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Insensitivity of Barley Endosperm ADP-Glucose Pyrophosphorylase to 3-Phosphoglycerate and Orthophosphate Regulation

Leszek A. Kleczkowski*, Per Villand, Ernst Lüthi, Odd-Arne Olsen, and Jack Preiss

Plant Molecular Biology Laboratory, The Agricultural Research Council of Norway (NLVF), P.O. Box 51, 1432 Ås, Norway (L.A.K., P.V., E.L., O.-A.O.); and Biochemistry Department, Michigan State University, East Lansing, Michigan 48824 (J.P.)

Crude extracts of starchy endosperm from barley (*Hordeum vulgare* cv Bomi) contained high pyrophosphorolytic activity (up to 0.5 μmol of glucose-1-P formed min^{-1} mg^{-1} of protein) of ADP-glucose pyrophosphorylase (AGP) when assayed in the absence of 3-phosphoglycerate (3-PGA). This high activity was observed regardless of whether AGP had been extracted in the presence or absence of various protease inhibitors or other protectants. Western blot analysis using antibodies specific for either the small or large subunit of the enzyme demonstrated that the large, 60-kD subunit was prone to proteolysis in crude extracts, with a half-time of degradation at 4°C (from 60 to 53 to 51 kD) on the order of minutes. The presence of high concentrations of protease inhibitors decreased, but did not prevent this proteolysis. The small, 51-kD subunit of barley endosperm AGP was relatively resistant to proteolysis, both in the presence or absence of protease inhibitors. For the crude, nonproteolyzed enzyme, 3-PGA acted as a weak activator of the ADP-glucose synthetic reaction (about 25% activation), whereas in the reverse reaction (pyrophosphorolysis) it served as an inhibitor rather than an activator. For both the synthetic and pyrophosphorolytic reactions, inorganic phosphate (Pi) acted as a weak competitive or mixed inhibitor of AGP. The relative insensitivity to 3-PGA/Pi regulation has been observed with both the nonproteolyzed crude enzyme and partially purified (over 60-fold) AGP, the latter characterized by two bands for the large subunit (molecular masses of 53 and 51 kD) and one band for the small subunit (51 kD). Addition of 3-PGA to assays of the partially purified, proteolyzed enzyme had little or no effect on the K_m values of all substrates of AGP, but it reduced the Hill coefficient for ATP (from 2.1 to 1.0). These findings are discussed with respect to previous reports on the structure and regulation of higher plant AGP.

AGP (EC 2.7.7.27) is the first committed enzymic step in the biosynthetic pathway leading to starch production in all plants (reviewed in Preiss, 1991, and in Kleczkowski et al., 1991). The higher plant enzyme, which is composed of two different subunit types (Morell et al., 1987; Okita et al., 1990), is characterized by a potent activation by 3-PGA and inhibition by Pi (Sanwal et al., 1968; Preiss, 1991). The activation (3-PGA) and inhibition (Pi) constants for AGP are usually on the order of micromolar (Sanwal et al., 1968; Sowokinos, 1981; Sowokinos and Preiss, 1982; Plaxton and Preiss, 1987), and the ratio of the two effectors is believed to play a key role in the control of starch biosynthesis (Neuhaus and Stitt, 1990; Preiss, 1991). The fine regulation by 3-PGA and Pi has been demonstrated for AGP from both photosynthetic and nonphotosynthetic plant tissues (Sanwal et al., 1968; Sowokinos, 1981; Sowokinos and Preiss, 1982; Plaxton and Preiss, 1987; Preiss, 1991).

Recently, a study of a low-starch mutant of *Chlamydomonas* (Ball et al., 1991) described an AGP activity that was defective in 3-PGA/Pi regulation. The enzyme from the low-starch mutants was only weakly activated by 3-PGA (2-fold, compared with 15-fold for the wild type) and was relatively insensitive to Pi inhibition. These unexpected properties have been ascribed to a mutation in either a structural gene of AGP or a regulatory gene responsible for switching the enzyme from a 3-PGA-insensitive to 3-PGA-sensitive form (Ball et al., 1991). A lower sensitivity to 3-PGA and Pi regulation has also been observed for a proteolytically modified AGP from developing maize seeds (Plaxton and Preiss, 1987). For both the *Chlamydomonas* and maize AGP, the decrease in sensitivity to 3-PGA activation was accompanied by a severalfold decrease in specific activity. Based on these results, it has been suggested (Ball et al., 1991) that the activation of the enzyme by 3-PGA represents an absolute requirement for substantial starch biosynthesis in plants.

In the present study, we report the isolation of AGP from barley (*Hordeum vulgare*) starchy endosperm, which is relatively insensitive to both 3-PGA and Pi regulation. This evidence is accompanied by an immunological characterization of the barley enzyme, both partially purified and in crude extracts, using antibodies specific for the small and large subunits of barley AGP. A possible effect of proteolytic modification on the regulatory characteristics of barley AGP is discussed.

