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Glucose 6-Phosphate Dehydrogenase Isozymes of Maize Leaves[1]

SOME COMPARATIVE PROPERTIES

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ABSTRACT

Two different forms of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) have been purified from etiolated and green leaves, respectively, of 6-day maize (*Zea mays* L. cv Fronica) seedlings. The procedure includes an ammonium sulfate step, an ion exchange chromatography, and a second gel filtration in Sephadex G-200 in the presence of NADP+ to take advantage of the corresponding molecular weight increase of the enzyme. The isozyme from etiolated leaves is more stable and has been purified up to 200-fold. Subunit molecular weight, measured by sodium dodecyl sulfate-gel electrophoresis, is 54,000. The active protein, under most conditions, has a molecular weight 114,000, which doubles to molecular weight 209,000 in the presence of NADP+. The association behavior of enzyme from green leaves is similar, and the molecular weight of the catalytically active protein is also similar to the form of etiolated leaves.

Glucose 6-phosphate dehydrogenase of dark-grown maize leaves isoelectric point (pI) 4.3 is replaced by a form with pI 4.9 during greening. The isozymes show some differences in their kinetic properties, K_m of NADP+ being 2.5-fold higher for pI 4.3 form. Free ATP (K_m = 0.64 millimolar) and ADP (K_m = 1.13 millimolar) act as competitive inhibitors with respect to NADP+ in pI 4.3 isozyme, and both behave as less effective inhibitors with pI 4.9 isozyme. Magnesium ions abolish the inhibition.

Glucose 6-P dehydrogenase in photosynthetic cells of most higher plants occurs as two distinct isozymes, localized in cytoplasm and chloroplasts, respectively, and with comparable activities (1, 3, 20). The plastid isozyme seems to resemble the cytoplasmic form in several general properties, including mol wt, kinetic properties, and control by certain effectors (1, 11, 14, 20). A single isozyme has, however, been reported for C_4 species of the 'malate formers' group (10). It will be shown in this paper that the isozyme of etiolated maize leaves is replaced by a different form during greening, in a way reminiscent of the substitution pattern of isozymes of NADP-dependent malic enzyme (18). The two glucose 6-P dehydrogenase isozymes show minor but distinctive kinetic differences. Subunit structure and association behavior of the purified enzyme are also described, and their resemblances to enzyme from animals and fungi are apparent.

MATERIALS AND METHODS

Maize plants (*Zea mays* L. cv Fronica) were germinated as described previously (18). Leaves and coleoptiles of 6-d seedlings, either dark- or light-grown (about 100 g) were excised, cut to pieces, and homogenized thoroughly in a Servall Omnimixer at full speed in an ice bath, in the presence of 3 volumes of buffer A (40 mM Tris-HCl, 1 mM EDTA, 14 mM 2-mercaptoethanol, 24 μM NADP+, 0.1 mM phenyl methyl sulfonyl fluoride, pH 7.5). All subsequent operations were carried out at 2 to 4°C. The filtered homogenate was centrifuged 30 min at 20,000*g*, and the supernatant was brought to 40% saturation by dissolving $(NH_4)_2SO_4$. After centrifuging (30 min, 20,000*g*), the supernatant was brought to 50% saturation in $(NH_4)_2SO_4$ and centrifuged. The sediment was suspended to 15 ml in buffer, and clarified. The preparation was desalted through a Sephadex G-25 column (2.5 × 30 cm) and equilibrated with buffer B (10 mM Tris-HCl, 1 mM EDTA, 14 mM 2-mercaptoethanol, pH 7.5). Pooled active fractions were applied to an equilibrated DEAE cellulose column (Whatman DE 52, 2 × 20 cm). Fractions of 7 ml were collected. The column was washed with 100 ml of buffer B, containing 100 mM KCl. The activity was eluted with 100 ml of buffer B containing 200 mM KCl. The DEAE cellulose step was omitted in some experiments (see "Results").

The active fractions from DEAE cellulose (or directly from Sephadex G-25) were concentrated to 3 to 4 ml by ultrafiltration (Amicon 52 cell, PM 10 membrane), or by precipitation with $(NH_4)_2SO_4$ to 70% saturation and resuspension in buffer. The clarified preparation was purified further by passage in a Sephadex G-100 or G-200 column of 2.6 × 60 cm, equilibrated with buffer B plus 100 mM KCl. The active fractions were desalted by ultrafiltration and used for the experiments.

