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ISOLATION AND PROPERTIES OF 5'-MONONUCLEOTIDASE FROM A MEMBRANE FRACTION OF BOVINE CEREBRAL CORTEX

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SUMMARY

1. From a membrane fraction of bovine cerebral cortex 5'-mononucleotidase (5'-ribonucleotide phosphohydrolyase, EC 3.1.3.5) was prepared and its properties were described.

2. A particulate enzyme which was prepared by deoxycholate treatment and gradient centrifugation showed a 240-fold higher specific activity than that of the original membrane fraction. The enzyme hydrolyzed exclusively nucleoside 5'-monophosphates; and the hydrolysis rates of pyrimidine–ribose nucleotides were 2-fold larger than those of purine–ribose nucleotides. K_m values for AMP and CMP were $2.9 \cdot 10^{-4}$ and $4.8 \cdot 10^{-4}$ M, respectively, in the presence of 2 mM $MgCl_2$.

3. The particulate preparation was active in the absence of extraneous divalent metal ions. Both Mg^{2+} and Mn^{2+} stimulated the activity, but other tested divalent cations were inhibitory. Among nucleosides, adenosine was the most potent inhibitor regardless of the base type of the substrate.

4. Although the particulate enzyme contained phospholipids (33% of total protein), they did not seem to be involved in the activity.

5. A soluble 5'-mononucleotidase was also prepared from acetone powder of the same source, using detergent treatment and gel-filtration chromatography. The enzyme appeared to be an acidic protein possessing a strong tendency to aggregate. The apparent particle weight of the enzyme was estimated to be 190000 by gel-filtration chromatography.

INTRODUCTION

The fact may not be simply accidental that various types of enzymes acting on nucleic acids are located in the cell membrane, such as Mg^{2+}-activated ATPase, (Na^+, K^+, Mg^{2+})-activated ATPase, adenylate cyclase, phosphodiesterase, and 5'-mononucleotidase. It is likely that there is a functional interrelation between these enzymes. For instance, it has been reported that adenosine greatly increases the amount of cyclic AMP of cerebral cortex slices of guinea pigs[1]. An adenosine-sensitive regulatory unit has been suggested to exist on the adenyl cyclase molecule[2]. In order to understand the relationship of membrane enzymes with other components of the membrane and to clarify chemical architecture of the neuronal membrane, we have studied the proper-

ties of enzymes isolated from nervous membranes[3,4]. In this line of our investigation, 5′-mononucleotidase of bovine cerebrum was examined.

The properties of 5′-mononucleotidase of brain tissues of sheep, rat, *etc.* have been reported by Reis[5], Ipata[6], and Bosmann and Pike[7]. It is very interesting that their properties vary to a large extent with the origin of the nucleotidase. Since we have been using bovine brain as a material source for our study on the relationship between membrane composites, bovine cerebral cortex was also used as an enzyme source in the present study. The properties of the bovine enzyme were found to be greatly different from those of the previously reported brain mononucleotidases of other animals. With an emphasis on its difference in properties from the enzyme of other sources, 5′-mononucleotidase partially purified from a membrane fraction of bovine cerebral cortex will be described in this report.

METHODS AND MATERIALS

Enzyme activity determination

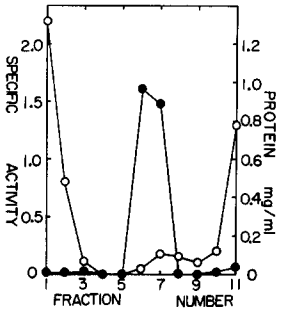
The standard reaction mixture contained 50 mM Tris buffer, pH 8.6, 2 mM $MgCl_2$, 2 mM nucleoside monophosphate, and approximately 10 μg of the enzyme protein in a final volume of 2.0 ml. AMP was routinely used as a substrate. The incubation was usually at 37 °C and for 40 min. The rate of P_i liberation from the substrates was linear up to, at least, 60 min, and under the standard conditions the hydrolyzed substrate did not exceed 25% of the initial amount. When the substrate concentration was low for Michaelis constant determination, the incubation period was shortened in such a manner that the enzyme did not hydrolyze the substrate more than one-fourth of the initial amount. The values obtained were corrected during calculation. After trichloroacetic acid was added to stop the reaction and the precipitate was removed by centrifugation, P_i in the clear supernatant was determined by Gomori's method[8].

