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Dissociation, Reassociation, and Purification of Plastid and Cytosolic Phosphoglucose Isomerase Isozymes[1]

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ABSTRACT

The plastid and cytosolic isozymes of the dimeric enzyme phosphoglucose isomerase (EC 5.3.1.9) from spinach (*Spinacia oleracea*) and cauliflower (*Brassica oleracea*) were purified to apparent homogeneity. The isozymes from sunflower (*Helianthus annuus*) and *Clarkia xantiana* were partially purified. When subunits from two electrophoretically distinguishable cytosolic isozymes, either from the same or from different species, were dissociated and allowed to reassociate in each other's presence, an active hybrid enzyme, consisting of one subunit of each type, was formed in addition to the two original homodimers. Active hybrid enzymes were also formed by dissociation and reassociation of plastid isozymes. Hybrid molecules were not produced between the plastid and cytosolic subunits, suggesting that they are not able to bind with each other. Additional differences between the plastid and cytosolic isozymes are described.

A number of enzymes in vascular plants exist as cytosolic and plastid isozymes (20, 21). In the cases investigated, both isozymes are coded by nuclear genes (1, 27), and both mRNAs appear to be translated on free cytoplasmic ribosomes (16). Many of these enzymes are oligomeric, consisting of subunits which presumably collide and associate with each other after synthesis. Because the subunits of both plastid and cytosolic isozymes initially occupy the same subcellular compartment, hybrid molecules could, in principle, form between them. However, such hybrids have not been reported for any plastid/cytosolic isozyme pair, and an explanation for their absence has not been presented.

We investigated this problem by performing dissociation/reassociation experiments on the isozymes of PGI[2] from various species. PGI has been studied from a number of standpoints. The subcellular localization of the two isozymes has been verified in several species (19, 20, 21, 26, 27). The mode of inheritance and the independent assortment of the coding genes has been demonstrated (4, 25, 27). The isozymes have been partially purified from several species (17–19), and one enzyme was purified in a crystalline form from peas (22). Kinetic studies have been reported, primarily on the cytosolic isozyme (5, 19, 22), and polymorphism for both isozymes has been reported in natural populations (6, 7). Finally, the duplication and divergence of the gene coding subunits of the cytosolic isozyme has been studied in the genus *Clarkia* (Onagraceae) (4, 6). However, a purification procedure for both isozymes has not been previously presented. Because purified isozymes were required for the controls of the dissociation/reassociation experiments, we developed, and report herein, a simple and gentle procedure for their purification from several plant species.

MATERIALS AND METHODS

Fresh spinach (*Spinacia oleracea*) was purchased locally. Sunflower (*Helianthus annuus*) and cauliflower (*Brassica oleracea*) were grown in garden plots on the Davis campus of the University of California. *Clarkia xantiana* was grown in growth chambers as described previously (4). Spinach and *Clarkia* were used, because they were available all year in large quantities. Sunflower and cauliflower were selected after electrophoretic examination of a large number of species had shown that these two possessed plastid PGIs with the most disparate mobilities. Enzymes with distinct mobilities were required for the dissociation/reassociation experiments.

Localization of Isozymes. Plastid and cytosolic isozymes of spinach and *Clarkia* have been identified previously (19, 27). Cytosolic isozymes of the other species were identified by comparing the electrophoretic patterns of soaked pollen and leaf extracts, as described previously (26). Because the cauliflower plants did not produce sufficient pollen, pollen from broccoli (*B. oleracea* cv. Premium Crop Hybrid) was used. The plastid isozyme in cauliflower was identified by preparation of a chloroplast pellet, disrupting the pellet and subjecting the extract to starch gel electrophoresis (26). Ireland and Dennis (11) have shown that, in sunflower, the PGI isozyme exhibiting the greater mobility after PAGE is located in the plastids.

Isozyme Purification Procedure.

Tissue Homogenization and Isozyme Separation. All procedures were performed in the cold (5–8°C), and all buffers contained 42 mM ME, unless stated otherwise. For each species, approximately 100 g fresh leaf tissue were homogenized for 60 s in a Waring Blendor in 300 ml 50 mM Trizma-HCl (pH 8.3) containing 1 mM EDTA and 4 g insoluble polyvinylpolypyrrolidone. The resulting slurry was filtered through four layers of Miracloth intercalated with layers of glass wool. The filtrate was centrifuged for 60 min at 49,000*g* on a Sorvall RC 5 refrigerated centrifuge. The supernatant was applied to a 5- × 20-cm DE52 column equilibrated in 50 mM Trizma-HCl (pH 8.3). The column was washed with 1 volume of extraction buffer, and the PGI activity was then eluted with a 1,000-ml, 50 to 500 mM KCl gradient in extraction buffer. Two peaks of activity were discernible in all species tested. The fractions containing the first peak of PGI activity (cytosolic isozyme) were pooled and dialyzed overnight against 15 mM Hepes (pH 7.0). The fractions containing the second PGI activity peak

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[2] Abbreviations: PGI, phosphoglucose isomerase; PAGE, polyacrylamide gel electrophoresis; ME, 2-mercaptoethanol; HAP, hydroxyapatite; ELISA, enzyme-linked immunosorbent assay; GPI, glucose phosphate isomerase.

