Plant Physiol. (1992) 99, 1626–1634
0032-0889/92/99/1626/09/$01.00/0

Received for publication January 2, 1992
Accepted March 17, 1992

The *rb* Mutation of Peas Causes Structural and Regulatory Changes in ADP Glucose Pyrophosphorylase from Developing Embryos[1]

Christopher Hylton and Alison M. Smith*

John Innes Institute, John Innes Centre for Plant Science Research, Colney Lane, Norwich NR4 7UH, United Kingdom

ABSTRACT

A mutation at the *rb* locus of pea (*Pisum sativum* L.) alters the shape, reduces the starch content, and increases the lipid and sucrose contents of the seed. These effects are probably all consequences of a reduction of up to 40-fold in the maximum catalytic activity of ADP glucose pyrophosphorylase in the developing embryo of the mutant relative to the wild type. We have investigated how the mutation brings about this reduction in activity. The purified enzyme from mutant embryos has a specific activity about 10-fold lower than that from wild-type embryos, and it is much more sensitive to the effectors inorganic phosphate and 3-phosphoglycerate than the wild-type enzyme. Both wild-type and mutant enzymes consist of polypeptides of around 50 kilodaltons. One of the polypeptides of the purified wild-type enzyme is missing from the mutant enzyme. We deduce that in the wild-type embryo this protein may interact with other subunits to confer a high specific activity and a low susceptibility to effectors on the enzyme.

The aim of this work was to investigate the effect of a mutation at the *rb* locus of peas (*Pisum sativum* L.) on the activity of ADP glucose pyrophosphorylase in developing embryos. The mutation has multiple phenotypic effects on the seed. It changes its shape from round to wrinkled, reduces starch content from 50 to about 25% of the final dry weight, and increases sucrose and lipid contents from 5 to 9% and 2 to 4%, respectively (4, 8, 25). These effects are very similar to those of the well-characterized mutation at the *r* locus of pea, which are consequences of a reduction in the rate of starch synthesis in the developing embryo resulting from a reduced activity of starch-branching enzyme (1, 8, 22). We have shown previously that the mutation at the *rb* locus results in a 10- to 40-fold reduction in the activity of ADP glucose pyrophosphorylase during embryo development, and has no effect on the activities of other enzymes catalyzing the conversion of sucrose to starch (23). This reduction almost certainly accounts for the reduced rate of starch synthesis (23) and, hence, the reduced starch content of the embryo. By analogy with the effects of the mutation at the *r* locus, it is likely that the reduced rate of starch synthesis accounts for many, if not all, of the other phenotypic effects. To discover how the *rb* mutation reduces the activity of ADP glucose pyrophosphorylase, we have studied the properties of the enzyme purified from developing embryos of two mutant and one wild-type line of peas. One of the mutant lines is near-isogenic to the wild-type line, hence, any differences between the two lines are most likely to be effects of the *rb* locus.

[1] This work was funded by a grant-in-aid from the Agriculture and Food Research Council to the John Innes Institute.

MATERIALS AND METHODS

Plant Material

One line of pea (*Pisum sativum* L.) of genotype *RbRb* and two of genotype *rbrb* were used. The *RbRb* line was BC1/9 RR (developed from JI 430 [John Innes Germplasm Collection] according to ref. 7). The *rbrb* lines were JI 399 (John Innes Germplasm Collection), and a line BC2/1 rbrb, developed from the near-isoline BC1/9 RR (C. Hedley, T. Wang, John Innes Institute, unpublished data). Plants were grown either in a controlled-environment room or in a greenhouse. Freshly harvested developing embryos were used in all experiments.

Assay of ADP Glucose Pyrophosphorylase (EC 2.7.7.27)

Glucose 1-Phosphate Synthesis

The production of glucose 1-phosphate was assayed spectrophotometrically at 25°C (23). The assay contained 75 mM Hepes (pH 7.9), 1.5 mM Na PPi, 5 mM $MgCl_2$, 0.8 mM NAD, 1 mM ADP glucose, 3 units glucose 6-phosphate dehydrogenase (NAD-linked, from *Leuconostoc mesenteroides*), 1 unit phosphoglucomutase and 7- to 35-μL of extract in a final volume of 700 μL. The activity was measured as NAD reduction dependent upon the presence of both ADP glucose and PPi. The rate was proportional to the amount of extract added and was linear for at least 5 min.

ADP Glucose Synthesis

Radioactive Assay. The assay contained 80 mM Hepes (pH 7.9), 1 mM ATP, 5 mM $MgCl_2$, 0.5 units inorganic pyrophosphatase, and 1 mM [U-^{14}C]glucose 1-phosphate at 35 GBq·mol^{-1}. Reactions were started by the addition of purified enzyme (in 100 mM Hepes [pH 7.9], 1 mM EDTA, 1 mM DTT, 5% [v/v] ethanediol), incubated for 10 min at 25°C, then terminated by heating to 100°C for 3 min. Glucose 1-phos-

phate was hydrolyzed and the radioactivity in ADP glucose was determined according to ref 6.

Spectrophotometric Assay. Production of PPi was linked to oxidation of NADH via PPi-dependent phosphofructokinase, aldolase, and glycerol 3-phosphate dehydrogenase, and monitored at 25°C and 340 nm. The assay contained, in a final volume of 1 mL, 70 mM Hepes (pH 7.9), 1 mM ATP, 5 mM $MgCl_2$, 5 mM fructose 6-phosphate, 1 mM glucose 1-phosphate, 0.18 mM NADH, 0.1 unit PPi-dependent phosphofructokinase (Sigma Chemical Co., Poole, Dorset, UK, from *Proprionibacterium freudenreichii*), 1 unit aldolase, 10 units triose phosphate isomerase, 0.3 unit glycerol 3-phosphate dehydrogenase, and 2 to 5 μL of purified enzyme. The rate was proportional to the amount of enzyme added up to approximately 0.008 units, and was linear with respect to time for at least 3 min. This assay is suitable only for highly purified enzyme.

