Multiple Forms and Intracellular Localization of Uridine Diphosphate Glucose Pyrophosphorylase in *Avena sativa*¹

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WILLIAM C. GORDON² AND LAWRENCE ORDIN

Department of Biochemistry, University of California, Riverside, California 92502

ABSTRACT

Uridine diphosphate glucose pyrophosphorylase was isolated separately from *Avena sativa* leaves, roots, and etiolated coleoptiles and purified by ammonium sulfate fractionation, DEAE-Sephadex chromatography and polyacrylamide gel electrophoresis. There was no difference in the enzyme from the different tissue types with respect to properties exhibited during the purification procedure. A small portion of the enzyme from all three sources was found to be particulate when homogenized in aqueous sucrose media. Characterization of the particulate form by discontinuous sucrose density gradient showed the enzyme to be located at two different densities, one of which corresponded to chloroplasts in the leaves and plastids in the coleoptiles and roots. Homogenization and fractionation of the oat leaves using the nonaqueous media, hexane, and carbon tetrachloride, resulted in 50 to 60% of the enzyme being associated with chloroplasts and the remainder being associated with other membranous material. These data indicate that the enzyme from oat leaves, roots, and etiolated coleoptiles has multiple intracellular locations, and it is suggested that compartmentation of this enzyme may be a mechanism for regulation of uridine diphosphate glucose metabolism in oats.

Uridine diphosphate glucose pyrophosphorylase (UTP: α-D-glucose-1-P uridylyltransferase, EC 2.7.7.9) is involved in plants in the production of UDP-glucose, which is important for reactions which utilize UDP-glucose as glucose donor in the synthesis of cellulose (18–20), callose (8, 18, 19, 21, 27), sucrose (4), and steryl glucosides (17). UDP-glucose is also the initial UDP-sugar in a number of interconversions producing other UDP-sugars and UDP-sugar acids which are used in production of cell wall polysaccharides and glycoproteins (9, 12, 23).

Several studies of UDP-glucose pyrophosphorylase in plant tissue have shown evidence for a subcellular localization of the enzyme that would correlate with its supposed metabolic role in that tissue. The enzyme from corn coleoptiles was shown to be located in high specific activity in the cell wall (15). At this site, it would be involved in the synthesis of cell wall polysaccharides which occurs at a high rate in this tissue. The enzyme isolated from etiolated mung beans was reported to have a significant lipid content (6) and was suggested to exist in vivo at the cell membrane in close proximity to sucrose synthetase. However, greater than 90% of the UDP-glucose pyrophosphorylase activity from etiolated oat coleoptiles was found to be associated with no subcellular particle (11). Various workers have reported the subcellular localization of UDP-glucose pyrophosphorylase in photosynthetic tissue after homogenization of the tissue in nonaqueous media. This technique prevents the possible solubilization of chloroplast components that occurs during homogenization in aqueous media (24). In this respect, the enzyme from tobacco leaves homogenized in hexane-CCl₄, was found to be contained entirely in the chloroplast, along with other enzymes involved in sucrose synthesis (3). Other workers concluded that UDP-glucose pyrophosphorylase was a cytoplasmic enzyme in spinach leaves, after experiments where the tissue was homogenized in light petroleum-CCl₄, (13). The same technique also showed the enzyme to be located in the cytoplasm in *Euglena* (22).

Participation of UDP-glucose in several metabolic pathways in oats may require compartmentation of UDP-glucose pyrophosphorylase as a regulatory mechanism. Modulation of the catalytic activity by varying concentrations of certain metabolites has been suggested for UDP-glucose pyrophosphorylases from other plant species (10, 14). The present studies investigated the possibility of UDP-glucose pyrophosphorylase iso-enzymes occurring in oat leaves, roots, and etiolated coleoptiles, and determined the subcellular localization of the enzyme in these tissues.

