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Purification and characterization of thiamine triphosphatase from bovine brain

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Soluble thiamine triphosphatase (EC 3.6.1.28) of bovine brain has been purified 68000-fold to an electrophoretically homogeneous state with an overall recovery of 5.5% by hydrophobic chromatography on Toyopearl HW-60, Sephadex G-75 gel filtration, DEAE-Toyopearl 650M chromatography and Blue Sepharose CL-4B chromatography. The enzyme has an absolute specificity among thiamine and nucleoside phosphate esters for thiamine triphosphate and shows no nonspecific phosphatase activities. Thiamine triphosphatase is composed of a single polypeptide chain with molecular mass of 33900 kDa as estimated by Sephadex G-100 gel filtration and SDS-polyacrylamide gel electrophoresis. The enzyme has a pH optimum of 8.7 and is dependent on divalent metal ions. Mg^{2+} has been found to be the most effective among cations tested. A study of the reaction kinetics over a wide range of thiamine triphosphate concentrations has revealed a biphasic saturation curve being described by higher-degree rational polynomials.

Introduction

Thiamine triphosphate has been shown to be present in microorganisms, plants, and a wide variety of animal tissues [1–4], however its physiological function remains obscure. It is assumed that ThTP plays a role in nerve conduction and transmission, and these processes are accompanied by the dephosphorylation of this derivative of vitamin B_1 [5–7]. Little is known about the enzymes of ThTP hydrolysis. The first evidence for the existence of specific hydrolases for ThTP came to light in 1972 when Hashitani and Cooper [8] described a cytosolic enzyme activity catalyzing the hydrolysis of thiamine triphosphate and Barchi and Braun [9] reported a membrane-associated ThTPase activity in rat tissues. Soluble ThTPase from rat brain was partially purified [8]. The purified enzyme was specific for ThTP among thiamine phosphate compounds, hydrolyzing the triphosphate to ThDP and P_i, but exhibited a slight activity toward nucleoside triphosphates. Penttinen and Uotila using a polyacrylamide gel electrophoresis technique separated ThTPase activity of various rat tissues from alkaline phosphatase, acid phosphatase and other nonspecific phosphatase activities [10]. The partially purified ThTPase from brain and liver was highly specific for ThTP. ThTPase activities were also detected in *Escherichia coli* [11] and in the main electric organ of *Electrophorus electricus* [12]. Membrane-bound triphosphatase of *E. coli* was purified to a homogeneity; the enzyme had a broad substrate specificity [13].

The aim of the present study was to purify ThTPase from bovine brain and to study some its properties and substrate specificity.

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Materials and Methods

Chemicals

Sephadex G-75, Sephadex G-100 and Sepharose CL-4B were from Pharmacia, Uppsala, Sweden; DEAE-Toyopearl 650M and Toyopearl HW-60 were from Toyo Soda Co., Tokyo, Japan; Cibacron Blue F3GA, thiamine diphosphate, Coomassie Brilliant Blue R-250 and G-250, 2-mercaptoethanol, cytochrome *c*,

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Abbreviations: ThTPase, thiamine triphosphatase; ThTP, thiamine triphosphate; ThDP, thiamine diphosphate; ThMP, thiamine monophosphate.

myoglobin, ovalbumin, chymotrypsinogen A were from Serva, Heidelberg, Germany; thiamine monophosphate and sodium dodecyl sulfate were from Fluka, Buch, Switzerland; all other chemicals were from Reanal, Budapest, Hungary. Thiamine triphosphate was synthesized as described by Chernikevich et al. [14]; Cibacron Blue F3GA-Sepharose CL-4B was prepared by the method of Dean and Watson [15].