Purification of ADP Glucose Pyrophosphorylase

Preparation of Crude Extract

About 80 g of embryos (350–600 mg fresh weight each) were homogenized with 160 mL of ice-cold medium A (50 mM KPi [pH 7.0], 20% [v/v] ethanediol, 1 mM DTT, 1 mM EDTA [disodium salt]) in a Waring blender. The homogenate was squeezed through four layers of cheesecloth and centrifuged for 10 min at 10,000*g*, 4°C. All subsequent steps were carried out at 4°C.

PEG Fractionation

A solution of PEG (average mol wt 6000, 50% [w/v] in medium A without DTT) was added to the supernatant to a final PEG concentration of 15%, stirred for 20 min, then centrifuged at 40,000*g* for 30 min. The pellet was discarded.

DEAE-Sepharose Chromatography

The supernatant was mixed with approximately 100 mL of a thick slurry of DEAE-Sepharose Fast Flow (Pharmacia, Uppsala, Sweden) equilibrated with medium B (as medium A but with 10% ethanediol), and shaken gently for 30 min. The Sepharose was washed on a Buchner funnel with five 100-mL aliquots of medium B, then poured into a column (2.6 cm i.d.) and eluted with a 100-mL gradient of 0 to 1 M KCl in medium A at a flow rate of 1 mL·min^{-1}. Fractions of 3 mL were collected and assayed. The five or six fractions with the highest activities were pooled and dialyzed overnight against 2 L of medium B.

Blue Sepharose Chromatography

The dialysate was applied to a column (16 cm long, 1.5 cm i.d.) of Blue Sepharose equilibrated with buffer B. The column was washed with 35 mL of medium B, then eluted with a 60-mL gradient of 0 to 1 M KCl in buffer B at a flow rate of 1 mL·min^{-1}. Fractions of 2 mL were collected and assayed. The four or five fractions with the highest activities were pooled and diluted to 50 mL with medium B.

Mono Q Chromatography

The diluted fractions were applied to a Mono Q anion-exchange column (Pharmacia Fast Protein Liquid Chromatography system) equilibrated with medium B. The column was washed with 10 mL of medium B, then eluted with a 50-mL gradient of 0 to 0.5 M KCl in medium B at a flow rate of 1 mL·min^{-1}. Fractions of 1 mL were collected and assayed. The fraction with the highest activity is referred to as the purified enzyme.

Superose 12 Chromatography

Samples of 0.2 mL of purified enzyme were applied to a column of Superose 12 (Pharmacia Fast Protein Liquid Chromatography system) and eluted at a flow rate of 0.3 mL·min^{-1}. In some experiments, the sample was in medium B and the column was equilibrated and eluted with medium B that contained 0.15 M NaCl. In other experiments, the column was equilibrated and eluted with 50 mM Bis-Tris propane (pH 7.4), 0.15 M NaCl, 10% (v/v) ethanediol, 1 mM DTT, 1 mM EDTA, and the sample was desalted on a column of Sephadex G-25 equilibrated with 50 mM Hepes (pH 7.4), 10% (v/v) ethanediol, 1 mM DTT, 1 mM EDTA prior to application.

Gel Electrophoresis and Immunoblotting

SDS-PAGE

All samples were dialyzed against water, freeze-dried, and dissolved in the appropriate sample buffer prior to electrophoresis. Proteins were separated by SDS-PAGE as previously described (10, 23) and were either stained with Coomassie brilliant blue R or electroblotted onto nitrocellulose filters.

Two-Dimensional Electrophoresis

Freeze-dried, purified enzyme was dissolved in isoelectric focusing sample buffer (15) at a concentration of 1 mg·mL^{-1} and samples containing 15 μg of protein were focused in isoelectric focusing tube gels as in ref. 15 except that the ampholine concentration was 1.48% (v/v), pH range 5 to 8, and 0.52% (v/v), pH range 3 to 10. The second dimension was SDS-PAGE on gels 120 mm long, 1 mm thick.

Native Gel Electrophoresis

Samples were applied in 100 mM Tris (pH 7.0), 10% (v/v) glycerol. Gels (7.5% polyacrylamide) and electrophoresis buffer were exactly as for SDS-PAGE except that SDS was omitted. Gels were run at 4°C.

Immunoblotting

Nitrocellulose filters were incubated either with crude rabbit serum or with the immunoglobulin fraction of rabbit serum followed by alkaline-phosphatase-conjugated goat anti-rabbit antiserum (Sigma, Poole, Dorset, UK) as in Blake *et al.* (2), except that the initial blocking step contained 3% (w/v) BSA and 2% (w/v) dried milk powder, and both incubations with antisera contained 3% (w/v) BSA.

Preparation of Antiserum

The purified enzyme was subjected to two-dimensional electrophoresis, and, after staining with Coomassie brilliant blue R, the polypeptide unique to the enzyme from *RbRb* embryos was excised from the gel. Pieces containing this polypeptide from several gels were pooled and electroeluted. The protein was freeze-dried and redissolved in 0.5 mL of PBS, mixed with 0.5 mL of Freund's complete adjuvant, and administered subcutaneously into a rabbit after collection of preimmune serum. This immunization was repeated 2 and 8 weeks later. A total of approximately 75 μg of protein was used to immunize the rabbit. Antisera were collected from 3 weeks after the third injection and the immunoglobulin fraction was purified (1).

Antiserum to ADP glucose pyrophosphorylase holoenzyme from spinach leaf (13) was the kind gift of Prof. J. Preiss, Michigan State University.

Immunoprecipitation of Enzyme Activity

A sample of 0.03 unit of ADP glucose pyrophosphorylase activity (as a 15% [w/v] PEG fraction of an extract of BC1/9 RR embryos, see above) was mixed with 3 mg of preswollen protein A Sepharose (at 62.5 $mg \cdot mL^{-1}$ in 20 mM Tris, pH 7.2) and 10 μL of serum containing varying amounts of antiserum diluted into preimmune serum, in a final volume of 0.25 mL. The mixture was incubated for 2 h at room temperature on a rotating wheel, then centrifuged at 12,000*g* for 5 min at 4°C. The supernatants were assayed for ADP glucose pyrophosphorylase.