MATERIALS AND METHODS

Reagents

All chemicals used in the present study, unless stated otherwise, were from Sigma Chemical Co. [U-^{14}C]Glucose-1-P was from DuPont Co. (Wilmington, DE). Phosphoglucomutase (rabbit muscle) and glucose-6-P dehydrogenase (*Leuconostoc mesenteroides*) were from Sigma.

* Corresponding author; fax 47–9–941465.

Abbreviations: AGP, ADP-glucose pyrophosphorylase; 3-PGA, 3-phosphoglycerate; TPCK, tosyl phenylalanyl chloromethyl ketone.

Plant Material

Barley (*Hordeum vulgare* L. var *disticum* cv Bomi) plants were grown in the field. Grains were harvested 20 d postanthesis, and the starchy endosperm was squeezed out into liquid nitrogen. The isolated endosperm was either immediately used for experiments or was stored at −80°C. Mature potato (*Solanum tuberosum* L.) tubers were from field-grown plants.

Buffers for Extraction and Purification

Buffer A: 40 mM Mops, pH 7.4, 2 mM $MgCl_2$, 1 mM EDTA, and 2 mM DTT. Buffer B: 30 mM Mops, pH 7.4, 1 mM EDTA, 14 mM 2-mercaptoethanol, 10 mM K_2HPO_4, and 20% (w/v) sucrose. SDS buffer: 4% (w/v) SDS and 4% (v/v) 2-mercaptoethanol.

Protein Extraction and Purification

Unless otherwise indicated, all extraction and purification procedures were carried out at 0 to 4°C, and centrifugations were at 10,000*g* for 10 min.

For studies on the regulation of crude AGP, a small amount of endosperm (approximately 0.4 g) was rapidly homogenized with a chilled mortar and pestle using buffer A supplemented with protease inhibitors (see legend to Table I), followed by centrifugation at 10,000*g* for 30 s. The resulting supernatant fluid was immediately added to assays for AGP activity.

For immunological studies, small amounts of endosperm tissue (up to 0.8 g) were rapidly homogenized with a chilled mortar and pestle. Crude AGP was extracted either with buffer A alone or with buffer A supplemented with various protease inhibitors as described in the legend to Figure 2. The extracts were immediately mixed (1:1) with SDS buffer and frozen at −20°C. Extraction in the presence of TCA was followed by washes with acetone, as described by Wu and Wang (1984).

For the partial purification of AGP, barley endosperm (23 g) was homogenized using a chilled mortar and pestle with 100 mL of buffer A supplemented with 5 μM leupeptin, 1 mM benzamidine, and 1 mM aminocaproic acid. The homogenate was squeezed through one layer of Miracloth (Calbiochem, La Jolla, CA) and centrifuged. The resulting supernatant fluid was fractionated with solid ammonium sulfate, and the fraction precipitating between 29 and 45% saturation was collected by centrifugation. The pellet was resuspended in buffer B. Following centrifugation to clarify the suspension, the supernatant fluid was heated to 59°C in a water bath and maintained at this temperature for 8 min. Then the fraction was put on ice and later centrifuged. To the resulting supernatant fluid a solution of 2.5 M sodium phosphate (pH 6.7) was added to a final concentration of 1 M. The enzyme (about 10 mL) was slowly loaded on top of a 1.5 × 3 cm column of aminopropyl-agarose (Sigma) equilibrated with 1 M sodium phosphate (pH 6.7). After the column was washed with 50 mL of equilibration buffer, the enzyme was eluted with 0.75 M sodium phosphate (pH 6.7). The eluate was concentrated under pressure using a Diaflo PM30 filter (Amicon Co., Danvers, MA) and then diluted at least 200-fold with buffer B. The desalted enzyme was loaded onto a 1.5 × 5 cm column of DEAE-cellulose (Whatman DE-52) equilibrated with buffer B. Following extensive washing with buffer B, the column was eluted with a 0 to 0.3 M gradient of NaCl in buffer B (total volume, 200 mL). Fractions containing AGP activity were pooled and concentrated using the Diaflo PM30 filter. Glycerol and $MgCl_2$ were added to the enzyme to final concentrations of 40% (v/v) and 2 mM, respectively, and the preparation was stored at −20°C.

Enzyme Activity

Assay A

In the direction of ADP-glucose synthesis, assays (0.2 mL) contained 200 mM Tes, pH 8.0, 7 mM $MgCl_2$, and, unless otherwise indicated, 0.4 mM [U-^{14}C]glucose-1-P (specific radioactivity, 833 cpm/nmol) and 2.5 mM ATP. After 10 to 15 min at 37°C, the reactions were stopped by boiling for 30 to 40 s. ADP-glucose was adsorbed onto DE81 paper (Whatman International, Maidstone, England), and the adsorbed ^{14}C-radioactivity was counted by liquid scintillation spectroscopy (see Sanwal et al. [1968] for other technical details). Under these assay conditions, the activity was linear with respect to time and amount of extract added. One unit of activity is defined as the amount of enzyme required to produce 1 μmol of ADP-glucose/min at 37°C.