MATERIALS AND METHODS

**Plant Material.** Oat seedlings (*Avena sativa* L. var. Segre-havre) were grown for 88 hr on vermiculite at 26°C under red-orange light and were watered daily with distilled water. Harvesting of etiolated coleoptiles was carried out in diffuse daylight at this time. For nonetiolated tissue, the plants were allowed to grow for 4 more days at 23°C under 13 ft-c white light and harvesting of tissue was performed under these conditions. Coleoptiles were selected and defoliated before homogenization. Leaves were harvested by excising the tissue just above the coleoptile. Seedlings were rinsed in distilled water to remove vermiculite before roots were harvested.

**Enzyme Assay.** UDP-glucose pyrophosphorylase was assayed in the direction of UDP-glucose synthesis, using glucose-1-P and UTP as substrates. Reaction mixtures contained 40 mM tris-HCl (pH 7.5), 3.75 mM MgCl₂, 0.5 mM glucose-1-P.
(30 μCi/μmole), 2.0 mM UTP, and inorganic pyrophosphatase (yeast) at 1 unit/ml of reaction medium. Incubations were carried out in 50-μl cuvettes at 30 °C, and the reaction was initiated by adding 5 μl of enzyme. A 5-μl aliquot was removed at specified times and spotted on a Whatman No. 3MM chromatogram at a point where 5 μl of 20 mM HgCl₂ was previously applied and dried. The enzyme reaction was thus terminated, and the spot was dried in an air stream. The chromatogram was chromatographed in an ascending direction for 10 cm in isopropanol-0.05 N HCl (3:2), which separates glucose-1-P from UDP-glucose and from several other possible compounds. The spots were located by autoradiography, cut out of the chromatogram, and the radioactivity was counted by liquid scintillation. Per cent conversion of glucose-1-P to UDP-glucose was then calculated and converted to nanomoles of glucose-1-P converted per min.

**Enzyme Purification.** UDP-glucose pyrophosphorylase was partially purified from coleoptil of etiolated seedlings and leaves and roots from nonetiolated seedlings and compared by polyacrylamide gel electrophoresis. Five to ten grams of each tissue type were harvested and homogenized with Kontes Dual tissue grinders in 50 mM tris-HCl (pH 7.5), 1 mM EDTA, and 10 mM 2-mercaptoethanol and squeezed through a layer of Miracloth. A 45 to 70% (w/v) ammonium sulfate fraction was prepared by the addition of solid ammonium sulfate to the homogenate. The dialyzed sample was then applied to a 1.5 × 3 cm DEAE-Sephadex (A50) column, and eluted with the 50 mM tris buffer. The column was then eluted stepwise by adding NaCl to the initial buffer in the following sequence: 0.05 M, 0.1 M, 0.2 M, and 1.0 M. The concentrated enzyme was dialed overnight against 2 liters of 0.1 M tris-HCl (pH 7.5), 1 mM EDTA, and 10 mM 2-mercaptoethanol.

Comparative polyacrylamide gel electrophoresis was conducted in the discontinuous system of Davis and Ornstein (5, 20). The system was modified in that the reservoir buffers were diluted 4-fold. Six per cent gels of 85 mm length were prepared in 12.5 × 110 mm glass tubes which were divided in half lengthwise by a glass slide sealed with paraffin between the slide and the tube. The stacking gel on top was also divided, and samples were subjected to electrophoresis side by side at 280 v and at 4 °C. Following electrophoresis, UDP-glucose pyrophosphorylase was measured after each half of the gel was sectioned and homogenized.