RESULTS

Purification of the Enzyme

Attempts to purify the enzyme with methods involving its precipitation with either ammonium sulfate or PEG were unsuccessful. At least 70% of enzyme activity was lost during precipitation steps, and the preparation lost a large proportion of its remaining activity during subsequent ion-exchange chromatography. However, when the procedure did not involve precipitation of the enzyme, recovery of activity was good and losses during ion-exchange chromatography were small. The enzyme was considerably more stable in phosphate than in organic buffers, and it could be stored for several days at 0°C without loss of activity.

ADP glucose pyrophosphorylase from maize endosperm is subject to proteolytic degradation during purification unless the protease inhibitors chymostatin and PMSF are present throughout the purification procedure (18). Inclusion of these inhibitors at 10 $\mu g \cdot mL^{-1}$ and 1 mM, respectively, did not affect the specific activity, kinetic properties, size and number of putative subunits, and recovery through extraction of the enzyme from pea embryos. Inclusion of the inhibitors pepstatin and leupeptin in the early stages of purification (both at 1 $\mu g \cdot mL^{-1}$) also had no effect on the recovery and kinetic properties of the enzyme. To check further the possibility that the enzyme is subject to proteolysis during extraction, embryos were extracted in an SDS-containing gel sample buffer (10). Proteolysis should be prevented by this treatment. The size, number, and relative intensity of bands revealed by SDS-PAGE on long, one-dimensional gels and immunoblotting of this extract (not shown) was exactly the same as for extracts prepared without SDS (see Fig. 2). This was true for both *RbRb* and *rbrb* embryos. These experiments suggest that our results are not affected by proteolysis of the enzyme during extraction and purification.

The enzyme was purified 400- to 1000-fold from both *RbRb* and *rbrb* embryos (range of four purifications). The purified enzyme from *RbRb* embryos had a specific activity of 39 to 58 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein (range of four purifications). In contrast, the enzyme from *rbrb* embryos of both JI 399 and the near-isogenic line had a specific activity of only 2 to 5.7 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein (range of four purifications). Typical purifications are shown in Table I and Figure 1.

Physical Properties of the Enzyme

After gel filtration chromatography, preparations of the purified enzyme from both *RbRb* and *rbrb* embryos consisted almost exclusively of polypeptides of about 50 kD (Fig. 1). On long (120 mm) SDS-polyacrylamide gels the *RbRb* enzyme resolved into two bands of protein, differing in apparent size by about 1 kD (Fig. 2). These could be further resolved into four polypeptides with isoelectric points in the pH range of 6.4 to 7.2 by two-dimensional electrophoresis. Although the precise relative positions of the polypeptides on two-dimensional gels varied slightly with experimental conditions, we emphasize that four polypeptides were observed in all of the preparations of the enzyme examined. Typical results from separate experiments on separate preparations of the enzyme are shown in Figure 3, A and D. In contrast, purified *rbrb* enzyme from both JII 399 and the near-isogenic line appeared as only one band of protein on long SDS-polyacrylamide gels (Fig. 2), and was resolved by two-dimensional electrophoresis into only three polypeptides. These three polypeptides were identical in isoelectric point and size to three of the polypeptides of the *RbRb* enzyme. We emphasize that one polypeptide that was always associated with the *RbRb* enzyme was never present in preparations of the *rbrb* enzyme. Typical results from two independent preparations of the *rbrb* enzyme from JII 399 and the near-isogenic line are shown in Figure 3, B and C.

On native gels, the purified *RbRb* enzyme appeared as a single band of protein (not shown). When this band was eluted from the gel and subjected to SDS-PAGE, all of the protein appeared as a band of about 50 kD (not shown). The purified native enzyme from both *RbRb* and *rbrb* embryos behaved as a dimer when subjected to gel filtration chromatography in phosphate-buffered media on a column of Superose 12 (Fig. 4). The apparent molecular mass of the enzyme, from estimates made on three separate preparations, was 110.5 ± 1.5 kD for *RbRb* preparations and 108.5 ± 3.3 kD for *rbrb* preparations (means ± SE). The apparent molecular mass of the purified enzyme and the stability of its activity were reduced when the enzyme was stored in phosphate-free media. Activity was stable for at least 48 h at 0°C in media that contained 50 mM phosphate, but declined by at least 50% in 24 h in phosphate-free media. This decline

Table I. *Purification of ADP Glucose Pyrophosphorylase from Developing Pea Embryos*

ADP glucose pyrophosphorylase was purified from about 80 g of freshly harvested embryos (350–600 mg fresh weight). Activity and protein were measured in the supernatant from the crude extract and after each stage of the purification. Samples from each stage were desalted on a column of Sephadex G-25 equilibrated with 50 mM Hepes (pH 7.9), 10% (v/v) ethanediol, 1 mM EDTA, and 1 mM DTT immediately prior to assay. Values for the DEAE-Sepharose step were measured after the dialysis that followed the step. Values for the Mono Q step are for the fraction that contained the highest activity. Purification at each step is the factor by which specific activity is increased relative to initial specific activity. A, Purification from embryos of the *rbrb* line JI 399. B, Purification from embryos of the *RbRb* line BC1/9 RR.

