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THE PURIFICATION AND CHARACTERIZATION OF ADP-GLUCOSE PYROPHOSPHORYLASE *A* FROM DEVELOPING MAIZE SEEDS *

ROY L. FUCHS ** and JAMES D. SMITH

Genetics Section, Plant Sciences Department, Texas A&M University, College Station, TX, 77843 (U.S.A.)

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Summary

ADPglucose pyrophosphorylase *A* (ATP:α-D-glucose-1-phosphate adenylyltransferase, EC 2.7.7.27) from developing maize (*Zea mays*) endosperm was purified 129 fold to apparent homogeneity. The molecular weight estimated by gel filtration and by polyacrylamide gel electrophoresis was 375 000 and 400 000, respectively. The preparation gave a single protein band after SDS-polyacrylamide gel electrophoresis suggesting a monomer mol. wt. of 96 000. It was concluded that ADPglucose pyrophosphorylase *A* in maize endosperm is a tetramer of four similar molecular weight subunits. Values for the K_m for glucose 1-phosphate and ATP were $3.8 \cdot 10^{-5}$ and $1.8 \cdot 10^{-4}$ M, respectively (using the homogeneous preparation).

Introduction

ADPglucose pyrophosphorylase (ATP:α-D-glucose-1-phosphate adenylyltransferase, EC 2.7.7.27) are important because of their possible role in the regulation of starch biosynthesis. These enzymes are activated by photosynthetic intermediates (i.e., fructose 1,6-diphosphate, fructose 6-phosphate, 3-phosphoglycerate, etc.) and inhibited by orthophosphate and ADP [1—5].

Recently, Hannah and Nelson [6] separated two forms of ADPglucose pyrophosphorylase from maize endosperm by protamine sulfate fractionation. The protamine sulfate soluble enzyme was labeled ADPglucose pyrophosphorylase *A* (no molecular weight was reported) and the protamine sulfate-precipitable enzyme ADPglucose pyrophosphorylase *B* (M_r = 237 000 and 253 000). Parti-

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** Present address: Charles F. Kettering Research Laboratory, 150 East South College Street, Yellow Springs, Ohio 45387, U.S.A.

ally purified preparations of both showed very similar kinetics. These authors [7] provided kinetic and genetic evidence to support the thesis that both enzymes were products of the same loci. Additionally, evidence was given that the *sh*-2 and *bt*-2 loci (*sh*-2 and *bt*-2 are starch deficient mutants) were the structural loci for the ADPglucose pyrophosphorylases.

We report the purification of ADPglucose pyrophosphorylase *A* to homogeneity, and the determination of some of the physical and biochemical characteristics of the purified enzyme.

Materials and Methods

Materials. Seeds of field-grown starchy maize (*Zea mays* varieties TX 40 and TX 601) were harvested 19 days post-pollination and stored at −20°C until use.

Adenosine diphosphate[U-^{14}C]glucose, α-D-[U-^{14}C]glucose 1-phosphate and omnifluor were purchased from New England Nuclear (Boston, Mass., U.S.A.). LKB, (Durham, N.C., U.S.A.) was the source for Ultrogel ACA 22 and ampholine carrier ampholytes (pH range 5—8). DEAE-cellulose (preswollen grade DE-52) and DE-81 paper were obtained from Whatman (Clifton, N.J., U.S.A.). Sigma Chemical Co. (St. Louis, Mo., U.S.A.) supplied the alkaline phosphatase, inorganic pyrophosphatase, apoferritin, lactate dehydrogenase, β-amylase, thyroglobulin and protamine sulfate. Glutamate dehydrogenase and catalase were obtained from Calbiochem (La Jolla, Calif., U.S.A.). Myosin, *c*-protein, α-actinin, M_α and M_β, prepared according to Rhome [8], were generous gifts of L.D. Yates (Texas A&M University).

Enzyme assay. ADPglucose synthesis was assayed by the method of Hannah and Nelson [6]. The reaction mixture contained 2 mM HEPES buffer (pH 8.0), 2 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM [U-^{14}C]glucose 1-phosphate ($3—4 \cdot 10^5$ cpm/mol), 50 μg bovine serum albumin, 2 μmol 3-phosphoglycerate, 1 unit inorganic pyrophosphatase activity, and enzyme with a total vol. of 0.2 ml. The control assays were run without ATP or with a boiled enzyme preparation. All reactions were initiated with the addition of enzyme, and run at 37°C for 30 min for the purification steps, or for 5 min for kinetic studies. The reaction was terminated by placing the reaction mixture in a boiling water bath for 45 s. Alkaline phosphatase (0.03 mg) was added to the cooled reaction mixture to cleave the phosphate from the remaining glucose-1-*P*. Separation of the ADPglucose and glucose followed the method of Hannah and Nelson [6]. Labelled ADPglucose and glucose-1-*P* were incubated with boiled enzyme to determine the percent of ADP-glucose retained (77%) and the percent of glucose-1-*P* eluted (99.8%) with the above system. One unit of enzyme activity was defined as the amount of enzyme required to synthesize 1 μmol ADPglucose.

