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# Purification of Spinach Leaf ADPglucose Pyrophosphorylase[1]

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## ABSTRACT

**ADPglucose pyrophosphorylase from spinach leaves has been purified to homogeneity by hydrophobic chromatography carried out in 1 molar phosphate buffer. After polyacrylamide gel electrophoresis, the preparation showed only one protein staining band that coincided with a single activity stain. The enzyme appears to be composed of two subunits with molecular weights of 44,000 and 48,000, respectively, as determined by SDS polyacrylamide gel electrophoresis. Thus ADPglucose pyrophosphorylase of spinach appears to be comprised of subunits which are similar in size to the subunits of ADPglucose pyrophosphorylase isolated from bacterial sources. In contrast, a subunit molecular weight of 96,000 has been reported for the maize endosperm ADPglucose pyrophosphorylase (Fuchs RL and JO Smith 1979 Biochim Biophys Acta 556: 40–48). The purified enzyme retains similar allosteric and catalytic properties as reported previously and is more sensitive to phosphate inhibition under "dark"-simulated conditions than under "light"-simulated conditions.**

The synthesis of ADPglucose from ATP and glucose-1-P, catalyzed by ADPglucose pyrophosphorylase (glucose-1-P adenylyl transferase, E.C. 2.2.7.27), is one of the main regulatory steps in the biosynthesis of starch in plants (5, 18, 22) and glycogen in bacteria (7, 13, 17, 19). Extensive studies have been carried out on the kinetic and regulatory properties of ADPglucose pyrophosphorylase from a wide range of sources and these have been reviewed recently (17). The enzyme has been purified to homogeneity from several bacteria and the chemical and physical properties investigated. A mol wt of 185,000 has been found for ADPglucose pyrophosphorylase from *Escherichia coli* and the enzyme shows only one protein band of 50,000 mol wt following SDS-polyacrylamide electrophoresis (7). A mol wt of approximately 200,000 has been found for ADPglucose pyrophosphorylase from a number of other sources (13), including spinach leaves (21) and maize endosperm (6). In another report it has been suggested that the ADPglucose pyrophosphorylase of maize endosperm occurs in two forms, with mol wts of 200,000 and 400,000, with the subunit mol wt of the maize enzyme being 96,000 (3). It would therefore be of high interest to know the subunit molecular weight of other plant ADPglucose pyrophosphorylases, particularly those very sensitive to activation and inhibition by various effector molecules.

ADPglucose pyrophosphorylase from spinach leaves has been previously purified to a high degree using preparative gel electrophoresis (21) but this method has several disadvantages. A new purification procedure which includes a hydrophobic chromatography step carried out in 1 M phosphate buffer is described herein for ADPglucose pyrophosphorylase from spinach leaves.

## MATERIALS AND METHODS

**Reagents.** [$^{14}$C]Glucose-1-P and PPi-$^{32}$P were obtained from New England Nuclear. Insoluble PVP obtained from Sigma was washed by boiling for 10 min in 10% HCl and then washed with $H_2O$ until the pH of the wash reached 7.0. 3-Aminopropyl-Sepharose (Seph-$C_3$-$NH_2$)[3] was prepared as described by Shaltiel and Er-El (23).

The AGATP-C8 column was obtained from the PL-laboratories. The hydroxylapatite used, Biogel HT, was obtained from BioRad. DEAE-cellulose grade DE-52 was purchased from Reeve-Angel. All other chemical reagents used were obtained from commercial sources at the highest purity possible.

**Assay of ADPglucose Pyrophosphorylase.** Pyrophosphorolysis of ADPglucose was followed by the formation of $^{32}$P-ATP in the presence of $^{32}$P-Pi. The reaction mixture contained 20 μmol of glycylglycine buffer (pH 7.5), 1.5 μmol $MgCl_2$, 0.25 μmol ADPglucose, 0.5 μmol $^{32}$P-PPi (1.0 to 5.0 × $10^6$ cpm/μmol), 100 μg crystalline BSA, 0.2 μmol 3-P-glycerate and enzyme in a final volume of 0.25 ml. The reaction mixture was incubated at 37 C for 10 min and the reaction terminated by the addition of 3 ml of cold 5% trichloroacetic acid. $^{32}$P-ATP was isolated and measured as previously described (24). This assay was used to quantitate the enzyme during the purification procedure. A unit of ADPglucose pyrophosphorylase activity is defined as that amount of enzyme catalyzing synthesis of 1 μmol ATP/min under the reaction conditions described.

