PROTAMINE KINASE INDEPENDENT OF ADENOSINE 3',5'-MONOPHOSPHATE FROM RAT BRAIN CYTOSOL*

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SUMMARY A protein kinase was obtained from rat brain cytosol which phosphorylated preferentially protamine and to some extent histone. This enzyme was independent of adenosine 3',5'-monophosphate (cyclic AMP) and was not identical with the catalytic unit of cyclic AMP-dependent protein kinase. The enzyme and cyclic AMP-dependent protein kinase from this tissue were distinguishable from each other in their kinetic and catalytic properties, and phosphorylated different seryl and threonyl residues of protamine and histone.

Adenosine 3',5'-monophosphate (cyclic AMP)-dependent protein kinases which are capable of phosphorylating histone and protamine have been shown in a wide variety of tissues (1,2), and the mode of action of cyclic AMP on the protein kinases has been clarified (3-7). Preceding reports from this laboratory have described that in many mammalian tissues multiple cyclic AMP-dependent protein kinases may be distinguished which are all dissociated by the cyclic nucleotide into various regulatory units and common catalytic unit (8,9). The present communication will briefly describe another type of protein kinase from rat brain cytosol which phosphorylates preferentially protamine rather than histone. This protein kinase is independent of cyclic AMP, and is distinguishable from the catalytic unit of

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cyclic AMP-dependent protein kinases from the same tissue.

Wistar albino rats (100-150 g) maintained *ad libitum* on CLEA laboratory chow were employed for the present studies. Protein kinase activity was assayed by measuring the radioactivity of [γ-^{32}P]ATP incorporated into salmon sperm protamine or calf thymus histone under the conditions specified previously (10). Other experimental procedures are described in the tables and figures.

Brains (20 g) were removed immediately after decapitation and homogenized with 5 volumes of 0.25 M sucrose containing 6 mM 2-mercaptoethanol and 5 mM diisopropyl fluorophosphate.[1/] All manipulations were carried out at 0-4°. The homogenate was centrifuged for 60 min at 105,000 x g, and ammonium sulfate (35 g) was added to the supernatant (55% saturation). After centrifugation for 20 min at 20,000 x g, the precipitate was dissolved in 20 ml of 0.01 M Tris-Cl at pH 7.5 containing 6 mM 2-mercaptoethanol and 10% (v/v) glycerol (TMG buffer), and was dialyzed for 15 hours against a large volume of TMG buffer. The enzyme solution was chromatographed on a DEAE-cellulose (DE-52) column (12 x 2 cm) equilibrated with TMG buffer. Elution was carried out with a 360-ml linear concentration gradient of NaCl (0-0.4 M) in TMG buffer. When fractions (4 ml each) were assayed with either protamine or histone as phosphate acceptor two peaks of enzyme activity appeared at 0.075 M NaCl (peak I) and at 0.18 M NaCl (peak II). Peak I (tube No. 13 through 26) was applied directly to a hydroxylapatite column (3 x 2 cm) equilibrated with 0.03 M potassium phosphate at pH 7.5 containing 6 mM 2-mercaptoethanol. After the column was washed with 30 ml of the same buffer, elution was carried out with a 320-ml linear concentration gradient (0.03 M-0.25 M) of potassium phosphate at pH 7.5. Again,

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1/ Diisopropyl fluorophosphate was added to block proteolysis of cyclic AMP-dependent protein kinases during the isolation procedure (9).

two peaks of enzyme activity appeared (Fig. 1); the first peak (peak Ia) was stimulated by cyclic AMP, whereas the second peak (peak Ib) did not respond to the cyclic nucleotide. When peak Ia was subjected to isoelectrofocusing electrophoresis, one major peak with a shoulder was detected (Fig. 2A); the major peak showed an isoelectric point (pI) of pH 5.3 and was stimulated by cyclic AMP, and the shoulder found at pH 5.8 was not stimulated nor inhibited by the cyclic nucleotide. When the same preparation of peak Ia was mixed first with 10^{-6} M cyclic AMP and then subjected to the electrophoresis, the cyclic AMP-dependent protein kinase disappeared and two components appeared at pH 7.4 and 8.2 (Fig. 2B). Under these conditions the protein kinase at pH 5.8 was not affected by the cyclic nucleotide. The latter kinase will be referred to tentatively as protamine kinase, since it reacted with protamine as a preferable substrate as described below. The pI 7.4 and 8.2 components were also obtained from peak Ib upon isoelectrofocusing electrophoresis in the absence of cyclic AMP (Fig. 2C).[2/]

