A PROENZYME OF CYCLIC NUCLEOTIDE-INDEPENDENT PROTEIN KINASE AND ITS ACTIVATION BY CALCIUM-DEPENDENT NEUTRAL PROTEASE FROM RAT LIVER*

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Received June 6,1977

SUMMARY: A proenzyme of protein kinase was found in rat liver soluble fraction. Upon limited proteolysis with Ca^{2+}-dependent neutral protease or trypsin, the proenzyme was converted to an active protein kinase which was able to phosphorylate calf thymus histone. The proenzyme was inert in this capacity but appeared to show protamine kinase activity. The protein kinase was independent of cyclic nucleotides. The proenzyme showed an approximate molecular weight of 7.7×10^4. The protease required Ca^{2+} for its activity with an optimum concentration of 2 to 3 mM. It showed a pH optimum of 7.5 to 8.5 and a molecular weight of about 9.3×10^4 as estimated by sucrose density gradient analysis. The method of purification of the proenzyme and protease from rat liver is described.

Two species of protein kinases have been thus far well characterized which phosphorylate preferentially histone and protamine: these are cyclic AMP-dependent protein kinase (protein kinase A) and cyclic GMP-dependent protein kinase (protein kinase G) (1,2). An additional species of protein kinase has now been found in the soluble fraction of rat liver which also favors histone and protamine as

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* This investigation has been supported in part by research grants from the Scientific Research Fund of the Ministry of Education, Science and Culture, Japan (1975-1977), the Intractable Diseases Division, Public Health Bureau, the Ministry of Health and Welfare, Japan (1976-1977), the Yamanouchi Foundation for Research on Metabolic Disorders, and from the Research Foundation for Cancer and Cardiovascular Diseases.

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ISSN 0006-291X

substrates. This enzyme is entirely independent of cyclic nucleotides and differs from protein kinases A and G. A series of evidence is available indicating that the enzyme is produced from its own proenzyme upon limited proteolysis with Ca^{2+}-dependent protease. Purification and some properties of the proenzyme and protease from rat liver will be described in this communication. Similar results have been obtained also for other tissues and organs such as brain and muscle, and details will be described elsewhere. The active protein kinase newly produced from the proenzyme is tentatively referred to in this paper as protein kinase M.

EXPERIMENTAL PROCEDURES

Sprague-Dawley rats were employed for the present studies. Protein inhibitor of bovine cerebellum and the regulatory subunit of rat liver protein kinase A were prepared by the methods of Donnelly et al. (3) and Kumon et al. (4), respectively. Calf thymus whole histone was prepared by the method of Johns (5). Salmon sperm protamine (Grade I, histone free) was obtained from Sigma. Bovine casein (Hammarsten) and egg yolk phosvitin were obtained from Merck AG-Darmstadt and Mann, respectively. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (6). Other materials were obtained from commercial sources.

Protein kinase M was assayed in the mixture (0.25 ml) which contained 5 μmol of Tris-HCl at pH 7.5, 7.5 μmol of 2-mercaptoethanol, 1.5 nmol of [γ-^{32}P]ATP (2 to 10 x 10^4 cpm/nmol), 100 μg of calf thymus whole histone as phosphate acceptor, 18.75 μmol of magnesium acetate, and an enzyme preparation. Incubation was carried out for 5 min at 30°. The reaction was stopped by the addition of 10% trichloroacetic acid, and the acid-precipitable radioactivity was determined as described previously (7). The proenzyme was quantitated by measuring the formation of protein kinase M after preincubation with either trypsin or Ca^{2+}-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 Ca^{2+}-dependent protease plus 0.2 μmol of $CaCl_2$, and a proenzyme preparation to be assayed. After preincubation at 30° for the period indicated in each experiment, a 10 μl aliquot of the mixture was taken and immediately assayed for protein kinase M under the conditions 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 $CaCl_2$ was omitted during the preincubation, but the protein kinase assay was made under the same conditions. Ca^{2+}-dependent protease was assayed under analogous conditions with an excess amount of the proenzyme and a protease preparation to be assayed. Detailed conditions are given in each experiment.

