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Isoenzymes of Glucose 6-Phosphate Dehydrogenase from the Plant Fraction of Soybean Nodules[1]

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ABSTRACT

Two isoenzymes of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) have been separated from the plant fraction of soybean (*Glycine max* L. Merr. cv Williams) nodules by a procedure involving $(NH_4)_2SO_4$ gradient fractionation, gel chromatography, chromatofocusing, and affinity chromatography. The isoenzymes, which have been termed glucose 6-phosphate dehydrogenases I and II, were specific for $NADP^+$ and glucose 6-phosphate and had optimum activity at pH 8.5 and pH 8.1, respectively. Both isoenzymes were labile in the absence of $NADP^+$. The apparent molecular weight of glucose 6-phosphate dehydrogenases I and II at pH 8.3 was estimated by gel chromatography to be approximately 110,000 in the absence of $NADP^+$ and double this size in the presence of $NADP^+$. The apparent molecular weight did not increase when glucose 6-phosphate was added with $NADP^+$ at pH 8.3. Both isoenzymes had very similar kinetic properties, displaying positive cooperativity in their interaction with $NADP^+$ and negative cooperativity with glucose 6-phosphate. The isoenzymes had half-maximal activity at approximately 10 micromolar $NADP^+$ and 70 to 100 micromolar glucose 6-phosphate. NADPH was a potent inhibitor of both of the soybean nodule glucose 6-phosphate dehydrogenases.

The process of symbiotic nitrogen fixation in root nodules of legumes is energetically costly to the host plant, with carbon substrates being required for the bacteroids and the assimilation of fixed nitrogen. To meet these demands, nodules have a high capacity for carbohydrate metabolism: in soybeans, the specific activities of most of the enzymes involved in the conversion of imported sucrose to organic acids via the glycolytic pathway are substantially higher in nodules than in roots (3). An alternative route for the breakdown of hexose monophosphates could be provided by the pentose phosphate pathway, which functions mainly to generate NADPH and precursors for various biosynthetic processes. This pathway has been shown to operate in root nodules (21), but information on its contribution to the metabolism of carbohydrates is limited. In an early study, Laing *et al.* (10) suggested that the involvement of the pentose phosphate pathway in the oxidation of glucose in excised lupin nodules was relatively minor. More recently, it has been proposed that this pathway may have a greater role in the metabolism of carbohydrates in nodules of ureide-exporting legumes, such as soybeans, than in amide-exporters (7).

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G6PD[2] (EC 1.1.1.49) catalyzes the first step in the pentose phosphate pathway and is a strategic point for controlling the flux through this sequence of reactions. In plants, where G6PD occurs in the cytoplasmic and plastid compartments, the enzyme has been studied most extensively from leaves and regulation of its activity has been considered mainly in relation to photosynthetic metabolism (5, 11, 12, 17, 18, 22). In comparison, there have been relatively few studies of G6PD from nonphotosynthetic tissues (2, 15). G6PD activity has been demonstrated in the plant cytosolic and plastid fractions of soybean nodule extracts (7), but the enzymes concerned have not been studied. In this report we describe the separation of two isoenzymes of G6PD from the plant fraction of soybean nodules. Their physical and kinetic properties have been investigated to obtain information on the regulation of the pentose phosphate pathway and its involvement in the metabolism of carbohydrates in association with symbiotic nitrogen fixation.

MATERIALS AND METHODS

Materials

Soybean (*Glycine max* L. Merr. cv Williams) seeds were inoculated with *Bradyrhizobium japonicum* CB 1809 and grown in Perlite as described previously (14). Fractogel TSK HW(55) was obtained from E. Merck and 2′,5′-ADP agarose from Sigma Chemical Co. Other chromatographic media, chemicals, and biochemicals were from Sigma Chemical Co. or Boerhinger Mannheim GmbH.