**Assay of ADPglucose Synthesis.** Synthesis of ADPglucose was measured as previously described (5). The reaction mixture contained 20 μmol Hepes buffer (pH 7.3 or 8.0), 1 μmol $MgCl_2$, 0.1 μmol [$^{14}$C]glucose-1-P (1.0 × $10^6$ cpm/μmol) 0.2 μmol ATP, 0.2 μmol 3-P-glycerate, 50 μg crystalline BSA, and enzyme in a final volume of 0.2 ml. The reaction mixture was incubated at 37 C for 10 min and the reaction terminated by heating in a boiling $H_2O$ bath for 30 s. In reaction mixtures measuring synthesis of ADPglucose in the absence of activator, the amount of glucose-1-P and ATP were increased to 0.2 and 0.5 μmol, respectively.

**Determination of Protein.** Protein concentration was determined by the method of Lowry *et al.* (14).

**Polyacrylamide Gel Electrophoresis.** Electrophoresis in SDS was done in slab gels using the electrophoretic buffer system of Laemmli (12). The gels were 10% acrylamide and 1-mm thick. The proteins were stained by the procedure of Fairbanks *et al.* (2). The protein standards used were phosphorylase a (mol wt, 95,000), BSA (mol wt, 68,000), goat γ globulin, heavy subunit (mol wt,

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[3] Abbreviations: Seph-$C_3$-$NH_3$, 3 aminopropyl-Sepharose; AGATP-C8, a gel consisting of 8-(6 aminohexyl)-amino-ATP coupled to cyanogen bromide activated 4% beaded agarose.

50,000), light subunit (mol wt, 25,000) and myoglobin (mol wt, 17,000).

The native enzyme was also studied in the Ornstein-Davis system (4) using 5 and 7.5% acrylamide gels. Enzyme activity in the acrylamide gel was determined by using a staining procedure previously described (16). Protein staining was done with Coomassie blue (1).

**Sucrose Density Gradient Ultracentrifugation.** Sucrose density ultracentrifugation was done according to the procedure of Martin and Ames (15). Lactate dehydrogenase (mol wt, 140,000) and pyruvate kinase (mol wt, 237,000) were used as marker enzymes.

## RESULTS

**Purification of ADPglucose Pyrophosphorylase from Spinach Leaves.** Spinach leaves, 2,400 g, obtained from a local supermarket, were deveined, washed in tap water and 3 times in distilled $H_2O$, were then homogenized for 3 min in a Waring Blendor with an equal volume of a cold solution containing 50 mM Tris-HCl (pH 7.5), 2 mM GSH, and 2 mM EDTA. The homogenate was filtered through two layers of filter pads and centrifuged at 16,000*g* for 20 min. Pi (1 M [pH 7.2]) was added to the supernatant to give a final concentration of 25 mM and the extract (in 1-liter batches) was heated to 60 C in an 80 C bath. The temperature was reached in about 5 to 6 min. After a further 5 min at 60 C, the extract was chilled rapidly in ice and centrifuged at 16,000*g* for 20 min. All subsequent procedures were done at 0 to 4 C unless noted. Solid $(NH_4)_2SO_4$ was added to the supernatant to give a concentration of 30% saturation. The mixture was centrifuged at 16,000*g* for 20 min and the pellet discarded. Further $(NH_4)_2SO_4$ was added to the supernatant to give 60% saturation. The precipitate was collected by centrifuging at 16,000*g* for 20 min and dissolved in 0.1 M Tris-succinate buffer (pH 7.2), containing 1 mM EDTA and 1 mM GSH (volume of dissolved pellet ~50 ml/kg spinach leaves). The preparation was dialyzed against 20 vol of 20 mM Tris-succinate buffer (pH 7.2), containing 1 mM EDTA, 1 mM GSH, and 20% sucrose for 1 h and then dialyzed for another h against 20 volumes of the fresh Tris-succinate-EDTA-GSH-sucrose buffer.

**PVP Treatment.** The dialyzed $(NH_4)_2SO_4$ fraction was percolated through a column of 500 ml of insoluble PVP that had been equilibrated with at least 5 volumes of 20 mM Tris-succinate buffer (pH 7.2), containing 20% sucrose, 1 mM EDTA, and 1 mM GSH. This buffer was also used to elute the enzyme from the PVP. The first 100 ml of eluant collected was discarded. The next 400 ml contained about 80% of the enzyme activity added.

**DEAE-Cellulose Chromatography.** The PVP eluant was loaded onto 200 ml of a DEAE-cellulose DE-52 column equilibrated with 10 mM Pi (pH 7.4), and 20% sucrose. The column was washed with 50 ml equilibration buffer and eluted with a linear gradient containing 1,650 ml of 10 mM Pi buffer (pH 7.4), and 20% sucrose in the mixing chamber and 1,650 ml of 50 mM Pi buffer (pH 6), 20% sucrose and 0.3 M NaCl in the reservoir. Fractions of 21 ml were collected. The fractions containing activity were pooled and concentrated to about 40 ml using an Amicon thin channel ultrafiltration system with a PM 10 membrane. The concentrated fraction was dialyzed overnight against 2 liters of 50 mM Hepes buffer (pH 7), containing 20% sucrose, 1 mM EDTA, and 30 mM Pi.