Assay of enzyme activity

The reaction mixture (1 ml) contained 50 mM Tris-HCl (pH 8.7), 5 mM $MgSO_4$, 0.15 mM ThTP and 0.37 ng of the purified enzyme or an aliquot of each fraction which showed the activity corresponding to that of 0.37 ng of the purified enzyme. Incubation was carried out at 37 °C for 20 min and the reaction was terminated by dilution of the reaction mixture with 4 ml of 0.5 M sodium phosphate buffer (pH 6.7). The amount of ThDP formed was determined enzymatically after recombination of 0.1 ml aliquot of the incubation mixture with apopyruvate decarboxylase from brewer's yeast at pH 6.8 for 30 min [16]. The activity of holopyruvate decarboxylase produced was measured by the decrease of NADH absorbance in the presence of alcohol dehydrogenase, recording the changes of optical density at 340 nm. The standard graph was prepared using chromatographically pure ThDP preparation. One unit of the enzyme was defined as the amount of enzyme catalyzing the formation of 1 μmol of ThDP per s. Specific activity was expressed in units per mg of protein.

Determination of substrate specificity and phosphatase activities

The reaction mixture for determination of substrate specificity contained 50 mM Tris-HCl (pH 8.7), 5 mM $MgSO_4$, 18 ng of the purified enzyme and phosphate compounds at concentrations both 0.15 mM and 3 mM. Inorganic phosphate liberated during 60 min incubation at 37 °C was determined by the method of Sapru et al. [17]. ATPase, nucleoside phosphatases and thiamine phosphatases were assayed in the same manner except that both pHs 7.5 and 9.0 were employed, and nucleoside phosphates and thiamine phosphates served as substrate at 5 mM.

Electrophoresis and molecular mass determination

Native gel electrophoresis was performed as described by Davis [18] in 10% polyacrylamide gel at pH 8.9. Denaturing disc electrophoresis in the presence of sodium dodecyl sulfate was performed on 15% polyacrylamide gels according to the method of Weber and Osborn [19]. Before electrophoresis, the samples were boiled for 20 min in 20 mM sodium-phosphate buffer (pH 7.2), 2% SDS, 5% marcaptoethanol. For protein detection, the gels were stained with Coomassie Brilliant Blue R-250. Standard proteins used for estimation of molecular mass were cytochrome *c* from horse heart (12400), hemoglobin from horse blood (15500), chymotrypsinogen A from bovine pancreas (25000), pyruvate kinase from rabbit muscle (57000) and bovine serum albumin (67000). The migration distance of each protein was measured relative to that of Bromphenol blue.

The molecular mass of the native enzyme was determined by gel filtration as described by Andrews [20] on a Sephadex G-100 superfine column (1.8 × 90 cm) equilibrated with 25 mM Tris-HCl buffer (pH 7.5), 0.1 M NaCl and calibrated with the following standards: cytochrome *c* (12400), myoglobine equine from skeletal muscle (17800), ovalbumin (45000) and bovine serum albumin (67000). The elution volume (V_e) of each fraction was determined from the absorbance at 280 nm and the enzyme activity. The void volume (V_0) was determined by dextran blue exclusion volume. ThTPase molecular mass was calculated by plotting the log molecular mass versus the log V_e/V_0 ratio.

Other methods

Protein was determined according to the method of Bradford [21] with bovine serum albumin as a standard or from the absorbance at 280 nm as described by Layne [22]. Analysis of the reaction product was performed by HPLC [23] with a slight modification.

Results

Purification of the enzyme

All purification steps were carried out at 4 °C.

Step 1. Crude extract. 1 kg of freshly prepared bovine brain was homogenized with a blender for 1 min in 2000 ml of 25 mM Tris-HCl (pH 7.5), containing 0.2 mM EDTA and 0.15 M KCl. The mixture was centrifuged at 5000 × *g* for 60 min. The resulting supernatant was used as the crude extract.

Step 2. Toyopearl HW-60 hydrophobic chromatography. Solid $(NH_4)_2SO_4$ was added to the extract with constant stirring to give 30% saturation. After 30 min the resulting precipitate was removed by centrifugation at 5000 × *g* for 30 min and discarded. The resulting supernatant was passed through a column (3.5 × 20 cm) of Toyopearl HW-60 equilibrated with 30% saturation $(NH_4)_2SO_4$ in 25 mM Tris-HCl buffer (pH 7.2), containing 0.2 mM EDTA and 50 mM NaCl. After the column was thoroughly washed with the same buffer, elution was carried out with a 600 ml linear gradient of $(NH_4)_2SO_4$ (30 to 0% saturation) in the same buffer at a flow rate of 40 ml/h. The active fractions eluted at approx. 0.49 M $(NH_4)_2SO_4$ were combined and adjusted to 50% saturation, the precipitate was collected by centrifugation.

