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Purification and Properties of Nonproteolytic Degraded ADPglucose Pyrophosphorylase from Maize Endosperm[1]

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ABSTRACT

ADPglucose pyrophosphorylase from developing endosperm tissue of starchy maize (*Zea mays*) was purified 88-fold to a specific activity of 34 micromoles α-glucose-1-P produced per minute per milligram protein. Rabbit antiserum to purified spinach leaf ADPglucose pyrophosphorylase was able to inhibit pyrophosphorolysis activity of the purified enzyme by up to 90%. The final preparation yielded four major protein staining bands following sodium dodecyl sulfate polyacrylamide gel electrophoresis. When analyzed by Western blot hybridization only the fastest migrating, 54 kilodaltons, protein staining band cross-reacted with affinity purified rabbit antispinach leaf ADPglucose pyrophosphorylase immunoglobulin. The molecular mass of the native enzyme was estimated to be 230 kilodaltons. Thus, maize endosperm ADPglucose pyrophosphorylase appears to be comprised of four subunits. This is in contrast to the respective subunit and native molecular masses of 96 and 400 kilodaltons reported for a preparation of maize endosperm ADPglucose pyrophosphorylase (Fuchs RL and JO Smith 1979 Biochim Biophys Acta 556: 40–48). Proteolytic degradation of maize endosperm ADPglucose pyrophosphorylase appears to occur during incubation of crude extracts at 30°C or during the partial purification of the enzyme according to a previously reported procedure (DB Dickinson, J Preiss 1969 Arch Biochem Biophys 130: 119–128). The progressive appearance of a 53 kilodalton antigenic peptide suggested the loss of a 1 kilodalton proteolytic fragment from the 54 kilodalton subunit. The complete conservation of the 54 kilodalton subunit structure following extraction of the enzyme in the presence of phenylmethylsulfonyl fluoride and/or chymostatin was observed. The allosteric and catalytic properties of the partially purified proteolytic degraded *versus* nondegraded enzyme were compared. The major effect of proteolysis was to enhance enzyme activity in the absence of added activator while greatly decreasing its sensitivity to the allosteric effectors 3-P-glycerate and inorganic phosphate.

Regulation of the activity of ADPglucose pyrophosphorylase (ATP:α-glucose-1-P adenyl transferase, EC 2.7.7.27) is believed to play a vital role in controlling the biosynthesis of α-1,4-glucans in plants and bacteria (20, 21). This enzyme catalyzes the reversible synthesis of ADPglucose and PP; from ATP and glucose-1-P and has had its kinetic and physical properties studied from a wide variety of sources (see Refs. 20–22, for reviews). Common kinetic features of most ADPglucose pyrophosphorylases from both photosynthetic and nonphotosynthetic plant sources are their extreme sensitivity to allosteric activation and inhibition by 3-P-glycerate and Pi, respectively. The purified enzyme from the bacteria, *Escherichia coli* (10) and *Salmonella typhimurium* (17), spinach leaves (3) and potato tubers (28) is tetrameric with native and subunit mol wt about 200,000 and 50,000 kD, respectively. In contrast, the extensively studied enzyme from developing maize endosperm (4, 6, 9) has been reported to have very unique kinetic and physical properties. First, its sensitivity to the allosteric effectors, 3-P-glycerate and Pi, was found to be far lower than those determined all for other plant ADPglucose pyrophosphorylases (21). Second, unlike any other plant or bacterial ADPglucose pyrophosphorylase examined to date, the maize endosperm enzyme purified to apparent homogeneity by Fuchs and Smith (6) was reported to have a subunit molecular mass of 96 kD, and to occur in two native forms, a 200 kD dimer and a 400 kD tetramer.

In this communication, we describe further studies on maize endosperm ADPglucose pyrophosphorylase and demonstrate that when extracted and partially purified under conditions which prevent its proteolytic degradation it shows kinetic properties which are, in fact, very closely related to the enzyme from photosynthetic and nonphotosynthetic plant tissues. In addition, through the use of immunological and electrophoretic techniques, compelling evidence is obtained which suggests that the subunit structure of the maize endosperm enzyme is very similar to that of other plant and bacterial ADPglucose pyrophosphorylases and not as reported by Fuchs and Smith (6).

MATERIALS AND METHODS

Chemicals and Seeds. All biochemicals, coupling enzymes, ribulose bisP carboxylase, acrylamide, bis-acrylamide, SDS, ethyl agarose, and butyl agarose were purchased from Sigma. DEAE-cellulose (DE-52) was from Whatman, BCA[3] protein reagent was from Pierce Chemical Co., nitrocellulose membranes (BA-85; 0.45 μm) were from Schleicher and Schull, and protein molecular mass standards were from Pharmacia. α-[^{14}C]Glucose-1-P was obtained from New England Nuclear Corporation. All buffers were degassed and adjusted to their respective pH at 22°C. Developing maize seeds (variety W64A) were harvested at 22 d postpollination, frozen with dry ice, and stored at −80°C until used.

Assay of ADPglucose Pyrophosphorylase. *Assay A.* The pyrophosphorolysis of ADPglucose was coupled with phosphoglucomutase and glucose-6-P dehydrogenase and assayed by measuring NADH production at 340 nm (27). Temperature for all assays was maintained at 37°C. Standard reaction mixtures con-

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[3] Abbreviations: BCA, bicinchoninic acid; PMSF, phenylmethylsulfonyl fluoride; NEM, *N*-ethylmaleimide; PABA, *p*-aminobenzamidine; PCMA, *p*-chloromercuribenzoate; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethylketone.

tained in 0.5 ml: 40 μmol of Hepes-NaOH buffer (pH 7.4), 5 μmol of $MgCl_2$, 5 μmol of 3-P-glycerate, 0.1 mg of BSA, 0.5 μmol of ADPglucose, 1 μmol of sodium pyrophosphate, 0.3 μmol of NAD^+, 5 nmol of glucose-1,6-bisP, and 1 unit each of dialyzed rabbit muscle phosphoglucomutase and *Leuconostoc mesenteroides* glucose-6-P dehydrogenase. Assays were initiated by addition of PPi. Negligible NADH was produced by any of the maize endosperm extracts in the absence of PPi or ADPglucose. The rate of NADH production was linear to both time and concentration of pyrophosphorylase used in all enzymic studies. A unit of activity is defined as that amount resulting in the production of 1 μmol glucose-1-P/min at 37°C. Specific activity is defined as unit/mg protein.

