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Characterization of ADPglucose pyrophosphorylase from a starch-deficient mutant of *Arabidopsis thaliana* (L.)*,†

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ABSTRACT

A starch-deficient mutant of *Arabidopsis thaliana* (L.) has only ~ 5% of ADPglucose pyrophosphorylase activity but accumulates ~ 40% of starch as the wild-type. ADPglucose pyrophosphorylase from the mutant and wild-type leaves was partially purified and characterized. The activity of each enzyme was inhibited by an antibody raised against purified spinach-leaf ADPglucose pyrophosphorylase. When analyzed by Western-blot hybridization after SDS-PAGE, the partially purified mutant enzyme was deficient in the larger of the two subunits observed in the wild-type enzyme. However, by Western-blot hybridization after native PAGE, the mutant enzyme demonstrated two equally stained cross-reactive bands instead of a single band for the wild-type enzyme. Of the mutant enzyme activity, >95% was found in the lower band. The mass of the native enzyme from either wild-type or mutant was ~ 210 kD, as determined by gel filtration, and those of the subunits were ~ 54 and 48 kD for the wild-type enzyme and 48 kD for the mutant enzyme. The mutant ADPglucose pyrophosphorylase, just as the wild-type enzyme, was activated allosterically by 3-P-glycerate and inhibited by P_i. However, the mutant enzyme required higher concentrations of 3-P-glycerate for maximal activation and was more sensitive to inhibition by P_i than the wild-type enzyme. In the presence of saturating concentrations of 3-P-glycerate, the K_m values for the mutant enzyme for ATP, Glc-1-P, and Mg^{2+} were ~ 6-, ~ 5-, and ~ 2-fold higher, respectively, than those of the wild-type enzyme. Changes in the kinetics of the mutant enzyme may be due to a deficiency of one of the two subunits, and/or modification of the remaining subunit.

INTRODUCTION

The biosynthesis of starch involves three steps catalyzed by ADPglucose pyrophosphorylase (EC 2.7.7.27), starch synthase (EC 2.4.1.21), and branching enzyme (EC 2.4.1.18). ADPglucose pyrophosphorylase plays a pivotal role in the control of starch biosynthesis in plants[1] since it catalyzes the synthesis of ADPglucose and PP_i from α-D-glucose 1-phosphate (Glc-1-P) and ATP, and is activated allosterically by 3-P-glycerate and inhibited by inorganic phosphate. Evidence accumulated[1] shows that the rate of synthesis of starch in plant cells is regulated by the ratio[2] of 3GPA to P_i via the allosteric control of ADPglucose pyrophosphorylase *in vivo*.

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Plant ADPglucose pyrophosphorylase proteins characterized to date have masses of ~200 kD and appear to be tetramers with two different subunits[1]. The purified spinach-leaf enzyme has a mass of 206 kD and is composed[3,4] of two different subunits of 51 and 54 kD. Immunological studies of leaf enzyme extracts from *Arabidopsis*[5], wheat, rice, and maize[6], and of maize endosperm extract[7] also demonstrated the presence of two subunits of different size in these plants.

A starch-deficient mutant of *Arabidopsis thaliana* (TL46) contained[8] only 5% of ADPglucose pyrophosphorylase activity but accumulated ~40% of the starch produced by the wild-type, and different kinetic and/or regulatory properties of the mutant enzyme from the wild type were expected. Lin *et al.*[8] analyzed the TL46 mutant leaf enzyme by Western-blot hybridization with the anti-spinach leaf ADPglucose pyrophosphorylase antibodies and showed that the mutant enzyme in the crude extract appeared to contain no detectable cross-reactive band of the larger subunit of the two subunits observed in wild-type extract. In order to confirm and study the nature of the mutation on the ADPglucose pyrophosphorylase protein, the enzyme from TL46 leaves had to be purified further and this study is now reported.

EXPERIMENTAL

Plant material. — The Columbia wild-type and a starch-deficient mutant (TL46) of *Arabidopsis thaliana* (L.) Heynh were grown for 12 h at ~22° under cool-white fluorescent illumination (~200 $\mu E.m^{-2}.s^{-1}$) on a 1:1:1 perlite–vermiculite–sphagnum mixture irrigated with a solution of mineral nutrients[9]. Leaves of 4–6-week old plants were harvested, frozen with liquid nitrogen, and stored at −80°.

Purification of ADPglucose pyrophosphorylase. — All steps were carried out at 0–4° unless noted otherwise.

(*a*) *Crude extract.* Frozen leaves (~140 g) were ground with a pestle and mortar in ice-cold 50mM Hepes–NaOH buffer (400 mL, pH 7.5) containing 2mM EDTA. The homogenate was filtered through four layers of cheesecloth and the solid residue was reground. The filtrates were combined and centrifuged at 30 000*g* for 20 min.