Protein estimation. Protein was estimated by the method of Lowry et al. [10] with crystalline bovine serum albumin as standard. For monitoring column chromatography, protein was estimated by absorbance at 280 nm.

Enzyme purification. All procedures were carried out at 0—4°C. 50 g whole seed from TX 40 were ground in a mortar and pestle with 50 ml 30 mM potassium phosphate buffer, (pH 7.0), 0.1 mM mercaptoethanol, 0.5 mM EDTA, 5 mM $MgCl_2$. The extract was filtered through two layers of cheesecloth and then centrifuged 39 000 × *g* for 20 min.

For separation of ADPglucose pyrophosphorylase *A* and *B*, 3/10 vol. 1% protamine sulfate in H_2O was slowly added to the supernatant fraction, stirred 15 min in an ice bath and then centrifuged at 23 000 × *g* for 20 min. The resulting supernatant fraction contained ADPglucose pyrophosphorylase A, while ADPglucose pyrophosphorylase *B* was precipitated by the protamine sulfate.

ADPglucose pyrophosphorylase *A* was precipitated at 30—45% $(NH_4)_2SO_4$ saturation, recovered by centrifugation at 13 300 × *g*, suspended and dialyzed against two changes of 10 mM potassium phosphate buffer (pH 7.0), 0.1 mM mercaptoethanol, 0.5 mM EDTA, 5 mM $MgCl_2$.

The dialyzed preparation was adsorbed onto a DEAE-cellulose column (6 × 1.3 cm), and the column eluted with 30 and 50 mM potassium phosphate buffer, followed by a 100 ml linear gradient of 0—0.2 M KCl in 50 mM phosphate buffer (2.5-ml fractions). Active fractions of ADPglucose pyrophosphorylase were pooled and dialyzed against 10 mM potassium phosphate buffer.

This preparation was added to a 100 ml LKB 8101 isoelectric focusing column in a sucrose gradient as described in the LKB instruction manual. 2.5-ml fractions were collected and the pH of each determined immediately. After the column was completely eluted, the fractions were assayed for ADPglucose pyrophosphorylase without further preparation. Neither the sucrose nor the ampholines affected enzymatic activity. Ampholines were removed by the method of Vesterberg [11].

For the preparation of ADPglucose pyrophosphorylase *B*, the protamine sulfate precipitate was suspended in 30 mM potassium phosphate, (pH 7.0), precipitated between 23—43% $(NH_4)_2SO_4$ saturation, recovered by centrifugation and dialyzed overnight against this buffer. The dialyzed material served as the source of ADPglucose pyrophosphorylase *B* for the molecular weight and isoelectric point estimation.

Polyacrylamide gel electrophoresis. The method of Davis [12] was used to follow the purification. Gels were stained with either Coomassie Brilliant Blue R-250 or Amido black. The activity stain of Weaver et al. [13] was used to locate enzyme activity in the gels. Gels were stained for lipoprotein by the methods of Ressler et al. [14] and Smithies [15]. The periodic acid fuchsin sulfite staining method of Zaccharius et al. [16] was used to visualize glycoproteins.

Molecular weight estimations. Molecular weight estimation of the homogeneous ADPglucose pyrophosphorylase *A* was done electrophoretically in polyacrylamide gels, according to Hedrick and Smith [17]. Protein standards were lactate dehydrogenase (M_r = 135 000), β-amylase (M_r = 215 000), catalase (M_r = 240 000), and apoferritin, monomer (M_r = 450 000). All standards were initially checked for protein homogeneity with Amido black stain on polyacrylamide gels.

Ultrogel AcA 22, was used for a second estimate of the molecular weight of ADPglucose pyrophosphorylase *A* and an initial estimate for ADPglucose pyrophosphorylase *B*. The column (1.5 × 91.6 cm) was prepared as described in the LKB instruction manual. The gel was poured (bed volume 162 ml) and equilibrated with 50 mM sodium phosphate (pH 7.0) buffer. The absorbance at 215

nm was used to monitor elution of the purified enzyme and the ADPglucose pyrophosphorylase assay was used to confirm these results. Protein standards were catalase, apoferritin, glutamate dehydrogenase (M_r = 320 000), and thyroglobulin (M_r = 670 000).