Protein was determined by a minor modification of the method of Lowry *et al.*[9].

Enzyme preparation

The cerebral cortex of the domesticated beef cattle was used as an enzyme source. A membrane fraction (or heavy microsomal fraction) was separated by differential centrifugation as reported elsewhere[10]. The whole preparation was carried out at 0–5 °C. Three different preparations were used in this study;

(*a*) *Particulate preparation.* After the fraction was washed by centrifugation with 10 vol. of 50 mM Tris, pH 7.4, and twice with 10 vol. of water, the residue was treated with 1.5% sodium deoxycholate, pH 8: and the ratio of the protein to deoxycholate was kept at one. The insoluble protein which had been spun down was suspended in water and separated by centrifugation at 200000 $\times$ *g* for 60 min on a continuous sucrose gradient of concentrations from 5 to 15% (SW 40 Ti rotor, Beckman Instruments). Both the protein profile and the specific activity pattern are shown in Fig. 1. Two fractions with high specific activity were combined and the protein was centrifuged down. The precipitate was suspended in a small amount of water and was again subjected to gradient centrifugation under the same conditions as the first one. At this stage the specific activity was 27 μmoles P_i per min per mg of protein: and it was 240-fold higher than that of the original membrane fraction. In order to remove a trace of phosphat-



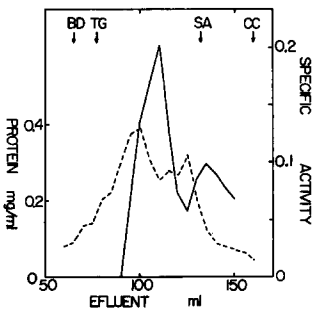


Fig. 1. Protein and specific activity pattern after gradient centrifugation. The figure illustrates the pattern of protein (○—○), and the specific activity of the enzyme (μmoles P_i liberated per min per mg protein) (●—●) obtained after the first gradient centrifugation (sucrose, 5–15%). The fraction volume was 1 ml. AMP was used for activity determination. Two fractions with high specific activity (fractions No. 4 and 5 in this case) were collected and subjected to the second gradient centrifugation.

Fig. 2. Column chromatography of solubilized enzyme. The enzyme protein extracted from acetone powder was chromatographed on a column of Bio-Gel A-5m (83 cm × 1.5 cm). The eluting solvent contained 1% deoxycholate, 0.1 mM AMP, 0.1 mM $MgCl_2$, and 50 mM NaCl, adjusted to pH 7.4. The protein content (mg/ml) and the specific activity (μmole P_i liberated per min per mg protein) of the fractions are shown by a broken line and a solid line, respectively. The fraction volume was 5 ml and AMP was used for activity determination. The molecular weight standards are: BD, Blue Dextran 2000; TG, bovine thyroglobulin; SA, bovine serum albumin; and CC, cytochrome *c*. The protein standards exhibited sharp peaks. Because the used preparation of Blue Dextran gave a sharp peak *plus* a large tailing, suggesting contamination of lower polymers, the peak was used as a reference point.

ases contaminating the preparation, it was heated in the presence of 1 mM AMP at 55 °C for 5 min, in which period the nucleotidase lost 6% of the initial activity. This preparation was mainly used in the present work.

(*b*) *Solubilized preparation.* Acetone powder was prepared from the membrane fraction by a conventional manner. The powder was extracted with a quantity of water 40-fold larger than its weight by continuous stirring for 60 min, and the water extract was disposed. From the residue the enzyme protein was solubilized by sonication for 8 min in 1% Triton X-100 (a half volume of the amount of water used in the previous step). After removal of insoluble residue by centrifugation, a portion of the clear supernatant was chromatographed on a Bio-Gel A-5m column (83 cm × 1.5 cm) with an eluting solvent containing 1% deoxycholate, 0.1 mM AMP, 0.1 mM $MgCl_2$, and 50 mM NaCl, adjusted to pH 7.4. An identical chromatographic procedure was used for determination of the apparent particle weight of the solubilized preparation according to Andrews[11,12]. The chromatographic patterns of proteins and of their specific activities are illustrated in Fig. 2. The increase in the specific activity was 16-fold at this stage as compared to that of the original membrane fraction; and the contaminating phosphatase activity was approximately 1% of the 5′-nucleotidase activity, when determined in the presence of *p*-nitrophenyl phosphate as a substrate.

