Thiamin Phosphorylation by Thiamin Pyrophosphotransferase during Seed Germination

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ABSTRACT

Thiamin pyrophosphotransferase activity was present in seedling extracts from several monocot and dicot species of agronomic as well as noncultivated plants. Changes in thiamin pyrophosphotransferase activity and thiamin pyrophosphate content were followed for 6 days in soybean (Merr.) seedlings. Maximum enzyme activity occurred 48 to 96 hours from imbibition. Thiamin pyrophosphate content peaked sharply at 36 hours and was preceded by increased thiamin pyrophosphotransferase activity. Addition of pyrithiamin, an inhibitor of in vitro thiamin pyrophosphotransferase activity, to the imbibition medium at various times inhibited subsequent fresh weight gains of soybean seedlings. These results indicated, although not among the earliest phosphorylation events after initiation of water imbibition by soybean seeds, a substantial increase in thiamin pyrophosphate content did precede the onset of rapid seedling growth and development. Since both enzyme activity and thiamin appear to be available in unimbibed soybean seeds, ATP or other nucleoside triphosphate concentration may represent an important factor in modulating thiamin phosphorylation during early seedling development.

Information on thiamin phosphorylation during the maturation and germination of seeds is insufficient and conflicting. TPP apparently is present in mature peanut seeds at a concentration of approximately 2% of total thiamin content (4). Conversely, Yusa (11) reported that phosphorylated forms of thiamin were absent from mature ungerminated seeds of corn, soybean, pea, and rice. The differences may stem in part from differential degradation of the phosphorylated derivatives of thiamin during seed maturation. Thiamin accumulates in maturing sunflower seeds, partially at the expense of TPP which is rapidly dephosphorylated during late maturation (2).

Although unimbibed seeds of corn, soybean, pea, and rice apparently contained only thiamin, only phosphorylated forms were detected after seeds germinated (11). In bean sprouts both thiamin synthetase and TPTase activities have been observed (7), and in 48-h germinated soybean seedlings thiamin phosphorylation is largely, if not exclusively, confined to TPTase activity (8). TPP may serve to regulate the activity of certain enzymes. The

pyruvate dehydrogenase complex from mitochondria of broccoli florets reversibly binds a metal-TPP complex (10). The steady-state reaction velocity of the pyruvate dehydrogenase complex-metal-TPP complex was reached only after a TPP-dependent lag phase and was related to the final concentration of metal-TPP. Hence, it was proposed that rapid dissociation of the metal-TPP complex from the enzyme might serve to regulate pyruvate dehydrogenase complex activity (10).

The presence of antithiamin compounds in seeds, such as specific phenols, could attenuate the rate of seedling growth by lowering the availability of thiamin and TPP for necessary metabolic reactions (C. G. Wilkerson and R. C. Fites, unpublished data).

TPP plays a crucial role in the energy and intermediary metabolism of living organisms, including seeds, by serving as a necessary coenzyme for δ-keto acid dehydrogenases, transketolases, and decarboxylases (3). Thus, the enzyme(s) controlling TPP formation during seed germination and seedling establishment could conceivably coordinate the reactivation of essential metabolic pathways through the timely synthesis of TPP. Alternatively, sufficient TPP may remain associated with the apoenzymes requiring this coenzyme so that their reactivation occurs independent of any initial TPP formation. In this case, the increased activity of these pathways that occurs during rapid seedling growth would be dependent on an adequate provision of TPP.

In soybean seedlings, only the latter alternative appears to apply. The data that support such a conclusion constitute the basis for this report.

MATERIALS AND METHODS

Seedling Growth. Except for tobacco, seedlings were grown as previously described (8) for varying periods according to experimental intent. Tobacco seeds were incubated at 26 °C in the dark in Petri dishes containing two Whatman No. 1 filter papers and 5 ml deionized H2O. For the growth regulator experiment, each compound (100 μM) was added at the onset of imbibition by soybean seeds.

TPTase Assay. Ammonium sulfate extracts (crude extracts in the case of tobacco and cotton seedlings) and TPTase assays were carried out as previously described (8). For the more detailed studies with soybean seedlings, 105 seeds or seedlings were harvested at each of the indicated times instead of 50 g tissue (8).