Isolation of Enzymes

Nodules (5–10 g) were harvested from 6 to 10 week old plants, rinsed in distilled water, and homogenized with a mortar and pestle in 40 mL of 100 mM Tris-Pi (pH 8.5), 20 mM 2-mercaptoethanol, and 4 mM EDTA which contained 0.15 g of insoluble PVP per g of nodules. The homogenate was centrifuged at 20,000*g* for 20 min and the supernatant retained as the crude extract from the plant fraction of the nodules. Extracts were prepared from soybean roots in a similar manner.

The crude nodule extract was fractionated by $(NH_4)_2SO_4$ gradient solubilization according to the method of King (8). Solid $(NH_4)_2SO_4$ was added to the 20,000*g* supernatant to give 70% saturation with $(NH_4)_2SO_4$, followed by celite (3 g) that had been washed previously with 70% saturated

[2] Abbreviation: G6PD, glucose 6-phosphate dehydrogenase.

$(NH_4)_2SO_4$ solution. The resulting slurry of approximately 60 mL was poured into a 2.5 cm diameter chromatography column which contained a plug of celite (5 cm) that had been equilibrated previously with 20 mM Tris-Pi (pH 8.5), 4 mM 2-mercaptoethanol, 1 mM EDTA, 10 μM $NADP^+$, and 10% (v/v) glycerol (buffer A), which contained $(NH_4)_2SO_4$ at 70% saturation. The column was washed with this solution to remove unprecipitated proteins and eluted at a flow rate of 1 $mL \cdot min^{-1}$ with a gradient formed by introducing 150 mL of buffer A into 150 mL buffer A containing $(NH_4)_2SO_4$ at 70% saturation. Fractions of 3 mL were collected and those containing G6PD activity were pooled and additional solid $(NH_4)_2SO_4$ added to increase the degree of saturation by 30%. The precipitate was collected by centrifugation at 20,000*g* for 15 min, dissolved in 5 mL of 20 mM Tris-Pi (pH 8.5), 4 mM 2-mercaptoethanol, 1 mM EDTA, 10 μM $NADP^+$, 100 mM KCl, and 25% (v/v) glycerol (buffer B) and chromatographed through a Fractogel TSK HW-55(F) column (2.2 × 58 cm) in buffer B using a flow rate of 1 $mL \cdot min^{-1}$. Fractions (3 mL) with G6PD activity were pooled, concentrated to 4 mL by ultrafiltration in an Amicon stirred cell fitted with a Diaflo PM 10 membrane and dialysed against 20 mM Tris-Pi (pH 7.2), 1 mM EDTA, 10 μM $NADP^+$, 4 mM 2-mercaptoethanol, and 25% (v/v) glycerol (buffer C).

The dialyzed preparation was applied to a Polybuffer exchanger 94 column (1 × 12 cm) that had been equilibrated previously with buffer C. The column was eluted with 160 mL of Polybuffer 74 that had been diluted 10-fold, adjusted to pH 3.9 with 1 N HCl, and had 10 μM $NADP^+$, 1 mM EDTA, 4 mM 2-mercaptoethanol, and 25% (v/v) glycerol added. Fractions of 3 mL were collected using a flow rate of 0.2 $mL \cdot min^{-1}$. The two peaks of G6PD activity that were eluted were pooled separately, dialyzed against 500 mL of 20 mM Tris-Pi (pH 7.6), 4 mM 2-mercaptoethanol, 1 mM EDTA, and 25% (v/v) glycerol (buffer D), and applied at a flow rate of 0.3 $mL \cdot min^{-1}$ to a 2′,5′-ADP agarose column (0.9 × 5.0 cm) that had been equilibrated previously with buffer D. After unbound proteins had been washed from the column, G6PD was eluted with 0.2 mM $NADP^+$ in buffer D. Active fractions were pooled, concentrated by ultrafiltration and dialyzed against 500 mL of buffer C that had been adjusted to pH 8. Preparations of this type were free of 6-P-gluconate dehydrogenase activity and were used in the experiments described.