Stage	Total Protein	Total Activity	Specific Activity	Purification
	mg	*μmol·min⁻¹*	*μmol·min⁻¹·mg⁻¹ protein*	*-fold*
A				
Crude extract	1994	5.75	0.003	
PEG fractionation	746	9.70	0.011	3.7
DEAE-Sepharose	58	8.33	0.145	48
Blue Sepharose	2.9	2.82	0.98	326
Mono Q	0.38	0.94	2.47	823
B				
Crude extract	1614	240	0.15	
PEG fractionation	551	330	0.60	4.0
DEAE-Sepharose	57	313	5.45	36
Blue Sepharose	3.4	110	32.6	217
Mono Q	0.37	20.9	56.5	377

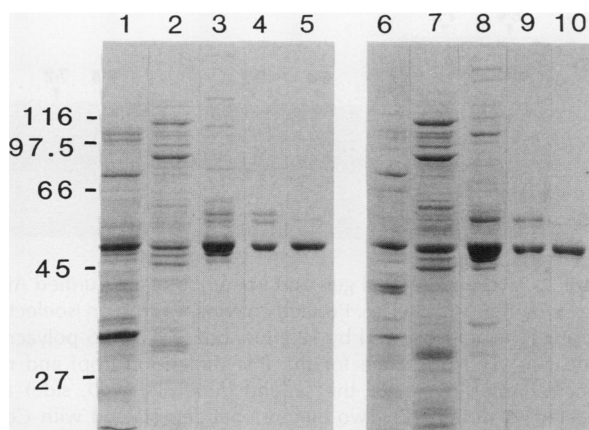

Figure 1. Purification of ADP glucose pyrophosphorylase from *RbRb* and *rbrb* developing embryos. Proteins from stages through the purification were denatured, run on 10% SDS-polyacrylamide gels, and visualized with Coomassie brilliant blue R. Molecular masses are indicated in kD. Lanes 1 through 5 are from *RbRb* embryos, lanes 6 through 10 are from *rbrb* embryos. Lanes 1 and 6, Crude extract; lanes 2 and 7, after DEAE-Sepharose chromatography; lanes 3 and 8, after Blue Sepharose chromatography; lanes 4 and 9, after Mono Q chromatography; lanes 5 and 10, after Superose 12 chromatography.

was accompanied by the appearance of a monomeric form of the enzyme in gel filtration chromatography (Fig. 4) and very low recoveries of activity from the column. The extent of dissociation increased with time and varied from one preparation to another, but was observed for both the *RbRb* and the *rbrb* enzyme.

Experiments with Antisera

The polypeptide unique to the enzyme from *RbRb* embryos was excised and electroeluted from two-dimensional gels of the *RbRb* enzyme and used to immunize a rabbit. The antiserum precipitated activity of ADP glucose pyrophosphorylase from partially purified preparations of the enzyme (Fig. 5). Activity was not precipitated in controls containing preimmune serum alone.

On immunoblots, the antiserum recognized all four polypeptides on two-dimensional gels and both the upper and the lower bands of protein on SDS-polyacrylamide gels of the purified enzyme from *RbRb* embryos, and the single band of protein on SDS-polyacrylamide gels of the purified enzyme from *rbrb* embryos (Figs. 2 and 3). It also recognized two bands of proteins on western blots of long SDS-polyacrylamide gels of crude extracts of *RbRb* embryos, and one band of the same size as the lower band from *RbRb* embryos in extracts of *rbrb* embryos (Fig. 2). Preimmune serum did not recognize polypeptides in either semipurified enzyme or crude extracts of embryos.

Antiserum to ADP glucose pyrophosphorylase from spinach leaf (13) recognized two of the four polypeptides of the

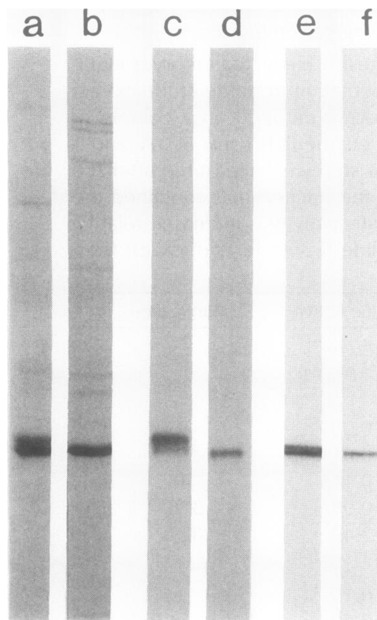

Figure 2. Gels and immunoblots of purified ADP glucose pyrophosphorylase and crude extracts from *RbRb* and *rbrb* embryos. Proteins from purified ADP glucose pyrophosphorylase or crude extracts of embryos of about 350 mg fresh weight were denatured and run on 120-mm long, 7.5% SDS-polyacrylamide gels. Lanes a and b, Gels of purified enzyme from *RbRb* embryos (a) and *rbrb* embryos (b) (JI 399). Lanes c and d, Immunoblots of gel shown in a (c) and gel shown in b (d) developed with the immunoglobulin fraction of serum containing antibodies to the polypeptide unique to the enzyme from *RbRb* embryos. Lanes e and f, Immunoblots of gels of crude extracts of *RbRb* embryos and *rbrb* embryos (JI 399) developed with antibodies as above. The relative intensities of the immunoreactive bands were subject to some experimental variation, but the pattern of banding was always as shown here. The immunoglobulin fraction of preimmune serum did not recognize any proteins on blots identical to those in lanes c–f (not shown).

enzyme from *RbRb* embryos (Fig. 3), and the same two polypeptides of the enzyme from *rbrb* embryos (not shown).

Kinetic and Regulatory Properties of the Enzyme

The purified enzyme from both *RbRb* and *rbrb* embryos displayed hyperbolic kinetics with respect to ADP glucose, ADP glucose in the presence of 1 mM 3PGA[2] (typical results for a single preparation shown in Fig. 6), glucose 1-phosphate, and pyrophosphate (not shown). High concentrations of ADP glucose inhibited the enzyme. The K_m for ADP glucose was not significantly affected by the presence of 1 mM 3PGA (Table II). Values of K_m for the *RbRb* and *rbrb* enzymes were very similar except for the K_m for ADP glucose in the presence of 1 mM 3PGA, which was twofold greater for the *rbrb* than for the *RbRb* enzyme ($P < 0.05$, Student's *t* test; Table II).

