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Enzymology of the Reduction of Hydroxypyruvate and Glyoxylate in a Mutant of Barley Lacking Peroxisomal Hydroxypyruvate Reductase[1]

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ABSTRACT

The use of LaPr 88/29 mutant of barley (*Hordeum vulgare*), which lacks NADH-preferring hydroxypyruvate reductase (HPR-1), allowed for an unequivocal demonstration of at least two related NADPH-preferring reductases in this species: HPR-2, reactive with both hydroxypyruvate and glyoxylate, and the glyoxylate specific reductase (GR-1). Antibodies against spinach HPR-1 recognized barley HPR-1 and partially reacted with barley HPR-2, but not GR-1, as demonstrated by Western immunoblotting and immunoprecipitation of proteins from crude leaf extracts. The mutant was deficient in HPR-1 protein. In partially purified preparations, the activities of HPR-1, HPR-2, and GR-1 could be differentiated by substrate kinetics and/or inhibition studies. Apparent K_m values of HPR-2 for hydroxypyruvate and glyoxylate were 0.7 and 1.1 millimolar, respectively, while the K_m of GR-1 for glyoxylate was 0.07 millimolar. The K_m values of HPR-1, measured in wild type, for hydroxypyruvate and glyoxylate were 0.12 and 20 millimolar, respectively. Tartronate and P-hydroxypyruvate acted as selective uncompetitive inhibitors of HPR-2 (K_i values of 0.3 and 0.4 millimolar, respectively), while acetohydroxamate selectively inhibited GR-1 activity. Nonspecific contributions of HPR-1 reactions in assays of HPR-2 and GR-1 activities were quantified by a direct comparison of rates in preparations from wild-type and LaPr 88/29 plants. The data are evaluated with respect to previous reports on plant HPR and GR activities and with respect to optimal assay procedures for individual HPR-1, HPR-2, and GR-1 rates in leaf preparations.

In photosynthetic tissues, metabolism of hydroxypyruvate and glyoxylate is thought to be intimately associated with the operation of the glycolate pathway (the oxidative photosynthetic carbon cycle), which results in photorespiration (5, 9). The pathway, which starts with P-glycolate formation by ribulose-1,5-bisP oxygenase, involves several cell compartments and is the main route for metabolism of two-carbon and some three-carbon compounds during photosynthesis (9). Leaves contain at least three distinct reductases reactive with hydroxypyruvate and/or glyoxylate, which are believed to be involved in the flow of carbon through the glycolate pathway: (a) NADH-preferring hydroxypyruvate reductase (HPR-1[3]), (b) NADPH-preferring HPR (HPR-2), both enzymes capable of utilizing glyoxylate as a non-specific substrate (1, 11, 14, 15, 17, 26, 28, 33), and (c) NADPH-preferring glyoxylate-specific reductase (GR-1) (6, 13, 15, 16). Leaf HPR-1, HPR-2, and GR-1 have different immunological, kinetic, and physical characteristics (11, 14, 15). HPR-1 is compartmentalized exclusively in the peroxisomes (23, 26, 28), whereas both HPR-2 and GR-1 are found predominantly in the cytosol (6, 12). HPR-1 is believed to be involved in the main route of carbon flow through the glycolate pathway (5, 9, 28, 31), while HPR-2 and GR-1 have been proposed to serve as backup or auxillary reactions utilizing hydroxypyruvate and/or glyoxylate exported or 'leaked' from the peroxisomes to the cytosol (6, 11–16).

Studies on the function and importance of enzymes of photorespiration have been given a strong stimulus following pioneering work of Somerville and Ogren (25), who developed procedures for identifying mutants deficient in essential proteins involved in the glycolate pathway. Mutants with defects in photorespiratory metabolism have been isolated by exploiting the properties of ribulose-1,5-bisP oxygenase which is inhibited at high CO_2 concentrations and serves then solely as a carboxylase in the reductive photosynthetic carbon pathway (Calvin-Benson cycle) (5, 9). The mutants were unable to survive or grew poorly in air, but could thrive in atmospheres containing a high concentration of CO_2 (or low [O_2]), *i.e.* when the glycolate pathway and photorespiration are limited (3, 19, 25). Recently, a mutant of barley, termed LaPr 88/29, was isolated that lacks HPR-1 activity (21). The mutant, although lacking an enzyme in the main carbon pathway of photorespiration, was capable of a CO_2 fixation rate in air equivalent to 75% of that of the wild type. There was only little disruption in photorespiratory metabolism in LaPr 88/29, with the notable exception of serine, which accumulated at levels several fold over those characteristic of wild-type plants. The data indicated that an isozyme of HPR-1, most probably analogous to HPR-2, contributes to the carbon flow