**Seph-$C_3$-$NH_2$ Hydrophobic Chromatography.** The dialyzed DEAE-cellulose fraction was made about 1 M with respect to Pi by addition of 0.6 volume of 2.5 M Pi (pH 7.0). The fraction was then adsorbed onto 8 ml of Seph-$C_3$-$NH_2$ gel equilibrated with 1 M Pi buffer (pH 7.0). Fifteen-ml fractions were collected. The column was washed with 90 ml of 1 M Pi buffer (pH 7.0), followed by 0.9 M Pi buffer until $A_{280}$ decreased to about 0.05. The enzyme was eluted with 0.2 M Pi buffer (pH 7.0) and was eluted essentially in one fraction. This fraction was dialyzed overnight against 1 liter of 50 mM Hepes buffer (pH 7.0), containing 20% sucrose, 1 mM EDTA, and 30 mM Pi. Figure 1 shows the hydrophobic chromatographic patterns of the enzyme on the Seph-$C_3$-$NH_2$ column.

**AGATP-C8 Affinity Chromatography.** The Seph-$C_3$-$NH_2$ enzyme fraction was dialyzed for 2 h against 500 ml of 50 mM Hepes buffer (pH 7.0) to remove sucrose and Pi which interfere with the binding of the enzyme to the AGATP-C8 column. $MgCl_2$, 0.1 M, was added to the dialyzed enzyme to give a final concentration of $Mg^{2+}$ of 5 mM. The enzyme was then adsorbed onto 6 ml of the AGATP-C8 column that had been equilibrated with 50 mM Hepes buffer (pH 7.0), containing 5 mM $MgCl_2$. The column was washed with 1 bed volume of the equilibration buffer and with 3 bed volumes of equilibration buffer containing 10 mM Pi. Fractions collected were 6 ml in volume. The enzyme was eluted with 10 bed volumes of equilibration buffer containing 150 mM Pi. The active fractions were pooled and concentrated to about 15 ml in an Amicon ultrafiltration cell containing a PM 10 membrane. The enzyme was dialyzed overnight against 50 mM Hepes buffer (pH 7.0), containing 20% sucrose, 1 mM EDTA, and 5 mM Pi.

**Hydroxylapatite Chromatography.** The AGATP-C8 fraction was adsorbed onto a 15-ml bed volume of Biogel HT-hydroxylapatite that was equilibrated with 50 mM Hepes (pH 7) buffer containing 20% sucrose and 1 mM EDTA. The column was washed with one bed volume of equilibrating buffer, two bed volumes of equilibrating buffer containing 10 mM Pi, one bed volume of equilibrating buffer containing 20 mM Pi, and then eluted with four bed volumes of equilibrating buffer containing 50 mM Pi. The active fractions were pooled and concentrated to about 10 ml in an Amicon ultrafiltration cell as described before. Table I summarizes the purification of the enzyme. The overall purification achieved is about 1,300-fold.

The PVP fractionation does not give an increase in specific activity of the enzyme. However, a large amount of pigmented material is removed from the extract by PVP and this procedure greatly improves the yield and stability of the enzyme in subsequent purification steps. The enzyme is most stable in buffer containing 20% sucrose and 30 mM Pi losing no activity when stored either frozen or at 4 C. For kinetic studies, the hydroxylapatite fraction was dialyzed against 200 volumes of a 50 mM Hepes buffer (pH 7.0), containing 20% sucrose, 0.2 mM DTE, and 1 mM EDTA. In this buffer the enzyme was stable for about 2 weeks but lost about 60% activity in a month when left at 4 C.

**Gel Electrophoresis.** Purified ADPglucose pyrophosphorylase run in the Tris/glycine system of Ornstein (4) with gel acrylamide concentrations of 5 and 7.5% showed only one band of protein. Representative results are seen in Figure 2 for the 5% gels. This band also coincided with enzyme activity as seen by an activity stain (Fig. 2). When the purified hydroxylapatite fraction was denatured and subjected to electrophoresis according to the procedure of Laemmli (12), two protein bands staining with about equal intensity were observed (Fig. 3). The mol wt of the two bands were determined to be 44,000 and 48,000, respectively, using the standard protein markers as indicated in Figure 4.

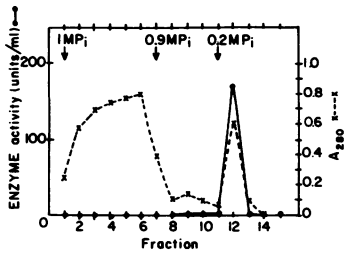


FIG. 1. Seph-$C_3$-$NH_2$ hydrophobic chromatography of spinach leaf ADPglucose pyrophosphorylase. Details of the procedure are described in the text.

