A Membrane-associated Thiamine Triphosphatase from Rat Brain

PROPERTIES OF THE ENZYME

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SUMMARY

A membrane-associated thiamine triphosphatase in subcellular fractions of the rat brain is described. This enzyme is shown to be different from a less active soluble thiamine triphosphatase previously reported. It is also shown to be distinct from ATPases and from a general nucleoside triphosphatase in the tissues examined. The membrane-associated thiamine triphosphatase activity has an absolute divalent cation requirement which is satisfied by Mg\(^{2+}\), Ca\(^{2+}\), or Mn\(^{2+}\). Maximal activation occurs at a cation to substrate ratio of 1:1. At its optimal pH of 6.5, the enzyme exhibits a \(K_m\) of 1.0 to 2.0 mM and a \(V_{max}\) of 4.0 to 8.0 \(\mu\)moles per mg of protein per hour. The enzyme is heat-labile and is inactivated by low concentrations of sodium deoxycholate. ADP (10\(^{-4}\) to 10\(^{-7}\) M) strongly inhibits the enzyme. A specific inhibitor of the enzyme, the \(\beta-\gamma\)-methylene phosphonate analog of thiamine triphosphate, has been synthesized, and its inhibitory characteristics are described.

EXPERIMENTAL PROCEDURE

Thiamine Triphosphate—ThTP was obtained as a gift from the Sankyo Co. Ltd., Japan. Purity was determined by paper electrophoresis and by chromatography on Dowex 1-X-4 anion exchange resin to be greater than 98.5% ThTP. Inorganic phosphate and pyrophosphate contamination was less than 0.5%, as determined by phosphate assay and by assay with inorganic pyrophosphatase.

Subcellular Fractionation—Brain tissue was fractionated by the method of Kataoka and DeRobertis (11). Adult female Wistar rats (200 to 400 g) were killed by decapitation, and the brains were rapidly removed to cold 0.32 M sucrose. All further procedures were carried out at 0–3\(^{\circ}\). Brains were homogenized in 20 volumes of cold 0.25 M sucrose with a glass homogenizer fitted with a Teflon pestle. The homogenate was then subjected to centrifugation (900 \(\times\) g, 10 min) to yield a crude nuclear pellet. This fraction and all subsequent particulate fractions were washed three times with cold 0.25 M sucrose. The combined

1 R. Barchi, manuscript in preparation.

2 The abbreviations used are: ThTP, thiamine triphosphate; ThMP-P-C-P, \(\beta-\gamma\)-methylene phosphonate analog of ThTP; NTP, nucleoside triphosphate.
supernatants were then progressively fractionated by centrifugation to yield a crude mitochondrial fraction (7,500 x g, 20 min), a crude nerve ending fraction (20,000 x g, 30 min), and a crude microsomal fraction (100,000 x g, 60 min). Supernatants were pooled as the soluble fraction (180,000 x g supernatant). Membrane fractions were diluted with 0.25 M sucrose to protein concentrations of 0.5 to 2.0 mg/ml for enzyme assay.

**Determination of Thiamine Triphosphatase Activity**—Hydrolysis of ThTP was measured by determining the release of inorganic phosphate from the substrate. Unless otherwise indicated, the standard reaction mixture contained 2-(N-morpholino)ethane sulfonate buffer (Sigma Chemical Co.), pH 6.5 (50 mM); MgCl₂ (5 mM); ThTP (5 mM); and tissue (0.1 to 0.3 mg of protein), in a final volume of 1.0 ml. Substrate was added to initiate the reaction following a 5-min preliminary incubation at 37°. After a further 30-min incubation at 37° with shaking, the reaction was terminated by the addition of cold 5% trichloroacetic acid (0.5 ml). Aliquots of deproteinized supernant were monitored concurrently with the assay. Protein was estimated according to the procedure of Hess and Lewin (13).

**Nucleoside Triphosphate and Mg²⁺ ATPase—**NTPase activity was determined in the same manner as ThTPase with the following exceptions: Tris buffer (pH 7.5) was employed, and CTP, GTP, or UTP (5 mM) served as substrate. Mg²⁺-ATPase was also assayed at pH 7.5, with Tris-ATP (5 mM) as substrate.

**Product Identification**—Thiamine phosphate esters were separated on a column (0.5 x 25 cm) of Mallinckrodt CG-50 cation exchange resin in the H⁺ form. Deproteinized samples were extracted with ether to remove trichloroacetic acid, adjusted to pH 4.0 with sodium acetate buffer, and applied to the column. Elution with acetate buffer, pH 4.0 (5 mM), removed ThTP in the void volume; thiamine-PP was slightly retarded, whereas thiamine-P and thiamine remained on the column. Thiamine-P and thiamine were eluted with 0.1 M HCl. The column effluent was monitored at 250 nm.