(plastid isozyme) were combined and placed in dialysis tubing and concentrated using PEG to a volume of approximately 4 ml.

Further Purification of the Plastid Isozyme. The concentrated plastid PGI fraction was applied to a 2.5- × 100-cm Sephacryl S-200 column. Column buffer was 10 mM EDTA (pH 7.2) containing 25 mM NaCl. Flow rate was 50 ml/h. Fractions containing PGI activity were pooled and dialyzed extensively against 6 mM sodium phosphate (pH 7.0) containing 50 mM NaCl. The dialyzed sample was placed on a 2- × 45-cm HAP (Bio-Rad) column equilibrated in the same buffer used for the dialysis. The column was washed with 100 ml of the phosphate buffer, and the PGI activity was eluted with a 400-ml, 6 to 200 mM sodium phosphate gradient at pH 7.0 containing 50 mM NaCl. The fractions containing PGI activity were pooled; they could be stored at 5°C for several weeks without loss of activity. The plastid PGI of sunflower was not purified beyond this step, and the preparation was designated 'partially purified plastid isozyme.'

Preparative PAGE for the Plastid Isozymes of Spinach and Cauliflower. The pooled fractions (approximately 40 ml) collected from the HAP column were placed in dialysis tubing and concentrated against PEG to a volume of 1 ml. The solution was then dialyzed for 30 min against 10 mM EDTA (pH 7.2) to reduce the salt concentration. This preparation was subjected to PAGE on 6% gels using a Hedrick-Smith system (9). After electrophoresis, the PGI band was located by applying briefly a Kimwipe (Kimberly-Clark, Neenah, WI) moistened with activity stain (26) on the surface of the gel and observing the location of the precipitated tetrazolium dye. The portion of the gel beneath the stain was excised with a razor blade. For work in which active enzyme was not required, the gel was stained with Coomassie brilliant blue R-250, and the major protein band was cut out. The protein obtained from this slice was judged homogeneous, because only a single protein band was visible after reelectrophoresis on 8 and 12% SDS gels.

Further Purification of the Cytosolic Isozyme. The 15 mM Hepes solution containing the cytosolic isozyme was applied to a second DE52 column (2 × 30 cm) equilibrated in the same Hepes buffer. The column was washed with 50 ml of buffer, and the PGI activity was eluted with a 400-ml, 15 to 200 mM Hepes gradient at pH 7.0. The activity peak eluting from this column was very sharp, effecting both significant purification and concentration of the enzyme. The two or three 4.0-ml fractions containing the majority of the PGI activity were individually applied to the 2.5- × 100-cm Sephacryl S-200 column and eluted under the conditions described earlier. For the spinach and cauliflower samples, the fractions which contained PGI activity were tested for purity by subjecting an aliquot to PAGE and staining for protein. Those fractions which exhibited a single protein band were pooled and designated 'purified cytosolic isozyme.' This preparation exhibited only a single band when 10 μg were subjected to PAGE. For sunflower and *Clarkia*, the entire activity peak off the Sephacryl column was combined and labeled 'partially purified cytosolic isozyme.' Both the purified and the partially purified preparations were stable in the EDTA buffer for several weeks and could be frozen for extended periods in 50 mM Hepes (pH 7.5) without loss of activity.

Enzyme Activity Assay. PGI activity was measured at 25°C by a coupled enzyme system in which fructose-6-P was added as substrate and glucose-6-P dehydrogenase was added as the coupling enzyme (5). The production of NADPH by the glucose-6-P dehydrogenase reaction was monitored at 340 nm on a Zeiss PM6 recording spectrophotometer. The standard reaction mixture contained 0.1 M Tris-HCl (pH 8.0), 0.5 mM NADP, 10 mM fructose-6-P, 10 mM $MgCl_2$, and 0.5 units/ml glucose-6-P dehydrogenase. The reaction was initiated by addition of a suitable aliquot of PGI extract. Usually, this was a 25-μl sample in a 475-μl assay mix; however, for samples with high PGI activity, either a smaller aliquot was used or a portion of the sample was diluted before assaying the activity.

