Regulation of Starch Biosynthesis in Plant Leaves: Activation and Inhibition of ADPglucose Pyrophosphorylase

Ghirdhar Gopal Sanwal, Elaine Greenberg, Jeanne Hardie, Erma C. Cameron, and Jack Preiss

Department of Biochemistry and Biophysics, University of California, Davis, California 95616

Received October 3, 1967.

Abstract. The ADPglucose pyrophosphorylases of 7 plant-leaf tissues were partially purified and characterized. In all cases the enzymes showed stability to heat treatment at 65°C for 5 minutes in the presence of 0.02 M phosphate buffer, pH 7.0. The leaf ADPglucose pyrophosphorylases were activated 5 to 15-fold by 3-phosphoglycerate. Fructose-6-phosphate and fructose 1, 6-diphosphate stimulated ADPglucose pyrophosphorylase to lesser extents. The $A_{0.2}$ (concentration of activator required to give 50% of the observed maximal activation) of 3-phosphoglycerate for the barley enzyme was $7 \times 10^{-6}$ M while for the sorghum enzyme it was $3.7 \times 10^{-4}$ M. Inorganic phosphate proved to be an effective inhibitor of ADPglucose synthesis. The $I_{0.5}$ (concentration of inhibitor that gave 50% inhibition of activity for the various leaf enzymes varied from $2 \times 10^{-5}$ M (barley) to $1.9 \times 10^{-4}$ M (sorghum). This inhibition was reversed or antagonized by the activator 3-phosphoglycerate. These results form the basis for an hypothesis of the regulation of leaf starch biosynthesis.

The biosynthesis of the $\alpha$-1,4 glucoside linkage of starch from UDPglucose and ADPglucose in many plant extracts have been the subject of many reports (1, 2, 5, 8, 16, 29, 36) since the initial observations by Leloir's group (7, 23, 38). In all extracts the rate of transfer of glucose from ADPglucose (reaction 1) to the polysaccharide primer was many-fold faster than the rate of transfer from uridine diphosphate glucose (UDPGlucose) (reaction 2).

1. ADPglucose + $\alpha$-1,4 glucan $\rightarrow$ ADP + $\alpha$-1,4 glucosyl glucan
2. UDPglucose + $\alpha$-1,4 glucan $\rightarrow$ UDP + $\alpha$-1,4 glucosyl glucan

Indeed the nucleoside diphosphate glucose: $\alpha$-1,4 glucan transferases isolated from leaf extracts or Chlorella were virtually specific for the sugar nucleotides, adenosine diphosphate glucose and deoxyadenosine diphosphate glucose.

The importance of the sugar nucleotide ADPglucose for the biosynthesis of starch therefore prompted an investigation of its biosynthesis. Espada (6) reported the presence of ADPglucose pyrophosphorylase, (reaction 3) in wheat flour extracts.

3. ATP + $\alpha$Glucose 1-P $\rightarrow$ ADPglucose + PP$_1$

Results in our laboratory have shown that the enzyme was present in chloroplasts of spinach leaf, barley leaf, and butter lettuce, in potato tubers, carrot roots, avocado mesocarp, and Chlorella pyrenoidosa (15-17, 35, 40). A very common property of the ADPglucose pyrophosphorylases isolated from these tissues was that their catalytic activities for either synthesis or pyrophosphorylization of ADPglucose were activated by 3PGA, a primary CO$_2$ fixation product of photosynthesis, as well as by other intermediates of the carbon cycle such as fructose 6-P and fructose 1,6-diP. In detailed kinetic analyses of the spinach leaf ADPglucose pyrophosphorylase (17) it was found that P$_1$ was a potent inhibitor of the enzyme and that this inhibition was reversed by the activator, 3PGA. On the basis of these results a mechanism was postulated for the regulation of starch synthesis in the chloroplast (17, 35). Since ADPglucose is the sole glucosyl precursor for leaf starch synthesis, it appears that starch synthesis may be controlled by the regulation of ADPglucose synthesis. The levels of the metabolites, P$_1$, ATP, 3PGA (as well as other carbon cycle intermediates as fructose 6-P and fructose 1,6-diP) would then control the rate of starch synthesis. It is well known that sucrose and starch are the major products of chloroplasts, resulting from carbon assimilation during photosynthesis. Therefore a mechanism for regulation of leaf starch synthesis involving 3PGA (the primary CO$_2$ fixation product of photosynthesis) would be of general importance to green plants.

However Nomura et al. (34) have reported that the ADPglucose pyrophosphorylase activities from rice or bean leaves were not stimulated by 3PGA, P-enolpyruvate or fructose 1,6-diP. These results would suggest that the mechanism of regula---

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1 This investigation was supported by research grant AI-05520 from the United States Public Health Service.
2 Present Address: Department of Biochemistry, University of Lucknow, Lucknow, U.P. India.
3 Abbreviations: 3PGA, 3-phosphoglycerate.
tion that was observed in spinach leaf tissue would not be a universal type. In view of this it was decided to examine in some detail the ADPglucose pyrophosphorylases associated with various leaf tissues. The results in this report will show that in all 7 leaf tissues studied of which rice and kidney beans were included, it was found that the ADPglucose pyrophosphorylases were activated from 5 to 17-fold by 3PGA. Activation by some other glycolytic intermediates also occurred to lesser extents. Furthermore these enzymes were inhibited by P₁ and this inhibition was reversed by 3PGA. It seems therefore that the leaf ADPglucose pyrophosphorylases generally are activated by 3-phosphoglycerate and inhibited by P₁.

