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Separation, Purification, and Comparative Properties of Chloroplast and Cytoplasmic Phosphoglycerate Kinase from Barley Leaves[1]

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ABSTRACT

The chloroplast and cytoplasmic isoenzymes of phosphoglycerate kinase (PGK) (EC. 2.7.2.3) from *Hordeum vulgare* leaves have been separated and purified for the first time to apparent homogeneity. The method for purifying the isoenzymes is described here and consists of DEAE Sephacel chromatography followed by affinity chromatography on ATP Sepharose. This consistently provided a 500- to 900-fold purification of each isoenzyme. Most of the total PGK in green barley leaves was found to be in the chloroplasts with only 10% in the cytoplasm. The immunological properties of the two isoenzymes were compared. The antisera raised to the separate isoenzymes showed cross-reactivity, although there is evidence that each isoenzyme possesses some distinct epitopes. The isoenzymes differ in overall charge with isoelectric points at 5.2 and 5.4 for the chloroplast and cytoplasmic isoenzymes, respectively. Molecular mass estimations by gel filtration and sodium dodecyl sulfate polyacrylamide gel electrophoresis provided similar values of approximately 38 kilodaltons for each isoenzyme, some 4 to 5 kilodaltons less than the values calculated from the cDNA sequences of the wheat isoenzymes. The isoenzymes have broadly similar pH optima of pH 7 to 8. The cytoplasmic isoenzyme is more thermally stable than the chloroplast isoenzyme. Further studies are now in progress to compare both the regulatory properties of the isoenzymes and also their three-dimensional structures as compared with the yeast enzyme.

PGK[3] is an important enzyme which functions in photosynthetic carbon metabolism, glycolysis, and gluconeogenesis. In green plants the enzyme has been shown to exist in the chloroplasts and cytoplasm (16). It was shown by Anderson and Advani (3) that there are two distinct isoenzymes in these compartments which could be separated on the basis of their isoelectric points. The pea leaf isoenzymes were partially purified and shown to be kinetically similar (23). However, the present results suggest that the two isoenzymes may have been incompletely separated as the cytoplasmic isoenzyme was purified from the postchloroplast supernatant after mechanical disruption of pea leaves to release the chloroplasts. The results shown here indicate that 90% of the PGK in green barley leaves is found in the chloroplasts with the remaining 10% in the cytoplasm.

The reaction catalyzed is as follows:

$$\text{3-Phosphoglycerate} + \text{MgATP}^{2-} \rightleftharpoons \text{1,3-diphosphoglycerate} + \text{MgADP}^{-}$$

The flux for the reaction is predominantly as shown during photosynthetic carbon reduction involving the chloroplast isoenzyme. The cytoplasmic isoenzyme is thought to be primarily involved in glycolysis, for which the flux is predominantly in the opposite direction to that shown.

In this laboratory, developmental studies on the appearance of PGK activity in *Phaseolus vulgaris* and of the PGK isoenzyme proteins in barley have consistently indicated that both isoenzymes are coded on nuclear genes and synthesized on cytoplasmic ribosomes (2, 4, 11). Confirmation has come from the use of antisera to the barley PGK isoenzymes to identify, and then to clone and sequence, cDNA coding for both isoenzymes in wheat (18). Each of the three haploid sets of chromosomes in hexaploid wheat contains a single copy of the chloroplast and cytoplasmic PGK genes (9). Data base searches have failed to detect any PGK-like sequences in organellar genomes of plants. The synthesis of the total enzyme in greening bean leaves is controlled by phytochrome (7). It is not yet known how the synthesis of different amounts of the two isoenzymes is regulated. The chloroplast isoenzyme is transported into the organelle by means of a transit peptide (18). This mechanism of transport is not yet understood.

Previous work in this laboratory involved the study of the total soluble proteins of green barley leaves. The total PGK was purified by the method of Kuntz *et al.* (13) and added in excess to extracts of total soluble proteins in order to locate the two PGK isoenzymes after two-dimensional PAGE (2). This system was used to compare the relative amounts of PGK isoenzymes produced both by a mutant barley which lacked plastid ribosomes and also barley grown at a nonpermissive temperature which prevented plasmid ribosome assembly. It was found that the plastid isoenzyme was reduced in the mutant (4) but not in the barley grown at high temperature (2).

Most of the work on the enzymic regulation of PGK has been performed on the yeast enzyme (see *e.g.* 14, 27, 30). The enzyme has a very complex regulatory mechanism which involves hinge-bending of the protein to allow catalysis. Pre-

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[3] Abbreviation: PGK, phosphoglycerate kinase.

liminary studies on the regulation of the barley PGK isoenzymes suggest that the regulation of the two plant isoenzymes is equally complex. There appear to be some differences in the regulation of the two plant isoenzymes and also between the plant and yeast enzymes (20).

The present separation and purification method was developed to allow a more comprehensive study of the distribution, structure, functional roles and regulation of the two distinct isoenzymes of PGK which occur in green leaf tissue.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Green leaf material was harvested from barley seedlings (*Hordeum vulgare* cv Golden Promise) which had been grown in trays of wet vermiculite at 20°C ± 1°C in a controlled environment cabinet under continuous illumination of approximately 40 $\mu E \cdot m^{-2} \cdot s^{-1}$.

Chemicals

Pharmacia supplied the chromatography gels, the isoelectric point calibration kit and the Ultrodex and the Ampholine carrier ampholytes. Onozuka R10 (cellulase) was purchased from R. W. Unwin and Co., Welwyn, Hertfordshire. Glyceraldehyde phosphate dehydrogenase from rabbit muscle, ATP, and NADH, were from Boehringer Mannheim. Seakem agarose (LE) was from Miles Scientific, Stoke Poges, Slough, U.K. All other chemicals were obtained from BDH Chemicals Ltd., Essex, or Sigma and were usually 'Analar' grade.

