Plant Physiol. (1997) 114: 1413–1419

In Vivo and in Vitro Studies of Glucose-6-Phosphate Dehydrogenase from Barley Root Plastids in Relation to Reductant Supply for NO_2^- Assimilation[1]

Derek P. Wright[2]*, Heather C. Huppe, and David H. Turpin

Department of Biology, Queen's University, Kingston, Ontario, Canada K7L 3N6

Pyridine nucleotide pools were measured in intact plastids from roots of barley (*Hordeum vulgare* L.) during the onset of NO_2^- assimilation and compared with the in vitro effect of the NADPH/NADP ratio on the activity of plastidic glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) from N-sufficient or N-starved roots. The NADPH/NADP ratio increased from 0.9 to 2.0 when 10 mM glucose-6-phosphate was supplied to intact plastids. The subsequent addition of 1 mM $NaNO_2$ caused a rapid decline in this ratio to 1.5. In vitro, a ratio of 1.5 inactivated barley root plastid G6PDH by approximately 50%, suggesting that G6PDH could remain active during NO_2^- assimilation even at the high NADPH/NADP ratios that would favor a reduction of ferredoxin, the electron donor of NO_2^- reductase. Root plastid G6PDH was sensitive to reductive inhibition by dithiothreitol (DTT), but even at 50 mM DTT the enzyme remained more than 35% active. In root plastids from barley starved of N for 3 d, G6PDH had a substantially reduced specific activity, had a lower K_m for NADP, and was less inhibited by DTT than the enzyme from N-sufficient root plastids, indicating that there was some effect of N starvation on the G6PDH activity in barley root plastids.

Several studies have correlated increased carbohydrate oxidation and/or OPPP activity with the onset of NO_2^- reduction in the plastids of heterotrophic tissues (Sarkissian and Fowler, 1974; Emes and Fowler 1979, 1983; Oji et al., 1985; Bowsher et al., 1989). These observations support a role for the OPPP in supplying reductant to NO_2^- assimilation in these tissues (Lee, 1980; Turner and Turner, 1980; Copeland and Turner, 1987). G6PDH (EC 1.1.1.49), which catalyzes the first reaction of the OPPP, represents a strategic point for control of the pathway. In green algae an increased activation of G6PDH has been demonstrated at the onset of NO_3^- assimilation in the dark (Vanlerberghe et al., 1992; Huppe et al., 1994), and the type and quantity of N supplied to the cells affects the specific activity of this enzyme (Huppe and Turpin, 1996).

Despite the potential importance of G6PDH in root NO_3^- metabolism, there is little information about the kinetic or regulatory properties of the plastidic enzyme from heterotrophic tissue. In photosynthetic tissues the chloroplastic G6PDH is redox-modulated via the Fd-thioredoxin system (Buchanan, 1991), whereas the possible redox sensitivity of the cytosolic isozymes is still controversial (Anderson et al., 1974; Fickenscher and Scheibe, 1986; Yuan and Anderson, 1987; Graeve et al., 1994). Both plastidic and cytoplasmic enzymes have been shown to be strongly inhibited by high ratios of NADPH/NADP (Lendzian and Bassham, 1975; Lendzian, 1980; Srivastava and Anderson, 1983; Fickenscher and Scheibe, 1986; Scheibe et al., 1989). It has been suggested that relatively high concentrations of NADPH are necessary to drive FNR reduction of Fd, the electron donor required by NiR. We have examined this apparent conflict between G6PDH regulation and NO_2^- reduction using intact plastids from barley (*Hordeum vulgare* L.) roots to determine the response of NADP(H) pools during NO_2^- provision. Furthermore, using plastids from both N-sufficient and N-starved barley roots, we have investigated the regulatory response of the plastidic G6PDH to varying NADPH/NADP ratios and to redox modulation by DTT to determine whether the regulatory properties are consistent with a role for G6PDH in providing reducing power for NO_2^- assimilation.

MATERIALS AND METHODS

Barley (*Hordeum vulgare* L. cv Klondike) seeds were allowed to imbibe in aerated, distilled water for 24 h at room temperature. The seeds were transferred onto plastic mesh fitted into plexiglass discs (80–100 seeds per disc), placed in a seed tray, and covered with damp sand (Siddiqi and Glass, 1983). After 2 d of germination in the dark, discs of seedlings were transferred to plexiglass hydroponic tanks (approximately 50-L capacity) containing modified Johnson's nutrient solution. To starve plants of N, discs were transferred, after the roots were washed in three changes of distilled water, to a second identical tank containing N-free nutrient solution 6 d after imbibition. The composition of the modified Johnson's solution was as follows: 1600 μM N (as NO_3^-), 800 μM K, 300 μM P, 800 μM Ca, 350 μM S, and 100 μM Mg; micronutrients: 5 μM Cl, 2.5 μM B, 0.2 μM Mn,

[1] This work was supported by the Natural Sciences and Engineering Research Council of Canada.

[2] Present address: Robert Hill Institute, Department of Animal and Plant Sciences, University of Sheffield, Western Bank, Sheffield S10 2TN, UK.

* Corresponding author; e-mail d.p.wright@sheffield.ac.uk; fax 44–114–276–0159.

