Enzymes of $\alpha,\alpha$-Trehalose Metabolism in Soybean Nodules

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ABSTRACT

Metabolism of trehalose, $\alpha,\alpha$-glucopyranosyl-$\alpha,\alpha$-glucopyranoside, was studied in nodules of Bradyrhizobium japonicum-Glycine max [L.] Merr. cv Beeson 80 symbiosis. The nodule extract was divided into three fractions: bacteroid soluble protein, bacteroid fragments, and cytosol. The bacteroid soluble protein and cytosol fractions were gel-filtered. The key biosynthetic enzyme, trehalose-6-phosphate synthetase, was consistently found only in the bacteroids. Trehalose-6-phosphate phosphatase activity was present both in the bacteroid soluble protein and cytosol fractions. Trehalase, the most abundant catabolic enzyme, was present in all three fractions and showed two pH optima: pH 3.8 and 6.6. Two other degradative enzymes, phosphotrehalase, acting on trehalose-6-phosphate forming glucose and glucose-6-phosphate, and trehalase phosphorylase, forming glucose and $\beta$-glucose-1-phosphate, were also detected in the bacteroid soluble protein and cytosol fractions. Trehalase was present in large excess over trehalose-6-phosphate synthetase. Trehalose accumulation in the nodules would appear to be predicated on spatial separation of trehalose and trehalase.

Trehalose, $\alpha,\alpha$-glucopyranosyl-$\alpha,\alpha$-glucopyranoside, is one of the major carbohdrates synthesized by cultured Bradyrhizobium japonicum, and every species of Rhizobium examined so far shows trehalose accumulation (26). This disaccharide has also been reported in yeast (1, 5), in fungi (12, 22), as well as in bacteria (3), mainly in actinomycetes (15). In soybean plants trehalose appears to be restricted to the nodule tissue (23). Its presence elsewhere in higher plants has not been conclusively established (7). Previous work with soybean nodules suggests that trehalose is synthesized by the microsymbiont (20).

The pathway for trehalose synthesis was first elucidated in Saccharomyces cerevisiae (5) (Fig. 1). The biosynthetic enzymes have also been reported in Lilium longiflorum pollen (8).

There appears to be more than one way of catabolizing trehalose (Fig. 1). Phosphotrehalase has been reported in Bacillus poviillia (3) and trehalose phosphorylase in Euglena gracilis (17). Trehalase, the most widely occurring catabolic enzyme has also been found in higher plants (6, 9, 27).

Despite the presence of large amounts of trehalose in legume nodules its possible role is not known. To approach this question, information on the metabolism of trehalose itself is helpful. In this paper we report on the enzymes of synthesis and breakdown of trehalose in extracts of soybean nodules.

*Supported in part by the United States Department of Agriculture under agreement No. 83-CR-1-1306. Salaries and research support were provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center and the Ohio State University. Journal Article No. 181-85.

MATERIALS AND METHODS

Plant Material. Soybeans, Glycine max [L.] Merr. cv Beeson 80, were grown in a greenhouse in 26 cm wide and 27 cm deep glazed pots of autoclaved silica sand with 20 plants/pot. Seeds were inoculated at planting with water cultures of either B. japonicum strain USDA 110 (low trehalose accumulator) or 61A76 (high trehalose accumulator) (26). Plants were irrigated with nutrient solution (24) three times daily.

Nodule Fractionation. After 5 to 6 weeks the plants were harvested, and subsequently all operations were conducted at 0 to 2°C. Five g of nodules were ground in a mortar with a pestle in 10 ml (added in 2 ml aliquots) of grinding medium, 0.1 M sodium phosphate buffer (pH 7.5) containing 0.15 M mannitol, 2 mM DTE, and 1 mM EDTA. The homogenate was filtered through two Miracloth discs in a 50 ml syringe into a 15 ml centrifuge tube.

The Miracloth filtrate was then centrifuged at 500g for 10 min. The resultant starch pellet was discarded and the supernatant fluid recentrifuged at 6,000g for 10 min to obtain the bacteroid pellet. The supernatant fluid was centrifuged at 48,000g for 15 min and the supernatant, cytosol fraction, was saved for gel filtration.

The bacteroid pellet was resuspended in 8 ml of grinding medium and centrifuged at 6,000g for 15 min. The pellet was suspended in 5 ml of wash medium, 2 drops (16 ml) of 10% (v/v) Triton X-100 were added, and the suspension was sonicated for 2 min at 0°C. The sonicated sample was centrifuged at 48,000g for 15 min. The supernatant was called bacteroid soluble protein fraction. The pellet was suspended in 7 ml of wash medium and called bacteroid fragment fraction.