Assay of PGK

The enzyme was assayed by a linked reaction with glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.2) (11). The rate of decrease in absorbance at 344 nm as measured on a Cecil 505 double beam spectrophotometer is proportional to the consumption of NADH and to the amount of enzyme activity. One unit of activity is defined as the amount of enzyme which consumes 1 μmol of substrate/min at 30°C. The 1 mL assay mixture contained the following concentrations of reactants: 10 mM $MgCl_2$, 4.8 mM EDTA, 3 mM ATP, 5 mM PGA with approximately 2.7 units of rabbit muscle glyceraldehyde phosphate dehydrogenase (Boehringer) and 0.14 mM NADH. The mixture was buffered in 50 mM-Tris (pH 7.8). A modified reaction mixture with a low level of heavy metals was prepared for the kinetic studies on the two isoenzymes (20).

Separation of the Chloroplast and Cytoplasmic Isoenzymes by Ion-Exchange Chromatography on DEAE Sephacel

The method was a modification of that described by Mann *et al.* (19) for the separation of glutamine synthetase isoenzymes. One kilogram of barley leaves was extracted in 1.5 L ice-cold, 0.3 M Tris HCl buffer (pH 8), in a Waring blender. The homogenate was squeezed gently through three layers of muslin and then centrifuged at 30,000*g* for 30 min. All extraction procedures were performed in a cold room at 5°C ± 1°C. Following centrifugation, the extract was dialyzed first for 2 h and then overnight against 50 mM Tris (pH 8). The dialyzed extract was then centrifuged once more at 30,000*g* for 1 h, adjusted to 5 mM $MgCl_2$ and applied to a 500 mL column of DEAE Sephacel (5.2 × 24 cm) equilibrated with 40 mM Tris, 5 mM $MgCl_2$ (pH 8). The gradient used for elution was 0 to 0.4 M NaCl in the same buffer.

Affinity Chromatography

The method used was essentially that of Kuntz *et al.* (13). The chloroplast and cytoplasmic isoenzyme preparations were each dialyzed against 50 mM Tris (pH 8) made up to 5 mM-$MgSO_4$ and applied to separate 30 mL columns of ATP Sepharose with a flow rate of 1 mL/min. Each column was washed with 200 mL of 50 mM Tris, 5 mM $MgSO_4$ (pH 8), (running buffer) followed by 100 mL 0.7 M NaCl, 50 mM Tris (pH 8), and the PGK was then eluted with 50 mL 40 mM ATP, 40 mM $MgSO_4$, 50 mM Tris (pH 8). For preparations containing both isoenzymes, the active fractions were eluted with 0.7 M NaCl. The gel was regenerated by washing in 4 M urea, 2% SDS, rinsing with distilled H_2O followed by 50 mM Tris, 5 mM-$MgSO_4$ (pH 8) and stored at −20°C in a solution of 30% glycerol in the latter buffer. Following thawing, the glycerol was removed by washing in running buffer, and this enabled the gel to be reused several times.

Gel Electrophoresis

An LKB 2117 Multiphor horizontal electrophoresis system was employed for all the electrophoresis methods used in this work.

Nondissociating PAGE

For slab gels, samples were prepared by dialysis against 0.01 M Tris-glycine buffer (pH 8.9) before concentration. Electrophoresis was performed in 7% acrylamide in 0.1 M Tris-glycine buffer (pH 8.9).

Isoelectrofocusing

The gel employed in this work was a commercially prepared LKB Ampholine PAG plate (pH 3.5–9.5). The samples were dialyzed overnight against 1% glycine to remove salt.

Immunological Methods

Preparation of Antisera

Antibodies to PGK were raised in New Zealand White rabbits. Samples containing both isoenzymes were prepared by elution from ATP Sepharose with 0.7 M NaCl and were further purified on DEAE Sephadex A25. A total volume of approximately 0.2 mL of solution containing 6.3 mg protein/mL was mixed with 0.2 mL of Freund's complete adjuvant and injected either into the foot or shoulder muscle of the rabbit on d 1. Further injections containing the same amount of protein mixed with incomplete adjuvant were administered on d 8, 22, and 29. The resulting antiserum was designated (Ab B).

In preparing antisera to the separately purified PGK isoenzymes, the rabbits were injected subcutaneously at 14 d intervals with 0.5 mL doses of purified enzyme containing 120 to 490 μg protein mixed in an emulsion with 0.5 mL of Freund's complete adjuvant. After injections, the animals were massaged to stimulate the lymph nodes and spread the antigen. The rabbits were bled from the ears 2 months after the initial injection followed by a second bleeding 7 weeks later. Blood samples were allowed to coagulate for 2 h in a refrigerator followed by centrifugation on a bench-top centrifuge to precipitate the blood cells. The filtrates were retained and divided into aliquots which were stored at −20 or −80°C.

Crossed-Immunoelectrophoresis

The method used was essentially that described by Clarke and Freeman (10). The first dimension gel was cast on a glass plate and did not contain any antisera. The first dimension electrophoresis was performed for 1.5 h at 10 V/cm. After electrophoresis, the gels were cut into slices with a sharp scalpel. Each slice of gel contained one antigen sample. The slices were transferred to sheets of 'gel-bond' for agarose gels (LKB instruments) for the second dimension, with the original sample application position at the left side of the cathode end of the plate. Silver staining was performed according to the method of Porro *et al.* (24). To prepare the gels for staining, they were subjected to pressing and washing with 0.1 M NaCl, followed by drying with a hairdryer to produce a thin transparent film. The second dimension gels consisted of 8 mL of agarose with an additional 0.8 mL of antiserum. The gel was cooled to 45°C before the antiserum was added. The mixture was immediately cast next to the slice of gel on the plastic plate. After allowing the gel to set, electrophoresis was performed overnight at 2 V/cm.

