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THIAMINE PYROPHOSPHATASE AND NUCLEOSIDE DIPHOSPHATASE IN RAT BRAIN

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SUMMARY: Two types of nucleoside diphosphatase were found in rat brain. One (Type L) had similar properties to those of the liver microsomal enzyme with respect to its isoelectric point, substrate specificity, Km values, optimum pH, activation by ATP and molecular weight. The other (Type B), which separated into multiple forms on isoelectric focusing, had lower Km values and a smaller molecular weight than the Type L enzyme, and was inhibited by ATP. The Type B enzyme catalyzed the hydrolysis of thiamine pyrophosphate as well as those of various nucleoside diphosphates at physiological pH, while Type L showed only nucleoside diphosphatase activity at neutral pH. These findings suggest that the two enzymes play different physiological roles in the brain.

Thiamine pyrophosphatase (TPPase) and nucleoside diphosphatase (NDPase) catalyze the hydrolyses of the terminal phosphates of thiamine pyrophosphate (TPP) and nucleoside diphosphate (NDP), respectively. Histochemical studies on rat brain have shown that TPPase activity is predominantly present in neurons, while NDPase activity is abundant in glial cells (1,2), suggesting that TPPase and NDPase are different enzymes. On the other hand, biochemical studies on liver and brain have demonstrated that TPPase is identical with microsomal NDPase and that it is activated in the presence of ATP (3,4). Therefore, we investigated whether the hydrolyses of TPP and NDP in the brain are catalyzed by the same enzyme protein. During this investigation, we found that rat brain contains two types of enzyme, which are separable by DEAE-cellulose column chromatography. This paper reports the separation and properties of these two types of enzyme.

MATERIALS AND METHODS

Enzyme assay; TPPase and NDPase were assayed with TPP and inosine diphosphate (IDP), respectively, as substrates. The standard assay system contained 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 4 mM substrate and enzyme solution in a final volume of 0.5 ml. After incubation for 20 min at 37° C, the reaction was stopped by adding 0.5 ml of ice-cold 10 % trichloroacetic acid. After removal of coagulated proteins by centrifugation, inorganic phosphate (Pi) was measured

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by the method of Heinonen and Lahti (5). One unit of enzyme activity was defined as the amount which produced 1 μmole of Pi per min under the standard assay conditions. Protein was determined by the methods of Lowry et al. (6) and Bradford (7) before and after enzyme solubilization, respectively.

Subcellular fractionation; Male Wistar strain rats (250-300 g) were killed by decapitation and their brains were homogenized in cold 0.32 M sucrose. The homogenate was passed through 8 sheets of gause and subjected to differential centrifugation to yield a crude nuclear fraction (1,000 x g, 10 min), a crude mitochondrial fraction (10,000 x g, 15 min), a microsomal fraction (80,000 x g, 60 min) and a supernatant fraction. NDPase activity was highest in the microsomal fraction (203 mU/mg) like that of the liver enzyme. On the other hand, TPPase activity was high in the mitochondrial fraction (9.4 mU/mg) as well as the microsomal fraction (12.7 mU/mg). Therefore, we used a mixture of the crude mitochondrial and microsomal fractions for further studies.

Isoelectric focusing; Isoelectric focusing in a polyacrylamide gel column was carried out, as described previously (8). Enzyme activity was stained by a modification of the cytochemical method of Novikoff and Goldfischer (9). After electrophoresis, the gels were washed 4 times with 0.2 M Tris-maleate (pH 7.2) for 10 min periods to allow equilibration with the solution. Then they were incubated at 37° C for 2 h in incubation medium containing 2 mM substrate, 0.1 M Tris-maleate (pH 7.2), 0.12 % $Pb(NO_3)_2$ and 5 mM $MgCl_2$. After incubation, the gels were rinced 3 times with 10 mM Tris-Maleate buffer (pH 7.2) and shaken slowly in the same buffer overnight. They were then immersed in 1 % $(NH_4)_2S$ to convert the band of lead phosphate to a detectable band of PbS. Isoelectric points were determined with pI marker proteins (Pharmacia).

Measurement of molecular weight; Molecular weight was determined by Sepharose CL-6B column chromatography. The column (2 x 90 cm), previously equilibrated with 20 mM Tris-HCl (pH 8.0) containing 0.1 M KCl, was eluted with the same buffer at a flow rate of 0.8 ml/min. The marker proteins used were cytochrome c (12,500), ovalbumin (68,000), aldolase (150,000) and ferritin (450,000).