TPP Content. TPP levels in germinating soybean seedlings were estimated by the procedure of Holzer et al. (5). Several different volumes of extract were tested to ensure that apoppyruvate decarboxylase, purchased from Sigma as pyruvate decarboxylase and purified to isolate apoppyruvate decarboxylase according to Holzer et al. (5), was not saturated by TPP beyond the 25% level. To extract TPP, 40 soybean seeds or seedlings were homogenized in 10 ml 16% HClO4 using a Sorvall Omni-Mixer (setting 10 for 1 min). Supernatant solutions were saved after centrifuging the homogenates at 20,000g for 15 min. Resulting 20,000g pellets were
twice extracted and recentrifuged, each time with additional 10-
mL aliquots of 16% HClO4. Supernatant fractions were pooled,
neutralized with KOH, and centrifuged a final time to remove
KClO4 precipitates. The final supernatant solutions were adjusted
first to 32 mL with distilled H2O and then to 40 mL by the addition
of 250 mM Tris-HCl buffer (pH 7.6).

Oxygen Consumption. Rates were measured at hourly intervals
over a 24-h period. Four seeds were placed in each chamber (with
three simultaneous replications) of a microrespirometer. Filter
paper saturated with KOH was used as a CO2 absorbent.

RESULTS AND DISCUSSION

Seedling TPTase Activity. Seedlings from eight plant species,
including three cultivars of soybeans, were extracted and examined
for TPTase activity. As indicated in Table I, all preparations were
active, ranging in TPTase activity from 4.96 to 25.56 nmol TPP
formed/g fresh weight-h. The highest activities were found in
preparations from pea and soybean seedlings, whereas those from
corn, tobacco, Iva, and Uniola contained considerably less TPTase
activity (Table I).

Growth Regulator Effects. If the onset of thiamin phosphoryl-
lation during seed germination serves as a coordinating signal for
the reactivation of essential metabolic pathways, then environ-
mental and growth regulator cues might conceivably act to modu-
late TPTase activity and, thereby, alter the rate of seed germi-
nation. Although this aspect was not examined in detail, the
addition of IAA, GA3, ABA, or kinetin (100 μM) to imbibing
soybean seeds had no effect on extractable TPTase activity from
seedlings germinated for 48 h (data not shown). Similarly, ger-
mination of tobacco seeds in the light or soybean seeds at 5 C
from 36 to 48 h did not result in significant alterations in TPTase
activities (data not shown).

Seedling Changes. Fresh weight changes in germinating soy-
bean seedlings exhibited three phases (Fig. 1). A rapid gain in
fresh weight occurred during the first 24 h of imbibition (phase I),
followed by a slow gain from 24 to 48 h (phase II), and then by a
sustained and substantial increase corresponding with rapid root
growth for the remainder of the experimental period (phase III).
Except for temporal differences, the fresh weight phases during
soybean seed germination (Fig. 1) were similar to those for ger-
minating radish seed (9).

Low levels of TPTase activity were present in preparations from
unimbibed seeds (Fig. 1). On a seedling basis, enzyme activity

Table 1. Thiamin Pyrophosphotransferase Activity from Germinating Seedlings

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Fresh Weight</th>
<th>Time Germinated</th>
<th>TPTase Activity in Thiamin Pyrophosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td>h</td>
<td>nmol g⁻¹ h⁻¹</td>
</tr>
<tr>
<td>Soybeans</td>
<td>cv Bragg</td>
<td>50</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16.88</td>
</tr>
<tr>
<td></td>
<td>cv Ransom</td>
<td>52</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15.88</td>
</tr>
<tr>
<td></td>
<td>cv Dare</td>
<td>42</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12.30</td>
</tr>
<tr>
<td>Mungbean</td>
<td>cv Jumbo</td>
<td>50</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10.75</td>
</tr>
<tr>
<td>Cotton</td>
<td>cv Coker 413</td>
<td>50</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12.94</td>
</tr>
<tr>
<td>Pea</td>
<td>cv Alaska</td>
<td>50</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>25.56</td>
</tr>
<tr>
<td>Tobacco</td>
<td>cv Coker 411</td>
<td>1</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.96</td>
</tr>
<tr>
<td>Corn</td>
<td>cv Yellow Dent</td>
<td>50</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6.00</td>
</tr>
<tr>
<td>Iva imbricata Walter</td>
<td>5</td>
<td>48</td>
<td>5.89</td>
</tr>
<tr>
<td>Uniola paniculata L.</td>
<td>4</td>
<td>48</td>
<td>8.03</td>
</tr>
</tbody>
</table>

increased rapidly for the first 36 h of imbibition, remained at
nearly the same level from 48 to between 96 and 120 h, and then
dropped somewhat (Fig. 1).

As indicated under “Materials and Methods,” TPTase content was
estimated by first HClO4-extracting the seedlings and then assaying
neutralized preparations by combining aliquots of the latter with
apoppyruvate decarboxylase (5). In contrast to Yusa’s report
(11), when the Bragg cultivar of soybean was examined, mature
unimbibed seeds proved to contain significant levels (approximately
45 nmol/seed) of TPT (Fig. 1). During preliminary experi-
mentation, we attempted to repeat Yusa’s findings using a similar
approach (11) but were unable to do so because of the presence in
soybean extracts of substances which interfered with fluorescent
thiochrome determination.