Materials and Methods

Growing of Plant Material. Barley seeds were germinated in moist vermiculite and grown for 10 days under fluorescent lighting. Sorghum seeds were soaked in water overnight and germinated in moist vermiculite and grown for 7 days in the light. Rice and kidney bean plants were grown in soil in a greenhouse for about 14 to 30 days. Sugar beet and tomato plants were grown in the university fields and were 1 to 2 months old when used. Tobacco seeds were sown in seedling pots in vermiculite. In 2 to 3 weeks after sowing seedlings were transplanted to individual plastic pots containing soil and grown for 3 to 4 weeks. The plants were maintained in a greenhouse throughout their growth period.

Reagents. ADPglucose-¹⁴C, UDPglucose-¹⁴C and TDPglucose-¹⁴C were prepared as described previously (42). Glucose-¹⁴C-1-P and inorganic pyrophosphate-³²P were obtained from New England Nuclear Corporation, Boston, Massachusetts. GDPglucose-¹⁴C was obtained from International Nuclear Corporation, California. All other chemical reagents used were obtained from commercial sources.

Assay of ADPglucose Pyrophosphorylase. Pyrophosphorolysis of ADPglucose was followed by the formation of ATP-³²P in the presence of ATP-³²P. The reaction mixture, which contained 20 µmole of tris-HCl buffer (pH 7.5) 1.5 µmole of MgCl₂, 0.2 µmole of ADPglucose, 0.5 µmole of pyrophosphate-³²P (specific activity 1.0 - 5.0 x 10⁶ cpm/µmole), 2.5 µmole of NaF, 50 µg of crystalline bovine serum albumin, 0.25 µmole of 3PGA and the enzyme in a final volume of 0.2 ml was incubated at 37° for 10 minutes. The reaction was stopped by the addition of 3 ml of cold 5% trichloroacetic acid. The ATP-³²P formed was isolated by adsorption onto Norit A and was measured as described previously (42). This assay was used to quantitate the enzyme during purification procedures. A unit of ADPglucose pyrophosphorylase activity was defined as that amount of enzyme that catalyzed the formation of 1 µmole of ATP-³²P under the conditions described above.

Assay for the Synthesis of ADPglucose. Sugar nucleotide synthesis was measured as described previously (17). The reaction mixture, which contained 20 µmole of N-2 hydroxethylpiperazine-¹⁴N-2 ethanesulfonic acid (HEPES) buffer (pH 7.5) (18), 1 µmole of MgCl₂, 0.1 µmole of glucose-¹⁴C-1-P (specific activity 1.0 x 10⁶ cpm/µmole), 0.2 µmole of ATP, 50 µg of crystalline bovine serum albumin, 0.2 µmole of 3PGA and enzyme in a final volume of 0.2 ml was incubated at 37° for 10 minutes. The reaction was terminated by heating the mixture in a boiling water bath for 30 seconds. Then 0.1 mg of Escherichia coli alkaline phosphatase was added and the mixture was incubated for 40 minutes. The unreacted glucose-¹⁴C-1-P was hydrolyzed to glucose-¹⁴C and the sugar nucleotide product remained intact. A portion (0.1 ml) of this phosphatase treated reaction mixture was adsorbed onto a DEAE-cellulose paper disk (2.5 cm in diameter), and the glucose-¹⁴C was washed off with four 150-ml portions of water. The paper disks were dried and the adsorbed radioactivity was counted by a liquid scintillation technique as described previously (37, 41). This assay was used primarily to measure ADPglucose synthesis in the partially purified fractions. With all purified enzyme fractions the assay was linear with protein concentration up to at least 7 µmole of ADPglucose formed in 10 minutes. The assay was linear with protein concentration up to 12 µmole of ADPglucose formed in 10 minutes with the tobacco enzyme and up to 18 µmole of ADPglucose formed in 10 minutes with the tomato enzyme.

Assay of ADPglucose: α-1,4-Glucan Transferase. The reaction mixture, which contained 40 µmole of ADPglucose-¹⁴C (1.07 x 10⁶ cpm/µmole), 10 µmole of tris-HCl buffer, pH 8.5, 5 µmole of KCl, 2 µmole of GSH, 1 µmole of EDTA, 500 µg of corn amylepectin and enzyme fraction in a total volume of 0.2 ml, was incubated at 37° for 15 minutes. The reaction was terminated by the addition of 2 ml of 75% methanol containing 1% KCl. The mixture was then centrifuged and the amylopectin precipitate was washed twice with 2 ml of the methanol-KCl solution. The amylopectin was then dissolved in 1 ml of water, and a 1 ml portion was counted with 10 ml of Bray’s solution (4) in a liquid scintillation counter. One unit of enzyme activity was defined as being equal to the transfer of 1 µmole of glucose-¹⁴C to amylepectin in 15 minutes at 37° under the above conditions.

Paper Chromatography and Electrofhoresis. The following solvent systems were used in descending paper chromatography on Whatman No. 1 filter paper: Solvent A, 95% ethanol-M ammonium acetate, pH 3.8 (5:2); Solvent B, isobutyric acid-M NH₄OH-1 M EDTA (pH 7.2) (10:6:0.16), Solvent C, ethyl acetate-pyridine-water (3.7:1.0:1).
ADPglucose. Paper electrophoresis was done in 0.05 M citrate buffer, pH 3.9, in a GME Electrophorator.