Estimates of subunit molecular weight were obtained by SDS-polyacrylamide gel electrophoresis [18], following subunit dissociation of the homogeneous ADPglucose pyrophosphorylase *A* [19]. Protein standards were: myosin (M_r = 200 000), M_α (M_r = 165 000), M_β (M_r = 155 000), *c*-protein (M_r = 140 000) and α-actinin (M_r = 95 000).

K_m determinations. Initial velocities were determined for 15 concentrations of glucose-1-*P* and ATP. Each substrate was varied independently of the other while all other reaction components remained unchanged. K_m estimates were obtained in the presence of 3-phosphoglycerate. The method of least-squares fit of Lineweaver-Burk plots was used to calculate the K_m for each substrate.

Results

Assay

Inorganic pyrophosphatase has been used as a reaction component for assaying ADPglucose pyrophosphorylases in bacterial [20—23] and plant leaf enzyme [24,25] system but not in the plant endosperm system [1,2,6,7,26]. Linear relationships of activity vs. time, with different reaction rates, resulted when endosperm ADPglucose pyrophosphorylase was assayed with an without pyrophosphatase. These differences could result from the inhibition of ADPglucose pyrophosphorylase activity by the accumulated pyrophosphate. Amir and Cherry [26], reported 1 mM quantities of pyrophosphate completely inhibit enzyme activity. However, a more probable explanation is that pyrophosphatase prevented the back reaction (pyrophosphate + ADPglucose → ATP + glucose-1-*P*) which was shown to be thermodinamically favored (in vitro) over the forward reaction [26]. Thus, inorganic pyrophosphatase was routinely incorporated in our standard assay.

Purification of ADPglucose pyrophosphorylase A

Typical purification results are shown in Table I. The enzyme was purified 129-fold relative to the dialyzed crude extract. The yield was 17.7% of the suspended 45% $(NH_4)_2SO_4$ precipitate step. Both ADPglucose pyrophosphorylase *A* and *B* were present in the crude extract, as evidenced by the two ADPglucose pyrophosphorylase bands visualized on polyacrylamide gel electrophoresis. In contrast to the finding by Hannah and Nelson [6], aging of buffer had no significant effect on the two enzymatic forms. Apparent enzymatic activity in the crude extract was low, probably due to interferring reactions and/or the presence of inhibitors. The ADPglucose pyrophosphorylase *A* fraction recovered after continued purification by $(NH_4)_2SO_4$ precipitation, had more than 2.8 times the total enzyme activity of the crude supernatant fraction. Therefore, reference to percent yield is based on the $(NH_4)_2SO_4$ step of purification.

The protamine sulfate fractionation effectively separated ADPglucose pyrophosphorylase *A* from B. Each contributed approximately equally to the activ-

TABLE I

PURIFICATION OF ADP-GLUCOSE PYROPHOSPHORYLASE *A* FROM MAIZE

Fraction	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield * (%)	Purification (-fold)
Crude extract (dialyzed)	60	31.9	96.0	0.332		1
Protamine sulfate supernatant	64	37.6	64.1	0.59		1.8
Protamine sulfate precipitate	32	48.2	43.4	1.11		
$(NH_4)_2SO_4$ precipitate	7.8	91.0	27.3	3.33	100.0	10.0
DEAE-cellulose	89.0	71.6	3.65	19.78	78.7	59.6
Isoelectric ** focusing	15.0	20.2	.38	42.90	17.7	129.2

* Due to the presence of both ADPglucose pyrophosphorylase *A* and *B* and interfering reactions in the crude supernatant, the fraction from the $(NH_4)_2SO_4$ step was used as the basis of enzyme yield.

** Corrected for the actual amount of DEAE-cellulose eluant applied to the isoelectric focusing column (82%).

ity of the crude preparation. Only the ADPglucose pyrophosphorylase *A* form was purified to homogeneity.