(*b*) *Heat treatment.* The supernatant solution was heated to 60°, maintained thereat for 5 min, then chilled rapidly in ice–water, and centrifuged at 30 000*g* for 20 min.

(*c*) *Fractionation with* $(NH_4)_2SO_4$. Solid $(NH_4)_2SO_4$ was added slowly to the supernatant solution to 25% saturation. The mixture was stirred for 20 min, then centrifuged at 20 000*g* for 20 min, and the concentration of $(NH_4)_2SO_4$ in the supernatant solution was increased to 60%. The mixture was stirred for 20 min, then centrifuged as above, and a solution of the pellet in a small volume of extraction buffer was dialyzed twice against 50mM Hepes–NaOH buffer (1 L, pH 7.5) containing 50mM NaCl and 20% of sucrose for a total 6 h.

(*d*) *Chromatography on DEAE-cellulose.* The product from (*c*) was loaded onto a column (1.5 × 17 cm) of DEAE-Cellulose equilibrated with 50mM Hepes buffer (pH 7.5) containing 50mM NaCl and 20% of sucrose. The column was washed with 2 bed

volumes of the buffer and then eluted with a linear gradient (6 times the bed volume) of 0.05→0.35M NaCl in the buffer. Fractions of 5.0 mL were collected and those that had high enzyme activity were combined and concentrated by ultrafiltration with a YM 10 membrane (Amicon). This partially purified enzyme was stable for at least several months when stored frozen.

(*e*) *Chromatography on Mono-Q.* The product from (*d*) was dialyzed against 20mM Hepes buffer (500 mL, pH 7.7) containing 20% of sucrose for 6 h with two changes, then loaded onto a column of Mono-Q (HR 5/5) equilibrated with 20mM triethanolamine (pH 7.7) containing 10% of sucrose. The enzyme was eluted with a linear gradient of 0→0.4M NaCl in the same buffer. Fractions with high enzyme activity were combined and concentrated.

(*f*) *Chromatography on Sephacryl S-300.* An aliquot of the product from (*e*) was loaded onto a column (1.5 × 95 cm) of Sephacryl S-300, using 50mM Hepes–NaOH (pH 7.5) containing 150mM NaCl as the equilibration and elution buffer. Fractions of 1.5 mL were collected and assayed for ADPglucose pyrophosphorylase activity. The apparent mass of the enzyme was estimated from a plot of K_{ae} (partition coefficient) *vs.* log mol. wt. of standard proteins.

Assay of ADPglucose pyrophosphorylase. — (*a*) *Assay A.* Pyrophosphorolysis of ADPglucose was followed by the formation of [^{32}P]ATP in the presence of [^{32}P]PP_i. The reaction mixture contained[5] 20 μmol of glycylglycine buffer (pH 7.6), 1.5 μmol of $MgCl_2$, 0.25 μmol of ADPglucose, 0.5 μmol of [^{32}P]PP_i (1000–3500 c.p.m./nmol), 10 μg of BSA, 0.25 μmol of 3 PGA, and enzyme preparation in a total volume of 0.25 mL. The reaction was carried out at 37° for 10 min and then terminated by addition of cold aq. 5% trichloroacetic acid (3 mL) containing acid-washed Norit (15 mg) and 10 μmol of $NaPP_i$. After two more washes with cold aq. 5% trichloroacetic acid (3 mL), the Norit-absorbed [^{32}P]ATP was boiled in M HCl (1 mL) for 10 min, and centrifuged. The supernatant solution (0.5 mL) was counted in 5 mL of the Safety-Solve scintillation cocktail in a liquid scintillation counter. This assay was used to measure ADPglucose pyrophosphorylase activity throughout the enzyme purification procedure.

(*b*) *Assay B.* The synthesis of ADPglucose was measured[10] in the presence of [^{14}C]Glc-1-P. The standard mixture contained 20 μmol of Bicine–NaOH (pH 7.6), 1 μmol of $MgCl_2$, 0.2 μmol of ATP, 50 μg of bovine serum albumin (BSA), 0.1 μmol of [^{14}C]Glc-1-P (1000–3500 c.p.m./nmol), 0.2 μmol of 3PGA, and enzyme in a final volume of 0.2 mL. For the mutant enzyme assay, the amounts of 3PGA, Glc-1-P, ATP, and Mg^{2+} were increased to 1.0, 0.2, 0.4, and 2.0 μmol, respectively. The reaction mixture was incubated for 10 min at 37° and terminated by heating in a boiling water bath for 30 s. The unreacted [^{14}C]Glc-1-P was hydrolyzed for 1 h at 37° with *E. coli* alkaline phosphatase.