**Nonaqueous Homogenization and Fractionation.** Tissue was homogenized in nonaqueous media using modifications of the methods of Stocking (24). Oat leaves were harvested and frozen by immersing the tissue in a glass vessel in Dry Ice-acetone. The frozen tissue (about 5 g) was then lyophilized at 0 to 5 °C for 20 hr and stored desiccated at -15 °C until used. The tissue was homogenized in nonaqueous media by gently grinding in a mortar and pestle with hexane-CCl₃, (d = 1.32 at 0 °C) as the grinding medium, and then squeezed through a layer of Miracloth. The homogenate was fractionated according to the scheme shown in Figure 1, and all procedures were performed at the temperature of ice. A hexane-CCl₃ density of 1.32 was found to be more dense than most of the chloroplasts of oat leaves subjected to these growing conditions. All pellet fractions were resuspended in hexane-CCl₃, (1:1) of equal volumes to ensure similar times of exposure to solvent and then dried down under a stream of nitrogen. After drying, the particulate fractions were dissolved in 25% (w/v) sucrose, 0.1 M tris-HCl (pH 7.5), 5 mM EDTA, and 10 mM 2-mercaptoethanol and the two solvent fractions were dissolved in 0.25 M sucrose, 0.1 M tris-HCl, 5 mM EDTA, and 10 mM 2-mercaptoethanol. The samples dissolved in the 25% sucrose were then divided into a fraction sedimenting to the bottom of the medium and a fraction layering on the top. The fractions were examined under a Leitz Dialux phase microscope.

Chlorophyll was estimated by the method of Arnon (2), and phosphoglycolate phosphatase was assayed following the release of Pi (1). Phosphate was detected using FeSO₄-ammonium molybdate reagent (25) and measuring absorbance at 710 nm. Protein was estimated by the method of Lowry et al. (16) after exhaustive dialysis of the samples versus distilled water.

**RESULTS AND DISCUSSION**

The nature of the involvement of UDP-glucose pyrophosphorylase in the production of UDP-glucose may vary in the leaf, root, and coleoptile, depending on the importance of the UDP-glucose-requiring reactions in that tissue. In this respect, the regulation of the utilization of UDP-glucose in the synthesis of cell wall polysaccharides, sucrose, or steryl glucosides is determined by the stage of development of the tissue and whether or not it is involved in photosynthesis. It has been suggested that UDP-glucose pyrophosphorylase is also involved in the pyrophosphorylation of UDP-glucose in some tissue (6, 28). The participation of different UDP-glucose pyrophosphorylase species in different branches of the metabolic pathway was investigated in the various tissue types.

**UDP-Glucose Pyrophosphorylase in Different Tissue Types.** UDP-glucose pyrophosphorylase was isolated from oat leaves, roots, and etiolated coleoptiles and compared during purification with respect to the determination of the possibility of the existence of isoenzymes. Greater than 95% of the enzyme activity from all three sources was contained in a 45 to 70% ammonium sulfate fraction. Several fold purification was realized with all three tissue types and the enzyme was thus stabilized against loss of activity, either in the presence or absence of the ammonium sulfate. The elution pattern of the (NH₄)₂SO₄ purified enzyme from a DEAE-Sephadex column by a stepwise NaCl gradient was identical for the leaf, coleoptile, and root.
enzymes. Figure 2 shows UDP-glucose pyrophosphorylase from coleoptile being eluted with 0.1 mM NaCl after the 0.05 mM NaCl step in a 50 mM tris-HCl (pH 7.5), 1 mM EDTA, and 10 mM 2-mercaptoethanol buffer system. Five- to 10-fold purification was realized with the various preparations with 85 to 90% enzyme yield.

The concentrated enzymes purified on DEAE-Sephadex were subjected to comparative polyacrylamide gel electrophoresis on a gel divided in half lengthwise by a glass partition. Control experiments with this system showed that samples migrate identically and that no mixing occurs between samples. Three gels were necessary to compare the enzyme preparations from the three tissue types, as the following samples were run: (a) root and coleoptile, (b) root and leaf, (c) coleoptile and leaf.