**Synthesis of ThMP-P-C-P**—ThMP-P-C-P was synthesized by a modification of the method of Smith and Khorana (14) for nucleotide compounds. Modifications were necessary because of the instability of thiamine compounds at alkaline pH.

The phosphopiperidizate of thiamine-P was formed by the reaction of thiamine-P (10 mmoles) with piperazine (50 mmoles) at pH 6.5 in aqueous solution in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (20 mmoles), added in 200- mg portions over a period of 8 hours. The reaction was allowed to proceed with continuous stirring for another 12 hours or until all of the thiamine-P had reacted (as determined by paper electrophoresis). The phosphopiperidizate was recovered as the guanidinium salt by lyophilization. It was partially purified by gel filtration on a Sephadex G-10 column (3 x 20 cm). The fractions containing the product were taken to dryness by vacuum evaporation and rendered rigorously anhydrous by repeated vacuum evaporation from absolute ethanol and then from benzene. One gram of the dried product was dissolved in 100 ml of anhydrous dimethyl sulfoxide and mixed with 4 g of methylene phosphonate (as the monomethylethanolamine salt rigorously dried by repeated evaporation from ethanol and benzene) dissolved in 50 ml of the same solvent. The turbid reaction mixture was stirred for 48 hours in a tightly sealed vessel. Acetone (5 volumes) was then added to ensure complete precipitation of the product.

The precipitate was collected by centrifugation, washed with acetone and ether, dissolved in a minimal amount of water, and chromatographed on a Dowex 1-X-4 column (formate form, 2 x 25 cm). The eluate was monitored at 250 nm. The column was washed with water until no further ultraviolet-absorbing material appeared in the eluate. The ThMP-P-C-P was then eluted with a linear gradient (0 to 1 M) of ammonium formate. The peak fractions eluting between 0.6 and 0.7 M salt were combined and concentrated by vacuum evaporation. The product was salted on a Sephadex G-10 column (3 x 20 cm), and the eluted product was adjusted to pH 2 with HCl. Acetone-ether (1:1) was added until the solution became slightly cloudy. The white crystals of ThMP-P-C-P which appeared over a 24-hour period at 4° were collected, washed with ether, and dried in vacuo.

**Enzyme Unit**—One unit of ThTPase is defined as that amount which produces 1 μmole of P₁ per hour under standard assay conditions. Specific activity is expressed in units per mg of protein.

**RESULTS**

Hydrolysis of ThTP in the presence of Mg²⁺ with resultant release of P₁ was detected in the crude membrane fraction of the brain and other tissues of the rat. Preservation of activity was observed after further subcellular fractionation in the rat brain. The reaction was found to be linear with respect to protein concentration in the assay (0.05 to 0.5 mg of protein). The release of P₁ with respect to time was linear for 60 min under the conditions used in the assays.

Products of the hydrolysis reaction were determined for the two most active particulate subcellular fractions of the rat brain: the nuclear fraction and the microsomal fraction. Aliquots of each reaction mixture were deproteinized and applied to a Mallinckrodt CG-50 column. Elution profiles at 0, 15, and 30 min of reaction with the nuclear subcellular fraction are shown in Fig. 1. Similar results were obtained with the microsomal fraction. The amount of thiamine-PP detected in the reaction mixture increases linearly with time, in quantitative agreement with the rate of hydrolysis as indicated by measurement of P₁ released. We have already demonstrated that the enzymatic hydrolysis of thiamine-PP to thiamine-P and P₁ proceeds very slowly in these tissues at pH 6.5 relative to

![Fig. 1: Identification of products of the ThTPase reaction.](http://www.jbc.org/) The nuclear fraction was used under standard assay conditions at pH 6.5. Samples were removed at the indicated times during the reaction, and thiamine compounds were separated on a column of CG-50. ThDP, thiamine diphosphate.
TABLE I
Subcellular distribution in rat brain of membrane-associated and soluble ThTPases