For complete purification of enzyme from etiolated maize, the more active fractions from this first gel filtration were treated with 0.24 mM NADP+ overnight, concentrated (PM 10) and applied to a Sephadex G-200 column previously equilibrated with gel filtration buffer (above) containing 3 mM 2-mercaptoethanol and 24 μM NADP+. The active peak of mol wt about 200,000 was concentrated. In some experiments, the enzyme was filtered a third time in the same column, in the absence of NADP+ (enzyme mol wt about 100,000). Procedures for SDS-gel electrophoresis (23) and for determination of marker enzymes in chromatographic eluates have been described (18, 19, 22). For preparative electrofocusing, an LKB 8100 (110 ml) column was loaded with sucrose gradient (40 to 5%). The solution to be mixed also contained, in most experiments, Ampholines pH 4 to 6 (40%), 1.5 ml; pH 3.5 to 10, 1 ml; pH 2.5 to 4, 0.5 ml; 2-mercaptoethanol, 50 mM; NADP+, 0.1 mM. Slightly different Ampholine mixtures were used in early experiments. Focusing was run 22 h at 500 v (4°C). Enzyme activity was assayed spectrophotometrically (340 nm) in a mixture containing 100 μmol Tris-HCl, 1 μmol EDTA, 2.5 μmol glucose 6-P, 0.12 μmol NADP+ (pH 7.5) and enzyme, in a 1.0 ml volume. Proteins were measured by a Coomassie blue method (6) with slight modifications, using BSA as standard.

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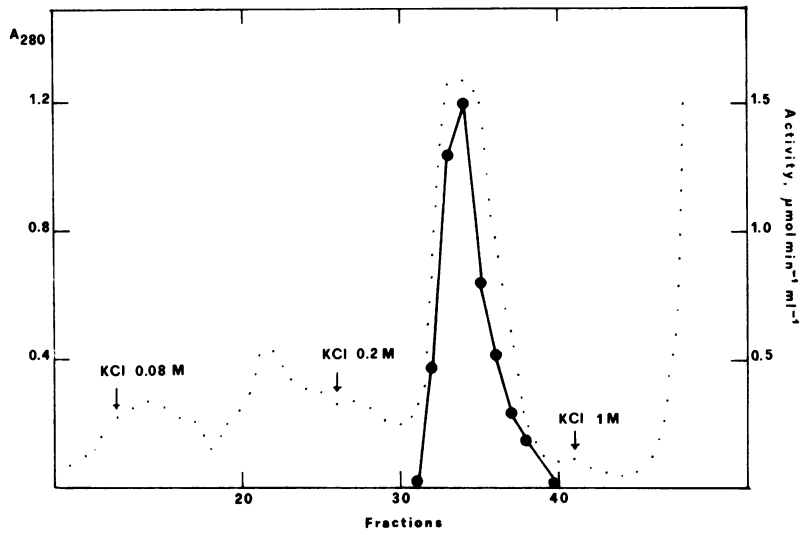

FIG. 1. DEAE cellulose chromatography of glucose 6-P dehydrogenase from dark-grown maize leaves (98 g) using a KCl step gradient, pH 7.5. The preparation was previously purified by ammonium sulfate fractionation and Sephadex G-25 filtration (83 mg protein). Fractions of 7 ml. A_{280} (· · · ·); enzyme activity (●), μmol min^{-1} ml^{-1}.

