Isolation and Characterization of Thiamin Pyrophosphotransferase from Glycine max Seedlings

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ABSTRACT

Thiamin:ATP pyrophosphotransferase (EC 2.7.6.2) activity from soybean (Merr.) seedlings grown for 48 hours was determined by measuring the rate of [2-14C]thiamin incorporation into thiamin pyrophosphate. With partially purified (11-fold) enzyme, optimal activity occurred between pH 7.1 and 7.3, depending on the buffer system that was used. Assays were routinely conducted at a final pH of 8.1 in order to minimize interference from competing reactions. Enzyme activity required the presence of a divalent cation, and a number of nucleoside triphosphates proved to be active as pyrophosphate donors. Apparent \( K_m \) values of 18.3 millimolar and 4.64 micromolar were obtained for Mg-ATP and thiamin, respectively. Among the compounds tested, thiamin and thiamin pyrophosphate were most effective in inhibiting thiamin pyrophosphotransferase activity. Based on Sephadex G-100 gel filtration, soybean thiamin pyrophosphotransferase has a molecular weight of 49,000.

Thiamin:ATP pyrophosphotransferase (EC 2.7.6.2) catalyzes the following reaction:

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\text{thiamin} + \text{ATP} \rightarrow \text{thiamin pyrophosphate} + \text{AMP}.
\]

This reaction has been demonstrated and characterized in extracts from mammalian (6, 12), microbial (17, 19), and botanical (13, 15) systems. However, there is a dearth of information on higher plant TPTases\(^8\), especially as to their possible significance in seedlings. Some survey work that indirectly implies the presence of TPTases in other plant species has been reported (22), but the bulk of available information is concerned with the enzyme from parsley leaves (13–15).

Many seeds are purportedly devoid of the phosphorylated forms of thiamin (22) but we have not found this to be true for soybeans (16). The apparent lack of TPP in some seeds (22) may result partially from the rapid dephosphorylation of TPP during seed maturation as reportedly occurs in sunflower (2). Since TPP serves as an important cofactor in the energy and intermediary metabolism of living organisms, changes in the level(s) of the enzyme(s) that control the formation and utilization of TPP during seed germination could conceivably influence the rate and vigor of that process. This possibility is addressed elsewhere (16). The purpose of the work reported here was: (a) to ascertain the reaction(s) for thiamin pyrophosphate formation in germinating soybean seeds and (b) to determine some of the properties of the enzyme(s) involved in thiamin phosphorylation.

MATERIALS AND METHODS

Plant Material. Seeds of soybean (Glycine max [L.] Merr. cv. Bragg) were surface-sterilized for 5 min in 10% Clorox, wrapped in moist paper towels, and germinated for 48 h in the dark at 26 C.

Compounds and Reagents. [2-14C]Thiamin-HCl (specific radioactivity, 14.0 mCi/mmol, 41.7 \( \mu Ci/\text{mg} \)) was obtained from the Amersham Corp. Thiamin, TP, pyritihiamin, nucleotides, oxothiamin, Sephadexes, and phosphatase substrate were purchased from Sigma. All other chemicals were reagent grade.

Cell-free Extract. All extraction and purification procedures were carried out at C. Fifty g of 48-h-germinated soybean seedlings were surface-sterilized with 10% Clorox for 1 min and washed in distilled H\(_2\)O. The seedlings were homogenized in buffer consisting of 50 mM Tris-HCl (pH 7.6), 10 mM \( \beta \)-mercaptoethanol, and 1.0 mM EDTA for 1 min in a Sorvall Omni-Mixer (setting 6). The homogenate was passed through a layer of nylon cloth before centrifugation at 20,000 g for 20 min. The supernatant fraction was decanted and brought to 100 ml with the extraction buffer (Fraction I).

Ammonium Sulfate. Fraction I was further fractionated with (NH\(_4\))\(_2\)SO\(_4\). The protein that precipitated between 30 and 50% saturation was collected by centrifugation at 20,000 g for 10 min and then dissolved (final volume, 18 ml) in 50 mM Tris-HCl (pH 7.6), 15% sucrose, and 10 mM \( \beta \)-mercaptoethanol (Fraction II).