Protein Measurement. Protein concentrations were determined with the Bio-Rad Protein Assay solution (Coomassie brilliant blue G-250) by measuring the *A* at 595 nm of the assay solution after addition of a known volume of sample. BSA (Bio-Rad) was used as the standard. Yeast PGI (Type X; Sigma) gave a standard curve similar to that obtained for BSA.

Mol Wt Determination. The mol wt of the various PGI isozymes were assessed by gel filtration on the Sephacryl S-200 column. An aliquot of the purified or partially purified isozyme was placed on the column in a total volume of no more than 2 ml. Triplicate determinations were performed in most cases, and all determinations contained 0.5 mg hemoglobin as an internal standard. The column was calibrated using glyceraldehyde 3-P dehydrogenase (143,000), yeast PGI (120,000), hemoglobin (66,000), and ovalbumin (43,000). The mol wt of the subunits were measured by SDS-PAGE on 10% gels, using a Weber-Osborn continuous system (24). Standards were phosphorylase b (94,000), BSA (68,000), yeast PGI (60,000), and ovalbumin (43,000).

Temperature Sensitivity Studies. Samples of the homogeneous spinach isozymes or the partially purified isozymes from other species were diluted in 15 mM Hepes (pH 7.0). Each diluted sample was divided into two aliquots, one being placed on ice and the other incubated at the test temperature. After specified time intervals, the PGI activity in each aliquot was determined using the standard assay conditions.

Zinc Inhibition Studies on Spinach Isozymes. To study the effect of zinc, the colorimetric assay of Roe (15) had to be employed, because the coupling enzyme, glucose-6-P dehydrogenase, in the normal assay, was found to be severely inhibited by the zinc concentration used. The standard assay mix (1.0 ml) contained 0.8 ml 0.1 M Hepes-KOH (pH 7.2), 0.1 ml of a 1% glucose-6-P solution, and 0.1 ml purified isozyme solution. Reactions were initiated by adding the substrate. Reaction conditions and color development were as described by Roe (15), and the *A* was measured at 490 nm on the Zeiss PM6 spectrophotometer. For zinc inhibition experiments, 0.05 ml of a 0.1 M $ZnCl_2$ solution was mixed with 0.75 ml of the Hepes buffer and 0.1 ml isozyme solution before addition of substrate. Final concentration of zinc in this solution was 5 mM; the final pH was 7.0.

Dissociation/Reassociation Procedure. These experiments were performed using a modification of the procedure of Tamaki *et al.* (23). Dissociation of the isozymes into their subunits was accomplished by adding a solution containing 5 to 25 μg of the desired isozyme or mixture of isozymes to an equal volume (usually 0.5 ml) of cold 10 M guanidine-HCl containing 0.1 M ME. After brief mixing, the solution was allowed to stand on ice for 10 min. The guanidine-HCl was then removed by passing the solution through a 1- × 10-cm Sephadex G-25 column equilibrated in 0.1 M triethanolamine hydrochloride (pH 7.2), containing 10 mM DTT and 10% (v/v) glycerol. The 2.0 ml of eluate immediately following the void volume contained the subunits, and this fraction was collected and allowed to stand overnight at 5°C to permit the reassociation of the subunits. Reassociation was assessed by starch gel electrophoresis, followed by staining for PGI activity (26).

The reassociation mix of purified plastid and cytosolic isozymes from cauliflower and a similar mix with purified spinach isozymes were investigated for the presence of hybrid enzymes by three methods. An aliquot of the solution was subjected to PAGE on three identical 6% tube gels, using a Hedrick and Smith buffer system (9). One gel was stained for PGI activity, and the second gel was stained for protein. The third gel was sliced into 0.25-cm sections, and each section was tested to determine whether it contained protein that was cross-reactive with antiserum raised in rabbits against purified plastid PGI and antiserum raised against purified cytosolic PGI (N. F. Weeden, R. C. Higgins, L. D. Gottlieb, in preparation). Each gel section was soaked overnight

in 0.2 ml 10 mM EDTA (pH 7.2) containing 25 mM NaCl and 14 mM ME. Duplicate 25-μl aliquots of each soaking solution were placed in wells on Dynatech plates (Dynatech, Alexandria, VA), and the cross-reactivity of each was measured by ELISA according to Engvall and Perlman (3), except that peroxidase-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) was used.

Biochemicals. Except where noted, all reagents and proteins were obtained from Sigma Chemical Co.