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[3] Abbreviations: HPR, hydroxypyruvate reductase; HPR-1, NADH-preferring HPR; HPR-2, NADPH-preferring HPR; GR, glyoxylate reductase; GR-1, NADPH-preferring glyoxylate specific GR.

through the glycolate pathway in LaPr 88/29. The mutant also contained considerable NADPH-GR activity (21), which could reflect GR-1 reaction.

LaPr 88/29 provides a means for an unrestricted study of HPR-2 and GR-1 enzymes. Previous work on crude or partially purified HPR-2 or GR-1 has been hampered by presence of HPR-1 which shows non-specific rates with either NADPH and glyoxylate (11, 17, 28). In the present study, we used both wild-type and LaPr 88/29 barley to characterize the three reductases with respect to their immunological and kinetic properties. The non-specific contributions of HPR-1 reactions in assays of HPR-2 or GR-1 from wild type were quantified and conditions to minimize these interferences were established. Immunological and kinetic data on the reductases from both wild-type and LaPr 88/29 barley were compared to previous evidence obtained for analogous purified or partially purified enzymes from a variety of plants.

MATERIALS AND METHODS

Plant Material

Isolation of the LaPr 88/29 mutant of barley (*Hordeum vulgare* L.), lacking the peroxisomal HPR-1, was previously described by Murray *et al.* (20). Barley plants, of both the mutant and wild type, were grown in a growth chamber under a 14 h light and 10 h dark photoperiod (at 25 and 18°C, respectively) in an atmosphere enriched with 0.5% CO_2. The light intensity at the top of the plants was about 500 μE $m^{-2}s^{-1}$. Leaves were harvested from 30 to 40 d old seedlings.

Reagents

All reagents were of analytical grade. Only enzymatically reduced NADPH from Sigma (catalog No. N-6505) was used in assays of NADPH-HPR and NADPH-GR activities. Purified spinach HPR-1 (commercial names—'glyoxylate reductase' or 'glycerate dehydrogenase') was from Sigma.

Preparation of Crude and Partially Purified Enzymes

Leaf extracts were prepared by homogenization of chopped leaf slices in an Osterizer blender (Pulse-matic) at full speed for 30 s. The extraction medium contained 40 mM Tricine (pH 7.8), 2 mM $MgCl_2$, 1 mM EDTA, and 5 mM DTT. The procedure was carried out at 0 to 4°C, and the ratio of leaf material to buffer was 1:3 (w/v). The extract was filtered through several layers of cheesecloth and centrifuged at 14,000*g* for 15 min. In the case of small leaf samples (less than 10 g), leaves were frozen in liquid nitrogen, and the enzymes were extracted by grinding with mortar and pestle in the extraction medium supplemented with 2% PVP (solid). The extract was filtered and centrifuged as described above. Ammonium sulfate fractionation was carried out as previously described (11). Pellets formed after fractionation with 30 to 45 and 45 to 60% saturated ammonium sulfate were resuspended in small volumes of the extraction media. Activities of HPRs and GRs in these fractions were stable when stored at −20°C.

Immunoblots and Immunoprecipitation

Rabbit polyclonal antibodies against spinach HPR-1 were prepared as previously described (15). Serum was stored in batches at minus 20°C. Western blotting was performed according to Towbin *et al.* (29). Bound antibodies were detected by incubating the nitrocellulose with goat antirabbit IgG-alkaline phosphatase conjugate in Tris buffered saline Tween (polyoxyethylenesorbitan) (pH 7.5; dilution 1:1000). Alkaline phosphatase activity was detected using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. SDS-PAGE, using 10% acrylamide slab gels, was carried out according to Laemmli (18).

