ENZYMIC FORMATION OF THIAMINE PYROPHOSPHATE IN PLANTS

Hisateru Mitsuda, Yukio Takii, Kimikazu Iwami, and Kyoden Yasumoto

Laboratory of Nutritional Chemistry, Faculty of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606
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Summary Evidence was presented by paper chromatographic analysis on the occurrence of an enzyme capable of catalyzing a pyrophosphate transfer from ATP to thiamine in green leaves of various plants. The exclusive localization of the enzyme activity in the 105,000 × g supernatant (in a soluble form) was demonstrated by differential centrifugation of a cell homogenate in 0.25 M sucrose. The enzyme was purified by column chromatography with DEAE-cellulose and by gel filtration with Sephadex G-150. The partially purified preparation, while contaminated with detectable activity of acid phosphatase, lost the ability of utilizing thiamine monophosphate as the substrate in place of thiamine. These findings lead to the conclusion that thiamine pyrophosphate is formed in green leaves of plants through a direct pyrophosphorylation of thiamine in the presence of ATP and Mg.

Three types of thiamine phosphates are known to exist in animals (1), plants(2) and microorganisms (3,4), however, the physiological significance of thiamine triphosphate (TTP) is still obscure. Thiamine pyrophosphate (TPP) functions as a cofactor for dehydrogenase, carboxylase and transketolase. Thiamine and its monophosphate (TMP) serve as the cofactor for such enzymes only after conversion to the pyrophosphate form. It has been well established in many investigations on the biosynthesis of thiamine that a final condensation product from pyrimidine and thiazole is not a free form of thiamine but thiamine monophosphate (5–8). However, partially purified enzymes from baker’s yeast (9,10) and from rat liver (11) are unable to utilize TMP as a substrate for the TPP formation. It has been confirmed with the use of ATP labeled with 32P that thiamine pyrophosphate in baker’s yeast (12,13) is formed by a direct transfer of a pyrophosphate group from ATP to thiamine.

1 満田久織，滝井幸男，岩見公和，安本教伝
2 pyrimidine, 2-methyl-4-amino-5-hydroxymethylpyrimidine.
3 thiazole, 4-methyl-5,β-hydroxyethylthiazole.
On the other hand, it has been recently demonstrated by Nishino et al. (14) that an enzyme preparation from E. coli can exclusively utilize TMP but not thiamine itself for TPP formation. The present authors have reported that the biosynthesis of TMP from pyrimidine and thiazole and its activation by ATP (15) do occur in higher plants, and that the requirement of thiamine on the growth of tobacco callus is replaced by thiazole (16).

The present paper deals with the distribution of enzymes catalyzing synthesis of thiamine mono- and di-phosphates in plants and the mechanism of thiamine phosphorylation in parsely (Petroselinum sativum Hoffm.).

MATERIALS AND METHODS

Materials. Fresh vegetables were obtained from a local market as needed. ATP, thiamine and its phosphates were obtained from Sigma Chemical Co., and phosphates were purified to remove contaminants by recrystallization from 50% acetone. Apoprotein of pyruvate decarboxylase [EC 4.1.1.1] was prepared from baker’s yeast according to the procedure of Aoshima (17). DEAE-cellulose and Sephadex G–150 were products of Serva Co. and Pharmacia Fine Chemicals. Other chemicals, of analytical grade, were purchased from commercial sources and used without further purification.

Preparation of enzyme. All operations were carried out below 4°C. One hundred gram portions of the fresh leaves of vegetables were ground in a Waring blender with 150 ml of 0.05 M Tris-HCl buffer (pH 7.5) containing 10^-5 M EDTA and 10^-2 M 2-mercaptoethanol. The homogenate was filtered through a layer of gauze and the resultant filtrate was centrifuged at 5,000 × g for 15 min. The supernatant was brought to a 70% saturation with solid ammonium sulfate. The precipitate obtained after centrifugation was dialyzed overnight against 2 liters of the Tris buffer. After insoluble materials were removed by centrifugation, the supernatant obtained was utilized in preliminary experiments as the enzyme preparation. For further purification, an aliquot of the supernatant was subjected to DEAE-cellulose and Sephadex G–150 column chromatography. Active effluent fractions were condensed in a collodion bag by reverse osmosis.

Assay of activity against thiamine^4 or thiamine monophosphate kinase(s). The reaction mixture contained 4 mmoles of thiamine (or TMP), 4 μmoles of ATP, 20 μmoles of MgCl₂ and an aliquot of the enzyme preparation in 2 ml of 0.05 M Tris-HCl buffer (pH 7.5). When TMP was used as alternative substrate, 200 μmoles of KCl was added to the mixture. The reaction was carried out at 37°C for 60 min and terminated by adding 2 ml of 1.0 M citrate buffer (pH 6.0) and immediately heating the mixture for 5 min at 90°C. After removal of denatured proteins by centrifugation, the TPP content was determined with a Warburg manometer by

^4 thiamine kinase, thiamine pyrophosphokinase [APT: thiamine pyrophosphotransferase, EC 2.7.6.2]
measuring carbon dioxide evolved from pyruvate during the course of reaction at 30°C for 30 min. The assay conditions were as follows: 200 μmoles of sodium pyruvate, 30 μmoles of MnCl₂, an aliquot of specimens to be analyzed for TPP content and 0.1 ml of a freshly prepared apocarboxylase in final 3.0 ml of 0.5 M (pH 6.0) citrate buffer. The kinase activity was expressed in nmoles of TPP formed per 60 min of the reaction period.

