assayed for proenzyme employing either 0.2 pg of trypsin or 7 pg of Ca+ dependent protease under the conditions described in the legend to Fig. 2. B, proenzyme II was subjected to a Sephadex G-200 column (91 x 2.5 cm) equilibrated with Buffer B. Elution was carried out upward with the same buffer at a flow rate of 20 ml/h. Fractions of 0.5 ml each were collected. A 10-p1 aliquot of each fraction was assayed for proenzyme employing either 0.2 pg of trypsin or 7 pg of Ca+ dependent protease under the conditions described in the legend to Fig. 2 except that the active protein kinase produced was assayed for 10-min incubation.

Protein kinase activity after preincubation with (O- - -O) and without (O- - -O) 0.2 pg of trypsin in a solution (0.1 ml) which contained 10 mM Tris/HCl at pH 8.0 and 5 mM 2-mercaptoethanol. Then, the solution was mixed with 2 pg of trypsin inhibitor and assayed for protein kinase at 75 mM Mg+ under the conditions for protein kinase M except that the incubation was made for 10 min. Another 10-p1 aliquot of each fraction was first preincubated for 10 min at 30° with (O- - -O) and without (O- - -O) 5 mM CaCl2 in a solution (0.1 ml) which contained 10 mM Tris/HCl at pH 8.0, 5 mM 2-mercaptoethanol, and 7 pg of purified Ca+ dependent protease.

Then, protein kinase was assayed at 75 mM Mg+ under the conditions for protein kinase M. CaCl2 did not affect protein kinase itself at this concentration. B, A 10-p1 aliquot of each fraction was assayed for Ca+ dependent protease with 0.5 pg of purified proenzyme I under analogous conditions. Protease activity in the presence (O- - -O) and absence (O- - -O) of 5 mM CaCl2 in the preincubation mixture.

large volume of Buffer B containing 20% sucrose, and concentrated to 2 ml (2.8 mg of protein) by ultrafiltration. This preparation was relatively stable and could be stored at 0° for at least 1 month.

**Purification of Proenzyme II** Fractions 25 through 30 in Fig. 2 were pooled and concentrated to 5 ml by ultrafiltration. After centrifugation for 10 min at 20,000 x g, the proenzyme (22 mg of protein) was subjected to gel filtration on a Sephadex G-200 column under the conditions described in the legend to Fig. 3B. When each fraction was assayed for proenzyme, two peaks appeared (Fig. 3B). The first peak was enhanced only slightly by preincubation with Ca+ dependent protease plus Ca+ or with trypsin, whereas the second peak was markedly increased. The active fractions of the second peak were collected and concentrated to 10 ml (6.0 mg of protein). The proenzyme was subjected to isoelectrofocusing electrophoresis as described above except that carrier ampholytes (pH 3.5 to 10, 1.5%) were used. The proenzyme appeared as multiple peaks in Fractions 9 through 21 with isoelectric points of about 4.0 to 5.6. These fractions were pooled, dialyzed against a large volume of Buffer B containing 20% sucrose, and was concentrated to 2 ml (3.0 mg of protein) by ultrafiltration. This preparation was relatively stable and could be stored at 0° for at least 1 month.

It was practically difficult to calculate the exact recovery and purification of proenzymes since the crude extract seemed to contain some unknown materials which interfered with the complete conversion from proenzymes to protein kinase M. Nevertheless, proenzymes I and II were purified at least 40- and 25-fold, respectively, and with an overall recovery of, all together, about 50% starting from the crude extract.
Effect of CaCl₂ and trypsin on protein kinase in rat brain soluble fraction

The soluble fraction of rat brain (310 µg of protein) was preincubated for 5 min at 20°C in a solution (0.5 ml) containing 10 mM Tris/ HCl at pH 8.0, 5% 2-mercaptoethanol, and either CaCl₂ or trypsin as indicated. Then, a 25-µl aliquot of the solution was taken and assayed for protein kinase at 5 or 75 mM Mg²⁺ under the standard conditions except that 10 µg of trypsin inhibitor was added to the assay mixture. CaCl₂ (0.5 mM) carried from the preincubation conditions except that 10 pg of trypsin inhibitor was added as indicated. The numbers represent picomoles of phosphate incorporated into acid-precipitable materials.