Two drops of blue dextran solution were added to the bacteroid soluble protein fraction to permit visualization of protein (leg-hemoglobin in the cytosol fraction served the same purpose). Each fraction was loaded onto a Sephadex G-25 column and eluted with 0.014 M Na-phosphate buffer (pH 7.5) containing 1 mM DTE.

Isolation of bacteroids using Percoll gradients was carried out according to Reibach et al. (19), except that the grinding medium was 0.2 M Na-phosphate buffer (pH 7.5) with 2 mM DTE.

Isolation of free-living bacteria from liquid cultures (26) was accomplished by centrifuging the cultures at 6000g for 15 min and subsequently treating the bacterial pellet in the same way as the bacteroid pellet above.

Enzyme Assays. All controls and assays were run in triplicate. Uridine diphosphoglucose pyrophosphorylase (UTP: $\alpha$-glucose-1-P uridyltransferase EC 2.7.7.9) was assayed according to Bergmeyer (2). Trehalose-6-phosphate synthetase (UDP-glucose: $\alpha$-glucose-6-P 1-glucosyltransferase EC 2.4.1.15) assay was based on the method of Roth and Sussman (22). In the spectrophotometric assay the reaction mixture (0.8 ml) contained 100 mM Tris buffer (pH 7.5), 8 mM UDP-glucose, 30 mM glucose-6-P, 100 mM MgCl2, 3 mM EDTA, 25 mM KCl, and 0.2 ml of nodule extract. The control tubes lacked glucose-6-P. After 30 or 45 min
at 30°C the reactions were stopped by heating at 100°C for 2 min. The samples were centrifuged at 2000g for 10 min and portions of the supernatants were assayed spectrophotometrically for UDP, as follows: To a cuvette was added 1.07 ml 50 mM Tris buffer (pH 7.0), 0.1 ml 10.5 mM phosphoenolpyruvate, 0.1 ml 500 mM MgCl₂, 0.1 ml 30 mM NADH, 0.1 ml 5 mM DTE, 10 μl 100 mM ATP, 10 μl (10 units) dinucleotidephosphokinase. After a baseline at 340 nm had been determined, 10 μl of a mixture of pyruvate kinase (10 units) and lactic dehydrogenase (10 units) was added and the decrease in absorbance recorded.

The radioassay for trehalose-6-P synthetase was based on the method of Lapp et al. (13). The reaction mixture (0.4 ml) consisted of 0.25 μCi [U-¹⁴C]UDPG-glucose (205 Ci/mol, ICN, Irvine, CA) in a final UDP-glucose concentration of 25 mM, 5 mM glucose-6-P, 4 mM MgCl₂, 50 mM Tris (pH 7.5). The reaction was stopped by boiling the mixtures for 5 min. The tubes were then centrifuged at 3000g for 10 min, and the supernatant saved. The precipitate was washed with 0.4 ml of Tris buffer (pH 8.0), recentrifuged as above and the supernatant fluid was added to that from the first centrifugation. One unit of alkaline phosphatase was added and the tubes incubated for 2.5 h at 37°C. The reaction was stopped by boiling for 5 min. The tubes were centrifuged at 2000g for 10 min, and each supernatant was put through a column of mixed bed ion exchange resin. The samples were eluted with 5 ml H₂O, and the eluate which was evaporated to dryness and taken up in 100 μl H₂O. Aliquots were then spotted on silica gel (Whatman K6) TLC plates and developed two times in 95% ethanol:NH₄OH:H₂O (5:3:1, v/v). The plates were dried, sprayed lightly with a saturated KI solution, heated at 40 to 50°C for 15 min, cooled and sprayed with a solution containing 1 ml saturated AgNO₃ + 200 ml acetone to which H₂O is added just until the precipitate dissolves. After drying, the plates were sprayed with a solution containing 2.5 ml saturated NaOH in 100 ml of 95% ethanol to which H₂O is added just until the precipitate dissolves. Trehalose spot was identified, scraped into a liquid scintillation vial, and the radioactivity determined.

Trehalose 6-P phosphatase (trehalose 6-P phosphohydrolase EC 3.1.3.12) was assayed by the method of Mitchell et al. (18). The Pi was determined according to Heinonen and Lahti (10). Phosphotrehalase activity was measured spectrophotometrically by the method of Bhumiratana et al. using glucose 6-P dehydrogenase as a coupling enzyme (3). Trehalase (trehalose 1,6-dehydropyrophosphorylase EC 3.2.1.28) was assayed according to Veluthambi et al. (27), and the glucose was determined by glucose oxidase assay (14). Trehalose phosphorlase (proposed name: α-D-glucopy-
the bacteroid soluble protein fractions, but in some of the experiments a significant proportion of activity (as high as 32% at total activity) was also found in the bacteroid fragments. This could indicate that the enzyme was normally associated with membranes but was solubilized during sonication in the presence of Triton X-100. Alternatively, it could also be indicative of incomplete breakage of bacteroids during sonication, or enzyme associating with the bacteroid fragments during centrifugation following sonication. The strain 61A76 had 1.8 times the activity found in USDA 110 bacteroids. This is consistent with the relatively high trehalose accumulation seen with 61A76 (26).