The *RbRb* and *rbrb* enzymes behaved differently with respect to the effects of 3PGA and phosphate on activity. The *RbRb* enzyme was slightly stimulated by 3PGA and inhibited by phosphate. In three separate preparations, addition of 1 mM 3PGA stimulated the enzyme by 21, 18, and 35% in the direction of glucose 1-phosphate synthesis. Stimulation in the direction of ADP glucose synthesis was 50 to 140% (two preparations, radioactive and spectrophotometric assays). The enzyme was about 30 to 50% inhibited by 1 mM phosphate in the direction of glucose 1-phosphate synthesis. Inhibition by phosphate was relieved by 3PGA, so that 1 mM phosphate was not inhibitory in the presence of 2 mM 3PGA. Typical results are shown in Figure 6. The *rbrb* enzyme was strongly stimulated by 3PGA and inhibited by phosphate to a much greater extent than the *RbRb* enzyme. In three separate preparations, addition of 1 mM 3PGA stimulated the enzyme by 325, 272, and 456% in the direction of glucose 1-phosphate synthesis. In a single preparation, the enzyme was stimulated by about 400% in the direction of ADP glucose synthesis (radioactive and spectrophotometric assays). Inhibition by phosphate was strong, and only partly relieved by 3PGA. In a typical preparation (Fig. 6), the enzyme was 85%

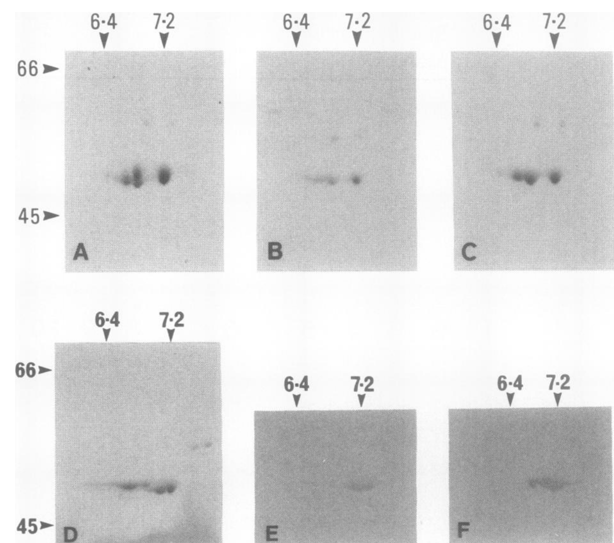

Figure 3. Two-dimensional gels and immunoblots of purified ADP glucose pyrophosphorylase. Purified enzyme was run on isoelectric focusing tube gels followed by 120 mm long, 7.5% SDS-polyacrylamide gels. The pH range for the first dimension (top) and the molecular mass range for the second dimension (kD, side) are indicated. A through D, Two-dimensional gels stained with Coomassie brilliant blue R. A and D, Enzyme from *RbRb* embryos; B, enzyme from embryos of the *rbrb* line JI 399. C, Enzyme from embryos of the *rbrb* line BC2/2 rbrb. E and F, Immunoblots of two-dimensional gels of the enzyme preparation shown in D. E, Developed with a 1/5000 dilution of serum containing antibodies to spinach leaf holoenzyme. F, Developed with the immunoglobulin fraction of serum containing antibodies to the polypeptide unique to the enzyme from *RbRb* embryos. The precise spacings of the polypeptides on two-dimensional gels was reproducible within one experiment, but showed some variation with slight variations in experimental conditions. A through C are from one batch of two-dimensional gels, and D through F are from another. These variations did not affect either the number or the relative positions of the polypeptides.

[2] Abbreviation: 3PGA, 3-phosphoglycerate.

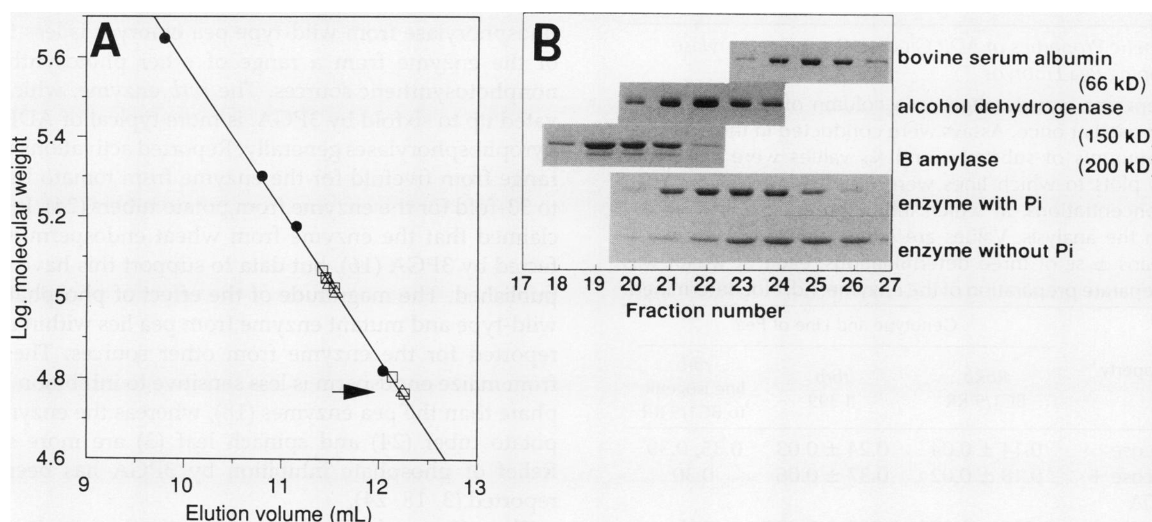

Figure 4. Elution of purified ADP glucose pyrophosphorylase from a Superose-12 gel filtration column. Left, Elution volumes of purified enzyme and marker proteins. Samples of 0.2 mL of purified enzyme were applied to a Superose-12 gel filtration column and eluted at a flow rate of 0.3 $mL \cdot min^{-1}$. ●, Molecular mass markers. These were apoferritin (443 kD), β-amylase (200 kD), alcohol dehydrogenase (150 kD), and BSA (66 kD). □, Enzyme purified from *RbRb* embryos. Δ, Enzyme purified from *rbrb* embryos (JI 399). Each sample was taken from a separate preparation of the enzyme. All samples were in a medium buffered with 50 mM phosphate and were chromatographed in this medium, except for the cluster of three samples indicated by an arrow. These were held in a phosphate-free medium at 0°C for 24 to 48 h prior to chromatography, and were chromatographed in a phosphate-free medium. They eluted as two peaks of protein: the elution volume of the second peak to emerge is plotted here. The phosphate content of the chromatography medium had no effect on the elution volume of the marker proteins. Right, SDS-PAGE of fractions eluted from a Superose-12 gel filtration column. Fractions of 0.3 mL were collected, dialyzed against water, and freeze-dried prior to SDS-PAGE.