<table>
<thead>
<tr>
<th>Preincubation with</th>
<th>At 5 mM Mg²⁺</th>
<th>At 75 mM Mg²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>CaCl₂, 5 mM</td>
<td>102</td>
<td>103</td>
</tr>
<tr>
<td>Trypsin, 0.5 µg</td>
<td>103</td>
<td>103</td>
</tr>
<tr>
<td>Trypsin, 1.0 µg</td>
<td>105</td>
<td>105</td>
</tr>
</tbody>
</table>

Purification of Protease—Fractions 31 through 48 in Fig. 2 were pooled and concentrated to 5 ml by ultrafiltration. After centrifugation for 10 min at 20,000 × g, the protease (56 mg of protein) was subjected to a Sephadex G-200 column (89 × 2.5 cm) equilibrated with Buffer B. Elution was performed upward with Buffer B at a flow rate of 15 ml/h. Fractions of 4.8 ml each were collected. Under these conditions protein kinase, which contaminated the enzyme preparation, appeared as a single peak in Fractions 27 through 36. To detect Ca²⁺-dependent protease, each fraction was preincubated with an excess amount of proenzyme I in the absence of Ca²⁺, and then assayed for protein kinase at 75 mM Mg²⁺. A new peak appeared in Fractions 43 through 53. This peak was not detected when Ca²⁺ was omitted from the preincubation mixture. Similar results were obtained with proenzyme II instead of proenzyme I.

Fractions 43 through 53 (20 mg of protein) were pooled and subjected to a DE52 column (5.2 × 2.2 cm) equilibrated with Buffer B. Then, the column was washed with 200 ml of Buffer B containing 0.1 M NaCl and 1 × 10⁻⁴ M cyclic AMP. By this procedure, protein kinase A, which slightly contaminated the preparation, was dissociated and removed from the column. The protease was subsequently eluted from the column with a 240-ml linear concentration gradient of NaCl (0.1 to 0.35 M) in Buffer B. Fractions of 8 ml each were collected. When each fraction was assayed for protease with protamine as substrate as described above, a single peak appeared in Fractions 15 through 20. An identical pattern was obtained with proenzyme II instead of proenzyme I.

Properties of Proenzyme I—Proenzyme I by itself was practically inactive for histone over a wide range of Mg²⁺ concentrations (5 to 100 mM). Cyclic nucleotides showed no effect. The proenzyme exhibited no glycerogen phosphorylase kinase activity, but was always associated with enzymatic activity to phosphorylate protamine without prior treatment with trypsin. The proenzyme exactly coincided with "protamine kinase" during the purification procedures, including DE52 column chromatography, gel filtration on a Sephadex G-100 column, and isoelectrofocusing electrophoresis. The Stokes radii and sedimentation coefficients of the proenzyme and "protamine kinase" were well matched; these were estimated to be about 42 Å and 5.1, respectively, which corresponded to a molecular weight of 7.7 × 10⁴. In addition, when preincubated with Ca²⁺-dependent protease, the protamine kinase was converted to a smaller molecular form which again was coincided with the newly produced active protein kinase (see below). In short, all attempts to separate the proenzyme and protamine kinase were unsuccessful thus far.

In the next set of experiments kinetic properties of protamine kinase were explored with protamine as substrate. The reaction was independent of cyclic nucleotides. The Kₐ value for ATP was about 3 × 10⁻⁵ M. The optimum pH was about 7.5 to 8.0 with 40 mM Tris/HCl as a test buffer. A divalent cation such as Mg²⁺ or Mn²⁺ was essential for the activity with the optimum concentration of about 20 and 5 mM, respectively, as shown in Fig. 4. The regulatory subunit and protein inhibitor of protein kinase A did not inhibit the reaction.