For the immunoprecipitation study, 200 μL of crude leaf proteins from either wild type or LaPr 88/29 were mixed with 50 μL of the antibodies and allowed to equilibrate for 30 min (21°C). Following incubation, the mixtures were centrifuged at 10,000*g* for 4 min, and aliquots of the supernatants were assayed for HPR activity.

Enzymatic Assays

Enzymes were assayed spectrophotometrically at 340 nm (21°C) in a 1.0 mL volume. Unless otherwise indicated, assays of HPRs contained 100 mM Mes (pH 6.5), 0.2 mM NAD(P)H, and 0.5 mM hydroxypyruvate, while assays of GRs contained 100 mM Mops (pH 7.1), 0.2 mM NAD(P)H and 1 mM glyoxylate. Unless otherwise indicated, assays were started with enzyme, and initial rates were taken for calculations of enzymatic rates. In all cases, control assays containing all the components of the reaction but hydroxypyruvate or glyoxylate were run to correct for nonspecific oxidation of NAD(P)H. One unit of activity was defined as the amount of enzyme required to oxidize 1 μmol of NAD(P)H per min.

Other Methods

Determination of protein was carried out according to Bradford (4).

RESULTS AND DISCUSSION

Immunological Studies

Leaf proteins present in crude extracts of wild type and LaPr 88/29 were resolved by SDS-PAGE/Western blotting and then probed with the anti-spinach HPR-1 antibodies (Fig. 1). Immunoblots of wild-type proteins showed two bands, at about 38 and 43 kD, which were similar to the previously established subunit molecular masses of spinach HPR-2 and HPR-1 (38 and 41 kD, respectively, [15]). Leaf extracts of LaPr 88/29 were deficient in the 43 kD protein.

Although the antibodies against spinach HPR-1 recognized protein bands corresponding to both barley HPR-1 and HPR-2 (Fig. 1), the immunoreaction was much stronger for the HPR-1 protein. Immunoprecipitation by the antibodies of crude leaf proteins from wild-type barley (see "Materials and Methods" for the details) resulted in over 95% decrease of NADH-HPR activity but only about 30% decrease of NADPH-HPR rate; the corresponding activities from LaPr 88/29 decreased merely by 5 to 10% each (data not shown). Previous data, based on immunoblot studies, have indicated that the same antibodies only weakly recognized HPR-2 from maize and rye, and did not react with the HPR-2 protein from spinach, pea, and wheat (15). The basis for this appar-

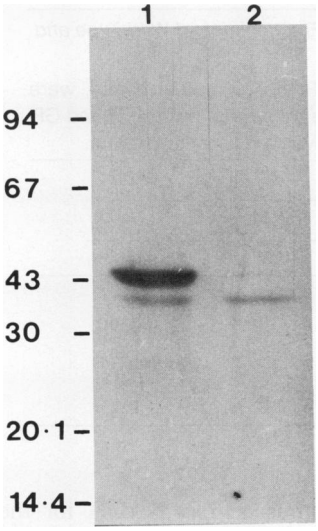

Figure 1. Immunoblot of proteins from crude leaf extracts of wild-type and LaPr 88/29 barley probed with antibodies against spinach HPR-1. The 50 μg aliquots of protein of wild type (*lane 1*) and LaPr 88/29 (*lane 2*) were loaded. The position of mol wt markers is shown.

ently species-dependent recognition of HPR-2 by the antibodies is unknown at present. Leaf GR-1 is not recognized by the antibodies against spinach HPR-1 (13–15).

Recently, it has been demonstrated that the amino acid sequence of HPR-1 from cucumber displayed homology to *Escherichia coli* phosphoglycerate dehydrogenase (7, 8) and to a yet unidentified gene product also from *E. coli* (7). These three proteins have been proposed to form a new group of 2-hydroxyacid dehydrogenases, distinct from other oxidoreductases of known amino acid sequences (27). Based on the immunological similarity between barley HPR-1 and HPR-2 (Fig. 1), it seems likely that the latter belongs to the same group, even though its amino acid sequence is not known.

