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The Subunit Structure of Potato Tuber ADPglucose Pyrophosphorylase[1]

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ABSTRACT

ADPglucose pyrophosphorylase has been extensively purified from potato (*Solanum tuberosum* L.) tuber tissue to study its structure. By employing a modified published procedure (JR Sowokinos, J Preiss [1982] Plant Physiol 69: 1459–1466) together with Mono Q chromatography, a near homogeneous enzyme preparation was obtained with substantial improvement in enzyme yield and specific activity. In single dimensional sodium dodecyl sulfate polyacrylamide gels, the enzyme migrated as a single polypeptide band with a mobility of about 50,000 daltons. Analysis by two-dimensional polyacrylamide gel electrophoresis, however, revealed the presence of two types of subunits which could be distinguished by their slight differences in net charge and molecular weight. The smaller potato tuber subunit was recognized by antiserum prepared against the smaller spinach leaf 51 kilodalton ADPglucose pyrophosphorylase subunit. In contrast, the anti-54 kilodalton raised against the spinach leaf subunit did not significantly react to the tuber enzyme subunits. The results are consistent with the hypothesis that the potato tuber ADPglucose pyrophosphorylase is not composed of a simple homotetramer as previously suggested, but is a product of two separate and distinct subunits as observed for the spinach leaf and maize enzymes.

ADPglucose pyrophosphorylase (ATP: α-glucose-1-P adenylyl-transferase, EC 2.7.7.27) catalyzes an important regulatory step in the biosynthesis of starch and glycogen in plants and bacteria, respectively (2, 16–20). This enzyme mediates the synthesis of ADPglucose and PPi from ATP and glucose-1-P; the product, ADPglucose, serving as the glucosyl donor in α-glucan synthesis. Both the plant and bacterial enzymes are subject to allosteric regulation by small effector molecules which accumulate during normal carbon metabolism in these organisms (17–20). Plant ADPglucose pyrophosphorylases are activated by 3PGA[4] and inhibited by Pi (1, 2, 11, 15, 23). Studies using intact leaf and isolated chloroplast systems support the role of the ratio of 3PGA:Pi in regulating starch biosynthesis via the modulation of ADPglucose pyrophosporylase activity (4, 5, 22).

In *Escherichia coli* and *Salmonella typhimurium*, ADPglucose pyrophosphorylase is encoded by a single gene locus which gives rise to a homotetramer with native and subunit mol wt of 200,000 and 48,000, respectively (19). Recent studies (1, 11, 20) have shown that the leaf enzyme from higher plants displays much more structural complexity than those present in bacterial cells. The homogeneous purified spinach leaf enzyme possesses a mol wt of 206,000 which resolves into two dissimilar size subunits of 54,000 and 51,000 in denaturing SDS-PAGE (1, 11). These spinach leaf subunits are further distinguished by their unique N-terminal amino acid sequences, tryptic peptide maps, and immunological reactivity to different antibodies (11). Western blot studies indicate that dissimilar size ADPglucose pyrophosphorylase subunits are present in *Arabidopsis* (9), wheat, maize, and rice leaf tissue (6). A recombinant cDNA clone for the 51 kD spinach leaf subunit has been isolated and displays significant homology to the bacterial enzyme (20). Moreover, two independently segregating defective leaf ADPglucose pyrophosphorylase mutants, one completely lacking the 54 kD subunit, have been isolated from *Arabidopsis* (8, 9). The overall biochemical and genetic evidence is consistent with the view that the leaf ADPglucose pyrophosphorylase is encoded by two distinct genes (9, 11, 20).

In contrast to the leaf enzyme, the ADPglucose pyrophosphorylases from nonphotosynthetic tissue appeared to be a simple homotetramer as observed for the bacterial enzymes. The potato tuber enzyme has been extensively purified and observed to have a native mol wt of about 200,000 with subunits of 50,000 (23). Comparable biochemical observations have been obtained for ADPglucose pyrophosphorylase from maize endosperm tissue (15). Recent results (18, 21), however, suggest that the maize endosperm ADPglucose pyrophosphorylase is also composed of two distinct polypeptides of 54 and 60 kD. As shown first for the spinach leaf

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[4] Abbreviations: 3PGA, 3-phosphoglycerate; IgG, immunoglobulin G; 2-D, two-dimensional.