Fractionation on Sephadex G-150. Two ml of Fraction II were applied to the top of a column (1.5 \( \times \) 30.0 cm) consisting of 5 cm Sephadex G-25 placed atop 25 cm Sephadex G-150. The Sephadexes had been equilibrated with 50 mM Tris-HCl (pH 7.6) containing 10 mM \( \beta \)-mercaptoethanol; elution with the same buffer was performed at a flow rate of 6.0 ml/h. Those fractions (1 ml each) containing TPTase activity were combined (Fraction III).

TPTase Assay. TPTase was assayed by a procedure similar to that of Deus et al. (3). The assay buffer consisted of 50 mM Tris-HCl (pH 8.4) and 10 mM \( \beta \)-mercaptoethanol. The standard assay (final volume, 505 \( \mu l \)) was as follows: 300 \( \mu l \) assay buffer, 100 \( \mu l \) ATP-MgCl\(_2\) (100:125 mm) solution (pH 8.4), 5 \( \mu l \) 10 mM [2-14C]-thiamin (25 \( \mu Ci/ml \)), and 100 \( \mu l \) partially purified enzyme (pH 7.6). The final pH of the mixture was 8.1. The reaction was initiated by the addition of labeled thiamin, incubated for 2 h at 37 C, and stopped by heating the assay mixture for 5 min in a boiling water bath. A measured aliquot (10 \( \mu l \)) of reaction mixture was spotted on unwashed Whatman No. 1 paper and chromatographed (descending) with authentic thiamin and its phosphate esters for 12 h in a solvent system (18) of 1-propanol-H\(_2\)O-1.0 M-

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4 Abbreviations: TPTase: thiamin pyrophosphotransferase; TP: thiamin monophosphate; TPP: thiamin pyrophosphate; pCM, p-chloromercuribenzoic acid.
RESULTS AND DISCUSSION

TPTase Assay Requirements. The requirements for the pyrophosphorylation of thiamin by soybean TPTase included ATP and Mg^{2+} (data not shown). Consistent reaction rates (3.45 ± 0.25 nmol TPT formed/mg protein-h) were obtained from complete assay mixtures, whereas omission of any single component (thiamin, ATP, Mg^{2+}, or protein) resulted in a total cessation of TPTase activity. Similar requirements have been reported for the TPTases from a number of sources (6, 11, 15, 19). Reaction rates remained linear for at least 2 h (r^2 = 0.97 at 95% confidence interval), similar to the TPTases from parsley leaves (15) and rat brain (6). The initial reaction velocity remained linear with amount of added protein to at least 0.2 mg protein (r^2 = 0.97 at 95% confidence interval).

Product (TPP) Identification. Using [14C]thiamin as substrate and Fraction I or Fraction II enzyme preparations from 48-h-germinated soybean seedlings, 10-μl aliquots from complete reaction mixtures (incubated for 2 h at 37 C; 0.5 ml total volume) and controls (containing heat-inactivated enzyme) were chromatographed on unwashed Whatman No. 1 paper. Chromatograms were developed for 12 h, air-dried, and then sprayed to produce long-wave UV fluorescent spots which were subsequently marked. With [14C]thiamin as substrate and using standard assay conditions, radioactivity was found only in spots which cochromatographed with authentic thiamin and TPP. [14C]TPP was not detected in boiled controls.

Putative [14C]TPP from several chromatograms was pooled and combined with authentic nonlabeled TPP. The mixture was chromatographed in three solvent systems (data not shown) and subjected to polyacrylamide strip electrophoresis (Fig. 1). In each case, only one radioactive fluorescing spot, with an R₅ identical to that of authentic TPP, was detected. The specific radioactivity remained nearly constant (44, 48, and 45 cpm/mg TPP, respectively) through three successive recrystallizations and separations using a propanol solvent system.

In addition, when putative [14C]TPP product was incubated with Fraction III protein in 50 mm citrate buffer (pH 5.5), increasing amounts of a compound were liberated over a 30-min incubation period that had fluorescent and R₅ properties identical to that of authentic thiamin monophosphate (Table I). As discussed in further detail below, this finding provided further indication of the presence of phosphatase activity in the Fraction III preparations. Nominal Pi was released from authentic samples of TPP.