Assay B. α-[^{14}C]Glucose-1-P was used to measure the synthesis of [^{14}C]ADPglucose. Standard reaction mixtures contained in 0.2 ml:16 μmol of Hepes-NaOH buffer (pH 7.4), 50 μg of BSA, 2 μmol of $MgCl_2$, 0.2 of μmol ATP, 0.1 μmol of α-[^{14}C]glucose-1-P (1100 cpm/nmol), and 0.12 unit of inorganic pyrophosphatase. 3-P-Glycerate (0.2 μmol) was added when the enzyme was assayed in the presence of activator. Assays were initiated by the addition of enzyme. Reaction mixtures were incubated for 10 min at 37°C and terminated by heating for 1 min in a boiling water bath and [^{14}C]ADPglucose assayed as previously described (7).

Assay of Other Enzyme Activities. Phosphoglycerate mutase, phosphoglucose isomerase, phosphoglucomutase, and adenylate kinase were assayed as previously described (1).

Kinetic Studies. All kinetic studies were performed using assay B. $S_{0.5}$, $A_{0.5}$, 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 (12, 15). All kinetic parameters are the mean of at least two determinations and are reproducible to within at least ±10%.

Purification of ADPglucose Pyrophosphorylase. All steps were carried out between 0 and 4°C. Assay A was used to monitor enzyme activity throughout the purification.

Preparation of Crude Extract. Corn endosperm (105 g) was dissected free of pericarp and embryo and homogenized with 65 ml of ice-cold 50 mM K-phosphate (pH 7.0) containing 5 mM EDTA and freshly added PMSF (1.5 mM; 50 mM stock solution was prepared in ethanol), 10 μg/ml chymostatin, and 1 mM DTE using a chilled mortar and pestle. The homogenate was filtered through Miracloth and cheesecloth and the residue reextracted with 2 × 20 ml of homogenization buffer. Filtrates were combined and centrifuged at 26,000*g* for 20 min.

PEG Fractionation. PEG (average mol wt 7 kD; 50% w/v dissolved in 50 mM Hepes-NaOH [pH 7.0] containing 1 mM EDTA) was slowly added to the supernatant fluid to bring the final PEG concentration to 10% (w/v). The solution was stirred for 20 min and centrifuged as above. Pellets were discarded and the supernatant adjusted to 20% (w/v) with PEG (50% w/v), stirred and centrifuged as above. The pellets containing ADPglucose pyrophosphorylase were resuspended in 20 ml of 15 mM K-phosphate (pH 7.0) containing 1 mM EDTA, 1 mM DTE, and 10% glycerol (buffer A) and centrifuged as above.

DEAE-Cellulose Chromatography. The clear supernatant was absorbed onto a column of DEAE-cellulose (1.5 × 10.5 cm; 18.5 ml resin bed volume) which had been preequilibrated with buffer A. The column was eluted with buffer A (70 ml) and then with 50 mM K-phosphate (pH 7.0) containing 1 mM EDTA, 1 mM DTE, and 10% glycerol (buffer B) until the A_{280} decreased to 0.05. The enzyme was eluted with a linear 0 to 200 mM gradient of KCl (200 ml total volume) in buffer B. Five ml fractions were collected. Pooled peak fractions were concentrated with an Amicon PM-30 ultrafilter.

C_4-Butyl Agarose Chromatography. A solution of $(NH_4)_2SO_4$ (100% saturation; pH 7.0) was slowly added to the pooled peak DEAE-cellulose concentrate to bring the final ammonium sulfate concentration to 27.5% saturation. The solution was stirred for 15 min and centrifuged as above. The clear supernatant was absorbed onto a column of C_4-butyl agarose (1.0 × 6.5 cm; 5.0 ml resin bed volume) which had been preequilibrated with 50 mM K-phosphate (pH 7.0) containing 27% saturated $(NH_4)_2SO_4$, 1 mM DTE, 1 mM EDTA, and 10% glycerol (buffer C). The column was washed with buffer C until the A_{280} decreased to 0.02. The enzyme was eluted with a descending linear gradient of $(NH_4)_2SO_4$ which consisted of 30 ml of buffer C in the mixing flask and 30 ml of buffer A in the reservoir flask. Two ml fractions were collected. Pooled peak fractions were concentrated as above.

C_2-Ethyl Agarose Chromatography. The pooled peak C_4-butyl agarose concentrate was dialyzed for 1.5 h *versus* 1.0 L of buffer A containing 30% saturated $(NH_4)_2SO_4$ (buffer D) and centrifuged as above. The clear supernatant was absorbed onto a column of C_2-ethyl agarose (1.0 × 3.2 cm; 2.5 ml resin bed volume) which had been preequilibrated in buffer D and the volume was washed with buffer D until the A_{280} decreased to 0.02. The column was then washed with buffer A containing 25% (saturation) $(NH_4)_2SO_4$ (buffer E) until the A_{280} dropped to 0.02. The enzyme was eluted with a descending linear gradient of $(NH_4)_2SO_4$ which consisted of 25 ml of buffer E in the mixing flask and 25 ml of buffer A in the reservoir flask. Two ml fractions were collected. Pooled peak fractions were concentrated as above and divided into 0.5 ml aliquots which were frozen in liquid N_2 and stored at −80°C. The partially purified enzyme was stable for several months when stored frozen.

Sephadex G-50 Chromatography. For kinetic studies 0.5 ml aliquots of the final preparation were desalted on a column of Sephadex G-50 (6 ml resin bed volume) using 40 mM Hepes-NaOH (pH 7.2) containing 40 mM KCl, 0.5 mg/ml BSA, 15% sucrose, 0.5 mM EDTA, and 1.0 mM DTE as the equilibration/elution buffer. Fractions of 0.5 ml were collected. Pooled peak fractions (100% yield) were divided into 80 μl aliquots, frozen in liquid N_2, and stored at −80°C until used.