Proenzyme II appeared to be heterogeneous upon isoelectrofocusing electrophoresis and behaved differently from proenzyme I on DE52 column chromatography as described above. However, these proenzymes showed the same sedimentation coefficients and Stokes radii, and were indistinguishable from each other in their kinetic and catalytic properties. Proenzyme II was also associated with protamine kinase activity. The exact nature of such heterogeneity as well as the correlation between these proenzymes has remained unknown.

Properties of Protease—The purified enzyme preparation described above hydrolyzed casein to produce trichloroacetic acid-soluble tyrosine-reacting materials. The enzyme was greatly stimulated by Ca²⁺ as judged by the formation of the active protein kinase as well as by the hydrolysis of casein. The enzyme was most active at 2 to 5 mM Ca²⁺, but was
4.8. The sedimentation coefficient was estimated to be about 3.9 with a molecular weight of 9.3. The Stokes radius was about 47 Å.

Protease obtained from rat brain was also capable of activating the time course of activation of proenzyme I. When Ca²⁺-glycogen phosphorylase kinase was activated by a soluble protease by Huston and Krebs (19). Table III shows that the protein factor which was later identified as Ca²⁺-dependent protease is highly specific for and was previously (20). The numbers indicate nanomoles of glucose 1-phosphate produced.

<table>
<thead>
<tr>
<th>Cations</th>
<th>Increase in protein kinase</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>149</td>
<td>100</td>
</tr>
<tr>
<td>SrCl₂</td>
<td>36</td>
<td>24</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BaCl₂</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table III

Activation of muscle glycogen phosphorylase kinase by Ca²⁺-dependent protease

Glycogen phosphorylase kinase (47 μg) was incubated for 2 min at 30° with 5 μg of purified Ca²⁺-dependent protease in a solution (0.1 ml) containing 10 mM Tris/Cl at pH 7.5, 5 mM 2-mercaptoethanol, and 2 mM CaCl₂. In the control incubation, CaCl₂ was omitted. After incubation, a 10-μl aliquot of the solution was diluted with 0.75 ml of an ice cold 50 mM glyceral 1-phosphate at pH 6.8 containing 2 mM EDTA and 10 mM 2-mercaptoethanol. Then, a 10-μl aliquot of the resulting solution was added quickly to the reaction mixture (0.2 ml) for phosphorylase kinase assay which contained 8 μmol of glyceral 1-phosphate at pH 6.8, 2 μmol of magnesium acetate, 0.4 μmol of ATP, 40 nmol of CaCl₂ (both experiment and control incubation), 1.5 μmol of 2-mercaptoethanol, and 25 μg of phosphorylase b free of 5'-AMP. After incubation for 5 min at 30°, a 20-μl aliquot of the mixture was directly added to another reaction mixture (0.4 ml) for phosphorylase assay which contained 10 μmol of glyceral 1-phosphate at pH 6.8, 2 μmol of P, (300,000 cpm), 0.8 μmol of EDTA, and 2 mg of glycogen. The mixture was incubated further for 10 min at 30°. The mixture was then directly added to another reaction mixture (0.4 ml) for phosphorylase assay which contained 10 μmol of glyceral 1-phosphate at pH 6.8, 2 μmol of 5'-AMP, (300,000 cpm), 0.8 μmol of EDTA, and 2 mg of glycogen. The mixture was incubated further for 10 min at 30°. The reaction was stopped and radioactive glucose 1-phosphate produced was determined as described previously (20). The numbers indicate nanomoles of glucose 1-phosphate produced.

Table IV

Effect of various divalent cations on protease activity

The protease activity was assayed under the standard conditions by measuring the increase in protein kinase activity by incubating 7 μg of purified proenzyme I with 6 μg of purified Ca²⁺-dependent protease in the presence of 2 mM cation as indicated.

