Regulation of Starch Synthesis in the Bundle Sheath and Mesophyll of *Zea mays* L.\(^1\)

INTERCELLULAR COMPARTMENTALIZATION OF ENZYMES OF STARCH METABOLISM AND THE PROPERTIES OF THE ADPglucose PYROPHOSPHORYLASES

Received for publication July 31, 1986 and in revised form October 25, 1986

STEVEN R. SPILATRO AND JACK PREISS*
Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824

**ABSTRACT**

The intercellular localization of enzymes involved in starch metabolism and the kinetic properties of ADPglucose pyrophosphorylase were studied in mesophyll protoplasts and bundle sheath strands separated by cellulase digestion of *Zea mays* L. leaves. Activities of starch synthase, branching enzyme, and ADPglucose pyrophosphorylase were higher in the bundle sheath, whereas the degradative enzymes, starch phosphorylase and amylase, were more evenly distributed and slightly higher in the mesophyll. ADPglucose pyrophosphorylase partially purified from the mesophyll and bundle sheath showed similar apparent affinities for Mg\(^{2+}\), ATP, and glucose-1-phosphate. The pH optimum of the bundle sheath enzyme (7.0–7.8) was lower than that of the mesophyll enzyme (7.8–8.2). The bundle sheath enzyme showed greater activation by 3-phosphoglycerate than did the mesophyll enzyme, and also showed somewhat higher apparent affinity for 3-phosphoglycerate and lower apparent affinity for the inhibitor, orthophosphate. The observed activities of starch metabolism pathway enzymes and the allosteric properties of the ADPglucose pyrophosphorylases appear to favor the synthesis of starch in the bundle sheath while restricting it in the mesophyll.

Metabolic processes of photosynthesis in 4-carbon pathway (C-4) type plants are partitioned between two cell types, the bundle sheath and mesophyll (12). Storage of starch occurs principally in the bundle sheath, although the amount of starch synthesis which occurs in the mesophyll is species dependent (4, 9). In maize (Zea mays), a NADP-ME\(^2\) type C-4 plant, synthesis of starch occurs only in the bundle sheath during normal photosynthesis (8).

Regulation of starch synthesis in leaves of maize and other C-4 plants is unclear. Experimental manipulation of starch synthesis in the mesophyll has shown that compartmentalization of starch metabolism in the maize bundle sheath is not absolute. Maize plants grown in continuous light produced starch in the mesophyll (8), suggesting that availability of photosynthate was limiting. The levels of enzymes of starch metabolism and their localization in the mesophyll and bundle sheath of maize may be an important factor determining the ability of these cells to synthesize starch. Previous studies (10, 23) have suggested that the greater level of ADPglucose pyrophosphorylase activity in the bundle sheath than in the mesophyll may lead to greater starch accumulation in the bundle sheath.

Starch synthesis in plants is believed to be regulated by the enzyme ADPglucose pyrophosphorylase (ATP:α-glucose-1-P adenylyltransferase, EC 2.7.7.27), which catalyzes the synthesis of ADPglucose, the substrate for starch synthase (ADPglucose, 1,4-α-D-glucan 4-α-glucosyl transferase, EC 2.4.1.21). ADPglucose pyrophosphorylase from leaves of C-3 and CAM plants (11, 25, 26), and from nonphotosynthetic tubers and endosperm (7, 28) are activated by 3PGA and inhibited by Pi. The interaction of these effector metabolites suggests that, in vivo, the rate of ADPglucose synthesis is controlled by the stroma 3PGA/Pi ratio, and thereby couple starch production to the rate of photosynthesis. Results from previous studies using a variety of experimental approaches have provided support for this mechanism (for review, see Preiss [22]).

In this study, the regulation of starch synthesis in maize leaves was investigated using highly purified mesophyll and bundle sheath cell fractions. Previous studies of localization of starch metabolizing enzymes have been reported (8, 10, 14). However, enzyme recovery and cell purity were not completely documented. In one case (10), complete separation of cells was not achieved. Localization of starch metabolism pathway enzymes were reinvestigated here. The properties of maize leaf ADPglucose pyrophosphorylase have been previously described. This enzyme in whole leaf extracts of sorghum, also a NADP-ME type C-4 plant, was activated by 3PGA and inhibited by Pi (25). However, the regulatory properties of bundle sheath and mesophyll specific enzymes were not investigated. The allosteric properties of ADPglucose pyrophosphorylases in each of these cells may play a role in determining the rate of starch synthesis.