Estimation of Mol Wt

The apparent mol wt of the G6PD isoenzymes was estimated by gel filtration in a Fractogel TSK HW(F)-55 column (2.2 × 58 cm) in 20 mM Tris-HCl (pH 8.3 or pH 7.2), 25% (v/v) glycerol, and 100 mM KCl, with additions as indicated. The flow rate was 0.75 $mL \cdot min^{-1}$ and ferritin (M_r 450,000), catalase (M_r 240,000), aldolase (M_r 158,000), BSA (M_r 68,000), chymotrypsinogen A (M_r 25,000), Cyt *c* (M_r 12,500), and Blue Dextran 2000 were used to calibrate the column.

Electrophoresis

Gel electrophoresis was performed at 4°C in polyacrylamide slabs using a method based on that of Gaal *et al.* (6). The stacking gel (2.8% acrylamide) was prepared in 50 mM Tris-Pi (pH 6.3) and 20% sucrose and the separating gel (5% acrylamide) in 0.75 M Tris-Pi (pH 8). The electrophoretic buffer contained 5 mM Tris, 80 mM aspartate, and 20 μM $NADP^+$ at pH 7.4. Gels were stained for G6PD activity at 30°C in a solution which contained, in a volume of 20 mL, 1.2 mmol Tris-Pi (pH 8.5), 25% (v/v) glycerol, 30 μmol glucose-6-P, 4 μmol $NADP^+$, 6 mg *p*-nitroblue tetrazolium, and 0.5 mg phenazine methosulfate.

Assay of Enzyme Activity

All enzyme assays were carried out at 30°C. G6PD activity was determined in a continuous assay by monitoring the increase in A_{340} due to the reduction of $NADP^+$. Standard reaction mixtures contained, in a volume of 1 mL, 50 μmol Tris-HCl (pH 8.3 or pH 7.2), 2.5 μmol glucose-6-P, 0.2 μmol $NADP^+$, 25% (v/v) glycerol, and an appropriate amount of enzyme. Reactions were initiated by adding the enzyme and rates were linear for at least 5 min. For assays of crude extracts, reactions were initiated with glucose-6-P and 2 units of 6-P-gluconate dehydrogenase were added to reaction mixtures. Activity of 3-hydroxybutyrate dehydrogenase was determined as described previously (23). A unit of activity is defined as the amount of enzyme which formed 1 μmol of product $\cdot min^{-1}$. Protein concentration was determined with Coomassie brilliant blue reagent (Bio-Rad) according to the manufacturer's instructions, using BSA as a standard.

RESULTS

Two isoenzymes of G6PD were separated from soybean nodules using the procedure summarized in Table I. The bacteroid enzyme 3-hydroxybutyrate dehydrogenase was not detected in the crude nodule extracts, which indicated that both forms of G6PD were derived from the plant fraction of the nodules. All of the G6PD activity in the crude nodule extracts was precipitated by $(NH_4)_2SO_4$ at 70% saturation and a single peak of G6PD activity was solubilized when a gradient of decreasing $(NH_4)_2SO_4$ concentration was applied to the precipitated proteins. Maximum G6PD activity was solubilized between 50 and 45% saturation with $(NH_4)_2SO_4$. Two forms of G6PD were separated by chromatofocusing in a Polybuffer exchanger 94 column that was eluted with Poly-

Table I. *Separation of Isoenzymes of G6PD from Plant Fraction of Soybean Nodules*

Nodules (10 g) were extracted as described. The data are from one of five experiments.