Elution of ADPglucose pyrophosphorylase *A* from DEAE-cellulose produced the elution pattern shown in Fig. 1. Maximum activity was found in fraction

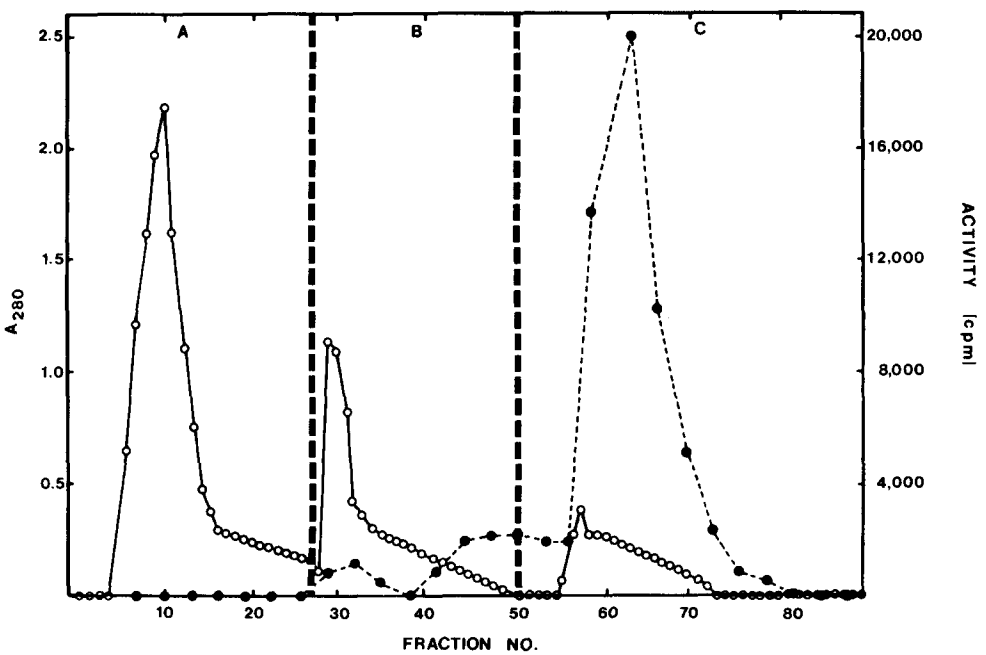

Fig. 1. DEAE-cellulose elution profile of ADPglucose pyrophosphorylase *A*. The dialyzed $(NH_4)_2SO_4$ precipitated preparation was absorbed and washed with 30 mM potassium phosphate buffer (A), followed by 50 mM potassium phosphate (B), and eluted with a 100 ml 0—0.2 M KCl linear gradient in 50 mM potassium phosphate (C). Protein was monitored by absorbance at 280 nm (———) and activity was measured by radioactivity assay (- - - - - -).

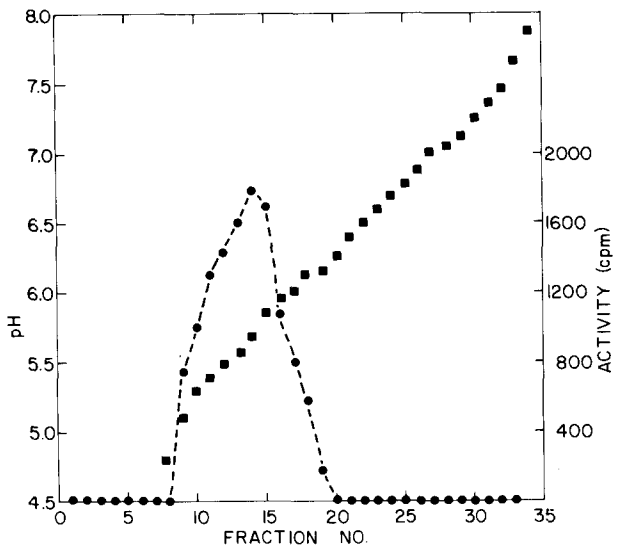

Fig. 2. Isoelectric focusing profile of ADPglucose pyrophosphorylase *A*. Eluates from the DEAE-cellulose column were combined and incorporated into a 110 ml isoelectric focusing column and run at 0—4°C for 42—45 h. A linear pH gradient (pH 5.3—7.5) was established with a maximum voltage of 700 V. 2-ml fractions were collected. Activity (●) was measured by the radioactive assay and pH (■) was measured with a glass electrode immediately after collecting and mixing the eluates.

No. 48 with a purification of nearly 6 fold over the previous step.