ADPglucose pyrophosphorylase (2), both the potato tuber (23) and maize endosperm (15) enzymes are regulated by 3PGA and Pi. In light of the similar catalytic and regulatory properties displayed by the ADPglucose pyrophosphorylases in leaf and nonphotosynthetic tissues, the subunit structure of the potato tuber enzyme was reexamined. Here, we report that a near homogeneously purified preparation of tuber ADPglucose pyrophosphorylase resolves into two immunologically distinct subunits on 2-D polyacrylamide gels. The smaller tuber subunit displays homology to the 51 kD spinach subunit, whereas the larger tuber subunits are less similar to the spinach leaf subunit based on immunological reactivity to antibodies.

MATERIALS AND METHODS

Reagents

[^{32}P]PPi and ^{125}I-protein A were obtained from New England Nuclear. Amino-propyl agarose, DEAE-Sephacel, Pharmalytes pH 3–10 and pH 5–8, and ADPglucose were purchased from Sigma Chemical Co. Mono Q HR 5/5 and Superose 12 HR 10/30 were from Pharmacia. Nitrocellulose filters (0.45 μm) were obtained from Schleicher and Schuell. Adjuvant used for antibody production was from RIBI Immunochem Research, Inc. All other reagents used in this study were of the highest quality available.

Plant Material

The potato variety Norchip (*Solanum tuberosum* L.) was used as a source of enzyme. Acetone powders of developing tuber tissue were prepared as previously described (23).

Assay of ADPglucose Pyrophosphorylase

The pyrophosphorylysis reaction of ADPglucose was monitored by the production of [^{32}P]ATP in the presence of [^{32}P]PPi (11). The reaction mixture contained 20 μmol glycylglycine buffer, pH 8.0, 1.25 μmol $MgCl_2$, 0.75 μmol DTT, 2.5 μmol NaF, 0.5 μmol ADPglucose, 0.38 μmol [^{32}P]PPi (1.0 to 5.0×10^6 cpm/μmol), 50 μg BSA, and 2 μmol of 3PGA in a total volume of 0.25 ml. The reaction was incubated for 10 min at 37 °C and then terminated by the addition of 3 mL of cold 5% TCA followed by the addition of 0.1 ml of 15% activated charcoal to adsorb the [^{32}P]ATP. After 10 min on ice the charcoal was collected by low speed centrifugation, washed twice with 5% TCA, and once with water. The charcoal was resuspended in 1 ml of water and the amount of radioactivity quantified by liquid scintillation counting. Counting efficiency for ^{32}P under these conditions was 15%. A unit of ADPglucose pyrophosphorylase activity is defined as that amount of enzyme which catalyzes the synthesis of 1 μmol of ATP/min.

Purification of Tuber ADPglucose Pyrophosphorylase

The tuber ADPglucose pyrophosphorylase was purified using a modified procedure developed by Sowokinos and Preiss (23). One hundred thirty-two g of acetone powder, prepared from about 600 g of fresh tubers, were slowly mixed with 1.32 L of extraction buffer. The extraction buffer was the same as previously described (23) except that it contained, in addition, 0.03% sodium metabisulfite, 1 μg/mL of leupeptin, 0.5 μg/mL pepstatin, and 0.1 mM PMSF. The extract was then heat treated and subjected to ammonium sulfate precipitation as described earlier (23). After dialysis in 10 mM K^+ phosphate (pH 7.4), 20% sucrose, 0.1 mM PMSF, and 1 mM glutathione for 12 h at 4 C, the sample was fractionated on a 50 mL DEAE-Sephacel column using a 500 mL linear buffer gradient as described by Sowokinos and Preiss (23). Active enzyme fractions were pooled, treated with 1 μg/mL leupeptin and 0.5 μg/mL pepstatin, and concentrated to about 7 mL using an Amicon PM30 membrane. The concentrated enzyme fraction was then passed through a 5 mL amino-propyl agarose column and fractionated as described previously (23). Active enzyme fractions were pooled, concentrated to about 3 mL, and then fractionated on a Mono Q column. Peak fractions were pooled and stored on ice.

PAGE

Native enzyme was analyzed by the Ornstein-Davis buffer system and assayed for enzyme activity as previously described (23). SDS-PAGE was performed according to Laemmli (7) on 10% polyacrylamide slab gels. Protein standards utilized in this study were α-lactalbumin (14,200), trypsin inhibitor (20,100), trypsinogen (24,000), carbonic anhydrase (29,000), glyceraldehyde-3-P dehydrogenase (36,000), egg albumin (45,000), and bovine albumin (68,000). Equilibrium and nonequilibrium high resolution 2-D PAGE were conducted as outlined by O'Farrell (12) and Phillips (14), respectively. Polyacrylamide gels were treated with Coomassie brilliant blue R-250 as described previously (13).