	Activity	Protein	Specific Activity	Recovery
	units	*mg*	*units·mg⁻¹ protein*	%
Crude extract	47.8	239	0.20	100
$(NH_4)_2SO_4$ gradient	34.3	42	0.82	72
Fractogel	28.6	6.6	4.3	60
Chromatofocusing				
G6PD I	6.1	0.033	185	13
G6PD II	14.6	0.089	164	31
2′,5′-ADP agarose				
G6PD I	3.7	0.011	333	8
G6PD II	10.6	0.023	461	22

buffer 74 (Fig. 1). The two isoenzymes, which were termed G6PD I and G6PD II, were eluted at pH 5.8 and pH 5.1, respectively, and were purified further by affinity chromatography using 2′,5′-ADP agarose. Approximately 8% of the G6PD activity in the crude extract was recovered in isoenzyme I and 22% in isoenzyme II. Polyacrylamide gel electrophoresis confirmed that preparations of G6PD I contained little G6PD II, and vice versa (Fig. 2). Two bands of G6PD activity, with similar mobility to G6PD I and G6PD II, were detected when crude extracts from the plant fraction of soybean nodules and soybean roots were subjected to polyacrylamide gel electrophoresis at pH 7.4 (Fig. 2). No G6PD activity was detected in gels when electrophoresis was performed at pH 8.3.

G6PD I and G6PD II were labile in the absence of $NADP^+$, losing 50% of their activity after 2 h at 4°C in 20 mM Tris-HCl at pH 8 and 90% after 12 h. Approximately 80% of the activity lost after 12 h was recovered when 10 μM $NADP^+$ was added to the buffer. The addition of $MgCl_2$ (10 mM), KCl (50 mM), 2-mercaptoethanol (5 mM), glucose-6-P (5 mM), or glycerol (25% v/v) did not restore activity. However, the addition of glycerol improved the stability of G6PD I and II during long-term storage. The purified isoenzymes could be stored at 4°C without significant loss of activity over 2 months in buffer C which had been adjusted to pH 8.

The isoenzymes of G6PD from soybean nodules had an apparent mol wt of approximately 110,000 at pH 8.3 and both aggregated to forms double this size in the presence of 50 μM $NADP^+$ (Fig. 3). With 10 μM $NADP^+$, the apparent mol wt of G6PD II was also doubled, but aggregation of G6PD I appeared to be only partial. The elution volume of G6PD I from the Fractogel column in the presence of 10 μM $NADP^+$ corresponded to an apparent mol wt of 174,000 (Table II). The apparent mol wt of G6PD I and II did not

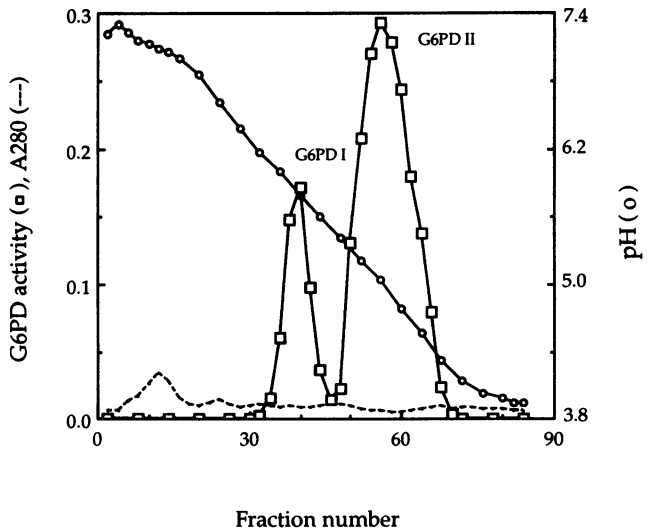

Figure 1. Chromatofocusing of G6PD isoenzymes from the plant fraction of soybean nodules. Chromatofocusing was performed as described in a Polybuffer exchanger 94 column using Polybuffer 74 as eluent. G6PD activity is given as the change in $A_{340} \cdot min^{-1} \cdot mL^{-1}$.