(*c*) *Water extract.* After the acetone powder was extracted by 8 min sonication with a quantity of water 40-fold larger than the powder weight, a clear supernatant containing 5′-nucleotidase activity was obtained by centrifugation at 140000 × *g* for 60 min.

Treatment with phospholipases

(a) *Phospholipases C and D.* The particulate preparation (protein, 1.3 mg) was incubated at 37 °C for 20 min with 50 μg of either one of the phospholipase preparations in the presence of 6 mM $MgCl_2$ and 10 mM $CaCl_2$, followed by centrifugation to collect the treated enzyme protein after the addition of 10 mg of bovine serum albumin.

(*b*) *Snake venoms.* The heat-treated venom[13] (100 μg) of either *Naja naja* or *Crotalus adamanteus* was incubated with the particulate preparation (1.3 mg of protein) at 4 °C for 17 h in 50 mM Tris, pH 8.0, and the enzyme was collected by centrifugation as above.

Chromatography of lipids

The phospholipids contained in the enzyme preparations were extracted with a chloroform–methanol mixture (2;1, v/v) at room temperature, and were identified by thin-layer chromatography using a solvent system of either Skidmore and Entenman[14] or Skipski *et al.*[15]. The amount of phospholipid was estimated from the amount of total phosphorus in the chloroform–methanol extract of each spot.

Materials

Nucleotides, nucleosides, phospholipases C and D, and venoms of *Naja naja* and *Crotalus adamanteus* were purchased from Sigma Chemical Co.; sodium deoxycholate from Schwarz-Mann; and Triton X-100 from Rohm and Haas Co.

RESULTS AND DISCUSSION

Substrate specificity

A particulate preparation (see Methods) of nucleotidase of bovine cerebral cortex showed approximately 2-fold higher activity against pyrimidine–ribose 5'-nucleotides than against purine nucleotides (Table I). The preference of pyrimidine

TABLE I

SUBSTRATE SPECIFICITY

The reaction mixture contained 2 mM substrate, 2 mM $MgCl_2$, 50 mM Tris buffer, pH 8.6, and 0.1 ml of the enzyme preparation in the final volume of 2 ml. The incubation was 37 °C and for 20 min. The activity is expressed in terms of percentage of the hydrolysis rate of 5'-AMP. The figures are average of 4 values agreeing within 6%.

Nucleotides	*Relative hydrolysis rate* (%)
AMP	100
GMP	114
IMP	112
CMP	213
UMP	173
dAMP	96
dGMP	36
dCMP	58
dUMP	99

nucleotides was peculiar to the present preparation. The order of hydrolysis rate of deoxyribose nucleotides was dUMP > dAMP > dCMP > dGMP. Thus, it seems a general rule that the hydrolysis rates are entirely unrelated between ribose nucleotides and deoxyribose nucleotides, as has also been shown with the 5′-nucleotidase preparations from calf intestine[16], rat liver[17], and rat cerebellum[7]. The hydrolysis rates of 2′- and 3′-AMP, GMP, CMP, and UMP, and ADP were between 0 and 1% of that of AMP with the present preparation. ATP was not hydrolyzed.

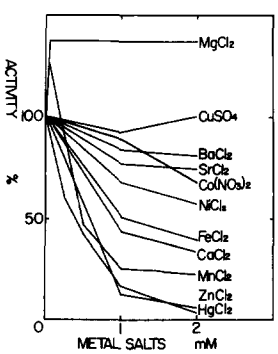
Michaelis constants

K_m for AMP was estimated to be $2.9 \cdot 10^{-4}$ M in the presence of 0.2–2 mM $MgCl_2$, and K_m for CMP $4.8 \cdot 10^{-4}$ M in the presence of 2 mM $MgCl_2$ (Fig. 5). K_m of the present preparation for AMP was larger than those of 5′-nucleotidases of rat cerebellum ($8 \cdot 10^{-5}$ M)[7], bull seminal plasma ($2.6 \cdot 10^{-5}$ M)[18], and sheep brain ($7.5 \cdot 10^{-6}$ M)[6], whereas K_m for CMP was similar to that of rat cerebellum ($9.2 \cdot 10^{-4}$ M)[7].