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Membrane-associated ThTPase (pH 6.5)</th>
<th>Soluble ThTPase (pH 9.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activity</td>
<td>Activity</td>
</tr>
<tr>
<td></td>
<td>units/mg protein</td>
<td>%</td>
</tr>
<tr>
<td>Homogenate</td>
<td>1.12</td>
<td>340</td>
</tr>
<tr>
<td>Nuclear</td>
<td>4.80</td>
<td>143</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>0.45</td>
<td>37</td>
</tr>
<tr>
<td>Nerve ending</td>
<td>1.25</td>
<td>94</td>
</tr>
<tr>
<td>Microsomal</td>
<td>2.01</td>
<td>80</td>
</tr>
<tr>
<td>Soluble</td>
<td>0.30</td>
<td>7.5</td>
</tr>
<tr>
<td>Total recovery</td>
<td>301.5</td>
<td>101</td>
</tr>
</tbody>
</table>

Fig. 2. ThTPase activity as a function of pH. Membrane-associated enzyme (nuclear fraction), ○; soluble enzyme (supernatant fraction), ●.

th the ThTPase activity (15). ThTPase activity was, therefore followed in most experiments by assaying the release of Pi.

Subcellular Distribution of Thiamine Triphosphatase Activity—The optimal pH values for the soluble and membrane-associated ThTPases differ markedly (see below), and there is little overlap in their peak activity ranges. Subcellular distribution of the two activities was determined by assaying for the hydrolysis of ThTP at pH 6.5 and 9.5 in each fraction (Table I). In the crude homogenate, membrane-associated ThTPase was found to have a specific activity 34% higher than soluble ThTPase. The highest percentage (39%) of the pH 6.5 enzymatic activity was observed in the nuclear fraction of the homogenate, whereas less than 7% of the total pH 9.5 activity was found here. Conversely, only 4.7% of the pH 6.5 activity was found in the soluble fraction which contained the highest percentage (37%) of the pH 9.5 activity. A 4.3-fold increase in the specific activity of the pH 6.5 enzyme could be demonstrated when the homogenate was fractionated. The specific activity of the triphosphatase at pH 9.5, on the other hand, increased only 1.4-fold on fractionation of the homogenate.

Kinetic Parameters of Hydrolysis—Some basic properties of the membrane-associated enzyme catalyzing ThTP hydrolysis in the rat brain were then examined. Enzymatic activity in the nuclear and microsomal fractions showed a pH maximum at 6.5 (Fig. 2), although significant hydrolysis occurred over a rather broad range between pH 5.0 and 8.0. The slight activity detected in the soluble fraction, on the other hand, showed a pH optimum of 9.5, similar to that reported by Hashitani and Cooper (10) for the partially purified soluble enzyme.

The membrane-associated phosphorolytic activity exhibited an absolute divalent cation requirement which could be satisfied by Ca2+, Mg2+, and Mn2+. Plots of hydrolysis rate versus cation concentration for these ions are given in Fig. 3. Ca2+ and Mg2+ provided maximal activity in the concentration range of 3 to 5 mM. Higher concentrations (above 10 mM) showed progressive inhibition of activity. Mn2+ had very sharp activation peak at 1 to 2 mM and rapidly became inhibitory at higher concentrations.

The activation of the membrane-associated thiamine triphos-
phatase as a function of Mg\textsuperscript{2+} ion concentration was studied at a number of different substrate concentrations. Increasing substrate levels progressively shifted the Mg\textsuperscript{2+} activation curve towards higher cation concentrations (Fig. 4a). A plot of substrate concentration versus the Mg\textsuperscript{2+} concentration which yields maximal activity (Fig. 4b) indicates that an approximately 1:1 ratio of divalent cation to substrate is necessary for optimal activation of the triphosphatase.

In the absence of Mg\textsuperscript{2+}, Ca\textsuperscript{2+} (2 to 4 mM) induced significantly higher hydrolysis activity in brain particulate fractions than did Mg\textsuperscript{2+} alone. However, in the presence of optimal Mg\textsuperscript{2+} concentrations, addition of Ca\textsuperscript{2+} did not result in the expected increase in activity. For all triphosphatase assays described below, Mg\textsuperscript{2+} or Ca\textsuperscript{2+} concentrations were kept sufficiently high to be in the plateau region of their respective activation curves, but well below concentrations at which inhibition became apparent.

\( K_m \) and \( V_{max} \) values for the hydrolysis of ThTP by nuclear and microsomal brain particulate fractions were determined (Figs. 5 and 6). In the nuclear fractions, \( K_m \) and \( V_{max} \) values obtained

The ThTPase in the microsomal fraction was inhibited 35% by 0.04% sodium deoxycholate, whereas the Mg\textsuperscript{2+}ATPase in the same fraction was unaffected by this detergent at the same concentration.