Partial Purification of Enzymes. Rice Leaf ADPglucose Pyrophosphorylase. Rice leaves, 84 grams, were frozen in liquid N₂ and ground in mortar and pestle. The ground leaves were then homogenized for 3 minutes with 400 ml of 0.05 M tris-HCl buffer (pH 7.5) containing 2 mM EDTA, 10 mM dithiothreitol, and 5 mM potassium phosphate. The resultant suspension was filtered through a pad of cheese cloth in a Buchner funnel and centrifuged at 15,000 × g for 30 minutes. The precipitate was washed with 11 ml of the same buffer and the wash and supernatant fraction were combined. The combined supernatant fraction (370 ml) was heated in a boiling water bath at 65° for 5 minutes, quickly cooled and then centrifuged at 15,000 × g for 10 minutes at 2°. The denatured protein precipitate was discarded and to the supernatant fluid was added solid ammonium sulfate to a final concentration of 0.75 saturation [100% saturation = 70 grams (NH₄)₂ SO₄ per liter]. The resultant suspension was centrifuged and the precipitate was dissolved in 0.1 M tris-succinate buffer (pH 7.2) containing 1 mM EDTA and 1 mM dithiothreitol.

Sorghum Leaf ADPglucose Pyrophosphorylase. Sorghum leaves, 60 grams, were frozen in liquid N₂ and then ground with 200 ml of 0.05 M tris-HCl buffer (pH 7.5) containing 1 mM EDTA and 1 mM dithiothreitol. The suspension was centrifuged at 10,000 × g for 10 minutes. The particles were washed once with 10 ml of the above buffer and the wash was then combined with the supernatant fraction. The combined fraction was heated at 65° for 5 minutes in the presence of 20 mM phosphate buffer (pH 7.0) and chilled and centrifuged. The resultant supernatant was fractionated with solid ammonium sulfate and the 0 to 0.59 saturated fraction was dialyzed for 6 hours against 20 mM tris-succinate buffer (pH 7.2) containing 1 mM EDTA and 1 mM dithiothreitol.

Barley Leaf ADPglucose Pyrophosphorylase. Barley leaves, 67 grams, were ground with an equal weight of sand and 67 ml of 50 mM tris-HCl buffer (pH 7.5) containing 1 mM EDTA and 5 mM GSH in a chilled mortar. The resultant suspension was filtered through cheese cloth and centrifuged at 10,000 × g for 15 minutes. To the supernatant fraction was added sufficient M potassium phosphate buffer (pH 7.0) to give a final concentration of 0.02 M. The supernatant fraction was then heated at 65° for 5 minutes, quickly cooled and centrifuged. The precipitate was discarded and the clear supernatant fraction was fractionated with crystalline ammonium sulfate. The activity was concentrated in the 0 to 0.40 saturated fraction which was dialyzed against 20 mM glycylglycine buffer (pH 7.0) containing 1 mM EDTA and 5 mM GSH.

Tomato Leaf ADPglucose Pyrophosphorylase. Tomato leaves, 50 grams, were homogenized with 100 ml of 0.05 M tris buffer (pH 7.5) containing 2 mM EDTA and 10 mM dithiothreitol in a Waring blender for 1 minute. The homogenate was filtered through a pad of cheese cloth in a Buchner funnel and centrifuged at 15,000 × g for 30 minutes. The particulate material was washed with 10 ml of the above buffer and centrifuged again. The wash and first supernatant fraction were combined. Sufficient M phosphate buffer (pH 7.0) was added to 118 ml of the supernatant to give a final concentration of 0.02 M phosphate. The supernatant fluid was subsequently heated in a water bath at 65° for 5 minutes, rapidly chilled and centrifuged at 16,000 × g for 15 minutes. To the clear supernatant was added solid ammonium sulfate to give a concentration of 0.75 saturation. The resultant suspension was centrifuged at 16,000 × g for 15 minutes. The precipitate was dissolved in 0.1 M tris-succinate buffer, pH 7.2, containing 1 mM EDTA and 1 mM dithiothreitol and dialyzed for 6 hours in 0.02 M tris-succinate (pH 7.2) containing 1 mM EDTA and 1 mM dithiothreitol. The precipitate formed during dialysis was centrifuged off at 18,000 × g for 15 minutes and the clear supernatant fluid was used as a source of enzyme.

Tobacco ADPglucose Pyrophosphorylase. Turkish tobacco, 29 grams, was homogenized for 3 minutes in 40 ml of 0.05 M tris-HCl buffer (pH 7.5) containing 2 mM EDTA and 2 mM GSH. The homogenate was centrifuged at 15,000 × g for 30 minutes. To the supernatant fluid was added M potassium phosphate buffer (pH 7.0) to a final concentration of 20 mM. The supernatant fluid was then heated in a water bath at 65° for 5 minutes. The denatured protein was separated by centrifugation and to the supernatant fraction was added solid ammonium sulfate to a concentration of 0.75 saturation. The resultant suspension was centrifuged and the precipitate which contained the ADPglucose pyrophosphorylase was dissolved in 0.1 M tris-succinate buffer, pH 7.2, containing 1 mM EDTA and 1 mM GSH and dialyzed against 500 ml of 20 mM tris-succinate buffer (pH 7.2) containing 1 mM GSH and 1 mM EDTA.

Sugar Beet Leaf ADPglucose Pyrophosphorylase. Sugar beet leaves, 124 grams, were homogenized in a Waring blender for 2 minutes with 98 ml of 0.05 M tris-HCl buffer, pH 7.5, containing 10 mM dithiothreitol and 2 mM EDTA. The suspension was centrifuged at 10,000 × g for 10 minutes and the precipitate which was obtained was washed with 10 ml of buffer and recentrifuged as above. The wash and original supernatant fluid were combined and brought to a concentration of 0.02 M phosphate by adding a solution of M phosphate buffer (pH 7.2). The supernatant fluid was then heated in a water bath at 65° for 5 minutes. The heat denatured protein was discarded by centrifugation and to the supernatant fluid was added
sufficient solid ammonium sulfate to give 0.70 saturation. The precipitate, which was obtained, was dissolved in a minimal volume of 0.1 M tris-succinate, pH 7.2, containing 2 mM dithiothreitol and 2 mM EDTA and dialyzed for 6 hours against 500 ml of 20 mM tris-succinate (pH 7.2) containing 2 mM dithiothreitol and 2 mM EDTA.