Assay of thiamine phosphate pyrophosphorylase. In the routine assay, the reaction mixture contained 500 nmoles of OMP-PP⁵, 500 nmoles of Th-P⁶, 60 μmoles of MgCl₂ and 0.5 ml of the enzyme preparation in 2 ml of 0.05 M Tris-HCl buffer (pH 7.5). After incubation at 37°C for 60 min, the reaction was stopped by adding 10 ml of 0.1 N H₂SO₄ and followed by heating at 80°C for 15 min. The resultant mixture was adjusted to pH 4.5 with 0.6 ml of 4 M sodium acetate, and incubated overnight at 38°C in the presence of 2% Takadiastase (0.6 ml) and toluene (0.1 ml). The incubate was passed through a column of permutit. The amount of free thiamine in the effluent was determined by the thiochrome method (18).

Assay of acid phosphatase. p-Nitrophenylphosphate was used as a principal substrate for determination of the acid phosphatase activity. The reaction was allowed to proceed at 37°C in 3.5 ml of pH 5.5 citrate buffer containing 17.5 μmoles of p-nitrophenyl phosphate. The reaction was terminated by adding 0.5 ml of 1 N NaOH. The amount of p-nitrophenol liberated was estimated by measuring the absorbance at 410 nm. The enzyme activity was expressed as the amount of p-nitrophenol (μmoles/ml) released per 60 min under standard assay conditions.

Other assays. The activity of TMP monophosphatase was estimated from the amount of thiamine content from TMP, which was determined according to the thiochrome method (19). Protein was estimated by the procedure of LOWRY et al. using Lab-Trol as a standard (20).

RESULTS

Chromatographic evidence for enzymic formation of TPP

Figure 1 depicts chromatograms of the reaction mixtures incubated with thiamine and the enzyme preparation from plants in the presence of ATP and Mg²⁺. Spots on the chromatogram were visualized under UV irradiation after spraying with a mixture (2: 1: 0.05, v/v) of ethanol, 10% NaOH and 2.5% K₃Fe(CN)₆ (21). A major spot and a minor one represented thiamine pyrophosphate formed and remaining thiamine, respectively. It is ascertained by comparing these chromatograms that thiamine pyrophosphate is a single main product and is the cofactor for which apocarboxylase from baker's yeast shows a strict specificity.

Distribution of thiamine synthetase and kinase(s) in plants

In Table 1 the experimental results on distribution of thiamine synthetase and

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⁵ OMP-PP, pyrimidine pyrophosphate.
⁶ Th-P, thiazole monophosphate.
Fig. 1. Paper chromatogram for enzymatically derived phosphates from thiamine. The enzyme reaction was performed under the standard assay conditions. The product was chromatographed on Toyo No. 51A filter paper using n-propanol/water/1 m acetate buffer of pH 5.0 (70:20:10, v/v) as developing solvent. The $Rf$ value was compared with that of authentic thiamine and phosphates.

Table 1. Distribution of thiamine kinase(s) and synthetase in a variety of plants. The assay conditions of individual enzyme activities were the same as described in "MATERIALS AND METHODS." Values are the means of three replicates.

<table>
<thead>
<tr>
<th>Plants</th>
<th>TPP formed (nmol/mg protein)</th>
<th>TMP synthetase (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>from thiamine</td>
<td>from TMP</td>
</tr>
<tr>
<td>Parsley</td>
<td>1.9</td>
<td>1.4</td>
</tr>
<tr>
<td>Honewort</td>
<td>1.6</td>
<td>0.1</td>
</tr>
<tr>
<td>Spinach</td>
<td>0.9</td>
<td>0.3</td>
</tr>
<tr>
<td>Celery</td>
<td>2.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Japanese radish</td>
<td>1.1</td>
<td>—</td>
</tr>
<tr>
<td>Bean sprout</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>Carrot</td>
<td>1.6</td>
<td>0.0</td>
</tr>
<tr>
<td>Welsh onion</td>
<td>0.4</td>
<td>0.0</td>
</tr>
<tr>
<td>Cabbage</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>Leek</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Chinese cabbage</td>
<td>0.6</td>
<td>0.8</td>
</tr>
</tbody>
</table>
kinase(s) in a variety of vegetables are summarized. A relative high activity of thiamine kinase was found in celery, parsley, honewort, carrot and leek. The ability of TPP formation from TMP was detected in a few vegetables. An enzyme preparation from parsley and leek seemed likely to utilize both thiamine and its monophosphate as the substrates for the TPP formation. On the other hand, the synthetase activity in green leaves of plants was much less than that of baker's yeast. The high activity for synthetase in bean sprout suggests an increasing requirement of TPP in the carbohydrate metabolism in seedlings of plants.