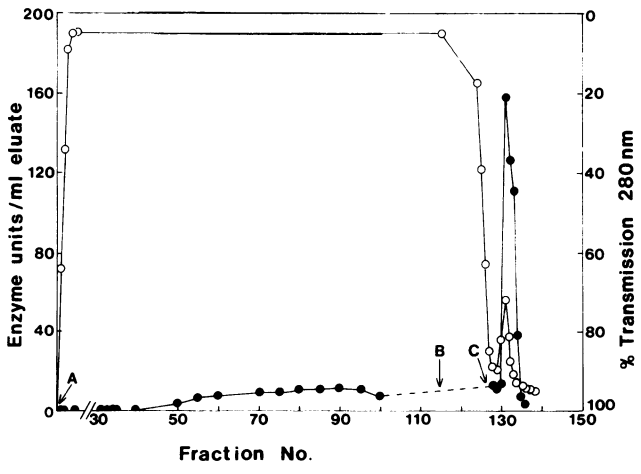

Figure 1. Purification of PGK by affinity chromatography on ATP Sepharose. A 1.5 L extract of 1 kg of barley leaves was made up to 50 mM Tris (pH 8.0), 5 mM $MgSO_4$. A, Extract applied; B, washed with 50 mM Tris, 5 mM $MgSO_4$ (pH 8.0); C, eluted with 50 mM Tris, 0.7 M NaCl (pH 8); (●), PGK activity; (○), % transmission at 280 nm. Fraction size, 10 mL. Column, 60 mL ATP Sepharose in 50 mM Tris, 5 mM $MgSO_4$ (pH 8).

Table I. *Purification of Total PGK from 14 D-Old Barley Leaves*

Purification Step	Total Protein	Total Activity	Recovery	Specific Activity	Purification
	mg	*units*	%		
Crude extract	12,112	15,624	100	1.29	1
ATP-Sepharose chromatography	11	8,714	56	794	615
DEAE-Sephadex chromatography[a]	8.8	8,009	51	914	717

[a] From a different preparation whose crude extract had a similar specific activity (1.27 compared with 1.29 units PGK:mg protein).

Immunotitration

The method was a modification of that described by Krüger and Schnarrenberger (12). A variable volume of antiserum in the range of 2 to 100 μL was mixed with 0.1 M-Tris buffer (pH 7.8), to give a constant volume of 100 μL of solution. This was placed in an Eppendorf tube with 100 μL of plant extract or purified enzyme solution which had previously been assayed for enzyme activity. The mixture was incubated at 30°C for 30 min. The tubes were capped and mixed on a Whirlimixer, (Fisons U.K.), and then centrifuged for 5 min at 8000*g* on an Eppdendorf centrifuge. The supernatant was shaken into a clean tube, taking care not to remove the soft precipitate, and assayed for PGK activity. A control sample treated in the same manner as described but without the addition of antiserum, was taken as 100% activity. The residual activity remaining in all the other samples was calculated as a percentage of the control. During the course of this work, it was discovered that the rabbit serum contained endogenous PGK activity. Control experiments were performed for each batch of antiserum without added barley enzyme. These control values were subtracted from all the results.

Ouchterlony Double Diffusion

Following the overnight diffusion, the gels were washed, pressed, and dried as described for the crossed immunoelectrophoresis gels. Staining was carried out with 0.5% Coomassie blue R250 in 45% ethanol, 10% acetic acid for 5 min followed by rinsing with distilled water. The plates were destained for 10 min in 45% ethanol, 10% acetic acid. After drying, the stained gels were stored in plastic bags.

RESULTS AND DISCUSSION

Purification of Total PGK by Affinity Chromatography

Figure 1 and Table I show typical results of the large scale preparation of total PGK from green barley leaves which consistently yielded a 600- to 800-fold purification with a recovery of 44 to 56% of the total activity. In the present work, the barley PGK isoenzymes were always eluted with 0.7 M NaCl except after separation of the chloroplast and cytoplasmic isoenzymes in which case each particular isoenzyme was eluted with $MgATP^{2-}$, (Fig. 2). There appeared to be a low level of contaminating kinases in the purified barley PGK preparation. This was demonstrated by assaying a peak fraction containing 65 units/ml barley PGK which was puri-

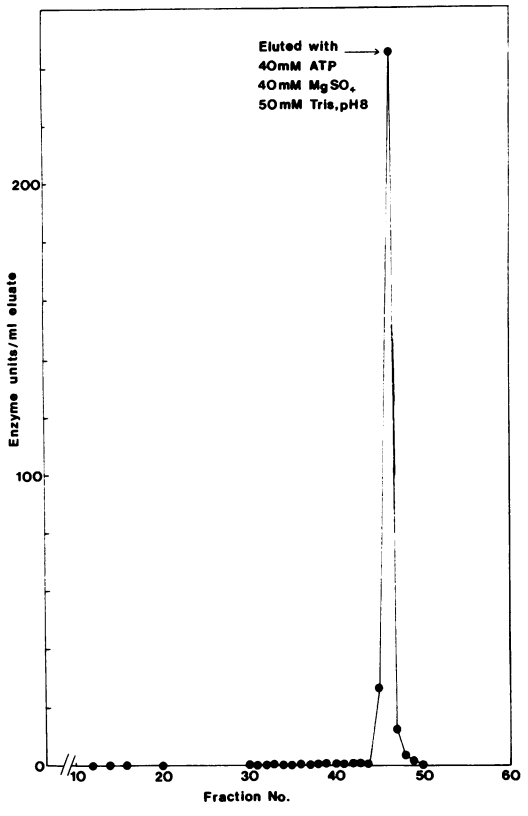

Figure 2. Purification of the chloroplast isoenzyme of PGK by affinity chromatography on an ATP Sepharose column. The chloroplast isoenzyme had been separated on a DEAE Sephacel column and was dialyzed against several changes of 50 mM Tris (pH 8.0), before being adjusted to 5 mM $MgSO_4$ and applied to the affinity column.

fied during a preliminary experiment. The following enzymes were assayed with their corresponding activities for the purified/crude extracts shown in parentheses, in enzyme units/mL: hexokinase (0.003/0.003), phosphofructokinase (0.003/0.016), pyruvate kinase (not detectable/0.052), and adenylate kinase (0.20/0.24). Thus, there was a reduction in all the enzyme activities examined with the exception of hexokinase which gave the same value in both the crude extract and the purified PGK fraction.