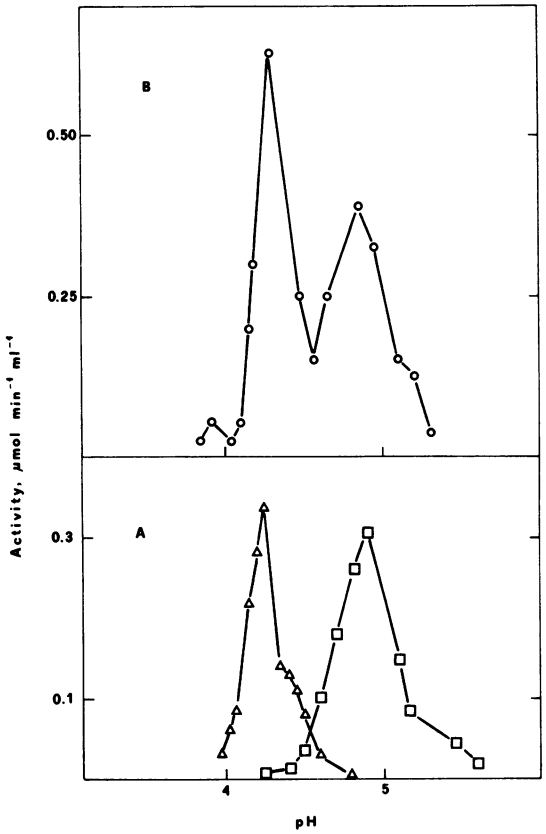

FIG. 2. Isoelectrofocusing of glucose 6-P dehydrogenase in a 110-ml column (sucrose gradient). A. (△), Enzyme partially purified from etiolated leaves (10.2 mg protein of a DEAE cellulose step); (□), enzyme from green leaves (13.3 mg). B. (○) A mixture of clarified extracts from green and etiolated leaves was prepared, containing equal activities from both sources (12.8 μmol min^{-1} each). This preparation was then processed (33 mg protein) and focused as explained for part A. See "Materials and Methods" and Table III for more details of purification and focusing procedures.

Reagents were analytical grade. Substrates and proteins used as markers were from Sigma, unless stated otherwise.

RESULTS

In preliminary studies, glucose 6-P dehydrogenase from etiolated or green maize leaves was partially purified to specific

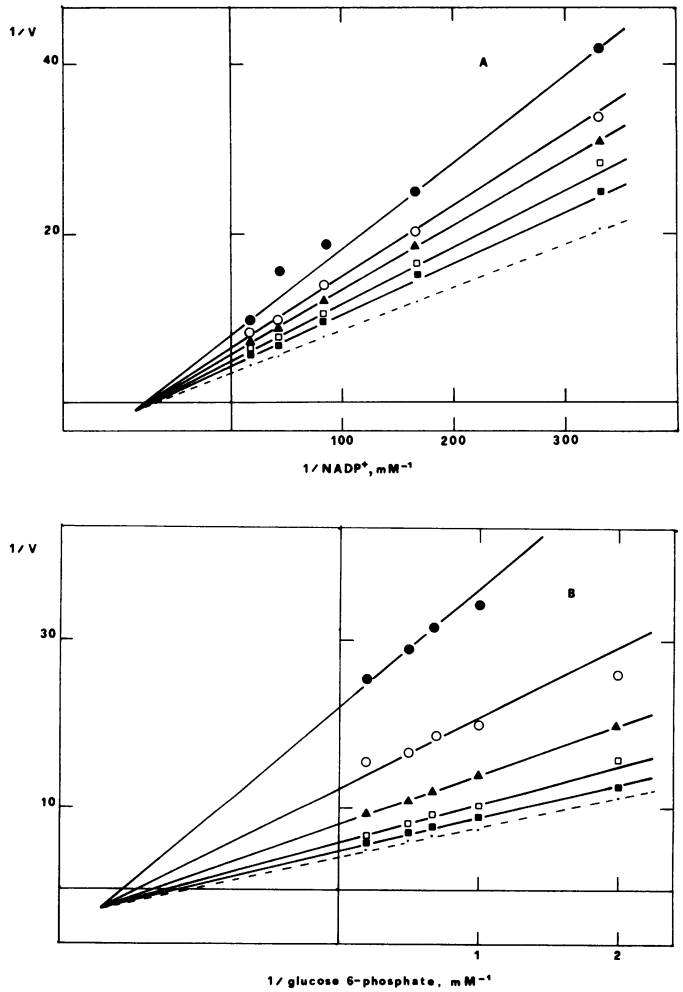

FIG. 3. Double reciprocal plots of a single set of velocity (μmol min^{-1} ml^{-1}) *versus* substrate data. Enzyme (170 μg per assay) from dark-grown maize leaves, purified up to first Sephadex G-200 filtration. A, $NADP^+$ concentration varied, glucose 6-P treated as nonvaried substrates. Glucose 6-P concentrations: (■), 5 mM; (□), 2 mM; (▲), 1.5 mM; (○), 1.0 mM; (●), 0.5 mM. B, Glucose 6-P varied at several fixed $NADP^+$ concentrations: (■), 60 μM; (□), 24 μM; (▲), 12 μM; (○) 6 μM; (●), 3 μM.