RESULTS

Separation of two types of enzyme; The brain homogenate in 0.32 M sucrose was centrifuged at 1,000 x g for 10 min and the resulting supernatant was centrifuged at 65,000 x g for 90 min. The pellet was washed with 20 mM Tris-HCl (pH 8.0) containing 1 mM PMSF by centrifugation (65,000 x g, 90 min) and suspended in 20 mM Tris-HCl, 10 % glycerol and 0.25 % Triton X-100 at a protein concentration of 3.5 mg/ml. The suspension was stirred for 30 min and centrifuged at 65,000 x g for 90 min, and the supernatant was adjusted to 0.1 M KCl and then applied to a column of DE-52 (Whatman), previously equilibrated with 20 mM Tris-HCl (pH 8.0) and 0.1 M KCl. Unadsorbed proteins were removed by washing the column with the equilibrating buffer, and then material was eluted with a linear gradient of 0.1 to 0.35 M KCl in 20 mM Tris-HCl (pH 8.0). As shown in Fig. 1, two enzyme fractions with NDPase and TPPase activities were obtained from the DE-52 column. The unadsorbed fraction showed similar activities of the two enzymes, whereas the adsorbed fraction showed high NDPase

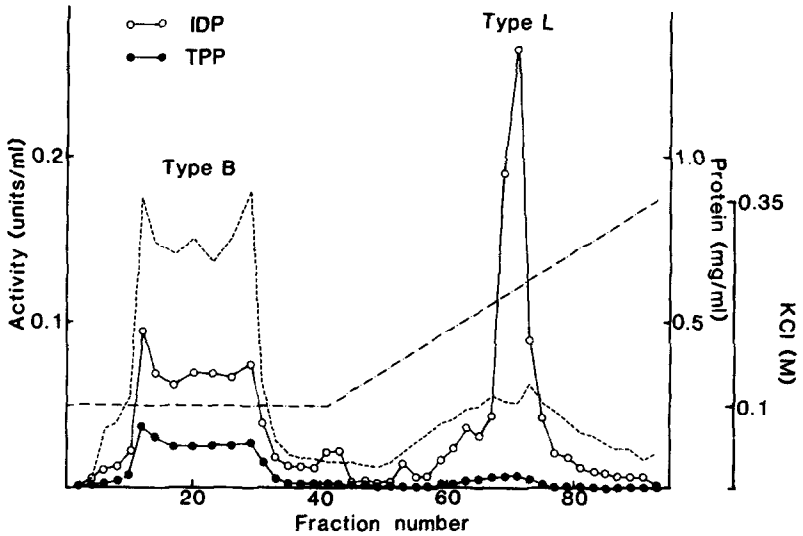

Fig. 1. DE-52 column chromatography. The enzyme preparation, solubilized with 0.25 % Triton X-100 from the microsomal and mitochondrial fractions, was applied to a DE-52 column (2 x 24 cm) as described in the text. o——o: NDPase activity. ●——●: TPPase activity. - - - - - - - : protein concentration. -·-·-·-: KCl concentration.

activity but only a little TPPase activity under the standard assay conditions. We named these two types of enzyme Type B and Type L, respectively, because Type B enzyme seemed to be specific for the brain whereas Type L resembled the liver enzyme, as described later.

Isoelectric points; Type L enzyme gave a single band at pH 4.6 and this pI value was similar to that of the liver microsomal enzyme (10). On the other hand, Type B enzyme gave 9 bands between pH 5.4 and 7.1, the bands with higher pI values being more intense, as shown in Fig. 2. When GDP, UDP, CDP or TPP was used instead of IDP as a substrate, 9 bands were again seen, indicating that all the enzyme fractions on the gel had the same substrate specificity. No bands of either Type L or Type B enzyme were detected when ADP or dTDP was used as substrate.

Substrate specificity; As shown in Table I, Type B enzyme was much more effective than Type L in catalyzing the hydrolysis of TPP. Like the liver microsomal enzyme (3), Type L enzyme hydrolyzed IDP, GDP and UDP well, but did not hydrolyze ADP. Type B enzyme also hydrolyzed IDP and GDP quite efficiently, but hydrolyzed UDP and CDP only slowly. Neither type of enzyme hydrolyzed thiamine monophosphate (TMP).