Protein was determined by the method of Lowry et al. (8) with bo-

vine serum albumin as a standard. S value of proteins was estimated by sucrose density gradient ultracentrifugation as described previously (9) except that the gradient was established in Buffer I (20 mM Tris-HCl at pH 7.5 containing 50 mM 2-mercaptoethanol and 0.5 mM EGTA[1]) Stokes radius of proteins was determined by gel filtration as described previously (10).

RESULTS AND DISCUSSION

All manipulations were performed at 0 to 4°. Rat liver (12 g) was quickly removed after decapitation, and homogenized in Teflon-glass homogenizer with 5 volumes of 20 mM Tris-HCl at pH 7.5 containing 0.25 M sucrose, 2 mM EDTA, and 10 mM EGTA. The homogenate was centrifuged for 90 min at 80,000 x g. The supernatant (58 ml, 795 mg of protein) was applied to a DEAE-cellulose (DE-52) column (8 x 3 cm) equilibrated with Buffer II (20 mM Tris-HCl at pH 7.5 containing 50 mM 2-mercaptoethanol, 2 mM EDTA, and 5 mM EGTA). After the column was washed with 600 ml of Buffer II, the proenzyme and protease were eluted by a 720-ml linear concentration gradient of NaCl (0 to 0.4 M) in Buffer II. Fractions of 15 ml each were collected. When each fraction was directly assayed for protein kinase in the presence of 75 mM Mg^{2+}, practically no enzymatic activity was found.[2] If, however, each fraction was treated with a limited amount of trypsin prior to assay, a single peak of protein kinase appeared in Fractions 8 through 13 as shown in Fig. 1A. These fractions, which contained proenzyme, were pooled and concentrated to 5 ml by an Amicon ultrafiltration cell equipped with PM-10 filter. The proenzyme (52 mg of protein) was subjected to gel

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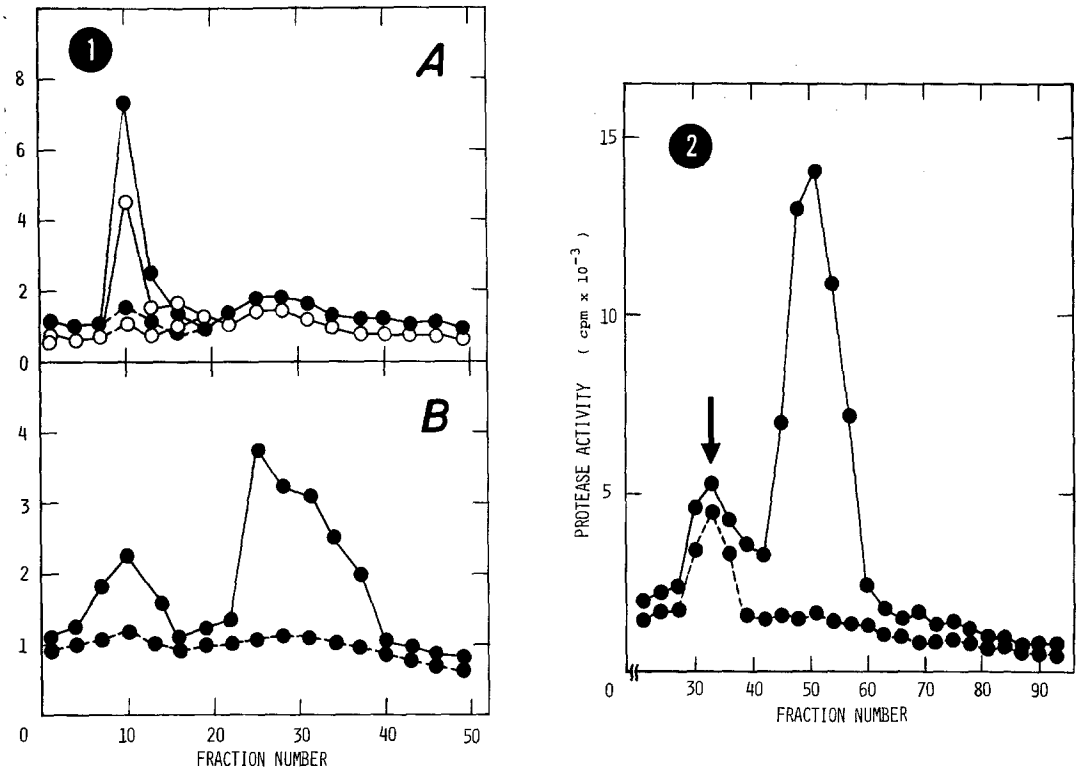
1/ Abbreviation used is: EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