Activities in Leaf Extracts

Leaf extracts of LaPr 88/29 contained less than 4% of NADH-HPR activity of the wild type, while rates of NADPH-HPR and GR were only slightly lower in the mutant (80–96% of rates in wild type) (Table I). Thus, under the assay conditions employed, HPR-1 activities contributed only weakly to overall rates of NADPH-HPR and GRs in leaf extracts. The activities were assayed at 0.5 mM hydroxypyruvate or 1 mM glyoxylate, *i.e.* under conditions which minimize nonspecific activities of HPR-1 (11), and which are near-optimal for HPR-2 or GR-1, respectively, as found for the purified enzymes (14–16). The GR activities in LaPr 88/29 most probably corresponded to the sum of rates of GR-1 and HPR-2 (glyoxylate-dependent), while HPR rates reflected HPR-2 reactions alone.

On the basis of estimated rates of photorespiratory CO_2 evolution in wild-type barley of about 0.028 $\mu mol \cdot min^{-1} \cdot (mg\ protein)^{-1}$ (21), HPR-2 appears to have sufficient activity (based on NADPH-HPR rates in LaPr 88/29, Table I) to deal with the whole flux of carbon through the glycolate pathway. It should be pointed out that a previously reported value of 0.016 $\mu mol \cdot min^{-1} \cdot (mg\ protein)^{-1}$ for NADPH-HPR activity from LaPr 88/29 (21) was determined at 5 mM hydroxypyruvate, *i.e.* under conditions of strong substrate inhibition of HPR-2. Despite its relatively high rate in leaf extracts, the contribution of HPR-2 to the glycolate pathway *in vivo* is still uncertain. LaPr 88/29 plants show lower photosynthetic rates than wild type, and they accumulate serine, the glycolate pathway intermediate (21). Apparently, HPR-2 activity *in vivo* is limited, perhaps by availability of its substrates or by some yet unknown regulatory mechanism.

Substrate Kinetics

After fractionation of crude leaf extracts with ammonium sulfate (Table II), most of HPR-1 activity was found in the 30 to 45% fraction (based on NADH-HPR rates in wild type), while NADPH-HPR and GR activities were largely confined to the 45 to 60% precipitate. Analogous distribution of the respective reductase activities following the ammonium sulfate procedure has previously been demonstrated for HPRs and GRs from a variety of plant species (11, 13–15, 33).

Apparent K_m values (hydroxypyruvate and glyoxylate) for NADH-HPR and NADH-GR activities from the 30 to 45% fraction of wild-type were 0.12 and 16 mM, respectively (data not shown). These values are typical for plant HPR-1 (1, 9–11, 13, 17, 26, 28). Barley HPR-1 could also use NADPH as a cofactor, especially with hydroxypyruvate serving as the second substrate (K_m of 10 mM, data not shown), similarly to HPR-1 from maize or spinach (11, 28).

Although the 45 to 60% fraction of wild type contained only a small percentage of leaf HPR-1 (Table II), presence of the enzyme greatly contributed to the estimates of apparent K_m values of HPR-2 for hydroxypyruvate, especially with respect to the NADPH-dependent activity (Table III, Fig. 2). Presence of HPR-1 lowered the estimated apparent K_m and decreased the extent of substrate inhibition of NADPH-HPR with hydroxypyruvate (Fig. 2). The substrate inhibition by hydroxypyruvate was previously demonstrated for HPR-2 from maize and spinach (11, 14). The NADH-HPR reaction of HPR-1 from the 30 to 45% fraction of wild type was also substrate inhibited by hydroxypyruvate, but the inhibition became apparent at a higher (millimolar) concentration of this reactant (data not shown, see also [1, 10, 11]).

The K_m of 0.7 mM for hydroxypyruvate, determined for

Table I. *Activities of HPRs and GRs in Leaf Extracts of Wild-Type and LaPr 88/29 Mutant Barley*

The enzymes were extracted from 2 g of leaves of either wild type or LaPr 88/29. Assays of both HPR and GR activities were carried out at pH 7.1 and contained 0.5 mM hydroxypyruvate and 1 mM glyoxylate, respectively.