Table I. *Some Kinetic Constants of Maize Leaf Isozymes*

Average of data from three different preparations, each purified up to at least the first gel chromatography step.

	Etiolated Leaf Isozyme	Green Leaf Isozyme
	mM	
K_m ($NADP^+$)	0.008 ± 0.002[a]	0.019 ± 0.002
K_m (glucose 6-P)	0.57 ± 0.06	0.33 ± 0.08
K_i (ATP)	0.64 ± 0.07	1.4 ± 0.1
K_i (ADP)	1.13 ± 0.05	3.5 ± 0.3

[a] Mean ± SD.

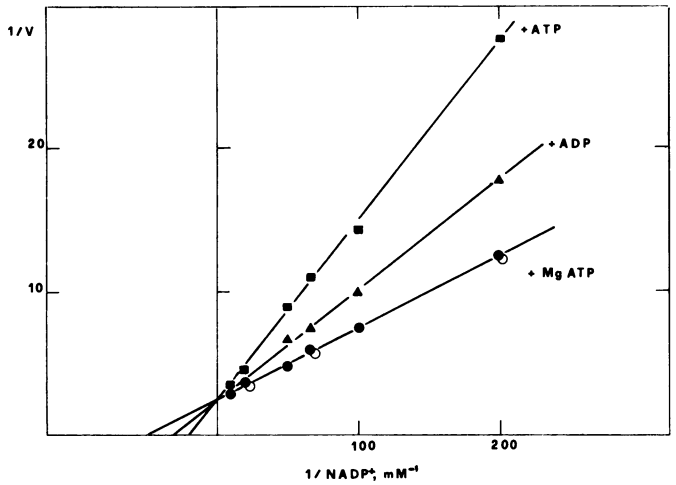

FIG. 4. Double reciprocal plots of velocity *versus* $NADP^+$ concentration, in the presence or absence of adenine nucleotides. (■), $NADP^+$ varied in the presence of 2 mM ATP (neutralized); (▲), of 2 mM ADP; (○), of 2 mM ATP and 10 mM $MgCl_2$; (●), without additions. Green leaf isozyme, referred to 0.5 mg protein per assay.

activities of 1-2 μmol min^{-1} mg^{-1} by a combination of $(NH_4)_2SO_4$ precipitation, desalting through Sephadex G-25, DEAE cellulose chromatography (Fig. 1), and a first Sephadex G-200 gel filtration. The yield was variable due to losses at the ion exchange chromatography step, and was usually higher (33% *versus* 8%) for preparations from etiolated material. The DEAE cellulose step, therefore, was omitted in some experiments aimed at assessing the native isozyme profile by column isoelectrofocusing.

Etiolated maize leaves contained a single glucose 6-P dehydrogenase peak with pI[2] 4.3 (Fig. 2A), similar to the value reported for the cytosolic enzyme from pea leaves (1). The form of green leaves showed a dominant pI 4.9 peak, also similar to the pea chloroplast dehydrogenase. The isoelectric points of the isozymes were reproducible on a second and third focusing and their respective profiles were distinguishable when both were mixed in crude extracts and focused together (Fig. 2B).

Both partially purified isozymes presented hyperbolic kinetics toward $NADP^+$ and glucose 6-P under conditions of assay. Michaelis constants were somewhat dependent on, and increasing with, concentration of nonvaried substrate, and linear double reciprocal plots were characterized by an intersection point located slightly below the abscissae in the lower left quadrant (Fig. 3, A and B). This value may be related to the reciprocal of the dissociation constant of enzyme-substrate complex (Cleland's K_i, [7]). The limiting K_m for $NADP^+$ was 2- to 3-fold lower in preparations from etiolated tissue (pI 4.3 form) that, however, may exhibit slightly lower affinity for glucose 6-P than pI 4.9 isozyme does (Table I). The values fall within the range observed

[2] Abbreviation: pI, isoelectric point.