Table II shows previously recorded methods for the purifications of PGK from various plant sources. Some of these isolations involved multiple procedures which did not always lead to the recovery of a highly purified enzyme. It can be seen that the affinity chromatography technique described in the present work compares very favorably with previous methods. It provided a relatively easy technique for the initial purification of both isoenzymes of PGK. A comparative nondissociating polyacrylamide gel was run of the preparations of purified PGK from peas, barley, and spinach (data not shown). Each preparation produced two main protein bands. The fastest migrating band for each pair is presumed to be the chloroplast isoenzyme as determined by Allsop *et al.* (2) for the barley isoenzymes. There were differences in the electrophoretic mobilities of the enzymes, the fastest migrating proteins being those extracted from peas. The difference between the migration of chloroplast and cytoplasmic isoenzymes results from a charge difference. The wheat isoenzymes, for example, have a charge difference of 3^+(18), whereas the isoelectric points of the barley isoenzymes are 5.2 and 5.4 for the chloroplast and cytoplasmic isoenzymes, respectively. Pea leaves had already been shown to possess two distinct isoenzymes (3). In contrast to this, the previously reported purifications of the spinach enzyme did not report the presence of isoenzymes (17, 13). However, in the former case, the cytoplasmic isoenzyme may have been discarded during one of the many stages of the purification procedure. In the latter case the purity of the enzyme was assessed by SDS-PAGE, which conceals differences in charge and therefore would not detect the presence of isoenzymes.

The purification of the spinach enzyme as described in the original method of Kuntz *et al.* (13), gave a specific activity of 480 with a final specific activity of 670 enzyme units/mg protein after purification on Sephadex G75. The assay conditions used in that preparation differed in many respects from the present work. Larsson-Raźnikiewicz also used affinity chromatography to obtain total PGK from spinach and wheat leaves (15).

Further Purification of a Preparation Containing both Isoenzymes of PGK by Ion-Exchange Chromatography on DEAE Sephadex A25

An initial attempt was made to separate the two isoenzymes by ion-exchange chromatography on DEAE Sephadex A25. This method had been used successfully on the PGK isoenzymes from developing castor oil seeds (21). The technique has been described as ion-sievorptive chromatography and involves the use of an ion-exchange resin with small pore size which allows the exclusion of all proteins. There is a weak ionic interaction with the gel. The procedure allows the use of fast flow rates and the weak binding of the proteins results in minimal denaturation.

A preparation of purified PGK was made which utilized both the affinity chromatography followed by the ion-exchange chromatography (Table I). The ion-exchange chromatography yielded a high peak of enzyme at the start of the gradient, suggesting that there was very little interaction with the gel matrix. A nondissociating gel was prepared to compare fractions from either side of the peak. The gel was stained with Coomassie blue G250 (Fig. 3). It is clear from the purified samples on the gel, that both isoenzymes were eluted together from the ion-exchange column. These samples appeared to contain no other detectable contaminants. It was decided to use this preparation to raise antibodies to both the isoenzymes together. These antibodies were then used for crossed-immunoelectrophoretic analysis of subsequent preparations to detect the presence of one or both of the isoenzymes.

Separation of Chloroplast and Cytoplasmic PGK

Preparative Isoelectrofocusing

Several attempts were made to separate the two isoenzymes by isoelectrofocusing on a preparative scale in a granulated gel. This procedure has been evaluated by Radola (25) as a

Table II. *Some Examples of Previous Purifications of Plant PGK*

Plant Material	Main Methods	Recovery	Specific[a] Activity	Final Purification	Reference
		%	*units/mg protein*		
Pea seeds	$(NH_4)_2SO_4$ fractionation	15	64[b]	10.49	5
Spinach leaves	Fractional precipitation, Biogel, DEAE cellulose	7.8	78	260	17
Pea leaves	$(NH_4)_2SO_4$ fractionation, DEAE Sephadex, gel filtration on Sephadex G50	28[c] 13.2[d]	22.5[c] 8.8[d]	40[c] 23[d]	23
Silverbeet leaves	$(NH_4)_2SO_4$ fractionation, chromatography on DEAE cellulose, Cibacron blue Sephadex, gel filtration on Sephadex G75	34	980	142	8
Spinach leaves	ATP-Sepharose affinity chromatography, gel filtration on Sephadex G75	102	670	103	13

[a] Assay conditions vary between authors. [b] Hydroxylamine assay—O.D. units at 490 nm/biuret units. [c] Chloroplast isoenzyme. [d] Cytosolic isoenzyme.

convenient method for preparing large quantities of purified proteins. In the present work, this method resulted in loss of enzyme activity due to the low pH incurred during the separation procedure. After preparative isoelectrofocusing, portions of the resulting gel could be shown to contain measurable amounts of enzyme. It was concluded that the enzymes had precipitated at their isoelectric points and remained in an insoluble form within the gel matrix. This precipitation has been shown to occur for some proteins during isoelectrofocusing (28) but is thought to be more common in density gradient systems. In some cases, this problem can be overcome by raising the pH of the zone containing the protein of interest, or by the addition of urea or other solubilising agents such as detergents. Raising the pH was not effective in the present work and the use of further solubilizing techniques was not attempted. PGK has been purified from human erythrocytes by isoelectrofocusing with the addition of DTT as a stabilizing agent (1). The isoelectric point of this enzyme is 8.75 which is a much more favorable pH for the preservation of PGK activity than the low pI values of the barley isoenzymes. The pI values for chloroplast and cytoplasmic isoenzymes were 5.2 and 5.4, respectively. These isoelectric points are in the range where the enzymes precipitate and become denatured. It was therefore necessary to find a more suitable method for the separation which could produce samples of soluble and enzymically active isoenzymes.

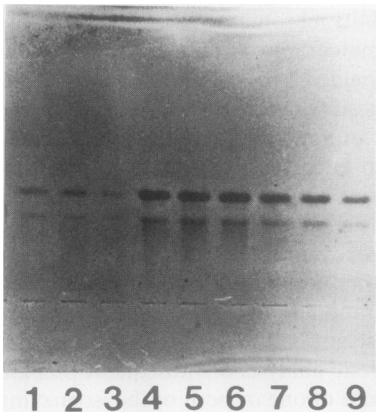

Figure 3. Nondissociating PAGE of barley PGK which had been purified by ATP Sepharose affinity chromatography prior to further purification on Sephadex A25. Each of lanes 1–3, 4–6, and 7–9, respectively, contains 10 μL of the PGK peak fractions 15, 17, and 19, amounting to 26, 71, and 44 μg protein, from the Sephadex A25 column. The chloroplast isoenzyme is the faster-running and more dense band.