Specific Inhibition of ThTPase—We synthesized the \( \beta\gamma\text{-methylene} \) analog of ThTP in an attempt to produce a specific inhibitor of ThTP hydrolysis. Since solubilization and isolation of membrane-associated enzymes is often difficult, and since
the ThTPase consistently resisted attempts at solubilization, we confirmed the substrate specificity of this enzyme by using this specific substrate analog. Fig. 7 shows the effect of increasing concentrations of ThMP-P-C-P on the hydrolysis of ThTP by brain particulate fractions. Inhibition occurred at concentrations equimolar with ThTP and increased progressively with increasing analog concentrations. Over the same concentration range, no inhibition of NTP hydrolysis was observed. In other experiments, ThMP-P-C-P, also at these concentrations, did not inhibit Mg<sup>2+</sup>-ATPase activity in subcellular fractions of homogenates. Inhibition, therefore, appears to be specific for the enzyme catalyzing phosphorolysis of ThTP.

Determinations of substrate concentrations versus rate of ThTP hydrolysis in the presence of several different concentrations of ThMP-P-C-P were carried out. The results, shown in Fig. 8, indicate a progressive increase in the $K_m$ whereas the $V_{max}$ remains constant. This kinetic behavior is indicative of a simple competitive inhibition of the ThTPase by the thiamine analog. The $K_i$ value calculated from these data is 3 mM.

Adenosine diphosphate at concentrations of 10<sup>-6</sup> to 10<sup>-3</sup> M strongly inhibits the hydrolysis of ThTP by the membrane-associated enzyme. Hydrolytic activity is decreased 50% by 5 x 10<sup>-5</sup> M ADP. This phenomenon will be described in detail in a subsequent communication. The soluble ThTPase activity was not affected by these low levels of ADP in similar experiments.

Heat Inactivation—Sensitivity of the particulate ThTPase activity to inactivation by heat was tested in the nuclear and microsomal fractions. Exposure of these fractions to a constant temperature of 50° resulted in 50% inactivation within 2 to 4 min. Ten minutes of treatment reduced ThTPase activity in the crude nuclear fraction to 2% of control levels; similar treatment of the microsomal fraction reduced activity to 27% of control value. The time course of inactivation is qualitatively similar in these two membrane fractions.

Tissue Distribution of ThTPases—The membrane-associated enzyme catalyzing the hydrolysis of ThTP was determined under optimal conditions in a number of different organs of the rat. All tissues were homogenized in 20 volumes of sucrose and centrifuged at 100,000 × g for 1 hour to produce a crude particulate fraction and a soluble fraction. Soluble ThTPase activity at pH 9.5 was also monitored, as was nonspecific NTP. Specific activity of particulate ThTPase in various organs ranged from 0.7 μmole of Pi per hour per mg of protein in skeletal muscle to 7.6 μmoles of Pi per hour per mg of protein in the small intestine. The specific activities for Mg<sup>2+</sup>-activated and Ca<sup>2+</sup>-activated ThTP hydrolysis in seven tissues of the rat are given in Fig. 9. In all tissues examined, the levels of activity for hydrolysis in the presence of Ca<sup>2+</sup> and Mg<sup>2+</sup> were comparable and Ca<sup>2+</sup>-activated phosphorolysis proceeded slightly more rapidly than Mg<sup>2+</sup>-activated phosphorolysis in the crude membrane preparation.

Specific activities determined for soluble ThTPase (pH 9.5) in these tissues show a distribution which parallels that for the particulate enzyme. Specific activity for the particulate enzyme in a given tissue was found to be 1.3 to 5 times greater than the specific activity of the soluble enzyme. The soluble activity also catalyzes ThTP hydrolysis in the presence of Ca<sup>2+</sup> as well as Mg<sup>2+</sup>, but in most tissues studied Mg<sup>2+</sup> was slightly more effective than Ca<sup>2+</sup> in stimulating this hydrolysis.

Fig. 9 also shows the observed specific activity for general nucleoside triphosphatase activity in these tissues. It can be seen that no obvious correlation exists between these values and the values obtained for ThTPase in the same tissues.