The three species of protein kinase obtained as described above, namely protamine kinase and pI 7.4 and 8.2 components, were fully active in the absence of cyclic AMP, and none of these kinases was stimulated or inhibited by the cyclic nucleotide. Available evidence indicated that both pI 7.4 and 8.2 components were catalytic units of cyclic AMP-dependent protein kinases, and that the two components were indistinguishable from each other in their kinetic and catalytic properties and phosphorylated the same specific sites of histone and protamine as judged by a fingerprint procedure. In

2/ Peak II (tube No. 30 through 60) from the DEAE-cellulose column described above was a mixture of multiple cyclic AMP-dependent protein kinases with isoelectric points ranging from pH 4.5 to 5.3. However, these protein kinases were all dissociated by cyclic AMP, each resulting in the release of two components of isoelectric points of pH 7.4 and 8.2. The preparation of peak II was essentially free of protamine kinase. The detailed experimental results will be described elsewhere.

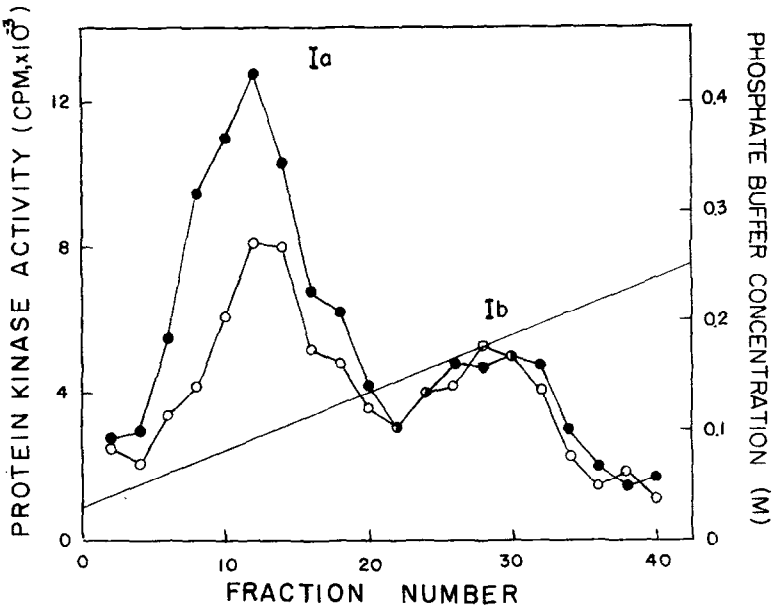

Fig. 1. *Hydroxylapatite column chromatography of rat brain protein kinases (Peak I).* The detailed experimental conditions were described in the text. The protein kinase activity was assayed with 100 µg of calf thymus whole histone as substrate in the presence (●——●) and absence (o——o) of cyclic AMP (1 µM) under the conditions described previously (10).

contrast, protamine kinase showed different properties and was not identical with any of the catalytic units of cyclic AMP-dependent protein kinases obtained from this tissue.

Table I shows the relative rates of reactions catalyzed by these kinases with histone and protamine as substrates; protamine was less active than histone for both pI 7.4 and 8.2 components but was far more active than histone for protamine kinase. The latter kinase was practically inactive towards casein and phosvitin, and was clearly distinguished from phosphoprotein kinases previously described (14,15). Acid hydrolysis of protamine and histone fully phosphorylated by each protein kinase resulted in the formation of phosphoserine and phosphothreonine. However, the amount of phosphothreonine produced by either one of these components was less than 3% of phosphoserine, whereas that by protamine kinase was more than