**MATERIALS AND METHODS**

**Materials.** Dent corn (*Zea mays* L.), inbred W64A\(^{HT}\), was grown in a greenhouse under natural light supplemented with 400 W high pressure sodium lamps providing a 16 h photoperiod. Leaves were collected at 12 d post-germination.

**Buffer Solutions.** A: 20 mm Mes (pH 5.5), 0.5 mm sorbitol, 5.0 mm MgCl\(_2\), 1.0 mm CaCl\(_2\); B: 5.0 mm Hepes (pH 7.5), 0.2 mm sorbitol, 5.0 mm MgCl\(_2\), 5.0 mm Hepes (pH 7.5), 0.5 mm succrose, 5 mm MgCl\(_2\), 15% dextran T-40; D: 5.0 mm Hepes (pH 7.5), 0.5 mm succrose, 5 mm MgCl\(_2\), 5.5% dextran T-40; E: 5.0 mm Hepes (pH 7.5), 0.43 mm succrose, 5 mm MgCl\(_2\), 70 mm KCl; F: 20 mm Hepes (pH 7.5), 5.0 mm MgCl\(_2\), 0.5 mm EDTA; G: 30 mm Tris-HCl, 5 mm MgCl\(_2\), 0.5 mm EDTA, 10% glycerol; H: 40 mm Tris-HCl (pH 8.0), 5 mm MgCl\(_2\), 0.5 mm EDTA, 3 mm CaCl\(_2\).

**Homogenization of Whole Leaves.** Leaves were homogenized
with a mortar and pestle and then with a Potter-Elvehjem homogenizer in buffer H (6 ml g fresh weight⁻¹). The homogenate was passed through Miracloth and centrifuged at 27000g for 15 min, and used for determination of whole leaf enzyme activities.

**Preparation of Mesophyll Protoplasts.** One and 300 g of leaves were used for enzyme localization and ADPglucose pyrophosphorylase isolation studies, respectively. Leaves were sliced (0.5-1.0 mm) and washed in buffer A prior to digestion.

The leaf segments were incubated in buffer A (10 ml g⁻¹ leaf) containing 2% cellulase ("Cellulysin," Calbiochem; for amylase localization studies, Cooper-Biomedical cellulase, 33 units mg⁻¹, was used) and 0.5% BSA for 1.5 h at 25°C on a shaker at 70 rotations min⁻¹. The buffer was then replaced with fresh buffer, and the digestion continued for 1.5 to 2 h as above, except that the shaker speed was 40 rotations min⁻¹. The crude digest was passed through a 80 μm nylon mesh. The partly digested segments were twice washed with gentle shaking in buffer A and refiltered.

The protoplasts were purified using a discontinuous dextran T-40 (Pharmacia) gradient similar to that previously described (20). A Sorvall HS-4 rotor was used for centrifugations, and all procedures were carried out at 4°C. Aliquots (35 ml) of the pooled filtrate were layered over 4 ml of buffer C in 50 ml centrifuge tubes and centrifuged at 1000 rpm for 3 min. The upper layer was discarded and the green interface layer was suspended in the subtending buffer. This suspension was overlaid with 10 ml of buffer D and then with 5 ml of buffer E, and centrifuged at 1500 rpm for 15 min. Protoplasts at the upper interface were collected, mixed with an equal volume of buffer C, overlaid with 5 ml of buffer E, and centrifuged as above. The protoplast layer was collected, resuspended in 3 volumes of buffer E, and centrifuged at 2500 rpm for 3 min. The pellet was resuspended in buffer H and used immediately for enzyme localization studies, or resuspended in a minimum volume of buffer F containing 0.5 μm sucrose, frozen in liquid N₂, and stored at -80°C.

**Preparation of Bundle Sheath Strands.** For enzyme localization studies, bundle sheath strands and mesophyll protoplasts were obtained from the same leaf digest as described above. Bundle sheath strands for ADPglucose pyrophosphorylase purification were isolated with a leaf digestion procedure which differed in that two 1 h digestion periods were used and the shaker speed was maintained at 70 rotations min⁻¹. In either case, the bundle sheath strands were separated from the undigested leaf segments which collected on the 80 μm mesh.