RESULTS

Enzyme Purification. A representative purification for both PGI isozymes from spinach is summarized in Table I. The plastid PGIs from spinach and cauliflower were judged to be homogeneous after the preparative PAGE step, because only one band was observed when these preparations were analyzed by electrophoresis on nondenaturing 6% gels and on denaturing Laemmli (12) 8% gels. The cytosolic isozymes obtained after Sephacryl S-200 column chromatography exhibited a similar degree of homogeneity. To maximize yield and minimize proteolysis, we found it necessary to perform the initial steps of the extraction procedure (through the Sephacryl column for the plastid PGI and to the dialysis against Hepes buffer for the cytosolic PGI) within 24 h. Degradation of PGI isozymes was monitored by starch gel electrophoresis. Initially, the PGI activity bands were sharp, without any anodal shadow; however, if the raw extract or the pooled fractions from the first DE52 column were allowed to stand overnight, the activity bands exhibited a prominent anodal blur. Maintaining a ME concentration of 42 mM in all buffers retarded the development of such shadows.

PGI activity was not observed in the wash of the first DE52 column, suggesting that all forms present in the crude extract had bound to the resin. From preliminary studies using step gradients on this column, it was determined that the cytosolic isozyme (first peak) eluted at approximately 100 mM KCl and the plastid isozyme at about 200 mM KCl. The exact position of the activity peak of a particular isozyme correlated with the mobility of that isozyme as determined by starch gel electrophoresis. An increase in mobility was almost invariably accompanied by a shift of the activity peak to a higher KCl concentration. The cytosolic isozymes eluted from the Sephacryl column immediately after a larger mol wt contaminant. Usually, the initial fractions of this PGI peak contained a significant quantity of the contaminating protein. However, the trailing one-half of the activity peak for both the spinach and the cauliflower extractions appeared free of this contaminant.

The specific activity of the cytosolic isozymes from both spinach (Table I) and cauliflower was approximately 700 IU/mg, which is similar to that found for yeast PGI (13). The plastid isozymes exhibited a specific activity of less than one-half this value. No previous data is available for this isozyme.

The plastid PGI from *Clarkia* could not be purified using the described procedure, because it coeluted from the first DE52 column with a viscous contaminant, which caused poor resolution of proteins on both the Sephacryl and the HAP columns. The plastid PGI could be freed of the viscous material by a 20 to 40% ammonium sulfate precipitation after the DE52 column; however, this resulted in a significant loss of activity.

Dissociation/Reassociation Experiments. Evidence that the PGI molecules had been dissociated and reassociated was obtained by two methods. First, a complete loss of enzymic activity was observed after addition of the guanidine-HCl, and this activity did not reappear until after the solution had been passed through the Sephadex G-25 column. Second, an additional activity band of intermediate mobility was observed after electrophoresis of certain reassociated samples, which originally contained only two enzymes. These novel bands were interpreted as hybrid molecules, consisting of one subunit from each of the two tested enzymes.

This interpretation was verified by experiments involving the three cytosolic PGI allozymes that could be isolated from sunflower (Fig. 1A). Genetic investigations (N. F. Weeden, unpublished) established that these enzymes were generated by the three possible combinations (FF, FS, and SS) of two allelic products, F (fast mobility) and S (slow mobility). Following electrophoresis on starch gels, the FF homodimer has the most anodal mobility; the FS heterodimer migrates slightly more slowly; and the SS homodimer has the slowest mobility (Fig. 1A). Subjecting either the FF homodimer or the SS homodimer to the dissociation/reassociation procedure should produce only a single activity band

Table I. *Representative Preparation of Phosphoglucose Isomerase Isozymes from Spinach*

Fraction	Volume	Total Protein	Total Activity	Specific Activity	Purification	Yield
	ml	*mg*	*IU*	*IU/mg protein*	*-fold*	*%*
Trizma-HCl extract	285	570	340	0.6		
First DE52 column						76[a]
Plastid PGI Isolation						
Second peak from DE52 column	73	61	94	1.5	2.5	76[b]
Sephacryl column chromatography	42	4.4	59	13	22	48
Hydroxyapatite column	44	0.62	50	81	134	40
PAGE and soak	4	0.08	24	300	500	20
Cytosolic PGI Isolation						
First peak from DE52 column	73	19	165	8.7	14	76[b]
DE52 chromatography in Hepes	17	0.6	110	183	306	51
Sephacryl column chromatography	44	0.07	48	690	1100	22[c]

[a] Yield based on calculated sum of total activity from both peaks from DE52.

[b] Yield for both isozymes at this step was taken as 76%, as we could not determine the true yield for each isozyme. Subsequent yield values were calculated on the basis that 100% yield for the plastid isozyme would have been 124 IU and 100% yield for the cytosolic isozyme would have been 217 IU.

[c] The yield of the Sephacryl column could be increased by taking the whole activity peak; however, the contaminating proteins would be included (see text).

