CHARACTERIZATION OF THIAMINE DIPHOSPHATASE IN RAT SMALL INTESTINE

Toshio MATSUDA, Sadaaki MAEDA, Akemichi BABA, and Heitaroh IWATA

Department of Pharmacology, Faculty of Pharmaceutical Sciences, Osaka University, Yamada-kami, Suita-shi, Osaka 565, Japan
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Summary  The properties of thiamine diphosphatase (TDPase) and p-nitrophenylphosphatase (p-NPPase) in rat small intestine were investigated. TDPase activity, like p-NPPase activity, was high in the mucosa and in the proximal region. Both activities were high in the membrane-associated fractions of the duodenal mucosa. Furthermore, TDPase had the same properties as intestinal alkaline phosphatase (al-Pase). These results suggest that thiamine diphosphate (TDP) and p-nitrophenyl-phosphate (p-NPP) are hydrolyzed by a single enzyme, al-Pase, in the intestine.

Recently, we (1) found that TDPase from rat intestine was purified with p-NPPase by n-butanol extraction, ethanol precipitation, cellulose ion-exchange chromatography and gel filtration and that the TDPase activity in the purified material was competitively inhibited by p-NPP, a substrate of p-NPPase. From these results it seemed likely that the enzymatic hydrolysis of TDP might be catalyzed by al-Pase in the intestine.

This paper describes the distributions of TDPase and p-NPPase in rat small intestine and the characterizations of the two enzymes. The results support our previous idea that TDPase is identical with p-NPPase in the intestine.

EXPERIMENTAL

Male Sprague-Dawley rats, weighing 150–300 g, were used throughout. The rats were killed and the duodenal segment (the proximal 12 cm), jejunal segment (the middle 15 cm) and ileal segment (the distal 20 cm) of the intestine were removed. The mucosa, obtained by gently scraping it off the underlying tissue with a glass slide, was homogenized with 30 volumes of ice-cold 0.25 M sucrose–5 mM EDTA (pH 7.4).

1 松田敏夫，前田定秋，馬場明道，岩田平太郎
Subcellular fractions were prepared from the mucosal homogenate of the duodenum by the procedure of NORMAN et al. (2). TDPase and p-NPPase were purified from the mucosa of the whole small intestine by a slight modification (1) of the procedure of SAINI et al. (3, 4).

TDPase activity was measured as reported previously (5) except that the reaction was performed for 10 min. The electrophoretic and fluorometric determination of the reaction mixture of intestinal TDPase showed that the reaction produced thiamine monophosphate (TMP) equimolar to inorganic phosphate, but not thiamine (data not shown). p-NPPase activity was determined by a slight modification (6) of the method of RUSSELL et al. (7). Sucrase (8) and Mg²⁺-ATPase (9) activities were assayed as previously reported. Protein was determined by the method of LOWRY et al. (10) with bovine serum albumin as a standard.

Nucleotides and TDP were obtained from Sigma Chemical Co. Thiamine triphosphate (TTP) and TMP were kindly donated by Takeda Chemical Industries Ltd., Osaka. TDP was purified by chromatography on a column of Amberlite IRC-50 (H⁺) (II). All other reagents were of highest analytical grade available.

RESULTS

1. Distributions of TDPase and p-NPPase activities in the intestine

TDPase activity, like p-NPPase activity, was located in the mucosa of the duodenum (Table 1). Table 2 shows the distributions of TDPase and p-NPPase activities along the small intestine. Both activities were highest in the proximal region with a sharp decrease in the distal region, whereas Mg²⁺-ATPase activity was almost constant throughout the intestine. The subcellular distributions of

| Enzyme activity | Sucrase |
|-----------------|---------|
| **TDPase**      | **p-NPPase** |
| Mucosa          | 0.23±0.06 (6)  | 3.01±0.21 (3)  |
| Underlying tissue| 0.06±0.00 (6)  | 0.13±0.02 (3)  |

Table 1. TDPase and p-NPPase activities in rat duodenum. Enzyme activity is expressed as μmoles/mg protein/min. Values are means±S.E. of those in the numbers of experiments shown in parentheses. The mucosa and underlying tissue were homogenized with 30 vol. of ice-cold 0.25 M sucrose-5 mM EDTA (pH 7.4).

| Enzyme activity | Mg²⁺-ATPase |
|-----------------|-------------|
| **TDPase**      | **p-NPPase** |
| Duodenum        | 0.50±0.08   | 2.39±0.45   |
| Jejunum         | 0.20±0.03   | 0.04±0.01   |
| Ileum           | 0.08±0.01   | <0.005      |