2/ Protein kinase M was most active at 50 to 100 mM Mg^{2+}. In the presence of 75 mM Mg^{2+} protein kinase A was practically inactive (9), although it was eluted as two peaks in Fractions 7 through 16 and 20 through 35. On the other hand, protein kinase G was also active at 75 mM Mg^{2+}, but the amount of this protein kinase in rat liver was usually very small. Consequently, in the absence of added cyclic nucleotides and at 75 mM Mg^{2+}, neither protein kinase A nor G interfered with the assay of protein kinase M under the present conditions.



g. 1. Resolution of proenzyme and Ca^{2+}-dependent protease on DEAE-cellulose
)E-52) column chromatography. Detailed experimental conditions are described
the text. A. A 10 µl aliquot of each fraction was first preincubated for 3
n at 30° with (o——o) and without (o-----o) 0.5 µg of trypsin in a solution
.1 ml) which contained 10 mM Tris-HCl at pH 8.0 and 5 mM 2-mercaptoethanol.
en, the solution was mixed with 2 µg of trypsin inhibitor and assayed for pro-
in kinase at 75 mM Mg^{2+} under the conditions for protein kinase M. Another 10
aliquot of each fraction was first preincubated for 5 min at 30° with (•——•)
d without (•-----•) 5 mM $CaCl_2$ in a solution (0.1 ml) which contained 10 mM Tris-
1 at pH 8.0, 5 mM 2-mercaptoethanol, and 10 µg of the purified Ca^{2+}-dependent
otease. Then, protein kinase was assayed at 75 mM Mg^{2+} under the conditions for
otein kinase M. $CaCl_2$ did not affect protein kinase itself at this concentration.
A 10 µl aliquot of each fraction was assayed for Ca^{2+}-dependent protease with
µg of the purified proenzyme under analogous conditions. Protease activity in
e presence (•——•) and absence (•-----•) of 5 mM $CaCl_2$ in the preincubation
xture.

g. 2. Purification of Ca^{2+}-dependent protease by gel filtration on a Sephadex
00 column. Detailed experimental conditions are described in the text. A 50
aliquot of each fraction was directly assayed for protein kinase at 75 mM Mg^{2+}
----•). Another 50 µl aliquot of each fraction was assayed for Ca^{2+}-dependent
tease by measuring the formation of protein kinase M from 39 µg of the purified
enzyme after preincubation in the presence of 5 mM $CaCl_2$ (•——•). The assay
ditions were the same as described in the legend to Fig. 1.

filtration on a Sephadex G-100 column (92 x 2.5 cm) equilibrated with Buffer I. Elution was performed with the same buffer. Fractions of 5 ml each were collected. When each fraction was assayed for proenzyme using trypsin, a single peak appeared in Fractions 35 through 44. These fractions were pooled and concentrated to 10 ml (39 mg of protein) by ultrafiltration. The proenzyme was then subjected to isoelectrofocusing electrophoresis under the conditions specified earlier (11) 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 10 μM cyclic AMP.[3] After the electrophoresis, fractions of 2 ml each were collected. The proenzyme appeared as a single peak in Fractions 14 through 22 with an isoelectric point of about 5.6. These fractions (8.7 mg of protein) were pooled, and dialyzed against a large volume of Buffer I containing 20% sucrose.

Next, using the purified preparation of proenzyme, Ca^{2+}-dependent protease was assayed by measuring the appearance of protein kinase M in the presence of Ca^{2+}. When each fraction of DE-52 column chromatography (Fig. 1) was assayed, two peaks, major and minor, of protease were found as plotted separately in Fig. 1B. The first peak was not studied here. The second peak, Fractions 25 through 37, were pooled and concentrated to 5 ml by ultrafiltration. This preparation (61 mg of protein) was then subjected to a Sephadex G-200 column (94 x 2.5 cm) equilibrated with Buffer I. Elution was performed with the same buffer, and fractions of 4.8 ml each were collected. When each fraction was directly assayed for protein kinase at 75 mM Mg^{2+}, a small peak was found in Fractions 30 through 38 as indicated by an arrow in