Crude bundle sheath strands were twice washed by resuspension in buffer B (6.7 ml per original g leaf) and collected on an 80 μm mesh. This material was again resuspended in buffer B and bundle sheath strands were allowed to settle through a 800 μm silk screen with gentle shaking. The bundle sheath strands which passed through the screen were collected on a 80 μm mesh, and treated as described above for the purified protoplasts.

**Cell Homogenization.** For enzyme localization studies freshly prepared mesophyll and bundle sheath cell fractions (0.4-0.7 mg Chl/ml buffer H) were disrupted using a Heat Systems-Ultrasonics model W-220F Cell Disintegrator with a prechilled probe until chloroplast breakage was achieved. The homogenate was diluted to a final Chl concentration of 0.15 mg ml⁻¹ with buffer H, and centrifuged at 27000g for 15 min. For purification of ADPglucose pyrophosphorylase, the cell fractions were homogenized as above in buffer F, diluted with buffer F to 0.3 mg mg⁻¹ Chl for mesophyll, or 0.4 mg mg⁻¹ Chl for bundle sheath, and centrifuged as above.

**ADPglucose Pyrophosphorylase Purification.** The procedures described below apply to the bundle sheath and mesophyll enzyme preparations and all procedures were carried out at 4°C, except where indicated otherwise.

Polyethylene glycol fractionation was performed by adding 50% PEG-6000 (Sigma) in buffer F to the centrifuged homogenate to final PEG concentrations of 5, 10, and 20% for mesophyll, and 5, 12, and 18% for bundle sheath. After each addition of PEG the solution was allowed to equilibrate 15 min and centrifuged 27,000g for 15 min. ADPglucose pyrophosphorylase precipitated with the third pellet and was dissolved in a minimum volume of buffer G, centrifuged as above and stored at -80°C for further purification. ADPglucose pyrophosphorylase in the third PEG pellet fraction was further purified using (FPLC; Pharmacia) with a Mono-Q (5/5) anion exchange column at room temperature. The mesophyll sample was chromatographed sequentially in three aliquots containing 7 to 14 mg protein, whereas the bundle sheath sample was chromatographed in a single run. The samples were loaded onto the column preequilibrated with buffer G, and the column was washed with buffer G until the A at 280 nm returned to baseline. The column was eluted in buffer G with a 0 to 500 mM NaCl linear gradient, 10 mM M, at a flow rate of 1 ml min⁻¹. One ml fractions were collected (0.5 ml for activity containing fractions), and those fractions containing activity were pooled and concentrated using an Amicon ultrafiltration cell containing a PM-30 membrane.

**Preparation of Antibody against Spinach Leaf ADPglucose Pyrophosphorylase.** After collection of preimmune sera, homogenous spinach leaf ADPglucose pyrophosphorylase (0.25 mg) (6) in complete Freund adjuvant was injected into a New Zealand rabbit subcutaneously between the shoulder blades every week for 3 weeks and then once a month for 3 months. One week after the final injection antiserum was collected and stored at -80°C in 0.05% NaN₂.

Antigenic peptides were detected by binding goat anti-rabbit IgG conjugated alkaline phosphatase secondary antibody and staining with nitro-blue tetrazolium (18).

**Assay of ADPglucose Pyrophosphorylase.** Pyrophosphorylase direction assay-ADPglucose pyrophosphorylase was determined by measuring the formation of [³²P]ATP from 32Pi and ADP-glucose (11). The assay reaction mixture contained 20 μmol of glycyglycine buffer (pH 7.5), 1.5 μmol of MgCl₂, 0.25 μmol of ADP, 0.1 mg of BSA, 0.5 μmol of ³²P (0.5-3.0 x 10⁵ cpm nmol⁻¹), 0.25 of μmol for (bundle sheath) or 2.5 μmol (for mesophyll) of 3 PGA, enzyme and H₂O in a total volume of 0.25 ml. The assay mixture was incubated at 37°C for 10 min and the reaction was terminated by adding 3 ml of 5% TCA containing 0.1 mg of acid washed norit and 10 μmol NaPPI (pH 8.0). [³²P]ATP was isolated by as previously described (11) except that after two washes with cold 5% TCA, the norit was boiled in 2 ml of 1 N HCl for 10 min and centrifuged in a clinical centrifuge. The supernatant (1 ml) was counted in 5 ml of ScintFlo scintillation fluid with a Packard model 3 liquid scintillation counter. One unit of activity was defined as 1 μmol ATP formed min⁻¹ mg⁻¹ protein. This assay was used to measure activity in whole leaf homogenates and during enzyme purification.