Step 3. Sephadex G-75 gel filtration. The precipitate from Step 2 was dissolved in 25 mM Tris-HCl (pH 7.4), containing 0.2 mM EDTA, 50 mM NaCl and placed on a column (2.5 × 110 cm) of Sephadex G-75 equilibrated with the same buffer. Elution was performed with the above buffer solution at a flow rate of 60 ml/h. Fractions with the highest activity were combined and glycerol was added to make a final concentration of 20%.

Step 4. DEAE-Toyopearl 650M chromatography. The combined fractions were applied to a column (1.3 × 8 cm) of DEAE-Toyopearl 650M equilibrated with 25 mM Tris-HCl (pH 7.4), containing 0.2 mM EDTA, 50 mM NaCl and 20% glycerol. After washing the column elution was carried out with a 300 ml linear gradient of NaCl (50 to 400 mM) in the same buffer at a flow rate of 18 ml/h. Fractions showing the highest enzyme activity (eluted at approx. 73 mM NaCl) were pooled.

Step 5. Cibacron Blue F3GA-Sepharose CL-4B chromatography. The DEAE fraction was dialyzed against 1.5 l of 10 mM Tris-maleate buffer (pH 6.5), containing 0.2 mM EDTA, 50 mM NaCl and applied to a Cibacron Blue F3GA Sepharose CL-4B column (0.5 × 3 cm) equilibrated with the same buffer. After the column was washed, the enzyme was eluted with a linear gradient of pH and glycerol formed from 15 ml of the above buffer in the mixing chamber and 15 ml of 25 mM Tris-HCl (pH 8.1), containing 0.25 mM EDTA, 50 mM NaCl and 40% glycerol. The elution was carried out at a flow rate of 5.6 ml/h. Figure 1 shows the elution profile of protein and enzyme activity. Fractions with the highest specific activity were combined and stored at −20 °C.

TABLE I

Purification of thiamine triphosphatase from bovine brain

Step	Protein (mg)	Activity (μmol $\cdot s^{-1}$)	Specific activity (μmol $\cdot s^{-1} \cdot mg^{-1}$)	Purification (fold)	Yield (%)
Crude extract	18868	3.01	$1.6 \cdot 10^{-4}$	–	100
Hydrophobic chromatography on Toyopearl HW-60	141.4	1.03	$7.3 \cdot 10^{-3}$	46	34
Sephadex G-75	10.9	0.71	$6.5 \cdot 10^{-2}$	405	23
DEAE-Toyopearl 650 M	0.24	0.27	1.12	7000	8.9
Blue Sepharose	0.015	0.17	10.8	68000	5.5

The results of the purification procedure are summarized in Table I. By this procedure thiamine triphosphatase was purified 68000-fold with a yield of 5.5%. The specific activity of the purified enzyme was 10.8 μmol $\cdot s^{-1} \cdot mg^{-1}$.

Purity of the enzyme preparation

In disc electrophoresis using Tris-HCl buffer (pH 8.9), the purified enzyme was found to show a single band which migrated to the anode, no traces of contaminating proteins were detected (Fig. 2A). After electrophoresis at 4 °C one gel was stained and the