Kidney Bean ADPglucose Pyrophosphorylase. Kidney bean leaves, 110 grams, were homogenized for 2 minutes in a Waring blender with 190 ml of 0.05 M tris buffer, pH 7.5, containing 10 mM dithiothreitol and 2 mM EDTA. The resultant suspension was centrifuged for 30 minutes at 15,000 × g. The precipitate which was obtained was washed with 20 ml of the above buffer and the wash and supernatant fluid were combined. To the combined supernatant fraction was added 1 mM phosphate buffer (pH 7.0) to give a final concentration of phosphate of 0.02 M. The combined supernatant fraction was then heated in a water bath at 65° for 5 minutes and then quickly cooled. The heat denatured protein precipitate was discarded after centrifugation and to the supernatant fraction was added solid ammonium sulfate to a final concentration of 0.65 saturation. The precipitate which was obtained was dissolved in 0.1 M tris-succinate buffer (pH 7.2) containing 1 mM EDTA and 1 mM dithiothreitol and dialyzed against 20 mM tris-succinate buffer (pH 7.2) containing 1 mM EDTA and 1 mM dithiothreitol.

Preparation of ADPglucose α-1,4 Glucan Transferases. The partial purification and properties of the ADPglucose: α-1,4 glucan transferases from leaf extracts of tobacco (10), spinach (5,15, 16), butter lettuce (35), and soybeans (29) have been previously reported. The soluble ADPglucose: α-1,4 glucan transferases from various leaf extracts of rice, tobacco, barley, tomato, kidney bean, sorghum and sugar beet were prepared in this laboratory in the following manner:

Barley Transferase. Barley leaves, 55 grams, were frozen in liquid N₂ and then ground up with mortar and pestle. The ground leaves were then homogenized with 140 ml of 0.05 M tris- HCl (pH 7.5) buffer containing 2 mM dithiothreitol. The resultant suspension was filtered through a pad of cheese cloth on a Buchner funnel and then centrifuged at 30,000 × g for 30 minutes. The precipitate was washed with 10 ml of the above buffer and then resuspended in the same buffer. The wash was combined with the supernatant fluid and used as a source of the enzyme since it contained over 98 % of the transferase activity. To the supernatant fraction was added solid ammonium sulfate to give a final concentration of 0.4 saturation. The suspension was centrifuged and the precipitate which contained 98 % of the transferase activity was dissolved in 0.1 M tris-succinate buffer, pH 7.2, containing 1 mM EDTA and 10 mM dithiothreitol and dialyzed for 6 hours against 20 mM tris-succinate buffer, pH 7.2, containing 1 mM EDTA and 10 mM dithiothreitol. This ammonium sulfate fraction also contained ADPglucose pyrophosphorylase activity.

Rice, Tobacco, Tomato, Kidney Bean, Sorghum, and Sugar Beet Transferases. The crude extracts were prepared in the same manner as those described for the preparation of the ADPglucose pyrophosphorylases from the respective leaves. The particulate fraction of the crude extract was resuspended in the same buffer used for the preparation of the extract. The supernatant fraction from each leaf source was fractionated with solid ammonium sulfate. In each case practically all the transferase activity precipitated at a concentration of ammonium sulfate of 0.4 saturation. The precipitates obtained at this concentration of ammonium sulfate were dissolved in 0.1 M tris-succinate buffer, pH 7.2, containing 10 mM dithiothreitol and 1 mM EDTA. The 0 to 0.4 fraction when prepared in this manner from the crude extracts of the various leaf sources also contained almost all of the ADPglucose pyrophosphorylase of the leaf extracts.

Results

General Properties of ADPglucose Pyrophosphorylases. Table I summarizes the partial purification of the various ADPglucose pyrophosphorylases. The enzymes are purified over the crude extract from 2- to 30-fold. One remarkable property of the leaf ADPglucose pyrophosphorylases is that they are stable to heat treatment at 65° for 5 minutes in 20 mM phosphate buffer. The most labile enzymes in the heat treatment were from kidney bean and sugar beet. The activities recovered from these leaf sources were 69 and 77 % respectively. The activities recovered of the other leaf ADPglucose pyrophosphorylases after heat treatment were over 86 %. The purification obtained was usually due to the heat treatment. The ammonium sulfate fraction was usually viewed as a concentration step.

The crude extracts also contained UDPglucose and TDPglucose pyrophosphorylase activity. However very little or none of these activities were found after the heat treatment step at 65°. The pyrophosphorylase assay (ATP-P32 formation) was absolutely dependent on Mg²⁺ and ADPglucose either in the presence or absence of 3PGA. The synthesis assay was also dependent on Mg²⁺ as well as ATP. The radioactive product formed in the synthesis reaction was identified as ADPglucose by its co-chromatography with authentic ADPglucose (Cal Biochem) in solvent systems A and B and its comigration with ADPglucose on paper electrophoresis at pH 3.9. For each leaf enzyme preparation the product behaved similarly to ADPglucose. Hydrolysis of the 14C-product in 0.02 N HCl for 10 minutes at 100° yielded a radioactive compound.