**ADPglucose Synthesis Direction Assay.** The kinetic properties of ADPglucose pyrophosphorylase were investigated measuring the reaction catalyzed in the synthesis direction (11). The basic reaction mixture contained 20 μmol Hepes buffer (at the indicated pH), 1 μmol of MgCl₂, 0.2 μmol of ATP, 0.05 mg of BSA, 0.12 unit of inorganic pyrophosphatase (Sigma), 0.1 μmol [³²Ci-glucose-1-P (1-2.5 x 10⁵ cpm nmol⁻¹); 0.2 μmol of 3PGA, and enzyme and H₂O to a final volume of 0.2 ml. The concent-
trations of 3PGA, ATP, glucose-1-P, and Mg$^{2+}$ were varied as indicated for investigations of enzyme kinetic properties. The assay mixture was incubated at 37°C, and terminated by boiling for 1 min. $[^{14}]$C-ADPglucose was measured as previously described (11).

All kinetic studies were performed using the synthesis assay. S$_{0.5}$, A$_{0.5}$, and I$_{0.5}$ values, corresponding to the concentrations giving 50% maximal activity, activation and inhibition, respectively, and Hill coefficients were calculated from Hill plots (16).

**Assay of Other Enzyme Activities.** RuBP carboxylase was assayed according to Bahr and Jensen (2) and PEP carboxylase was measured as for RuBP carboxylase, except that 10 mM PEP was the substrate, 2 mM glucose-6-P was present, and the preincubation period was eliminated. Activities of primed starch synthase (13; assay A), branching enzyme (13), starch phosphorylase (24; except that Hepes buffer [pH 7] was used), amylase (21), P-glyceraldehyde mutase and P-glucomutase (3) were assayed as previously described. A change in 3PGA concentration during the ADPglucose synthesis assay was used as a measure of 3PGA phosphatase activity. 3PGA was measured by using P-glyceraldehyde and enolase and coupling via lactate dehydrogenase to NADH oxidation measured spectrophotometrically at 340 nm.

**Other Procedures.** Chl was determined according to Arnon (1). Protein was assayed as described using BSA as a standard (27).

### RESULTS

**Efficacy of Cell Separation.** High cell yield and purity were objectives in the fractionation of maize leaf cells, and were particularly critical for characterization of ADPglucose pyrophosphorylase, which had much lower activity in the mesophyll than in the bundle sheath. Gentle shaking and replacement of digestion buffer optimized yield of mesophyll protoplasts, whereas recovery of bundle sheath strands required a short incubation time and stronger shaking. Activities of the marker enzymes RuBP carboxylase and PEP carboxylase (Table I), and visual monitoring with a light microscope indicated that cell contamination was less than 1%. Cell yields were 0.16 and 0.17 mg Chl g leaf$^{-1}$ for mesophyll and bundle sheath, respectively.

**Localization of Starch Metabolizing Enzymes.** Activities of starch metabolizing enzymes in bundle sheath and mesophyll are presented in Table I. Enzymes involved in starch synthesis, ADPglucose pyrophosphorylase, starch synthase, and branching enzyme, were localized predominantly in the bundle sheath. On a protein basis, only 5% of the ADPglucose pyrophosphorylase and 25% of starch synthase and branching enzyme were in the mesophyll. In contrast, enzymes of starch degradation, starch phosphorylase, and amylase showed higher activities in the mesophyll. The percent of the activities in the bundle sheath was 40 and 47% for starch phosphorylase and amylase, respectively. Enzyme activities in the cell fractions were influenced by the type cellulase used for isolation. Amylase activity was higher when Cooper-Biomedical cellulase was used, whereas Calbi-ochem "Cellulysin" resulted in higher activities of ADPglucose pyrophosphorylase, starch synthase, and branching enzyme.

**Purification of ADPglucose Pyrophosphorylase.** Table II summarizes the partial purification of bundle sheath and mesophyll ADPglucose pyrophosphorylase. Both enzyme activities were found to be stabilized by PEG or glycerol during the purification. Both enzymes eluted from the Mono-Q column at 0.28 to 0.3 mM NaCl. Final purifications of 74-fold and 53-fold with 49% and 25% recoveries were obtained for the bundle sheath and mesophyll, respectively. Optimal purification from the FPLC step was higher than indicated for the mesophyll. The data in Table II reflects three FPLC experiments which differed in enzyme purification, but which were pooled to maximize the overall enzyme recovery.