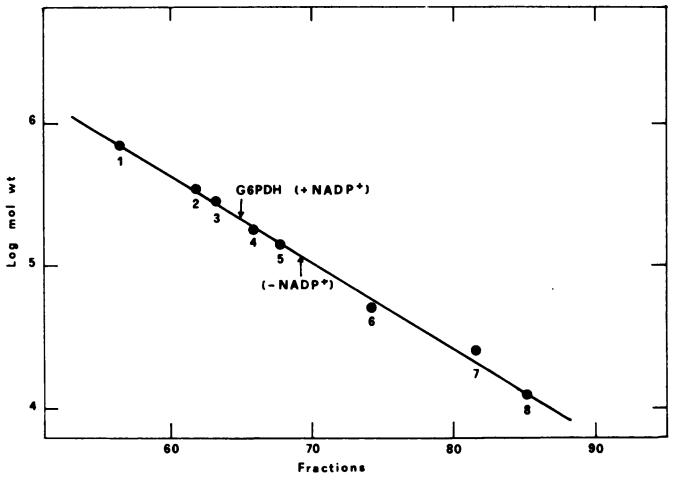

FIG. 5. Mol wt determination for enzyme from green leaves (indicated as G6PDH), a preparation previously purified by DEAE cellulose and Sephadex G-100 gel chromatography. Samples of 0.5 mg protein were chromatographed through a Sepharose 6B column (2.6 × 58 cm) in the presence or absence of 30 μM $NADP^+$ in the buffer. Fractions of 3.1 ml were collected. Markers: 1, Beet chloroplast glyceraldehyde 3-P dehydrogenase (assumed mol wt 700,000); 2, ferritin (340,000); 3, NADP-dependent malic enzyme from green maize (280,000); 4, beet leaf glyceraldehyde 3-P $NADP^+$ reductase (180,000); 5, beet leaf glyceraldehyde 3-P dehydrogenase, NAD-specific (140,000); 6, peroxidase, horseradish (50,000); 7, chymotrypsinogen A (25,000); 8, Cyt *c* (12,500). Markers 1, 3, 4, 5 were detected in eluates by enzyme activity; markers 2, 6, and 8 by A_{410}; marker 7 by A_{280} (5 mg).

Table II. *A Comparison of Some Structural Properties of Maize Glucose 6-P Dehydrogenase*

pI was determined by column isoelectrofocusing, mol wt by analytical Sephadex G-200 chromatography. Subunit mol wt was calculated from SDS-acrylamide gel electrophoresis of purified protein from etiolaed material. Several different preparations from etiolated and green leaves were used.

	Etiolated Leaf Isozyme	Green Leaf Isozyme
pI	4.29 ± 0.05[a]	4.87 ± 0.06
Apparent mol wt ($NADP^+$ absent), kD	114 ± 2	116 ± 1
Apparent mol wt ($NADP^+$ present), kD	209 ± 6	212 ± 3
Mol wt (monomers), kD	54 ± 2	

[a] Mean ± SD.

for other plant species.

Figure 4 illustrates the effects of free ATP and ADP as competitive inhibitors, using $NADP^+$ as variable substrate. Both nucleotides inhibited the isozyme from etiolated leaves more effectively than the isozyme from green leaves (Table I), the ratio of respective inhibition constants being, again, ½ to ⅓. Addition of excess Mg ions in the assay prevented the inhibition completely.

Mol wt and association behavior of the isozymes were investigated by gel exclusion chromatography. The active dehydrogenases were found to have an apparent mol wt of 114,000 to 116,000 when filtered in buffer B containing 100 mM KCl, as seen in Figure 5 and Table II. This value first obtained for preparations from etiolated or photosynthetic leaves, chromatographed in the presence of 3 to 14 mM 2-mercaptoethanol and 1 to 2 mM EDTA, was not changed by addition of DTE (up to 5 mM) or NADPH (0.02–0.03 mM) to the filtration buffer. In contrast, the pI 4.3 enzyme chromatographed in the presence of $NADP^+$ (0.02–0.03 mM) had an average mol wt 209,000, and pI 4.9 isozyme had mol wt 212,000 (Fig. 5; Table II). Thus purified