Reaction	Activity	
	Wild type	LaPr 88/29
	units/mg protein	
NADH-HPR	0.652	0.023
NADPH-HPR	0.068	0.054
NADH-GR	0.015	0.013
NADPH-GR	0.059	0.054

Table II. *Distribution of HPR and GR Activities after Ammonium Sulfate Fractionation of Wild-Type and LaPr 88/29 Mutant Barley*

Leaf extracts, prepared from 8.5 g leaves of either wild-type or LaPr 88/29 mutant barley, were fractionated with 30 to 45% and 45 to 60% saturated ammonium sulfate. Assays of both HPR and GR activities were carried out at pH 7.1, and contained 0.5 mM hydroxypyruvate or 1 mM glyoxylate.

Reaction	Total Activity					
	Wild type			Mutant		
	Crude	30–45%	45–60%	Crude	30–45%	45–60%
	units					
NADH-HPR	32.1	18.5	2.8	1.2	0.17	0.61
NADPH-HPR	3.5	0.80	2.3	2.8	0.50	1.9
NADH-GR	0.60	0.12	0.39	0.52	0.08	0.31
NADPH-GR	2.5	0.43	1.6	2.4	0.39	1.4

NADPH-HPR activity from LaPr 88/29, was similar to that of purified spinach HPR-2 [0.8 mM (14)], while the K_m of 0.08 mM for the activity from wild type was comparable to the values of 0.04 to 0.12 mM determined for NADPH-HPR from partially purified (by ammonium sulfate fractionation) preparations of spinach and other plants (11, 13). Thus, the use of LaPr 88/29 has allowed for an unequivocal demonstration that the differences in K_m values between partially purified and purified HPR-2 result from the interference by HPR-1.

Kinetics of NADPH-GR from the 45 to 60% fraction were biphasic, both for wild type and LaPr 88/29, yielding two K_m values of 0.07 and 1.1 mM (Table III). The former value most probably corresponds to GR-1, which shows high affinity for glyoxylate (13, 15), while the higher K_m likely reflects the nonspecific glyoxylate-dependent rate of HPR-2 (11, 14) (see also refs. 10 and 33). These tentative conclusions were reinforced by results of the inhibition studies.

Is HPR-2 Activated by NADPH?

Previous data on partially purified maize HPR-2 suggested that the enzyme was activated by preincubation with NADPH (11). However, we could not rule out an artefact caused by presence of endogeneous phosphatase which would hydrolyze NADPH and, thus, supply NADH for the contaminating HPR-1 enzyme (11). The NADPH-phosphatase, which is ubiquitous in plant tissues, copurifies with HPR-2 during ammonium sulfate fractionation of leaf extracts (11).

Our present study with the use of LaPr 88/29 (Fig. 3) provided unequivocal evidence that the NADPH-dependent activation was, in fact, an artefact. Incubation with NADPH, either in the absence or presence of 15 mM NaF (a phosphatase inhibitor), had no effect on NADPH-HPR from LaPr 88/29, while it caused a two- to three-fold increase of the corresponding activity from wild-type plants (Fig. 3). A similar NaF-insensitive increase in NADPH-HPR activity during incubation with NADPH was observed for partially purified preparations from maize (11). Apparently, endogeneous rates of the phosphatase were sufficient for an effective coupling to HPR-1 activities, even in the presence of NaF.

Incubation with NADPH (or NADH) had no effect on activity of purified spinach HPR-1 (data not shown).

Inhibition by Acetohydroxamate, Tartronate, and P-Hydroxypyruvate

Rates of HPR and GR reactions in LaPr 88/29 were differently affected by 2 mM concentrations of acetohydrox-

Table III. *Apparent Michaelis-Menten Constants (K_m) for Hydroxypyruvate and Glyoxylate of HPR and GR Activities from Wild-Type and LaPr 88/29 Mutant Barley*

The activities were assayed in the 45 to 60% precipitate fractions after ammonium sulfate fractionation. Assays of HPR activities contained a 0.027 to 2.7 mM range of hydroxypyruvate concentrations, while for GR reactions glyoxylate was varied from 0.04 to 8 mM.

Reaction	K_m	
	Wild type	LaPr 88/29
	mM	
NADH-HPR	0.13	0.22
NADPH-HPR	0.08	0.71
NADH-GR	1.3	1.3
NADPH-GR	0.07 (1.1)[a]	0.07 (1.1)[a]

[a] Second K_m.