Degradation of ADPglucose Pyrophosphorylase. Whole maize seeds (6.0 g) were homogenized with 15 ml of 50 mM K-phosphate buffer (pH 7.0) containing 1 mM EDTA using a chilled mortar and pestle. The homogenate was filtered through 2 layers of Miracloth and centrifuged for 6 min at 15,600*g* in an Eppendorf microcentrifuge. A 1.0 ml aliquot of the crude supernatant was incubated at 30°C and at 0 and 310 min a 50 μl aliquot was removed, frozen in liquid N_2, and stored at −80°C for later examination of potential pyrophosphorylase degradation via SDS-PAGE/Western blotting. Individual additions of various protease inhibitors were also added to separate 1.0 ml aliquots of the crude supernatant. The mixtures were incubated at 30°C and at 310 min 50 μl aliquots were removed, frozen, and later analyzed as described above.

Antibody Production and Neutralization of Enzyme Activity. After collection of preimmune sera, homogenous spinach leaf ADPglucose pyrophosphorylase (0.25 mg) (3) 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% NaN_3. Rabbit antispinach leaf ADPglucose pyrophosphorylase IgG was affinity purified from the immune sera according to the procedure of Smith and Fisher (24).

Neutralization of enzyme activity was tested by mixing 0.05 unit of the purified maize endosperm ADPglucose pyrophosphorylase with 3 μmol of Hepes-NaOH (pH 7.1), containing 10 μg of BSA, 2.5 μmol of Pi, 0.1 μmol of DTE, 5 mg of sucrose, and 30 μl of serum containing varying amounts of antispinach leaf ADPglucose pyrophosphorylase immune sera diluted into

preimmune serum in a total volume of 0.1 ml. The mixture was incubated for 30 min at 30°C and then for 2 h on ice prior to centrifuging for 5 min at 15,600*g* in an Eppendorf microcentrifuge. Enzyme activity in the supernatant was assayed using Assay A.

SDS Electrophoresis and Immunological Detection of ADPglucose Pyrophosphorylase via Western Blotting. SDS-PAGE was performed using a discontinuous system based on the procedure of Laemmli (16). Final monomer concentration in the 1.0 mm thick slab gel was 4.5% (w/v) for the stacking gel and 10% (w/v) for the separating gel. All samples were boiled for 3 min in the presence of 0.1% (w/v) SDS and 5% (v/v) 2-mercaptoethanol prior to loading on the gel. Gels were run with 50 V (constant voltage) overnight. Following electrophoresis, gels were stained for protein using Coomassie blue R-250 or electroblotted onto nitrocellulose membranes using a Transphor TE 42 electroblotting apparatus (Hoeffer Scientific Instruments) according to Burnette (2). Following electroblotting nitrocellulose membranes were treated with affinity purified rabbit antispinach leaf ADPglucose pyrophosphorylase IgG (primary antibody) and the antigen-antibody complex visualized via treatment with alkaline phosphatase linked goat antirabbit IgG (secondary antibody) followed by histochemical staining with the 5-bromo-4-chloro-3-indoyl phosphate and nitrobluetetrazolium procedure as described (26). Immunological specificity was confirmed by performing Western blots in which rabbit preimmune serum was substituted for affinity purified rabbit antispinach leaf ADPglucose pyrophosphorylase IgG.

For determination of subunit mol wt using SDS-PAGE, a plot of relative mobility *versus* log mol wt was constructed for the standard proteins: β-galactosidase (116 kD), phosphorylase b (97.5 kD), BSA (66 kD), ribulose-1,5-bisP carboxylase (large subunit, 55 kD), ovalbumin (45 kD), and carbonic anhydrase (29 kD).

Native Molecular Mass Estimation. Molecular mass estimations were made on a column of Sephacryl S-300 (1.5 × 46.5 cm; 82 ml resin bed volume) using 1.0 ml sample volumes and 25 mM bis-Tris-propane (pH 7.0) containing 40 mM KCl, 0.5 mM EDTA, and 10% sucrose as the equilibration/elution buffer. Fractions (1.0 ml) were collected and assayed for A_{280} and ADPglucose pyrophosphorylase or rabbit muscle pyruvate kinase activity. The native molecular mass of ADPglucose pyrophosphorylase was determined from a plot of K_{av} (partition coefficient) *versus* log molecular mass for the standard proteins: thyroglobulin (669 kD), ferritin (440 kD), catalase (232 kD), rabbit muscle pyruvate kinase (228 kD), and aldolase (158 kD). Void volume was determined using a 1 mg/ml solution of blue dextran 2000.

Other Procedures. Protein concentration was measured by the method of Smith *et al.* (25) using the Pierce Chemical Co. prepared BCA reagent and BSA as the standard.

Descending paper chromatography on Whatman 1 filter paper was performed with the following solvent system: 95% ethanol: 1 M ammonium acetate (pH 3.8) (5:2) (solvent A).

RESULTS

Purification and Physical Properties. Approximately 99% of the ADPglucose pyrophosphorylase activity found in intact maize seeds is localized in the endosperm (4). Thus, enzyme purification was performed using endosperm dissected free of pericarp and embryo. The use of PMSF and chymostatin in the homogenizing media was important in preventing proteolytic degradation of the enzyme during its extraction and purification (see below). Table I summarizes the purification of ADPglucose pyrophosphorylase from maize endosperm. The enzyme was purified about 90-fold to a final specific activity of 34 unit/mg. When the final preparation was subjected to SDS-PAGE four major protein staining bands were revealed (Fig. 1A, lanes 7 and 8). However, when an SDS gel of the same preparation was analyzed by Western blot hybridization, only the lower protein staining band cross-reacted with the affinity-purified rabbit antispinach leaf ADPglucose pyrophosphorylase IgG (Fig. 1B, lanes 3 and 4). No immunological reactivity of this band was observed when rabbit preimmune sera was substituted for the spinach leaf ADPglucose pyrophosphorylase IgG. The molecular mass of the immunoreactive band was determined to be 54 ± 0.2 kD (n = 4) (Fig. 1). The native molecular mass of the enzyme as estimated by gel filtration of the final preparation of Sephacryl S-300 was found to be 230 ± 7.1 kD (n = 3) (unpublished results).