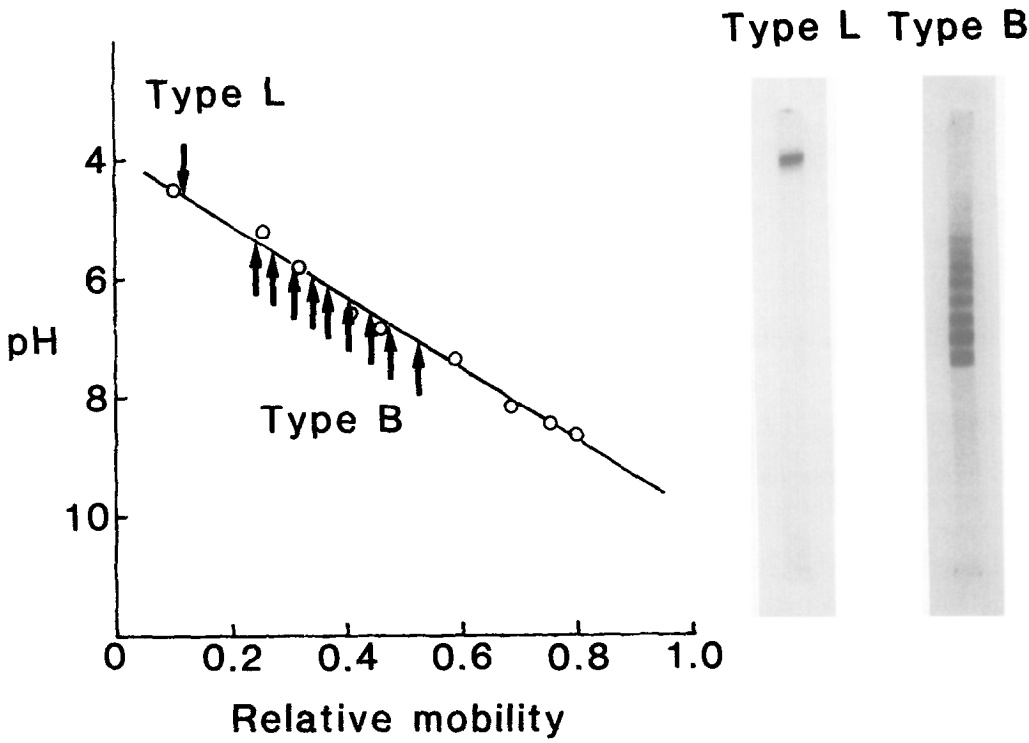

Fig. 2. Isoelectric focusing in polyacrylamide gel. Type B and L enzymes were prepared by DE-52 column chromatography and ammonium sulfate fractionation. In this case, however, the enzyme preparation solubilized by ultrasonic vibration (20 kHz, 20 W) for 2 min was used for DE-52 column chromatography. About 5 mU of the enzyme was loaded on a polyacrylamide gel column, and activity with IDP as substrate was stained. The nine bands of Type B enzyme correspond to pI 5.4, 5.6, 5.8, 6.0, 6.1, 6.3, 6.6, 6.8 and 7.1, respectively.

Km values; The Km values for substrates were determined from Lineweaver-Burk plots (11). The Km values of NDPase activity with IDP were 4.2 mM for Type L and 0.69 mM for Type B. The Km values with TPP were 17 mM for Type L and 2.0 mM for Type B. Thus the Type B enzyme showed lower Km values than the Type L enzyme with both substrates. Type B may be mainly responsible for the TPPase activity in the brain, since the Type L enzyme had a higher Km value for TPP

Table I. Substrate specificities of the two types of enzyme. Enzyme activities were determined with 4 mM substrate and values are shown relative to that with IDP.