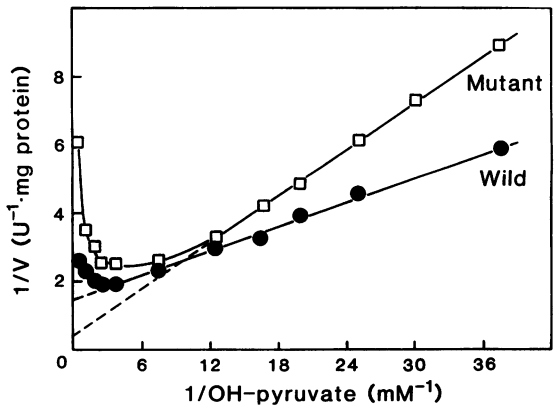

Figure 2. Substrate kinetics of the NADPH-HPR activity from wild-type and LaPr 88/29 barley. The activity was assayed in the 45 to 60% fraction, following ammonium sulfate fractionation of leaf extracts from wild type and LaPr 88/29. Hydroxypyruvate concentration was varied from 0.027 to 2.7 mM.

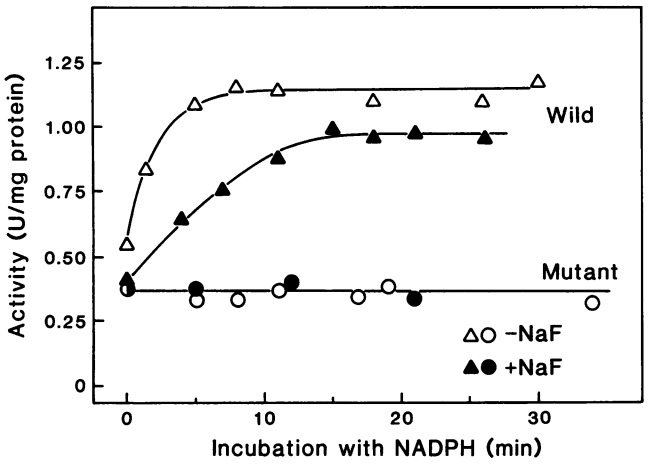

Figure 3. Effect of NADPH-preincubation on the NADPH-dependent rates of HPR from wild-type and LaPr 88/29 barley. The activity was assayed in the 45 to 60% fraction, following ammonium sulfate fractionation of leaf extracts from wild type and LaPr 88/29. The enzyme was incubated with NADPH in the reaction mixture containing all assay components (+15 mM NaF) but hydroxypyruvate. Reactions were initiated with hydroxypyruvate after indicated period of incubation. At time zero, reactions were initiated with enzyme.

amate, tartronate and P-hydroxypyruvate (Table IV). Acetohydroxamate, a specific inhibitor of GR-1 (11, 16), had no effect on HPR activities but caused 25 to 47% inhibition of NADPH-GR, depending on glyoxylate concentration. In contrast, both tartronate and P-hydroxypyruvate strongly (56–83%) inhibited HPR rates and caused 37 to 41% inhibition of NADPH-GR at 1 mM glyoxylate, but were ineffective at 0.1 mM glyoxylate (Table IV). Interestingly, 2 mM concentrations of malonate and maleate, which are analogs of tartronate, had no effect on rates of barley HPRs and GRs (data not shown). Activities of the reductases were also not affected by 0.25 mM ATP, a potent inhibitor of leaf lactate dehydrogenase (2). This enzyme can nonspecifically utilize hydroxypyruvate or glyoxylate as substrates (2, 9, 10) and should be considered in studies of leaf HPR and GR activities. Either crude or partially purified preparations of barley proteins contained only negligible activities of lactate dehydrogenase (measured with pyruvate and NADH).