Abbreviations: FNR, Fd:NADP oxidoreductase; G6PDH, Glc-6-P dehydrogenase; NiR, NO_2^- reductase; OPPP, oxidative pentose phosphate pathway; PEPC, PEP carboxylase; 6PG, 6-phosphogluconate; 6PGDH, 6-phosphogluconate dehydrogenase.

0.2 μM Zn, 0.05 μM Cu, 0.05 μM Mo, and 2 μM Fe. For the N-free solution all of the concentrations were identical except for 500 μM Ca and 850 μM S, and there was no N source. The composition of the nutrient solution in the tank was maintained by a continuous supply of a concentrated stock solution using a peristaltic pump. The nutrient solution in the tank was mixed by an immersion circulator and was aerated by two aquarium pumps. The plants were maintained in a growth chamber at 20°C on a 16-h photoperiod. Light (300 μmol m^{-2} s^{-1}) was provided by a combination of fluorescent and tungsten bulbs.

Preparation of Root Plastids

Plastids were prepared from roots of N-sufficient or N-starved barley plants 9 d after imbibition following a modification of the method of Emes and England (1986). For N-starved plants only root material produced after transfer to the N-free tank was used. All procedures were carried out on ice. Approximately 30 to 50 g of roots was rapidly sliced using a razor blade, transferred to a mortar and pestle, and gently ground with sand in 50 mM Tricine (pH 7.9), 330 mM sorbitol, 4 mM EDTA, 1 mM $MgCl_2$, 0.1% (w/v) BSA, and 200 μM PMSF (approximately 1.5 mL/g tissue). The homogenate was filtered through four layers of cheesecloth, one layer of 100-μm nylon mesh, and one layer of 20-μm nylon mesh. The filtrate was centrifuged at 200*g* for 6 min using a swing-out rotor. The 200*g* pellet was resuspended in 50 mM Tricine (pH 7.9), 330 mM sorbitol, 4 mM EDTA, and 1 mM $MgCl_2$ (resuspension buffer). Aliquots (15–20 mL) of the 200*g* supernatant were underlaid with a resuspension buffer containing 22% (v/v) Percoll (Sigma) and centrifuged in a swing-out rotor at 4000*g* for 7 min. The 4000*g* pellets (4000*g* plastid fraction) were recombined in 2 to 4 mL of resuspension buffer and aliquots were frozen at −80°C until use.

Enzyme Analysis

G6PDH activity was routinely measured in a standard buffer containing 100 mM Tris-HCl (pH 7.9), 200 μM NADP, and 2 mM Glc-6-P (1 mL total volume) at 340 nm using a dual-wavelength spectrophotometer (Sigma-Eppendorf ZFP22, Hamburg, Germany). To ensure that all plastids were ruptured, aliquots were treated with 0.1% (v/v) Triton X-100 and then centrifuged (16,000*g* for 20 s) to remove particulate material prior to use. To determine the apparent K_m(NADP) the concentration of NADP used in the standard buffer was varied between 10 and 240 μM. In experiments to determine the effect of the NADPH/NADP ratio on the activity of G6PDH, NADP was omitted and replaced by 10 or 30 μM NADPH. An artificial NADPH/NADP ratio was created by the addition of NADP to the assay cuvette to a final concentration between 10 and 240 μM and the activity was assayed immediately. Preincubation at a given NADPH/NADP ratio for up to 5 min did not alter the measured activity. The K_i(NADPH) against NADP was calculated from a plot of apparent K_m(NADP)/V_{max} against varying [NADPH]. Reductive inactivation of G6PDH was measured by the addition of DTT. Aliquots of the plastid preparation were incubated with different concentrations of DTT or with a buffer blank at 4°C, and the activity was determined using the standard buffer to which an equivalent concentration of DTT had been added.

When the simultaneous effect of DTT and the NADPH/NADP ratio on the activity of G6PDH was determined, the extract was preincubated with DTT for 30 min prior to an assay. The effect of various metabolites (1 mM) on the activity of G6PDH was determined using the standard assay buffer or at 10 μM NADP and 2 mM Glc-6-P in 100 mM Tris-HCl (pH 7.9). All metabolite solutions were adjusted to pH 7.0 as appropriate immediately prior to use. In all of the experiments spontaneous inactivation of G6PDH with time was corrected for by using a suitable control assay that was repeated regularly throughout the duration of the experiment. The spontaneous rate of inactivation of G6PDH from N-sufficient and N-starved plastids resulted in a decrease of 5 to 7% of the initial maximum activity over 1 h. To check that the measured activity of G6PDH was not limited by the activity of 6PGDH in the assay cuvette, 6PG was added at the end of an assay to confirm that 6PGDH activity was not saturated. No corrections for simultaneous 6PGDH activity were made in the reported values.

Total activities of marker enzymes were determined in aliquots of the 200*g* supernatant, the 200*g* pellet, the supernatant just above the Percoll interface (4000*g* Percoll supernatant), and the 4000*g* plastid fraction after the addition of 0.1% (v/v) Triton X-100. G6PDH was assayed using the standard assay buffer described earlier. 6PGDH was determined by the change in rate following addition of 2 mM 6PG to this mixture. PEPC was assayed according to the method of Stitt et al. (1989). Alkaline inorganic pyrophosphatase and NiR (except 100 mM Tricine, pH 7.9, was used instead of 100 mM KPO_4) were determined, as described by Borchert et al. (1993). Fumarase was assayed according to the method of Hill and Bradshaw (1969).