Substrate	IDP	GDP	UDP	CDP	dTDP	ADP	TPP	TMP
Type B	100	95	59	43	0	0	64	0
Type L	100	95	88	8	14	0	4	0

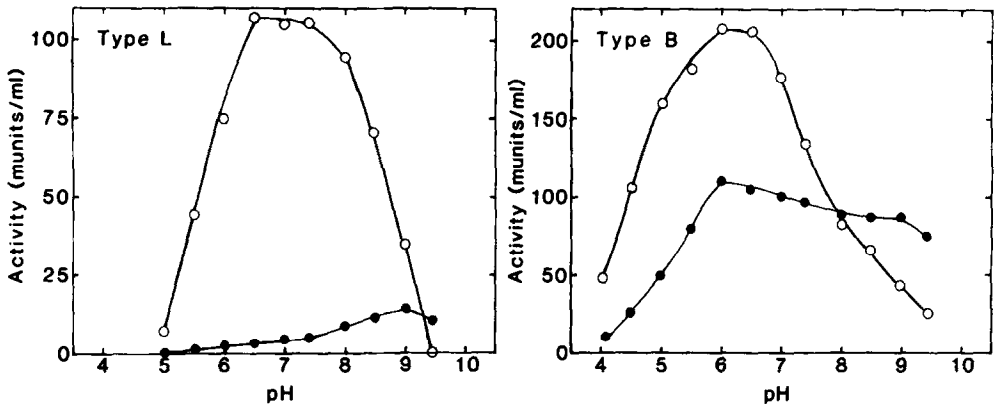

Fig. 3. Effect of pH on the enzyme activities. NDPase and TPPase activities were measured at various pH values with sodium acetate buffer (pH 4.0 - 5.5), MES-NaOH buffer (pH 5.5 - 7.4) and Tris-HCl buffer (pH 7.4 - 9.4). o——o: NDPase activity. ●——●: TPPase activity.

and showed little TPPase activity at a physiological pH.

Effect of pH; The optimum pH of the Type L enzyme activity was 6.5 with IDP and 9.0 with TPP, as shown in Fig. 3. This pH-dependency of the activity was similar to that of the liver microsomal enzyme (3). On the other hand, the optimum pH of the Type B enzyme was 6.0 with both IDP and TPP. In addition, the Type B enzyme showed considerable TPPase activity throughout the pH range examined, whereas the Type L enzyme showed TPPase activity only in the alkaline region.

Effect of ATP; The Type L enzyme activities with IDP and TPP were both markedly enhanced by ATP like those of the liver microsomal enzyme (3); addition of 0.2 mM ATP increased the NDPase and TPPase activities about 2-fold and more than 10-fold, respectively, at substrate concentrations of 0.5 mM. On the contrary, the Type B enzyme activity was inhibited by ATP; about 0.07 mM ATP inhibited the activity 50 %.

Molecular Weight; The molecular weights of the Type L and Type B enzymes were estimated as 140,000 and 60,000 by gel filtration. The value for the Type L enzyme is similar to that of 130,000 reported by Ohkubo et al. (10) for the enzyme from rat liver microsomes.

DISCUSSION

Yamazaki and Hayaishi (3) reported that NDP and TPP are hydrolyzed by the same enzyme in the liver. However, two types of enzyme have been demonstrated

histochemically in the nervous system (1,2). In addition, TPPase activity from brain showed two pH optima, while those from liver and kidney showed only one in an alkaline region (12). Therefore, the enzyme with an acidic pH optimum seems specific to brain (13,14). In this paper, we demonstrated two types of enzyme in rat brain and separated them by DEAE-cellulose column chromatography (Fig. 1).

The isoelectric point of the Type L enzyme was 4.6, like that of the liver microsomal enzyme. Ohkubo et al. (10) reported a pI value of 4.85 for the liver enzyme, but in our experiments the brain Type L and the liver enzymes were electrofocused at the same position of pH 4.6. On the other hand, the Type B enzyme consisted of multiple forms with different pI values of between 5.4 and 7.1 (Fig. 2). On treatment with neuraminidase, these multiple forms showed a tendency to converge to a form(s) with a neutral pI (data not shown), suggesting that the differences in pI values are due to differences in sialic acid contents.

Histochemical studies (1,2,15,16) demonstrated that TPPase is mainly located in the Golgi apparatus, axons, synaptic membranes and synaptic vesicles of neurons. It was also reported (1,2) that the distribution of NDPase activity is similar to that of TPPase activity, but that glial cells contain higher activity of NDPase than of TPPase. In view of these histochemical findings, it seems likely that the Type B enzyme may exist predominantly in neurons while the Type L enzyme is mainly present in glial cells.

Itokawa and Cooper (17) reported that thiamine is released from nervous tissues by various stimuli and that its release is due to dephosphorylation of thiamine phosphate ester. Therefore, the Type B enzyme may participate in nervous activity by catalyzing dephosphorylation of TPP. However, the physiological roles of the two types of enzyme require further investigation.

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