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PURIFICATION AND PROPERTIES OF 2'-NUCLEOTIDASE FROM MAMMALIAN BRAIN

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Summary

A nucleotidase specific for 2'-nucleotides was localized in both the soluble and the synaptosomal fractions of rat brain. The enzyme was partially purified from the soluble fraction of bovine brain. The $s_{20,w}$ was 4.9 S with an estimated molecular weight of about 70 000. The optimum pH was 8.0 and K_m value for 2'-AMP was $5.5 \cdot 10^{-4}$ M. The substrate and inhibitor specificities of the enzyme were examined. The nucleotidase has an absolute requirement for Mg^{2+} but neither Fe^{3+} nor Ca^{2+} acted as replacement ions. In fact, Mn^{2+} and Ca^{2+} inhibited the Mg^{2+}-dependent 2'-nucleotidase.

Introduction

Among various mononucleotide phosphatases in mammalian tissues, 5'-nucleotidase has been purified either from liver plasma membranes [1—3] or from supernatant fractions of brain and liver [4,5]. The lysosomal fraction of liver or spleen contains an acid phosphomonoesterase which hydrolyzes 3'- and 2'-nucleotides [6,7]. However, a nucleotide phosphomonoesterase specific for 2'-nucleotide had not been detected in mammalian tissues. Jacob and Sontag [8] purified NADP phosphatase which also exhibited 2'-nucleotidase activity from the serum of *Heavea brasiliensis*.

Drummond et al. [9] found an enzyme which catalyzed the cleavage of 3'-phosphodiester bond of cyclic 2',3'-AMP, producing 2'-AMP. The enzyme is localized mainly in the myelin fraction of rat brain [10]. In this paper, we report the purification and characterization of a 2'-nucleotidase from mammalian brain.

Materials and Methods

Chemicals. Authentic 2′-AMP and 3′-AMP were purchased from the Sigma Chemical Company. 3′-GMP was prepared from RNA by digesting with ribonuclease T_1 according to the method described by Takahashi [11] and purified by Dowex-1 column chromatography. For regular use 2′-AMP and 3′-AMP (Kojin Chemical Co.) were separated according to Cohn [12] with some modifications. The mixture of 2′- and 3′-AMP (500 mg) was neutralized with NaOH and placed on a column of Dowex-1 (formate form, X2, 200—400 mesh, 45 × 1.2 cm). The column was washed with H_2O and the nucleotides eluted by the application of a concave gradient formed by dripping 0.4 N formic acid into a reservoir containing 300 ml H_2O. 2′-AMP appeared from 290 to 350 ml and 3′-AMP appeared from 390 to 440 ml. The eluate was dried and the residue was washed 3 times with water. Other 2′- or 3′-nucleotides were prepared by Dowex-1 column chromatography using different concentrations of formic acid.

Fractionation of the subcellular components of rat brain. Male Wistar rats were killed by decapitation and the brain immediately removed. 2 g whole brain were minced at 4°C and gently homogenized in 40 ml 0.32 M sucrose containing 10 mM Tris-acetate (pH 7.3). Subcellular fractionation was performed according to the method of Whitaker [13] with a modification.

Determinations. Protein and inorganic phosphate were determined by the methods of Lowry et al. [14] and Lowry and Ropez [15], respectively. Molecular weight was estimated from Sephadex G-200 column chromatography, using Blue Dextran, malate dehydrogenase, lactate dehydrogenase, glyceraldehyde dehydrogenase and aldolase as internal standards. The sedimentation constant was calculated by the method of Martin and Ames [16]. Centrifugation was carried out at 4°C for 14 h at 100 000 × *g* on a 5—20% linear sucrose density gradient, using catalase, alcohol dehydrogenase, serum albumin and cytochrome *c* as standards.