Assay B

The pyrophosphorolytic activity of AGP was assayed spectrophotometrically by monitoring the formation of NADH at 340 nm and 25°C. A standard assay mixture (1 mL) contained 100 mM Mops, pH 7.4, 1 mM PPi, 0.5 mM ADP-glucose, 5 mM $MgCl_2$, 2 mM DTT, 0.2 mg of BSA, 0.6 mM NAD, and 2 units each of phosphoglucomutase and glucose-6-P dehydrogenase. Control assays were run without ADP-glucose or PPi to correct for nonspecific reduction of NAD. Assays were linear with time and amount of extract added. One unit of activity is defined as the amount of enzyme required to reduce 1 μmol of NAD/min at 25°C.

Kinetic Studies

The K_m for ATP was determined from a Hill plot (see Fig. 4). The K_m values for all other substrates of AGP were determined from double-reciprocal plots using near-saturating concentrations of the nonvaried substrate (Segel, 1975). K_i values for Pi were determined by varying the Pi concentration at several fixed concentrations of ATP (synthesis reaction) or ADP-glucose (pyrophosphorolysis). In these experiments, the concentrations of glucose-1-P (synthesis) and PPi (pyrophosphorolysis) were kept constant at 0.5 and 1 mM, respectively. The K_i values of Pi for both directions of the AGP reaction were estimated from Dixon plots (Segel, 1975). Unless otherwise indicated, the assay concentration of 3-PGA was 2.5 mM.

Preparation of Antibodies and Immunological Methods

A short (17 amino acids) peptide, which was based on a conserved cDNA sequence for the large subunit of AGP from barley (P. Villand, unpublished results) and wheat endosperm (Olive et al., 1989), was custom synthesized by Multiple Peptide Systems (San Diego, CA). The peptide (Fig. 1) corresponded to amino acid numbers 428 to 444 in the full-length small subunit AGP from rice seeds (Anderson et al., 1989). The synthetic peptide was custom conjugated (Multiple Peptide Systems) through the sulfhydryl group of its N-terminal cysteine (maleimidobenzonic acid-*N*-hydroxysuccinimide ester method) to rabbit serum albumin, which served as a carrier protein for immunization. About 100-μg aliquots of the conjugate were injected into a rabbit at biweekly intervals. Before each injection, a 3- to 4-mL aliquot of blood was collected. Antibodies collected 5 months after the first immunization were used in the present study. They were precipitated with 50% saturation ammonium sulfate and then dissolved to their original volume in 100 mM Mops, pH 7.4. The final preparation was kept at −20°C.

Antibodies raised against the small subunit of spinach leaf AGP were prepared and affinity purified as described by Morell et al. (1987).

Transfer of the SDS-PAGE-resolved proteins onto nitrocellulose was carried out according to Towbin et al. (1979) using either a horizontal Multiphor Electrophoresis Unit (LKB-Pharmacia) or a Hoefer SE 600 vertical set. To decrease or eliminate nonspecific binding of the antibodies to the nitrocellulose, 0.05% (v/v) Nonidet P-40 (Sigma) was added to the antibody buffer, and 0.05% (v/v) Tween 20 (Sigma) was included in the washes following exposure to the antibodies. For the detection of the specific antigen-antibody complexes on nitrocellulose, an alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (Sigma) was used as secondary antibody, followed by histochemical staining with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium.

Other Methods

Slab-gel electrophoresis (10 or 12.5% acrylamide gels) was done according to Laemmli (1970), using a Hoefer SE 600 vertical apparatus. For molecular mass determination by SDS-PAGE, a protein marker kit from Pharmacia was used.

peptide	CIIDMNARIGRDVVISN
barley Ls	CIIDMNARIGRDVVISN
potato Ls	CIIDKNAKIGKNVSIIN
barley Ss	AIIDKNARIGYNVMIIN
potato Ss	AIIDKNARIGDNVKIIN

Figure 1. The design and sequence of the synthetic peptide that was used for production of antibodies specific for the large subunit of barley endosperm AGP. Aligned are the corresponding sequences for the large subunit (*Ls*) of AGP from barley endosperm (P. Villand, unpublished results) and potato tubers (Nakata et al., 1991), and for the small subunit (*Ss*) of AGP from barley endosperm (P. Villand, unpublished results) and potato tubers (Müller-Röber et al., 1990). Amino acids identical to those present in the synthetic peptide are shaded. The peptide sequence is 100% identical to the corresponding sequence for the large subunit of wheat endosperm AGP (Olive et al., 1989).