Table I. *Purification of ADPglucose Pyrophosphorylase*

| Fraction | Volume | Protein | Activity | Specific Activity | Recovery |
|---|---|---|---|---|---|
| | *ml* | *mg* | *units* | *units/mg* | % |
| Crude extract | 2,100 | 9,870 | 1,200 | 0.12 | 100 |
| Heat treated | 2,020 | 5,252 | 1,370 | 0.26 | 115 |
| $(NH_4)_2SO_4$ | 95 | 3,420 | 883 | 0.26 | 74 |
| PVP | 400 | 3,120 | 696 | 0.22 | 58 |
| DEAE-cellulose | 39 | 69 | 507 | 7.3 | 42 |
| Seph $C_3NH_2$ | 8.7 | 9.2 | 378 | 40.9 | 32 |
| AGATP-C8 | 9 | 2.3 | 198 | 88 | 18 |
| Hydroxylapatite | 10.5 | 0.71 | 111 | 156 | 9 |

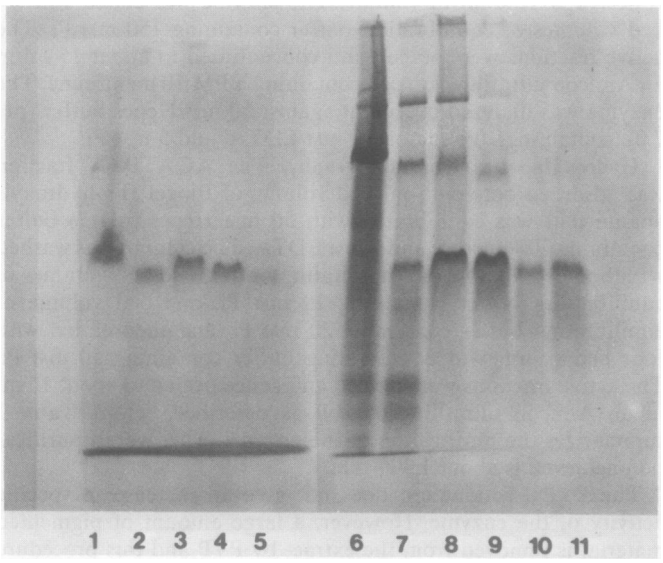


FIG. 2. Gel electrophoresis of ADPglucose pyrophosphorylase fractions in the Tris-glycine (pH 8.8), Ornstein system. The resolving gel polyacrylamide concentration was 5.0%. Lanes 1 through 5 are fractions subjected to activity stains (16) and lanes 6 through 11 are fractions stained with Coomassie blue. The amount of proteins and fractions applied to the various lanes are; lanes 1 and 6, ammonium sulfate fraction, 180 μg; lanes 2 and 7, DEAE-cellulose fraction, 17.7 μg; lanes 3 and 8, Seph-$C_3$-$NH_2$ fraction, 21 μg; lanes 4 and 9, AGATP-C8 fraction, 10.2 μg; lane 10, hydroxylapatite fraction, 2.7 μg, and lanes 5 and 11, hydroxylapatite fraction, 5.4 μg.

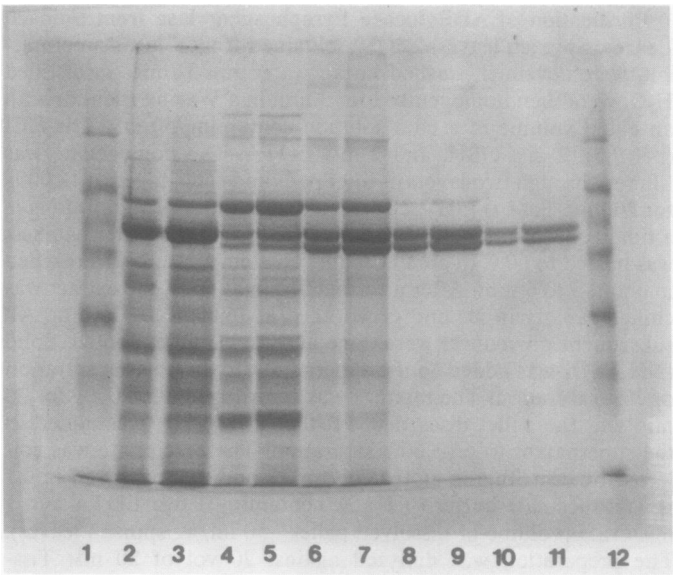


FIG. 3. Gel electrophoresis of ADPglucose pyrophosphorylase fractions in the Tris-glycine (pH 8.8) SDS system of Laemmli (12). Lanes 1 and 12 contain the various protein standards described under "Materials and Methods." Lanes 2 and 3 contain 180 and 360 μg of protein, respectively, of the ammonium sulfate fraction. Lanes 4 and 5 contain 17.7 and 35.4 μg of protein, respectively, of the DEAE-cellulose fraction. Lanes 6 and 7 contain 16 and 32 μg, respectively, of the Seph-$C_3$-$NH_2$ fraction. Lanes 8 and 9 contain 5 and 10 μg, respectively of the AGATP-C8 column and lanes 10 and 11 contain 2.7 and 5.4 μg, respectively, of the hydroxylapatite fraction.