Enzyme assay. The activity of 2′-nucleotidase was determined by two assay methods. Methods 1 and 2 measure the liberation of P_i and adenosine from 2′-AMP, respectively. For method 1, the reaction mixture contained 1 μmol 2′-AMP, 50 μmol Tris-acetate (pH 8.0), 2 μmol $MgCl_2$ and enzyme in a final volume of 0.5 ml. As control, 3′-AMP was substituted for 2′-AMP, since nonenzymic dephosphorylation from 2′-AMP was not detected under the standard assay condition. The reaction was carried out for 30 min at 37°C and terminated by addding 0.1 ml 25% trichloroacetic acid. The supernatant solution obtained by centrifugation was used for the determination of P_i. The unit of activity is defined as 1 μmol P_i released/min at 37°C. For method 2, the reaction was performed in the same manner as described above. The supernatant solution was neutralized with NaOH and put on a 1 ml (bed volume) column of Dowex-1 (formate form, X2, 200—400 mesh). Adenosine was eluted with 5 ml 5 mM formic acid, while 2′-AMP remained on the column. The amount of adenosine was determined from ultraviolet absorption at 260 nm (molar absorbance: 15 000).

The increase in P_i (method 1) coincided with the increase in adenosine (method 2). The reaction proceeded linearly with time up to 90 min and with

enzyme amount up to 0.02 unit.

The activity of L-aromatic amino acid decarboxylase was determined by the method of Ichiyama and Nakamura, with modifications [17]. The incubation mixture contained the enzyme, 0.1 μmol pyridoxal phosphate, 10 μmol L-[^{14}C]DOPA (20 000 cpm/μmol) and 100 μmol potassium phosphate (pH 6.6). Incubation was carried out at 37°C for 60 min and radioactive CO_2 was determined as described before [17].

Results

Subcellular distribution in rat brain

In Table I are shown 2'-nucleotidase activities of subcellular fractions of rat brain. Enzyme activity was distributed mainly in the soluble fraction, but an appreciable amount of 2'-nucleotidase activity was detected in fraction P_2. When fraction P_2 was centrifuged on a sucrose density gradient, 2'-nucleotidase activity was detected in fraction P_2S which corresponds to the synaptosomal fraction [13].

Purification of the soluble enzyme

Bovine brain was obtained at a slaughterhouse and chilled in ice. All subsequent steps were performed at 4°C. Cerebral cortex and cerebellum were removed and the remaining part of the brain chopped with a knife. 125 g brain were homogenized with 2500 ml cold 0.85% NaCl in a Waring Blendor for 1 min. The homogenate was filtered through cheesecloth and was centrifuged for 60 min at 50 000 × *g*. To the supernatant solution, 7.7 ml of 2 N acetic acid were added and the solution was brought to pH 3.5. The suspension was centrifuged immediately for 10 min at 10 000 × *g*. The supernatant solution was brought to pH 7.4 by adding 1.5 M Tris solution. The precipitate forming

TABLE I

SUBCELLULAR DISTRIBUTION OF 2'-NUCLEOTIDASE

Homogenate was centrifuged at 4°C for 10 min at 1500 × *g*, separating the precipitate (P_1). The supernatant solution was centrifuged for 20 min at 13 000 × *g* to obtain supernatant solution (S_2) and pellet (P_2). An aliquot (2.2 ml) of P_2 suspended in 40 ml 0.32 M sucrose was layered over a discontinuous sucrose density gradient containing 1.5 ml 1.2 M sucrose and 1 ml 0.8 M sucrose. After centrifugation for 60 min at 33 000 rev./min in the SW 40 rotor (Hitachi), three bands were observed; the white layer between 0.32 and 0.8 M sucrose (P_2A), suspended material over 1.2 M sucrose (P_2S) and pellet (P_2B). Each layer was separated and washed with four volumes of H_2O. Fraction S_2 was centrifuged for 60 min at 100 000 × *g* and supernatant solution (S_3) and precipitate (P_3) were obtained.

Subcellular fractions	Activity (U/mg wet wt.)	Protein (mg/g wet wt.)	Specific activity (U/μg protein)	Aromatic amino acid decarboxylase (U/μg protein)
Homogenate	115.1	177	0.65	0.95
P_1	15.4	55	0.28	0.48
P_2	13.9	37.5	0.37	0.87
P_2A	0.06	5.8	0.01	0.32
P_2S	10.4	12.2	0.85	2.28
P_2B	6.5	16.7	0.39	0.41
P_3	0.23	11.7	0.02	0.37
S_3	94.1	49.5	1.90	2.18