Neutralization of Enzyme Activity by Antispinach Leaf ADPglucose Pyrophosphorylase Immune Serum. The effect of rabbit antispinach leaf ADPglucose pyrophosphorylase immune sera on the activity of ADPglucose pyrophosphorylase purified from maize endosperm and spinach leaves is shown in Figure 2. Increasing amounts of the spinach leaf ADPglucose pyrophosphorylase antiserum inhibited both ADPglucose pyrophosphorylases. The amount of antiserum required for 50% inhibition was about 46 and 175 μl of antiserum per unit of spinach leaf and maize endosperm enzyme, respectively.

Enzyme Degradation. A crude extract was prepared from whole maize seeds in the absence of added protease inhibitors, incubated at 30°C from 0 to 310 min and subjected to SDS-PAGE. Western blot hybridization of the SDS gel was then performed using affinity-purified rabbit antispinach leaf ADPglucose pyrophosphorylase IgG. At t = 0 min only a single immunoreactive polypeptide of 54 kD was observed (Fig. 3A, lane 1). However, incubation of the crude supernatant at 30°C for 310 min caused the appearance of a 53 kD antigenic peptide (Fig. 3A, lane 2). A variety of protease inhibitors were tested for their ability to prevent this apparent proteolytic degradation of maize endosperm ADPglucose pyrophosphorylase. The following substances had no effect as judged by SDS-PAGE/Western blot hybridization: PABA, TLCK, NEM, PCMB, and benzamidine (all 0.5 mM); leupeptin and pepstatin (10 μg/ml); and 5 mM EDTA. However, the serine protease inhibitors 0.5 mM PMSF and 10 μg/ml chymostatin both prevented any proteolytic degradation of the enzyme following incubation of crude supernatants at 30°C for up to 310 min (Fig. 3A, lanes 3 and 7).

ADPglucose pyrophosphorylase was also partially purified from 50 g of whole maize kernels using the protamine sulfate,

Table I. *Purification of Maize Endosperm ADPglucose Pyrophosphorylase*

Fraction	Volume	Protein	Activity	Specific Activity	Fold Purification	Yield
	ml	*mg*	*units*	*units/mg*		%
Crude supernatant	148	931	357	0.38		100
Polyethylene glycol	21.5	288	519	1.8	4.7	145
DEAE-cellulose	32	43.5	315	7.2	19	88
C_4-butyl agarose	9.5	19.5	236	12.1	32	66
C_2-ethyl agarose	6.8	4.5	151	33.6	88	42

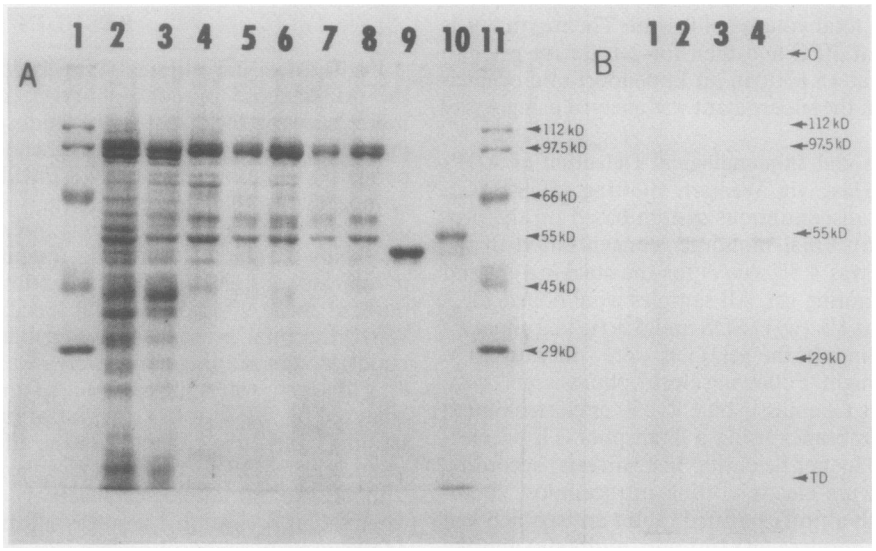

FIG. 1. A, Gel electrophoresis of ADPglucose pyrophosphorylase fractions in the Tris-glycine (pH 8.8) SDS system of Laemmli (16). Lanes 1 + 11 contain 12 μg of the various protein standards described under "Materials and Methods." Lane 2 contains 45 μg of protein of the crude supernatant fraction. Lane 3 contains 60 μg of protein of the PEG fraction. Lane 4 contains 15 μg of protein of the DEAE-cellulose fraction. Lanes 5 + 6 contain 7.5 and 15 μg, respectively, of the C_4-butyl agarose fraction. Lanes 7 + 8 contain 4 and 8 μg, respectively, of the C_2-ethyl agarose fraction. Lane 9 contains 5 μg of purified *E. coli* ADPglucose pyrophosphorylase, whereas lane 10 contains 5 μg of spinach leaf ribulose-1,5-bisP carboxylase. B, Immunological detection of ADPglucose pyrophosphorylase. Samples were subjected to SDS-PAGE and blot transferred to nitrocellulose. The blot was probed using affinity-purified rabbit anti-spinach ADPglucose pyrophosphorylase IgG as described under "Materials and Methods"; phosphatase staining was for 15 min at room temperature. Lane 1 contains 15 μg of protein of the crude supernatant fraction. Lane 2 contains 4 μg of protein of the DEAE-cellulose fraction. Lanes 3 and 4 each contain 2 μg of protein of the C_2-ethyl agarose fraction. O, origin; TD, tracker dye front.