Pyridine Nucleotide Determinations

Intact plastids extracted from N-sufficient roots were diluted using the resuspension buffer and were incubated in a cuvette surrounded by a water jacket maintained at 20°C. The suspension was mixed by continuous bubbling with air. Glc-6-P (10 mM) and $NaNO_2$ (1 mM) were added either together or sequentially, as indicated in Figure 1. Samples (100 μL) were killed and processed as described by Vanlerberghe et al. (1992), except that the volume after Speedvac (Labconco, Kansas City, MO) centrifugation was adjusted to 500 μL and the sample volume used during the cycling of each nucleotide was 20 μL.

Intactness of Plastids

Intactness of plastid preparations was determined by assaying 6PGDH (100 mM Tris-HCl [pH 7.9], 0.2 mM NADP, and 2 mM 6PG) and enolase (Smith et al., 1992) in the presence and absence of 0.1% (v/v) Triton X-100. All reagents were osmotically buffered with 330 mM sorbitol.

Protein Determination

Protein was determined using a commercial Bio-Rad protein assay based on the method described by Bradford (1976) using γ-globulins as the protein standard.

RESULTS

Preparation of Plastids

Attempts to separate root G6PDH isozymes by column chromatography were unsuccessful despite inclusion of numerous inhibitors or cofactors; therefore, we chose to separate plastids to assess the activity of the plastidic enzyme. The activity of organelle-specific marker enzymes was determined to indicate the purity, intactness, and yield of plastid preparations (Table I). Approximately 5 to 8% of the total activity of the plastidic marker enzymes NiR and alkaline pyrophosphatase were recovered in the plastid fraction of N-sufficient and N-starved barley roots. Contamination of these fractions by the cytosolic marker enzyme PEPC was between 0.01 and 0.1%, whereas the activity of the mitochondrial marker fumarase recovered from N-sufficient and N-starved tissue was 12 and 3%, respectively. The latency of 6PGDH and enolase indicated that intactness of the plastid preparations was between 75 and 85%. After the activity of G6PDH was determined, addition of 6PG to the assay cuvette stimulated the rate of reaction, indicating that the activity of 6PGDH did not limit the measured activity of G6PDH under any experimental condition.

Pyridine Nucleotide Pool Changes during Glc-6-P-Dependent NO_2^- Uptake

The NADPH pool increased and the NADP pool decreased upon addition of 10 mM Glc-6-P to intact N-sufficient plastids (Fig. 1a) so that the NADPH/NADP ratio increased significantly. When 1 mM $NaNO_2$ was subsequently added the NADPH pool decreased and the NADP pool increased, resulting in an overall decrease in the NADPH/NADP ratio (Fig. 1a). An increase in the NADPH/NADP ratio also occurred when Glc-6-P and $NaNO_2$ were added simultaneously (Fig. 1b). The NAD pool steadily decreased after the addition of Glc-6-P either alone or simultaneously with $NaNO_2$ (data not shown). When $NaNO_2$ was added after Glc-6-P, however, there was a slight increase in NADH that was not found when the compounds were added simultaneously (data not shown). Unfortunately, attempts to repeat these measurements with N-starved plastids were unsuccessful because of plastid fragility.

Kinetic Properties of G6PDH from N-Sufficient and N-Starved Plastids

G6PDH extracted from isolated plastids from plants that had been deprived of N for 3 d had a significantly lower maximum specific activity than the enzyme from N-sufficient plant plastids (16.8 nmol NADP reduced mg^{-1} protein min^{-1} from N-sufficient compared with 2.8 nmol NADP reduced mg^{-1} protein min^{-1} for N-starved tissue) (Table II). The enzyme from N-starved tissue also exhibited a slightly lower apparent K_m(NADP) compared with the N-sufficient enzyme. Inclusion of NADPH caused a substantial increase in the apparent K_m(NADP) of the N-sufficient and N-starved enzyme but had little effect on the V_{max} of either enzyme (data not shown). The K_i(NADPH) against NADP for the N-sufficient enzyme was not significantly different from that of the N-starved tissue (11.0 ± 1.8 μM from N-sufficient compared with 20.9 ± 8.5 μM from N-starved tissue) (Table II).

Several metabolites were investigated to determine their effect on G6PDH activity assayed using limiting concentrations of NADP (10 μM). NADPH (30 μM) inhibited the N-sufficient (24%) and N-starved (36%) enzymes. In addi-

Table I. *Distribution of organelle marker enzyme activities resulting from the preparation of barley root plastids*

Marker enzymes are presented as total activity measured in each fraction. The values are the means ± SE (n = 8–10). The figures in parentheses represent the activity recovered in the 4000*g* plastid fraction as a percentage of the 200*g* supernatant.