Analysis by paper chromatography (11; solvent B) indicated that the product of the synthesis direction reaction for both enzyme preparations to be ADPglucose. No degradation of ADPG was observed when $[^{14}]$C-ADPglucose was substituted for $[^{14}]$C-glucose-1-P in the assay mixture. P-glyceraldehyde and P-glucomutase activities were not found in either enzyme preparation, and negligible activity of 3PGA-phosphatase was found.

**SDS-PAGE (Fig. 1)** showed prominent peptides of mol wt of 55,000 and 51,000 in the FPLC purified bundle sheath and spinach leaf ADPglucose pyrophosphorylase samples. Western blot hybridization of SDS-PAGE resolved peptides with antisera to ADPglucose pyrophosphorylase antibodies (Fig. 1) showed prominent antigenic peptides of the same mol wt in these samples as well as the mesophyll. A smaller peptide of mol wt of 40,000 in the bundle sheath sample also reacted with the antibody but not the preimmune serum, and may represent degraded ADP-glucose pyrophosphorylase peptide.

**pH Optimum.** The pH optimum of ADPglucose synthesis for the bundle sheath enzyme was slightly lower and broader than that for the mesophyll enzyme (Fig. 2, A and B). A pH optimum of 7.8 to 8.2 was observed for the mesophyll while that of the bundle sheath was around 7.0 to 7.8. In the range of the pH optimum, the mesophyll enzyme gave very similar activity for Hepes, glycylglycine, Tricine, and imidazole, whereas the bundle sheath enzyme activity was lower in the presence of imidazole. Activities and pH optimum of both enzymes were lower in Pipes buffer.

**Kinetic Properties.** Substrate Saturation Kinetics. Values reported represent the average of at least two determinations. In the presence of 1 mM 3PGA the $K_m$ values for ATP were 64 and 67 $\mu$m for the bundle sheath and mesophyll enzymes, respectively. Concentrations of glucose-1-P which gave half maximal velocity were 0.02 and 0.03 mM for the bundle sheath and mesophyll, respectively. Glucose-1-P and ATP saturation curves were essentially hyperbolic in shape. Both enzyme activities had an absolute requirement for Mg$^{2+}$. S$_{0.5}$ values for MgCl$_2$ were 1.5 and 1.8 mM, for the bundle sheath and mesophyll enzymes, respectively. In contrast with the substrate saturation curves, the activation by Mg$^{2+}$ was markedly sigmoidal. The Hill coefficient, n, was 3.2 and 3.1 for the bundle sheath and mesophyll enzymes,

**Table I. Activities of Starch Biosynthetic Enzymes in Bundle Sheath and Mesophyll Cell of Maize Leaf**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Whole Leaf</th>
<th>Bundle Sheath</th>
<th>Mesophyll</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol min$^{-1}$</td>
<td>mg$^{-1}$</td>
<td>protein</td>
</tr>
<tr>
<td>RuBP carboxylase</td>
<td>160</td>
<td>180</td>
<td>0.8</td>
</tr>
<tr>
<td>PEP carboxylase</td>
<td>710</td>
<td>6</td>
<td>958</td>
</tr>
<tr>
<td>Starch synthase</td>
<td>22</td>
<td>21</td>
<td>13</td>
</tr>
<tr>
<td>Branching enzyme</td>
<td>160</td>
<td>720</td>
<td>320</td>
</tr>
<tr>
<td>ADPglucose pyrophosphorylase</td>
<td>70</td>
<td>180</td>
<td>9.7</td>
</tr>
<tr>
<td>Starch phosphorylase</td>
<td>31</td>
<td>18</td>
<td>37</td>
</tr>
<tr>
<td>Amylase</td>
<td>915</td>
<td>170</td>
<td>260</td>
</tr>
</tbody>
</table>

*One unit of enzyme is defined as 1 $\mu$mol ATP formed min$^{-1}$.