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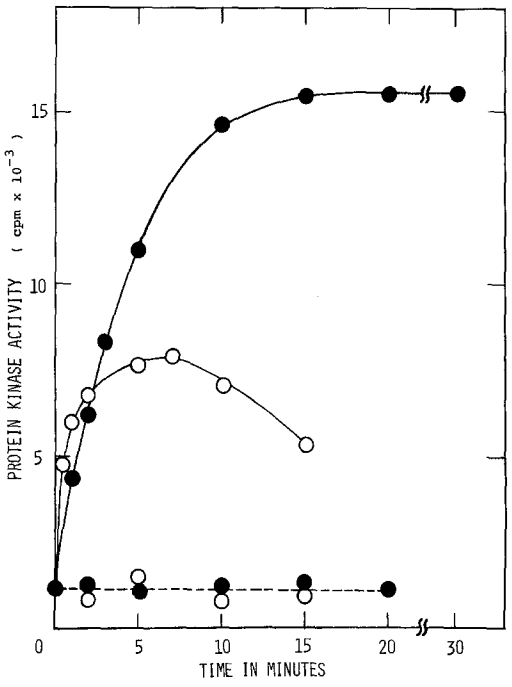
3/ In the presence of cyclic AMP, protein kinase A which slightly contaminated the preparation was dissociated and completely separated from the proenzyme. The isoelectric point of the catalytic subunit of protein kinase A was 7.4 and 8.2 (11).

Fig. 2.[4/] If, however, each fraction was preincubated with the proenzyme and $CaCl_2$, a new major peak appeared in Fractions 43 through 57. This major peak was not found when the proenzyme or $CaCl_2$ was omitted. This protease fraction (Fractions 43 through 57, 16 mg of protein) was subjected again to a DE-52 column (4.3 x 2.5 cm) equilibrated with Buffer I. Then, the column was washed with 300 ml of Buffer I containing 0.1 M NaCl and 10 μM cyclic AMP. By this procedure protein kinase A, which slightly contaminated the preparation, was dissociated and completely removed from the column. The protease was subsequently eluted from the column with a 360-ml linear concentration gradient of NaCl (0.1 to 0.35 M) in Buffer I. Fractions of 12 ml each were collected. When each fraction was assayed for protease with the proenzyme as substrate, a single peak appeared in Fractions 17 through 21. Active fractions (3.3 mg of protein) were pooled, and dialyzed against Buffer I containing 20% sucrose.

Using the purified preparation of protease, the proenzyme was again quantitated in each fraction of DE-52 column chromatography (see Fig. 1), gel filtration on a Sephadex column, and of isoelectrofocusing electrophoresis. The peaks obtained in this way always coincided exactly with those obtained with trypsin.

Fig. 3 shows the time course of activation of proenzyme by Ca^{2+}-dependent protease and by trypsin. When Ca^{2+}-dependent protease was employed, protein kinase M was produced with time, reached a plateau, and maintained its activity for a prolonged incubation. If, however, trypsin was used, the active protein kinase once produced disappeared rapidly presumably due to further proteolysis of the enzyme. Trypsin inhibitor blocked this process. The rate of appearance and disappear-

/ This protein kinase was purified further and was shown to belong to another ntity of protein kinase. The purification and properties of this protein kinase ill be described in detail elsewhere.



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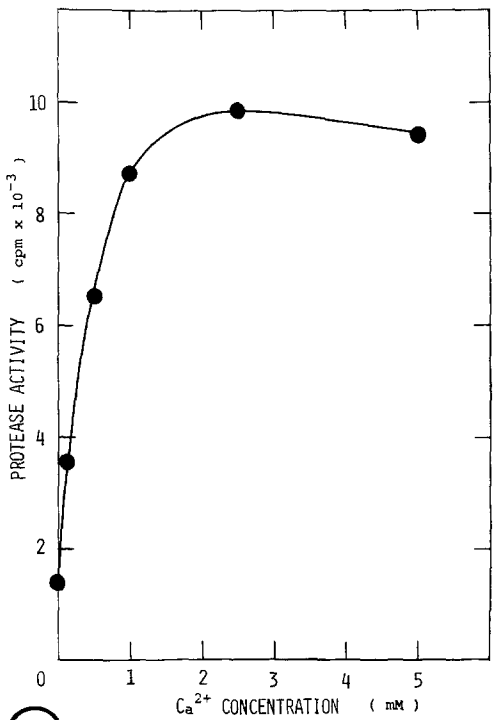