Treatment	G6PDH (Plastid)	6PGDH (Plastid)	NiR (Plastid)	Alkaline Pyrophosphatase (Plastid)	PEPC (Cytosol)	Fumarase (Mitochondrion)
			nmol NADP reduced min^{-1}			
N-sufficient						
200*g* Supernatant	6960 ± 795	16,238 ± 1,640	22,522 ± 1,217	10,250 ± 1,359	3125 ± 233	3.12 ± 0.16
200*g* Pellet	157 ± 21	270 ± 36	1,419 ± 66	387 ± 71	21 ± 6	ND[a]
4000*g* Percoll supernatant	4145 ± 458	11,023 ± 1,143	18,269 ± 1,191	9,192 ± 1,213	2750 ± 311	2.31 ± 0.10
4000*g* Plastid fraction	497 ± 51 (8.1 ± 0.9)	994 ± 98 (6.7 ± 0.7)	1,586 ± 116 (7.1 ± 0.5)	720 ± 58 (8.0 ± 0.7)	1.4 ± 0.6 (0.09 ± 0.02)	0.38 ± 0.04 (11.8 ± 0.5)
N-starved						
200*g* Supernatant	4332 ± 439	10,554 ± 983	15,445 ± 1,153	10,957 ± 1,372	2510 ± 206	6.9 ± 0.5
200*g* Pellet	48 ± 5	126 ± 8	1,146 ± 61	325 ± 32	7.5 ± 2.5	0.09 ± 0.02
4000*g* Percoll supernatant	3962 ± 440	9,877 ± 969	14,401 ± 1,063	9,808 ± 1,353	2419 ± 190	5.9 ± 0.6
4000*g* Plastid fraction	107 ± 19 (2.6 ± 0.5)	333 ± 28 (3.2 ± 0.4)	654 ± 59 (4.4 ± 0.5)	627 ± 54 (6.1 ± 0.5)	2.6 ± 0.8 (0.11 ± 0.03)	0.23 ± 0.04 (3.38 ± 0.72)

[a] ND, Not detected.

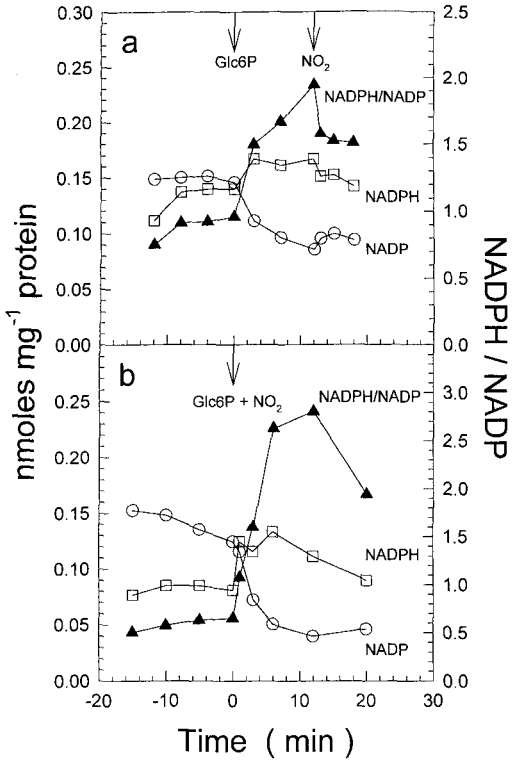

Figure 1. Response of the NADP (○), NADPH (□), and the NADPH/NADP ratio (▲) to the sequential addition of 10 mM Glc-6-P at time 0 and 1 mM $NaNO_2$ after 12 min (a) or the simultaneous addition of both 10 mM Glc-6-P and 1 mM $NaNO_2$ at time 0 (b). The data shown are representative of several experiments that produced similar results.

tion, ATP, UDP, and citrate (all at 1 mM) inhibited both enzymes between 12 and 18%, whereas acetyl-CoA (1 mM) caused a 25 to 27% reduction in activity (data not shown). The following metabolites (at 1 mM) showed no significant effect on G6PDH from plastids of N-sufficient or N-starved roots: Gln, glutamate, α-ketoglutarate, ADP, pyruvate, PEP, 3-phosphoglyceric acid, dihydroxyacetone phosphate, Rib-5-P, and malate. NADPH (20–25%), acetyl-CoA (12%), and citrate (10%) showed some inhibition of the enzymes from both N-sufficient and N-starved plastids when enzymes were assayed under saturating substrate concentrations (200 μM NADP and 2 mM Glc-6-P).

Effect of DTT on G6PDH Activity

Reductive inhibition by DTT on G6PDH extracted from N-sufficient and N-starved roots was both time and concentration dependent (Fig. 2). Incubation with 15 mM DTT decreased plastidic G6PDH activity from N-sufficient and N-starved roots within 17 min (Fig. 2). Higher concentrations of DTT caused greater inactivation of the enzyme from N-sufficient tissue; however, increasing DTT concentrations had little effect on the enzyme from N-starved plants. In both tissues activity decreased most rapidly during the initial 17 min after addition of DTT. The initial rapid inactivation was followed by a slow decline in activity throughout the remainder of the time course. Incubation (5 min) with an equimolar concentration of the oxidizing agent sodium tetrathionate at the end of the time course caused reactivation to approximately 80% of the initial activity (data not shown).