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Table I. Partial Purification of ADP Glucose Pyrophosphorylases from Leaf Extracts

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Rice Units/ml</th>
<th>Units/mg-protein</th>
<th>Barley Units/ml</th>
<th>Units/mg-protein</th>
<th>Sorghum Units/ml</th>
<th>Units/mg-protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>370</td>
<td>396</td>
<td>0.43</td>
<td>102</td>
<td>152</td>
<td>0.35</td>
</tr>
<tr>
<td>Heat treated extract</td>
<td>370</td>
<td>340</td>
<td>0.84</td>
<td>100</td>
<td>155</td>
<td>5.9</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>37</td>
<td>278</td>
<td>0.8</td>
<td>4.6</td>
<td>150</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>19</td>
<td>37</td>
</tr>
<tr>
<td>Sugar Beet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tobacco</td>
<td>180</td>
<td>738</td>
<td>0.48</td>
<td>48</td>
<td>192</td>
<td>1.05</td>
</tr>
<tr>
<td>Tomato</td>
<td>162</td>
<td>567</td>
<td>1.15</td>
<td>47</td>
<td>165</td>
<td>2.6</td>
</tr>
<tr>
<td>Tomato</td>
<td>18</td>
<td>316</td>
<td>1.16</td>
<td>4.8</td>
<td>118</td>
<td>2.7</td>
</tr>
<tr>
<td>Kidney Bean</td>
<td>285</td>
<td>664</td>
<td>0.50</td>
<td>118</td>
<td>2.7</td>
<td>1.47</td>
</tr>
<tr>
<td>Heat treated extract</td>
<td>284</td>
<td>455</td>
<td>0.89</td>
<td>118</td>
<td>2.7</td>
<td>1.47</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>15</td>
<td>285</td>
<td>1.05</td>
<td>118</td>
<td>2.7</td>
<td>1.47</td>
</tr>
</tbody>
</table>

that migrated the same as did glucose in solvent systems C and D.

 Activation of ADP-glucose Synthesis by 3-Phosphoglycerate. Figures 1 and 2 show that all the ADP-glucose pyrophosphorylases of the various leaves were activated by 3PGA. The extent of activation under the conditions used varied from 5.5-fold (observed with tomato enzyme) to 14.5-fold (observed with the rice enzyme). The activation of the highly purified spinach leaf enzyme activity by 3PGA was reported to be about 10-fold at pH 7.5 (35). The concentrations of 3PGA needed for half-maximal stimulation of ADP-glucose synthesis for the various leaf enzymes are summarized in the first column of table II. Barley leaf ADP-glucose pyrophosphorylase is quite sensitive to 3PGA concentration in that 7 μM causes half-maximal stimulation. Concentrations of 3PGA required to cause half-maximal stimulation of ADP-glucose synthesis with the other leaf enzymes ranged from 20 μM.

![Graphs](https://www.plantphysiol.org)
Table II. Kinetic Constants of Leaf ADPglucose Pyrophosphorylases

The abbreviations $K_{0.5}$ and $I_{0.5}$ are those of Koshland et al. (25). $K_{0.5}$ was the concentration of activator required to give 50% of the observed maximal stimulation while $I_{0.5}$ was the concentration of inhibitor needed for 50% inhibition. The data in this table were calculated from the curves of figures 1 through 4 and reference (35). The last column pertains to the concentration of 3-phosphoglycerate present in determining the $I_{0.5}$ in the next to the last column.

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
<th>Common name or variety</th>
<th>$K_{0.5}$ (3PGA)</th>
<th>$I_{0.5}$ (-3PGA)</th>
<th>$I_{0.5}$ (+3PGA)</th>
<th>Conc of 3PGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solanaceae</td>
<td>Nicotiana</td>
<td>tabacum</td>
<td>Turkish tobacco</td>
<td>0.045</td>
<td>0.03</td>
<td>1.01</td>
<td>1.0</td>
</tr>
<tr>
<td>Solanaceae</td>
<td>Lycopersicon</td>
<td>esculentum</td>
<td>Red cherry tomato</td>
<td>0.09</td>
<td>0.08</td>
<td>0.88</td>
<td>2.5</td>
</tr>
<tr>
<td>Gramineae</td>
<td>Hordeum</td>
<td>vulgare L</td>
<td>Barley</td>
<td>0.007</td>
<td>0.02</td>
<td>2.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Gramineae</td>
<td>Sorghum</td>
<td>hybrid</td>
<td>Sorghum</td>
<td>0.37</td>
<td>0.19</td>
<td>0.41</td>
<td>2.2</td>
</tr>
<tr>
<td>Chenopodiaceae</td>
<td>Beta</td>
<td>vulgaris</td>
<td>Sugar beet USH7</td>
<td>0.19</td>
<td>0.05</td>
<td>0.43</td>
<td>0.87</td>
</tr>
<tr>
<td>Gramineae</td>
<td>Oryza</td>
<td>sativa</td>
<td>Rice</td>
<td>0.18</td>
<td>0.06</td>
<td>0.27</td>
<td>1.0</td>
</tr>
<tr>
<td>Chenopodiaceae</td>
<td>Spinacia</td>
<td>oleracea</td>
<td>Spinach</td>
<td>0.020</td>
<td>0.06</td>
<td>1.2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

3PGA to 370 μM (sorghum leaf).

Specificity of Activation. Table III shows that in addition to 3PGA other glycolytic intermediates are capable of activating ADPglucose synthesis.

In all cases activation was observed with fructose diP and fructose-6-P. The stimulation observed in each case however was never as great as with 3PGA being anywhere from 1.4- to 7.8-fold. In most preparations 2-P-glycerate and P-enolpyruvate also stimulated ADPglucose synthesis. Very little or no stimulation with these compounds was observed with the barley leaf and kidney bean leaf preparations. It is quite possible that activation by 2-P-glycerate and P-enolpyruvate in these rather impure enzyme preparations may be due to their conversion to 3PGA by contaminating enolase and phosphoglyceromutase activities. It should be mentioned that the highly purified spinach leaf enzyme preparation which does not contain these contaminating enzyme activities is activated by the fructose 6-P 3.6-fold, fructose diP 2.2-fold, P-enolpyruvate 3.5-fold, but is not activated by 2-P-glycerate. Compounds which were tested at 1 mM concentration and did not show any stimulation of ADPglucose synthesis for any of the leaf enzymes were glucose 6-P, glyceraldehyde-3-P, dihydroxyacetone-P, sucrose, pyruvate, di-malate, oxaloacetate, DPNH, and TPNH.