**Molecular Weight Determinations.** The mol wt of the enzyme was determined to be 206,000 ± 18,000 by ultracentrifugation in sucrose density gradients (15) and is similar to that previously reported (21). The presence of 30 mM Pi, an allosteric inhibitor of the enzyme, had no effect on the migration of the enzyme in sucrose density gradients.

**Kinetic Properties of ADPglucose Pyrophosphorylase.** Table II lists various kinetic parameters found for the substrates and the effectors of spinach leaf ADPglucose pyrophosphorylase. The range of concentrations of substrates, activator, and inhibitor studied were the same studied previously (5, 18). The presence of 1 mM 3-P-glycerate decreases the $K_m$ value for both ATP and glucose-1-P. The values obtained are similar to those observed earlier (5, 18) under slightly different reaction conditions.

The activator specificity of the purified ADPglucose pyrophosphorylase was similar to that reported previously for less purified enzymes (5, 18) with 3-P-glycerate, the most potent activator. Other compounds that activated to a much lesser extent were fructose-6-P, P-enolpyruvate, and fructose-1,6-$P_2$.

Figure 5 shows the 3-P-glycerate activation curve for the purified enzyme. The reaction was run at two different pH values reported to be found in chloroplast stroma in dark (pH 7.3) and

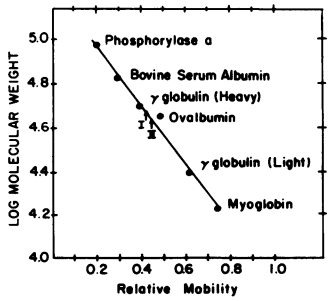


FIG. 4. Determination of the molecular weights of the ADPglucose pyrophosphorylase subunits. I and II are the protein-staining bands observed in lanes 10 and 11 of Figure 5. The straight line is obtained from the plots of relative mobility against the log of molecular weights of the subunits of the protein standards (lanes 1 and 12 of Fig. 5). Relative mobility was calculated from the distance the protein band migrated divided by the distance the bromophenol blue dye migrated.

Table II. *Kinetic Parameters of ADPglucose Pyrophosphorylase*

| Substrate/Effector | pH 7.3 | | | | pH 8.0 | | | |
|---|---|---|---|---|---|---|---|---|
| | $S_{0.5}$ | $A_{0.5}$ | $I_{0.5}$ | $\bar{n}$ | $S_{0.5}$ | $A_{0.5}$ | $I_{0.5}$ | $\bar{n}$ |
| | | μM | | | | μM | | |
| 3-P-glycerate | | 30 | | 1.0 | | 51 | | 1.0 |
| 3-P-glycerate in presence of 0.2 mM Pi | | 130 | | 1.8 | | 218 | | 1.8 |
| ATP (+ 1 mM 3-P-glycerate) | 45 | | | 1.0 | 62 | | | 0.9 |
| ATP (in absence of 3-P-glycerate) | 320 | | | 0.9 | 380 | | | 0.9 |
| Glc-1-P (+ 1 mM 3-P-glycerate) | 31 | | | 1.0 | 35 | | | 1.0 |
| Glc-1-P (in absence of 3-P-glycerate) | 105 | | | 0.9 | 120 | | | 0.9 |
| Pi | | | 64 | 1.1 | | | 45 | 1.1 |
| Pi in presence of 1 mM 3-P-glycerate | | | 1,270 | 3.0 | | | 970 | 3.7 |

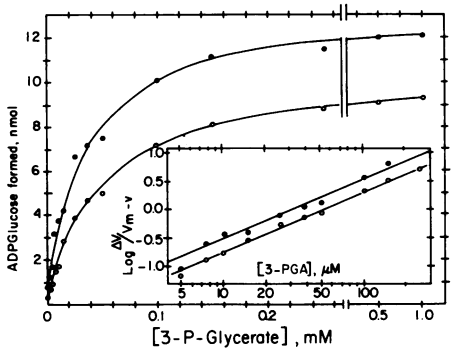


FIG. 5. The activation of ADPglucose pyrophosphorylase by 3-P-glycerate. The synthesis reaction mixture is described under "Material and Methods" except that the concentration of 3-P-glycerate was varied as indicated. (—●—), reactions run at pH 7.3 and (—○—), reactions run at pH 8.0.