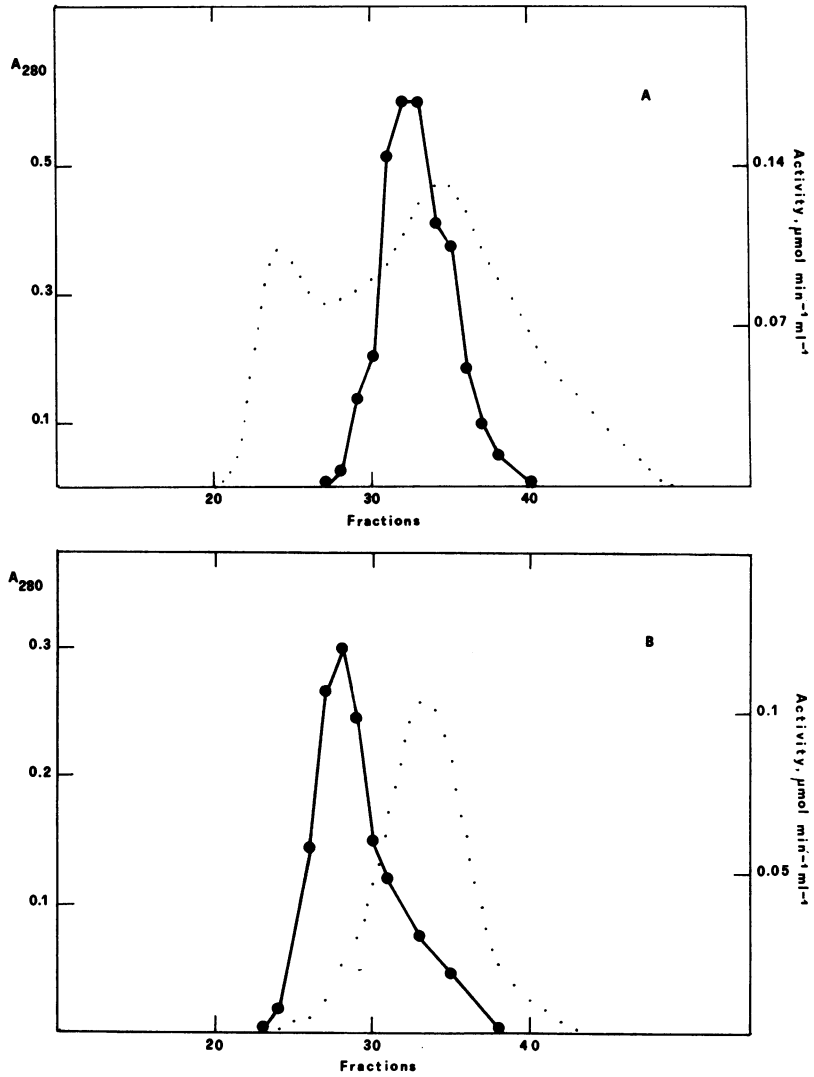

FIG. 6. Chromatographic profiles of pI 4.9 isozyme in a Sephadex G-200 column (2.6 × 61 cm, 6.2 ml fractions) under different conditions. A, A fraction from DEAE cellulose (28 mg, specific activity 0.55 μmol min^{-1} mg^{-1}) was applied in the absence of $NADP^+$. B, Fractions 31 to 36 from this first column were pooled, concentrated, treated with 0.2 mM $NADP^+$, and applied to the same column, now equilibrated with 24 μM $NADP^+$. (●), activity; (····), A_{280}.

preparations from etiolated or photosynthetic leaves did not appear to differ significantly in mol wt and association behavior. Enzyme pretreatment with $NADP^+$ failed to appreciably affect the subsequent association pattern during gel permeation chromatography, which depended only on the cofactor present in the column. This implies fast and reversible changes of enzyme molecular structure. Presence or absence of EDTA (2 mM) or Mg ions (10 mM) did not appear to affect the association state.