Modulation of G6PDH Activity by the NADPH/NADP Ratio

Increasing the NADPH/NADP ratio from 0 (no NADPH) to 1.5 caused an approximately 50% decrease in the activity of G6PDH from N-sufficient or N-starved tissue (Fig. 3c). The activity from N-starved tissue showed a greater degree of inhibition than the enzyme from N-sufficient roots at low NADPH/NADP ratios (Fig. 3c, compare ○ and ●). Increasing the ratio from 1.5 to 6.0 caused a further 10% decrease in activity of the enzyme from both tissues (data not shown). Reduction of the enzyme from N-sufficient plants enhanced the inhibition 20 to 25% at each NADPH/NADP ratio (Fig. 3a), a decrease similar to the maximum inhibition observed when this enzyme was treated with 15 mM DTT alone (Fig. 2). At low NADPH/NADP ratios, the enzyme from N-starved tissue responded to the reduction similarly to that from N-sufficient plastids, showing an additional 15 to 20% decrease in activity (Fig. 3b). At high NADPH/NADP ratios, however, treatment with 15 mM DTT had only a marginal additional effect on the N-starved plastidic enzyme (Fig. 3b). It is interesting that even at low NADPH/NADP ratios the additional effect of DTT on the N-starved enzyme was consistently smaller in magnitude than the inhibition observed when 15 mM DTT alone was added to extracts from N-starved plastids (Fig. 2).

DISCUSSION

There was a marked decrease in the NADPH/NADP ratio at the onset of NO_2^- assimilation in isolated plastids that had previously been given Glc-6-P. This response resembles the decreased ratio associated with OPPP activation at the onset of dark NO_3^- assimilation in N-limited

Table II. *Kinetic constants for G6PDH from plastids of N-sufficient and N-staved barley roots*

The apparent K_m (NADP) and V_{max} of G6PDH from N-sufficient and N-starved barley root plastids were determined under standard assay conditions.

Treatment	Apparent K_m(NADP)	K_i (NADPH)	V_{max}
	μM		*nmol NADP reduced mg^{-1} protein min^{-1}*
N-sufficient plastids	6.6 ± 2.4	11.0 ± 1.8	16.8 ± 0.1
N-starved plastids	3.4 ± 0.4	20.9 ± 8.5	2.8 ± 0.3

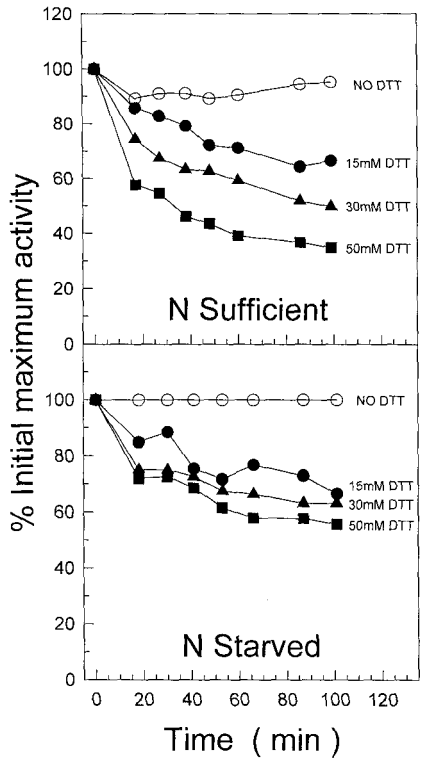

Figure 2. Time course of the change in activity when G6PDH extracted from N-sufficient or N-starved roots was treated with different concentrations of DTT. The data shown are representative of several experiments that produced similar results.

algae (Vanlerberghe et al., 1992). Activation of the OPPP in barley root plastids to supply energy for NO_2^- assimilation is consistent with reports using pea root, which indicated, by monitoring the release of $^{14}CO_2$ from Glc-6-P isotopically labeled in different C atoms, that there was an increased flow of C through the plastid-located OPPP during NO_2^- assimilation (Bowsher et al., 1989). In pea root only the simultaneous addition of Glc-6-P and NO_2^- caused changes in labeling consistent with increased OPPP activity, whereas Glc-6-P alone did not (Bowsher et al., 1989). In this study Glc-6-P added alone or simultaneously with NO_2^- caused an increase in the NADPH/NADP ratio (Fig. 1), which is consistent with the activation of G6PDH and the OPPP and would provide high NADPH/NADP ratios to support Fd reduction via FNR. Even when the addition of C and N sources was separated temporally, the NADPH/NADP ratio decreased to only approximately 1.5 upon NO_2^- addition, a value higher than before the addition of C (Fig. 1). These ratios, however, are greater than the ratios reported to inhibit G6PDH from several tissues (Lendzian and Bassham, 1975; Ashihara and Komamine, 1976).

Some potential explanations have been developed to explain the apparent conflict between a need for high NADPH concentrations to reduce Fd and the inhibition of G6PDH activity by high NADPH/NADP ratios. First, the FNR and Fd from roots may differ from their leaf counterparts so that high NADPH is not necessary to drive Fd reduction. In recent years several studies have demonstrated structural differences between these proteins from heterotrophic and leaf tissues. However, results from in vitro studies concerning a difference between FNR proteins in their ability to reduce Fd from NADPH are mixed (Ninomiya and Sato, 1984; Suzuki et al., 1985; Wada et al., 1986, 1989; Hirasawa et al., 1988, 1990; Morigasaki et al., 1990a, 1990b; Green et al., 1991). A second explanation is that, even if inhibited by high NADPH/NADP ratios, G6PDH might be active enough to support NO_2^- reduction. Bowsher et al. (1989) calculated that the rates of NO_2^- reduction observed in isolated pea root plastids would require as little as 8 to 15% of the maximum catalytic activity of G6PDH extracted from this tissue.