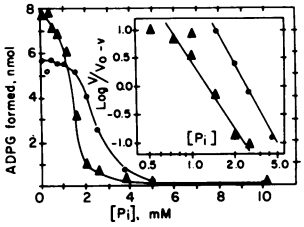


FIG. 6. Phosphate inhibition of spinach leaf ADPglucose pyrophosphorylase. The reaction mixtures contained in a volume of 0.2 ml the following: dark-simulated conditions (▲); 20 μmol Hepes buffer (pH 7.3), 0.04 μmol ATP, 0.04 μmol [$^{14}$C]glucose-1-P, 0.8 μmol glucose-6-P, 0.4 μmol fructose-6-P, 50 μg BSA, 0.32 μmol 3-P-glycerate, 1 μmol $MgCl_2$ and enzyme; light-simulated conditions (●); 20 μmol Hepes buffer (pH 8.0), 0.2 μmol ATP, 0.014 μmol [$^{14}$C]glucose-1-P, 0.27 μmol glucose-6-P, 0.13 μmol fructose-6-P, 50 μg BSA, 0.8 μmol 3-P-glycerate, 1 μmol $MgCl_2$ and enzyme. The ratios of glucose-6-P to fructose-6-P to glucose-1-P were based on the known equilibrium constants of the phosphohexose-isomerase and phosphoglucomutase reactions and the amounts added to the reaction mixture are based on the values obtained by Kaiser and Bassham (10).

light (pH 8.0) conditions (25). The concentration of 3-P-glycerate required for 50% of maximal activation was found to be 30 μM at pH 7.3 and 51 μM at pH 8.0. These values are similar to those previously reported with the DEAE-cellulose fraction assayed under slightly different conditions (5, 18). The activation curves are hyperbolic in shape with Hill plot slope values, $\bar{n}$, (9) of 1. However, they become sigmoidal in shape in the presence of 0.2 mM Pi, with the Hill plot slope increasing to 1.8 (Table II). Pi at 0.2 mM also increases the $A_{0.5}$ value of 3-P-glycerate approximately 4-fold.

Phosphate is a potent inhibitor of the enzyme with 64 μM causing 50% inhibition at pH 7.3 and 45 μM Pi causing 50% inhibition at pH 8.0. In the presence of 1 mM 3-P-glycerate the hyperbolic phosphate curve is changed to a sigmoidal form and the concentration of Pi required for 50% inhibition is increased about 20-fold.

The $I_{0.5}$ values for Pi are slightly lower at pH 8.0 than at pH 7.3, suggesting it is a more effective inhibitor at the higher pH. Conversely, 3-P-glycerate seems to be a better activator at pH 7.3 than at pH 8.0. These results appear to be in conflict with the observations that stromal pH during photosynthesis in the light is about 8.0 and is 7.3 in the dark (10, 25) and starch synthesis occurring in the light and being inhibited in the dark (8, 10). The phosphate inhibition was therefore studied under reaction conditions approximating the reported pH values and physiological concentrations of ATP, 3-P-glycerate, glucose-1-P, fructose-6-P, and glucose-6-P in the "light" and "dark" conditions (10). The last two phosphorylated sugars were included because of their occurrence in the chloroplast at high concentration. Figure 6 shows the effect of Pi under the simulated "light" and "dark" conditions. The inhibition curves are highly sigmoidal with Hill plot slope values, $\bar{n}$, being 4.6 under light conditions and 3.9 under the dark conditions. The concentration of Pi required for 50% inhibition under the light conditions is 2.5 mM while under the dark conditions the enzyme is more sensitive to inhibition with the $I_{0.5}$ value being 1.3 mM.

## DISCUSSION

The spinach leaf ADPglucose pyrophosphorylase can be obtained in homogenous form using the technique of hydrophobic chromatography. This step results in a 5.6-fold purification (Fig. 1). The specific activity is 156 μmol-min$^{-1}$-mg$^{-1}$ of protein and this is equivalent to the specific activity reported for purified bacterial ADPglucose pyrophosphorylases (7, 13, 17). The facile purification procedure should enable one to isolate substantial amounts of protein for further analysis at the molecular level. At all stages of the preparation only one band of ADPglucose pyrophosphorylase is seen on gels stained for activity (Fig. 2). The purified enzyme shows only one major protein band following electrophoresis in gels of 5 and 7.5% polyacrylamide, respectively (Fig. 2). Two bands which stained for protein with equal intensity were observed when the purified spinach ADPglucose pyrophos-