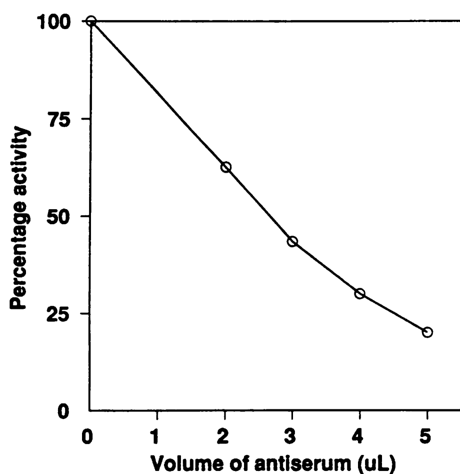

Figure 5. Inhibition of ADP glucose pyrophosphorylase activity by immunoprecipitation with antiserum to the polypeptide unique to the *RbRb* enzyme. Partially purified ADP glucose pyrophosphorylase from *RbRb* embryos was incubated with protein A Sepharose and varying amounts of antiserum, diluted into preimmune serum so that the total volume of serum in all incubations was the same. After centrifugation, the supernatant was assayed for ADP glucose pyrophosphorylase activity. Results are expressed as percentage of inhibition of activity relative to that in incubations containing preimmune serum alone.

inhibited by 1 mM phosphate, and 79% inhibited by 0.5 mM phosphate in the direction of glucose 1-phosphate synthesis. In the presence of 2 mM 3PGA, these values were 80 and 23%, respectively.

DISCUSSION

Kinetic and Regulatory Properties of the Enzyme

Purified ADP glucose pyrophosphorylase from *RbRb* embryos has a specific activity of 40 to 60 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein. This is within the range reported for the enzyme from other sources. For example, the specific activity of the semipurified enzyme from maize endosperm is 34 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein (16), and that of the enzyme from spinach leaf is 156 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein (3). However, such comparisons must be treated with caution because reported measurements for many organs were made in the presence of the activator 3PGA and at higher temperature than our measurements. The specific activity of the purified enzyme from *rbrb* embryos is about 10-fold less than that of the *RbRb* enzyme. This low value is likely to be an intrinsic property of the protein rather than the result of damage during extraction and purification. The recovery and degree of purification of the *RbRb* and *rbrb* enzymes were similar, and mixing experiments with the two sorts of embryo indicated that the enzyme was not degraded or inhibited during extraction (23). The fact that inclusion of protease inhibitors during the purification did not affect the recovery or properties of either enzyme indicates that the difference between the *RbRb* and

Table II. *Kinetic Properties of ADP Glucose Pyrophosphorylase from Developing Pea Embryos*

Purified enzyme was desalted on a column of Sephadex G-25 (Table I) and used at once. Assays were conducted in the presence of varying amounts of substrate, and K_m values were calculated from Hanes plots to which lines were fitted by regression. High substrate concentrations, at which inhibition occurred, were excluded from the analysis. Values are either individual determinations or means ± SE of three determinations, each of which was made on a separate preparation of the enzyme. nd, Not determined.

Kinetic Property	Genotype and Line of Pea		
	RbRb BC1/9 RR	*rbrb* JI 399	*rbrb* line isogenic to BC1/9 RR
K_m ADP glucose	0.14 ± 0.04	0.24 ± 0.03	0.35, 0.39
K_m ADP glucose + 1 mM 3PGA	0.18 ± 0.02	0.37 ± 0.06	0.30
K_m pyrophosphate	0.026 ± 0.002	0.022 ± 0.003	nd

rbrb enzymes is not artifactual. The low specific activity of the *rbrb* enzyme accounts for the low maximum catalytic activity of the enzyme in *rbrb* embryos, and this in turn is likely to account for the reduced rate of starch synthesis and alterations in composition and morphology of these embryos relative to the wild type (23).

The reduction in specific activity of ADP glucose pyrophosphorylase caused by the *rb* mutation is accompanied by changes in the regulatory properties of the enzyme. The K_m values of the enzyme for ADP glucose, pyrophosphate, and glucose 1-phosphate are barely affected by the mutation, but the sensitivity of the enzyme to regulation by 3PGA and phosphate is greatly increased. The *RbRb* enzyme is only slightly activated by 3PGA, whereas the *rbrb* enzyme is substantially activated. Inhibition by phosphate of the *RbRb* enzyme in the direction of glucose 1-phosphate synthesis can be completely relieved by 3PGA. The *rbrb* enzyme is more inhibited by phosphate than the *RbRb* enzyme, and the inhibition is much less readily overcome by 3PGA.

The extent of activation by 3PGA of ADP glucose pyrophosphorylase from wild-type pea embryos is less than that of the enzyme from a range of other photosynthetic and nonphotosynthetic sources. The *rbrb* enzyme, which is activated up to sixfold by 3PGA, is more typical of ADP glucose pyrophosphorylases generally. Reported activations by 3PGA range from fivefold for the enzyme from tomato leaves (21) to 33-fold for the enzyme from potato tubers (24). It has been claimed that the enzyme from wheat endosperm is not affected by 3PGA (16), but data to support this have not been published. The magnitude of the effect of phosphate on the wild-type and mutant enzyme from pea lies within the range reported for the enzyme from other sources. The enzyme from maize endosperm is less sensitive to inhibition by phosphate than the pea enzymes (18), whereas the enzymes from potato tuber (24) and spinach leaf (3) are more sensitive. Relief of phosphate inhibition by 3PGA has been widely reported (3, 18, 24).