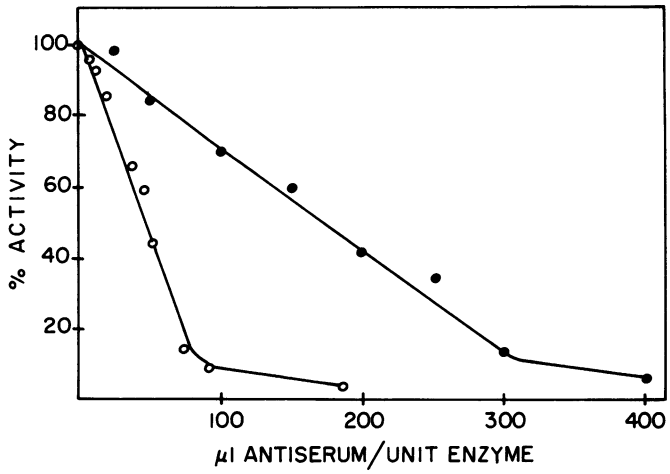

FIG. 2. Neutralization of maize endosperm and spinach leaf ADPglucose pyrophosphorylase by antispinach leaf ADPglucose pyrophosphorylase immune serum. Neutralizations were performed as described under "Materials and Methods." (○), Purified spinach leaf enzyme; (●), purified maize endosperm enzyme. The values for 100% activity (nmol glucose-1-P produced in 1 min) for the reaction mixtures were: spinach leaf enzyme, 45; maize endosperm enzyme, 21.

$(NH_4)_2SO_4$ and DEAE-cellulose procedure of Dickinson and Preiss (4) (no protease inhibitors were used) and the final preparation subjected to SDS-PAGE. Western blot hybridization of the SDS gel revealed two peptides of 54 and 53 kD which cross-reacted with affinity-purified rabbit antispinach leaf ADPglucose pyrophosphorylase IgG (Fig. 3B, lane 3).

The above findings strongly suggest that proteolytic degradation of maize endosperm ADPglucose pyrophosphorylase can occur not only during incubation of crude supernatants at 30°C, but also during the purification of the enzyme at 4°C. Thus, 1.5 mM PMSF and 10 μg/ml chymostatin were routinely added to the homogenization media used during development of the purification scheme described in Table I.

Catalytic Properties of ADPglucose Pyrophosphorylase from Maize Endosperm. Except where indicated, the desalted (Sephadex G-50) pooled peak C_2-ethyl agarose fraction was used in the experiments described below. Kinetic studies were performed using assay B with which the specific activity of the final preparation was approximately 11.2 (μmol ADPglucose synthesized/min)·mg when measured in the presence of saturating 3-P-glycerate. The product synthesized from α-[^{14}C]glucose-1-P and ATP was shown to be ADPglucose by paper chromatography using solvent A. The following enzymes' activities were tested for and not found: adenylate kinase, phosphoglycerate mutase, phosphoglucose isomerase, and phosphoglucomutase. Moreover, addition of 0.04 unit of ADPglucose pyrophosphorylase to the reaction mixture quantitatively converted the glucose-1-P to ADPglucose in 70 min. On continued incubation of the reaction mixture for an additional 70 min, no degradation of the radioactive ADPglucose was observed as determined by paper chromatography. Thus, the enzyme preparation was also free of ADPglucose degrading activity.

Effect of pH. The pH activity profile for the synthesis of ADPglucose was determined in the presence and absence of 1 mM 3-P-glycerate. To optimalize the activity of the unactivated enzyme elevated concentrations of 20 mM Mg^{2+}, 7.5 mM ATP, and 5 mM glucose-1-P were required; maximal enzyme activity was enhanced about 3-fold under these circumstances as compared to that obtained using the conditions which were optimal for the activated enzyme (assay B). With saturating concentrations of substrates and cofactors both the activated and unactivated enzyme showed a typically broad pH optima of about pH 7.4. Unless otherwise noted, all kinetic parameters for the purified enzyme have been determined at pH 7.4.