at 50—80% $(NH_4)_2SO_4$ saturation was prepared, dissolved in 50 ml 5 mM Tris-HCl (pH 7.0) and dialyzed twice against 5 mM Tris-HCl (pH 7.0) for 3 h each. A precipitate was removed by centrifugation and the supernatant solution was applied on a column (34 × 1.8 cm) of DEAE-cellulose equilibrated with 5 mM Tris-HCl (pH 7.0). The column was washed with H_2O, followed by 0.05 M NaCl/5 mM Tris-HCl (pH 7.0). The enzyme was eluted by applying a linear concentration gradient 0.05—0.25 M NaCl in Tris-HCl (pH 7.0). The bulk of protein was eluted by washing, but 2′-nucleotidase activity was observed in the fraction eluted with approx. 0.13 M NaCl. The enzyme solution was dialyzed against 5 mM Tris-HCl (pH 7.0) for 4 h, concentrated and then placed on a column (150 × 2.0 cm) of Sephadex G-200 equilibrated and eluted with 0.2 M NaCl in 5 mM Tris-HCl (pH 7.0). 2′-Nucleotidase activity was observed at 153—162 ml. Purification was 950-fold over the soluble fraction of brain; yield, 12% (Table II). Although the purified preparation in 0.2 M NaCl was relatively stable after storing at 0°C, a dialyzed preparation showed a loss of 2′-nucleotidase activity by 30% after storing at −20°C for 7 days. Addition of 2 mM dithiothreitol did not stabilise the activity.

Estimation of molecular weight

An estimation of molecular weight was carried out by gel filtration with Sephadex G-200. Assuming that the shape of 2′-nucleotidase is the same as that of the calibrating proteins, the position given by this enzyme corresponds to a molecular weight of about 70 000. The $s_{20,w}$ of 2′-nucleotidase was found to be 4.9 S by centrifugation on a sucrose density gradient.

Substrate specificity

The purified preparation showed phosphatase activity toward glycerol 2-phosphate, 2-phosphoglyceric acid and NADP as well as 2′-nucleotides. The ratio of activity toward glycerol 2-phosphate to that toward 2′-AMP was 0.70—0.85 throughout the purification. The purified 2′-nucleotidase did not hydrolyze 3′-nucleotides, 5′-ADP, 5′-ATP, glycerol 3-phosphate, phenylphosphate, *p*-nitrophenylphosphate, glucose 1-phosphate, glucose 6-phosphate, 6-phosphogluconate, 3-phosphoglyceric acid, glyceraldehyde 3-phosphate, dihydroxyacetonephosphate, CoA and cyclic 2′,3′-AMP. Cleavage of the phosphodiester bond of cyclic 2′,3′-AMP was not detected when the reaction mixture was analyzed by column chromatography on Dowex-1.

TABLE II

PURIFICATION OF 2′-NUCLEOTIDASE

Step	Volume (ml)	Total activity (units)	Specific activity (U/mg protein)	Yield (%)
Soluble fraction	1000	5140	0.024	100
Acid treatment	1025	4575	0.028	89
$(NH_4)_2SO_4$	50	3700	0.064	72
DEAE-cellulose	60	1440	0.917	28
Sephadex G-200	9	617	2.281	12

TABLE III

SUBSTRATE SPECIFICITY

The enzyme activity was measured using method 1 and 2 μmol of various substrates were added. Dephosphorylation of 2'-AMP was assigned a value of 100.

Substrate	Relative activity
2'-AMP	100
2'-GMP	32
2'-UMP	112
2'-CMP	25
3'-UMP	4
3'-CMP	2
Glycerol 2-phosphate	78
2-Phosphoglyceric acid	11
NADP	8

Effect of pH on activity

Maximum 2'-nucleotidase activity occurred at pH 8.5 in the presence of 0.2 M Tris-maleate and 4 mM $MgCl_2$ and at pH 8.0 in the presence of 0.1 M Tris-maleate and 10 mM $MgCl_2$ as shown in Fig. 1.

Kinetics

The reaction was assayed by method 2 and the K_m value was calculated from the double-reciprocal plot [18]. The K_m value for 2'-AMP was $5.5 \cdot 10^{-4}$ M and glycerol 2-phosphate showed a competitive inhibition on the hydrolysis of 2'-AMP ($K_i = 2.4 \cdot 10^{-3}$ M) (Fig. 2).