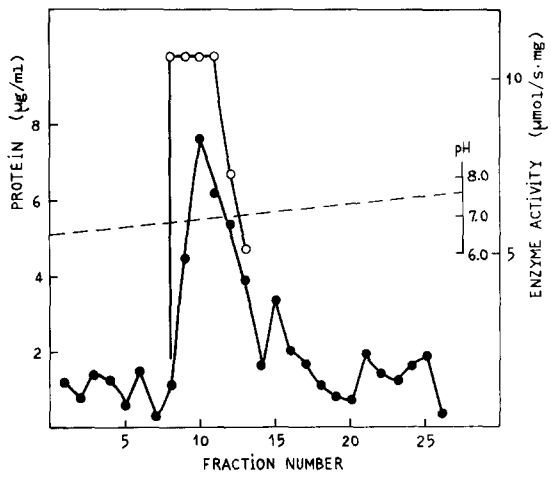

Fig. 1. Elution pattern of Blue Sepharose CL-4B chromatography. On a column (0.5×3 cm) of Blue Sepharose CL-4B the sample eluted from DEAE-Toyopearl 650M was applied after dialysis against 10 mM Tris-maleate buffer (pH 6.5), containing 50 mM NaCl, 0.2 mM EDTA. Elution was accomplished by a linear gradient of pH (from pH 6.5 to pH 8.1) and glycerol (0-40%) (only pH is shown) at a flow rate of 5.6 ml/h. About 0.8 ml fractions were collected. (●——●) Protein concentration, (○——○) enzyme activity, (— — —) pH-gradient.

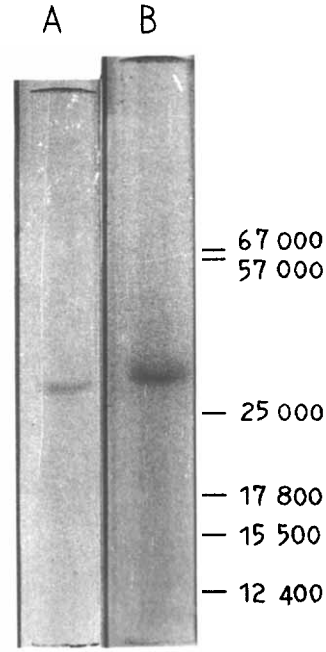

Fig. 2. Disc electrophoresis of purified ThTPase. A. Native gel electrophoresis in 10% polyacrylamide gel. B. Denaturing gel electrophoresis in 15% polyacrylamide gel in the presence of sodium dodecyl sulfate. The migration of molecular mass standards is indicated.

other was cut into 2 mm widths to extract the enzyme from the gel. Each fragment was homogenized in 25 mM Tris-HCl (pH 7.5), containing 50 mM NaCl, 0.2 mM EDTA and then centrifuged. The enzyme activity determined in the supernatant was found to be consistent with the location of the stained band. Purified ThTPase also did not show other phosphatase activities.

Molecular mass

Gel filtration of the purified ThTPase on Sephadex G-100 revealed a single zone containing all the enzymatic actiivty. The molecular mass of the enzyme obtained from its elution behaviour from Sephadex G-100 column (1.8 × 90 cm) was 33350. Electrophoresis of the denatured enzyme in polyacrylamide gel containing sodium dodecyl sulfate also revealed a single band (Fig. 2B) corresponding to a molecular mass of 33900, thus showing the absence of subunit structure.

Stability and storage

The enzyme was stable when prepared and stored in the absence of protective reagents for SH groups. Addition of exogenous sulfhydryl compounds such as dithiotrietol, 5 mM, or β-mercaptoethanol, 20 mM, did not effect on stability of the enzyme. Essentially full activity of ThTPase was retained for about 2 wk when stored at 4 °C. The enzyme was not inactivated by freezing and thawing, and kept at −20 °C for several month in 0.05 M Tris-HCl buffer (pH 7.5), without of loss of activity.

ThTPase assay requirements

The enzyme reaction showed an absolute dependence on ThTP and Mg^{2+}. The reaction proceeded linearly for at least 90 min, and the reaction velocity for 20 min was proportional to the amount of purified enzyme protein up to 10 ng.

Stoichiometry and product identification

The stoichiometry of the reaction was determined with two different concentrations of ThTP (Table II).