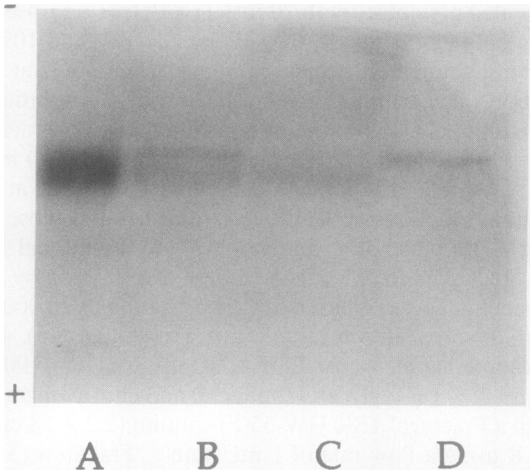

Figure 2. Polyacrylamide gel electrophoresis of G6PD I and G6PD II from the plant fraction of soybean nodules. Electrophoresis was performed and gels stained for G6PD activity as described. A, crude extract of soybean roots; B, crude extract of soybean nodules; C, G6PD II; D, G6PD I.

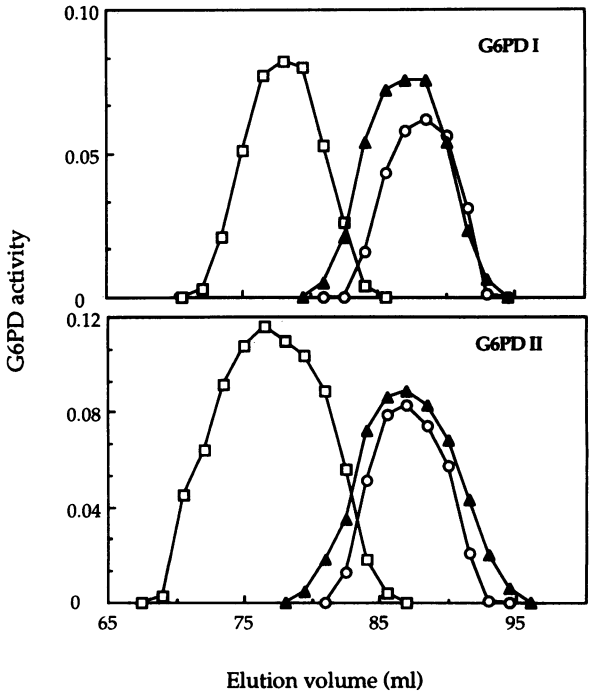

Figure 3. Gel filtration of G6PD I and G6PD II from the plant fraction of soybean nodules. G6PD I and II were chromatographed in a Fractogel TSK HW-55(F) column at pH 8.3 as described, with additions made to the buffer as follows: none (○), 50 μM $NADP^+$ (□), and 50 μM $NADP^+$ and 5 mM glucose-6-P (▲). G6PD activity is in units · mL^{-1}.

increase at pH 8.3 when 5 mM glucose-6-P was added with $NADP^+$, nor in the presence of 10 μM $NADP^+$ at pH 7.2 (Fig. 3, Table II).

G6PD I and II were optimally active at pH 8.5 and pH 8.1, respectively, with activities of 90% or more of the maximum occurring between pH 8 and pH 8.9 for G6PD I and between pH 7.8 and pH 8.5 for G6PD II. The properties of the enzymes were investigated at a pH close to the optimum (pH 8.3) and at a pH more likely to reflect physiological conditions (pH 7.2). Both enzymes were specific for $NADP^+$ and glucose-6-P. No activity was detected when $NADP^+$ was replaced in standard reaction mixtures at pH 8.3 by 0.6 mM NAD^+, nor when glucose-6-P was replaced by glucose-1-P, mannose-6-P, 6-P-gluconate, fructose-6-P (all 2 mM), glucose 1,6-bisP (1 mM), ribose-5-P, D-glucose, D-fructose, and D-mannose (all 5 mM). The addition of 10 mM $MgCl_2$ to standard reaction mixtures had no effect on either enzyme at pH 8.3 but at pH 7.2 stimulated activity of G6PD I by 72% and G6PD II by 36%.