We assessed the kinetic properties of plastidic G6PDH from barley roots in relation to NADPH/NADP ratios. G6PDH activity extracted from isolated root plastids was only 50% inhibited by an NADPH/NADP ratio of 1.5 (Fig. 3). Higher ratios, up to 6.0, caused little further inhibition (data not shown). Therefore, at the physiological NADPH/NADP ratio measured in the root plastids during NO_2^-

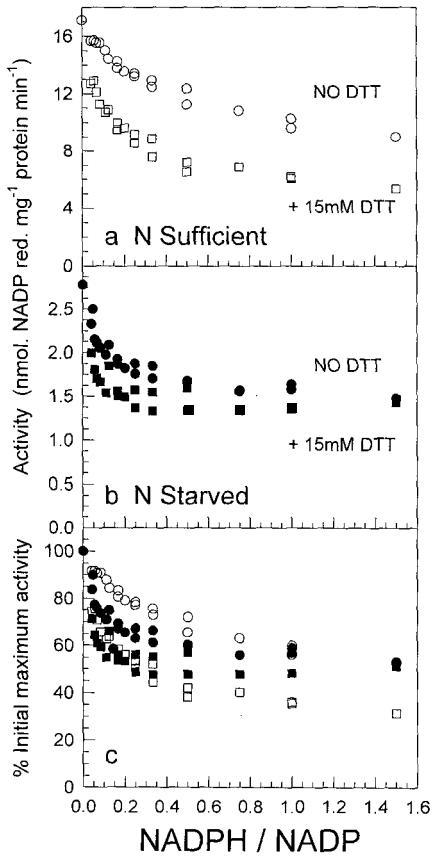

Figure 3. Inhibition of G6PDH from N-sufficient (a; ○, □) and N-starved (b; ●, ■) plastids at different NADPH/NADP ratios in the presence (□, ■) and absence (○, ●) of 15 mM DTT (c). The concentration of NADPH was held at 10 or 30 μM and the NADP concentration was varied to give the ratios indicated. Data are the means of two to three independent determinations using different plastid preparations. SES for N-sufficient and N-starved data were less than 10 and 15%, respectively.

assimilation, this plastidic G6PDH should remain reasonably active. A NADPH/NADP ratio of 1.5 has also been shown to inhibit the plant G6PDH isozyme from soybean nodules by approximately 50%; however, a higher ratio of 4 caused almost 80% inhibition of this enzyme (Hong and Copeland, 1991). The spinach chloroplast isozyme was completely inhibited by a ratio of 1.5 (Lendzian and Bassham, 1975).

Reductive inactivation is another major regulatory mechanism controlling G6PDH from photosynthetic plastids. The light modulation of the activity of G6PDH by the Fd-thioredoxin system can be mimicked in vitro by the addition of less than 5 mM DTT (Srivastava and Anderson, 1983; Scheibe et al., 1989; Farr et al., 1994; Huppe et al., 1994). Recently, the reductive regulation of the plastidic G6PDH from potato leaf was reported (von Schaewen et al., 1995). By contrast, the cytoplasmic isozyme of pea leaf or potato tuber showed no inactivation with 20 or 50 mM DTT, respectively (Fickenscher and Scheibe, 1986; Graeve et al., 1994). Incubation with DTT decreased the activity of N-sufficient barley root plastidic G6PDH and the effect of DTT was dependent on the concentration that was used (Fig. 2). However, even at 50 mM DTT substantial activity remained. Treatment with the oxidizing agent sodium tetrathionate largely reversed the effects of reduction, suggesting that the inactivation was due to a reversible reduction of disulfide bonds on the enzyme, similar to that described for the chloroplastic isozyme (Scheibe et al., 1989; Farr et al., 1994). Although the relationship of the plastidic enzymes from leaves and roots has not been determined, the effect of reduction on the barley root plastidic enzyme was much less dramatic than has been reported for chloroplastic isozymes from several different sources (Srivastava and Anderson, 1983; Scheibe et al., 1989; Farr et al., 1994; Huppe et al., 1994). The reductive inhibition of this N-sufficient barley root plastid enzyme was essentially additive to the effect of NADPH/NADP ratios (Fig. 3). In light of the recent report of a complex isozyme pattern in *Chlamydomonas reinhardtii*, which altered depending on the N source available (Huppe and Turpin, 1996), it cannot be discounted that multiple isoforms may exist in this plastid that show a differential response to reduction and/or NADPH/NADP ratios and that our results are an average of those enzyme responses.