Numerous minor protein contaminants remained in the preparation after DEAE-cellulose chromatography, as indicated by polyacrylamide gel electrophoresis. These contaminants were removed by isoelectric focusing as shown in Fig. 2. A linear pH gradient was formed between pH 5.3 and 7.5 and ADPglucose pyrophosphorylase *A* migrated to its isoelectric point of 5.60. (The average isoelectric point determined from four different isoelectric focusings was 5.59.) Aliquots from the tubes with the highest specific activity (11—15) showed a single protein band after polyacrylamide gel electrophoresis. Weaver's [13] stain showed that the band had ADPglucose pyrophosphorylase activity. Electrophoretic gels were overloaded with sample and stained with Amido black to show that no minor protein contaminants remained in the focused preparation. Only a single band was visualized when the focused preparation was run at five different acrylamide gel concentrations and at two different pH values, indicating the enzyme had been purified to homogeneity. The specific activity of this preparation was 42.9 μmol/30 min per mg protein, which was low compared to the specific activities of the other ADPglucose pyrophosphorylases that have been purified to homogeneity; *Escherichia coli* [27] *Rhodospirillum rubrum* [24], and *Spinacia oleracea* [28] with specific activities of 105, 54.5, and 93.5 μmol/min per mg protein, respectively. The relatively low specific activity might be explained if the enzyme was partially denatured or inactivated by isoelectric focusing; this is consistent with the low yield obtained. Also, the contamination of the focused sample with ampholytes caused by incomplete removal of the ampholines by the method used probably resulted in an increased estimated protein concentration and thus reduced estimated specific activity.

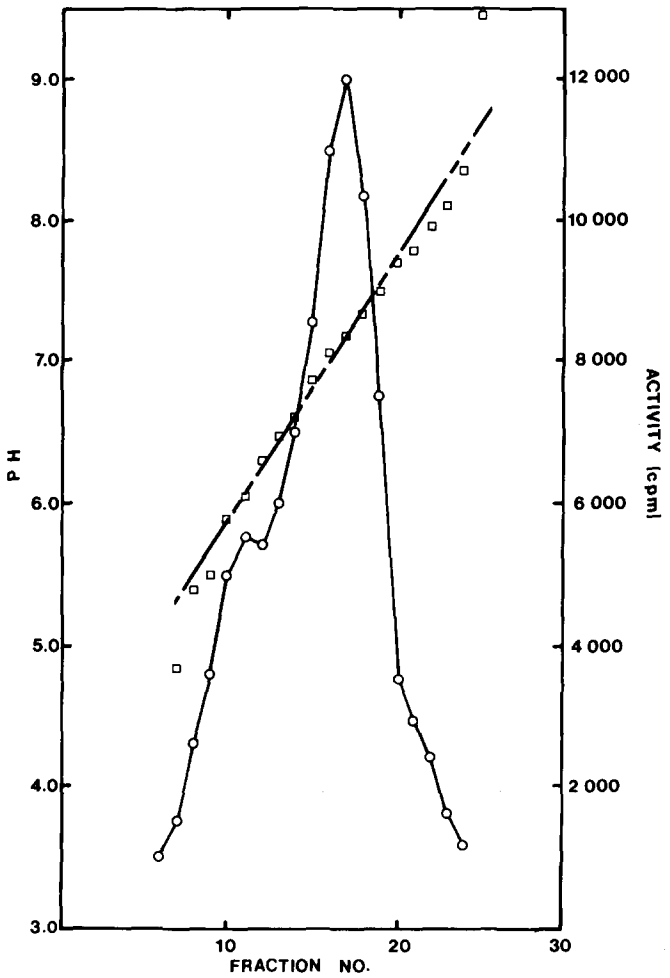

Fig. 3. Isoelectric focusing profile of ADPglucose pyrophosphorylase *B*. Resuspended, dialyzed protamine sulfate precipitate was applied to a pH 5—8 110 ml linear gradient. 2-ml fractions were collected. Activity (○○○) was measured by the radioactive assay and the pH (□□□) was measured immediately after collecting and mixing the eluants.

The dialyzed protamine sulfate precipitate which contained ADPglucose pyrophosphorylase *B* was applied to the pH 5—8 isoelectric focusing gradient and the isoelectric point was estimated at pH 7.02 as seen by the gradient profile in Fig. 3. A partial contamination by ADPglucose pyrophosphorylase *A* was evident, causing a second smaller peak in the lower pH range.

Molecular weight estimations

The molecular weight of ADPglucose pyrophosphorylase *A* was estimated at 400 000 on polyacrylamide gels and 375 000 by gel filtration. ADPglucose pyrophosphorylase *B* was also run on Ultrogel and a mol. wt. of 235 000 was estimated. This is in good agreement with the earlier values estimated by Hannah and Nelson [6].