**Table II. Purification of Maize Leaf ADPglucose Pyrophosphorylase**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Step</th>
<th>Volume</th>
<th>Protein</th>
<th>Activity</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ml</td>
<td>mg</td>
<td>units</td>
<td>units/mg protein</td>
</tr>
<tr>
<td>Bundle sheath</td>
<td>Crude</td>
<td>49.5</td>
<td>41</td>
<td>8.2</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>PEG</td>
<td>5.5</td>
<td>7.0</td>
<td>5.9</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>Mono-Q</td>
<td>0.6</td>
<td>0.25</td>
<td>4.1</td>
<td>16.5</td>
</tr>
<tr>
<td>Mesophyll</td>
<td>Crude</td>
<td>171</td>
<td>297</td>
<td>2.3</td>
<td>0.0079</td>
</tr>
<tr>
<td></td>
<td>PEG</td>
<td>9.3</td>
<td>36</td>
<td>1.8</td>
<td>0.050</td>
</tr>
<tr>
<td></td>
<td>Mono-Q</td>
<td>0.7</td>
<td>1.3</td>
<td>0.58</td>
<td>0.42</td>
</tr>
</tbody>
</table>
which did with activation of ADPglucose synthesis by enzyme.

enzymes, included 2-P-glycerate, pyruvate, glucose-1,6-bis-P, fructose-2,6-bis-P, and malate.

Allosteric Regulation by 3PGA and Pi. The kinetic data obtained for activation by 3PGA and inhibition by Pi of the bundle sheath and mesophyll enzymes are seen in Figures 3 to 6 and summarized in Tables IV and V. All experiments were repeated at least twice.

At pH 7.4, the activation by 3PGA was hyperbolic in the
absence of Pi (Figs. 3, 4; Table IV). The $A_{0.5}$ was slightly higher for the mesophyll enzyme. The bundle sheath ADPglucose pyrophosphorylase showed a 22-fold activation, whereas only an 8-fold activation was observed for the mesophyll enzyme. When the Pi concentration was increased from 0 to 0.4 mM, velocity was decreased, the activation curves became more sigmoidal, and the $A_{0.5}$ value for both enzymes increased 8- to 9-fold. At all concentrations of Pi studied, the $A_{0.5}$ was somewhat higher for the mesophyll enzyme. In the presence of 0.4 mM Pi, enzyme activation increased to 44-fold and 450-fold for the mesophyll and bundle sheath enzymes, respectively. The changes in fold activation resulted principally from a decrease in the unactivated velocities, since the presence of Pi decreased the velocity in the absence of activator. Both enzymes were sensitive to inactivation by Pi (Figs. 5, 6; Table IV). The $I_{0.5}$ values were slightly higher for bundle sheath at all concentrations of 3PGA investigated. 3PGA influenced the $I_{0.5}$, and the cooperativity of Pi site interaction. The $I_{0.5}$ increased 25-fold and 32-fold for the mesophyll and bundle sheath enzymes, respectively, as the 3PGA concentration was increased from 0.1 to 10 mM. Sigmoidicity of the inhibition curves also increased with increasing 3PGA concent-

---

**Table IV. Effect of 3PGA and Pi Concentration on Maize Leaf ADPglucose Pyrophosphorylase Activities at pH 7.4**

<table>
<thead>
<tr>
<th>[3PGA]</th>
<th>$I_{0.5}$</th>
<th>$V_0$</th>
<th>$I_{0.5}$</th>
<th>$V_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.040</td>
<td>1.1</td>
<td>0.050</td>
<td>1.6</td>
</tr>
<tr>
<td>0.5</td>
<td>0.12</td>
<td>1.5</td>
<td>0.16</td>
<td>2.3</td>
</tr>
<tr>
<td>2</td>
<td>0.36</td>
<td>1.7</td>
<td>0.5</td>
<td>3.0</td>
</tr>
<tr>
<td>10</td>
<td>1.0</td>
<td>2.0</td>
<td>1.6</td>
<td>3.1</td>
</tr>
</tbody>
</table>

**FIG. 5.** The effect of 3PGA on the inhibition by Pi of the maize leaf bundle sheath ADPglucose pyrophosphorylase at pH 7.4. Activity was measured in the synthesis direction (10 min incubation) as described under "Materials and Methods." The concentration of 3PGA was varied as indicated and the 100% activities (nmol ADPG formed) for each curve were 11 for 10 mM 3PGA (O); 11 for 2 mM 3PGA (O); 10 for 0.5 mM 3PGA (A); and 6.0 for 0.1 mM 3PGA (Δ).