Table I. *Effects of 3-PGA and Pi on the activity of crude AGP from barley endosperm*

The enzyme was extracted from 0.5 g of endosperm using buffer A that was supplemented with 0.1 mM leupeptin, 1 μM pepstatin, 50 μM chymostatin, 0.5 mM TPCK, and 2.5 mM PMSF. Activities were determined immediately after extraction for both the synthetic and pyrophosphorolytic reactions.

Effector	AGP Activity			
	Synthesis[a]		Pyrophosphorolysis[b]	
	units/mg of protein	%	*units/mg of protein*	%
None	0.032	100	0.38	100
3-PGA (10 mM)	0.040	125	0.10	26
Pi (20 mM)	0.027	84	0.26	68
3-PGA + Pi	0.028	88	0.16	42

[a] Determined at 37°C. [b] Determined at 25°C.

For estimation of the molecular mass of AGP on nitrocellulose following western transfer, prestained protein standards (Bio-Rad) were used. The latter standards were precalibrated on SDS gels against the Pharmacia markers to adjust for the increase in apparent mass due to the attached dye molecules.

Determination of protein was done using the Bio-Rad Protein Assay, with BSA as standard.

RESULTS

Activity in Crude Extracts

Extracts of barley endosperm were found to contain high pyrophosphorolytic activity of AGP (Table I) when assayed in the absence of 3-PGA, an essential activator of higher plant AGP (Sanwal et al., 1968; Preiss, 1991). Rates of up to 0.5 μmol of glucose-1-P formed min^{-1} mg^{-1} of protein (25°C) have been determined for several preparations of crude barley endosperm AGP. In the direction of synthesis of ADP-glucose, 3-PGA caused only 25% activation of AGP, whereas in the reverse direction (pyrophosphorolysis) 3-PGA acted as an inhibitor rather than an activator. For the pyrophosphorolytic reaction, a range of 3-PGA concentrations from 0.01 to 10 mM had either no effect (up to 0.2 mM) or inhibited the crude enzyme, both in the presence or absence of Pi (see Table I, and data not shown). Pi, a well-known inhibitor of AGP (Sanwal et al., 1968; Preiss, 1991), was relatively ineffective in inhibiting both barley AGP reactions. Under assay conditions of near-saturating concentrations of ATP or ADP-glucose, 20 mM Pi caused only about 15 and 30% inhibition of the forward and reverse reactions, respectively (Table I). The relative insensitivity of the barley endosperm enzyme to Pi and 3-PGA was also observed (data not shown) using a radiometric assay based on [^{32}P]PPi conversion to [^{32}P]ATP (Sanwal et al., 1968).

The high pyrophosphorolytic activity of barley AGP and its relative insensitivity to 3-PGA and Pi regulation have been observed regardless of the presence or absence of protease inhibitors in the extraction medium (see Table I and below). Addition of 2 mM NaF (phosphatase inhibitor) and/or desalting of the crude extracts on Sephadex G-25 also had no effect on AGP activity. The activity of the crude enzyme and its relative insensitivity to effectors remained stable for at least 3 h, whether stored at 0 to 4°C or room temperature.

Effects of Extraction Conditions on Structural Integrity of AGP Protein

The structural properties of barley endosperm AGP have been studied by western immunoblotting analysis using antibodies specific for the small and large subunits of AGP. The small subunit antibodies were raised against the 51-kD subunit of spinach leaf AGP (Morell et al., 1987), and antibodies against the large subunit were raised against a synthetic peptide based on a 51-nucleotide-long region of a cDNA encoding the large subunit of barley endosperm AGP (see "Materials and Methods" and Fig. 1).

When the endosperm was homogenized with TCA (or SDS buffer), electrophoresis of crude proteins, followed by immunodetection with specific antibodies (Fig. 2, lanes at 0 time), resulted in a single band at 51 kD reacting with the antibodies against the small subunit of AGP and a band at 60 kD that was recognized by the large subunit antibodies. Extraction of the enzyme under nondenaturing conditions, however, resulted in a rapid rate of endogeneous proteolysis