A potential difficulty with the apoppyruvate decarboxylase ap-
proach (5) is that the TPT extraction method is harsh enough that
TPT may be stripped (differentially) from apoenzymes. Thus,
when this approach is used, it is not possible to directly (or actu-
ally) distinguish between free TPT and that bound to in situ
apoenzymes prior to extraction. However, the TPT profile in
Figure 1 does suggest that, in all likelihood, mostly free TPT levels
were measured since it seems highly unlikely that the total tissue
content of TPT (free plus bound) would decline so substantially
during the period of most rapid seedling growth (i.e. the fresh
weight increases after 36 h germination).

The TPT content extracted from germinating soybean seed-
lings increased substantially during the 12 to 36 h period of
germination and then declined sharply (Fig. 1). Of particular
significance, the rise in TPT levels was preceded by increased
TPTase activity (Fig. 1) and also by enhanced rates of O2 con-
sumption (data not shown).

After 36 h germination, extractable TPT content declined dra-
matically even though maximal TPTase activity was maintained
until at least 96 h germination (Fig. 1). As indicated above,
accurate interpretation of the reasons for the TPT increase and
decline is hindered somewhat by a lack of understanding as to
whether the TPT data of Figure 1 clearly represent only free TPT
levels as opposed to a combination of free and previously bound
(to in situ apoenzymes) TPT.

Because the TPT content probably represented largely free TPT
from seedling tissues, the increase from 45 to 75 nmol TPT/
seedling (Fig. 1) during the first 36 h after imbibition apparently
represented an accumulation of TPT in free-form due to a paucity
of apoenzyme binding sites. Accordingly, the decline in TPT
content after 36 h was probably a reflection of an increased
demand and subsequent chelation of TPT by apoenzymes requir-
ing this coenzyme, possibly in combination with a decreased rate of TPP formation in situ due to reductions in available supplies of thiamin and ATP. It is also possible that TPTase became compartmentalized from these components after 36 h germination such that extractable enzyme activity (Fig. 1) did not accurately portray rates in situ. However, the rapid decline in TPP content was initiated near the end of the slow gain period (phase II) of fresh weight changes (Fig. 1); consequently, the rapid enlargement and development of seedling tissues that began shortly thereafter would have imposed a sharply increasing demand for both ATP and TPP.

Pyritthiamin Effects. Pyritthiamin is a potent inhibitor of partially purified soybean TPTase (8), similar to its effects on rat brain TPTase (6), where pyritthiamin acts as a competitive inhibitor with a $K_i$ equivalent to the $K_m$ for thiamin. Thus, it was of interest to determine the effects of pyritthiamin on germinating seedlings.

Soybean seeds were imbibed in distilled H$_2$O; pyritthiamin (5 mg/ml) was applied at 12, 24, 36, and 48 h after onset of imbibition (Fig. 2). Pyritthiamin depressed the rate of increase in seedling fresh weight, however, inhibition was not expressed until approximately 36 h after addition of pyritthiamin to the imbibition medium. Thus, the pyritthiamin-mediated decreases in fresh weight changes occurred during phase III germination, the period when TPTase is apparently needed most (Fig. 1).

Assuming that pyritthiamin acted primarily on TPTase activity, these results (Fig. 2), in combination with the observation that an increase in TPP content was preceded by enhanced TPTase activity (Fig. 1), indicated that TPP formation and utilization represent important facets of vigorous seedling growth, at least in soybeans.

The first few days of germination represent a period of intense metabolic activity directed toward seedling ecession. Peaks in O$_2$ uptake (data not shown), TPP formation, and TPTase activity (Fig. 1) all preceded the period of rapid fresh weight increases (phase III, Fig. 1). Compared to soybeans, germinating radish seedlings have a compressed temporal profile with regard to fresh weight changes (9). There is a large increase in ATP content at the expense of AMP and ADP during the first 90 min (phase I) of water imbibition (9). A similar increase in ATP (and decrease in AMP and ADP) content occurs in a number of soybean cultivars but does so over the initial 4 to 5 h (as opposed to 90 min in radish) of imbibition (ref. 1 and D. E. Moreland, personal communication). Thus, the increase in TPP content by 36 h that we observed (Fig. 1) also was preceded by a rise in seedling ATP content. During the course of characterizing soybean TPTase (8), we found, under our conditions of assay, that there was no detectable TPTase activity when ATP concentrations were below 1.0 mM. Consequently, an important modulator of thiamin phosphorylation by TPTase in situ may be the proximal concentration of ATP or other nucleoside triphosphates (8).

**LITERATURE CITED**

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