Ion-Exchange Chromatography

The initial attempts to separate the isoenzymes by ion-exchange chromatography on DEAE Sephadex A25 were unsuccessful. The chromatography was performed with 0.02 M imidazole buffer (pH 6.8) with a gradient of 0 to 0.5 M NaCl. The barley isoenzymes of chloroplast and cytoplasmic PGK were finally separated by ion-exchange chromatography on DEAE Sephacel in 40 mM Tris, 5 mM $MgCl_2$ (pH 8) (Fig. 4). It was found that the barley cytoplasmic PGK did not bind to the DEAE Sephacel column under the conditions described here. The isoenzymes could therefore be separated by preferential binding of the chloroplast isoenzyme to DEAE Sephacel. In several experiments, the cytoplasmic isoenzyme consistently comprised only 9 to 14% of the enzyme applied

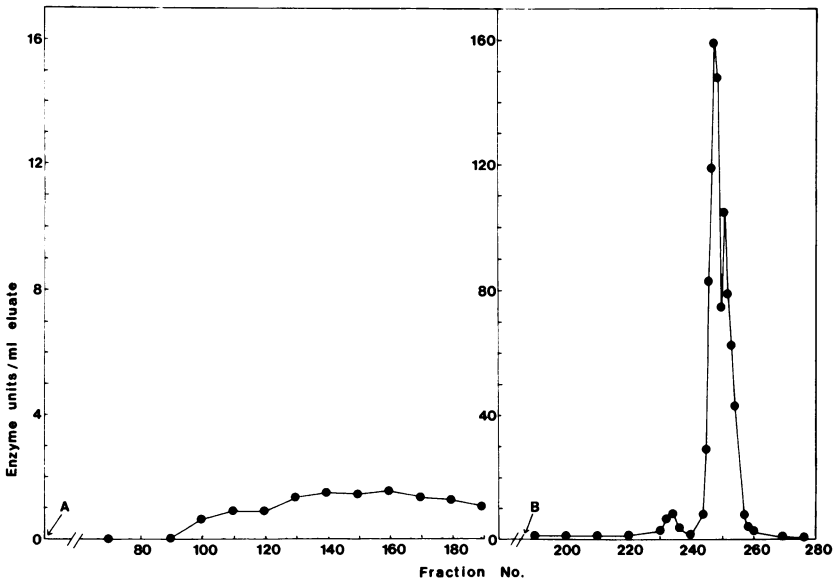

Figure 4. Recovery of both PGK isoenzymes by the ion-exchange chromatography of an extract of 1 kg barley leaves, in 50 mM Tris (pH 8.0), 5 mM $MgCl_2$, on a 500 mL column of DEAE Sephacel (5.2 × 24 cm). A, Application of the extract. The cytoplasmic isoenzyme did not bind and was collected in fractions 100–190. B, After washing with buffer the chloroplast isoenzyme was eluted by a gradient of 0–0.4 M NaCl in 40 mM Tris, 5 mM $MgCl_2$ (pH8).

to the DEAE columns, with the remaining enzyme activity forming the major chloroplast peak (see Table III). The chloroplast peak was further purified by affinity chromatography on ATP Sepharose. In this case, the isoenzyme could not be eluted by 0.7 M NaCl as described earlier when both isoenzymes were present. It was eluted as a sharp peak with 50 mM Tris, 40 mM $MgSO_4$, 40 mM ATP (pH 8) (Fig. 2). This difference in the requirements for elution of PGK from the affinity column occurred consistently. When a single isoenzyme was bound, elution required $MgSO_4$/ATP, but when both were bound, elution occurred in 0.7 M NaCl. Further purification of the cytoplasmic isoenzyme was carried out by affinity chromatography in a similar manner to that described for the chloroplast isoenzyme. The results of a preparation of both isoenzymes are shown in Table IV, in which separation of the isoenzymes was followed by their individual purification on ATP Sepharose. In this case, the final specific activity of the cytosolic isoenzyme was low although the purification factors for both isoenzymes were similar and no discrete impurities were found.

Immunological Studies

Crossed-Immunoelectrophoresis

This technique was chosen to analyze peak fractions of enzyme activity after various column electrophoresis procedures. In view of the low titer of antisera, silver staining was used to increase the sensitivity of the protein staining, and to conserve antigen samples. Figure 5 shows the resulting gel after crossed-immunoelectrophoresis of 5 μL of eluate after purification of total barley PGK on an ATP Sepharose column. The two isoenzyme peaks can be seen clearly, with disproportionate amounts of the two isoenzymes. The height of the peaks is proportional to the amount of antigen present in the sample, and inversely proportional to the antibody concentration in the gel. The sharp peaks indicate a high avidity of the antibodies in the gel for the antigens (6). The two isoenzyme peaks are joined, suggesting that the two precipitin lines represent related antigens. Further gels with successive peak fractions from the same column (not shown) indicated that the proportion of each isoenzyme in each fraction varied across the peak of enzyme activity. The peak heights on this gel do not therefore represent the actual proportion of the two isoenzymes present in the barley leaves used for the extraction. The peak which migrated furthest in the first dimension (the chloroplast isoenzyme), always appeared to be in excess. There were no other precipitin lines produced, indicating that the antiserum was specific for the two barley PGK isoenzymes. Crossed-immunoelectrophoresis was used to compare the cytoplasmic isoenzyme (which did not bind to the DEAE Sephacel column), with the chloroplast sample which did bind to the column. Both fractions were separately purified by ATP Sepharose affinity chromatography before the immunoelectrophoresis. The resulting gels are shown in Figure 6 and illustrate the complete separation of the chloroplast and cytoplasmic PGK isoenzymes.