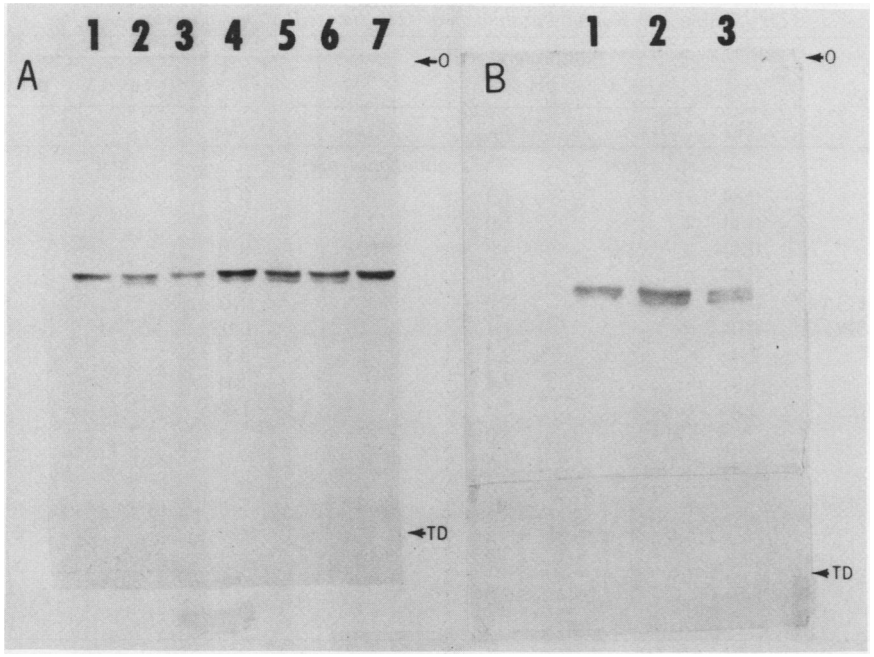

FIG. 3. A, The effect of various protease inhibitors on the subunit structure of ADPglucose pyrophosphorylase following incubations at 30°C of a crude supernatant obtained from whole maize seeds as judged by SDS-PAGE/western blot hybridization. Antigenic peptides were detected using affinity-purified rabbit anti-spinach leaf ADPglucose pyrophosphorylase IgG as described under "Materials and Methods"; phosphatase staining was for 15 min at room temperature. Lanes 1 and 2 each contain 25 μg of protein of a control crude supernatant (no additions) incubated for 0 and 310 min, respectively; lane 3 contains 25 μg of protein of a crude supernatant incubated for 310 min with 0.5 mM PMSF; lane 4 contains 25 μg of protein of a crude supernatant incubated for 310 min with 10 μg/ml leupeptin; lane 5 contains 25 μg of protein of a crude supernatant incubated for 310 min with 0.5 mM benzamidine; lane 6 contains 25 μg of protein of a crude supernatant incubated for 310 min with 0.5 mM NEM; lane 7 contains 25 μg of protein of a crude supernatant incubated for 310 min with 10 μg/ml chymostatin. O, origin; TD, tracker dye front. B, Immunological detection of ADPglucose pyrophosphorylase in various fractions obtained following the partial purification of the enzyme from whole maize kernels according to the procedure of Dickinson and Preiss (5). Samples were subjected to SDS-PAGE and blot transferred to nitrocellulose as described under "Materials and Methods." Antigenic peptides were detected as in 'A;' phosphatase staining was for 15 min at room temperature. Lane 1 contains 40 μg of protein of the crude supernatant reaction; lane 2 contains 30 μg of protein of the dialyzed $(NH_4)_2SO_4$ fraction; lane 3 contains 20 μg of protein of the DEAE-cellulose fraction. O, origin; TD, tracker dye front.

Activator Specificity of ADPglucose Synthesis. A variety of metabolites were tested for their ability to activate ADPglucose synthesis. Table II compares the activation ability of those compounds which were found to affect ADPglucose pyrophosphorylase. As is the case with ADPglucose pyrophosphorylases from all other plant sources examined to date (20), 3-P-glycerate proved to be the best activator, with lesser activation provided by fructose-6-P, glucose-6-P, P-enolpyruvate, and dihydroxyacetone-P. The following compounds had no effect on enzyme activity: glyceraldehyde-3-P, 2-P-glycerate, pyruvate, 2,3-diphosphoglycerate, fructose-1,6-bisP, fructose-2,6-bisP, and glucose-1,6-bisP (all tested at 0.5 and 5 mM). Bovine brain calmodulin (6.25 μM) with or without added Ca^{2+} (15 μM) also had no effect.

Kinetic Parameters of the Synthesis Reaction. Enzyme prepared during the course of the present study using the procedure of Dickinson and Preiss (4) has been shown to be proteolytically degraded (Fig. 3B). Table III compares the various kinetic parameters determined for substrates and effectors of maize endosperm

Table II. *Metabolic Activation of ADPglucose Synthesis Maize Endosperm ADPglucose Pyrophosphorylase*

Reaction mixtures were prepared as outlined in "Materials and Methods" (Assay B) and contained 5×10^{-3} unit of the desalted C_2-ethyl agarose fraction.

Metabolite	Concentration Tested	ADPG Formed	Activation
	mM	*nmol*	*-fold*
None		0.40	
3-Phospho-D-glycerate	0.5	8.25	20.6
	5.0	10.1	25.3
D-Fructose-6-phosphate	0.5	3.1	7.8
	5.0	6.8	17.0
D-Glucose-6-phosphate	0.5	1.4	3.5
	5.0	5.6	14.0
Phosphoenolpyruvate	0.5	0.56	1.4
	5.0	1.6	4.0
Dihydroxyacetonephosphate	0.5	0.34	
	5.0	1.3	3.2

Table III. *Kinetic Parameters of ADPglucose Pyrophosphorylase*

Substrate/Effector	Nonproteolyzed[a] pH 7.4					Proteolyzed[b] pH 6.8				
	$S_{0.5}$	$A_{0.5}$	$I_{0.5}$	n_H	V_{max}	$S_{0.5}$	$A_{0.5}$	$I_{0.5}$	n_H	V_{max}
	mM				μmol/min·mg^{-1}	mM				μmol/min·mg^{-1}
ATP	0.84			1.3	0.4	0.17			1.5	1.9
ATP (+1 mM 3PGA)	0.11			1.0	11.2	ND[c]			ND	ND
ATP (+10 mM 3PGA)	0.11			1.0	10.9	0.10			1.0	3.7
Glucose-1-P	0.67			0.9		0.10			1.0	
Glucose-1-P (+1 mM 3PGA)	0.03			1.0		ND			ND	
Glucose-1-P (+10 mM 3PGA)	0.04			1.1		0.05			1.0	
$MgCl_2$	2.4			2.7		3.1			2.2	
$MgCl_2$ (+1 mM 3PGA)	1.8			2.4		ND			ND	
MgCl2 (+10 mM 3PGA)	2.4			2.3		1.8			2.7	
3PGA		0.12		1.0			2.2		1.0	
3PGA (+1 mM Pi)		1.2		1.5			ND		ND	
3PGA (+2 mM Pi)		ND		ND			4.7		1.2	
3PGA (+6 mM Pi)		ND		ND			12.8		1.3	
3PGA (+8 mM Pi)		3.6		2.6			ND		ND	
Pi									3.0	ND
Pi (+1 mM 3PGA)			0.44	1.0					ND	ND
Pi (+10 mM 3PGA)			9.8	1.2					10.0	ND

[a] All values were determined using Assay B and are the mean of at least 2 separate determinations for the enzyme partially purified from maize endosperm tissue using protocol as described under "Materials and Methods." [b] These values are those reported by Dickinson and Preiss (1969) for the enzyme partially purified from whole maize seeds in the absence of added protease inhibitors. [c] Not determined.

ADPglucose pyrophosphorylase prepared as described in the "Materials and Methods" ('nonproteolyzed') to those previously determined for the same enzyme studied (4). The range of concentrations of substrates, activator, and inhibitor studied were the same in both cases. At pH 6.8 and 7.4 respective $A_{0.5}$ values for 3-P-glycerate of 1.6 and 1.2 mM were obtained for the proteolytic degraded enzyme. These values are thus in the same range as the value of 2.1 mM determined at pH 6.8 which was earlier reported (4) (Table III). It is therefore highly likely that the enzyme studied previously (4) was also proteolytically degraded and is hence referred to in Table III and elsewhere as 'proteolyzed.' The proteolyzed and nonproteolyzed preparations differed most strongly in their response to 3-P-glycerate, with the nonproteolyzed preparation having an $A_{0.5}$ for this activator 18-fold lower than that of the proteolyzed preparation (Table III).