The inhibitory effects of acetohydroxamate, tartronate and P-hydroxypyruvate were consistent with the presence of both GR-1 and HPR-2 in LaPr 88/29. An inhibitor of HPR-2 would be expected to affect both HPR and GR-dependent rates of this multisubstrate reductase, as was the case for tartronate and P-hydroxypyruvate. Both tartronate and P-hydroxypyruvate seem to be specific for HPR-2. They did not inhibit wild-type barley HPR-1 (isolated in the 30–45% ammonium sulfate precipitate) or purified spinach HPR-1 (data not shown) and had no effect on GR-1, as determined by the lack of inhibition at 0.1 mM glyoxylate, *i.e.* close to the K_m of this reductase (Table III). Judging by the effect of acetohydroxamate on NADPH-GR at 1 mM glyoxylate (25% inhibition), it appears that most of the NADPH-GR activity in crude extracts is due to HPR-2 rather than GR-1. Similar data were reported for NADPH-GR in maize (11) and some other species (13), but not for spinach or pea where about 75 to 91% of NADPH-GR activity in leaf extracts belonged to GR-1, based on acetohydroxamate inhibition (6, 13, 16). Assuming that rates of GR-1 in barley constitute at least 25% of total NADPH-GR activity measured at 1 mM glyoxylate, its specific activity in leaf extracts would be estimated as at least 0.014 μmol·min^{-1}·(mg protein)$^{-1}$ (Table I), which can be compared to rates of photorespiration in this species of 0.028 μmol·min^{-1}·(mg protein)$^{-1}$ (21). Thus, both HPR-2 and GR-1 have sufficient activity to contribute significantly to the flow of carbon through the glycolate pathway in barley.

Inhibition by tartronate and P-hydroxypyruvate was further examined using Dixon plots (Fig. 4). This approach allowed for calculation of apparent K_i values for tartronate and P-hydroxypyruvate at a given concentration of hydroxypyruvate or glyoxylate. True K_i values (21), calculated from plots of 1/hydroxypyruvate *versus* apparent K_i, were about 0.3 and 0.4 mM for tartronate and P-hydroxypyruvate, respectively, regardless of whether NADH-HPR or NADPH-HPR reactions were considered. Apparent K_i values determined for NADPH-GR activity at 4 mM glyoxylate were 1.7 and 1.9 mM for tartronate and P-hydroxypyruvate, respectively (Fig. 4). The NADPH-GR activity was inhibited by tartronate or P-hydroxypyruvate at 4 mM glyoxylate (Fig. 4, see also Table IV), reflecting the inhibition of HPR-2 (K_m of 1.1 mM for glyoxylate, Table III), but not at 0.1 mM glyoxylate where the rates corresponded almost entirely to GR-1 (K_m of 0.07 mM for glyoxylate, Table III). The parallel inhibition patterns observed for HPR reactions at different hydroxypyruvate con-

Table IV. *Effects of Acetohydroxamate (AHA), Tartronate (TA), and P-Hydroxypyruvate (PHA) on Activities of HPRs and GRs from LaPr 88/29 Mutant Barley*

The activities were assayed in the 45 to 60% precipitate fraction after ammonium sulfate fractionation of LaPr 88/29 proteins.

Reaction	Hydroxypyruvate or Glyoxylate	Activity			
		Control	+ 2 mM AHA	+ 2 mM TA	+ 2 mM PHA
	mM		*Δ OD/min*		
NADH-HPR	0.2	0.39	0.39 (100)[a]	0.12 (31)	0.17 (44)
NADPH-HPR	0.2	0.66	0.65 (99)	0.11 (17)	0.14 (21)
NADH-GR	1.0	0.12	0.11 (92)	0.10 (81)	0.10 (87)
NADPH-GR	0.1	0.21	0.11 (53)	0.21 (98)	0.21 (101)
	1.0	0.83	0.62 (75)	0.49 (59)	0.52 (63)

[a] Percent of uninhibited activity.