Figure 3 shows the comparisons of the enzyme activity profiles as measured on the gels. Some of the samples show two activity peaks while others show one. However, enzyme from the same source may show one peak on one gel and two peaks on another. Thus, the root preparation displayed two peaks on gel I but only the fast peak was evident on gel II. Also, the coleoptile preparation showed one peak on gel I and two peaks on gel III. Two peaks of enzyme activity were measured on both gels for the leaf enzyme, but the distribution of the two peaks was not the same. The samples of each tissue type run on the gels were from the same preparation and, therefore, the variance in the distribution of the two enzyme activity peaks observed during electrophoresis represented a transient interconversion between forms in the same preparation with time. A similar experiment with enzyme isolated from different batches of tissue produced the same results, i.e., identical migration of enzyme from each source during electrophoresis and display of multiple enzyme forms. In one attempt to determine contributing factors to this interconversion, the presence or absence of 2-mercaptoethanol or thioglycolic acid was found to have no effect and, therefore, changes in sulfhydryl groups on the enzyme were not involved. These investigations found no tissue specific differences in UDP-glucose pyrophosphorylase in oat seedlings.

**Particulate Form of the Enzyme.** The subcellular localization...
Localization of UDP-Glucose Pyrophosphorylase

Tissue was minced and cell wall was removed. Fractions were taken sequentially on the same homogenate. Measurements represent the average of duplicate samples.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Enzyme</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/min</td>
<td>%</td>
</tr>
<tr>
<td>Leaf</td>
<td></td>
<td></td>
</tr>
<tr>
<td>800g (15 min) pellet</td>
<td>1.681</td>
<td>5.6</td>
</tr>
<tr>
<td>10,000g (20 min) pellet</td>
<td>2.650</td>
<td>8.9</td>
</tr>
<tr>
<td>78,000g (2 hr) pellet</td>
<td>540</td>
<td>1.8</td>
</tr>
<tr>
<td>Soluble</td>
<td>25,040</td>
<td>83.7</td>
</tr>
<tr>
<td>Coleoptile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>800g (15 min) pellet</td>
<td>2,480</td>
<td>3.1</td>
</tr>
<tr>
<td>10,000g (20 min) pellet</td>
<td>2,923</td>
<td>3.7</td>
</tr>
<tr>
<td>78,000g (2 hr) pellet</td>
<td>949</td>
<td>1.2</td>
</tr>
<tr>
<td>Soluble</td>
<td>73,280</td>
<td>92.0</td>
</tr>
<tr>
<td>Root</td>
<td></td>
<td></td>
</tr>
<tr>
<td>800g (15 min) pellet</td>
<td>244</td>
<td>1.8</td>
</tr>
<tr>
<td>10,000g (20 min) pellet</td>
<td>896</td>
<td>6.4</td>
</tr>
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<td>78,000g (2 hr) pellet</td>
<td>340</td>
<td>2.4</td>
</tr>
<tr>
<td>Soluble</td>
<td>12,420</td>
<td>89.4</td>
</tr>
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</table>

The heaviest particles were characterized by discontinuous sucrose gradient centrifugation, and the distribution of UDP-glucose pyrophosphorylase activity in such a gradient is shown in Table II. Particulate matter was banded at every interface, but the majority of the enzyme activity was divided between a band at the 0.8 M/1.0 M sucrose interface and a heavier band at the 1.1 M/1.3 M sucrose interface. The latter band contained the plastid fraction, as all of the Chl in the leaf and protochlorophyll in the coleoptile was located at that interface. Cytochrome oxidase and catalase activity were also located there. UDP-glucose pyrophosphorylase was easily dissociated from both particulate forms upon resuspension and manipulation, suggesting this to be the reason that the majority of the enzyme activity was distributed in the soluble fraction during homogenization and differential centrifugation in aqueous media.