The possibility of contamination of the bacteroids by mitochondria appeared negligible based on a malate dehydrogenase assay described by Waters et al. (28). A rate of 41.3 ± 2.3 (mean ± se) μmol/min·g nodule fresh weight was measured for controls, whereas the corresponding rates using two inhibitors (1 mM) known to inhibit mitochondrial MDH were 36.3 ± 0.8 for iodoacetamide and 38.1 ± 1.2 for phenylmethylsulfonyl fluoride after 1 h preincubation of the bacteroid soluble protein with the inhibitors. These results would indicate that essentially no mitochondrial malate dehydrogenase was present (28).

Purification of bacteroids using Percoll gradients (19) gave confirming data for the localization of trehalose 6-P synthetase activity in the bacteroids. A rate of 10.2 ± 0.1 (mean ± se) nmol/min·g nodule fresh weight was obtained for the soluble protein fraction of 61A76, whereas the corresponding value for USDA 110 was 1.4 ± 0.1.

Trehalose 6-P phosphatase was present in both the bacteroid soluble protein and cytosol fractions. The level of activity in the bacteroid soluble protein fraction was higher in 61A76 than in USDA 110 which is consistent with a greater rate of trehalose synthesis of 61A76. The activities in the cytosol fraction were fairly evenly distributed between the two types of nodules.

The low activity of trehalose 6-P synthetase was of some concern. We, therefore, determined the activity of this enzyme in free-living cultures of 61A76 to see whether it would be sufficient to account for the observed levels of trehalose accumulation. Using data in Figure 1 from Streeter (26) a value of 10.3 μg trehalose·day⁻¹·(mg dry weight bacteria)⁻¹ was calculated from d 5 to d 7 of the growth period. For 5-d-old cultures we measured trehalose 6-P synthetase activity equivalent to 10.5 μg trehalose·d⁻¹·(mg dry weight bacteria)⁻¹.

Phosphotrehalase activity was found in both bacteroid soluble protein and cytosol fractions of both strains (Table III). The most active degradative enzyme, however, was trehalase. This enzyme exhibited two pH optima, at 3.8 and at 6.6 (25). Slightly higher activities were found at pH 6.6 (Table III). The ratio of activities between the two pH values was close to 2 (pH 6.6/pH 3.8) in the bacteroid fragment fractions and close to 1 in both nonparticulate fractions. In the bacteroid soluble protein fraction, 61A76 had only 50% of the activity found in USDA 110 at pH 3.8. At pH 6.6, the corresponding percentage was 63%. A lower activity in 61A76 cytosol fraction was also seen relative to USDA 110, although the difference was much less (74% at pH 3.8 and 83% at pH 6.6). Most of the trehalase activity was in the cytosol fraction.

Trehalase phosphorylase activity was identical between the two strains in the bacteroid soluble protein fraction. In the cytosol, the 61A76 nodules showed an over 4-fold increase relative to the activity in USDA 110. The activity in 61A76 nodules was highly variable as shown by the large standard error.

**DISCUSSION**

We have demonstrated the presence of the enzymes depicted in Figure 1 in soybean nodules. The key biosynthetic enzyme, trehalose 6-P synthetase was confined to the bacteroids, confirming an earlier report which indicated that trehalose is synthesized in the bacteroids (20). The higher activity of this enzyme in 61A76 than in USDA 110 is consistent with their respective trehalose levels in the nodules and in culture (26). Also, the pattern of trehalose 6-P phosphatase activity in the bacteroids of the two strains is proportional to the synthetase activities. The trehalose 6-P phosphatase activity in the cytosol could represent nonspecific phosphatase, as no effort was made to determine the specificity of the phosphatase in either fraction.

Although the trehalose 6-P synthetase activity was low, our calculations indicated that in cultured bacteria it was sufficient to account for the observed rates of accumulation of trehalose, assuming no trehalase activity was present. However, the rates we measured in bacteroids could account for only 25 to 30% of the trehalose accumulation in the nodules reported in Table 5 by Streeter (26) during 27 to 47 d after planting. This could mean that the enzyme activity (a) was not at its maximum at the time of harvest, (b) decreased during extraction and gel filtration, or (c) our reactions were not run under optimal conditions. We were unable to determine activity in the crude extracts due to high background. The bacteroid soluble protein fraction lost 7% of the activity when kept in ice bath for 1 h, indicating that at least the gel-filtered preparation was fairly stable.