phorylase was subjected to SDS-polyacrylamide gel electrophoresis (Fig. 3). The presence of two bands, which had mol wt of 44,000 and 48,000, respectively (Fig. 4), raises the possibility that ADPglucose pyrophosphorylase is comprised of two different subunits. In contrast, the ADPglucose pyrophosphorylase from *E. coli* (7) or *Salmonella typhimurium* (13) shows only one band of 50,000 mol wt following SDS-polyacrylamide gel electrophoresis. It is possible that in spinach leaf the 48,000 mol wt subunit has been subjected to proteolytic cleavage giving rise to the 44,000 mol wt subunit. If so, the proteolytic cleavage must have occurred early in the fractionation procedure as the two subunits are evident after the DEAE-cellulose chromatography step which is started in the 1st day of the purification procedure. The finding of subunits of molecular size in the order of about 50,000 indicates that the subunit size of the spinach leaf ADPglucose pyrophosphorylase is different from the subunit size of 96,000 reported for the maize endosperm ADPglucose pyrophosphorylase (3). Although it is possible that proteolytic cleavage of the spinach leaf enzyme was internal and produced two fragments of approximately equal size, one would suspect that such proteolysis would drastically reduce the catalytic activity and change the regulatory properties of the enzyme. Furthermore, there is no evidence for an appreciable amount of peptide of 90,000 (or higher) subunit mol wt in any of the enzyme fractions shown in Figure 3. The evidence thus far suggests that the spinach leaf ADPglucose pyrophosphorylase has a native mol wt of about 200,000 and is a tetramer comprised of two different subunits.

Evidence has accumulated to indicate that the ratio of 3-P-glycerate to Pi is critical in the regulation of starch accumulation. A high ratio increases the rate of starch accumulation while a low ratio appears to favor degradation (8, 10). Consistent with this have been the findings that plant, algal, and cyanobacterial ADPglucose pyrophosphorylases are activated by 3-P-glycerate and inhibited by Pi (5, 17–19, 21). Under the conditions approximating the *in vivo* or *in situ* concentrations of various metabolites reported to occur in spinach chloroplasts in light and dark conditions, the ADPglucose pyrophosphorylase is more sensitive to Pi inhibition in the dark conditions (Fig. 6). At 1.0 mM Pi, the rate of ADPglucose synthesis in the two conditions appears to be similar. However, at 2.0 mM Pi, there is considerable reduction (85%) of ADPglucose synthesis in the dark-simulated conditions while there is only 25% reduction in the light-stimulated conditions. The rate of ADPglucose synthesis at 1.5, 2.0, 2.5, and 3.7 mM Pi is 2.0, 4.6, 4.2 and 3.8 times greater under the light-simulated conditions than under the dark-simulated conditions.

The major reason for the increased sensitivity is due to the lower concentrations of 3-P-glycerate in the dark conditions (1.6 mM) compared to the light conditions (4.0 mM). If the concentration of 3-P-glycerate under the light-simulated conditions is decreased to 1.6 mM, then the Pi sensitivity obtained is very similar to that observed for the dark-simulated conditions (unpublished experiments). No change in Pi sensitivity is seen if the glucose-1-P concentration or pH or the hexose phosphate concentrations in the light-simulated conditions are changed to what is observed in the dark-simulated conditions (experiments not shown). However, if the ATP concentration is reduced from 1 to 0.2 mM (the concentration reported to be present in the dark-simulated conditions), a greater increase in sensitivity to Pi inhibition is observed. The possibility in the variations of ATP concentration in chloroplast as a controlling factor in ADPglucose and starch formation was first suggested by Kaiser and Bassham (11) and is consistent with previous (5) and recent unpublished studies that show that Pi considerably increases the $K_m$ value of ATP for spinach leaf ADPglucose pyrophosphorylase.

Thus, two factors appear to regulate the ADPglucose pyrophosphorylase activity. One is the 3-P-glycerate to Pi ratio and the other is the variation of ATP in the light and dark (10, 11). Kaiser and Bassham (10, 11) also propose light-mediated activation of the ADPglucose pyrophosphorylase since they observed a 5.7-fold stimulation of ADPglucose synthesis by high concentrations of DTT. However, the purified or crude extract ADPglucose pyrophosphorylases are not stimulated by either DTT or by various spinach leaf thioredoxins (given to us by Dr. Buchanan of the University of California, Berkeley) plus DTT. Kaiser and Bassham were measuring ADPglucose synthesis from glucose-6-P. Their assay system was dependent on phosphoglucomutase activity, an enzyme activity that may be affected by sulfhydryl group reagents (20). It may be possible that DTT activation of ADPglucose synthesis in their system reflected activation of the phosphoglucomutase activity. Further documentation is required to determine whether DTT does directly activate ADPglucose pyrophosphorylase activity.