TABLE II

Stoichiometry of thiamine triphosphatase reaction

Purified preparation of the enzyme (7 ng) was incubated at 37 °C for 60 min in the reaction mixture containing 50 μmol of Tris buffer (pH 8.7), 5 μmol of $MgSO_4$, and either 0.15 or 0.30 μmol of ThTP in a total volume of 1.0 ml. One aliquot of the reaction mixture was assayed for ThDP and another for inorganic phosphate. Data represent the mean ± SEM of three experiments.

Experiment	ThTP added (μmol)	ThDP formed (μmol)	P_i formed (μmol)
I	0.150	0.120 ± 0.002	0.112 ± 0.004
II	0.300	0.261 ± 0.003	0.242 ± 0.004

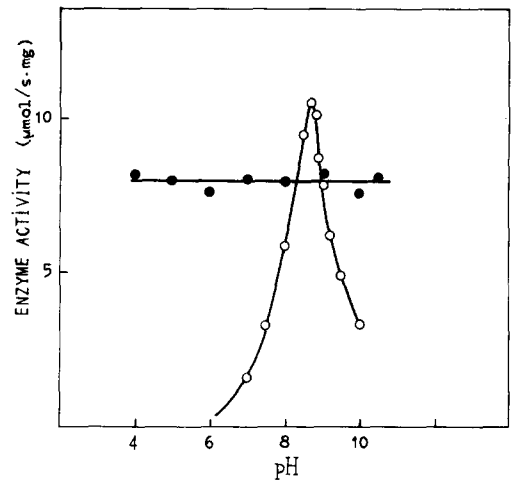

Fig. 3. Enzyme activity and stability as a function of pH. The enzyme was assayed under the standard experimental conditions except that the next buffers were used: pH 4.0–5.0, acetate (20 mM); 5.5–6.5, maleate (10 mM); 7.0, Tris-maleate (20 mM); 7.5–9.0, Tris-HCl (50 mM); 9.5–10.5, glycine (40 mM). To determine stability the enzyme was preincubated in solutions with various pH values and then assayed under the standard experimental conditions. (○——○) Enzyme activity, (●——●) stability.

The amount of ThDP detected enzymatically in the reaction mixture was in quantitative agreement with the rate of hydrolysis measured as release of inorganic phosphate. In addition, HPLC of the products of the reaction revealed that only ThDP was produced with no appearance of either ThMP or free thiamine. This indicates that the purified enzyme catalyzes the following reaction: ThTP → ThDP + P_i. The equilibrium lies far toward the right and the reaction is essentially irreversible.

Substrate specificity

Purified ThTPase showed no activity toward the next comopounds tested as substrates: ThDP, ThMP, ATP, ADP, AMP, GTP, GDP, GMP, UTP, UDP, UMP, CTP, CDP, CMP, NAD, NADP, ITP, adenosine-3′5′ cyclophosphate, guanosine-2′3′ cyclophosphate, phosphoenolpyruvate, dATP, creatine phosphate, fructose 1-phosphate.

pH optimum

The effect of variation in pH on the enzyme activity was determined over a pH range of 4.0 to 10.5 using acetate, maleate, Tris-maleate, Tris-HCl or glycine to buffer the assay mixture. ThTPase showed a sharp peak of activity at alkaline pH values and the highest activity was observed at pH 8.7 (Fig. 3). After preincubation in solutions with varying pH values for 30 min and then restoration to the initial neutral state, thiamine triphosphatase retained the activity over the range of pH from 4.0 to 10.5.

TABLE III

Effect of metal ions on ThTPase activity

The reaction mixture (1 ml) contained 50 mM Tris-HCl (pH 8.7), 0.15 mM ThTP, 7 ng of the enzyme and various cations at concentration of 5 mM. Incubation was carried out at 37 °C for 60 min and inorganic phosphate was assayed. Each value is mean ± SEM for three separate determinations.