The effects of the *rb* mutation on the activity of ADP glucose pyrophosphorylase are like those of the *shrunken-2* (*sh2*) and *brittle-2* (*bt2*) mutations that affect maize endosperm, in that the specific activity of the enzyme is considerably reduced (6). However, although both of the maize mutations decrease the extent of activation of the enzyme by 3PGA in the direction of glucose 1-phosphate synthesis (5), the *rb* mutation increases it. The maize mutations both substantially increase the K_m for glucose 1-phosphate (5), whereas the *rb* mutation has little effect on the K_m values of the enzyme.

Physical Properties of the Enzyme

Purified ADP glucose pyrophosphorylase from *RbRb* embryos consists of four polypeptides of about 50 kD. All of these polypeptides comigrate on a native gel, indicating that they are part of the same complex. One of the polypeptides, with an apparent molecular mass about 1 kD greater than the remaining three, is absent from the *rbrb* enzyme. We used an antiserum to this polypeptide to discover whether it is lost during the purification of the enzyme or is actually absent from the embryo. The antiserum recognized all four polypeptides of the purified *RbRb* enzyme, indicating that the poly-

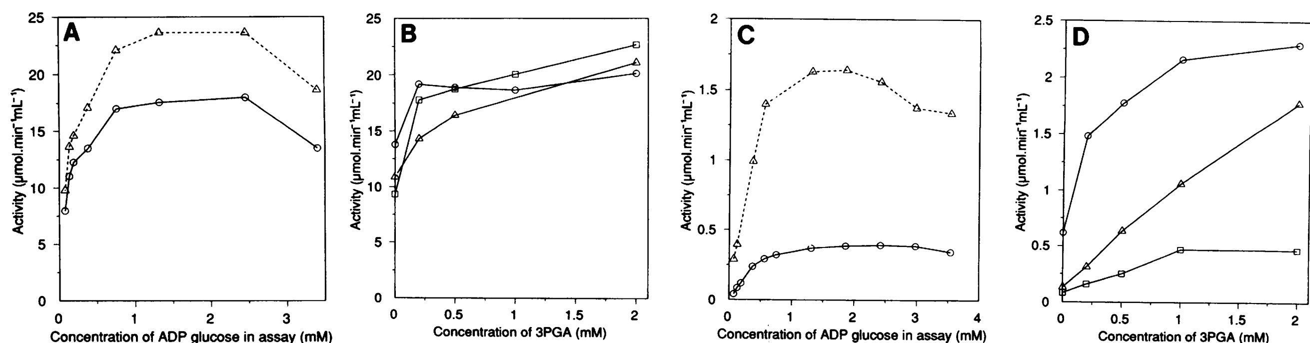

Figure 6. Kinetic properties of ADP glucose pyrophosphorylase purified from *RbRb* and *rbrb* embryos. Purified enzyme was desalted on a column of Sephadex G-25 as in Table I and used immediately. Assays were in the direction of glucose 1-phosphate synthesis. A and C, Effect of 1 mM 3PGA on the activity of the enzyme with respect to ADP glucose concentration. △, With 3PGA; ○, without 3PGA. B and D, Effects of 3PGA and phosphate on the activity of the enzyme at 1 mM ADP glucose. ○, 0 mM Pi; △, 0.5 mM Pi; □, 1 mM Pi. A and B, *RbRb* enzyme. C and D, *rbrb* enzyme.

peptide absent from the *rbrb* enzyme is antigenically related to other components of the enzyme. On long gels of crude extracts of embryos, the antiserum recognized two bands of protein of about 50 kD from *RbRb* embryos, and only one band of this size from *rbrb* embryos. We conclude that the *rb* mutation causes the loss from the embryo of a component of ADP glucose pyrophosphorylase.

ADP glucose pyrophosphorylase from a range of sources consists of 43- to 60-kD polypeptides (9, 11, 13, 16–18). It is generally believed that there are large and small subunits that are present in equal amounts (17). The small subunits of the enzyme from a range of sources have been found to be closely antigenically related, whereas the large subunits are much less highly antigenically conserved (17, 19, 20). However, two-dimensional gels have revealed the presence of more than two polypeptides in the enzyme purified from spinach leaf (13) and potato tuber (17). It is not clear whether these polypeptides are present *in vivo* and represent multiple forms of individual subunits. An antibody to the holoenzyme from spinach leaf recognizes two of the four polypeptides of the enzyme from pea embryos. Thus, it is likely that these two polypeptides are structurally similar to the small subunit of the spinach enzyme.

The *rb* mutation is superficially analogous to the *sh2* and *bt2* mutations of maize endosperm (20) and the *adg2* mutation of *Arabidopsis* leaves (12) in that these all eliminate one putative subunit of the enzyme. However, whereas the maize and *Arabidopsis* mutations eliminate a polypeptide that, in the wild type, represents approximately half of the enzyme protein, the *rb* mutation eliminates a polypeptide that represents considerably less than half of the protein of the wild-type enzyme.

Purified ADP glucose pyrophosphorylase from both mutant and wild-type pea embryos behaves as a dimer on a gel filtration column. It differs in this respect from the enzyme from other sources: the potato tuber (24), spinach leaf (2), and maize endosperm (18) enzymes all behave as tetramers. Our finding does not necessarily imply that the pea enzyme is actually active as a dimer, and its physiological significance is unclear. The pea enzyme dissociates into monomers in the absence of phosphate, and in this state has little or no activity. We do not know whether this behavior has any physiological importance, but it may explain the stabilizing effect of phosphate on the pea and spinach leaf (3) enzymes during purification and storage.

CONCLUSION

The *rb* mutation results in a 10-fold decrease in the specific activity of ADP glucose pyrophosphorylase from pea embryos, an increase in the sensitivity of the enzyme to regulation by 3PGA and phosphate, and the loss from the embryo of one of the polypeptides present in the wild-type enzyme. These changes are not like those conferred by mutations in other species that apparently eliminate one of two subunits of the enzyme. We do not yet know whether the polypeptide affected by the *rb* mutation is a different gene product from the other three polypeptides of the pea enzyme. We propose that this polypeptide confers a high catalytic activity and a relative insensitivity to effectors upon the enzyme. Its effects are strikingly similar to those caused by the reductive phosphopyridoxylation of the enzyme from spinach leaf (14). This chemical modification results in a sixfold higher specific activity in the absence of effectors, and a large reduction in sensitivity to 3PGA and phosphate. Pyridoxal phosphate binds primarily to the allosteric activator site of the enzyme (14). Whether the polypeptide eliminated by the *rb* mutation acts in a similar manner remains to be investigated.