Effects of divalent metal ions

The particulate preparation was active in the absence of exogenous divalent metal ions. Although Mg^{2+} stimulated the enzyme activity of this particular preparation by 35% at concentrations higher than 0.1 mM (Fig. 3), the stimulation varied in a range of 5–35% from preparation to preparation.

Most divalent cations were inhibitory to the particulate preparation as illustrated in Fig. 3, like many 5′-nucleotidase preparations from other origins, such as a variety of tissues of human, calf, rat, chicken, *etc.* Although Co^{2+} activates mononucleotidase of both calf intestine[19] and rat cerebellum[7], it was inhibitory to the present preparation of bovine cerebral cortex. Because Cu^{2+} had no effect, an amino acid residue such as histidine may not be involved in the activity of the present nucleo-



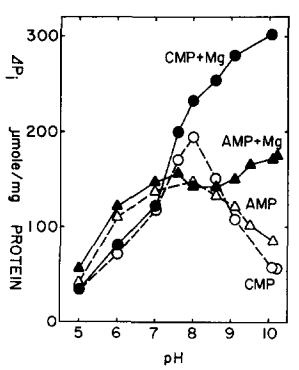


Fig. 3. Effects of divalent metal ions. The metal salts were added to the standard reaction medium (*minus* $MgCl_2$) at the indicated concentrations. It will be noted that the particulate enzyme preparation showed activity in the absence of extraneous metal ions. The enzyme activity in the presence of metal ions is expressed in terms of percentage of the activity obtained in the absence of metal ions.

Fig. 4. Effects of pH. The used buffers were 50 mM acetate–Tris for pH 5–6, and 50 mM Tris for pH 7–10.2. Either AMP (triangles) or CMP (circles) at 2 mM were used as the substrates in the presence (filled) and in the absence (open) of 2 mM $MgCl_2$.

tidase[20]. As reported with rat liver 5'-nucleotidase[21], only low concentrations of Mn^{2+} activated the bovine nucleotidase to a slightly smaller extent than Mg^{2+} did; high concentrations were inhibitory. Hg^{2+} inhibited the nucleotidase by 50% at 0.3 mM, whereas *p*-chloromercuriphenyl sulfonate showed 25% inhibition at 4 mM in the presence of AMP as a substrate, and at 2 mM in the presence of CMP. Similar results were obtained with *p*-chloromercuribenzoate. These findings suggest that most of the sites which bind Hg^{2+} and are involved in the enzyme activity are not exposed to the bulk water phase, being inaccessible to relatively larger organic mercury ions.

Effects of pH

The pattern of the pH–activity curve obtained in the absence of extraneous Mg^{2+} was different from that obtained in the presence of the ions (Fig. 4). When AMP was the substrate, the activity increase by Mg^{2+} was conspicuous at pH values higher than 8.6; when CMP was used the increment was marked at pH values higher than 7.6.

There was a small concavity in the pH–activity curve between pH values 8 and 9 in the presence of AMP *plus* Mg^{2+}. The overall patterns resembled those of mononucleotidase of bull seminal plasma[18], although in the presence of Mg^{2+} the activity peaks were located more to the alkaline side in this case than they are in the case of seminal plasma.