The reversible association properties of this enzyme were exploited for its complete purification, using preparations from etiolated material. Active fractions from the first gel filtration step (Fig. 6A) were collected, concentrated, treated with buffer containing 0.24 mM $NADP^+$ and 3 mM 2-mercaptoethanol, and loaded on a Sephadex G-200 column equilibrated with an $NADP^+$ medium inducing reassembly to the higher mol wt enzyme form (Fig. 6B). The head fractions of the peak were pooled. After this step, the dehydrogenase was found to be 70 to 80% pure, judged by SDS-gel electrophoresis (Fig. 7). The major band was not distinguishable in SDS electrophoresis from the single band of authentic *Torula* yeast glucose 6-P dehydrogenase (mol wt 53,000, [24]). Table III summarizes the procedure. The reverse purification protocol, *i.e.* first filtering the dehydrogenase in the presence of $NADP^+$, then in its absence, gave preparations with comparable specific activities (3–6 μmol min^{-1} mg^{-1}) but with a larger amount of minor bands.

The calculated mol wt of the subunits of pI 4.3 isozyme (Fig. 8) was 54,000 ± 2,000 similar to, although somewhat higher than, reported values for glucose 6-P dehydrogenases from nonplant sources (see below).

DISCUSSION

Although glucose 6-P dehydrogenase is an important control point for the oxidative pentose phosphate pathway of chloroplasts (3), little is known of its molecular structure in plant tissues. Schnarrenberger *et al.* (20) established a mol wt 105,000 (±10%) for both soluble and chloroplastic form of the spinach enzyme. These forms appeared to be similar in several properties, as has also been observed for preparations from pea by Anderson *et al.* (1). Muto and Uritani (16) reported on an increase of sedimentation coefficient of enzyme from sweet potato roots (for which a mol wt 110,000 was determined by gel chromatography) on treatment with $NADP^+$. We have been unable to find any more recent report on the molecular properties of plant enzymes.

The association behavior of two maize leaf isozymes has been investigated. Both usually show a mol wt of about 115,000 and can associate to mol wt 210,000 in the presence of $NADP^+$. This latter form is, therefore, the main catalytic conformer. EDTA (13) or divalent cations do not appear to alter this size distribution. Since the monomers of the isozyme from etiolated leaves seem to have mol wt 54,000, the 115,000 D form is a dimer and the 210,000 D form is a tetramer. Widely accepted values for the protein from a variety of sources, including red cell and yeast,

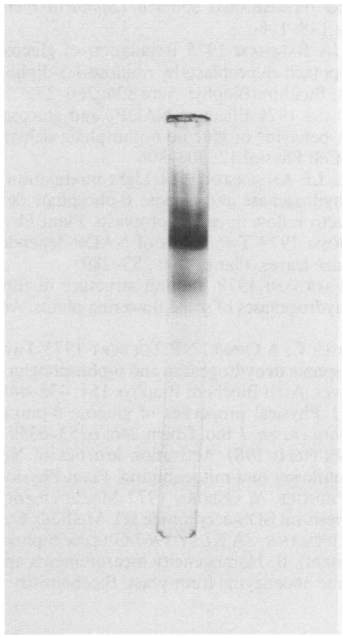

FIG. 7. Photograph of an SDS-polyacrylamide (7.5%) gel cylinder showing enzyme from etiolated leaves, 15 μg, stained with Coomassie brilliant blue. The gel is broken by the front of bromothymol blue.

Table III. *Summary of the Purification of Glucose 6-P Dehydrogenase of Etiolated Leaves*

Soluble proteins from 70-g leaves were precipitated between 40 and 50% $(NH_4)_2SO_4$ saturation, resuspended, clarified by centrifuging, then filtered through a Sephadex G-25, and finally subjected to DEAE cellulose chromatography (see "Methods"). Activity was further purified by Sephadex G-200 chromatography, first in the absence of $NADP^+$ and then in the presence of 24 μM $NADP^+$. Values in brackets represent the specific activities of the more active fractions.

Step	Protein	Activity	Specific activity	Purification
	mg	*μmol/min*	*μmol/min · mg*	*-fold*
Homogenate	850	35	0.04	1
$(NH_4)_2SO_4$ (40–50%)	170	26	0.15	3.7
DEAE cellulose	40	22	0.55	13.4
Sephadex G-200 I ($NADP^+$ absent)	13.7	11	0.80 (1.20)	19.5 (28.7)
Sephadex G-200 II ($NADP^+$ present)	0.80	2.6	3.25 (8.80)	78.5 (173.5)

are 51,000 ± 1,000 for monomers and 205,000 ± 1,000 for tetramers (4, 13). Other reported data (*e.g.* 21, 24) fall near this range.