Immunotitrations

The immunotitrations shown in Figure 7 were performed after the isoenzymes had been successfully separated and had been used to raise separate antisera to the chloroplast and cytoplasmic isoenzymes. In these experiments the Protein A was omitted and the antigen samples were incubated for a

Table III. *Separation of Chloroplast and Cytoplasmic Isoenzymes of PGK from Barley Leaves*

Purification Step	Total Activity	Recovery
	units	%
Crude extract	14,274	100
Cytoplasmic isoenzyme not bound to DEAE-Sephacel column	1,758	12.3
Chloroplast isoenzyme eluted from DEAE-Sephacel column	12,724	89.1

Table IV. *Purification of Both PGK Isoenzymes from 8 D-Old Barley Leaves*

Purification Step	Total Protein	Total Activity	Recovery	Specific Activity	Purification
	mg	*units*	*% of total activity*	*units/mg*	
Crude extract	18,585	24,327	100	0.13[a]	1
				1.18[b]	1
Cytoplasmic isoenzyme not bound to DEAE-Sephacel	16,545	1,820	7.5	0.11	0.85
ATP-Sepharose chromatography of cytoplasmic isoenzyme	16.1	1,110	4.6	68.9	530
Chloroplast isoenzyme eluted from DEAE Sephacel	1,043	20,130	82.7	19.3	16.4
ATP-Sepharose chromatography of chloroplast isoenzyme	14.1	9,080	37.3	644	545

[a] Estimated specific activity of cytoplasmic isoenzyme. [b] Estimated specific activity of chloroplast isoenzyme. Assuming a ratio of 9:1 chloroplast:cytoplasmic PGK activity in whole leaf extract.

longer period (30 min), with the particular antiserum. Under these conditions it was still possible to precipitate the antigen/antibody complexes, and the amount of endogenous enzyme activity was greatly reduced. The results confirm that there is a large degree of cross-reaction between the two isoenzymes. Each antiserum was demonstrated to be most effective at precipitating the particular antigen against which it was raised.

Ouchterlony Double Diffusion

The antiserum which was raised against the major chloroplast peak from the DEAE column, was of a low titer and only produced a clearly visible precipitin line in the gel with the chloroplast antigen against which it had been raised. (Fig. 8A). There was a very faint precipitin line produced against the cytoplasmic isoenzyme.

In contrast, the cytoplasmic antiserum was of a much higher titer, showing a strong reaction with the cytoplasmic isoenzyme, and also reacting with the chloroplast isoenzyme. The reaction appears to be a single dissimilarity reaction., as defined by Otterness and Karush (22). This definition is used to describe a reaction in which the antiserum recognises some common epitopes between two antigens, as seen by the fusion of the precipitin line from both antigen wells. There is, however, a visible spur which occurs toward the chloroplast well. This spur is indicative of the presence of at least one epitope which is present in the cytoplasmic isoenzyme, and absent from the chloroplast isoenzyme. The spur forms a slightly less densely staining band than the main precipitin line. Similarly, the mixed antisera gave evidence of a spur arising toward the well containing the cytoplasmic antigen. This indicates that the chloroplast antigen contains an epitope which is recognised by the chloroplast antiserum in the mixture and is absent in the cytoplasmic antigen. It was not possible to see the spur in the reaction which utilised the chloroplast antiserum alone, due to the low titer of these antibodies. No visible precipitin bands were observed with the preimmune sera and either of the two isoenzymes (results not shown).

Immunological Confirmation of the Identification of the Chloroplast and Cytoplasmic Isoenzymes

The identities of the barley chloroplast and cytoplasmic isoenzymes had been previously established by PAGE of extracts of isolated chloroplasts on native gels (2). Further confirmation was sought by immunological methods. A sample of barley tissue was subjected to cell wall degradation by pectolyase and cellulase. Chloroplasts were released from the resulting protoplasts and used to prepare a sample of purified chloroplast PGK on a 2 mL column of ATP Sepharose. The

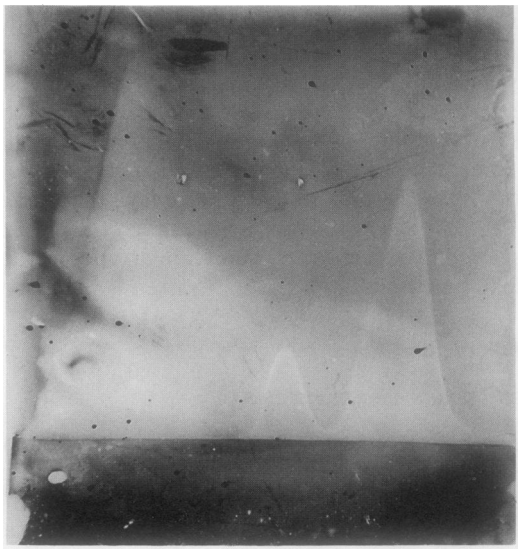

Figure 5. Crossed immunoelectrophoresis of the PGK obtained by ATP-Sepharose affinity chromatography of a barley leaf extract. A 0.9 μg amount of protein (0.9 unit of PGK) was subjected to nondissociating agarose gel electrophoresis in the first dimension. In the second dimension the gel strip was subjected to electrophoresis into a gel containing 10% rabbit anti-PGK. The protein was originally applied to the well in the bottom left hand corner. The chloroplast isoenzyme is the fastest running and larger peak, the cytoplasmic isoenzyme the slower and smaller peak.