Table 2. Distributions of mucosal TDPase and p-NPPase activities along the small intestine. Enzyme activity is expressed as μmoles/mg protein/min. Values are means±S.E. of those in 4–6 separate experiments.
Table 3. Subcellular distributions of TDPase and p-NPPase activities in the duodenal mucosa. The mucosa was homogenized with 30 vol. of ice-cold 0.25 M sucrose-5 mM EDTA (pH 7.4) and the indicated fractions were prepared by the method of Norman et al. (2). Values are averages of those in 3–6 separate experiments.

| Fraction                        | Protein (%) | TDPase | p-NPPase |
|---------------------------------|-------------|--------|----------|
| Crude nuclei and brush border   | 37.4        | 0.22   | 2.21     |
| (800×g, 10 min)                 |             | 42.3   | 43.7     |
| Mitochondria                    | 12.3        | 0.44   | 4.62     |
| (8,000×g, 20 min)               |             | 21.5   | 30.0     |
| Microsomes                      | 9.6         | 0.48   | 4.79     |
| (105,000×g, 1 hr)               |             | 23.7   | 24.3     |
| Supernatant                     | 40.7        | 0.06   | 0.09     |
|                                 |             | 12.5   | 2.0      |

S.A., Specific activity (μmoles/mg protein/min); T.A., Total activity (%).

TDPase and p-NPPase activities in the duodenal mucosa are shown in Table 3. The specific activities of the two enzymes were highest in the microsomal fraction and their total activities were highest in the nuclear and brush border fraction, but slight activities of the enzymes were observed in the supernatant.

2. Purification of intestinal TDPase and p-NPPase

We purified TDPase and p-NPPase from rat small intestine to study the properties of the enzymes in the intestine. The details in the enzyme purification steps were previously described (1). Figures 1 and 2 show the elution profiles of the TDPase and p-NPPase on DEAE cellulose, Sephadex G-200 and DE-32 cellulose. Both enzymes could not be separated by these procedures. In the

Fig. 1. Elution profiles of TDPase and p-NPPase on DEAE cellulose column chromatography. The ethanol precipitate fraction previously reported (1) was applied to a column (2.5×80 cm) of DEAE cellulose equilibrated with 5 mM Tris-HCl (pH 7.4)-2.5 mM MgSO₄. Elution was carried out with the same buffer containing 0–0.4 M NaCl (linear gradient) at a flow rate of 30 ml/hr and each 10 ml of effluent was collected.
following study, we used 2nd DE-32 cellulose fraction as a purified enzyme preparation.

Table 4 shows that the purified enzyme hydrolyzed various nucleotides and glucose-6-phosphate. Phosphorylated thiamines, such as TDP, TTP and TMP, were also hydrolyzed by the enzyme in that order. The hydrolyses of thiamine phosphoric esters were higher in experiment 2, under conditions for TDPase.

Table 4. Substrate specificity of the purified preparation. Experiment 1; 42 mM glycine-NaOH (pH 9.2), 0.83 mM ZnCl₂, 4.2 mM MgCl₂, enzyme protein and substrate. Experiment 2; 75 mM Tris-HCl (pH 9.0), 4 mM CaCl₂, enzyme protein and substrate. Enzyme activity is expressed as μmoles P₁/mg protein/min.

| Substrate             | Enzyme activity | Experiment 1 | Experiment 2 |
|-----------------------|-----------------|--------------|--------------|
| p-NPP                 | 516             | 353          |
| Glucose-6-phosphate   | 701             | 519          |
| AMP                   | 603             | 583          |
| IMP                   | 225             | 202          |
| UDP                   | 462             | 568          |
| IDP                   | 158             | 227          |
| ADP                   | 144             | 309          |
| GTP                   | 204             | 351          |
| ATP                   | 109             | 346          |
| TTP                   | 250             | 380          |
| TDP                   | 136             | 560          |
| TMP                   | 109             | 148          |
assay, than in experiment 1, under conditions for p-NPPase assay.

3. Comparison of TDPase and p-NPPase activities in the purified enzyme preparation

Table 5 shows that the $K_m$ values for the substrates of TDPase and p-NPPase in the purified preparation are dependent on the pH value. This increase in the $K_m$ value with increase in pH value is a characteristic property of intestinal al-Pase (12, 13). We previously reported that intestinal TDPase was competitively inhibited by p-NPP and the $K_i$ value was 0.1 mM. This value agreed with the $K_m$ value for p-NPP obtained in assay conditions for TDPase activity (Table 5).