Inhibition of ADPglucose Synthesis by Inorganic Phosphate. Figures 3 and 4 show that the leaf ADPglucose pyrophosphorylases are quite sensitive to inhibition by P_i in the absence of 3PGA. Concentrations needed for 50% inhibition ($I_{0.5}$) ranged from 20 mM (barley leaf) to 190 mM (sorghum leaf) (table II). In the presence of 3PGA the leaf enzymes were more resistant to phosphate inhibition (fig 3 and 4); i.e. higher concentrations of phosphate were required to give 50% inhibition (table II). Figures 1 and 2 show that increasing concentrations of 3PGA reverse or overcome the inhibitions observed with P_i. The presence of P_i in the reaction mixture causes the 3PGA saturation curves to change in shape. In the absence of phosphate they are hyperbolic in form while in the presence of phosphate the curves become sigmoidal. These phenomena are similar to what was observed

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Fig. 2. The effect of 3PGA on the rate of synthesis of ADPglucose. The contents of the reaction mixture were the same as described in the text (synthesis assay) except that the amounts of 3PGA were varied and phosphate was added as indicated in the figure.
Table III. Specificity of the Stimulation of Leaf ADP-glucose Pyrophosphorylases

The concentration of activators present in the reaction mixtures containing the sorghum enzyme was 5 mM. The concentration of activators present in the reaction mixtures containing the other enzymes was 1 mM. The abbreviations used are: 3PGA, 3 P-glycerate; 2PGA, 2 P-glycerate; PEP, P-enolpyruvate; FDP, fructose 1,6 diP; F6P, fructose 6-P.

<table>
<thead>
<tr>
<th>Ammonium sulfate fraction</th>
<th>None</th>
<th>3PGA</th>
<th>2PGA</th>
<th>PEP</th>
<th>FDP</th>
<th>F6P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco leaf</td>
<td>5.7</td>
<td>51.5</td>
<td>35.4</td>
<td>18.6</td>
<td>32.3</td>
<td>19.0</td>
</tr>
<tr>
<td>Tomato leaf</td>
<td>14.6</td>
<td>79.5</td>
<td>32.0</td>
<td>34.2</td>
<td>27.0</td>
<td>30.5</td>
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<tr>
<td>Sugar beet leaf</td>
<td>2.8</td>
<td>24.0</td>
<td>3.7</td>
<td>10.7</td>
<td>7.9</td>
<td>10.5</td>
</tr>
<tr>
<td>Sorghum leaf</td>
<td>0.56</td>
<td>4.0</td>
<td>2.7</td>
<td>0.8</td>
<td>1.2</td>
<td>1.0</td>
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<tr>
<td>Rice leaf</td>
<td>2.2</td>
<td>32.2</td>
<td>9.5</td>
<td>7.9</td>
<td>7.3</td>
<td>6.2</td>
</tr>
<tr>
<td>Barley leaf</td>
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<td>493</td>
<td>48</td>
<td>26</td>
<td>136</td>
<td>290</td>
</tr>
<tr>
<td>Kidney bean leaf</td>
<td>2.2</td>
<td>11.1</td>
<td>2.2</td>
<td>2.2</td>
<td>3.1</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Activator activity was in the supernatant fraction of the crude extract. The transferase activity could be concentrated in the 0 to 0.4 ammonium sulfate fraction. The properties of the tobacco transferase have already been reported by Frydman and Cardini (10). In general the properties of the soluble

with the highly purified spinach leaf enzyme (17, 35).

Properties of Leaf ADPglucose: α-1,4 Glucan Transferase from Leaves of Rice, Tobacco, Tomato, Kidney Bean, Sorghum, Barley, and Sugar Beet. In these tissues at least 80% of the transferase activity was in the supernatant fraction of the crude extract. The transferase activity could be concentrated in the 0 to 0.4 ammonium sulfate fraction. The properties of the tobacco transferase have already been reported by Frydman and Cardini (10). In general the properties of the soluble

Fig. 3. The inhibition of ADPglucose synthesis by P$_i$. The contents of the reaction mixtures were the same as those described in the text for the synthesis assay except that 3PGA and phosphate were added in the amounts indicated in the figure. One hundred percent activity pertains to the ADPglucose synthesis in the absence of inhibitor. The values for 100% activity (mumoles of ADPglucose formed in 10 min) for the reaction mixtures containing the different enzymes were: tobacco, plus 3PGA, 5.1; minus 3 PGA, 1.2; tomato, +3PGA, 10; −3PGA, 0.8; sugar beet, +3PGA, 5.7; −3PGA, 1.3; sorghum, +3PGA, 4.7; −3PGA, 1.1.
424 PLANT PHYSIOLOGY

Inorganic phosphate.

Fig. 4. The inhibition of ADPglucose synthesis by inorganic phosphate. The contents of the reaction mixtures were the same as those described in the text for the synthesis assay except that 3PGA and P_1 were added in the amounts indicated in the figure. One hundred percent activity pertains to the ADPglucose synthesis in the absence of inhibitor. The values for 100% activity (molecules of ADPglucose formed in 10 min) catalyzed by the different enzymes were: rice, +3PGA, 4.4; -3PGA, 0.9; barley, +3PGA, 13.2; -3PGA, 0.90.

transfases from tomato, kidney bean, sorghum, barley, and sugar beet were the same. The incorporation of glucose-14C from ADPglucose into the amylopectin primer requires the presence of a primer and is inhibited by the presence of 20 μg of α-amylase in the reaction mixture. The reaction is specific for ADPglucose: UDPglucose, TDPglucose, and GDPglucose were not active in the system. Glucose incorporation into amylopectin by the 7 leaf transferases was not stimulated or inhibited by 3PGA, fructose 6-P, fructose 1,6-diP, P-enolpyruvate, 2-P-glycerate, pyruvate, glucose 6-P, glyceraldehyde-3-P, dihydroxyacetone-P, oxaloacetate, sucrose, α-malate, or P_. Degradation of the radioactive, alcohol insoluble product with β-amylase at pH 5.0 yielded a radioactive material which cochromatographed with maltose in solvent systems C and D. This indicated that new α-1,4

glucosidic linkages were being formed by transfer of glucose-14C from ADPglucose to the primer amylopectin.