In the presence of saturating concentrations of 3-P-glycerate both enzyme preparations had very similar K_m values for ATP and glucose-1-P (Table III). However, in the absence of added activator the nonproteolyzed enzyme's K_m values for ATP and glucose-1-P were increased approximately 7-fold and 20-fold, respectively, whereas the unactivated proteolyzed enzyme's K_m values for ATP and glucose-1-P each increased only about 2-fold. Besides its effect on K_m values, the presence of saturating 3-P-glycerate also increased the V_{max} values by about 25- to 30-fold for the nonproteolyzed enzyme, but only by 3- to 4-fold for the proteolyzed enzyme (4). Both enzyme preparations had similar $S_{0.5}$ values for Mg^{2+} and showed a markedly sigmoidal binding pattern for this cofactor with or without added activator (Table III).

Modulation of ADPglucose Synthesis Activity by 3-Phosphoglycerate and Phosphate. In the absence of Pi the activation curve with 3-P-glycerate is hyperbolic in shape yielding a Hill plot slope value n_H, of 1.0 (Fig. 4). Pi at 1 and 8 mM increased the $A_{0.5}$ value of 3-P-glycerate 10- and 30-fold, respectively, and changed the shape of the saturation curve from a hyperbolic to a sigmoidal form (Fig. 4) with respective Hill slope coefficients of 1.5 and 2.6 (Table III). The affinity for 3-P-glycerate of the proteolyzed enzyme (4) was less sensitive to increasing concentrations of Pi as 2 and 6 mM Pi increased its $A_{0.5}$ value of 3-P-glycerate only 2- and 6-fold, respectively (Table III). Not surprisingly, the two enzyme preparations differed in their responses to inhibition by Pi. In the absence of added activator the nonproteolyzed enzyme showed little inhibition by Pi concentrations of up to 10 mM (Fig. 5), whereas the proteolyzed preparation showed one-half maximal inhibition by 3 mM Pi (Table III) and was maximally inhibited by over 75% (4). The presence of 1 mM 3-P-glycerate greatly increased the sensitivity of the nonproteolyzed enzyme to inhibition by Pi with one-half maximal inhibition occurring at 0.44 mM Pi and the extent of inhibition increasing to over 90% (Fig. 5; Table III). However, higher concentrations of 3-P-glycerate greatly reduced the inhibition by Pi. With 10 mM 3-P-glycerate both enzyme preparations showed one-half maximal inhibition by approximately 10 mM Pi, and were maximally inhibited to about 50% of the uninhibited activity (Table III; Fig. 5).

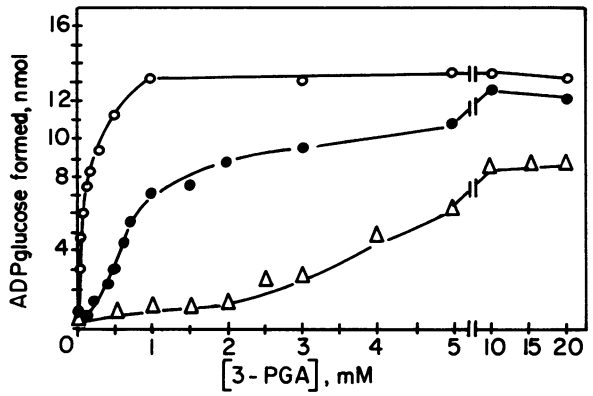

FIG. 4. The activation of maize endosperm ADPglucose pyrophosphorylase by 3-PGA. The synthesis reaction mixture is described under "Materials and Methods" except the concentration of Pi was varied as indicated: none (○); 1 mM Pi (●); 8 mM Pi (△).

DISCUSSION

Electrophoresis of the final preparation in the presence of SDS resulted in four major protein staining bands (Fig. 1A). The

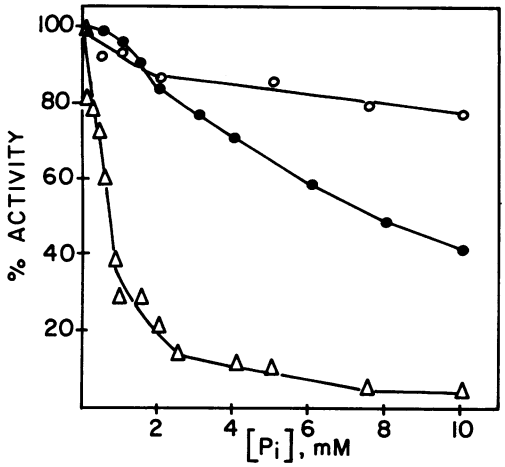

FIG. 5. Phosphate inhibition of maize endosperm ADPglucose pyrophosphorylase. The synthesis reaction mixture is described in "Materials and Methods" except the amount of 3-PGA was varied as indicated: none (○); 1 mM 3-PGA (△); 10 mM 3-PGA (●). One hundred percent activity pertains to the rate of ADPglucose synthesis in the absence of inhibitor. The values for 100% activity (nmol ADPglucose formed in 10 min) for the reaction mixtures were: minus 3-PGA, 0.3; plus 1 mM 3-PGA, 8.6; plus 10 mM 3-PGA, 8.3.