Subcellular localization of kinase activity in parsley and celery

It was demonstrated in Table 1 that the TPP formation from thiamine was most effectively catalyzed by an enzyme preparation from leaves of parsley and celery. As shown in Table 2, the successive centrifugation of cell homogenates in 0.25 M sucrose demonstrated that the kinase activity was located exclusively in the 105,000 x g supernatant. Thus, thiamine kinase from higher plants appears to be in a soluble state, while thiamine kinase from E. coli is located in the membrane fraction (22). Sodium fluoride, which is a specific inhibitor for phosphatase and other related enzymes, caused a significant decrease in the kinase activity. The decrease in the "TMP kinase" activity was relatively remarkable in comparison with that of thiamine kinase in the presence of 20 mM sodium fluoride; possibly indicating that dephosphorylation of TMP would be suppressed by fluoride. It is also of interest whether or not such TMP kinase as found in E. coli occurs in plants.

Table 2. Fractionation of thiamine kinase(s) in parsley and celery by different centrifugation.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Parsely from</th>
<th>Celery from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>thiamine</td>
<td>TMP</td>
</tr>
<tr>
<td>Homogenate</td>
<td>6.2</td>
<td>1.8</td>
</tr>
<tr>
<td>5,000 x g pellet (debris and nuclei)</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>105,000 x g pellet (microsome)</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>105,000 x g supernatant, soluble</td>
<td>7.2</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Data are indicated as means of three replicates.

Behavior of thiamine kinase(s) on column chromatogram with DEAE-cellulose and Sephadex G-150

The enzyme preparation fractionated with ammonium sulphate from parsely,
was applied to a DEAE-cellulose column (1.8 × 38 cm) which was previously equilibrated with 0.05 M Tris-HCl buffer (pH 7.5) containing 10⁻² M 2-mercaptoethanol. The column was washed with one liter of Tris-HCl buffer and subsequently eluted by a linear gradient to 0.5 M KCl. The eluate was collected in 5 ml aliquots. Figure 2 shows a chromatographic pattern with DEAE-cellulose. The enzyme activity of thiamine kinase(s) and acid phosphatase in each fraction was assayed in the same manner as described in the text, except for 5 μM of thiamine and its monophosphate. As shown in Fig. 2, the fraction showing a maximal activity of kinase(s) was apparently separated from a main active fraction for acid phosphatase. The active fractions were collected and concentrated to 2 ml in a collodion bag under reduced pressure. The enzyme condensate was furthermore analyzed by gel filtration with Sephadex G-150 column. The chromatogram is shown in Fig. 3. The protein elution was separated into three major peak fractions, and the kinase activity was found in the third one. While the fraction showing kinase activity was still contaminated with acid phosphatase, it was scarcely active enough to dephosphorylate thiamine monophosphate. Thiamine kinase devoid of acid phosphatase lost most of ability to utilize TMP as the substrate for the TPP formation.
Apocarboxylase from baker’s yeast absolutely requires TPP as the coenzyme. Alternately, the function of TPP could not be replaced by free thiamine and its monophosphate. The evidence was provided from paper chromatographic identification for that TPP was produced from thiamine by the action of an enzyme preparation from plants (Fig. 1). The enzyme activity capable of catalyzing formation of TPP from thiamine and its monophosphate was widely distributed in plants (Table 1). TMP seemed likely to serve as the prominent substrate for the TPP formation in a few plant sources. In the presence of NaF, however, the efficiency of TMP was inferior to that of thiamine (Table 2). A further purification of thiamine kinase by means of chromatography with DEAE-cellulose and Sephadex G-150, caused a remarkable decrease in the activity of “TMP kinase” as compared with thiamine kinase (Figs. 2 and 3). Separation of thiamine kinase from acid phosphatase was achieved to a certain extent by chromatographic techniques. Acid phosphatase from tobacco leaf has been reported to function not only as orthophosphoric monoester phosphohydrolase [EC 3.1.3.2] but also as pyrophosphate phosphohydrolase [EC 3.6.1.3] (23). Such non-specific phospho-
tase is presumed to be present in leaves of parsley and celery, since a higher activity of thiamine kinase was observed in the 105,000×g supernatant than in the homogenate (Table 2). Recently, an enzyme capable of catalyzing a direct phosphorylation of TMP to TPP, in the presence of ATP and Mg, has been found in the soluble fraction of E. coli K12 differing from thiamine kinase, whereas thiamine kinase was located in the spheroplast membrane of E. coli (22). Seedlings of plants were found to exhibit a extremely high thiamine phosphate pyrophosphorylase activity. A condensation product from thiazole and pyrimidine was characterized as TMP on the basis of the experimental results (15). It is not yet clear whether or not TMP is directly converted to TPP during germination. It seems reasonable to conclude from the findings so far presented that such TMP kinase as discovered in E. coli would not be involved at least in green leaves of plants.

Further purification of thiamine kinase from plant leaves and its enzymic properties will be presented in the following paper (24).

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REFERENCES