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Fig. 3. Time course of activation of proenzyme by proteolysis. The purified proenzyme (90 µg) was preincubated with either 2 µg of trypsin or 40 µg of the purified Ca^{2+}-dependent protease plus 2 mM $CaCl_2$ under the conditions described under "EXPERIMENTAL PROCEDURES". In control experiments 10 µg of trypsin inhibitor was added to or $CaCl_2$ was omitted from the respective preincubation mixtures. Protein kinase activity after preincubation with trypsin alone (o——o), trypsin plus trypsin inhibitor (o-----o), Ca^{2+}-dependent protease plus $CaCl_2$ (•——•), or with Ca^{2+}-dependent protease alone (•-----•).

Fig. 4. Effect of Ca^{2+} on protease activity. The protease activity was assayed by measuring the formation of protein kinase M from the purified proenzyme. The purified preparation of Ca^{2+}-dependent protease (11 µg) was incubated for 2 min at 30° with 18 µg of the purified proenzyme under the standard conditions except that various concentrations of $CaCl_2$ were added to the preincubation mixture (0.1 ml). Then, protein kinase was assayed at 75 mM Mg^{2+} under the conditions specified under "EXPERIMENTAL PROCEDURES".

ance, and the amount of protein kinase M produced varied with the quantity of trypsin employed.

The proenzyme itself showed practically no activity toward histone, but showed ability to phosphorylate protamine without prior treatment with protease. This activity was independent of cyclic

necleotides, and was always associated with proenzyme during the purification procedures.5/ The precise relation between these two entities will be explored after further purification. The proenzyme showed an S value of about 5.1 which corresponded to a molecular weight of 7.7 x 10^4. A Stokes radius was about 42 Å. On the other hand, protein kinase M produced in vitro showed an S value of 3.9 with a molecular weight of 5.1 x 10^4 and a Stokes radius of 38 Å.

The protease which activates the proenzyme required Ca^{2+}, and the effect of varying the concentration of this cation is shown in Fig. 4. The maximum activity was obtained at 2 to 3 mM. Among other cations tested, Sr^{2+} and Mn^{2+} were about 25% and 10% as active as Ca^{2+}, respectively. Mg^{2+}, Ba^{2+}, Cu^{2+}, and Zn^{2+} were totally inactive. The protease showed an optimum pH of 7.5 to 8.5, an S value of about 5.8 which corresponded to a molecular weight of 9.3 x 10^4, and a Stokes radius of about 47 Å.

The results presented above seem to indicate that in the soluble fraction of rat liver a precursor protein exists which may be converted to a protein kinase which actively phosphorylates histone in the absence of cyclic nucleotides. Plausible evidence suggests that this activation of the proenzyme is mediated through Ca^{2+}-dependent protease occurring in the same tissue. More detailed mechanism of such proteolytic activation will be clarified after further purification of the proenzyme and protease. Ca^{2+}-dependent neutral protease has been described first in rat brain (12), subsequently in rabbit skeletal muscle (13 to 15) and recently in rat uterus (16) and macrophages (17). The proteases from these tissues seem to show closely similar physical and kinetic properties to the hepatic protease presented above. This class of pro-

/ The proenzyme and "protamine kinase" always behaved together during sucrose density gradient analysis, electrophoresis, gel filtration, and several other chromatographic procedures. So far all attempts to dissociate these two activities have been unsuccessful.

tease appears to show rather broad substrate specificities and reacts with muscle phosphorylase kinase as well as glycogen synthetase resulting in the activation and inactivation of the respective enzymes (13, 18,19). Nevertheless, it remains unknown at this time whether the activation mechanism of the protein kinase described in this paper is physiologically significant, since the process is essentially irreversible and requires relatively high concentrations of Ca^{2+} up to 3 mM. Further exploration is necessary to establish the precise role of this Ca^{2+}-activating proenzyme-protein kinase system in controlling protein phosphorylation reactions in biological systems.

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

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