Both isoenzymes had very similar kinetic properties. When $NADP^+$ was the varied substrate, double reciprocal plots appeared to deviate only slightly from linearity at pH 8.3 but were distinctly concave upward at pH 7.2 (Fig. 4). The corresponding Hill plots at pH 8.3 and 7.2 had slopes of approximately 1.1 and 1.3, respectively, for G6PD I and 1.3 and 1.7 for G6PD II (Table III). Half-maximal activity of G6PD I and II at both pH 8.3 and 7.2 occurred at concentrations of $NADP^+$ near 10 μM (Table III). In contrast, when glucose-6-P was the varied substrate, double reciprocal plots at pH 8.3 and pH 7.2 were concave downward (Fig. 4). Slopes of Hill plots were less than 1, indicating negative cooperativity with this substrate. The concentrations of glucose-6-P which gave half-maximal activity of G6PD I and II at pH 8.3 and 7.2 were between 70 and 100 μM (Table III).

G6PD I and II were strongly inhibited by NADPH (Fig. 5). With $NADP^+$ at a concentration of 50 μM, both enzymes were inhibited 50% by 70 μM NADPH and more than 80% by 0.2 mM NADPH (Fig. 5). Plots of 1/v against the concentration of NADPH were concave upward (not shown), indicating that the nature of the inhibition was complex. G6PD I was inhibited 10 to 15%, and G6PD II 25 to 30%, by 1.5 mM ATP and 1 mM erythrose-4-P at pH 8.3. The following metabolites, when added to standard reaction mixtures at pH 8.3 at the concentrations indicated, had no effect on the activity of G6PD I or G6PD II: NADH (0.1 mM), 6-P-gluconate (2 mM), ADP, AMP, ribose-5-P, ribulose-5-P, succinate, fumarate, citrate, phosphoenolpyruvate, glyceraldehyde-3-P, allantoin, and allantoic acid (all 1 mM), malate (5 mM), fructose 2,6-bisP (0.1 mM), and glucose 1,6-bisP (0.2 mM).

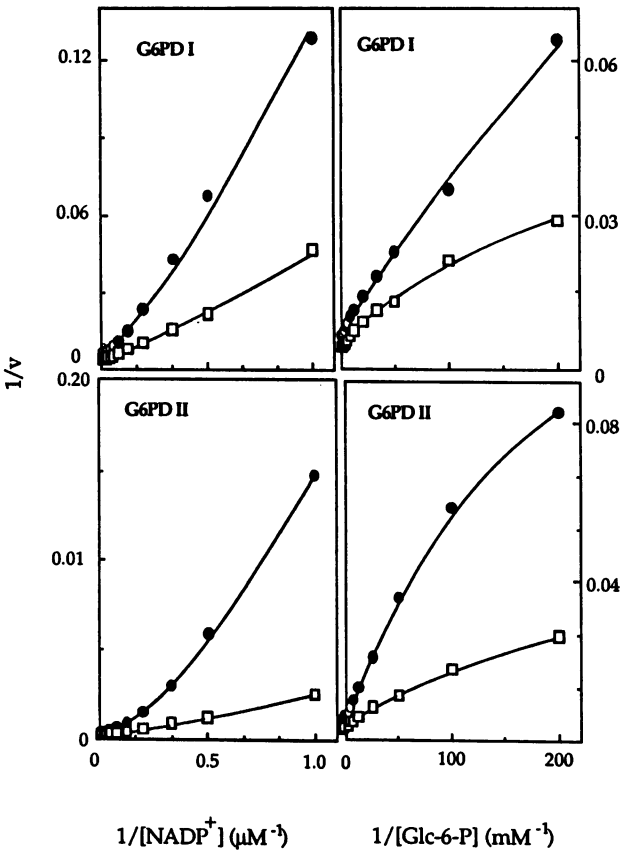

Figure 4. Effect of $NADP^+$ and glucose-6-P on initial velocity of G6PD I and G6PD II from the plant fraction of soybean nodules. Data are from one of three experiments carried out at pH 8.3 (□) and pH 7.2 (●). The concentrations of the fixed substrates were 0.2 mM for $NADP^+$ and 2.5 mM for glucose-6-P. Initial velocities (v) are in units · mg^{-1} protein.