CAtions	P_i formed (nmol)	Activity (%)
None	0	0
Mg^{2+}	120.0 ± 2.6	100
Ni^{2+}	43.1 ± 2.1	35.9
Mn^{2+}	39.2 ± 2.2	32.7
Zn^{2+}	37.6 ± 1.2	31.3
Co^{2+}	22.0 ± 1.9	18.3
Ca^{2+}	15.2 ± 0.4	12.7
Ba^{2+}	8.8 ± 0.6	7.3
Cu^{2+}	0	0
Fe^{2+}	0	0

Effect of metal ions

The purified enzyme exhibited an absolute divalent cations requirement. Mg^{2+} was the most effective among the cations tested. With Ni^{2+}, Mn^{2+} or Zn^{2+} the activity was about 30% as compared to that of magnesium, whereas the other cations exhibited little or no stimulating effect. The effect of various metal ions on the enzyme activity is shown in Table III. The studies on the effect of varying Mg^{2+} concentrations on the initial velocity showed that activation of ThTPase by Mg^{2+} did not occur when the concentration of free Mg^{2+} was below 0.1 mM (the concentrations for free Mg^{2+} were calculated using the dissociation constant for Mg^{2+}ThTP complex of $6.5 \cdot 10^{-5}$ M [24]). Figure 4 demonstrates the character of activation by free Mg^{2+} at different fixed concentrations of ThTP (only 0.05 and 0.5 mM are shown). At low ThTP concentrations of < 0.15 mM the addition of Mg^{2+} led to increasing rate of hydrolysis up to a certain optimal concentration, above this level a little decrease in activity was noted. When the ThTP concentrations were > 1.5 mM the saturation curves approximated to a hyperbola. Figure 4, inset, shows a Lineweaver-Burk plot for the enzyme with a ThTP concentration of 0.5 mM. The apparent K_m value for free Mg^{2+} under these conditions is 1.3 mM.

Effect of ThTP concentration

When the initial reaction velocity was plotted against the ThTP concentration in the range of 2 μM to 0.3 mM at a fixed saturating concentration of Mg^{2+} a hyperbolic curve was obtained. (The apparent K_m value for ThTP can be calculated to be 43 μM and the $V_{max} - 9.9$ $\mu mol \cdot s^{-1} \cdot mg^{-1}$; data not shown). However, when the concentration of ThTP exceeded 0.3 mM, the second sharp increasing in the velocity was observed. The biphasic saturation curve and the double-reciprocal plot for the enzyme over a wide range ThTP concentrations from 2 μM to 4 mM at a fixed Mg^{2+} concentration of 50 mM are depicted in Fig. 5. From these plots $S_{0.5}$ value and V_{max} may be calculated to be 0.3 mM and 19.6 $\mu mol \cdot s^{-1} \cdot mg^{-1}$, respectively. The data obtained indicate that ThTPase does not obey a classical Michaelis-Menten kinetics and has a high-degree mechanism [25].

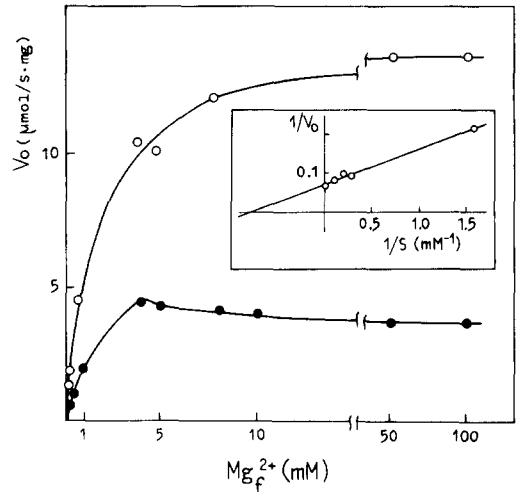

Fig. 4. Effect of varying Mg^{2+} concentrations on the initial reaction velocity. Conditions are as described under "Materials and Methods", except that the Mg^{2+} concentration was varied, and ThTP concentrations were 0.05 M (•) and 0.5 mM (○). Inset: Lineweaver-Burk plot for ThTP concentration of 0.5 mM.