All kinetic studies were performed with the DEAE-Cellulose enzyme preparations, using the synthesis assay.

Determination of protein. — Protein concentration was determined by the method of Smith *et al.*[11], using bicinchoninic acid reagent (Pierce Chemical Co.) with BSA as the standard.

Neutralization of enzyme activity[12]. — The neutralization mixture, which contained 5 μmol of Hepes buffer (pH 7.5), 10 μg of BSA, 5 mg of sucrose, 0.005 U of Mono-Q purified enzyme, and 30 μL of sera with various amounts of antispinach leaf ADPglucose pyrophosphorylase immune sera diluted into pre-immune serum, in a final volume of 0.1 mL, was incubated for 30 min at 30°. The mixture was placed for 2 h on ice and centrifuged. Enzyme activity in the supernatant solution was assayed using Assay B.

Gel electrophoresis and Western blotting. — 1D or 2D polyacrylamide gel electrophoresis (native tube PAGE and slab SDS-PAGE) was carried out as described[5]. The gel was electroblotted[13] onto a nitrocellulose membrane for 2 h at 0.5 A. The membrane was blocked in 50mM Tris–HCl (pH 7.5) containing 150mM NaCl and 1% of BSA (TBS + 1% of BSA) for 1 h at room temperature, then treated with rabbit antisera raised against spinach-leaf ADPglucose pyrophosphorylase or the 51- or 54-kD subunit of the enzyme[4]. The membrane was washed with 3 changes of TBS, then incubated in goat antirabbit IgG-horseradish peroxidase conjugate (1:3000 dilution). The cross-reacting peptides were visualized[14] by using a solution of 4-chloro-1-naphthol (30 mg) in MeOH (10 mL) plus TBS (40 mL) and 40 μL of aq. 30% H_2O_2.

Electroelution. — Native slab PAGE was carried out as described[5]. A line of the gel was cut and stained for proteins, using the silver stain[15]. The wild-type enzyme protein band was excised and incubated for 2 h in 50mM Tris–glycine buffer (10 mL, pH 8.3) containing 5% of 2-mercaptoethanol and 2% of SDS to denature proteins. The gel slice was washed several times in the sample-trap buffer [5mM Tris–glycine buffer (pH 8.3) containing 0.1% of SDS], diced into small pieces, and loaded onto ISCA sample traps. Electroelution was carried out overnight at 3 mA/trap, using 12.5mM Tris–glycine (pH 8.3) and 0.1% SDS as electrode compartment buffer[16].

RESULTS AND DISCUSSION

Purification of ADPglucose pyrophosphorylase. — The partial purifications of *Arabidopsis* ADPglucose pyrophosphorylase from wild-type and mutant TL46 are summarized in Table I; each purification was ~ 50-fold with a recovery of 25–30%. The enzyme was eluted as a single peak at 0.25–0.30M NaCl from DEAE–Cellulose and Mono-Q, and was stable after the former step. As previously indicated[8], the mutant leaf extract contained only ~ 3% of the ADPglucose pyrophosphorylase activity of the wild-type extract (Table I).

Optimum pH. — A typical pH activity curve of ADPglucose synthesis for the wild-type enzyme in the presence and absence of 3GPA is shown in Fig. 1 and a similar curve was obtained with the mutant enzyme (data not shown). The optimum was pH 7.5–8.0. In Tris buffer, less enzyme activity was noted. Unless otherwise stated, all the ADPglucose synthesis assays in this study were carried out at pH 7.5 using Bicine buffer.

Specificity of enzyme activation. — Various carbon metabolites at mM concentration were tested for their ability to activate ADPglucose synthesis. For both wild-type and mutant enzymes, 3PGA was the most effective activator (Table II), phosphoenol-

TABLE I

Purification of ADPglucose pyrophosphorylase from *Arabidopsis* leaves

Fraction	*Vol. (mL)*	*Protein (mg)*	*Specific activity (U/mg)*	*Total activity (U^a)*	*Purification (fold)*	*Yield (%)*
Wild type						
Crude	476	562	0.15	82	1.0	100
Heat treatment	469	285	0.29	82	1.9	100
$(NH_2)_4SO_4$	9.7	167	0.50	83	3.3	100
DEAE-cellulose	20	25	2.2	54.5	15	66
Mono-Q	6.2	2.9	7.7	22	52	27
TL46						
Crude	510	500	0.005	2.7	1.0	100
Heat treatment	495	242	0.011	2.7	2.1	100
$(NH_2)_4SO_4$	12.3	183	0.014	2.5	2.6	96
DEAE-cellulose	11.4	30	0.062	1.8	12	69
Mono-Q	5.2	3.1	0.25	0.78	48	29

[a] One unit of enzyme produces 1 μmol of ATP per min.