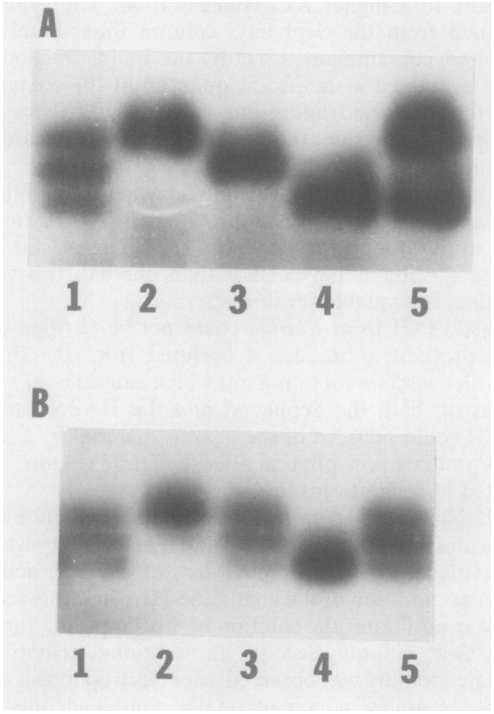

FIG. 1. Dissociation/reassociation of sunflower cytosolic PGI allozymes. A, Activity bands present after starch gel electrophoresis of allozyme preparations before dissociation/reassociation procedure. Lane 1, Raw extract (standard); lane 2, fast homodimer; lane 3, heterodimer; lane 4, slow homodimer; lane 5, mixture of fast and slow homodimers. B, Activity bands seen on starch gels after all preparations, except the raw extract, were subjected to the dissociation/reassociation procedure. The sequence on the gel is the same as that in A. Anode is at top of each photograph.

after electrophoresis, at a position identical to that of the original homodimer, whereas, if a sample containing only the FS heterodimer or a mixture of both homodimers were dissociated and reassociated, all three forms should be evident after electrophoresis. In every case, the observed activity bands conformed with the predicted results (Fig. 1B).

Active hybrid enzymes were also produced following dissociation and reassociation of cytosolic isozymes from different species. The combinations tested were spinach and *Clarkia*, cauliflower and *Clarkia*, and cauliflower and sunflower. Hybrid dimers of plastid-specific PGIs were also produced in two combinations (cauliflower and sunflower, sunflower and spinach). Following reassociation, the yields varied from 20 to 70% of the original PGI activity used in the test. Purer preparations and increased concentrations of the enzymes gave higher yields.

An additional activity band was not produced when a combination of a cytosolic isozyme and a plastid isozyme was subjected to the dissociation/reassociation procedure. To confirm that the cytosolic and plastid isozymes had both dissociated in such mixtures and, thus, had had the opportunity to form hybrid dimers, two control experiments were performed. In the first experiment, two distinguishable plastid isozymes and one cytosolic isozyme were placed in the same solution, and a dissociation/reassociation reaction was performed on an aliquot. Four activity bands were observed after electrophoresis of the reassociation mixture (Fig. 2), which corresponded to the three original homodimers and a novel fourth band which was the hybrid formed between the plastid subunits. The second control, performed under identical conditions, consisted of two isozymes, the cytosolic heterodimer from sunflower plus the cauliflower plastid isozyme. Again, four

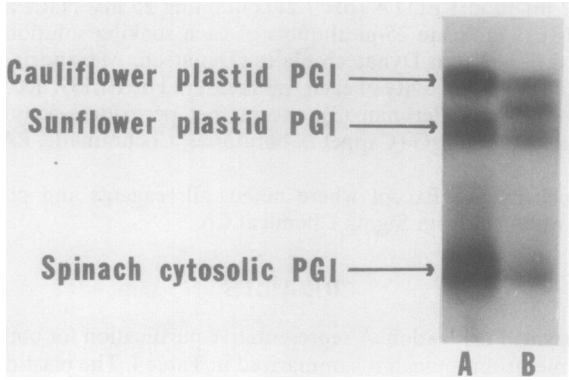

FIG. 2. Dissociation/reassociation experiment involving the plastid PGIs from cauliflower and sunflower and the cytosolic PGI from spinach. A, Mixture of the three enzymes before dissociation/reassociation; B, mixture after dissociation/reassociation. Anode is at top of figure.

activity bands were observed after electrophoresis, including the most anodal cauliflower plastid homodimer and the fast, hybrid, and slow allozymes of the sunflower cytosolic PGI described earlier (Fig. 1). These results demonstrate that both the plastid and the cytosolic isozymes dissociate in these mixtures but do not reassociate with each other to form active hybrid molecules.