Table II. *Effect of $NADP^+$ and Glucose-6-P on the Apparent Mol Wt of G6PD I and G6PD II from the Plant Fraction of Soybean Nodules*

The values are the means ± SE of three determinations.

Additions	Apparent Mol Wt	
	G6PD I	G6PD II
None	110,000 ± 8,000	114,000 ± 8,000
10 μM $NADP^+$	174,000 ± 7,000	229,000 ± 25,000
50 μM $NADP^+$	219,000 ± 12,000	226,000 ± 13,000
5 mM G6P	110,000 ± 10,000	115,000 ± 8,000
50 μM $NADP^+$, 5 mM G6P	109,000 ± 9,000	114,000 ± 9,000

DISCUSSION

Two isoenzymes of G6PD have been separated and purified from the plant fraction of soybean nodules. The specific activity of G6PD in the resulting preparations was comparable to that of G6PD obtained in highly purified form from other plant tissues (5, 15). In an earlier study, G6PD was shown to be present in the plastid and cytosolic fractions of soybean nodules (7) and it is possible that the isoenzymes isolated in this investigation were derived from these different subcellular compartments. Comparing the distribution of G6PD activity between the two compartments with the relative amounts of the two isoenzymes, we suggest that G6PD I may be from the plastids and G6PD II from the cytosol. Two forms of G6PD with electrophoretic mobility similar to G6PD I and II were also detected in crude extracts of soybean roots.

G6PD I and II from the plant fraction of soybean nodules aggregated in the presence of $NADP^+$ at pH 8.3. Glucose-6-P

Table III. *Kinetic Parameters of G6PD I and G6PD II from the Plant Fraction of Soybean Nodules*

The concentration of the substrates that gave half-maximal activity ($S_{0.5}$) and the slopes of Hill plots (n) were calculated from the data of Figure 4, using the intercept on the ordinate of the extrapolated double reciprocal plots as an estimate the apparent maximum velocity (V).

	NADP+			Glucose-6-P		
	V	$S_{0.5}$	n	V	$S_{0.5}$	n
	units·mg^{-1} protein	μM		units·mg^{-1} protein	μM	
G6PD I						
pH 7.2	175	12.3	1.26	166	69	0.85
pH 8.3	280	9.1	1.11	257	68	0.76
G6PD II						
pH 7.2	228	8.5	1.66	198	98	0.93
pH 8.3	372	6.8	1.15	341	76	0.83

overcame the effect of NADP+, but further studies are required to determine whether this was due to glucose-6-P acting directly on G6PD or due to it causing the concentration of NADP+ to fall. Both G6PD I and II were labile in the absence of NADP+, which suggests that the aggregated form of the enzymes is the more stable structure. G6PD from other plants and animals is also inactivated rapidly in the absence of NADP+ (5, 13, 18). NADP+ has been shown to promote aggregation of G6PD from sweet potato (16), maize leaves (22), and various animal and microbial sources (13): these enzymes are converted from dimers with apparent mol wt of 110,000 into tetramers. In contrast, the chloroplast and cytoplasmic forms of G6PD from pea leaves have an apparent mol wt of 240,000, which is not affected by NADP+ (5, 18).