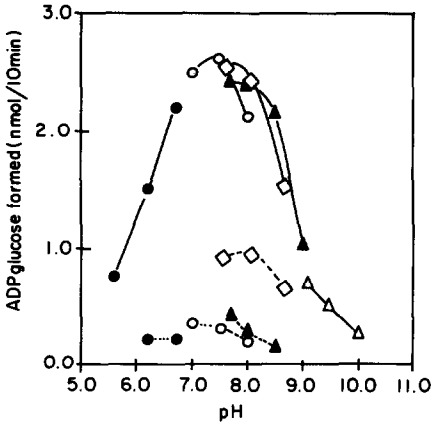

Fig. 1. Effect of pH on the activity of ADPglucose pyrophosphorylase, extracted from *Arabidopsis* wild-type leaves, in the presence (solid line) and absence (broken line) of 3PGA with Mes (●), Hepes (○), Bicine (◇), glycylglycine (▲), and glycine (△) buffers, each at 100mM.

TABLE II

Metabolic activation of ADPglucose synthesis[a]

Metabolite	*Concentration (mM)*	*Wild-type*		*TL46*	
		ADPG formed (nmol)	*Activation (fold)*	*ADPG formed (nmol)*	*Activation (fold)*
None	–	0.11	1.0	0.15	1.0
3-P-glycerate	1	2.7	24	2.9	19
3-P-glycerate	2			3.7	25
Phosphoenolpyruvate	1	1.45	13	1.47	9.8
Fructose-6-P	1	0.67	6.1	0.45	3.0
Dihydroxyacetone-phosphate	1	0.38	3.5	0.72	4.8
Glyceraldehyde-3-P	1	0.34	3.1	0.55	3.7
2,3-Bis-P-glycerate	1	0.88	8.0	0.49	3.3
Glucose-6-P	1	0.39	3.5	0.45	3.0
2-P-glycerate	1	1.3	12	0.44	2.9
Ribulose 1,5-bis-P	1	0.51	4.6	0.37	2.4
Fructose 1,6-bis-P	1	0.41	3.7	0.58	3.9
Fructose 2,6-bis-P	1	0.20	1.8	0.39	2.6
Glucose 1,6-bis-P	1	0.35	3.2	0.42	2.8
Pyruvate	1	0.27	2.5	0.40	2.7
AMP	1	0.19	1.7	0.30	2.0
ADP	1	0.14	1.2	0.13	0.9
P_i	1	<0.01	–	0.01	<0.1

[a] Assay *B* was used (see Experimental) with DEAE-cellulose enzyme preparations.

pyruvate activated the enzymes to a lesser extent, and the other metabolites had only weak effects.

AMP, ADP, and P_i are allosteric inhibitors of bacterial ADPglucose pyrophosphorylases[2]. AMP and ADP showed no effect on the *Arabidopsis* wild-type and mutant enzymes, but P_i was an effective inhibitor (Table II).

Allosteric regulation by 3PGA and P_i. — The mutant enzyme, as the wild-type enzyme, was modulated by 3PGA and P_i. For each enzyme, the activation curve with 3PGA was hyperbolic in the absence of P_i (Fig. 2). The presence of P_i inhibited the enzyme at lower concentrations of 3PGA and changed the shape of the saturation curves from a hyperbolic to a sigmoidal form. Increasing amounts of P_i also made the saturation curve more sigmoidal (Fig. 2 and Table III). Although the enzyme from either wild-type or mutant leaves showed a similar shape of saturation curve with respect to 3PGA and P_i, the mutant enzyme required a much higher concentration of 3PGA for maximal activation than the wild-type enzyme. The $A_{0.5}$ (50% maximal activation) value of the mutant enzyme for 3PGA was ~ 30-fold higher in the absence of P_i (Table III). In the presence of 0.5mM or more P_i, the affinity of the mutant enzyme for 3PGA was ~ 5-fold or less lower than that of the wild-type enzyme (Table III).