**FIG. 6.** The effect of 3PGA on the inhibition by Pi of the maize leaf mesophyll ADPglucose pyrophosphorylase at pH 7.4. Activity was measured in the synthesis direction as described under "Materials and Methods." Assay length was 20 min for assays containing 10 and 2 mM 3PGA, and 30 min for assays containing 0.5 and 0.1 mM 3PGA. The concentration of 3PGA was varied as indicated in Figure 5, and the 100% activities (nmol ADPG formed) were 3.9 for 10 mM 3PGA; 3.5 for 2 mM 3PGA; 4.4 for 0.5 mM 3PGA; and 3.2 for 0.1 mM 3PGA.

Qualitatively similar results were obtained at pH 8.2 although
for both enzymes the apparent affinity for 3PGA was lower, and apparent affinity for Pi was higher (Table V), than at the lower pH. Hill coefficients also tended to be greater than at pH 7.4, particularly for the bundle sheath enzyme and at higher Pi concentrations. In the absence of Pi, 3PGA activation increased to 56-fold and 22-fold for the bundle sheath and mesophyll enzymes, respectively, and resulted principally from a decrease in the unactivated velocities. The \( I_{0.5} \) value for Pi for the mesophyll enzyme was slightly lower at all 3PGA levels except at the 0.1 mM level, where the value for the bundle sheath enzyme was lower. As observed for the activation kinetics, inhibition curves were more sigmoidal at the higher pH.

**DISCUSSION**

The localization of the starch biosynthesis pathway enzymes and their activities in the mesophyll and bundle sheath have been previously reported (8, 10, 15). In this study, highly purified cell fractions were used, and enzyme activities generally equaled or exceeded those previously reported. In agreement with others (8, 10, 14), significant activities of starch synthase, branching enzyme, and ADPglucose pyrophosphorylase indicated that the pathway for starch synthesis was present in both the bundle sheath and mesophyll. Higher activities of these enzymes in the bundle sheath suggest a greater capacity for starch synthesis, in agreement with the localization of starch synthesis to the bundle sheath (8). Interestingly, both amylase and starch phosphorylase showed slightly higher activities in the mesophyll. Differences between studies in cell isolation procedure and extent of cell contamination may be responsible for the differences in reported values. Significantly lower activities reported in some other studies indicate that extensive enzyme inactivation may have occurred.

The availability of ADPglucose may be limiting to starch synthesis in maize mesophyll (8) and may relate to specific properties of the ADPglucose pyrophosphorylases in the mesophyll and bundle sheath. ADPglucose pyrophosphorylase from other plants has been shown to be a key enzyme regulating carbon flow into starch (22). While all plant ADPglucose pyrophosphorylases so far studied are activated by 3PGA and inhibited by Pi, the allosteric properties vary widely for this enzyme from different plant sources (25). Thus, in vivo rates of ADPglucose synthesis will depend on how levels of substrate and effector metabolites relate to the specific properties of the ADPglucose pyrophosphorylase.

The apparent affinities of the maize leaf ADPglucose pyrophosphorylases for ATP and glucose-1-P are comparable to values obtained for the purified spinach leaf enzyme (6). A requirement for Mg²⁺ and sigmoidal binding kinetics appears to be common to all plant ADPglucose pyrophosphorylases (7, 11, 26). Activation of the maize ADPglucose pyrophosphorylases by 3PGA and inhibition by Pi, suggest that fluctuations of in vivo levels of 3PGA and Pi control the rate of starch synthesis in maize leaves as in other plants. Sensitivity of the maize ADPglucose pyrophosphorylases to Pi was modulated by the concentration of 3PGA. Interaction between the 3PGA and Pi effector sites was observed for other plants, and suggested that the in vivo activity of ADPglucose pyrophosphatases is regulated by the ratio of 3PGA/Pi. A high ratio of 3PGA/Pi favors the synthesis of ADPglucose while a low ratio is inhibitory (16, 22). For the mesophyll and bundle sheath enzymes, the ratio of 3PGA/Pi giving half-maximal activation in the presence of pH 8.2, ranged from 9 to 16, and 7 to 10, respectively. In contrast, values of less than 1.5 were obtained for the spinach enzyme at a similar pH (11). Greater sensitivity to Pi inhibition for the maize enzymes may allow the rate of starch metabolism to be regulated in presence of high 3PGA levels (29). The allosteric properties of the maize enzymes appear to favor synthesis of starch in the bundle sheath while limiting it in the mesophyll. Three-fold greater sensitivity to 3PGA of the bundle sheath ADPglucose pyrophosphorylase is at least partially responsible for higher activity in this cell type. In the presence of 0.4 mM Pi this difference in sensitivities to 3PGA increases to an order of magnitude. Moreover, \( A_{0.5} \) and \( I_{0.5} \) values indicate that the mesophyll ADPglucose pyrophosphorylase requires a higher 3PGA/Pi for half-maximal activation than the bundle sheath, particularly at lower Pi levels.