Incorporation of Glucose-14C from Glucose-14C-1-Phosphate into Amylopectin via Reactions Catalyzed by ADPglucose Pyrophosphorylase and ADPglucose: α-1,4 Glucan Transferase. Since the activation of ADPglucose synthesis by 3PGA may be of physiological importance in the regulation of starch biosynthesis, it was of interest to determine whether the activation of glucose-14C transfer from glucose-1-P to starch would occur when the ADPglucose pyrophosphorylase reaction was coupled with the ADPglucose: α-1,4 glucan transferase reaction. Since the dialyzed 0 to 0.4 ammonium sulfate fraction from the crude extracts contained almost all of the ADPglucose pyrophosphorylase and glucan transferase activities of the crude extract, it was used as the source for these activities. The ammonium sulfate fractions of barley, rice and tobacco leaf tissue extracts were arbitrarily chosen for the experiment. In each case significant stimulation by 3PGA was observed (table IV). With the rice preparation where there was more ADPglucose pyrophosphorylase activity than transferase activity, the stimulation was only 2-fold. With the barley and tobacco ammonium sulfate preparations where the transferase activity was equal or greater than the pyrophosphorylase activity the stimulation by 3-phosphoglycerate was about 7-fold. The incorporation also required ATP and MgCl_2, requirements of the pyrophosphorylase reaction. This indicated strongly that glucose-14C incorporation from glucose-1-P occurred via the formation of the ADPglucose and then transfer of the glucosyl moiety from ADPglucose to the primer. The small incorporation observed with the tobacco and rice preparations in the absence of ATP and MgCl_2 may be due to the presence of phosphorylase. The reaction also required amylopectin and was inhibited about 35 to 40% in the presence of 0.5 mM P_1.

When the crude extract of rice leaf or tobacco or barley was used as an enzyme source for the coupling reaction no stimulation of glucose incorporation from glucose-1-P into amylopectin by 3PGA, ATP or MgCl_2 was observed. This may have been due to an endogenous supply of these substrates in the extracts or competing reactions present in the crude extracts which prevented the observation of requirements of the coupling reaction. The preparation of the dialyzed ammonium sulfate fraction serves to concentrate the 2 enzymes (pyrophosphorylase and transferase) as well as remove endogenous metabolites.

Stimulation of Spinach Leaf ADPglucose Pyrophosphorylase by 3-Phosphoglycerate at Physiological Concentrations of Inorganic Phosphate. Heber et al. (23, 39) have shown that the concentration of P_1 in the chloroplasts of spinach leaf in the dark is about 5 to 10 mM and decreases about 30%
Table IV. Incorporation of Glucose-14C From Glucose-14C-1-P Into Amylopectin Via Reactions Catalyzed by ADP-glucose Pyrophosphorylase and ADPglucose: Glucan Transerase

The complete reaction mixture consisted of 20 μmoles of tris-HCl buffer, pH 8.5, 2 μmoles of GSH, 5 μmoles of KCl, 0.2 μmole of ATP, 10 μmoles of MgCl₂, 0.1 μmole of glucose-1-P-14C (specific activity, 1.5 × 10⁴ cpm per mole), 50 μg of bovine plasma albumin, 0.5 mg of amylpectin, 0.2 μmole of 3PGA and ammonium sulfate fraction in a total volume of 0.2 ml. The reactions were incubated for 15 minutes at 37°C and terminated by the addition of 2 ml of a solution containing 75% methanol and 1% KCl. The amylpectin precipitate was washed and counted in the same manner as described in the test for the transerase assay. The units of ADPglucose pyrophosphorylase and ADPglucose α-1,4 glucan transerase added to the reaction mixture were respectively: barley, 0.043 and 0.093; rice 0.075 and 0.004; and tobacco 0.045 and 0.05.

<table>
<thead>
<tr>
<th>Reaction Mixture</th>
<th>Barley</th>
<th>Rice</th>
<th>Tobacco</th>
</tr>
</thead>
<tbody>
<tr>
<td>Omissions/Additions</td>
<td>mmoles incorporated per mg per 30 min</td>
<td>mmoles incorporated per mg per 30 min</td>
<td>mmoles incorporated per mg per 30 min</td>
</tr>
<tr>
<td>None</td>
<td>23.8</td>
<td>120</td>
<td>130</td>
</tr>
<tr>
<td>−3PGA</td>
<td>3.5</td>
<td>66</td>
<td>19</td>
</tr>
<tr>
<td>−ATP</td>
<td>0.02</td>
<td>7.6</td>
<td>10</td>
</tr>
<tr>
<td>−MgCl₂</td>
<td>0.04</td>
<td>9.0</td>
<td>12</td>
</tr>
<tr>
<td>−Amylopectin</td>
<td>0.06</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>+P₁, 0.1 mm</td>
<td>21.6</td>
<td>102</td>
<td>...</td>
</tr>
<tr>
<td>+P₁, 0.5 mm</td>
<td>14.0</td>
<td>...</td>
<td>86</td>
</tr>
</tbody>
</table>