Since al-Pase is a metallo-enzyme containing zinc (12), we studied the effects of EDTA on the TDPase and p-NPPase activities in the purified preparation (Fig. 3). The enzyme preparation (pH 7.4) was incubated with 2.5 mM EDTA (pH 7.4) at 37°C and then after the indicated times it was diluted 20-fold for measurement of activities. In this experiment, p-NPPase activity was determined in the absence of Zn$^{2+}$. Treatment with EDTA inhibited both enzyme activities. Since Fernly (12) reported that cysteine and cyanide inhibited the al-Pase activity by chelating with Zn$^{2+}$ in the enzyme, we next studied the effects of various SH-containing reagents and cyanide on the TDPase activity (Table 6). All the reagents tested

| pH  | $K_m$ (mm) TDP | $K_m$ (mm) p-NPP |
|-----|---------------|-----------------|
| 7.4 | 0.11          | 0.01            |
| 8.2 | 0.22          | 0.03            |
| 9.0 | 0.33          | 0.10            |

75 mM Tris-HCl buffers were used.

Fig. 3. Effect of treatment of purified enzyme preparation with EDTA on p-NPPase (A) and TDPase (B) activities. O, △: Control; ⬤, ▲: EDTA-treated.
Table 6. Effects of SH reagents and cyanide on TDPase activity in purified enzyme preparation. Values are averages of those in 2–4 separate experiments.

| Reagents                  | Conc. (−log m) | Activity (% of control) | 4 mM CaCl₂ | 4 mM CaCl₂+0.8 mM ZnCl₂ |
|---------------------------|----------------|-------------------------|------------|------------------------|
| Sodium cyanide           | 4              | 65                      | 96         |                        |
| Cysteine (Cys)           | 6              | 72                      | 98         |                        |
|                           | 5              | 20                      | 102        |                        |
|                           | 4              | 7                       | 84         |                        |
| Dithiothreitol           | 5              | 42                      | 96         |                        |
|                           | 4              | 9                       | 90         |                        |
| 2-Mercaptoethanol        | 3              | 34                      | 97         |                        |
| Glutathione (reduced)    | 4              | 81                      | 93         |                        |
| p-Chloromercuribenzoate  | 3              | 117                     |            |                        |
| N-Ethylmaleimide         | 3              | 96                      |            |                        |
| Diamide                  | 3              | 104                     |            |                        |
| Sodium tetrathionate     | 3              | 106                     |            |                        |
| Cys+Diamide              | 5+3            | 102                     |            |                        |
| Cys+Sodium tetrathionate | 5+3            | 106                     |            |                        |

Table 7. Effects of divalent cations on TDPase and p-NPPase activities in purified enzyme preparation treated with EDTA. Values are averages of those in 2 experiments. Purified enzyme preparations were preincubated for 15 min at 37°C in the absence (control) or presence (EDTA-treated) of 2.5 mM EDTA and the mixtures were diluted 20 times for enzyme assay.

| Ion (mm) | TDPase | p-NPPase |
|----------|--------|----------|
|          | Control EDTA-treated | 425 | 35 | 421 | 1 |
| Mg²⁺     | Zn²⁺   | Ca²⁺     | (μmoles/mg protein/min) | 491 | 38 | 437 | 30 |
| 0        | 0      | 0        | 420 | 60 | 451 | 238 |
| 0        | 0      | 4        | 463 | 97 | 402 | 221 |
| 0        | 0.1    | 0        | 453 | 281 | 563 | 331 |
| 0        | 0.8    | 0        | 433 | 300 | 542 | 377 |
| 0        | 0.1    | 4        | 462 | 413 | 518 | 265 |
| 0        | 0.8    | 4        | 441 | 299 | 456 | 259 |

Inhibited TDPase activity, but their inhibitions were completely reversed by the presence of SH-oxidizing reagents, such as diamide and sodium tetrathionate, indicating that the inhibition was due to SH groups. These compounds also inhibited the p-NPPase activity assayed in the absence of Zn²⁺, but not in the presence of Zn²⁺ (data not shown). Table 7 shows the effects of Mg²⁺, Zn²⁺ and Ca²⁺ on the TDPase and p-NPPase activities in the purified preparation treated with EDTA. Neither Mg²⁺ nor Ca²⁺ alone activated either enzyme. However, the addition of 0.1–0.8 mM Zn²⁺, with or without Mg²⁺ and Ca²⁺, restored the
Table 8. Effects of various inhibitors on TDPase and p-NPPase activities in purified enzyme preparation. Values are percentages of the control value and averages of those in 2-4 separate experiments.