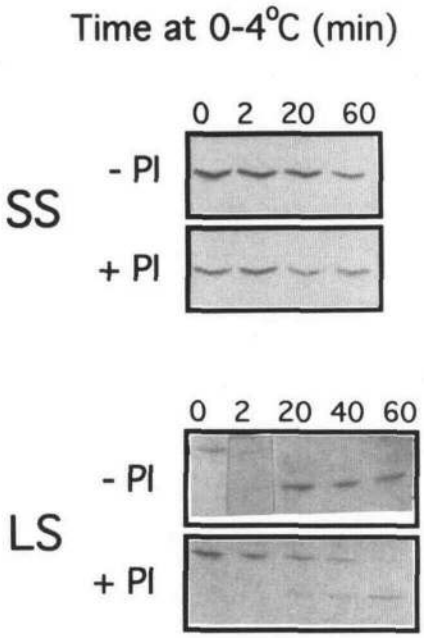

Figure 2. *Effects of extraction conditions on immunoblotting patterns of crude AGP from barley endosperm.* Proteins were extracted from barley endosperm following homogenization in buffer A alone (−PI) or buffer A that was supplemented with 0.1 mM TPCK, 0.1 mM leupeptin, 8 μM chymostatin, 1 μM pepstatin, and 1 mM PMSF (+PI). Proteins were also extracted (lanes at time 0) with TCA, as described by Wu and Wang (1984). Extracts were centrifuged for 30 s at 10,000g and then incubated at 0 to 4°C for the times shown. Incubations were terminated by dilution of the extracts into SDS buffer (1:1). The proteins were resolved by SDS-PAGE (10% polyacrylamide gel) and then transferred to nitrocellulose for immunostaining with the antibodies against the small (SS) or large (LS) subunit. In each case, the amount of crude protein loaded on the gel was approximately 100 μg. PI, Protease inhibitors.

of the large subunit from a 60- to 53-kD band. In buffer A lacking protease inhibitors, the half-time of degradation of the large subunit was approximately 1 to 2 min at 0 to 4°C (Fig. 2). Supplementation of the extraction buffer with TPCK (inhibitor of chymotrypsin-like serine proteases), leupeptin (inhibitor of trypsin-like serine and some cysteine proteases), chymostatin (inhibitor of chymotrypsin-like serine and some cysteine proteases), pepstatin (inhibitor of some aspartic proteases), and PMSF (inhibitor of serine proteases) decreased but did not prevent large-subunit proteolysis, with a half-time of degradation of approximately 40 min (Fig. 2). This proteolysis could not be prevented by addition of 5 mM EDTA, by extraction with 20% saturation ammonium sulfate followed by precipitation between 20 and 60% saturation ammonium sulfate, nor by addition of 2 mg/mL BSA or casein to buffer A (data not shown). For preparations isolated in buffer A alone, the appearance of the 53-kD peptide was followed by a slow accumulation of a 51-kD band. After storage at 0 to 4°C for 3 to 4 h, the 51- and 53-kD bands were about equally intense and their relative abundance changed little with storage at −20°C or during purification of AGP.

Compared with the large subunit, the rate of proteolysis of the small subunit of AGP was much lower (Fig. 2, upper panel). Only after several hours of incubation at 0 to 4°C was the appearance of a 49-kD band (in addition to the 51-kD polypeptide) observed (data not shown). When Nonidet 40, a nonionic detergent used during immunoblotting to decrease nonspecific interactions, was omitted from the antibody buffer during immunodetection of AGP in crude and partially purified preparations, the antibodies against the small subunit recognized an additional band of about 58 kD. Whether this protein is functionally related to AGP is unknown at present.

Physical Properties

Barley endosperm AGP was partially purified over 60-fold to a specific activity (pyrophosphorolysis) of 29 units/mg of protein (Table II). The final yield of about 15% was most affected by the ammonium sulfate precipitation procedure, where only 35% of the initial activity was recovered. However, this step allowed concentration of the enzyme and stabilized its activity, to some extent, during subsequent heat treatment (data not shown). The heat stability characteristics and the ability of the enzyme to bind to anion-exchange and hydrophobic chromatography matrices have previously been utilized for purification of AGP from several plant tissues (Sanwal et al., 1968; Dickinson and Preiss, 1969; Sowokinos and Preiss, 1982; Plaxton and Preiss, 1987; Okita et al., 1990; Ball et al., 1991).

SDS-PAGE analysis of the final preparation revealed a protein band at about 85 kD and four or five bands of about 47 to 53 kD (Fig. 3, lane A). Immunoblotting with antibodies against the small subunit of AGP revealed one band of about 51 kD (Fig. 3, lane B), and the use of antibodies directed against the large subunit indicated two immunologically related proteins at about 51 and 53 kD (Fig. 3, lane C). These data are consistent with the immunoblotting evidence presented for the crude enzyme (Fig. 2), where the small subunit

Table II. *Partial purification of AGP from barley starchy endosperm*
Activities were determined by monitoring pyrophosphorolysis of ADP-glucose (assay B) at 25°C.

Step	Protein	Total Units	Specific Activity	Yield	Purification
	mg	*µmol/min*	*units/mg of protein*	*%*	*-fold*
Crude extract	235	101	0.43	100	1.0
$(NH_4)_2SO_4$ fractionation	117	35	0.30	35	0.7
Heat treatment	16.5	28	1.7	28	4.0
Aminopropyl-agarose	1.8	22	12.2	22	28
DEAE-cellulose	0.5	14	29.1	14	68

(51 kD) was relatively resistant to proteolysis and the large subunit-derived 53-kD band was observed shortly after extraction, followed by the slow accumulation of a 51-kD protein (data not shown).

Although the immunoblot probed with antibodies against the large subunit yielded strong signals for barley endosperm AGP, there was no immunorecognition of AGP from potato tubers (data not shown). This is not surprising, however, because the antibodies were directed against a specific region of the large subunit of the barley protein. This region does differ from the corresponding sequences of the small and large subunits of potato AGP, and from the small subunit of the barley endosperm enzyme (Fig. 1).