Discussion

The above results indicate strongly that the leaf ADPglucose pyrophosphorylases generally have 3 common properties. Activation by 3PGA, inhibition by relatively low concentrations of P₁, and reversal by 3PGA of the inhibition caused by P₁. In addition it appears that the activity of the leaf ADPglucose pyrophosphorylases is quite stable to heat treatment at 65°C in the presence of 0.02 μM phosphate buffer, pH 7.0. The reason for the variance in results that we have obtained with rice leaf ADPglucose pyrophosphorylase as compared to Nomura et al. (34) is unexplained. However we should point out that the specific activity of the glucose-14C-1-P that we have used is 10-fold greater than that used by Nomura et al. (34), thus giving us greater sensitivity in our assays. The activation observed with our partially purified leaf ADPglucose pyrophosphorylase preparations is also observed in the crude extracts using either the synthesis or pyrophosphorylase assays.

Because of the great sensitivity of the leaf ADPglucose pyrophosphorylases to 3PGA, the primary CO₂ fixation product of photosynthesis, and P₁, it is suggested that they play a significant role in the regulation of starch biosynthesis. The levels of P₁ have been shown to decrease in leaves during photosynthesis [due to the process of photophosphorylation]. Glycolytic intermediates are known to increase in the chloroplast in the light. This situation would therefore contribute to conditions necessary for optimal starch synthesis via the increased rate of formation of ADPglucose. In the light the levels of ATP as well as reduced pyridine nucleotides are also increased for the formation of sugar phosphates from 3PGA. In the dark there is an increase in phosphate concentration with concomitant decreases in the levels of glycolytic inter-

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**Fig. 5.** The activation of ADPglucose synthesis by 3-phosphoglycerate in the presence of high concentrations of P₁. The contents of the reaction mixture were the same as those described in the text except that the 3PGA concentration was varied. The enzyme was the highly purified preparation from spinach leaf (17).
mediates, ATP and reducing pyridine nucleotides. This would lead to a situation where inhibition of ADP-glucose synthesis and therefore starch synthesis occurs. To support this formulation, knowledge of the concentrations of the various affecting molecules at the actual site of the ADP-glucose pyrophosphorylase should be known. However, no information of this sort is known and at present is impossible to obtain.

At best the results obtained by workers on the concentrations of the glycolytic intermediates (22), phosphate (23, 39) and ATP (39) in the chloroplast qualitatively support the hypothesis of regulation of starch synthesis by 3PGA, other glycolytic intermediates and phosphate levels. This hypothesis of course does not preclude other mechanisms participating in the regulation of starch synthesis. There may also be regulation at the site of synthesis of the 2 enzymes, ADP-glucose pyrophosphorylase and ADP-glucose: α-1,4 glucan transferase. Furthermore it is not clear whether the ADP-glucose pyrophosphorylases from other parts of the plant are also activated by 3PGA. Preliminary indications are that the ADP-glucose pyrophosphorylase from potato tubers, etiolated pea seedlings, and carrot roots are also activated by 3PGA.

It should be pointed out that the change in the shape of the saturation curve from hyperbolic to sigmoidal form in the presence of phosphate may be physiologically important. This is readily made clear in figure 5 where relatively small changes in $P_1$ (30-50%) at certain concentrations of 3PGA can cause changes in the rate of ADP-glucose synthesis anywhere from 4- to 20-fold. The nature of the sigmoidal curve makes such an effect possible and this phenomenon has been pointed out by Taketa and Pogell (43).

Recently Kortschak et al. (24) and Hatch et al. (19-21) have shown that corn, sugarcane, sorghum leaves and many tropical grasses during photosynthesis do not incorporate $^{14}$CO$_2$ into 3PGA directly but first incorporate it into the C-4 of the dicarboxylic acids. The label is then transferred to the C-1 of 3PGA. These results suggested a novel pathway of CO$_2$ fixation. Our results indicate nevertheless that the activator for sorghum leaf ADP-glucose pyrophosphorylase is still 3PGA. Preliminary results (unpublished results) indicate that corn leaf ADP-glucose pyrophosphorylase is also activated by 3PGA. Oxaaoacetate, malate, or aspartate did not activate the sorghum or corn leaf enzymes.

The leaf ADP-glucose pyrophosphorylases therefore belong to a group of enzymes known as regulatory or allosteric proteins where metabolites unlike the substrates or products affect the catalytic activity of the enzyme (3, 27, 28). These metabolites known as effector molecules act on the protein on sites other than the substrate or active site. Kinetic analyses of the highly purified spinach enzyme have shown that the activator 3PGA increases the $V_{max}$ of the enzyme activity while decreasing the $K_m$ for both ATP and glucose-1-P.

The leaf ADP-glucose pyrophosphorylase is therefore one of the few enzymes from plant origin that has been clearly established as an allosteric protein.

Acknowledgments

The authors are extremely grateful for the supply and/or assistance in the growth of plant materials from the following people: Rice leaves from Dr. T. C. Hsiao and Mr. G. Guinakaka of the Department of Water Science and Engineering; tobacco plants from Mr. Stephen Beer of the Department of Plant Pathology; sugar beet plants from Dr. R. S. Loomis and Mr. A. H. Hall of the Department of Agronomy; kidney bean plants, Phaseolus vulgaris, variant hamulits from Dr. P. A. Castelfranci of the Department of Botany; sorghum seeds from Dr. E. E. Como and Mr. H. Hendrickson of the Department of Biochemistry and Biophysics; barley seeds from Dr. P. K. Stumpf and Dr. M. Macey of the Department of Biochemistry and Biophysics; tomato plants from Dr. W. R. Anderson of the Department of Biochemistry and Biophysics (permanent address, Department of Botany, Brigham Young University).

Literature Cited