Effects of nucleosides

Nucleosides were tested for their inhibitory effects on the particulate preparation in a concentration range from 1 to 8 mM. When AMP was the substrate, adenosine inhibited the enzyme approximately 10% at 8 mM and the inhibition by other nucleosides was not marked. (Table II. The values obtained in the presence of 8 mM nucleosides only are shown.) When CMP was used, adenosine also inhibited the enzyme 45% whereas cytidine inhibition was insignificant and other nucleosides showed 10–30% inhibition at 8 mM. Thus, adenosine was the most effective inhibitor whichever the substrate was, and the effective inhibitor molecule did not share a common

TABLE II

EFFECTS OF NUCLEOSIDES

Either 2 mM AMP or 2 mM CMP was used as a substrate as indicated. The activities obtained in the absence of nucleoside were arbitrarily set as 100%. The activities shown are the values obtained in the presence of 8 mM nucleosides, and are average of 3 values agreeing within 7%.

Nucleosides	*Activity* (%)	
Substrate:	*AMP*	*CMP*
None	100	100
Adenosine	91	55
Guanosine	98	87
Inosine	95	81
Cytidine	101	95
Uridine	97	71

base moiety with the substrate molecule. This finding and the double reciprocal plot of the values obtained with CMP either in the presence or absence of adenosine (Fig. 5), indicate that the inhibitory mechanism of nucleosides is not a competitive type. With 5′-nucleotidases from other sources, guanosine and inosine are the most potent inhibitors and the inhibitory effects are stronger than they were with the present preparation[7,22,23].

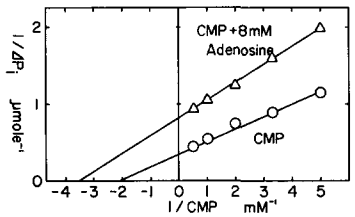


Fig. 5. Double reciprocal plot of CMP hydrolysis rate obtained in the presence and the absence of adenosine. The reaction conditions were described in Methods, except that the substrate was CMP in the presence of 8 mM adenosine as indicated in the figure. Each point represents an average of 3 separate experiments, and the agreement between them was within 8%.

Relationship with lipids

The particulate preparation contained various phospholipids, which were 33% of its protein. The phospholipids were lecithin (60% of total phospholipids), phosphatidyl serine (36%), and phosphatidylethanolamine (4%), and a trace of lysophospholipids.

A 5′-nucleotidase purified from rat liver membrane was reported to contain exclusively sphingomyelin and a close relation between the lipid and the enzyme activity was suggested[24]. The accompanying phospholipids with the present preparation do not seem to be involved in the activity, however, because neither extensive extraction with deoxycholate nor the addition of deoxycholate to the reaction medium inhibited the enzyme activity, and because the treatment with phospholipases C and D, and snake venoms containing phospholipase A did not affect the activity.

Other properties

When the water extract (see Methods) of bovine brain acetone powder was chromatographed on a Bio-Gel A-15m column (90 cm × 2.5 cm) whose exclusion limit was 15000000 daltons, using an eluting solvent containing 50 mM NaCl and 50 mM Tris, pH 7.4, 5′-nucleotidase activity was found in the fractions before the limit. Consequently, it is likely that the enzyme protein and other membrane proteins aggregate during the procedure. Because the specific activities of the precipitates were similar after $(NH_4)_2SO_4$ fractionation at concentrations of 25, 35, 45, and 60% saturation, the enzyme protein and other membrane proteins seem to aggregate in the presence of high concentrations of the electrolyte. Once precipitated by $(NH_4)_2SO_4$, the enzyme protein could hardly be dissolved in aqueous solutions again. The tendency of aggregation may be expected from the hydrophobic nature of the membrane proteins.

The enzyme protein appears to be an acidic one, inasmuch as adjustment of the pH of the water extract to 5 precipitated the proteins containing most of the enzyme

activity. This idea is supported also by the finding that, when small amounts of protamine sulfate were added to the water extract, proteins that showed the enzyme activity and amounted to 5-fold weights of the added protamine, were precipitated.

The aggregation of the enzyme protein was prevented by addition of detergents, such as deoxycholate and Triton X-100, to the extracting solvents. In the presence of 1% deoxycholate and 0.1 mM $MgCl_2$ the apparent particle weight of the enzyme was estimated to be approximately 190000 by gel-filtration chromatography using a Bio-Gel A-5m column (Fig. 2). The apparent particle weight was 1.4-fold larger than the molecular weight of a water-soluble 5′-nucleotidase of sheep brain[6].

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