The subunit molecular weight of ADPglucose pyrophosphorylase *A* was estimated at 96 000 by SDS-polyacrylamide gel electrophoresis.

Kinetic properties

Lineweaver-Burk plots for homogeneous ADPglucose pyrophosphorylase *A*, (spec. act. 42.9 units/mg) provided K_m estimates for ATP ($1.8 \cdot 10^{-4}$ M) and glucose-1-*P* ($3.8 \cdot 10^{-5}$ M). Our value for glucose-1-*P* agrees closely with those determined previously for maize endosperm: $6.0 \cdot 10^{-5}$ M [7], $4.0 \cdot 10^{-5}$ M [6], and $5.0 \cdot 10^{-5}$ M [1]. The K_m for ATP agrees with the estimate ($1.1—1.7 \cdot 10^{-4}$ M) of Dickinson and Preiss [1].

Discussion

ADPglucose pyrophosphorylase *A* was purified 129 fold, resulting in an enzyme preparation that was homogeneous on polyacrylamide gel electrophoresis.

The mol. wt. estimates of 375 000—400 000 are the highest yet recorded for an ADPglucose pyrophosphorylase. Ribereau-Gayon and Preiss [28] did report the presence of a faint band on polyacrylamide gel electrophoresis in a preparation of spinach leaf ADPglucose pyrophosphorylase corresponding to a mol. wt. of 453 000, but the major protein had a mol. wt. of 195 000—240 000. Other mol. wt. estimates range from 190 000 to 250 000; *Rs. rubrum*, 225 000—245 000 [24]; maize endosperm 237 000—253 000 [7]; *E. coli*, 204 000—210 000 [29]; *Serratia marcescens*, 186 000 with a second enzyme with a mol. wt. of 96 000 [22] and *E. coli* 210 000 [27].

Previously the subunit structure (number and molecular weight) had only been reported in *E. coli* [27,29]. These studies estimated the mol. wt. of the subunits as 47 000 and 53 000 [29] and 51 000 [27]. The authors concluded the holoenzyme was a tetramer with four identical subunuts. Subunit determinations made in our study using SDS disc gel electrophoresis showed a single protein band with a mol. wt. of 96 000. Thus, it was concluded that ADPglucose pyrophosphorylase *A* in maize endosperm existed as a tetramer with four subunits of identical or very similar molecular weights.

ADPglucose pyrophosphorylase *B* from maize endosperm had an estimated mol. wt. of 237 000 and 253 000 [6] and our results show 235 000. Based on the following considerations, the ADPglucose pyrophosphorylase *B* appears to be a dimer with two nearly identical subunits: (1) ADPglucose pyrophosphorylase *A* and *B* represent different aggregation states of a common protein and not different molecular species [6,7]; (2) *bt*-2 and *sh*-2 are probably structural genes coding for ADPglucose pyrophosphorylases in maize endosperm [6] and (3) genetic data indicate that normal subunits of both the *bt*-2 and *sh*-2 loci are required for both ADPglucose pyrophosphorylase *A* and *B* [2,6]. The molecular weight of the dimeric form might be larger than expected based on the tetrameric molecular weight and subunit molecular weight. Partially purified ADPglucose pyrophosphorylase *B* was subjected to electrophoresis and then stained with lipoprotein and glycoprotein stains to determine if a lipid or carbohydrate was attached to the protein; results were negative. No smaller molecular weight forms (dimers or monomers) were generated from the homo-

geneous preparation of ADPglucose pyrophosphorylase *A* stored at 0—4°C for more than a month, as determined electrophoretically. This suggests the tetrameric state of ADPglucose pyrophosphorylase was a stable enzyme form under the conditions used and that no spontaneous equilibrium existed between the tetramer and dimer (ADPglucose pyrophosphorylase *A* and *B*) during storage.

The evidence presented here that the ADPglucose pyrophosphorylase *A* corresponds to a tetramer and ADPglucose pyrophosphorylase *B* to a dimer in maize endosperm fits well with the suggestion of Hannah and Nelson [6] that *sh*-2 and *bt*-2 are structural mutants for the ADPglucose pyrophosphorylases. This would indicate that ADPglucose pyrophosphorylase *B* consists of one polypeptide encoded by each structural gene with a total of two polypeptides, similar enough in molecular weight that separation by SDS-polyacrylamide gel electrophoresis was not possible. ADPglucose pyrophosphorylase *A* would be the aggregation of two polypeptides from each structural gene with a total of four polypeptides.

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