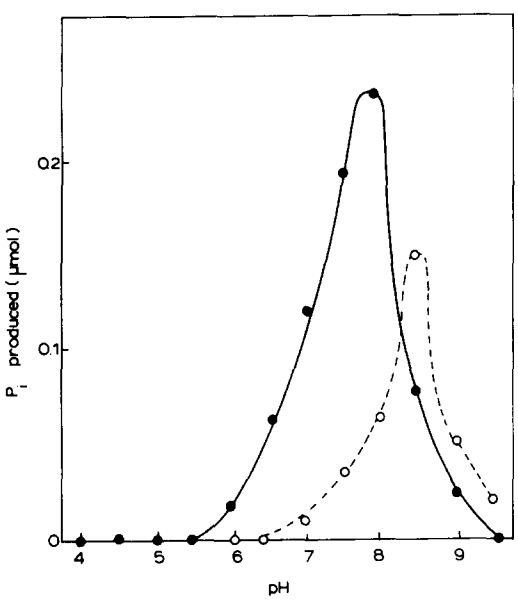

Fig. 1. Effect of pH on the activity of 2'-nucleotidase. The reaction was carried out under standard assay conditions except for 0.2 M Tris-maleate of various pH and 4 mM $MgCl_2$ (○) or 0.1 M Tris-maleate of various pH and 10 mM $MgCl_2$ (●) instead of 0.1 M Tris-acetate (pH 8.0) and 4 mM $MgCl_2$.

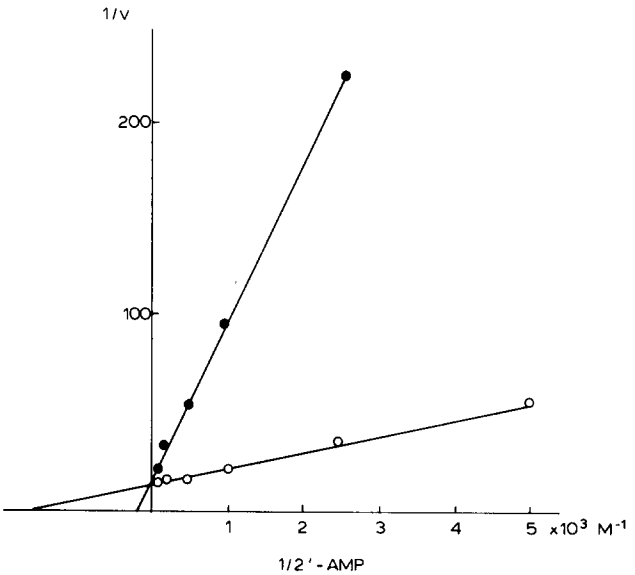

Fig. 2. Effect of concentration of 2′-AMP on 2′-nucleotidase activity. The reaction was carried out under standard assay conditions except for various amounts of 2′-AMP instead of 1 μmol 2′-AMP in the presence (●) or in the absence (○) of 2.5 μmol glycerol 2-phosphate.

Metal ions and inhibitors

2′-Nucleotidase reaction was assayed by method 2. The purified enzyme required Mg^{2+} and magnesium was partially replaced by manganese to as much as 10% (Table IV). Glycerol 2-phosphate was a competitive inhibitor to 2′-nucleotidase, but 5′-ATP, 5′-ADP, 5′-AMP, 3′-AMP, phenylphosphate, glycerol 3-phosphate or concanavalin A did not inhibit the enzyme at the concentration described in Table V. Dithiothreitol showed an inhibition on the purified 2′-nucleotidase.

Discussion

Jacob and Sontag [8] reported a 2′-nucleotidase from the cytoplasm of *H. brasiliensis*. The 2′-nucleotidase in mammalian brain differs from that in plant

TABLE IV

REQUIREMENT OF METALS

Assays were performed according to assay method 2, except 4 mM $MgCl_2$.

Compounds added	Concentration (mM)	Relative activity
None		0
$MgCl_2$	4	100
	2	93
	1	53
	0.5	16

TABLE V

EFFECT OF VARIOUS COMPOUNDS ON 2′-NUCLEOTIDASE ACTIVITY

Assays were performed according to assay method 2. Various compounds were added at the concentrations as indicated.