By analogy with other systems, it seems possible that some inhibitory effects on the plant enzyme, such as that of DTE (11, 14), of a probable photosynthetic electron carrier under light conditions (1, 17), or of a thioredoxin-like substance (2) may be mediated in part by the tetramer-dimer transition (reversed by $NADP^+$), followed by dimer dissociation to inactive subunits (5, 13). This general mechanism of eucaryotic glucose 6-P dehydrogenase would offer an additional explanation for the decreased activity of chloroplast enzyme in the light, and is not alternative to current interpretations based on simple competition by NADPH at the active center (8, 14, 15). By displacing $NADP^+$ from this center, NADPH would also enhance the dissociation and subsequent inactivation process indirectly.

It has been demonstrated above that free ATP (not MgATP, Fig. 4; Table I) is an effective competitive inhibitor for both

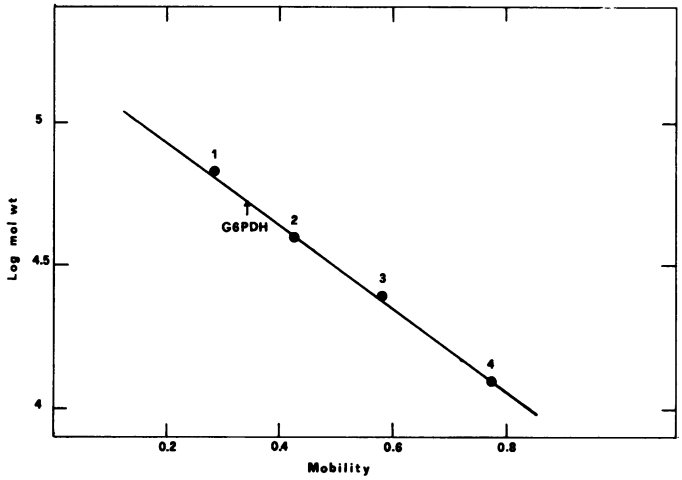

FIG. 8. Determination of subunit mol wt for the major band of pI 4.9 isozyme (see Fig. 7) by SDS-polyacrylamide gel electrophoresis. Markers: 1, BSA (mol wt 67,000); 2, aldolase (rabbit, 40,000); 3, chymotrypsinogen A (25,000); 4, Cyt *c* (12,500).

isozymes. Inhibition constants of 0.64 mM observed with the etiolated leaf species, and 1.4 mM with the green leaf species, may suggest ATP as *in vivo* modulator of glucose 6-P dehydrogenase, whereas no such role can be postulated for ADP. K_i values of ATP reported for a cyanobacterial enzyme were distinctly higher, 2 to 5 mM (8). The etiolated leaf isozyme shows a 2- to 3-fold higher affinity for $NADP^+$, as well as for the competitive inhibitors ATP and ADP, than its counterpart of developed leaves. At any rate the resemblances between the isozymes, already noticed by Schnarrenberger *et al.* (20), are confirmed and extended to their overall structure and reactions.

The possibility that cytosolic glucose 6-P dehydrogenase and 6-P gluconate dehydrogenase may disappear from developing leaves of some C_4 plants has been raised by the finding (10) that green leaves of maize and other species contain a single isozyme of each of these activities, confined to mesophyll cells. It is shown above that soluble glucose 6-P dehydrogenase of dark-grown leaves (pI 4.3) is replaced by a new form (pI 4.9) during photosynthetic differentiation of maize. Of course, this does not exclude that very minor amounts of other isozymes may exist in green maize leaves. The pI of the photosynthetic form is similar to that of pea chloroplast enzyme (4.7, [1]). The replacement patterns of NADP-dependent malic enzyme (18) and of PEP carboxylase (9) are similar, although chloroplast NADP-malic enzyme is associated with bundle sheath cells (12). If green leaf glucose 6-P dehydrogenase is particulate, NADPH production in the cytosol is probably effected in malate forming C_4 species by glyceraldehyde 3-P-$NADP^+$ reductase (EC 1.2.1.9), that increases considerably during maize greening.

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