Anti-ADPglucose Pyrophosphorylase Immunoglobulin Preparation

The highly purified ADPglucose pyrophosphorylase from the Mono Q chromatography step was centrifuged at 12,000g to remove any particulate matter, and the supernatant fluid was dialyzed overnight against 10 mM sodium phosphate, 100 mM NaCl (pH 7.0). The protein preparation was mixed with adjuvant to a concentration of 75 μg/mL and injected into New Zealand rabbits according to the adjuvant manufacturer's recommendations (RIBI Immunochem Research, Inc.). Immunization boosts were applied at 3-week intervals. The rabbits were bled after the third injection and observed to have ample antibodies reactive toward ADPglucose pyrophosphorylase. The IgG fraction was purified by ammonium sulfate precipitation, and passage in succession through DEAE-Sephadex and protein A columns.

Western Blotting

Proteins resolved by PAGE were transferred to nitrocellulose, incubated in succession with antipyrophosphorylase and ^{125}I-protein A, and visualized by autoradiography as described previously (13).

Table I. *Purification of ADPglucose Pyrophosphorylase*

Fraction	Volume	Protein	Activity	Specific Activity	Purification	Recovery
	mL	*mg*	*units*	*units/mg*	*-fold*	%
Crude	1500	3750	380	0.10		100
Heat	1380	2939	357	0.12	1.2	93
$(NH_4)_2SO_4$	47	470	188.3	0.74	7.4	49.5
DEAE	205	71.8	143	2.0	20	37
C3	107	8.97	101.2	11.3	113	27
Mono Q	1.65	0.355	20.2	56.9	569	5.3

RESULTS AND DISCUSSION

Purification of Tuber ADPglucose Pyrophosphorylase

Table I summarizes the fractionation steps employed in the purification of the potato tuber ADPglucose pyrophosphorylase. The purification protocol of Sowokinos and Preiss (23) was followed through the amino-propyl agarose (C_3-agarose) chromatography step, although somewhat higher enzyme yields and substantially higher enzyme activity were obtained in this study. We attribute the more active enzyme preparations, particularly at the early purification steps, to two factors. First, dialysis of the protein fractions during the course of the enzyme purification was kept to a minimum. The resuspended ammonium sulfate precipitate was dialyzed for about 12 h instead of the previously prescribed 20 h (23). Moreover, the dialysis step after the DEAE-Sephacel chromatography step was omitted, as the pooled fractions were concentrated with a Amicon PM30 membrane to about 6 mL and diluted with an equal volume of 2 M K-phosphate (pH 7.0) before application to the C_3-agarose column. Second, a Superflo-50 column (Sephagen Corp.) was employed at the ion exchange fractionation step. The higher flow rates attained with the radial flow column enabled us to complete the loading and elution of the enzyme in about 3 to 4 h. Application of these approaches permitted 4-fold and 9-fold increases in specific activity and recovery, respectively, over that achieved in the previous study (23).

The ADPglucose pyrophosphorylase fractions were monitored during the purification scheme by SDS-PAGE (Fig. 1). The C_3-agarose protein fraction showed a predominant band attributable to the purified enzyme migrating with a mol wt of 50,000, although several minor polypeptide bands of both higher and lower mobility were also readily observed. This protein fraction was subsequently purified by Mono Q chromatography using the same elution conditions as in the DEAE-Sephacel chromatography step. Analysis of the Mono Q purified fraction by native PAGE showed either a single broad band or two closely migrating bands exhibiting enzyme activity *in situ* (results not shown; see ref. 23) along with several other faint bands of higher mol wt (Fig. 1). Based on the extent of contamination as assessed by SDS-PAGE and Superose 12 fast protein liquid chromatography (results not shown), we estimate the purity of the ADPglucose pyrophosphorylase fraction after Mono Q chromatography to be about 80%. The final enzyme fraction had a specific activity of 56.9 μmol/min/mg protein. The purity of the tuber ADPglucose pyrophosphorylase was substantially higher than that obtained in an earlier study (23). The specific activity of the purified tuber enzyme is at least 2-fold lower than that reported for the spinach leaf enzyme (11), which may suggest that the catalytic turnover rates of the leaf and nonphotosynthetic enzymes differ. It should be pointed out, however, that the potato tuber enzyme was quite unstable during the Mono Q fractionation step, as about an 80% reduction in yield was evident (Table I). This reduction in activity could be due to proteolysis as evidenced by the extreme sensitivity of the maize endosperm ADPglucose pyrophosphorylase to endog-