Compounds	Concentrations (mM)	Relative activity
None		100
5′-ATP	5	102
5′-ADP	5	110
5′-AMP	5	97
3′-AMP	10	98
	5	101
Adenosine	5	100
Phenylphosphate	10	105
	5	96
Glycerol 2-phosphate	5	37
	2	62
Concanavalin A	1 *	123
	0.1 *	106
Dithiothreitol	2	71
$MnCl_2$	4	23
$CaCl_2$	4	4
$FeCl_3$	4	96

* Concentration in mg/ml.

serum by the following: (i) Purified brain 2′-nucleotidase exhibited a low activity toward NADP, while the plant enzyme showed 6-time more activity towards NADP than towards 2′-AMP. The plant enzyme might be more appropriately named NADP phosphatase. (ii) Maximum activity was observed at pH 8.0—8.5 with the mammalian enzyme and at pH 5.4 with the plant enzyme. (iii) The molecular weight of the brain enzyme was 70 000, whereas that of the plant enzyme was 115 000—120 000. (iv) The 2′-nucleotidase from mammalian brain was not inhibited by 5′-ADP, 5′-ATP or adenosine, all of which inhibited the enzyme from plant source.

Purified brain 2′-nucleotidase catalyzed the hydrolysis of phosphomonoester bond of 2′-nucleotides and the highest activitity was observed when 2′-UMP was used as substrate. The purified preparation did not hydrolyze 5′-nucleotides or 3′-nucleotides which are hydrolyzed by acid nucleotidase in lysosomes [6,7]. Cyclic phosphodiesterase from microorganisms shows 3′-nucleotidase activity [19,20]. Neither 2′-AMP nor 3′-AMP were produced when cyclic 2′,3′-AMP was incubated with the purified 2′-nucleotidase preparation and the incubation mixture analyzed as described above.

The results on substrate and inhibitor specificities suggest that 2′-nucleotidase catalyzes the hydrolysis of glycerol 2-phosphate or 2-phosphoglyceric acid, but possible contamination of specific glycerol 2-phosphatase cannot be eliminated, since the enzyme has not been yet purified to a single protein.

Appaji Rao et al. [21] reported that a specific glycerol 2-phosphatase from mung beans is activated by Fe^{3+}. Glycerol 2-phosphatase activity in brain was not enhanced by the addition of Fe^{3+}.

Although 5′-nucleotidase in plasma membrane [3] or in the soluble fraction [2] and 2′-nucleotidase from plant cytoplasm [8] were inhibited competitively by 5′-ADP, 5′-ATP, adenosine or 3′-AMP, the activity of 2′-nucleotidase from mammalian brain was not influenced by the addition of 5′-ADP, 5′-ATP, adenosine or 3′-AMP. It is worth noting that 2′-AMP was not an inhibitor to 5′-nucleotidase from plasma membranes and 5′-AMP was not an inhibitor to 2′-nucleotidase. 5′-Nucleotidase was inhibited by concanavalin A [22,23], but 2′-nucleotidase was not affected by this lectin.

Most 2′-nucleotidase activity was found in the soluble fraction; the activity detected in fraction P_2S cannot be explained by contamination of the soluble fraction. 2′-Nucleotidase is distributed both in the soluble and synaptosomal fractions, which resembles the distribution pattern of L-aromatic amino acid decarboxylase in the brain [24]. It is unlikely that 2′-nucleotidase is localized in lysosomes and the activity in the soluble fraction is derived from ruptured lysosomes since acid phosphatase activity was very small both in the soluble fraction (S_3) and in the fraction P_2S.

The physiological role of 2′-nucleotidase in the brain is still obscure, since the presence of free 2′-nucleotide has not been reported in the brain as far. However, it seems possible that 2′-nucleotide produced by cyclic 2′,3′-AMP 3′-phosphohydrolase [9,10] is dephosphorylated by a specific 2′-nucleotidase to adenosine, thus participating in RNA metabolism in the brain.

This specific 2′-nucleotidase will provide a useful tool for biochemists who are engaged in determining the position of phosphate in ribonucleotide or assaying cyclic 2′,3′-AMP 3′-phosphohydrolase.

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