Alterations in the activity of G6PDH and other OPPP enzymes have been shown when exogenous NO_3^- is supplied in addition to, or as a replacement for, more reduced sources of N (for a review, see Lee, 1980). Recently, we reported a large increase in the specific activity of G6PDH in algae grown on limited supplies of N (Huppe and Turpin, 1996). N starvation of barley caused a large decrease in the maximum specific activity of its root plastid G6PDH (Table II), and this enzyme showed a decrease in the apparent K_m(NADP) compared with enzyme from N-sufficient plastids. Values for both enzymes are within the 2.4 to 19 μM range that has been reported for plant G6PDH (Ashihara and Komamine, 1976; Srivastava and Anderson, 1983; Fickenscher and Scheibe, 1986; Scheibe et al., 1989; Hong and Copeland, 1991; Graeve et al., 1994). N starvation did not significantly alter the K_i(NADPH) of the root plastid G6PDH compared with the enzyme from N-sufficient root plastids. The K_i(NADPH) for G6PDH from both tissues is similar to previously reported values for pea chloroplasts ranging from 8 to 18 μM (Srivastava and Anderson, 1983; Scheibe et al., 1989) and for the cytoplasmic G6PDH from pea leaves and potato tubers at 11 μM (Fickenscher and Scheibe, 1986; Graeve et al., 1994). Minor inhibition of the activity of G6PDH by ATP, UDP, citrate, and acetyl-CoA was similar for both enzymes (data not shown) and is consistent with reports from other heterotrophic tissues (Muto and Uritani, 1972; Ashihara and Komamine, 1976; Mirfakhrai and Auleb, 1989; Graeve et al., 1994).

In conclusion, Glc-6-P addition to barley root plastids results in an increase in the NADPH/NADP ratio, which decreases when NO_2^- is added. The resulting NADPH/NADP ratio during Glc-6-P-dependent NO_2^- assimilation was higher than before the addition of Glc-6-P. The plastidic G6PDH in this tissue is not greatly inhibited at these NADPH/NADP ratios, indicating that the enzyme can remain active even if there is an increase in NADPH to reduce Fd for NO_2^- assimilation. The plastidic enzyme was partially inactivated by reduction, and this effect was additive to the inhibition by NADPH. N starvation decreased the specific activity of the root plastid isozyme, and the enzyme from this tissue had a lower apparent K_m(NADP) and was less sensitive to redox inhibition but was more sensitive to increasing NADPH/NADP ratios. Although there appear to be some changes in this enzyme under N starvation, the basis of these differences will require further study.

Received February 24, 1997; accepted May 20, 1997.

LITERATURE CITED

Anderson LE, Lim T-C, Park K-EP (1974) Inactivation of pea leaf chloroplastic and cytoplasmic glucose-6-phosphate dehydrogenase by light and dithiothreitol. Plant Physiol **53:** 835–839

Ashihara H, Komamine A (1976) Characterisation and regulatory properties of glucose-6-phosphate dehydrogenase from Black Gram (*Phaseolus mungo*). Physiol Plant **36:** 52–59

Borchert S, Harborth J, Schunemann D, Hoferichter P, Heldt HW (1993) Studies of the enzymic capacities and transport properties of pea root plastids. Plant Physiol **101:** 303–312

Bowsher CG, Hucklesby DP, Emes MJ (1989) Nitrite reduction and carbohydrate metabolism in plastids purified from roots of *Pisum sativum* L. Planta **177:** 359–366

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem **72:** 248–254

Buchanan BB (1991) Regulation of CO_2 assimilation in oxygenic photosynthesis: the ferredoxin/thioredoxin system. Perspective on its discovery, present status, and future development. Arch Biochem Biophys **288:** 1–9

Copeland L, Turner JF (1987) The regulation of glycolysis and the pentose phosphate pathway. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Vol 11. Academic Press, New York, pp 107–128

Emes MJ, England S (1986) Purification of plastids from higher plant roots. Planta **168:** 161–166

Emes MJ, Fowler MW (1979) Intracellular interactions between the pathways of carbohydrate oxidation and nitrate assimilation in plant roots. Planta **145:** 287–292

Emes MJ, Fowler MW (1983) The supply of reducing power for nitrite reduction in plastids of seedling pea roots (*Pisum sativum* L.). Planta **158:** 97–102

Farr TJ, Huppe HC, Turpin DH (1994) Coordination of chloroplastic metabolism in N-limited *Chlamydomonas reinhardtii* by redox modulation. I. The activation of phosphoribulosekinase and glucose-6-phosphate dehydrogenase is relative to the photosynthetic supply of electrons. Plant Physiol **105:** 1037–1042

Fickenscher K, Scheibe R (1986) Purification and properties of the cytoplasmic glucose-6-phosphate dehydrogenase from pea leaves. Arch Biochem Biophys **247:** 393–402

Graeve K, von Schaewen A, Scheibe R (1994) Purification, characterisation, and cDNA sequence of glucose-6-phosphate dehydrogenase from potato (*Solanum tuberosum* L.). Plant J **5:** 353–361

Green LS, Boihon C, Buchanan BB, Kamide K, Sanada Y, Wada K (1991) Ferredoxin and ferredoxin-NADP reductase from photosynthetic and non-photosynthetic tissues of tomato. Plant Physiol **96:** 1207–1213

Hill RI, Bradshaw RA (1969) Fumarase. Methods Enzymol **13:** 91–99

Hirasawa M, Chang K-T, Knaff DB (1990) Characterisation of a ferredoxin:$NADP^+$ oxidoreductase from a nonphotosynthetic plant tissue. Arch Biochem Biophys **276:** 251–258

Hirasawa M, Sung JD, Malkin R, Zilber A, Droux M, Knaff DB (1988) Evidence for the presence of a [2Fe-2S] ferredoxin in bean sprouts. Biochim Biophys Acta **934:** 169–176

Hong ZQ, Copeland L (1991) Isoenzymes of glucose-6-phosphate dehydrogenase from the plant fraction of soybean nodules. Plant Physiol **96:** 862–867