The ADPglucose pyrophosphorylase from wild-type and mutant leaves also

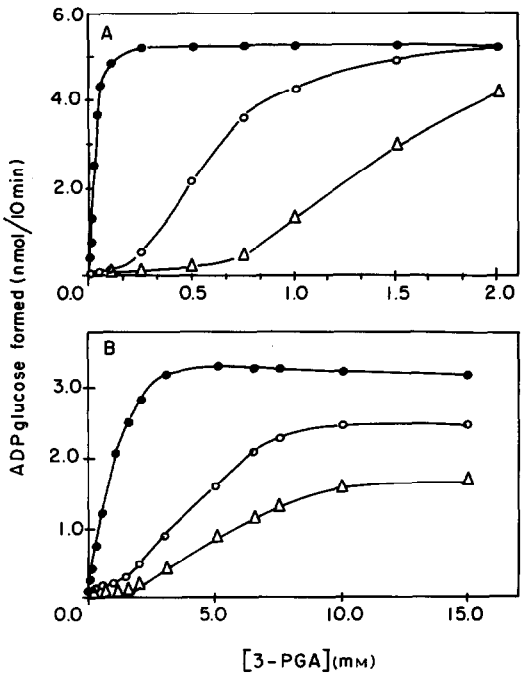

Fig. 2. Activation by 3PGA of ADPglucose pyrophosphorylase isolated from *Arabidopsis* wild-type (A) and mutant TL46 leaves (B). The ADPglucose synthesis was measured as described in the Experimental except that the 3PGA concentration was varied and P_i was present as indicated: none (●), 0.5mM (○), and mM (△).

differed in their responses to inhibition by P_i (Fig. 3). In the presence of various amounts of 3PGA, the mutant enzyme was much more sensitive to inhibition by P_i than the wild-type enzyme. With 1.0 mM 3PGA in the reaction mixture, mM P_i almost completely inhibited the mutant enzyme but only 75% of the activity of the wild-type enzyme (Fig. 3). In the presence of the same concentrations of 3 PGA, the mutant enzyme was about 5–6 times more sensitive to inhibition by P_i than the wild-type enzyme. The apparent higher sensitivity to inhibition by P_i for the mutant enzyme may be due to the requirement for a higher level of 3PGA to activate the enzyme. Higher concentrations of 3PGA could reduce sensitivity of the enzyme to inhibition by P_i. The $I_{0.5}$ value increased 10- and 13-fold for the mutant and wild-type enzymes, respectively, as the 3PGA concentration was increased from 0.2 to 5.0mM (Table III).

Kinetic parameters of the synthesis reaction. — The enzyme fractions obtained from DEAE-cellulose were used for the kinetic studies. Table III summarizes the various kinetic parameters of ADPglucose pyrophosphorylase for substrates and effectors of ADPglucose synthesis; the values reported represent the results of at least two separate experiments.

For both wild-type and mutant enzymes, the saturation curves for substrates Glc-1-P and ATP were essentially hyperbolic with or without added 3PGA, as the Hill

TABLE III

Kinetic parameters of ADPglucose pyrophosphorylase[a]

Substrate/effector	*Wild-type*		*TL46*	
	$S_{0.5}$ $A_{0.5}$ $I_{0.5}$ (*mM*)	n	$S_{0.5}$ $A_{0.5}$ $I_{0.5}$ (*mM*)	n
3-PGA	0.031 ±0.003	1.3	0.87 ±0.02	1.4
3-PGA (+0.5mM P_i)	0.69 ±0.01	2.4	3.4 ±0.20	2.3
3-PGA (+1.0mM P_i)	1.4 ±0.11	4.0	4.8 ±0.00	3.6
P_i	0.043	0.8	0.078	0.6
P_i (+0.2mM 3-PGA)	0.18 ±0.00	2.7	0.046 ±0.002	1.3
P_i (+1.0mM 3-PGA)	0.64 ±0.02	3.3	0.13 ±0.02	1.5
P_i (+5.0mM 3-PGA)	2.2 ±0.23	3.8	0.46 ±0.05	1.8
G1P	0.13 ±0.030	1.0	0.87 ±0.03	1.0
G1P (+1.0mM 3-PGA)	0.042 ±0.007	1.0		
G1P (+5.0mM 3-PGA)			0.19 ±0.00	1.0
ATP	0.69 ±0.005	1.0	0.15 ±0.04	1.0
ATP (+1.0mM 3-PGA)	0.048 ±0.005	1.0		
ATP (+5.0mM 3-PGA)			0.30 ±0.09	1.0
Mg^{2+}	3.1 ±0.29	6.0	3.6 ±0.11	4.2
Mg^{2+} (+1.0mM 3-PGA)	1.5 ±0.09	2.7		
Mg^{2+} (+5.0mM 3-PGA)			3.65 ±0.13	4.4

[a] Assay *B* was used (see Experimental) with the partially purified enzyme from the DEAE-cellulose. Values represent the mean ±s.e. (n = 2 or 3).

slope constants were ~1 (Table III). The presence of saturating concentrations of 3PGA decreased the K_m values for Glc-1-P ~3- or ~4-fold for the wild-type and mutant enzyme, respectively. The K_m value of the mutant enzyme for Glc-1-P was ~5-fold higher than that of wild-type enzyme. In the presence of 3PGA, the K_m value of wild-type enzyme for ATP decreased ~14-fold, whereas that of the mutant enzyme for ATP was slightly increased. The affinity of mutant enzyme for ATP was ~6-fold lower than that of the wild-type enzyme (Table III). Thus, the absence of large subunit not only affects the ability of activator, 3PGA, to overcome inhibition by P_i but also its ability to increase the affinity of the enzyme for its substrate, ATP.