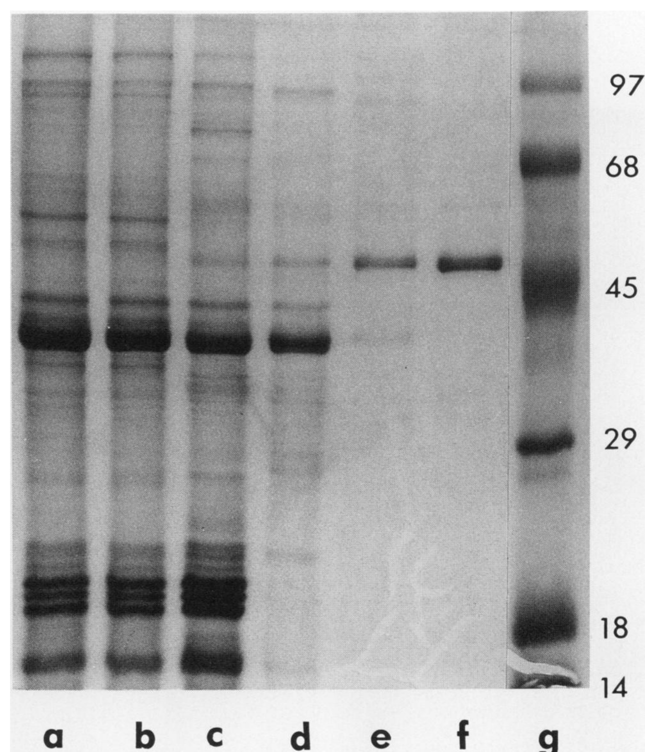

Figure 1. SDS-PAGE analysis of protein fractions during the purification of tuber ADPglucose pyrophosphorylase. Approximately 20 μg of protein were applied in lanes a to c, 10 μg of protein in lane d, while only 1.5 μg of protein were analyzed in lanes e and f. Numbers on the right side of the figure are the molecular sizes of the protein standards in kD. Lane a, crude extract; lane b, heat treated extract; lane c, 30 to 50% ammonium sulfate fraction; lane d, DEAE-Sephacel fraction; lane e, C_3-agarose fraction, lane f, Mono Q fraction; and lane g, protein standards.

enous proteases in crude extracts (15). Precautions for protein turnover, *i.e.* the inclusion of protease inhibitors, however, were included in this study particularly during the early stages of enzyme purification. Efforts are now being directed to improve the stability of the enzyme during this chromatography step which may substantially improve the recovery and specific activity of the enzyme.

Properties of IgG to ADPglucose Pyrophosphorylase

Antibodies to the Mono Q purified protein were raised in rabbits and tested for their reactivity with ADPglucose pyrophosphorylase by both Ouchterlony immunodiffussion and by Western blot analysis. Only a single sharply defined precipitate line was evident in Ouchterlony double diffusion tests when the IgG preparation was incubated with the purified enzyme fraction (results not shown). Western blot analysis, however, suggested that the immunological specificity of this antisera was not restricted to ADPglucose pyrophosphorylase alone (Fig. 2). Strong reactivity of the IgG fraction was readily evident to ADPglucose pyrophosphorylase subunits, but significant cross-reactivity was also observed to polypeptides present in the crude extract. Polypeptides of 40 kD and 22 kD, abundant in the crude extract, as well as several other minor species, showed varying degrees of reactivity with the antipyrophosphorylase IgG fraction. The employment of different blocking agents, *e.g.* BSA or detergents, at various concentrations yielded basically the same results. Similar polypeptide patterns were also visualized using a mouse antipotato tuber ADPglucose pyrophosphorylase (results not shown). In all instances, however, the IgG fraction showed the highest reactivity with the ADPglucose pyrophosphorylase subunits. It is not clear whether this cross-reactivity to nonpyrophosphorylase proteins is the result of slight contamination of these polypeptides in the enzyme preparation used for antibody production or whether they share common epitopes with pyrophosphorylase subunits. The cross-reacting polypeptide at 40,000 is probably patatin based on its electrophoretic mobility and abundance in crude extracts of tuber tissue (10). Comparison of the primary structures of patatin and the rice endosperm 54 kD subunit revealed no obvious sequence identity longer than four consecutive residues. Therefore, the slight reactivity of the IgG fraction to patatin may reflect the small stretches of residues that are common with ADPglucose pyrophosphorylase, although one cannot discount the possibility that some homology exists between patatin and the larger ADPglucose pyrophsophorylase subunit whose complete primary sequence is not known.