Discussion

Soluble ThTPase activity was detected in various animal tissues [10,12]. Hashitani and Cooper described

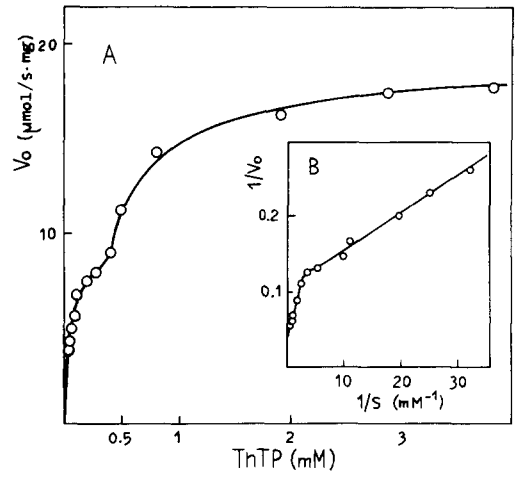

Fig. 5. Initial velocity as a function of ThTP concentration. A. Biphasic saturation curve. B. Double-reciprocal plot of the data. The enzyme was assayed under the standard experimental conditions except that the concentration of ThTP was varied at a fixed Mg^{2+} concentration of 50 mM.

the partial purification of soluble ThTPase from rat brain. Their purification procedure [8] utilized acid treatment, acetone fractionation and DEAE-cellulose chromatography to yield a final 120-fold purification with an overall recovery of 26%. The final preparation exhibited specific activity of 50 μmol of P_i per mg of protein for 30 min. Although Penttinen and Uotila successed to separate the activity of soluble thiamine triphosphatase from nucleoside phosphatase, alkaline and acid phosphatase activities in the extracts of various rat tissues, the completely purified enzyme was not obtained [10]. Until now the properties of the enzyme, such as molecular mass, subunit structure, substrate specificity and kinetic parameters have not been clear.

In this paper we described for the first time the purification of bovine brain soluble thiamine triphosphatase to electrophoretic homogeneity and data on its molecular properties.

Our preliminary experiments on ammonium sulfate fractionation of bovine brain crude extracts showed that ThTPase activity started to precipitate below 25% $(NH_4)_2SO_4$ saturation thus displaying a hydrophobic character. This observation permitted to devise the purification procedure including two steps based on hydrophobic properties of the enzyme (Step 2 and Step 5), while all our attempts to obtain a homogeneous preparation without using a hydrophobic character of ThTPase were a failure. By our procedure we could obtain 15 μg of the homogeneous enzyme from 1 kg of bovine brain in 5 steps with a final yield of 5.5%.

The purified enzyme revealed a single protein band in native polyacrylamide gel electrophoresis and the molecular mass of the native form has been estimated to be 33500 by gel filtration on Sephadex G-100. This value is in agreement with the value obtained (30000) for rat brain and liver soluble ThTPase [10]. In SDS-polyacrylamide gel electrophoresis the purified enzyme migrated as a single protein band showing the molecular mass of 33900. These results indicate that the native thiamine triphosphatase has a single polypeptide structure.

The partially purified ThTPase from rat brain and liver [8,10] had a pH optimum 8.5–9.0 and was highly specific for ThTP, but exhibited a slight activity toward some nucleoside triphosphates. We have found that soluble ThTPase from bovine brain has optimum pH at 8.7 and an absolute specificity for ThTP. Of the various nucleoside phosphates and other phosphorylated compounds tested as substrates only ThTP is utilized.

The enzyme has an absolute divalent metal ions requirement. Several divalent cations were tested for its ability to support ThTP hydrolysis and the reaction velocity was substantially higher when Mg^{2+} was used compared to any of the other cations. As ThTP has high chelating ability for Mg^{2+} one may suggest that Mg^{2+}ThTP complex is the true substrate for ThTPase [24]. However, Mg^{2+} takes part in the reaction not only as a component of $Mg^{2+}\cdot$ThTP complex. Free magnesium ions is an essential activator of the enzyme, and the reaction does not proceed with measured rate at calculated concentration of free Mg^{2+} below 0.1 mM. Activation of ThTPase by free Mg^{2+} ions has a complicated character and appears to be a function of ThTP concentration. At low fixed ThTP concentrations the maximal activation was obtained at the free Mg^{2+} concentration of 4 mM and then partial inhibition was observed. When the ThTP concentration was above 0.15 mM the saturation curves for free Mg^{2+} strived to a hyperbola.

