ROLE OF THIAMINE METABOLISM IN THE CENTRAL NERVOUS SYSTEM

I. BASIC PROPERTIES OF THIAMINE TRIPHOSPHATASE IN RAT BRAIN

Heitaroh IWATA, Akemichi BABA and Toshio MATSUDA
Department of Pharmacology, Faculty of Pharmaceutical Sciences, Osaka University, Toyonaka, Osaka, Japan

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Abstract - The properties of soluble and microsomal thiamine triphosphatase (TTPase) in rat brain were examined. The subcellular distributions and the pH optima of these enzyme activities differ markedly. TTPase seems to be distinct from a general nucleoside triphosphatase. The TTPase activities have an absolute divalent cation requirement which is fulfilled by Mg"⁺ or Ca"⁺ in microsomes and by Mg"⁺, but not Ca"⁺, in the soluble fraction. Addition of a physiological concentration of Ca"⁺ markedly inhibited the soluble TTPase activity.

von Muralt (1) first suggested the specific involvement of phosphorylated thiamine in nerve conduction. In support of this it has been demonstrated that neuroactive agents, at concentrations affecting conduction, caused release of thiamine from nerve membranes (2, 3) and that pyrithiamine, an antimetabolite of thiamine, affected the action potential of peripheral nerves (4). However, the relationship between these phenomena and the process of dephosphorylation of phosphorylated thiamines have not been elucidated.

In previous papers we reported some properties of thiamine diphosphatase in rat brain (5-7). More recently the existence and some properties of thiamine triphosphatase (TTPase) in rat brain were reported (8, 9). The possible significance of thiamine triphosphate (TTP) in nerve tissue was suggested by the demonstration (10) that TTP was not present in the brain of patients with subacute necrotizing encephalomyelitis, a fatal disease associated with an abnormality in thiamine metabolism. However, the function of TTPase in nerve tissue has not been clarified. As a part of our studies on the role of thiamine in the function of the central nervous system, we examined the basic properties of TTPase in rat brain and its interaction with Ca"⁺. The results are described herein.

MATERIALS AND METHODS

TTP was a gift from Sankyo Co., Ltd., Tokyo. Purity was determined by paper electrophoresis (11) to be 97% TTP. GTP, ITP, UTP and ATP were obtained from Sigma Chem. Co., St. Louis. TTP and nucleotides were neutralized with tris base before use in enzyme assays.

Subcellular fractionation: Adult male Sprague-Dawley rats weighing 200-250 g were sacrificed by decapitation and the brain was rapidly removed and homogenized in
10 vol. of ice-cold 0.25 M sucrose using a glass homogenizer fitted with a Teflon pestle. The homogenate was subjected to differential centrifugation to obtain a nuclear fraction (1000 \times g, 10 min), a crude mitochondrial fraction (14500 \times g, 20 min), a microsomal fraction (105000 \times g, 60 min) and the resulting supernatant fraction. Particulate fractions were washed three times in ice-cold sucrose, and then diluted with 0.25 M sucrose to protein concentrations of 2.0 to 3.5 mg/ml. Succinate dehydrogenase activity was determined by the method of King (12). DNA and RNA was determined by the method of Schmidt-Thannhauser-Schneider (13). Protein was determined by the procedure of Lowry et al. (14).

**Determination of TTPase activity:** Hydrolysis of TTP was measured by determining the release of inorganic phosphate by the method of Baginski et al. (15). Unless otherwise indicated the standard reaction mixture contained: for soluble TTPase, 100 mM tris buffer (pH 9.0), 6 mM MgCl₂, 3 mM substrate and about 300 μg/ml of protein; for membrane-associated TTPase, 100 mM tris-maleate buffer (pH 6.5), 3 mM MgCl₂, 3 mM substrate and about 600 μg/ml of protein in a final volume of 0.5 ml. After 5 min of pre-incubation, incubation was started by addition of TTP and carried out for 30 min at 37°C. The reaction was terminated by addition of cold trichloroacetic acid to a final concentration of 5%. Nucleoside triphosphatase activity was determined in the same way as TTPase activity except that ITP, GTP, UTP or ATP served as substrate and the incubation time was 15 min.

**Partial purification of soluble TTPase:** Unless otherwise stated, partially purified soluble TTPase was used. Partially purified soluble TTPase was obtained by a slight modification of the method of Hashitani and Cooper (8); material precipitated with between 55 to 80% acetone was suspended in 50 mM tris buffer (pH 7.8) and dialysed for 16 hr against the same buffer at 0°C. The specific activity was increased about 10-fold by this procedure.

**Electrophoretic and fluorometric determination of thiamine phosphate esters:** Thiamine phosphate esters in the reaction mixture of microsomal TTPase were determined by a slight modification of the method of Itokawa and Cooper (11); paper electrophoresis was carried out for 20 min in 50 mM acetate buffer (pH 3.8) using Whatman No. 3MM paper (80 V/cm, 2 mA/cm).
a) Activity is expressed as μ moles Pi/mg protein/h.