assignment of the faster migrating, 54 kD, protein staining band for ADPglucose pyrophosphorylase is indicated by the immunology experiments. Rabbit antispinach leaf ADPglucose pyrophosphorylase immune serum is able to inhibit the pyrophosphorolysis activity of the final enzyme preparation by up to 90% (Fig. 3). This indicates that polyclonal antibodies raised against a homogenous preparation of spinach leaf ADPglucose pyrophosphorylase do indeed cross-react with ADPglucose pyrophosphorylase from developing maize endosperm and shows that a certain degree of structural homology exists between the spinach and maize enzymes. A Western blot of a SDS gel of our final enzyme preparation revealed a single antigenic peptide which migrated with an apparent molecular mass of about 54 kD when probed using affinity-purified rabbit antispinach leaf ADPglucose pyrophosphorylase IgG (Fig. 1B). Furthermore, mouse antiserum raised against purified potato tuber ADPglucose pyrophosphorylase shows cross-reactivity with the same polypeptide (M Morell, personal communication), whereas no immunological reactivity of this band is seen when rabbit or mouse preimmune sera are substituted for the anti-ADPglucose pyrophosphorylase IgGs. Therefore, it is highly improbable that the subunit molecular mass of maize endosperm ADPglucose pyrophosphorylase is 96 kD, as reported by Fuchs and Smith (6) for an apparently homogenous enzyme preparation, but is actually about 54 kD and, therefore, in the order of that demonstrated for the *E. coli* (50 kD; 10), spinach leaf (44 and 48 kD; 3) and potato tuber (50 kD; 27) enzymes. As is the case with our final enzyme preparation (Fig. 1A, lanes 7 and 8), the final preparation of Fuchs and Smith (6) probably contained a major protein contaminant with a subunit molecular mass of about 96 kD and that possibly by underloading their SDS gel they reached the erroneous conclusions that: (a) they had a homogenous enzyme preparation, and (b) the subunit mol wt of the maize endosperm is 96 kD. That the final specific activity of their enzyme preparation was 8-fold lower than the one reported for the enzyme partially purified during the course of the present study would further suggest that their final preparation was impure.

The maize endosperm enzyme showed a native molecular mass of about 230 kD as determined by gel filtration on Sephacryl S-300, and thus appears to be a tetramer of 4 identical, or very similar subunits. This native molecular mass contradicts the value of about 400 kD reported for the enzyme purified by Fuchs and Smith (6), but is in the same range as the value of about 240 kD reported for the maize endosperm ADPglucose pyrophosphorylase studied by Hannah and Nelson (9). In contrast to the findings of Hannah and Nelson (9) and Fuchs and Smith (6), different aggregation states of the enzyme were not detected during enzyme purification or during the determination of the enzyme's native molecular mass.

The availability of spinach leaf ADPglucose pyrophosphorylase antibodies made it possible to show that proteolytic degradation of maize endosperm ADPglucose pyrophosphorylase can occur during incubation of crude extracts at 30°C (Fig. 3A), or during the partial purification of the enzyme at 4°C (Fig. 3B). Proteolytic degradation was prevented when the serine protease inhibitors PMSF and/or chymostatin were added to the homogenization buffer (Figs. 1B and 3A). Maize endosperm ADPglucose pyrophosphorylase thus joins the ranks of ribulose bisP carboxylase (large subunit) (8) and glutamate synthase (19) as an example of a plastid protein which is susceptible to degradation by a serine protease during its extraction. Based on the above findings it is quite possible that previous workers who have reported on the various *in vitro* properties of developing maize endosperm ADPglucose pyrophosphorylase (4, 6, 9) may have been studying an enzyme partially degraded by protease.

ADPglucose pyrophosphorylase from both photosynthetic and nonphotosynthetic tissues of C_3, C_4, and CAM plants has been shown to be a key regulatory enzyme in the conversion of photosynthate into starch (20–23). All enzymes examined to date are activated by 3-P-glycerate and inhibited by Pi and it is widely accepted that through variations in the ratio of plastidic [3-P-glycerate]/[Pi] the plant cell is able to fine tune the rate of starch synthesis via allosteric modulation of ADPglucose pyrophosphorylase synthetic activity (21, 22). Although the maize endosperm ADPglucose pyrophosphorylase purified and characterized by Dickinson and Preiss (4) shows the usual activation by 3-P-glycerate and inhibition by Pi (Table III), in a kinetic sense it seems to be in a class by itself as its sensitivity to these allosteric regulators is far less than that determined for all other plant ADPglucose pyrophosphorylases studied to date (21). Results of the present study show that these rather unique kinetic properties are a result of the proteolytic degradation which occurs when it is purified from extracts prepared in the absence of added serine protease inhibitors. In most respects the catalytic and regulatory properties of nonproteolytic degraded maize ADPglucose pyrophosphorylase conform to those shown by other plant ADPglucose pyrophosphorylases (20–23).

The significance of activation by 3-P-glycerate and inhibition by Pi of ADPglucose synthesis and its relationship to the regulation of starch synthesis in the amyloplasts of nonphotosynthetic reserve tissues is still unclear. Amyloplasts can in many respects be thought of as nonphotosynthetic chloroplasts, as both organelles share similar morphologies and metabolic functions (13, 14). In leaf tissue, 3-P-glycerate is the primary product of photosynthesis, and is known to be transported out of the chloroplast via the triose-P/Pi translocator (11). A similar mechanism involving the transport of 3-P-glycerate and/or dihydroxyacetone-P across wheat endosperm amyloplasts has been postulated (13). Studies on isolated maize amyloplasts indicate that metabolites (triose-P) formed in the cytosol from imported sucrose via sucrose synthetase and glycolytic reactions probably enter the amyloplast via similar triose-P/Pi translocation mechanisms (18). This would serve to elevate the amyloplastic [3-P-glycerate] /[Pi] ratio, thereby enhancing the rate of ADPglucose synthesis, the primary glucosyl donator for starch biosynthesis found in developing maize seeds (5). The opposite sequence of events would presumably occur during periods of reduced source to sink photosynthate flux.

Of interest is the finding that with 10 mM 3-P-glycerate both proteolyzed and nonproteolyzed maize endosperm ADPglucose pyrophosphorylase have similar K_m values for ATP and glucose-1-P and an identical $I_{0.5}$ for Pi of about 10 mM (Table III). However, in the absence of activator the nonproteolytic degraded enzyme becomes far less active with its K_m values for ATP and glucose-1-P increasing approximately 7- and 20-fold, respectively. In contrast, the unactivated proteolytically degraded enzymes' K_m values for these substrates were elevated only by about 2-fold (Table III). These results suggest that the approximate 1000 D peptide which is proteolytically clipped from the enzyme during its purification from extracts prepared in the absence of added serine protease inhibitors may play a key role in maintaining the unactivated enzyme in a catalytically 'inactive' conformation. Comparison of the primary, secondary, and tertiary structures of the nondegraded *versus* proteolytically degraded enzyme awaits their respective purifications to homogeneity and may provide useful insights as to the relationship between the structure and the function of this key enzyme of starch metabolism.

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