Under the assay conditions used in these studies, neither of the soybean nodule G6PD isoenzymes displayed typical Michaelis-Menten type kinetics. In view of the high degree of enrichment of G6PD I and G6PD II in the respective preparations, it seems unlikely that the nonlinear double reciprocal plots were due to the presence of a mixture of isoenzymes in the reactions. The isoenzymes displayed positive cooperativity when NADP+ was the varied substrate and, as indicated by the slopes of the Hill plots, the cooperativity was much more pronounced at pH 7.2 than at pH 8.3. On the other hand, the negative cooperativity observed with glucose-6-P appeared to be more marked at pH 8.3 than at pH 7.2. The concentrations of NADP+ and glucose-6-P at which soybean nodule G6PD I and II were half-maximally active were comparable to those for the enzyme from other plant sources. In general, G6PD from plants has been reported to have Michaelis-Menten kinetics when NADP+ is the varied substrate (5, 15, 22). However, the dimeric form of G6PD from human erythrocytes displayed sigmoidal kinetics with NADP+ at high concentrations of glucose-6-P (1). Negative cooperativity with glucose-6-P has been reported for G6PD from sweet potato (13), black gram (2), as well as for the enzyme from animal and microbial sources (13). Close similarities in the physical and kinetic properties of isoenzymes of G6PD, such as observed for G6PD I and II from the plant fraction of soybean nodules, have also been noted for the multiple forms of the enzyme in pea and maize leaves (17, 22).

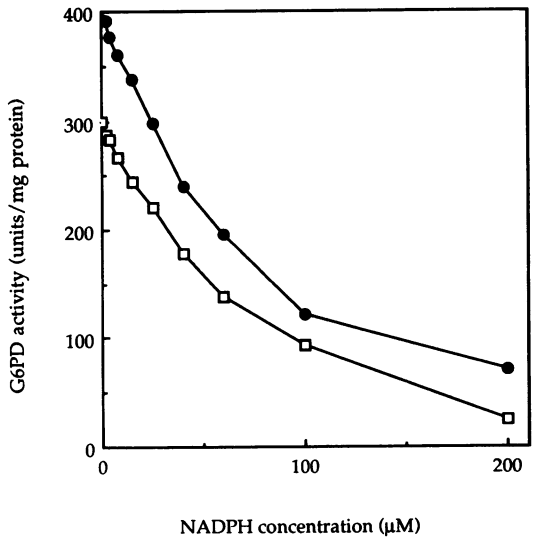

Figure 5. Inhibition of G6PD I and G6PD II of soybean nodules by NADPH. G6PD I (□) and G6PD II (●) were assayed under standard conditions at pH 8.3, except that the concentration of NADP+ was 50 μM and NADPH was added at the concentrations indicated.

G6PD I and II had regulatory properties which could be important in the fine control of the pentose phosphate pathway in the plant fraction of soybean nodules. Strong inhibition by NADPH, which has also been reported for G6PD from photosynthetic tissues (2, 5, 11, 18), may have significance for the regulation of the soybean nodule isoenzymes *in vivo*. Estimates of the concentrations of pyridine nucleotides indicate that the ratio of NADPH/NADP+ in soybean nodules could exceed 3 (20). Although the cellular and subcellular compartmentation of these metabolites need to be considered, under such conditions it seems likely that G6PD activity would be low. The inhibition of G6PD would be relieved and, in turn, the flux through the pentose phosphate pathway increased, in response to a fall in the NADPH/NADP+ ratio. This could occur when NADPH is utilized in biosynthetic processes or in the conversion of oxaloacetate to malate by NADP-dependent malate dehydrogenase activity (19). The latter reaction could provide dicarboxylic acids for uptake by the bacteroids (4). The ratio of NADPH/NADP+ changes in soybean nodules of different ages (21), which may mean that the contribution of the pentose phosphate pathway to the metabolism of carbohydrates varies during the different stages of development of nodules. Changes in the quarternary structure of G6PD I and II and the cooperative interactions with

the substrates could also be involved in regulation of the enzymes. Further studies are required to determine if these properties have physiological significance, but it is possible that positive cooperativity in the interaction of the isoenzymes with $NADP^+$, especially near pH 7, could enhance their response to a decline in the $NADPH/NADP^+$ ratio.

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