Nonaqueous Fractionation. Oat leaves were homogenized and fractionated in hexane-CCI, to avoid the solubilization or leaching out of chloroplast bound components. The technique was first utilized in plant systems by Stocking (24), and these methods were modified in the present investigation by further fractionation of the lipid-rich chloroplasts from other low density material. This additional step purified the chloroplasts and allowed for the conclusion that any enzyme activity distributed with this component was, in fact, a chloroplast contained enzyme. The majority of the chloroplasts of oat leaves with an age and history of those used in these experiments were found to be less dense than a hexane-CCI, mixture of d = 1.32 at 0 C. Chloroplasts were thus separated from nonchloroplast components of the cell, and enzymes contained in the chloroplast presumably remained in situ. Removal of the organic medium by evaporation, and resuspension of chloroplasts in a 25% sucrose buffer solution resulted in the further fractionation of two particle types. Organelles sedimenting to the bottom of the solution were identified as whole chloroplasts when examined under a phase microscope. The other fraction layered on top of the 25% sucrose and was seen to consist of membranous particles and vesicles. Material of the original homogenate which was more dense than 1.32 was then resuspended in hexane-CCI, of d = 1.38. Many of the remaining chloroplasts layered on top of this medium, and further fractionation with 25% sucrose resulted in two particulate components as in the first hexane-CCI, fractionation. Similarly, chloroplasts sedimented to the bottom of this solution and membranous vesicles layered on top.

The Chl contained in the various particulate fractions was considered to be a direct indication of the number and location of whole chloroplasts. Chl in the solvent phases was a measure of chloroplasts destroyed during homogenization and fractionation; this solubilized pigment was contained almost totally in the solvent phase of the first fractionation (d = 1.32). The yield of whole chloroplasts in these experiments was 62 to 72%, which was considerably higher than the reported yield for tobacco and spinach chloroplasts homogenized by essentially the same technique (3, 13). This difference is most likely reflective of the degree of fragility of the chloroplasts from the various plant species. The distribution of Chl, UDP-glucose pyrophosphorylase, and phosphoglycolate phosphatase during fractionation is shown in Table III. The co-fractionation of UDP-glucose pyrophosphorylase with Chl was calculated as 38 to 51% of the total enzyme activity after assuming the UDP-glucose pyrophosphorylase-Chl ratio in fraction III was the same as in the lower portion of fractions I and II.
Table III. Nonaqueous Homogenization and Fractionation of Oat Leaves in Hexane-CCl₄

| Fraction | Experiment I | | | | Experiment II | | | | | | Experiment III | | | |
|----------|--------------|----|----|----------------|--------------|----|----|----------------|--------------|----|----|----------------|--------------|----|----|
|          | UDGP pyrophosphorylase | Chl | µmol min | P-glycolate phosphatase | UDGP pyrophosphorylase | Chl | µmol min | P-glycolate phosphatase | UDGP pyrophosphorylase | Chl | µmol min | P-glycolate phosphatase |
| Fraction I (d < 1.32) | 180 | 0 | 0 | | | | | | | | | | |
| Upper | 941 | 719 | 277 | | | | | | | | | | |
| Lower | 1108 | 0 | 0 | | | | | | | | | | |
| Fraction II (d = 1.32 to 1.38) | 102 | 0 | 0 | | | | | | | | | | |
| Upper | 1764 | 721 | 76 | | | | | | | | | | |
| Lower | 117 | 0 | 0 | | | | | | | | | | |
| Fraction III (d > 1.38) | 351 | 2 | 1 | | | | | | | | | | |
| Percentage of UDGP pyrophosphorylase in chloroplast fractions | 38 | 50 | | | | | | | | | | |
| Noncorrected | 62 | 72 | | | | | | | | | | |
| Corrected for chloroplast loss | 64 | 66 | | | | | | | | | | |

1 Fractionation procedures are described under “Materials and Methods.”
2 Chl in particulate fractions/total Chl in tissue × 100.
3 Calculation described under “Results.”

Literature Cited

27. Tsai, C. M. and W. Z. Hassid. 1971. Solubilization and separation of uridine diphospho-\( \beta \)-glucose: \( \beta \)-\( \beta \)/(1\( \rightarrow \)4)glucan and uridine diphospho-\( \beta \)-glucan: \( \beta \)-\( \beta \)/(1\( \rightarrow \)3)glucan glucosyltransferases from coleoptiles of Avena sativa. Plant Physiol. 47: 740-744.