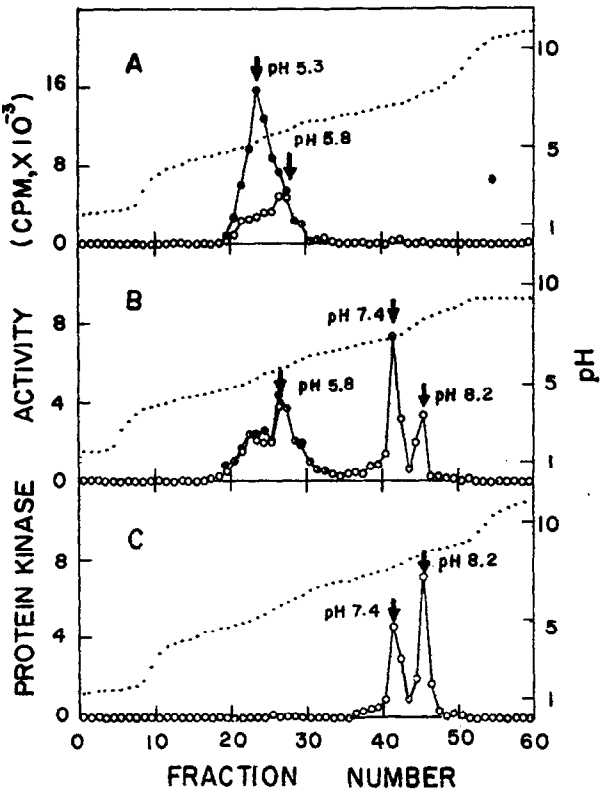

Fig. 2. *Isoelectrofocusing electrophoresis of protein kinases (peak Ia and Ib) in the presence and absence of cyclic AMP.* Isoelectrofocusing electrophoresis was performed at 4° by the method of Svensson (11) using a 110-ml column (LKB Instruments). The pH gradient (pH 3 through 10) was established during the electrophoresis following the sequential addition of ampholite solutions (0.8% W/V) in a 0-48% (W/V) sucrose gradient. The protein sample was applied in the central ampholite solution. Electrophoresis was continued for 42 hours at 500 volts. Fractions of 1.8 ml each were collected. ●——● and o——o, protein kinase activity assayed in the presence and absence of cyclic AMP (1 μM), respectively; ·····, pH. A: peak Ia; B: peak Ia mixed with cyclic AMP (1 μM) before being subjected to the electrophoresis; C: peak Ib.

10% of phosphoserine. In order to examine whether each protein kinase phosphorylated specific sites of the substrate proteins, the radioactive histone and protamine preparations were digested with trypsin and then subjected to high voltage paper electrophoresis followed by radioautography under the conditions specified earlier (9). As illustrated in Fig. 3, distinct radioactive peptide patterns were obtained with protamine kinase and with cyclic AMP-dependent protein kinase.

Table I

Relative rates of reactions catalyzed by protamine kinase and two components of the catalytic unit of cyclic AMP-dependent protein kinase

Protein kinase activity was assayed with 100 µg each of calf thymus whole histone or salmon sperm protamine as phosphate acceptor under the conditions specified previously (10). The reaction was stopped and acid-precipitable material was washed with 10% trichloroacetic acid containing 0.25% sodium tungstate (12). The specific activity of [γ-^{32}P]ATP was 3 x 10^4 cpm per nmole, and approximately 2 µg each of the protein kinases was employed. Protein was determined by the method of Lowry *et al.* (13) with bovine serum albumin as a standard.

Substrate and ratio	Protamine kinase	Catalytic unit of cyclic AMP-dependent protein kinase	
		pI 7.4 component	pI 8.2 component
Histone (a)	3,620 *cpm*	3,450 *cpm*	3,000 *cpm*
Protamine (b)	25,790 *cpm*	1,340 *cpm*	1,200 *cpm*
Ratio (a/b)	0.14	2.57	2.48

Table II

Effect of a regulatory unit on protamine kinase and two components of the catalytic unit of cyclic AMP-dependent protein kinase

Protein kinase activity was assayed with 100 µg of calf thymus whole histone as phosphate acceptor and acid-precipitable radioactivity was determined under the conditions described previously (10). Where indicated 20 µg of a regulatory unit which was prepared from rat liver as described previously (9), 1 nmole of cyclic AMP and approximately 2 µg each of the protein kinases were added. The regulatory unit used here was practically free of protein kinase activity in the presence and absence of cyclic AMP.

System	Protamine kinase	Catalytic unit of cyclic AMP-dependent protein kinase	
		pI 7.4 component	pI 8.2 component
Protein kinase alone	2,640 *cpm*	3,000 *cpm*	3,000 *cpm*
Protein kinase plus cyclic AMP	2,910	3,200	2,790
Protein kinase plus regulatory unit	2,700	950	900
Protein kinase plus cyclic AMP and regulatory unit	3,060	2,900	3,090