<table>
<thead>
<tr>
<th>Cations</th>
<th>(μmol) phosphate incorporated</th>
<th>% Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>149</td>
<td>100</td>
</tr>
<tr>
<td>SrCl₂</td>
<td>36</td>
<td>24</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BaCl₂</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 6. Gel filtration of proenzyme I before and after preincubation with Ca²⁺-dependent protease. A, purified proenzyme I (140 μg) was directly subjected to gel filtration on a Sephadex G-100 column (80 × 1.6 cm) equilibrated with Buffer B. B, purified proenzyme I (140 μg) was first preincubated for 10 min at 30° with Ca²⁺-dependent protease (120 μg) in the mixture (2 ml) containing 10 mM Tris/Cl at pH 8.0, 2 mM CaCl₂, and 5 mM 2-mercaptoethanol. The mixture was then directly subjected to gel filtration. In both A and B, elution was carried out downward with Buffer B at a flow rate of 10 ml/h, and fractions of 2.0 ml each were collected. A 75-μl aliquot of each fraction was first incubated for 3 min at 30° with 7 μg of Ca²⁺-dependent protease in the presence (●) and absence (○) of CaCl₂ in a solution (0.1 ml) containing 10 mM Tris/Cl at pH 8.0 and 5 mM 2-mercaptoethanol. A 15-μl aliquot of the solution was assayed for protein kinase at 75 mM Mg²⁺. Another 15-μl aliquot of each fraction was directly assayed for protein kinase with protamine as substrate (—) under the standard conditions for protamine kinase except that the incubation was made for 3 min. The arrows represent the void volume.

Almost inactive below 0.1 mM Ca²⁺. Among divalent cations thus far tested Ca²⁺ was most active as shown in Table II. Sr²⁺ and Mn²⁺ were slightly active but other metal ions were inactive to support the reaction. These metals themselves showed no effect on protein kinase activity under the conditions employed. The optimum pH was 7.5 to 8.5 with 40 mM Tris/acetate as a test buffer. The isoelectric point was about 5.8, corresponding to a molecular weight of 9.3 × 10⁴. The Stokes radius was about 47 Å.

It was previously shown by Meyer et al. (18) that muscle glycogen phosphorylase kinase was activated by a soluble protein factor which was later identified as Ca²⁺-dependent protease by Huston and Krebs (19). Table III shows that the protease obtained from rat brain was also capable of activating muscle phosphorylase kinase in the presence of Ca²⁺.

Conversion of Proenzyme to Protein Kinase —Fig. 5 shows the time course of activation of proenzyme I. When Ca²⁺-dependent protease was employed, the active protein kinase reached a plateau and maintained its activity for a prolonged incubation. However, when trypsin was employed, the active protein kinase once produced disappeared rapidly presumably due to further digestion with trypsin; the rate of appearance and disappearance, and amount of the protein kinase produced varied with quantity of trypsin employed.

In another set of experiments proenzyme I was subjected to gel filtration on a Sephadex G-100 column before being treated with protease. Each fraction was preincubated with Ca²⁺-dependent protease and subsequently assayed for protein kinase with whole histone as substrate. A single peak appeared in Fractions 24 through 28 as shown in Fig. 6A. Practically no activity was found without protease treatment prior to assay. When each fraction was assayed for protamine kinase without preincubation, a single peak appeared which coincided with the proenzyme. If, however, proenzyme I was preincubated first with Ca²⁺-dependent protease and subsequently assayed for protein kinase, the active protein kinase once produced appeared in Fractions 24 through 28 as shown in Fig. 6A. The mixture was then directly subjected to gel filtration. In both A and B, elution was carried out downward with Buffer B at a flow rate of 10 ml/h, and fractions of 2.0 ml each were collected. A 75-μl aliquot of each fraction was first incubated for 3 min at 30° with 7 μg of Ca²⁺-dependent protease in the presence (●) and absence (○) of CaCl₂ in a solution (0.1 ml) containing 10 mM Tris/Cl at pH 8.0 and 5 mM 2-mercaptoethanol. A 15-μl aliquot of the solution was assayed for protein kinase at 75 mM Mg²⁺. Another 15-μl aliquot of each fraction was directly assayed for protein kinase with protamine as substrate (—) under the standard conditions for protamine kinase except that the incubation was made for 3 min. The arrows represent the void volume.
TABLE IV
Substrate specificity of proenzyme I before and after proteolysis by Ca++-dependent protease