There is evidence in *Dicyostelium discoideum* that trehalose 6-P synthetase is highly regulated, and its kinetic behavior is suggestive of negative cooperativity (12). Mg²⁺, EDTA, and KCl were reported to be activators of this enzyme (12). On the basis of similar results (Table I) obtained using spectrophotometric (+EDTA, +KCl) and radioisotope (−EDTA, −KCl) assays, it would appear that EDTA and KCl do not have a major role in the bacteroid enzyme activity, at least under our experimental conditions. There was no effect of 2 mM HCO₃⁻, which has been shown to reverse inhibition of trehalose 6-P synthetase by mycorrhin in mycobacteria and yeast (15) (data not shown). We would expect a key enzyme to be rate limiting. UDP-glucose would be in excess and participate in other reactions, whereas trehalose 6-P synthetase is in fact the first enzyme specifically committed to trehalose synthesis.

The presence of phosphotrehalase activity in nodules was somewhat surprising because the enzyme has been reported previously only in one organism (3). The activity found was quite low, although it amounted to at least twice that of controls (minus trehalose 6-P). Because activity was so low relative to the activity of phosphotrehalase (Table II), it seems likely that most of the trehalose 6-P formed is converted to trehalose.

Trehalase activity in the bacteroid soluble protein fraction of

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**Table II. Enzymes of Trehalose Synthesis in Extracts of Nodules Formed by USDA 110 or 61A76**

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>USDA 110</th>
<th>61A76</th>
</tr>
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<tbody>
<tr>
<td>Bacteroid Soluble Protein</td>
<td>Cytosol</td>
<td>Cytosol</td>
</tr>
<tr>
<td>UDPG pyrophosphorylase</td>
<td>940 ± 230</td>
<td>641 ± 25</td>
</tr>
<tr>
<td>Trehalose-6-P-synthetase</td>
<td>3.9 ± 0.5</td>
<td>7.2 ± 1.0</td>
</tr>
<tr>
<td>Trehalose-6-P-phosphatase</td>
<td>32 ± 11</td>
<td>103 ± 24</td>
</tr>
</tbody>
</table>

Mean ± se of three experiments.

nmol·min⁻¹ (g nodule fresh wt)⁻¹
61A76 was much less than the same fraction of USDA 110, whereas in the cytosol the activities were more similar (Table III). The enzyme from the bacteroid soluble protein fraction of 61A76 did have a higher $K_m$ than the cytosolic enzyme at both pH values (data not shown). Also, the $K_m$ for trehalase from the bacteroid soluble protein fraction increased with an increase in pH, whereas in the case of the cytosolic enzyme the $K_m$ decreased. Whether the bacteroid enzyme is in fact different from the cytosol enzyme remains to be determined. The pH optima of 3.8 for trehalase would appear to lack physiological relevance. It is, however, possible that the enzyme could encounter such low pH if it were associated with vacuoles, as has been shown in yeast (11).

The other hydrolytic enzyme, trehalose phosphorylase, converts trehalose to glucose and $\beta$-glucose-1-P. This enzyme is readily reversible in vitro, and was, in fact, assayed in the reverse direction in this study, because of the difficulties encountered in trying to elute $\beta$-glucose-1-P from an ion exchange column quantitatively when the reaction was run in the hydrolytic direction. High trehalase activity would preclude glucose analysis. It has been suggested (17) that in vivo trehalose phosphorylase, like other phosphorylases, acts unidirectionally as a degradative enzyme, whereas the synthesis of trehalose would require a nucleotide diphosphate sugar as a glycosyl donor.

To our knowledge trehalose phosphorylase has previously been demonstrated only in Euglena (17) which lacks trehalase. Although the activity reported here was dependent on glucose and $\beta$-glucose-1-P (the $\alpha$-form showed only background activity in the presence or absence of glucose), some caution should be exercised in interpreting the results, until they can be verified using enzyme that has been at least partially purified from the nodules. Furthermore, considering the high trehalase activity in the nodules the physiological role of trehalose phosphorylase might well be questioned.

Because trehalase has been shown to be toxic in Cuscuta reflexa (27) it is perhaps not too surprising to find high levels of the catabolic enzymes in the cytosol. The abundance of the enzymes catalyzing trehalose in bacteroids and cytosol indicates that trehalase can be readily metabolized. The fact that it accumulates both in the bacteroids and cytosol requires sequencer of trehalase and the catabolic enzymes. A need for compartmentation is further emphasized by the greater activity of trehalase compared to trehalose-6-P synthetase, and a tight regulation of activity of the two enzymes may also be required for trehalose accumulation to occur.

Acknowledgment—We thank Paul DeVine for technical assistance.

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