Procedures used for subcellular fractionation and assay are described in Methods. Reaction mixture contained: for soluble TTPase, 100 mM tris buffer (pH 9.0), 6 mM MgCl₂, 3 mM TTP and 300 μg/ml of protein; for membrane-associated TTPase, 100 mM tris-maleate buffer (pH 6.5), 3 mM MgCl₂, 3 mM TTP and 600 μg/ml of protein in a final volume of 0.5 ml.

**Table 1. Subcellular distribution of thiamine triphosphatase activity in rat brain**

<table>
<thead>
<tr>
<th>Membrane-associated</th>
<th>Specific activity¹</th>
<th>Percent of total activity</th>
<th>Soluble</th>
<th>Specific activity¹</th>
<th>Percent of total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei</td>
<td>0.94</td>
<td>26</td>
<td>0.65</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.44</td>
<td>41</td>
<td>0.50</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Microsomes</td>
<td>0.75</td>
<td>27</td>
<td>0.86</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>0.17</td>
<td>4</td>
<td>1.06</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

¹ Activity is expressed as μ moles Pi/mg protein/h.

**Table 2. Subcellular distribution of protein, DNA, RNA and succinate dehydrogenase activity in rat brain**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (%)</th>
<th>DNA (%)</th>
<th>RNA (%)</th>
<th>Succinate dehydrogenase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei</td>
<td>15</td>
<td>97</td>
<td>31</td>
<td>5</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>48</td>
<td>2</td>
<td>26</td>
<td>80</td>
</tr>
<tr>
<td>Microsomes</td>
<td>19</td>
<td>0</td>
<td>28</td>
<td>3</td>
</tr>
<tr>
<td>Supernatant</td>
<td>14</td>
<td>1</td>
<td>27</td>
<td>10</td>
</tr>
</tbody>
</table>

**Fig. 1. Activities of soluble and microsomal TTPases at various pH values.**

- •, soluble; - ▲ -, microsomal

ases was found to be very different (Fig. 1). The soluble TTPase had one peak at pH 8.5-9.0, while the microsomal TTPase had two peaks at pH 6.5 and 7.8. Furthermore, it was found that the optimal pH of the microsomal nucleoside triphosphatase activity, determined using ITP as substrate, differed markedly for that of TTPase (Fig. 2).

The electrophoretic and fluorometric determination of the reaction mixture of micro-
somal TTPase at pH 6.5 or 7.8 was examined (Table 3). The production of thiamine diphosphate was equimolar to inorganic phosphate liberated at both pH values. A slight amount of thiamine monophosphate, a product of a further dephosphorylation of thiamine diphosphate, was detected at pH 7.8 reaction.

**Substrate specificity of partially purified soluble TTPase**

Partially purified soluble TTPase showed slight activity with GTP or ATP, but no activity with ITP or UTP (Table 4).

### Table 3. Identification of the products of the microsomal TTPase reaction

| Incubation condition is described in Methods. Reaction was terminated by the addition of cold 0.1 N HCl (final concentration, 0.05 N). After centrifugation one portion of the supernatant was used for the assay of Pi and another was diluted with acetate buffer (pH 3.8) and used for the electrophoretic and fluorometric determination of the reaction mixture. *Not detected (<0.005)* |
|---|---|---|---|---|
| Time (min) | Pi liberated (μ moles/mg protein) | TDP formed (μ moles/mg protein) | TMP formed (μ moles/mg protein) |
| pH 6.5 | 30 | 0.34 | 0.41 | N.D.* |
| | 60 | 0.63 | 0.70 | N.D.* |
| pH 7.8 | 30 | 0.43 | 0.48 | 0.04 |
| | 60 | 0.88 | 0.90 | 0.08 |

### Table 4. Substrate specificity of partially purified soluble TTPase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (μ moles Pi/mg protein/h)</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTP</td>
<td>9.79</td>
<td>100</td>
</tr>
<tr>
<td>ITP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GTP</td>
<td>1.27</td>
<td>13</td>
</tr>
<tr>
<td>UTP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ATP</td>
<td>0.39</td>
<td>4</td>
</tr>
</tbody>
</table>

TTP and various nucleotides (3 mM) were used as substrates. Assay conditions are described in Methods.
**Effect of Ca**\(^{++}\) on TTPase activity**

As shown in Fig. 3, the microsomal TTPase activity exhibited an absolute divalent cation requirement which was satisfied by Mg\(^{++}\) or Ca\(^{++}\) and maximum activations were observed with concentrations of about 3 mM cation. On the other hand, the divalent cation requirement of partially purified soluble TTPase activity was fulfilled by Mg\(^{++}\), but not by Ca\(^{++}\).