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Before proteolysis</th>
<th>After proteolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Whole histone</td>
<td>2.40</td>
<td>3.40</td>
</tr>
<tr>
<td>H1 histone</td>
<td>&lt;1</td>
<td>3</td>
</tr>
<tr>
<td>H2A histone</td>
<td>&lt;1</td>
<td>3</td>
</tr>
<tr>
<td>H2B histone</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>H3 histone</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>H4 histone</td>
<td>&lt;1</td>
<td>1</td>
</tr>
<tr>
<td>Protamine</td>
<td>41</td>
<td>43</td>
</tr>
</tbody>
</table>

The purified preparation of proenzyme I (0.35 μg) was directly assayed for protein kinase at 75 mM Mg++ under the standard conditions described for protein kinase M except that the substrate protein was used as indicated. In another experiment, 7 μg of proenzyme I were preincubated for 15 min at 30° in a solution (10.2 ml) containing 10 mM Tris/HC1 at pH 8.0, 5 mM 2-mercaptoethanol, 2 mM CaCl2, and 8 μg of purified Ca++-dependent protease. Then, a 10-μl aliquot (equivalent to 0.05 μg of proenzyme) was assayed for protein kinase at 75 mM Mg++. The reactions with H1 histone and protamine were stopped by 25% trichloroacetic acid and ice cold 10% trichloroacetic acid, respectively. The reactions with other substrates were stopped routinely at room temperature with 10% trichloroacetic acid. Acid-precipitable proteins were collected on a membrane filter. The numbers indicate picomoles of phosphate incorporated/min under these conditions.

The weight of 5.1 × 10⁴ and a Stokes radius of 38 Å. However, when Ca++-dependent protease was replaced by trypsin, an active protein kinase was produced which showed a sedimentation coefficient of 4.2, with a molecular weight of 5.7 × 10⁴, and a Stokes radius of 38 Å. In contrast, the active protein kinase, which was produced in the Ca++-containing crude enzyme system under the conditions described in Fig. 1, showed a sedimentation coefficient of 4.5 corresponding to a molecular weight of 6.4 × 10⁴, and a Stokes radius of 29 Å. Although the exact reason for such variation is unknown, this is presumably due to the difference of specificities of the proteases employed and/or to the presence of inert proteins which may interfere with the limited proteolysis of the proenzyme. Proenzyme II was similarly converted to an active protein kinase upon limited proteolysis with trypsin or Ca++-dependent protease. Nevertheless, all active protein kinases thus far obtained were indistinguishable from each other in terms of kinetic and catalytic properties described below.

Substrate Specificity before and after Proteolysis—Table IV shows relative effectiveness of various histone fractions to serve as phosphate acceptors for proenzyme I before and after treatment with Ca++-dependent protease. The proenzyme was practically inactive with histone fractions but phosphorylated protamine as described above, whereas the newly produced active protein kinase could react, to variable extents, with five species of histone fractions as well as protamine. It may be noted that the activity toward protamine was increased only slightly after limited proteolysis with the protease. Similar results were obtained with proenzyme II. Casein and phosphoethanolamine were practically inactive as substrates both before and after proteolysis.

Other Properties of Newly Produced Protein Kinase—The active protein kinase, which was newly produced as described above, showed essentially identical kinetic properties with those of protein kinase M which was partially purified from bovine cerebellum (1). These protein kinases were catalytically indistinguishable from each other, and appeared to phosphorylate the same specific seryl residues in H1 and H2B histone fractions as judged by the fingerprint procedure which was described in the accompanying paper (1). The active protein kinase thus produced was not susceptible to the regulatory subunit nor to protein inhibitor of protein kinase A.

Tissue Distribution of Proenzyme and Ca++-dependent Protease—The soluble fractions of various tissues and organs were prepared as described above, and the proenzyme and protease were fractionated and identified after DE52 column chromatography under the conditions similar to those described in Fig. 2. The results of such experiments showed that the proenzyme and protease appeared to be distributed in many tissues and organs including lung, liver, kidney, cerebellum, heart, skeletal muscle, and adipose tissue.