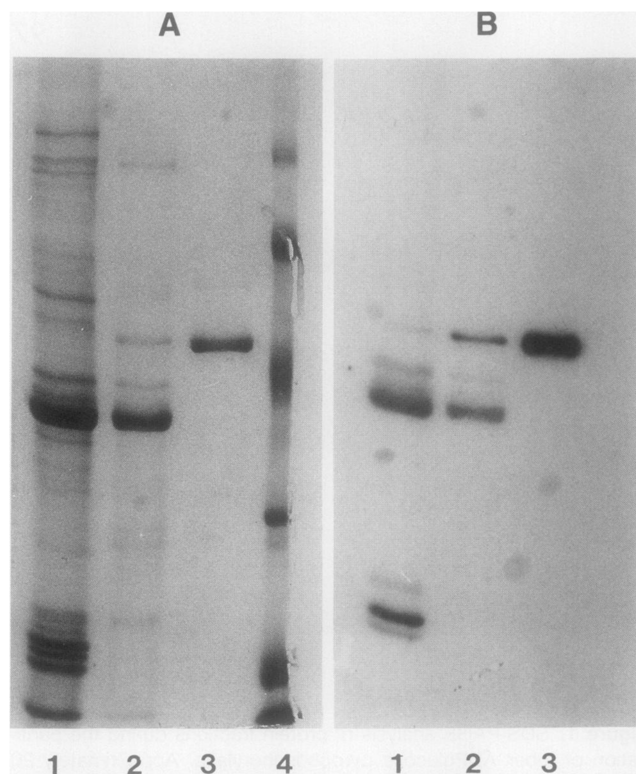

Figure 2. Western blot analysis of IgG prepared against the purified tuber ADPglucose pyrophosphorylase. Panel A depicts a Coomassie brilliant blue stained polyacrylamide gel while panel B shows a Western blot of panel A. Lane 1, crude extract (20 μg protein); lane 2, DEAE-Sephacel fraction (10 μg); lane 3, Mono Q fraction (1.5 μg); and lane 4, protein standards as listed in the Figure 1 legend.

Structural Analysis of ADPglucose Pyrophosphorylase

The purified ADPglucose pyrophosphorylase protein fraction was analyzed by high resolution 2-D PAGE. The purified enzyme resolved into two groups of polypeptides with nearly the same mol wt when subjected to denaturing isoelectric focusing under equilibrium conditions followed by SDS-PAGE (Fig. 3). Similar subunit patterns were also observed in nonequilibrium pH gradients during electrophoresis in the first dimension (results not shown). The slightly higher molecular mass subunits were more basic in net charge, and 3 to 5 isoforms were observed. The heterogeneous character of the basic subunits varied somewhat, as it appeared to be dependent on the age of the protein sample and isoelectric focusing conditions. The smaller subunit usually migrated as a single spot, although in some instances polymorphic forms had also been observed. Both subunits were present at equal molar amounts as viewed by the intensity of the Coomassie brilliant blue staining patterns. Western blot analysis by 2-D PAGE showed that both types of subunits were recognized by the antibody raised against the purified potato tuber ADPglucose pyrophosphorylase, although it appeared to have higher specificity to the basic subunits (Fig. 3B).

The immunological identity of the tuber subunits was assessed by their cross-reactivity to the anti-54 kD and anti-51 kD prepared against the purified spinach leaf ADPglucose pyrophosphorylase subunits (11). The anti-54 kD serum did not recognize the potato tuber subunits as analyzed by Western blotting (results not shown). Antibodies raised against the spinach 51 kD subunit, however, showed significant reaction to the smaller more acidic potato tuber subunit (Fig. 3C). This was further verified by stripping the blot incubated with the tuber antibodies (Fig. 3B) and reprobing with the anti-51 kD. Again, only the smaller tuber subunit showed reaction