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## LITERATURE CITED


1. CHRAMBACH A, RA REISFELD, M WYCKOFF, J AACCARI 1967 A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. Anal Biochem 20: 150–154
2. FAIRBANKS G, TL STECK, DFH WALLACE 1971 Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 10: 2606–2616
3. FUCHS RL, JD SMITH 1979 The purification and characterization of ADPglucose pyrophosphorylase A from developing maize seeds. Biochim Biophys Acta 566: 40–48
4. GABRIEL O 1971 Analytical disc gel electrophoresis. Methods Enzymol 22: 565–578
5. GHOSH HP, J PREISS 1966 Adenosine diphosphate glucose pyrophosphorylase. A regulatory enzyme in the biosynthesis of starch in spinach chloroplasts. J Biol Chem 241: 4491–4504
6. HANNAH LC, OE NELSON JR 1975 Characterization of adenosine diphosphate glucose pyrophosphorylases from developing maize seeds. Plant Physiol 55: 297–302
7. HAUGEN T, A ISHAQUE, J PREISS 1976 Biosynthesis of bacterial glycogen. Characterization of the subunit structure of *Escherichia coli* B glucose-1-P adenylyl transferase. J Biol Chem 251: 7880–7885
8. HELDT HW, CJ CHON, D MARONDE, A HEROLD, ZS STANKOVIC, D WALKER, A KRAMINER, MR KIRK, U HEBER 1977 Role of orthophosphate and other factors in the regulation of starch formation in leaves and isolated chloroplasts. Plant Physiol 59: 1146–1155
9. HILL AV 1910 The possible effects of the aggregation of the molecules of hemoglobin on its dissociation curves. J Physiol 40: IV–VII
10. KAISER WM, JA BASSHAM 1979 Light-dark regulation of starch metabolism in chloroplasts. I. Levels of metabolites in chloroplasts and medium during light-dark transition. Plant Physiol 63: 105–108
11. KAISER WM, JA BASSHAM 1979 Light-dark regulation of starch metabolism in chloroplasts. II. Effect of chloroplastic metabolite levels on the formation of ADPglucose by chloroplast extracts. Plant Physiol 63: 109–113
12. LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685
13. LEHMANN M, J PREISS 1980 Biosynthesis of bacterial glycogen: purification and properties of *Salmonella typhimurium* LT-2 adenosine diphosphate glucose pyrophosphorylase. J Bacteriol 143: 120–127
14. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
15. MARTIN RG, RN AMES 1961 A method for determining the sedimentation behaviour of enzymes: application to protein mixtures. J Biol Chem 236: 1372–1379
16. OZAKI H, J PREISS 1972 ADPglucose pyrophosphorylase from *Escherichia coli* B. Methods Enzymology 28: 279–281
17. PREISS J 1978 Regulation of ADPglucose pyrophosphorylase. *In* A Meister, ed, Advances in Enzymology and Related Areas of Molecular Biology. Chap 46. John Wiley and Sons, Inc, New York, pp 317–381
18. PREISS J, HP GHOSH, J WITTKOP 1967 Regulation of the biosynthesis of starch in spinach leaf chloroplasts. *In* T Goodwin, ed, Biochemistry of Chloroplasts Vol I. Academic Press, New York, pp 131–153
19. PREISS J, WK KAPPEL, E GREENBERG 1979 Comparative regulation of $\alpha$,1,4 glucan synthesis in photosynthetic and non-photosynthetic systems. *In* DE Atkinson, ed, ICN-UCLA Symposia on Covalent and Non-Covalent Modulation of Proteins. Academic Press, New York, pp 161–184
20. RAY WJ JR, EJ PECK JR 1972 Phosphomutases. *In* PD Boyer, ed, The Enzymes, 3rd Ed, Vol VI. Academic Press, New York, pp 407–477
21. RIBEREAU-GAYON G, J PREISS 1971 ADPglucose pyrophosphorylase from spinach leaf. Methods Enzymol 23: 691–695

22. SANWAL GG, E GREENBERG, J HARDIE, EC CAMERON, J PREISS 1968 Regulation of starch biosynthesis in plant leaves: activation and inhibition of ADPglucose pyrophosphorylase. Plant Physiol 43: 417–427
23. SHALTIEL S, Z ER-EL 1973 Hydrophobic chromatography: use for purification of glycogen synthetase. Proc Natl Acad Sci USA 70: 778–781
24. SHEN L, J PREISS 1964 The activation and inhibition of bacterial adenosine diphosphoglucose pyrophosphorylase. Biochem Biophys Res Commun 17: 424–429
25. WERDAN KW, HW HELDT, M MILONVANCEV 1975 The role of pH in the regulation of carbon fixation in the chloroplast stroma. Studies on $CO_2$ fixation in the light and dark. Biochim Biophys Acta 396: 276–292