Mg^{2+} was essential for both wild-type and mutant ADPglucose synthesis activities (data not shown) and the affinities were similar in the absence of 3PGA. In the presence of 3PGA, the $S_{0.5}$ value of mutant enzyme for Mg^{2+} was >2-fold higher than that of wild-type (Table III). The saturation curve of each enzyme for Mg^{2+} was highly sigmoidal with or without added activator. Mg^{2+} at >10mM inhibited the enzyme.

Neutralization of enzyme activity. — The Mono-Q purified ADPglucose pyrophosphorylases from wild-type and mutant TL46 leaves were each inhibited completely by rabbit antiserum raised against spinach-leaf ADPglucose pyrophosphorylase

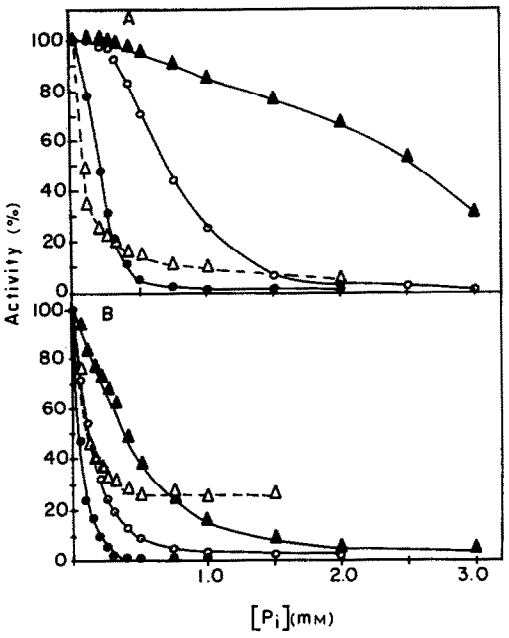

Fig. 3. Inhibition by P_i of ADPglucose pyrophosphorylase extracted from wild-type (A) and mutant TL46 leavens (B). The ADPglucose synthesis was determined as described in the Experimental except that the P_i concentration was varied and the amount of 3PGA was present as indicated: none (●), 0.2mM (△), mM (○), and 5.0mM (▲): 100% activity represents the rate of ADPglucose synthesis in the absence of P_i.

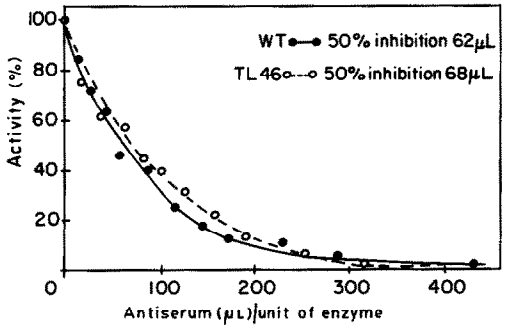

Fig. 4. Inhibition of activity of ADPglucose pyrophosphorylase from *Arabidopsis* wild-type (●) and mutant TL46 (○) leaves by anti-spinach-leaf ADPglucose pyrophosphorylase immune serum. The inhibition experiment was performed with Mono-Q purified enzyme preparations (see Experimental).

(Fig. 4) and the amount of antiserum required for 50% inhibition was ~62 and ~68 μL of antiserum per unit, respectively. In a previous report[12] using the same antibody, 50% of spinach-leaf enzyme activity was inhibited by ~46 μL of antiserum per unit of enzyme. Thus, the enzymes from spinach and *Arabidopsis* leaves are immunologically similar.

Neutralization of the Mono-Q purified enzymes was also tested with rabbit antisera raised against either the 51- or 54-kD subunit of spinach-leaf ADPglucose pyrophosphorylase. For both wild-type and TL46 enzymes, increasing amounts of the large-subunit antiserum had little effect on the enzyme activities, whereas increasing amounts of the small-subunit antiserum only gave slight inhibition of the enzyme activities (results not shown).