| Inhibitor           | Conc. (mM) | TDPase | p-NPPase |
|---------------------|------------|--------|----------|
| Theophylline        | 0.05       | 83     | 41       |
|                     | 0.1        | 69     | 32       |
|                     | 0.5        | 29     | 16       |
|                     | 1          | 10     | 10       |
| L-Histidine         | 10         | 0      | 33a      |
| L-Phenylalanine     | 5          | 13     | 69a      |
|                     | 10         | 3      | 22       |
| Imidazole           | 10         | 79     | 81       |
| Caffeine            | 1          | 103    | 100      |
| Theobromine         | 1          | 100    | 98       |
| Ouabain             | 0.1        | 105    | 101      |
| Pyrithiamine        | 1          | 103    | 97       |
| Oxythiamine         | 1          | 114    | 91       |

* 0.8 mM ZnCl₂ was added to the reaction mixture for TDPase.

two enzyme activities. A higher concentration of Zn²⁺ (4 mM) inhibited these activities (data not shown). These results on inhibition agree with those obtained in previous studies with purified al-Pase from other sources (14, 15).

Table 8 shows the effects of various inhibitors on the TDPase and p-NPPase activities in the purified preparation. Inhibitors of intestinal al-Pase, such as theophylline (16), L-histidine (17) and L-phenylalanine (18, 19), strongly inhibited these enzymes, but inhibitors of intestinal thiamine transport, such as ouabain (20, 21) and pyrithiamine (20-24) did not affect either enzyme. L-Histidine and L-phenylalanine greatly inhibited TDPase, but their effects were partly prevented by the addition of Zn²⁺. These results indicate that Zn²⁺ is involved in the inhibitory actions of these amino acids.

DISCUSSION

TDPase activity is observed in microsomal fraction containing Golgi apparatus of tissues (25, 26) and its properties in the liver and brain have been investigated. YAMAZAKI et al. (27) purified TDPase from bovine liver and showed that liver TDPase was identical with nucleoside diphosphatase. They also reported that liver TDPase was activated by ATP. In contrast, BARCHI et al. (28) and we (5) showed that brain TDPase was not influenced by ATP though the hydrolysis of TDP in the brain, like the liver, might be catalyzed by nucleoside diphosphatase. However, there is little information on intestinal TDPase despite its high activity.

Previously we showed that intestinal TDPase was purified with p-NPPase and that the TDPase in the purified preparation was competitively inhibited by
p-NPP (its $K_i$ value agreed with $K_m$ value of the al-Pase for p-NPP). In this work, we found that TDPase and p-NPPase had similar distributions in the intestine and showed similar sensitivities to various reagents. Furthermore, we found that TDPase has the characteristic properties of intestinal al-Pase, such as the requirement of $\text{Zn}^{2+}$ for activity and the dependency of its $K_m$ value for substrate on the pH value of the assay medium. Thus, it seems likely that intestinal TDPase and p-NPPase are the same enzyme. These observations also indicate that TDPase in the intestine is different from that in the liver and brain. Though the cause and significance of the differences are not understood, it is likely that TDPase has a different function in each tissue.

On the other hand, we previously obtained some differences in the ratios of the two enzyme activities in different steps of enzyme purification. In addition, our preliminary experiments showed that inhibition of the p-NPPase by phenylphosphate, $\beta$-glycerophosphate, AMP and glucose-6-phosphate was competitive, but that of the p-NPPase by TDP, TMP and AMP was not. These discrepant results may be explained by the evidence reported by SAINT et al. (4) that the purified material contains three main isozymes which differ with substrate specificity and each of them consists of a microheterogeneous set of glycoproteins. But, further studies are required to confirm our proposal that TDPase may be identical with al-Pase in the intestine.

Purified al-Pase could hydrolyze not only TDP, but also TMP and TTP. This result also suggests that al-Pase is important in dephosphorylation of phosphorylated thiamines in the intestine.

Intestinal TDPase is located in membrane-associated fractions. In general, the enzymatic hydrolysis of TDP seems to serve the important function of regulating the cellular content of this coenzyme. FERRARI et al. (29) suggested that free thiamine is probably a precursor of intracellular phosphorylated thiamine and found that free thiamine enters actively into intestinal epithelial cells. Thus, intestinal TDPase in membrane-associated fractions may be important in transport of this coenzyme by converting it, its more permeable product, thiamine. However, this enzyme did not seem to be directly relate to the carrier coupled with the active transport of thiamine, since this activity was not influenced by inhibitors of intestinal thiamine transport, such as ouabain and pyrithiamine. Further studies are required to elucidate the precise role of intestinal membrane-bound TDPase in the cellular transport of phosphorylated thiamines.

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