Effects of 3-PGA and Pi, and Kinetic Characteristics

In the direction of synthesis of ADP-glucose, substrate kinetics with ATP (in the absence of Pi and 3-PGA) were sigmoidal, with a Hill coefficient of 2.1 (Fig. 4). Another feature of the kinetics with ATP was substrate inhibition at concentrations of ATP exceeding 1 mM. This phenomenon was not observed when 3-PGA and/or Pi were included in the reaction mixtures (Fig. 4). 3-PGA caused about 30% activation of the synthetic reaction of the partially purified AGP, which compares with the 25% activation observed with the crude enzyme (Table I). Addition of 3-PGA changed the sigmoidal kinetics with respect to ATP ($n = 2.1$) to a hyperbolic response ($n = 1.0$) and decreased the K_m for ATP (Fig. 4, Table III). However, with Pi in the assays, the kinetics were sigmoidal ($n = 1.9$) in either the presence or absence of 3-PGA (Fig. 4). Pi, which was found to serve as a competitive or competitive-mixed inhibitor versus ATP (data not shown), diminished the activating effect of 3-PGA (Fig. 4), similar to its effect on crude AGP (Table I).

For the pyrophosphorolytic reaction, both 3-PGA and Pi served as relatively weak inhibitors of the partially purified enzyme (Fig. 5). 3-PGA at 10 mM caused about a 45% inhibition of the enzyme, and 24 mM Pi decreased the activity

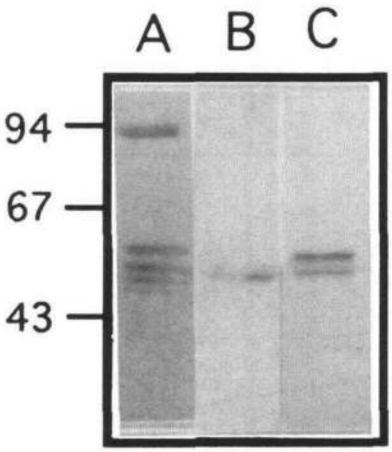

Figure 3. SDS-PAGE and immunoblot analyses of partially purified AGP from barley endosperm. The partially purified preparation (Table II) was subjected to SDS-PAGE, followed either by staining with Coomassie brilliant blue G (Sigma) or transfer to a nitrocellulose filter for immunoblotting. Lane A, SDS-PAGE of AGP (7 µg of protein). Lane B, Immunoblot of AGP (7 µg of protein) probed with antibodies against the small subunit of AGP; Lane C, Immunoblot of AGP (7 µg of protein) probed with antibodies against the large subunit of AGP. Values (kD) are the estimated positions of protein markers (Pharmacia).

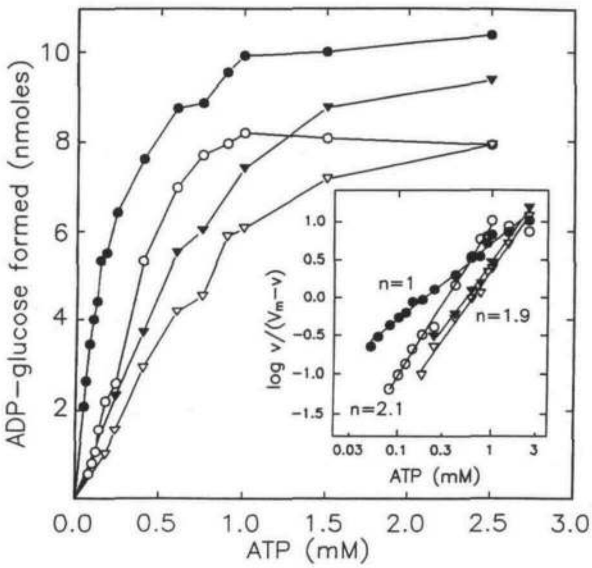

Figure 4. Substrate kinetics with respect to ATP and the mode of regulation by 3-PGA and Pi of partially purified barley endosperm AGP. Activities were determined by assay A (synthesis) using 0.5 mM glucose-1-P. Concentrations of 3-PGA and Pi were 2.5 and 6 mM, respectively. Inset represents Hill plots (Segel, 1975) of the data. ○, −3-PGA, −Pi; ●, +3-PGA, −Pi; ▽, −3-PGA, +Pi; ▼, +3-PGA, +Pi.