Immunological analysis. — Because the subunit of ADPglucose pyrophosphorylase has similar mobility on SDS-PAGE as the large subunit of Rubisco (ribulose bisphosphate carboxylase), and because Rubisco was always present in the ADPglucose pyrophosphorylase preparations even after gel-filtration chromatography, 2D gel electrophoresis (native tube PAGE and slab SDS-PAGE) was carried out prior to immunoblotting in order to remove the Rubisco peptide. Western-blot hybridization with the Mono-Q purified enzymes confirmed the previous reports[5,8] that the wild-type enzyme contained two subunits which cross-reacted with the rabbit antibody raised against native spinach-leaf ADPglucose pyrophosphorylase, whereas only the lower subunit of the mutant enzyme cross-reacted with the antibody (Fig. 5). A similar result was obtained when antisera produced against either the 51- or 54-kD subunit of spinach leaf ADPglucose pyrophosphorylase were used (data not shown). A good immunoreaction was obtained with the 51-kD antibody and none was observed with the 54-kD antibody. The small- and large-subunit antibodies each reacted well with the normal *Arabidopsis* ADPglucose pyrophosphorylase[5,8].

When the mutant ADPglucose pyrophosphorylase subunits were separated by native slab PAGE and then electroblotted for Western-blot hybridization, only one cross-reactive band was observed for the wild-type enzyme. In contrast, two equally

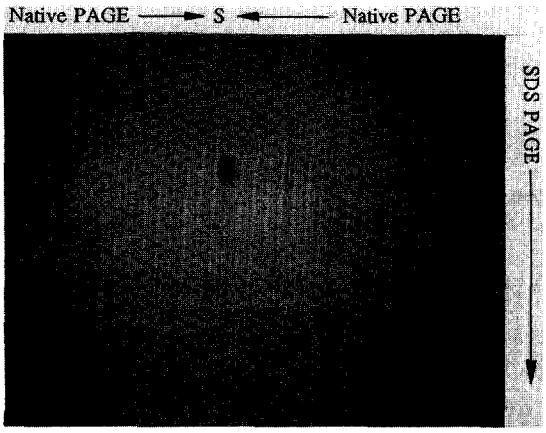

Fig. 5. Western-blot analysis of *Arabidopsis* ADPglucose pyrophosphorylase from wild-type (left) and mutant (right) leaves. Mono-Q purified enzymes (~ 15 and ~ 87 μg of protein, respectively, for wild-type and mutant) were subjected to 2D electrophoresis and detected with antiserum prepared against spinach-leaf ADPglucose pyrophosphorylase (see Experimental). The middle line is the purified spinach-leaf ADPglucose pyrophosphorylase (1 μg) which was run only in SDS-PAGE.

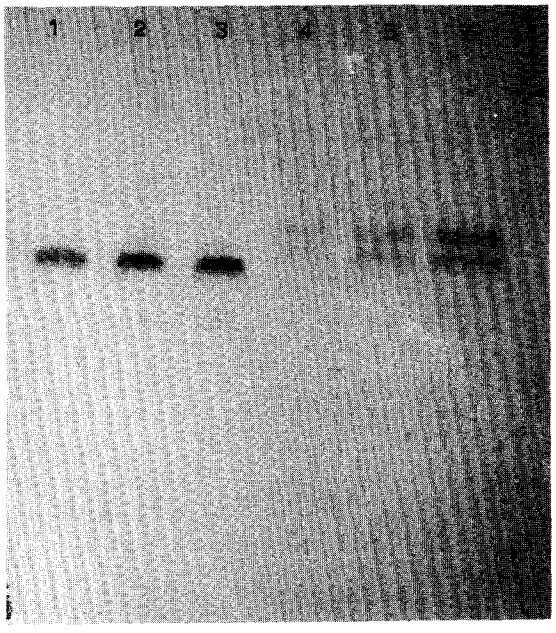

Fig. 6. Western blot of *Arabidopsis* native ADPglucose pyrophosphorylase from wild-type (1–3) and mutant TL46 (4–6) leaves. DEAE-cellulose-purified enzymes were separated in a native PAGE and detected with antiserum prepared against spinach-leaf ADPglucose pyrophosphorylase. Lines 1–6 contain about ~ 3.0, ~ 1.5, ~ 0.75, ~ 4.1, ~ 8.2, and ~ 16.4 μg of protein, respectively.

staining cross-reactive bands were observed for the mutant enzyme (Fig. 6); no bands were detected with pre-immune serum. When the gel was sliced and assayed for enzyme activity, >95% of the mutant enzyme activity was found in the lower band (Fig. 7). Further analysis of the mutant enzyme with antisubunit antisera showed that the 51-kD antiserum cross-reacted with each band, whereas the 54-kD antiserum failed to react with either band (result not shown). For the wild-type enzyme, both the 51- and 54-kD antiserum cross-reacted with the single band. These results indicate that the two bands of the mutant enzyme separated by PAGE have a similar immunological composition.