The allosteric poise of the maize ADPglucose pyrophosphorylases will depend, ultimately, on the levels of the effector metabolites. Partition of the photosynthetic processes in maize between the mesophyll and bundle sheath complicates the interrelation between the rates of photosynthesis and the levels of 3PGA and Pi. The level of 3PGA in the bundle sheath is likely to rise with the onset of photosynthesis, as in C-3 plants, whereas by diffusion from the bundle sheath, a subsequent increase would follow in the mesophyll (12). Recent measurements indicate that 3PGA levels may be 15 to 16 mM in the bundle sheath and 5 to 7 mM in the mesophyll (19, 29). These levels, especially in the bundle sheath, would be saturating for the maize ADPglucose pyrophosphorylases, although actual stromal concentrations are uncertain. The sigmoidicity of the curves may be of particular importance to the maize leaf enzymes in view of the reported high levels of 3PGA, since sigmoidal kinetics may shift the allosterically significant range of 3PGA/Pi toward those values encountered physiologically.

Physiological significance of the starch metabolism pathway in the mesophyll has not been shown, although starch synthesis in maize leaf mesophyll has been induced under experimental conditions (8). Diversification of properties for the maize leaf ADPglucose pyrophosphorylases indicates a necessity for regulation of their activities under distinct stromal environments, and starch synthesis may occur in the mesophyll when physiological conditions result in a stromal ratio of 3PGA/Pi favorable to the synthesis of ADPglucose. Starch synthesis in the mesophyll may be constrained at a number of levels, including the activities of enzymes of the starch biosynthesis pathway or the ratio of the synthetic to degradative activities, stromal ratio of 3PGA/Pi, and allosteric properties of ADPglucose pyrophosphorylase. All these factors appear to limit synthesis of starch in the mesophyll relative to the bundle sheath; whether this relationship changes with fluctuating physiological and developmental conditions to allow for starch synthesis in the mesophyll is yet to be determined.

Acknowledgment—The authors are very grateful to Dr. Nina Robinson for the preparation of rabbit antispinach leaf ADPglucose pyrophosphorylase immune sera.
LITERATURE CITED

5. BURNTETE WN 1981 "Western blotting" electrophoretic transfer of proteins from sodium dodeyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radiolodinated protein A. Anal Biochem 112: 195–203
7. DICKINson D, J PREISS 1969 ADPglucose pyrophosphorylase from maize endosperm. Arch Biochem Biophys 130: 119–128
13. HAWKER JS, JL OZBUN, H OZAKI, E GREENBERG, J PREISS 1974 Interaction of spinach leaf adenosine diphosphate glucose α,1,4-glucan α,4-glucosyl transferase and α,1,4-glucan α,1,4-glucan-6-glucosyl transferase in the synthesis of branched α-glucan. Arch Biochem Biophys 160: 530–551
18. LEARY JJ, DJ BRIGATI, DC WARD 1983 Rapid and sensitive colorimetric method for visualizing biotinated DNA or RNA probes hybridized to DNA or RNA immobilized on nitrocellulose: bio-blots. Proc Natl Acad Sci USA 80: 4045–4049
27. SMITH PK, RI KROHN, GT HERMANSON, AK MALLIA, FH GARNER, MD PROVENZANO, EK FUGIMOTO, NM GOREH, BJ OLSON, DC KLEIN 1985 Measurement of protein using bichrominic acid. Anal Biochem 150: 76–85
29. STITT M, HW HELDT 1985 Control of photosynthetic sucrose synthesis by fructose-2,6-bisphosphate. Intracellular metabolite distribution and properties of the cytosolic fructosidephosphatase in leaves of *Zea mays*. Planta 164: 179–188