Table III. *Kinetic constants of partially purified AGP from barley endosperm*

Kinetic Constant	Direction of AGP Reaction	
	Synthesis	Pyrophosphorolysis
	mM	
K_i (Pi)	2.1	2.3
K_m (ATP		
−3-PGA	0.31[a]	
+3-PGA	0.19	
K_m (Glc-1-P)		
−3-PGA	0.12	
+3-PGA[b]	0.12	
K_m (ADP-glucose)		
−3-PGA		0.13
+3-PGA[b]		0.14
K_m (PPi)		
−3-PGA		0.027
+3-PGA[b]		0.029

[a] Sigmoidal kinetics (n = 2.1 [see Fig. 4]). [b] 3-PGA at 2.5 mM.

by 60%. Pi acted as a linear competitive inhibitor versus ADP-glucose (data not shown); thus, the extent of inhibition by a given concentration of Pi depended greatly on the level of ADP-glucose. The inhibitory effect of 3-PGA was weaker for the partially purified AGP when compared with the enzyme from crude extracts (see Table I). The presence of Pi in the reaction mixtures containing 3-PGA had no marked effect on the extent of inhibition seen with 3-PGA alone. At all 3-PGA concentrations examined (0.2–10 mM), there was no indication of any activation of pyrophosphorolytic rates of barley AGP (Fig. 5).

With the exception of the kinetics with respect to ATP, the K_m values of the other substrates of AGP were not affected by 3-PGA (Table III). Saturation curves with these substrates were hyperbolic (n = 1.0), regardless of the presence or absence of 3-PGA (data not shown).

DISCUSSION

We report here the isolation and properties of barley endosperm AGP, which is relatively insensitive to 3-PGA/Pi regulation. The endosperm enzyme is composed of two subunit types of 51 and 60 kD (nonproteolyzed), which compares favorably with the values of about 50 to 56 kD (small subunit) and 51 to 60 kD (large subunit) reported for AGPs from other tissues (Sowokinos and Preiss, 1982; Morell et al., 1987; Plaxton and Preiss, 1987; Anderson et al., 1989; Preiss et al., 1989; Okita et al., 1990; Nakata et al., 1991; Nakamura and Kawaguchi, 1992; Smith-White and Preiss, 1992). The enzyme has high pyrophosphorolytic activity in crude extracts when assayed without 3-PGA and Pi (Table I); this activity is comparable to or higher than that previously reported for the 3-PGA-activated crude AGP from potato tubers (Sowokinos and Preiss, 1982; Okita et al., 1990), maize seeds (Plaxton and Preiss, 1987), or leaves of several species (Sanwal et al., 1968). The pyrophosphorolytic activity in the absence of effectors is about 4-fold higher than previously reported for the barley endosperm AGP (which was assayed with 10 mM 3-PGA) (Schulman and Ahokas, 1990).

Of particular interest is the effect of 3-PGA, which only weakly activates the rate of synthesis of ADP-glucose by barley endosperm AGP and serves as an inhibitor, rather than activator, for the pyrophosphorolytic reaction (Table I, Figs. 4 and 5). The lack of activation by 3-PGA has not previously been reported for any plant AGP, although it has been noted that maize seed AGP that had its small subunit proteolytically modified showed weaker activation when compared with the intact maize enzyme (Dickinson and Preiss, 1969; Plaxton and Preiss, 1987). However, the effect of 3-PGA was still very appreciable for the modified maize AGP, with rates stimulated by 5- and 2-fold for the synthetic and pyrophosphorolytic reactions, respectively (Dickinson and Preiss, 1969; Plaxton and Preiss, 1987). The 3-PGA-dependent activation of the pyrophosphorolytic reaction was most prominent for crude maize AGP (4- to 5-fold), and the sensitivity to 3-PGA decreased during purification of the protein (Dickinson and Preiss, 1969). In our hands, the crude barley enzyme was only 25% activated by 3-PGA for the synthetic reaction and was inhibited by this effector in the pyrophosphorolysis direction. The inhibitory effect of 3-PGA on barley AGP was weaker for the partially purified enzyme (Fig. 5) when compared with crude AGP (Table I), perhaps due to a different sensitivity of the enzyme following proteolysis of the 60-kD large subunit. On the other hand, the activating effect of 3-PGA on the synthetic reaction was similar for both the crude and partially purified enzymes (25 and 30% activation, respectively) (Table I, Fig. 4). Inclusion of 3-PGA changed the sigmoidal kinetics with respect to ATP to a hyperbolic response (Fig. 4), similar to its effect on AGP from maize seeds (Dickinson and Preiss, 1969; Plaxton and Preiss, 1987). Although relatively insensitive to 3-PGA regulation, the barley enzyme has substrate K_m values (Table III) that are similar to or lower than those previously reported for the 3-PGA-activated AGP from other plant tissues (Sowokinos, 1981; Sowokinos and Preiss, 1982; Plaxton and Preiss, 1987; Preiss, 1991).