Fig. 4 shows the effect of Ca\(^{++}\) on the microsomal and soluble TTPase activities in the presence of the optimal concentration of Mg\(^{++}\). Addition of 1.25 × 10\(^{-4}\) M EGTA [ethylene glycol-bis-(β-aminoethylether)-N,N-tetraacetic acid], had little effect on the

![Graph showing the effect of Ca\(^{++}\) on TTPase activity](image)

**Fig. 3. Effects of Ca**\(^{++}\) and Mg\(^{++}\) on microsomal and soluble TTPase activities.

- ○ ---, Mg\(^{++}\); × ---, Ca\(^{++}\)

![Graph showing the effect of Ca\(^{++}\) on TTPase activities in the presence of Mg\(^{++}\)](image)

**Fig. 4. Effects of Ca**\(^{++}\) on TTPase activities in the presence of Mg\(^{++}\).  
- ○ ---, soluble; - △ ---, microsomal; - ○ ---, soluble (in the presence of EGTA, 1.25 × 10\(^{-4}\) M); - △ ---, microsomal (in the presence of EGTA, 1.25 × 10\(^{-4}\) M). Control (100%) activities: soluble, 9.79 μ moles Pi/mg protein/h; microsomal, 0.71 μ moles Pi/mg protein/h.
activities of the soluble and microsomal TTPases. In the presence of 6 mM Mg\(^{++}\), Ca\(^{++}\) strongly inhibited the soluble TTPase activity at physiological concentrations of this cation. However, Ca\(^{++}\) did not inhibit the microsomal enzyme.

**DISCUSSION**

Hashitani and Cooper (8) found a specific TTPase, having an optimal pH of 9.0 in rat brain supernatant. A recent report by Barchi and Braun (9) showed that there is also an enzyme specifically associated with subcellular membrane fractions, which catalyses the same reaction and has an optimal pH of 6.5.

In the present work, we also found the highest specific activities of the soluble and membrane-associated TTPases in the supernatant and nuclear fractions, respectively. However, unlike Barchi and Braun (9) we could not detect high specific activity of membrane-associated TTPase. This may be due to differences in the methods used for enzyme assay or to differences in the procedures used for obtaining subcellular fractions. We also found that soluble TTPase has a pH optimum of 8.5–9.0 and that TTP is the specific substrate of this enzyme. This result is in good agreement with that of Hashitani and Cooper (8). On the other hand, the microsomal enzyme showed two optimal pH values, one at pH 6.5 and the other at pH 7.8, whereas the optimal pH for the microsomal nucleoside triphosphatase activity, measured using ITP as substrate, was pH 7.8.

Barchi and Braun reported that the membrane-associated TTPase in nuclear fraction of rat brain has a pH optimum of 6.5 (9). As thiamine diphosphatase in brain has an optimal pH at alkali ranges (7, 16, 17), it may be considered that the high activity of microsomal TTPase at pH 7.8 is due to a further dephosphorylation of thiamine diphosphate through thiamine diphosphatase. However, as shown in Table 3, the production of thiamine monophosphate was only about 10% of thiamine diphosphate at pH 7.8 reaction. Thiamine monophosphate was not detected in the reaction mixture of pH 6.5. We found that the microsomal enzyme is difficult to solubilize with deoxycholate, Triton X-100 or alkali treatment and has a wide substrate specificity for various nucleotides (data not shown), so it is still unknown whether or not the TTPase activity of the microsomal fraction is specific for TTP. However, from our results mentioned above and the finding of a specific inhibitor of the hydrolysis of TTP (9), the microsomal TTPase also seems to be distinct from a general nucleoside triphosphatase in the reaction at pH 6.5.

The hydrolysis of TTP by the soluble and microsomal enzymes have an absolute divalent cation requirement which is fulfilled by Mg\(^{++}\) or Ca\(^{++}\).

Previously, Hashitani and Cooper (8) reported that soluble TTPase was inhibited by Ca\(^{++}\). Furthermore, Barchi and Braun (18) reported the inhibition of membrane-associated TTPase by this cation, but in a later report (9) they stated it to be in error as a result of the contribution to the system of an inorganic pyrophosphatase which is inhibited by Ca\(^{++}\). In the present study, it was observed that physiological concentrations of Ca\(^{++}\) inhibited the soluble, but not the microsomal enzyme activity. Changes of the enzyme activities caused by contamination of the fractions with Ca\(^{++}\) were found to be slight because
the effect of addition of EGTA was not observed. These results suggest that Ca$^{+}$ may regulate TTP metabolism in nerve tissue. In elucidating the role of thiamine in the central nervous system, this effect of Ca$^{+}$ on TTPase activity is worthy of attention, since Ca$^{+}$ is known to have a specific role in nerve conduction (19, 20).

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REFERENCES