The apparent K_m values for ThTP were reported to be 1.2 mM and 0.5 mM for rat brain and liver soluble ThTPase, respectively [8,10]. One may assume such high values were obtained because of the enzyme was assayed by inorganic phosphate production, that, owing to the low sensitivity of the method, dose not allow to measure initial rates at low ThTP concentrations. We determined the ThTPase activity by formation of ThDP enzymatically using apopyruvate decarboxylase. This method is much more sensitive (1 pmol of ThDP [26]). Our kinetic studies over a wide range of ThTP concentrations from 0.002 to 4 mM revealed a stair-step saturation curve and non-linear double-reciprocal plot (Fig. 5). In mathematical terms such behavior means that the reaction has a complex mechanism being described by higher-degree rational polynomials [25] and in this sense does not obey Michaelis-Menten kinetics. Here is should be noted that being considered in isolation both sections of the curve can be satisfactorily linearized on the basis of transformations of a hyperbolic Michaelis-Menten equation. In this case for the second section of the curve obtained at high ThTP concentrations (the situation when an initial rate may be determined by inorganic phosphate) the apparent K_m value can be calculated to be 0.3 mM. This value does not strongly differ from that for the liver enzyme [10].

We are, however, interested the enzyme behavior at low substrate concentrations because of the low value of ThTP content in the brain (0.07 nmol/g wet weight [12] in rat brain). At the low ThTP concentrations ranging from 0.002 to 0.3 mM the saturation curve for bovine brain enzyme fits to a rectangular hyperbola and results in a linear Lineweaver-Burk plot giving the apparent K_m of 43 μM. This value seems high when compared with that of ThTP concentration of 0.7 μM in bovine brain (Makarchikov and Chernikevich, unpublished data), however this concentration is global and does not exclude spatial compartmentalization of the cell. Indeed, it has been found that ThTP is predominantly localized in synaptic membrane fraction in rat brain [27] and in nerve membrane fractions [28], that leads to much higher local concentrations of this

compound. One may, however, suggest that ThTPase is never saturated with the substrate in vivo and the reaction kinetics is first order with respect to the concentration of ThTP.

It is important that an enzyme should have somewhat higher the K_m value as compared with the physiological concentration of its substrate. In such a case slight changes of the substrate concentration cause comparatively great changes of the reaction rate, that is one of the way of the regulation of the enzyme activity. Also at any low substrate concentrations slight deviations of the K_m value produce relatively large changes of the reaction rate. This considerably enhances the possibilities of metabolic control of such rates.

As already mentioned, ThTP in the brain exists mainly as a membrane-bound form, whereas we discuss here the kinetics of soluble ThTPase. At first sight it seems to be strange, however, ultracytochemical determination of the activities of phosphatases related to thiamine in rat brain showed that ThTPase activity at pH 9.2 (pH optimum for rat brain soluble enzyme [8]) was localized in the region of the plasma membrane and the synapses [29].

It permits to consider that soluble ThTPase is a peripheral protein which is responsible for ThTP hydrolysis connecting with the realization of its biological function in the brain cells. This conclusion bases on the following points. First, the soluble enzyme has more high apparent affinity for ThTP (0.04 mM) as compared with an integral membrane-bound ThTPase (1–2 mM) [9,12]. Secondly, the soluble enzyme has an absolute specificity for ThTP, that is unpossible to say about membrane-associated phosphatase [9,13].

In conclusion, although it has been established for a long time that the processes of nervous activity are accompanied by dephosphorylation of ThTP, it is still unknown at what level of nervous activity ThTP may act, and in what manner the free energy of this compound hydrolysis is used.

In this paper we indicate the existence of the specific hydrolase for ThTP in bovine brain. Further investigations of ThTPase may help to understand the role of ThTP in the nervous system.

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