Huppe HC, Farr TJ, Turpin DH (1994) Coordination of chloroplastic metabolism in N-limited *Chlamydomonas reinhardtii* by redox modulation. II. Redox modulation activates the oxidative pentose phosphate pathway during photosynthetic nitrate assimilation. Plant Physiol **105:** 1043–1048

Huppe HC, Turpin DH (1996) Appearance of novel glucose-6-phosphate dehydrogenase isoforms in *Chlamydomonas reinhardtii* during growth on nitrate. Plant Physiol **110:** 1431–1433

Lee RB (1980) Sources of reductant for nitrate assimilation in non-photosynthetic tissues: a review. Plant Cell Environ **3:** 65–90

Lendzian KJ (1980) Modulation of glucose-6-phosphate dehydrogenase by NADPH, NADP and dithiothreitol at variable NADPH/NADP ratios in an illuminated reconstituted spinach (*Spinacia oleracea* L.) chloroplast system. Planta **148:** 1–6

Lendzian KJ, Bassham JA (1975) Regulation of glucose-6-phosphate dehydrogenase in spinach chloroplasts by ribulose-1,5-diphosphate and NADPH/NADP ratios. Biochim Biophys Acta **396:** 260–275

Mirfakhrai M, Auleb L (1989) Partial purification and kinetic characterisation of wheat germ glucose-6-phosphate dehydrogenase. J Plant Physiol **135:** 191–196

Morigasaki S, Takata K, Sanada Y, Wada K, Yee BC, Shin S, Buchanan BB (1990a) Novel forms of ferredoxin and ferredoxin-$NADP^+$ reductase from spinach roots. Arch Biochem Biophys **283:** 75–80

Morigasaki S, Takata K, Suzuki T, Wada K (1990b) Purification and characterization of a ferredoxin-$NADP^+$ oxidoreductase-like enzyme from radish root tissues. Plant Physiol **93:** 896–901

Muto S, Uritani I (1972) Inhibition of sweet potato glucose-6-phosphate dehydrogenase by NADPH and ATP. Plant Cell Physiol **13:** 377–380

Ninomiya Y, Sato S (1984) A ferredoxin-like electron carrier from non-green cultured tobacco cells. Plant Cell Physiol **25:** 453–458

Oji Y, Watanabe M, Wakiuchi N, Okamato S (1985) Nitrite reduction in barley root plastids: dependence on NADPH coupled with glucose-6-phosphate and 6-phosphogluconate dehydrogenases, and possible involvement of an electron carrier and a diaphorase. Planta **165:** 85–90

Sarkissian GS, Fowler MW (1974) Interrelationship between nitrate assimilation and carbohydrate metabolism in plant roots. Planta **119:** 335–349

Scheibe R, Geissler A, Fickenscher K (1989) Chloroplast glucose-6-phosphate dehydrogenase: K_m shift upon light modulation and reduction. Arch Biochem Biophys **274:** 290–297

Siddiqi MY, Glass ADM (1983) Studies of the growth and mineral nutrition of barley varieties. I. Effect of potassium supply on the uptake of potassium and growth. Can J Bot **61:** 671–678

Smith RG, Gauthier DA, Dennis DT, Turpin DH (1992) Malate- and pyruvate-dependent fatty acid synthesis in leucoplasts from developing castor endosperm. Plant Physiol **98:** 1233–1238

Srivastava DK, Anderson LE (1983) Isolation and characterisation of light- and dithiothreitol-modulatable glucose-6-phosphate dehydrogenase from pea chloroplasts. Biochim Biophys Acta **724:** 359–369

Stitt M, McLilley R, Gerhardt R, Heldt HW (1989) Metabolite levels in specific cells and subcellular compartments of plant leaves. Methods Enzymol **174:** 518–552

Suzuki A, Oaks A, Jacquot J-P, Vidal J, Gadal P (1985) An electron transport system in maize roots for reactions of glutamate synthase and nitrite reductase. Plant Physiol **78:** 374–378

Turner JF, Turner DH (1980) The regulation of glycolysis and the pentose phosphate pathway. *In* DD Davies, ed, The Biochemistry of Plants, Vol 2. Academic Press, New York, pp 279–316

Vanlerberghe GC, Huppe HC, Vlossak KDM, Turpin DH (1992) Activation of respiration to support dark NO_3^- and NH_4^+ assimilation in the green alga *Selenastrum minutum*. Plant Physiol **99:** 495–500

von Schaewen A, Langenkamper G, Graeve K, Wenderoth I, Scheibe R (1995) Molecular characterization of the plastidic glucose-6-phosphate dehydrogenase from potato in comparison to its cytosolic counterpart. Plant Physiol **109:** 1327–1335

Wada K, Onda M, Matsubara H (1986) Ferredoxin isolated from plant non-photosynthetic tissues. Purification and characterisation. Plant Cell Physiol **27:** 407–415

Wada K, Onda M, Matsubara H (1989) Amino acid sequences of ferredoxin isoproteins from radish roots. J Biochem **105:** 619–625

Yuan X-H, Anderson LE (1987) Changing activity of glucose-6-phosphate dehydrogenase from pea chloroplasts during photosynthetic induction. Plant Physiol **85:** 598–600