Following native slab PAGE, the ADPglucose pyrophosphorylase protein band was excised and electroeluted out of the gel in SDS solution and SDS-PAGE that revealed two major polypeptide bands (silver staining, data not shown). These two bands had the same mobility as the bands detected by Western-blot hybridization. Thus, the result further confirmed that the wild-type *Arabidopsis* ADPglucose pyrophosphorylase was composed of two different subunits.

Determination of molecular weights. — The masses of both the wild-type and mutant ADPglucose pyrophosphorylase were estimated to be ~ 210 kD by gel filtration of the Mono-Q preparations of Sephacryl S-300. The masses of the two subunits of the wild-type enzyme were determined to be ~ 54 and ~ 48 kD, respectively, by comparison with the standard protein markers on SDS-PAGE. The mutant enzyme contained only

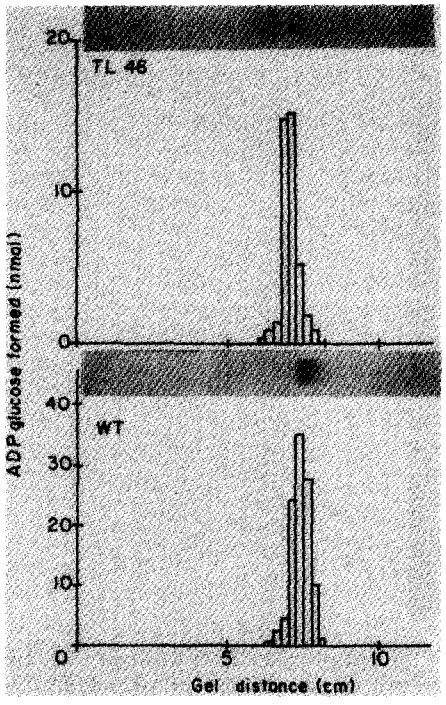

Fig. 7. Activity of ADPglucose pyrophosphorylase after PAGE. DEAE-cellulose-purified wild type (180 μg) and mutant (250 μg). The proteins were excised and assayed for enzyme activity (Assay B). The insets are Western-blot results.

one immunoreactive band of ~48 kD after SDS-PAGE. Thus, as found for all plant ADPglucose pyrophosphorylases studied, the ADPglucose pyrophosphorylase from *Arabidopsis* leaves appears to comprise four subunits, with the wild-type enzyme having two different subunits and the mutant enzyme being a homotetramer. It is unlikely that the presence of two bands was due to proteolytic degradation during extraction, as addition of protease inhibitors in extraction buffer gave the same result. Although the mutant enzyme had two equally stained cross-reactive bands in native PAGE, only the lower band showed enzyme activity (Fig. 7). The upper band could be the precursor protein of the enzyme, and/or lack of proper assembly of subunits. Further analysis of the mutant enzyme with anti-spinach 51- and 54-kD antisera revealed that each cross-reactive band contained only the 51-kD immunologically related polypeptide. This finding is consistent with the result that, in the denatured state, the mutant enzyme had only a single band of ~48 kD that cross reacted with the 51-kD antibody (Fig. 5). As the mass of the mutant enzyme was ~210 kD, the mutant enzyme may be a homotetramer.

Changes in the structure of the mutant enzyme may alter the regulatory and/or catalytic properties. All plant ADPglucose pyrophosphorylases studied so far[1], in-

cluding that from *Arabidopsis*, are activated by 3PGA and inhibited by P_i. Kinetic studies of the mutant enzyme showed that the enzyme required higher concentrations of 3PGA for maximal activation and was more sensitive to P_i inhibition than wild-type enzyme (Table III). Apart from the effect on regulatory properties, the mutation also reduced the apparent affinity of the mutant enzyme for the substrates. The K_m values for Glc-1-P and ATP were ~5 and ~6-fold higher, respectively, than those of wild-type enzyme (Table III).

Studies of maize-endosperm and spinach-leaf ADPglucose pyrophosphorylase showed that the two subunits of the ADPglucose pyrophosphorylases were most likely encoded by two different genes[4,7,16]. Similarly, Lin *et al.*[8] also showed that *Arabidopsis* ADPglucose pyrophosphorylase was controlled by at least two genes, and that the TL46 mutant lacked the *adg2* gene which codes for the 54-kD subunits. By quantifying the level of the small subunit immunochemically, the mutant enzyme seemed to contain only ~ 4% of the small subunit as compared to the wild-type[8]. Thus, changes in the kinetic properties of the mutant enzyme could be directly related to the absence of the *adg2* gene product, and/or the modification of the remaining polypeptides. It seems that the presence of two subunits in *Arabidopsis* native ADPglucose pyrophosphorylase is required for full enzyme activity.

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