Experiments which were designed to identify enzymically inactive as well as active molecules also indicate that hybrids between plastid and cytosolic PGI subunits do not form *in vitro*. The protein-stained gel showed only the same two bands as did the gel stained for PGI activity (Fig. 3). Results from the immunological tests indicated that protein cross-reacting with cytosolic PGI antiserum was located only in a single region coincident with the cytosolic PGI homodimer, and the antiserum raised against plastid PGI reacted only with protein localized in the plastid PGI activity peak. If hybrid enzymes had been formed, the antibodies would have cross-reacted with them, because the antisera had been raised against SDS-denatured isozymes and had recognized individual subunits as well as the native enzymes (N. F. Weeden, R. C. Higgins, L. D. Gottlieb, in preparation).

The variation in the background *A* on the gel stained for protein was low enough to permit densitometric observation of a peak 10% the height of the cytosolic PGI peak (Fig. 3). Similarly, in the ELISA test, a peak 2% the height of the homodimer cross-reactivity would have been observable above the background. Thus, if inactive heterodimers formed, they did so at a very low frequency. In the parallel tests using purified spinach isozymes, the protein stain and immunological probe again failed to produce evidence for the production of heterodimers. These tests were not as sensitive, however, because 5-fold less protein was applied to each gel.

Mol Wt Measurements. Gel filtration on Sephacryl S-200 indicated that the mol wt of spinach, cauliflower, and sunflower plastid PGIs were all about 140,000 daltons. The cytosolic PGI from each of these species, as well as that from *Clarkia*, was calculated to be 125,000 daltons (Fig. 4). In contrast, the subunits of both the cytosolic and the plastid isozymes of cauliflower and spinach exhibited indistinguishable mobilities on Weber-Osborn gels. This mobility was slightly less than that of yeast PGI subunits, indicating a mol wt for the plant PGI subunits of 63,000 daltons.

Temperature Stability of the Isozymes. The results of the heat sensitivity studies are given in Table II. All forms of PGI tested were stable at 31°C for 60 min. After 10-min incubation at 42°C, all of the plastid PGI isozymes exhibited some loss of activity, ranging from 14 to 100%. At this same temperature, none of the cytosolic isozymes displayed any loss of activity. The best temperature for differentiating cytosolic and plastid PGIs appeared to be 47°C. At this temperature, all plastid PGIs suffered marked loss