DISCUSSION

The experimental results described above are compatible with the supposition that in the soluble fraction of mammalian tissues a precursor protein exists which may be converted by limited proteolysis to an active protein kinase. The protein kinase thus produced is indistinguishable in the kinetic and physical properties from the enzyme which has been partially purified and characterized from bovine cerebellum as described in the accompanying paper (1). This conversion in vitro system is dependent on Ca++ and, therefore, seems to be mediated by Ca++-dependent neutral protease occurring in the same tissue, although a similar conversion may also be accomplished by limited proteolysis with trypsin. Another possible interpretation of the results presented in this paper might be that there is an inhibitor protein which is being inactivated by proteolysis during the preincubation. However, such a possibility may be excluded since the proenzyme did not inhibit protein kinase M under any condition so far tested. The best evidence thus far available for the proenzyme to enzyme conversion has been provided by the change in behavior upon gel filtration as well as sucrose density gradient centrifugation. Nevertheless, such proenzyme-enzyme relationship proposed may be definitely proved when a new NH₂-terminal residue is demonstrated after further purification of these protein molecules. It remains also unknown whether this mechanism of activation of protein kinase is physiologically significant since the proteolytic process is irreversible. In addition, the optimum concentration of Ca++ required for this activation is about 3 mM and, therefore, further explorations are necessary to establish the role of this activation mechanism in the regulation of protein phosphorylation reactions.

The active protein kinase phosphorylates preferentially histone and protamine but not casein and phosphoethanolamine. However, the enzyme is independent of cyclic nucleotides, and differs from the catalytic subunit of protein kinase A and also from the catalytic fragment of protein kinase G (1, 2). It may be emphasized that the enzyme may incorporate the terminal phosphate of ATP into the specific seryl residues in H1 and H2B histones which are all identified as the phosphorylation sites in bovine cerebellum (1). The optimum pH was 8.5 to 9.0. K₅ for 2-mercaptoethanol was about 6 mM. K₅ values for ATP and whole histone were 3.3 × 10⁻⁴ M and 150 μg/ml, respectively.
exact relationship between these three classes of protein kinases is inevitable for further investigations. The proenzyme, in contrast, is practically inactive for histone, but appears to phosphorylate protamine. Since the proenzyme and protamine kinase are always associated during various column chromatographic procedures, it is most likely that both activities originate from a single protein.

Ca\(^{2+}\)-dependent neutral protease has been first described by Guroff (14) in 1964 in the soluble fraction of rat brain. This protease has been shown to act on casein, oxidized ribonuclease, and the oxidized \(\beta\)-chain of insulin (14). In muscle, Ca\(^{2+}\)-dependent neutral protease has been described by Meyer et al. (18) which acts on muscle glycogen phosphorylase kinase. This muscle protease has been later purified highly by Huston and Krebs (19), Drummond and Duncan (21), Reddy et al. (22), and recently by Dayton et al. (23). The enzyme has been shown to act not only on muscle glycogen phosphorylase kinase but also on casein, muscle glycogen synthetase, and many components of myofibrils such as Z-discs, troponin, troponymosin, and C-protein (19, 21, 22, 24, 25). The rat brain protease first described by Guroff (14) has been later proved by Drummond and Duncan (21) to act on muscle phosphorylase kinase. The protease partially purified in the present studies also exhibits similar properties. Therefore, all Ca\(^{2+}\)-dependent proteases thus far described seem to belong to the same class of enzymes. This class of proteases appears to occur in various tissues and organs as described in this paper. The exact physiological role of this class of proteases has remained for further exploration.

Acknowledgments—We are grateful to Mrs. Sachiko Nishiyama and Miss Miwako Kuroda for their skillful secretarial assistance.

REFERENCES

Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium-dependent protease from rat brain.

M Inoue, A Kishimoto, Y Takai and Y Nishizuka