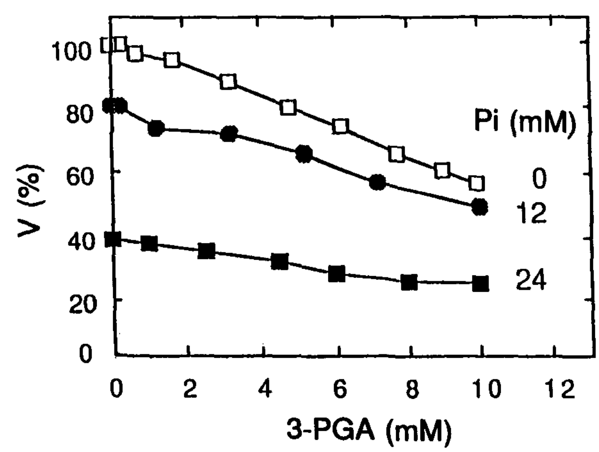

Figure 5. Inhibitory effects of 3-PGA and Pi on the pyrophosphorolytic reaction of partially purified barley endosperm AGP. Activities were determined using assay B. Concentrations of ADP-glucose and PPi were 0.35 and 1 mM, respectively. The 3-PGA concentration varied from 0.2 to 10 mM.

The properties of barley endosperm AGP, such as its relative insensitivity to effectors (Table I, Fig. 4), the inhibitory effect of 3-PGA on the pyrophosphorolysis reaction (Table I, Fig. 5), and the low K_m values in the absence of 3-PGA (Table III), clearly differentiate this AGP from the corresponding enzyme reported from other plant tissues. Whether these properties are intrinsic to the intact enzyme or are the result of its proteolytic modification is unclear at present. The activity of crude AGP, whether isolated in the absence or presence of protease inhibitors, is stable for several hours, even though immunoblotting studies indicated a high rate of proteolysis of the 60-kD large subunit (Fig. 2). The partially purified enzyme shows a relatively high specific activity (pyrophosphorolysis) of up to 29 units/mg of protein, which is only 2- to 3-fold lower than that for the 3-PGA-activated homogeneous, or near homogeneous, AGPs from spinach leaves (Morell et al., 1987) and potato tubers (Okita et al., 1990). Judging from the relative intensities of the stained protein bands following SDS-PAGE of the partially purified preparation (Fig. 3), AGP represents about one-third of the total protein in the analyzed sample. These two observations suggest that proteolytic modification of the large subunit has no appreciable effect on the specific activity (pyrophosphorolysis) of the endosperm enzyme. However, the possibility cannot be excluded that this proteolysis results in an enzyme "fixed" in an activated or partially activated state, characterized by a high specific activity without 3-PGA. Obviously, changes in the regulatory characteristics of the proteolytically modified enzyme would be irreversible, in contrast with the reversible effect of 3-PGA. Such a fixation in the activated state would be consistent with the high activity of barley AGP when measured in the absence of 3-PGA, and with little or no effect of 3-PGA on the K_m values of the enzyme (Table III).

In contrast with the enzyme from barley endosperm, AGP from barley leaves was reported to be activated by 3-PGA and strongly inhibited by Pi (Sanwal et al., 1968). We have confirmed these results using extraction and assay conditions similar to those used for the barley endosperm enzyme (data not shown). This evidence supports our recent finding (Villand et al., 1992), based on analysis of cDNAs from barley tissues, that different AGP genes are expressed in the endosperm and leaves of this species.

The present report, similar to a previous study by Plaxton and Preiss (1987) on maize seed AGP, underscores the necessity for careful examination of the extraction conditions for plant AGP. In the case of maize AGP (Plaxton and Preiss, 1987; Preiss et al., 1989), the authors studied degradation of the small subunit of the enzyme. For barley AGP, the small subunit is relatively resistant to proteolytic degradation and the large subunit is most affected, with a half-time for proteolysis at 0 to 4°C (60- to 53-kD band) on the order of minutes, even in the presence of various protease inhibitors (Fig. 2). Proteolysis of the large subunit of barley AGP could not be prevented by extraction in the presence of PMSF or chymostatin (inhibitors protecting against the degradation of the small subunit of maize AGP [Plaxton and Preiss, 1987]), nor by the use of other protectants. It is notable that in a recent report on the purification of rice seed AGP (Nakamura and Kawaguchi, 1992), the authors postulated the presence of multiple forms of AGP, characterized by small differences in mol wt of the component subunits. Because the extraction buffer in this latter study contained only 0.5 mM PMSF as a protease inhibitor, it is likely that the multiple bands are indicative of proteolytic products of AGP rather than of intact polypeptides. As pointed out by Wu and Wang (1984), extraction in the presence of denaturing agents, e.g. TCA or SDS, may be the only reliable way to examine the structural integrity of some proteins, especially in tissues known to contain potent proteolytic activities, such as seeds or flowers. Because of the documented sensitivity of plant AGP to proteolysis, based on our own evidence for the barley endosperm enzyme and that of Plaxton and Preiss (1987), any study of the properties of AGP from plant tissues should be accompanied by a careful analysis of the isolation conditions and a comparison of the intactness of both the large and small subunits from crude and purified preparations of AGP.

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