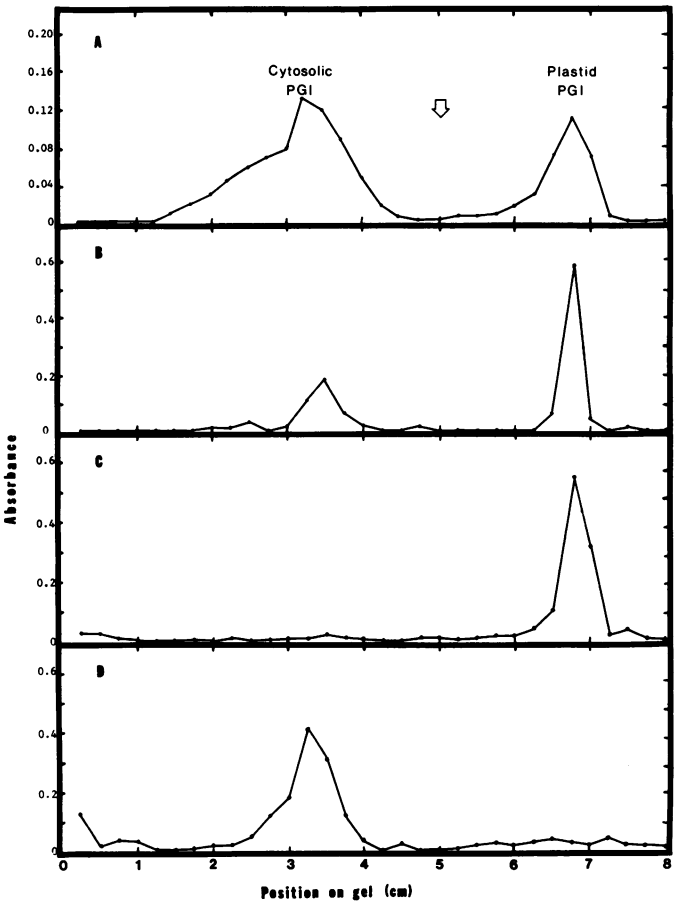

FIG. 3. Protein and immunological search for possible catalytically inactive hybrid enzymes following dissociation and reassociation of the cytosolic and plastid PGI isozymes from cauliflower. A, Densitometric tracing of a tube acrylamide gel stained for PGI activity. Arrow indicates the expected position of a hybrid enzyme. B, Densitometric tracing of a tube acrylamide gel stained for protein using Coomassie brilliant blue R-250. C, Cross-reactivity, as determined by ELISA, of protein in gel sections with antiserum raised against SDS-denatured spinach plastid isozyme. Cross-reactivity of material contained in a 0.25-cm segment of gel should be directly proportional to the *A* measurement. D, Cross-reactivity, as determined by ELISA, of protein in gel sections with antiserum raised against SDS-denatured spinach cytosolic isozyme. All graphs have the same abscissa. Densitometric scans were obtained on a Quick Scan Jr. (Helena Laboratories, Beaumont, TX) using a filter transmitting at 550 to 575 nm. Absorbance was measured on a Titertek Multiscan (Flow Laboratories, Rockville, MD).

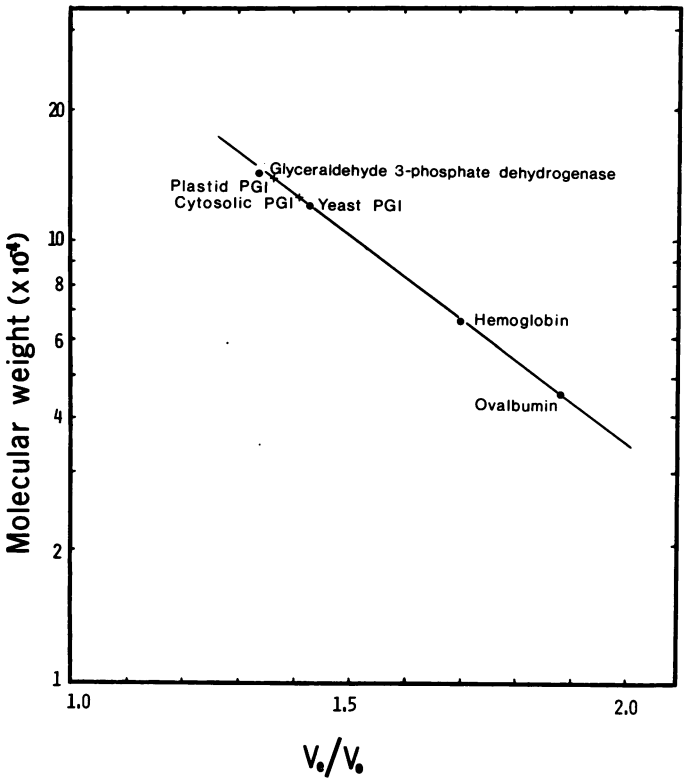

FIG. 4. Mol wt determination of plastid and cytosolic PGI isozymes using Sephacryl S-200 column chromatography. Standards are glyceraldehyde 3-P dehydrogenase (143,000), yeast PGI (120,000), hemoglobin (66,000), and ovalbumin (43,000).

of activity (35 to 100% inactivation) after 10 min, while most cytosolic isozymes were stable for 40 min.

Zinc Inhibition of Spinach PGI Isozymes. Purified spinach plastid PGI was completely inhibited by 5 mM Zn^{2+}. The activity of the cytosolic isozyme at this Zn^{2+} concentration was reduced to 49% of the activity of the untreated control. Higher Zn^{2+} concentrations could not be tested, due to the formation of a zinc hydroxide precipitate at pH 7. At 1 mM Zn^{2+}, less than 10% inhibition was observed for the plastid isozyme, and none was observed for the cytosolic.

DISCUSSION

The experiments reported in this paper demonstrate that both plastid and cytosolic PGI isozymes can be dissociated into their respective subunits, that plastid subunits from different plant species could be reassociated into active hybrid enzymes, that different cytosolic subunits could also be reassociated into active enzymes, but that neither active nor inactive hybrid molecules could be formed between plastid and cytosolic subunits. The dissociation/reassociation experiments were performed with rigorous controls. These included the use of a combination of isozymes in the same mixture, to confirm that all forms were dissociated, and the search for inactive hybrid molecules by protein staining and immunological techniques. The dissociation/reassociation procedure is probably applicable to various other proteins and can be used for similar studies on other isozyme systems.