**DISCUSSION**

There appear to be at least two specific enzymes in most rat tissues which catalyze the hydrolysis of ThTP to thiamine-PP and Pi. Hashitani and Cooper (10) have reported the partial purification of a soluble ThTPase having a pH optimum of 9.5. We also have observed this activity in our 100,000 × g supernatant fractions. However, an enzyme specifically associated with
subcellular membrane fractions also exists which catalyzes the same reaction with an optimal pH of 6.5. This enzyme shows little activity at pH 9.5. The membrane-bound enzyme cannot be easily rendered soluble and loses activity rapidly when exposed to the usual solubilizing agents. In the rat brain, hydrolytic activity towards ThTP at pH 6.5 is seen in all of the particulate subfractions, but is highest in the nuclear fraction, with only a small percentage detectable in the soluble fraction. Activity in the various organs is highest in the liver, kidney, and intestine, and lowest in skeletal and heart muscle. Activity in the brain is intermediate between these extremes.

Although we have previously reported in preliminary form the existence of this enzyme and its substrate specificity (16), these earlier studies were complicated by the presence in our subfraction preparation of small amounts of inorganic pyrophosphate. The hydrolysis of this contaminant by inorganic pyrophosphatase interfered with our assay for ThTPase. The present studies reported here circumvent this problem in that pyrophosphate-free ThTP was used throughout (see "Experimental Procedure"). This is supported by the stoichiometry observed between substrate hydrolyzed and products formed.

The soluble and membrane-associated ThTPases can be differentiated in several ways. First, the membrane-associated enzyme cannot be solubilized by simple techniques with detergents and is inhibited by low concentrations of these compounds, whereas the soluble enzyme is slightly activated by low concentrations of sodium deoxycholate. Second, the pH optima of the two enzyme activities are widely separated and show little overlap. Third, the soluble enzyme is strongly inhibited by Ca$^{2+}$ at levels above $10^{-4}$ m (10), whereas the membrane-associated enzyme is activated by the same cation at levels up to $10^{-2}$ m. An earlier preliminary study (16) also indicated a strong inhibition by Ca$^{2+}$, but we now know this to be in error as a result of the contribution to the system of an inorganic pyrophosphatase which is inhibited by Ca$^{2+}$. As above, this problem is obviated by the use of pyrophosphate-free ThTP. The membrane-associated ThTPase is inhibited by low concentrations of ADP which have no effect on the rate of hydrolysis by the soluble enzyme. Finally, Hashitani and Cooper (10) have reported that the soluble enzyme is most highly active at 50°; we have found, on the other hand, that the membrane-associated activity is rapidly inactivated at temperatures above 45°.

The membrane-associated hydrolytic activity in question is specific for ThTP. The synthesis of the $\beta$-methylene phosphonate analog of ThTP has enabled us to differentiate this enzyme from other enzymes catalyzing hydrolysis of ATP and of CTP and other nucleoside triphosphates in the presence of Mg$^{2+}$. Substrate level concentrations of ThMP-P-P-P have no effect on the hydrolysis of ATP and CTP, whereas under identical conditions ThTP hydrolysis in the same subcellular fractions is inhibited by 60%. In addition, the specific inhibition of the ThTPase has been shown to be due to simple competitive inhibition as would be expected if the analog were influencing an enzyme with an active site specific for ThTP.

Further support for the specificity of the ThTPase activity can also be given. As regards its differentiation from ATPase activities present under the assay conditions used, we have shown that ThTPase activity can be progressively reduced to 60% of control levels by the addition of sodium deoxycholate at concentrations which have no effect on the ATPase activity. Conversely, ATPase activity under these assay conditions can be progressively inhibited by levels of Ca$^{2+}$ which have no inhibitory effect on the membrane-associated ThTPase. Additional support for the separation of these activities is found in their markedly different subcellular activity distribution profiles and in their differing pH optima.

Hydrolysis of ThTP by the membrane-associated enzyme requires the presence of a divalent cation. Cation concentrations necessary for maximal activity vary sharply with the substrate concentration, but not with the amount of enzyme present. In light of the 1:1 ratio of cation to substrate required for maximum enzyme activity, it is probable that the true substrate for the enzyme is the ThTP-cation complex. The sharp activation peak and rapid inactivation seen in the presence of Mn$^{2+}$ may be related to the tendency of this ion to induce aggregate formation in solutions of pyrophosphate-containing compounds (17).

The data indicating the uniqueness and specificity of the membrane-associated ThTPase and its general distribution raise the question of an as yet undetermined function for the triphosphate ester of thiamine. We have been unable to discover any peculiarities about the brain particulate ThTPase which would lead us to believe that it is uniquely different from that found in other tissues of the body. The successful synthesis of a specific inhibitor for the enzyme, the methylene phosphonate analog of ThTP, may allow us to investigate more closely the role of this enzyme in vivo.

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REFERENCES
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