Fig. 1. Polyacrylamide strip electrophoresis profile of putative [14C]TPP. Labeled product from TPTase assays was mixed with authentic thiamin (T), thiamin monophosphate (TP), and 250 μg thiamin pyrophosphate (TPP). Separation of authentic compounds is indicated in the lower part of the figure and radioactivity distribution in the upper portion.
Table I. Hydrolysis of TPP by Fraction III Protein
Putative TPP was used as substrate for Fraction III protein. Assays were performed at pH 5.5 using 50 mM citrate buffer. Complete mixtures were incubated for the indicated periods before termination of the reaction by plunging tubes in a boiling water bath. Specific radioactivities were based on an original isotopic dilution of putative (labeled) TPP with authentic (unlabeled) TPP.

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<th>Incubation Duration</th>
<th>TPP cpm/μg thiamin phosphate ester</th>
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FIG. 2. Effect of varied concentration of Mg$^{2+}$, Mn$^{2+}$, Co$^{2+}$, and Cu$^{2+}$ ions on TPTase activity. The concentration of ATP was held constant at 20 mM. Except for cation substitutions, assay conditions were as indicated in Table II. Each of the data points represents the mean from six separate determinations. Cation Effects. A number of divalent cations (including Mg$^{2+}$, Co$^{2+}$, Cu$^{2+}$, Fe$^{2+}$, Zn$^{2+}$, Ni$^{2+}$, Mn$^{2+}$, Ba$^{2+}$, and Ca$^{2+}$) were added at 25 mM final concentration, with Cl$^{-}$ as the anion tested for their ability to support pyrophosphorylation of thiamin (data not shown). Substantially higher levels of TPTase activity were obtained when Mn$^{2+}$ was used compared to any of the other cations. When Mg$^{2+}$, Mn$^{2+}$, Co$^{2+}$, and Cu$^{2+}$ were tested over a range of concentrations, Mg$^{2+}$ (again at 25 mM) was still the most stimulatory in regard to TPTase activity (Fig. 2). At concentrations of 10 mM or less, Mn$^{2+}$ proved to be more stimulatory than Mg$^{2+}$ (Fig. 2). The TPTases from parsley leaf (15) and brewer's yeast (20) (data not shown) respond similarly to Mg$^{2+}$ and the other cations, whereas the enzyme from baker's yeast (7, 8) exhibits maximal activity with Mn$^{2+}$ compared to Mg$^{2+}$ and Co$^{2+}$. However, activity of the TPTase from baker's yeast varied markedly with pH, cation concentration, buffer system, and the type and concentration of nucleotides (7, 8).

Nucleotide Effects. Ribonucleoside tri-P, ADP, and AMP (20 mM final concentrations) were screened for their ability to serve as phosphorylating agents during enzymic formation of TPP (Table II). Except for AMP, all the compounds tested were active as substrates, but rates of TPP formation were reduced considerably when ADP and xanthosine tri-P were used in place of ATP. Similar results have been reported (11, 12, 17); however, after subsequently greater purification, TPTase activity sometimes is limited to specific nucleotides (8, 12). Soybean extracts may have been capable of catalyzing nucleotide interconversion. Mano (11, 12), for example, reported the presence of adenylyl kinase activity in animal tissue preparations and its presence cannot be ruled out here.

When purified to homogeneity, parsley leaf (14) and brewer's yeast (20) TPTases exhibited activity with all of the nucleoside tri-P. Consequently, Voskoboiev et al. (20) proposed that these TPTases be renamed thiamin:TPP pyrophosphotransferase, where the NTP refers to any nucleoside tri-P. The present findings with partially purified soybean TPTase also support such a revision.

Enzyme Purification. The purification protocol was similar in many respects to that of Mitsuda et al. (15). One change was the use of Sephadex G-150 column chromatography (Fig. 3) in place of DEAE-cellulose (15) as the final purification step (Table III). Even though considerable phosphatase activity co-eluted with TPTase in both procedures, filtration through Sephadex G-150 effected a greater purification with less variation per extraction (Table III). Fraction III protein separated into eight discernible bands during polyacrylamide gel electrophoresis (W. T. Molin, unpublished data), indicating the presence of enzymes other than phosphatase and TPTase. Attempts were not made to purify soybean TPTase further; we did find subsequently that a rapid, short-term heating of extracts (to 60°C) in the presence of thiamin (4) may prove fruitful in future purification studies.