The inability of plastid and cytosolic PGI subunits to bind each other *in vitro* provides an explanation for the absence of such hybrid dimers *in vivo* and reflects a significant divergence in the binding regions of the two polypeptides. It should be emphasized that the results do not preclude the possibility that other mechanisms are also controlling subunit association. The apparent absence of active plastid-specific isozymes in the cytosol indicates that such a mechanism may be preventing the association of the subunits of this isozyme before they enter the plastid compartment. The plastid PGI subunit may be synthesized initially as a larger precursor, as is the case for the small subunit of ribulose bisphosphate carboxylase (2, 10). The additional polypeptide segment on the original translation product may be necessary for transport of the precursor molecules across the plastid envelope (10). Such a segment may also alter the folding properties of the plastid PGI subunits, thus preventing dimerization. A similar suggestion has been made for nuclear-coded mitochondrial enzymes (8). In addition, such a mechanism may further inhibit any interaction between cytosolic subunits and the plastid subunits in the cytosol.

The plastid and cytosolic PGIs differed in several other characteristics. The mol wt determinations suggest that the cytoplasmic PGI is a more compact molecule than is the plastid isozyme. The two isozymes also exhibit considerable antigenic differences, as

Table II. *Temperature Stability of PGI Isozymes*

Incubation Temperature	Duration of Incubation	Percentage of Activity Remaining after Incubation							
		Plastid PGIs				Cytosolic PGIs			
		Spin[a]	Caul	Sunf	Clar	Spin	Caul	Sunf	Clar
°C	*min*								
31	60	86	100	100	100	100	100	100	100
42	10	0	86	85	42	100	100	100	100
47	10	0	4	65	20	87	100	100	100
47	40	0	0	26	0	63	100	100	100
50	10	0	0	ND[b]	ND	27	ND	ND	100

[a] Spin, spinach; Caul, cauliflower; Sunf, sunflower; Clar, *Clarkia.*
[b] Not determined.

the cross-reactivity of the heterologous antigen with either antiserum was very low (Fig. 3). Furthermore, the specific activity of the cytosolic form was over twice that of the plastid enzyme. That this difference was not an artifact produced by the extraction procedure was indicated by the observation that the ratio of activity to protein as determined by area under the curves for the cytosolic PGI peak in Figure 3 is 3 times that for the plastid PGI peak. The subunits able to reassociate to form these active enzymes were at least intact and probably unmodified by the extraction procedure, making this relative specific activity figure a very good approximation.

The plastid and cytosolic PGIs generally have distinct electrophoretic mobilities, with the plastid form always being faster (6, 7, 11). This difference provides a convenient method for the separation of the two isozymes using anion exchange chromatography (*e.g.* the first DE52 column in our procedure). It also makes it possible to identify the plastid and cytosolic isozymes in three previous studies, in which their intracellular locations were not reported. Thus, the 'GPI II' in maize (16) and the 'phosphoglucoisomerase III' in sweet potato (17) were most likely the plastid isozymes, because they eluted from a DEAE column at about 0.2 M salt concentration. The PGI enzyme purified from pea (22) is probably the cytosolic form, because it eluted from DEAE cellulose at 0.1 M NaCl.

This interpretation is supported by the different heat sensitivities of the two isozymes. Both the GPI II of maize (16) and the PGI III from sweet potato (17) were reported to be heat labile compared to the other isozyme(s), as was found for the plastid isozymes of spinach, cauliflower, sunflower, and *Clarkia.* Finally, the maize GPI II was reported to be inhibited by zinc, whereas the other forms were not (16). Based on our results with the spinach isozymes, this would again indicate that GPI II was the plastid isozyme.

Sweet potato extracts clearly show the plastid PGI isozyme when subjected to starch gel electrophoresis, although the activity of this form comprises only a small fraction of the total activity in the extract. The inability of Phillips *et al.* (14) to confirm the findings of Sasaki *et al.* (17) may reflect differences in the extraction procedure. The latter group subjected the raw extract to a 20 to 45% ammonium sulfate cut before column chromatography. We have found that such a fraction from spinach or *Clarkia* extracts contains primarily the plastid isozyme. At least 60% saturated ammonium sulfate solution is required to obtain good precipitation of the cytosolic form. Thus, it is likely that the extract placed on the DEAE cellulose column by Sasaki *et al.* was significantly enriched in the plastid isozyme. Phillips *et al.* (14) did not include an ammonium sulfate precipitation step and probably overlooked the smaller activity peak of the plastid isozyme.

Except for their catalysis and certain kinetic properties (4, 19), the cytosolic and plastid PGIs from dicotyledonous plants appear to be strikingly different molecules. The subunits differ in their binding regions and their immunological properties, while the intact enzymes display distinct heat sensitivity profiles, zinc inhibition properties, and apparent mol wt. These structural and kinetic disparities must reflect a considerable divergence between the genes coding the two subunits. It remains to be seen whether the heterogeneity exhibited by these two nuclear genes is characteristic of most plastid/cytosolic isozyme pairs or if the PGI isozymes are exceptional in their divergence.

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