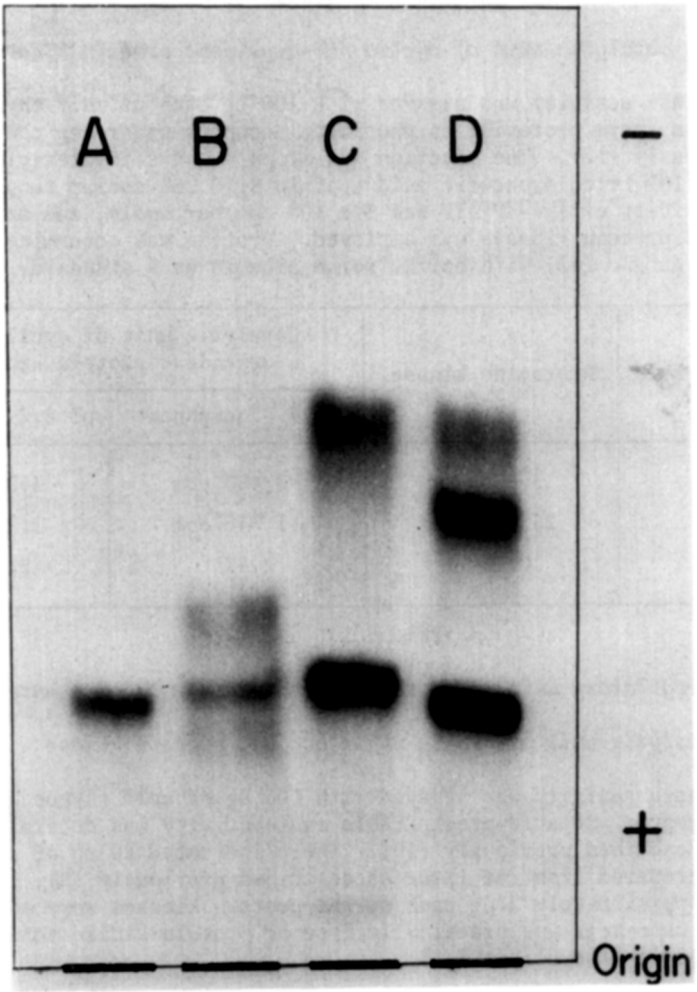

Fig. 3. *Radioautography of an electropherogram of the tryptic digests of histone and protamine phosphorylated by protamine kinase and catalytic unit of cyclic AMP-dependent protein kinase.* Calf thymus whole histone and salmon sperm protamine were fully phosphorylated by each protein kinase, and subjected to tryptic digestion followed by high voltage paper electrophoresis under the conditions described previously (16). A: histone with protamine kinase; B: histone with pI 7.4 component; C: protamine with protamine kinase; D: protamine with pI 7.4 component. The radioactive peptide patterns obtained with pI 8.2 component were essentially identical with those obtained with pI 7.4 component.

Both pI 7.4 and 8.2 components exhibited maximum activities at pH 7.0. Mg^{+2} ion was required absolutely and was saturated at 3 mM in 20 mM Tris-Cl. These components showed an identical Km value of

5×10^{-6} M for ATP.[3/] In contrast, protamine kinase showed optimum pH at 8.0 and 20 mM Mg^{+2} ion was needed for the maximum activity. The Km value for ATP of the latter kinase was 1.3×10^{-6} M.

A previous report demonstrated that the dissociation of cyclic AMP-dependent protein kinase into regulatory and catalytic units was reversible, and that the addition of regulatory unit from homologous as well as from heterologous tissue to catalytic unit converted the latter to a cyclic AMP-dependent form (16). Table II shows that both pI 7.4 and 8.2 components were inhibited significantly by a regulatory unit obtained from rat liver, and this inhibition was completely overcome by the addition of the cyclic nucleotide. However, protamine kinase was not susceptible to the regulatory unit under any condition thus far tested. These results indicate that protamine kinase presented in this paper is essentially independent of cyclic AMP and belongs to a different entity. The amount of the enzyme is less than 10% of the total activity which is capable of phosphorylating histone in the brain cytosol. Langan (17) has proposed recently that a cyclic AMP-independent protein kinase may exist in calf liver which phosphorylates a separate site in f_1-histone. The exact correlation of this kinase to protamine kinase is unknown, and the biological role as well as the natural substrates of this new species of protein kinase have remained unexplored.

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3/ The separate identity of pI 7.4 and 8.2 components was demonstrated only by the electrophoretic procedure, and the chromatographic procedure itself was not responsible for the resolution of the two components. Both components showed a practically identical molecular weight of 3.5×10^4, and exhibited same substrate specificities. The exact nature of this heterogeneity of the catalytic unit is currently under investigations.

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