Effect of pH. TPTase activity in Fraction III protein was...
determined over a pH range of 5.8 to 9.0 using Tris-maleate, Tris, or Tris-glycine to buffer the assay mixture (Fig. 4). Optimal activity occurred at pH 7.1 and 7.3 when TPTase was assayed in Tris-maleate and Tris buffers, respectively. Within the range of optimal pH for TPTase activity, Fraction III protein still exhibited considerable phosphatase activity when nitrophenol phosphate was used as substrate (Fig. 4). Furthermore, chromatograms of aliquots from TPTase incubation mixtures assayed between pH 7.0 and 7.6 contained low levels of TP (less than 0.1 nmol TP formed/mg protein-h).

Phosphatase activity was sufficiently high at the above pHs to account for all of the TP that was observed. Incubation of Fraction III protein preparations at pH 5.5 rapidly converted some TPP to TP (Table I). In preliminary experiments, we did not observe any monophosphotransferase activity that could account for the TP production. However, Phaseolus seed extracts do contain such activity and effectively transfer phosphate from flavin mononucleotide to thiamin (9). This reaction was maximal at pH 5.7 and ceased at pH 8.0.

In order to preclude the possibility of monophosphotransferase activity (9) and eliminate most of the phosphatase activity (Fig. 4), we routinely assayed for TPTase activity at pH 8.1. Under these conditions, TP formation was not evident.

**Kinetic Values.** Data were obtained by assaying for TPTase activity in Fraction III protein at five different concentrations of thiamin and nucleoside tri-P. These concentrations ranged between 0.13 to 2.18 times the apparent $K_m$ values for ATP and the other nucleoside tri-P and from 0.27 to 1.55 times the apparent $K_m$ for thiamin. $K_m$ and $V_{max}$ values were determined by linear regression analysis of Lineweaver-Burk plots (Fig. 5). Average values from six separate determinations are summarized in Table IV. $K_m$ values for thiamin and ATP were 4.64 μM and 18.3 mM, respectively. For thiamin, the apparent $K_m$ of soybean TPTase was about 34 times greater than that for parsley leaf (15) and rat brain (6) TPTases. The apparent $K_m$ for ATP (Table IV) was approximately 20-fold greater than that obtained for the parsley leaf enzyme but was equivalent to that determined for rat brain TPTase. The apparent $K_m$ values for CTP, UTP, and GTP were all significantly lower than that for ATP (Table IV). The low apparent $K_m$ for thiamin relative to those for the nucleoside tri-P (Table IV) suggests that, in soybean seedlings, the concentration of ATP or other nucleoside tri-P may play an important regulatory role in modulating the activity of TPTase. This is supported further by the observation that, under our assay conditions, soybean TPTase was completely inactive with respect to TPP formation in the presence of ATP concentrations of 1 mM or less.

**Inhibition of TPTase Activity.** A number of compounds were tested in a preliminary experiment at a final concentration of 0.5 mM for efficacy in inhibiting TPTase activity (Table V). Pyrithiamin, TPP and pCMB significantly reduced TPTase activity, whereas the other compounds were largely without effect. TPP is a noncompetitive inhibitor of parsley leaf TPTase (14), whereas pyrithiamin competitively inhibits rat brain TPTase (6). At 0.5 mM, pCMB inhibited soybean TPTase in variable fashion as indicated by the large standard deviation (Table V). Nevertheless, the reduction in TPTase activity in the presence of pCMB was...
indicative of the presence of sensitive or essential disulfide bonds and/or sulfhydryl groups similar to the findings for parsley leaf TPTase (15).

Molecular Weight. Soybean TPTase had an apparent mol wt of 49,000 when Fraction III protein was passed through Sephadex G-100 (data not shown). Parsley leaf TPTase reportedly has a mol wt of 30,000 (15), whereas the mol wt of the enzyme from yeast is 90,000 to 95,000 (21). Voskoboev et al. (21) suggested that the yeast TPTase is a tetramer having inactive subunits of 24,000 mol wt. Thus, the TPTases from higher plants may represent active monomer (parsley leaf) and dimer (soybean) forms.

LITERATURE CITED

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