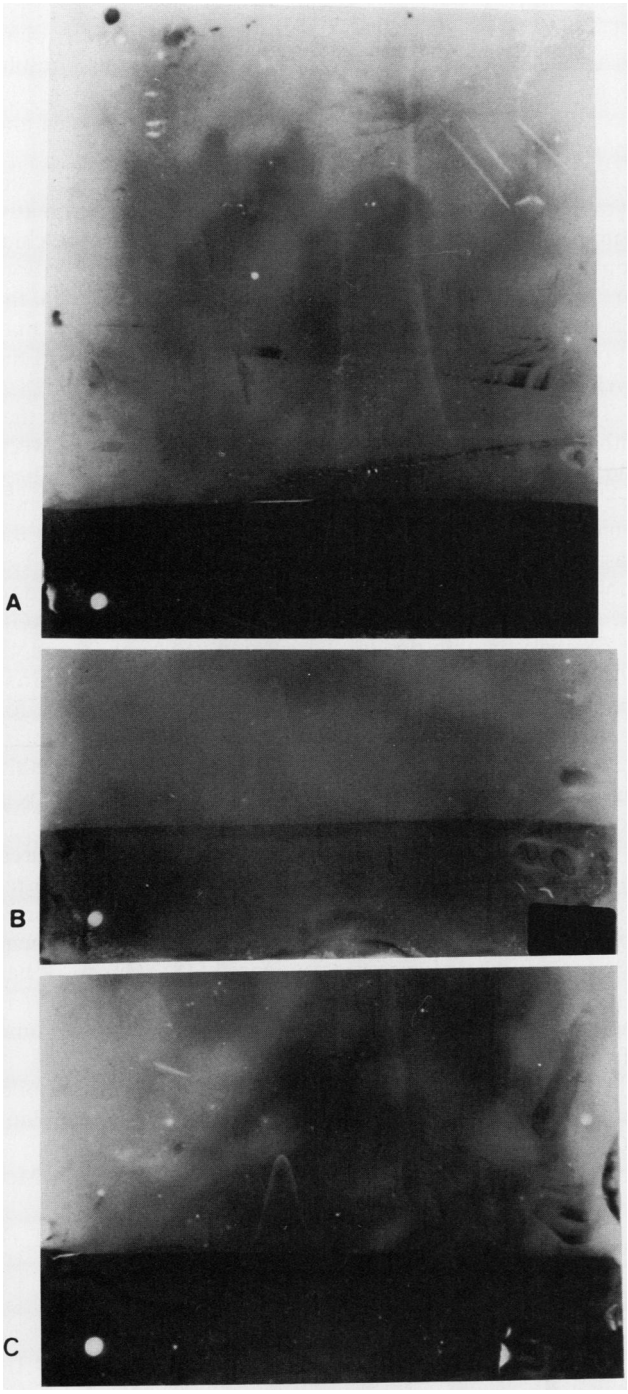

Figure 6. Comparison of chloroplast and cytoplasmic PGK by crossed-immunoelectrophoresis. A, 5 μL of chloroplast isoenzyme (1.23 units); B, 5 μL of cytoplasmic isoenzyme (0.29 unit); C, 2.5 μL of each isoenzyme. Other details as for Figure 5.

postchloroplast supernatant was used to prepare a sample of PGK which contained the cytoplasmic isoenzyme. The chloroplast and cytoplasmic PGK fractions were used in both immunotitrations, and Ouchterlony double diffusion gels. The immunological reactions of these fractions from the protoplast preparation, were compared with those of the previously purified isoenzymes, in order to establish the location of the two isoenzymes within the barley leaf.

Immunotitrations

Immunotitrations showed that the fraction of PGK purified from the chloroplast fraction was preferentially precipitated by the antiserum raised to the large peak of PGK which bound to the DEAE Sephacel column (Fig. 9A). The cytoplasmic fraction was preferentially precipitated by the antiserum to the small peak of PGK which did not bind to the DEAE Sephacel column (Fig. 9B). The amount of cross-reaction in Figure 9 is similar to that in Figure 7. The result confirms the identity of the chloroplast and cytoplasmic isoenzymes.

Ouchterlony Double Diffusion

A double diffusion gel compared the chloroplast and cytoplasmic fractions obtained from protoplasts with the antisera raised to the two separately purified PGK isoenzymes (Fig. 8B). The purified PGK from the cytoplasmic fraction of the protoplast preparation produced a similar precipitin line pattern to the small peak from the DEAE column. This reaffirms the conclusion that the fraction of PGK which does not bind to the DEAE Sephacel is the cytoplasmic isoenzyme. There was a large amount of cross-reaction between the cytoplasmic antiserum and both chloroplast and cytoplasmic fractions. Once again, a spur could be seen toward the chloroplast antigen well, indicating that the cytoplasmic PGK extract contained an extra epitope which was not present in the chloroplast PGK fraction. The spur is diffuse, and is also visible in the reaction between the mixed antisera and the chloroplast and cytoplasmic isoenzymes. The low level of titer for the chloroplast antiserum prevented the formation of a visible precipitate with either the chloroplast or cytoplasmic PGK antigens. The chloroplast and cytoplasmic PGK from the protoplast preparations were much more dilute in terms of protein content than the purified preparations shown in Figure 8A.

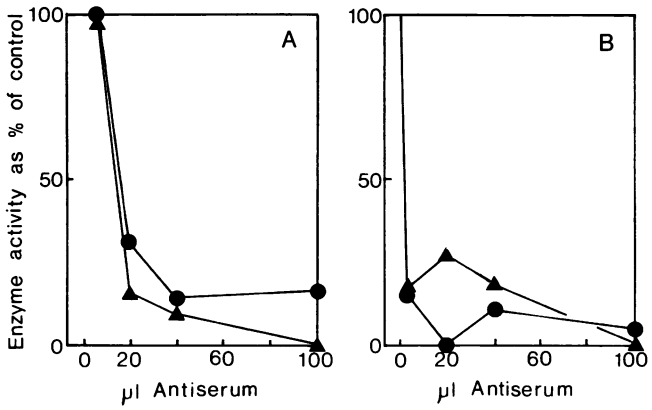

Figure 7. Immunotitration of chloroplast (▲) and cytoplasmic (●) PGK isoenzymes *versus* antisera raised against the chloroplast (A) and cytoplasmic (B) PGK isoenzymes.