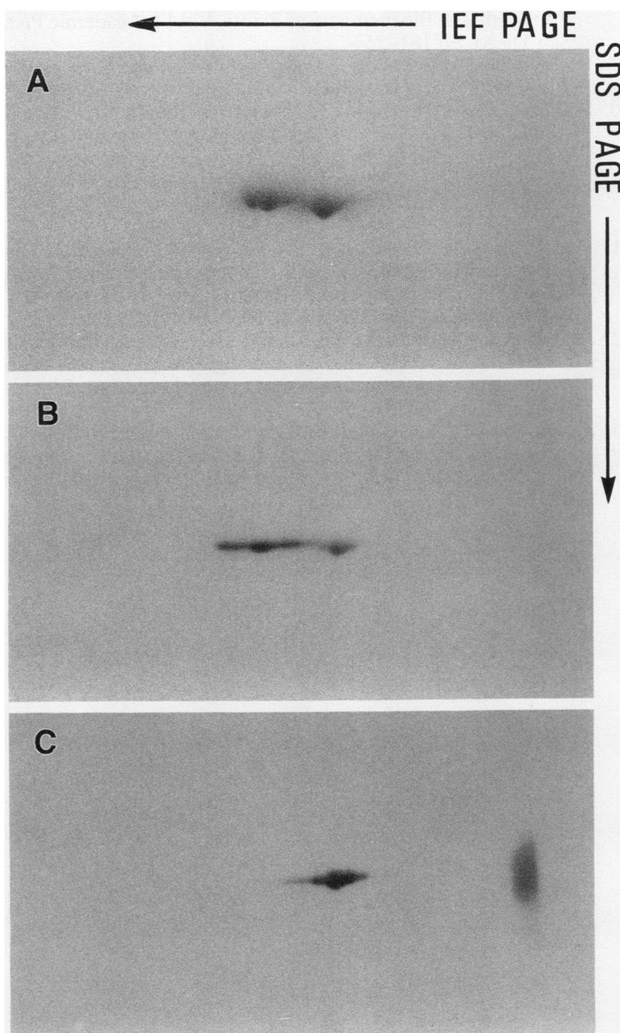

Figure 3. Structural analysis of purified tuber ADPglucose pyrophosphorylase by 2-D PAGE and Western blot analyses. Mono Q purified ADPglucose pyrophosphorylase (5 μg) was electrophoresed in a 2-D PAGE system as described by O'Farrell (12). Panel A depicts a Coomassie brilliant blue stained gel. Panel B shows a Western blot of panel A probed with IgG prepared against the purified tuber ADPglucose pyrophosphorylase. Panel C represents a Western blot of panel A incubated with the anti-51 kD of spinach leaf. The broad band on the right side of panel C is the protein standard, ovalbumin, which is reactive to the IgG fraction. The directions of the denaturing isoelectric focusing (acidic to basic) and SDS-PAGE runs are indicated.

with the spinach antibody (results not shown). These results indicate that the smaller tuber ADPglucose pyrophosphorylase subunit shares structural homology to the spinach leaf 51 kD subunit as initially suggested by Morell *et al.* (11). These workers (11) showed that antibodies raised against the tuber holoenzyme reacted with the spinach 51 kD polypeptide but only very weakly with the 54 kD spinach subunit. The conservation of the 51 kD component of ADPglucose pyrophosphorylase detected in different plant tissues has been recently corroborated by analysis of recombinant cDNA clones. The primary sequences of the rice endosperm subunit and spinach leaf 51 kD subunit reveal about 76% identity (20). Overall, these results (11, 15, 20), as well as those reported here, indicate that the 51 kD subunit remains structurally conserved. In contrast, the inability of the spinach leaf anti-54 kD to cross-react with the larger, more basic subunit of the tuber enzyme suggests that this subunit type present in these different plant ADPglucose pyrophosphorylases is more divergent and shares less sequence identity.

Similar observations were also made in studies of the maize enzyme. Western blot analyses of maize endosperm crude extracts or of partially purified maize ADPglucose pyrophosphorylase showed that antibodies prepared against the purified spinach leaf ADPglucose pyrophosphorylase 51 kD or 54 kD subunits cross reacted with the 54 kD and 60 kD maize subunits, respectively (21). The cross reaction with the antibody prepared against the spinach leaf 54 kD subunit gave a weaker reaction with the corresponding 60 kD subunit of the maize enzyme than the reaction observed with the anti 51 kD spinach antibody and the corresponding maize endosperm enzyme 54 kD subunit. Distinct subunits of the maize endosperm would account for the presence of the two unlinked maize mutants, *Shrunken-2* and *Brittle-2,* defective in ADPglucose pyrophosphorylase activity (3). Therefore, the available evidence indicates that the ADPglucose pyrophosphorylase from nonphotosynthetic tissues is not a simple homotetramer as found in bacterial cells but is structurally similar to the leaf enzyme.

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