ACKNOWLEDGMENTS

We are very grateful to Dr. Cliff Hedley and Dr. Trevor Wang for the gift of developing seeds of the *rbrb* near-isogenic line of peas, and Professor Jack Preiss (Michigan State University) for the gift of antiserum. We thank Drs. Ian Dry (CSIRO, Adelaide) and Cathie Martin for helpful comments during the course of this work, and Dr. Kay Denyer, Professor D.R. Davies, and Professor Jack Preiss for constructive criticism of the manuscript.

LITERATURE CITED

1. **Bhattacharyya MK, Smith AM, Ellis THN, Hedley C, Martin C** (1990) The wrinkled-seed character of peas described by Mendel is caused by a transposon-like insertion in a gene encoding starch-branching enzyme. Cell **60:** 115–122
2. **Blake MS, Johnston KH, Russell-Jones GJ, Gotschlich EC** (1984) A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on western blots. Anal Biochem **136:** 175–179
3. **Copeland L, Preiss J** (1981) Purification of spinach leaf ADP-glucose pyrophosphorylase. Plant Physiol **69:** 996–1001
4. **Coxon DT, Davies DR** (1982) The effect of the r_a and r_b loci on the lipid content of the seed of *Pisum sativum*. Theor Appl Genet **64:** 47–50
5. **Dickinson DB, Preiss J** (1969) Presence of ADP-glucose pyrophosphorylase in shrunken-2 and brittle-2 mutants of maize endosperm. Plant Physiol **44:** 1058–1062
6. **Hannah LC, Nelson OE** (1975) Characterization of adenosine diphosphate glucose pyrophosphorylases from developing maize seeds. Plant Physiol **55:** 297–302
7. **Hedley CL, Ambrose MJ, Smith CM, Cook S, Wang TL** (1986) An analysis of seed development in *Pisum sativum*. II The effect of the *r*-locus on the growth and development of the seed. Ann Bot **58:** 371–379
8. **Kooistra E** (1962) On the differences between smooth and three types of wrinkled peas. Euphytica **11:** 357–373
9. **Krishnan HB, Reeves CD, Okita TW** (1986) ADPglucose pyrophosphorylase is encoded by different mRNA transcripts in leaf and endosperm of cereals. Plant Physiol **81:** 642–645
10. **Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685
11. **Lin T-P, Caspar T, Somerville C, Preiss J** (1988) Isolation and characterization of a starchless mutant of *Arabidopsis thaliana* (L.) Heynh lacking ADPglucose pyrophosphorylase activity. Plant Physiol **86:** 1131–1135
12. **Lin T-P, Caspar T, Somerville C, Preiss J** (1988) A starch-deficient mutant of *Arabidopsis thaliana* with low ADPglucose pyrophosphorylase activity lacks one of the two subunits of the enzyme. Plant Physiol **88:** 1175–1181
13. **Morell MK, Bloom M, Knowles V, Preiss J** (1987) Subunit structure of spinach leaf ADPglucose pyrophosphorylase. Plant Physiol **85:** 182–187
14. **Morell M, Bloom M, Preiss J** (1988) Affinity labelling of the allosteric activator site(s) of spinach leaf ADP-glucose pyrophosphorylase. J Biol Chem **263:** 633–637

15. **O'Farrell P** (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem **250:** 4007–4021
16. **Olive MR, Ellis RJ, Schuh WW** (1989) Isolation and nucleotide sequences of cDNA clones encoding ADP-glucose pyrophosphorylase polypeptides from wheat leaf and endosperm. Plant Mol Biol **12:** 525–538
17. **Okita TW, Nakata PA, Anderson JM, Sowokinos J, Morell M, Preiss J** (1990) The subunit structure of potato tuber ADP-glucose pyrophosphorylase. Plant Physiol **93:** 785–790
18. **Plaxton WC, Preiss J** (1987) Purification and properties of nonproteolytic degraded ADPglucose pyrophosphorylase from maize endosperm. Plant Physiol **83:** 105–112
19. **Preiss J** (1988) Biosynthesis of starch and its regulation. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Vol 14. Academic Press, New York, pp 181–254
20. **Preiss J, Danner S, Summers PS, Morell M, Barton CR, Yang L, Nieder M** (1990) Molecular characterization of the brittle-2 gene effect on maize endosperm ADPglucose pyrophosphorylase subunits. Plant Physiol **92:** 881–885
21. **Sanwal GG, Greenberg E, Hardie J, Cameron EC, Preiss J** (1968) Regulation of starch biosynthesis in plant leaves: activation and inhibition of ADPglucose pyrophosphorylase. Plant Physiol **43:** 417–427
22. **Smith AM** (1988) Major differences in isoforms of starch-branching enzyme between developing embryos of round- and wrinkled-seeded peas (*Pisum sativum* L.). Planta **175:** 270–279
23. **Smith AM, Bettey M, Bedford ID** (1989) Evidence that the *rb* locus alters the starch content of developing pea embryos through an effect on ADP glucose pyrophosphorylase. Plant Physiol **89:** 1279–1284
24. **Sowokinos JR, Preiss J** (1982) Pyrophosphorylases in *Solanum tuberosum.* III. Purification, physical, and catalytic properties of ADPglucose pyrophosphorylase in potatoes. Plant Physiol **69:** 1459–1466
25. **Wang TL, Hadavizideh A, Harwood W, Welham TJ, Harwood WA, Faulks R, Hedley CL** (1990) An analysis of seed development in *Pisum sativum* L. XIII. The chemical induction of storage product mutants. Plant Breeding **105:** 311–320