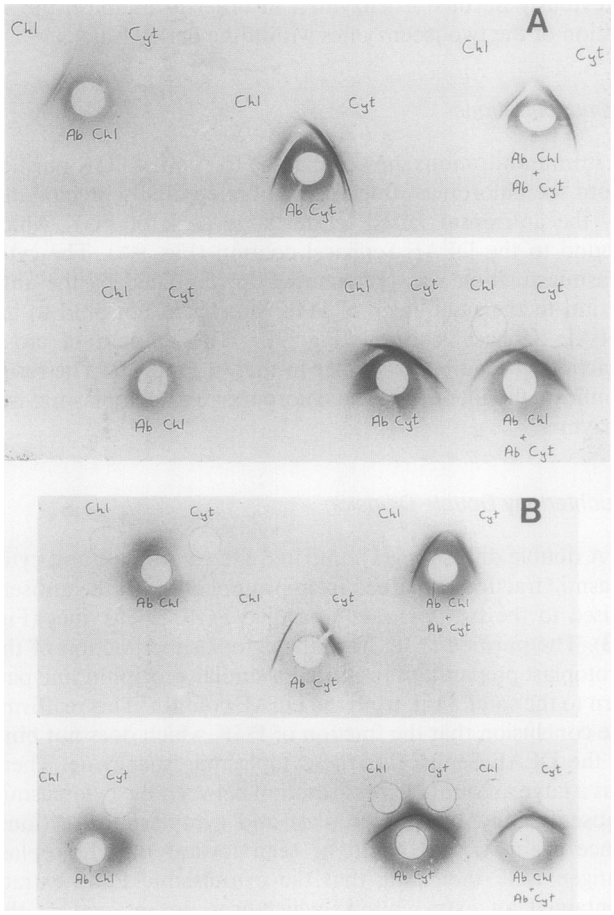

Figure 8. Ouchterlony double diffusion of antisera raised against chloroplast (Ab Chl) and cytoplasmic (Ab Cyt) isoenzymes of PGK *versus*: A, chloroplast isoenzyme (Chl) and cytoplasmic isoenzyme (Cyt); B, PGK purified from a chloroplast extract obtained from protoplasts (Chl) and PGK purified from the cytoplasmic fraction of a protoplast preparation (Cyt).

GENERAL DISCUSSION

The low level of cytoplasmic PGK in barley leaves is not thought to be a result of breakdown of this enzyme during the purification procedure. The cytoplasmic enzyme has been shown to be more stable than the chloroplast enzyme under similar conditions of pH and temperature as those described here (EM McMorrow, unpublished results). Other isoenzyme pairs have recently been shown to exist in similar proportions to the PGK isoenzymes, *e.g.* fructose-bisphosphate adolase from spinach, peas, wheat, and corn (26). In the case of the aldolase isoenzymes, considerable difficulties were encountered in resolving the small peak of cytoplasmic enzyme.

The early studies on PGK from pea leaves (3) indicated that the cytoplasmic enzyme was in excess in relation to the chloroplast enzyme which was only present as a minor fraction of the total activity. It is thought from examination of the methods used to prepare the chloroplast and cytoplasmic fractions, that the two samples described were both largely derived from the chloroplasts, and this would also explain the similar kinetic properties attributed to the pea isoenzymes. The exact proportions of the PGK isoenzymes in other plant material has not yet been investigated. The preliminary results from PAGE suggest that there is a larger amount of the isoenzyme with the lowest isoelectric point in both pea and spinach leaf extracts.

The high level of PGK in the chloroplasts relative to the cytoplasm may partly reflect the relative size of the two compartments. The enzymatic reaction of PGK in the chloroplasts is predominantly in the direction of ATP utilization. As this is the least energetically favorable reaction in the Calvin cycle, the high proportion of chloroplast enzyme may also be required to ensure a fast turnover of this phase of the cycle.

The results described in the present work suggest that although there is a high degree of structural homology between the two isoenzymes, there are also distinct differences. These are reflected in the isoelectric points of the two isoenzymes which allow the separations described to be performed on the basis of the differences in overall charge of the proteins. The molecular mass of both barley isoenzymes were estimated to be about 38 kD on the basis of gel filtration and SDS-PAGE in comparison with previously reported values of 47 and 48 kD reported for other plants (8, 17). The values deduced from the derived amino acid sequences for wheat are 42,122 for the cytoplasmic isoenzyme and 42,992 for the chloroplast mature protein (18). Gel filtration showed that all of the PGK was in the monomeric form in the extracts and that there was no evidence of aggregation.

The antisera described in the present work have been used to screen a wheat cDNA library to isolate the clones corresponding to the two PGK isoenzymes. The cDNAs corresponding to the complete coding regions for the two isoenzymes from wheat have been sequenced (18). This work has also led to the demonstration that each PGK isoenzyme is represented by a single nuclear gene in the haploid genome of wheat (9).

The derived amino acid sequences are being used to build three-dimensional models for the structures of the two plant PGK isoenzymes. The yeast structure of Watson *et al.* (29) is being used as a template for molecular modeling (EM Mc-

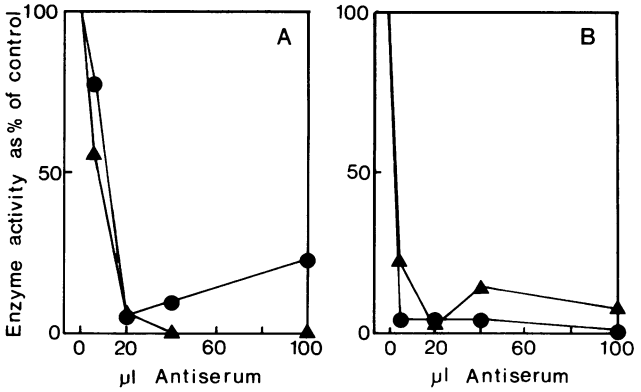

Figure 9. Immunotitration of antisera raised against the chloroplast (A) and cytoplasmic (B) isoenzymes of PGK *versus* the PGK purified from extracts of barley chloroplasts (▲) and cytoplasm (●) obtained from a protoplast preparation.

Morrow, BJ Sutton, JW Bradbeer, unpublished results). This confirms that there are considerable differences in the side chains of the chloroplast and cytoplasmic isoenzymes. Further work is in progress to attempt to relate differences observed in the three-dimensional structures with differences in the regulation of the isoenzymes.

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