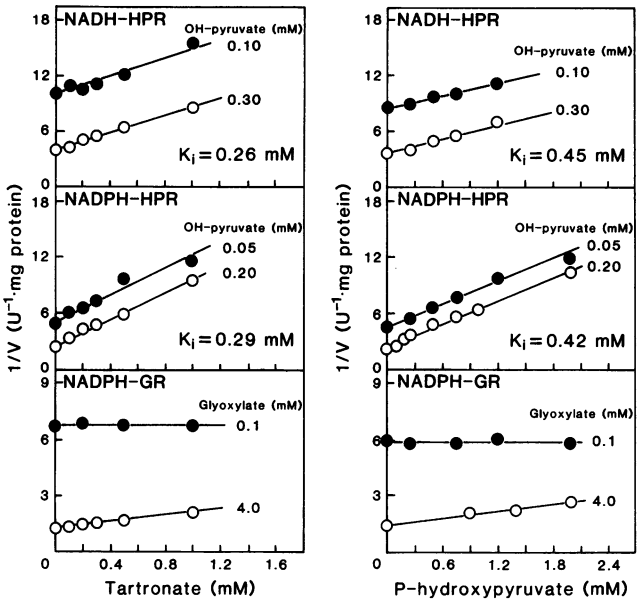

Figure 4. Tartronate and phosphohydroxypyruvate inhibition of HPR and NADPH-GR activities from LaPr 88/29 barley. The activities were assayed in the 45 to 60% fraction, following ammonium sulfate fractionation of leaf extracts from LaPr 88/29. Assay concentrations of tartronate and P-hydroxypyruvate varied in the range of 0 to 1 and 0 to 2 mM, respectively. True K_i values in assays involving HPR activities have been calculated according to Segel (24).

centrations (Fig. 4) indicate that both tartronate and P-hydroxypyruvate act as uncompetitive inhibitors of HPR-2, *i.e.* they bind exclusively to the enzyme-substrate form and not to the free enzyme.

P-Hydroxypyruvate was previously reported to be an uncompetitive inhibitor of an HPR enzyme from mammalian liver (22). This enzyme has been considered as analogous or identical to leaf HPR-1, but a recent study by Van Schaftingen *et al.* (30) demonstrated that the liver reductase preferred NADPH over NADH as the cofactor, similarly to leaf HPR-2.

Optimization of Assays of HPR-2 and GR-1

Our present results (Table II) indicate that, at low concentration of hydroxypyruvate (0.5 mM) or glyoxylate (1 mM), HPR-1 reactions contribute only slightly to NADPH-HPR and GR rates in crude extracts. At higher concentrations of substrates, the nonspecific rates mediated by the HPR-1 enzyme do increase, most markedly for NADPH-HPR and NADH-GR reactions (11). Maintenance of a low concentration of hydroxypyruvate is therefore of utmost importance for assays of NADPH-HPR activity of HPR-2, even in the absence of interferring HPR-1 enzyme, because of strong substrate inhibition at a concentration of hydroxypyruvate as low as 1 mM (Fig. 2). Another precaution, which cannot be overemphasized, is the need to initiate the assays with enzyme rather than with hydroxypyruvate to prevent a nonspecific "coupling" of endogeneous NADPH-phosphatase and HPR-1 (Fig. 3, see also [11]). Previously reported "bursts" of activity following short preincubation of crude extracts with NADPH reflect NADH-HPR rate of HPR-1 which uses NADH accumulated due to the phosphatase action (11). Steady-state rates after the "burst" can still be used for a fairly accurate measure of HPR-2: such an approach has been used for assays of HPR-2 during subcellular localization studies in pea and spinach leaf protoplasts (12).

Previous estimates of the contribution of HPR-2 to overall NADPH-HPR rates in leaf extracts ranged from 13 to 26%, with most of the activity attributed to non-specific action of HPR-1 (13, 21). The assessments were based on assays which were initiated with hydroxypyruvate, *i.e.* allowing for some preincubation of enzymes with NADPH. These earlier conclusions need to be revised and should be compared to the data in Tables I and II, where assays were initiated with enzyme, and where NADPH-HPR activity in LaPr 88/29 constituted at least 80% of that in wild-type plants. Presence of HPR-1 and the NADPH-dependent phosphatase may also interfere with the accuracy of assays of NADPH-GR activity of HPR-2 and/or GR-1 (11). The interference, however, is usually of much smaller magnitude (a few percent), even after several minutes incubation with NADPH, probably because the NADH-GR reaction of HPR-1 has a high K_m for glyoxylate [6-25 mM (1, 9–11, 15, 17, 26, 28, 32)]. HPR-1 itself shows very low, usually negligible, rates of the NADPH-GR reaction (11, 28, 32).

The finding that tartronate and P-hydroxypyruvate strongly and selectively inhibited reactions of HPR-2 (Table IV, Fig. 4) indicated that these compounds could be very useful for differentiation of HPR-2 activities from those of HPR-1 and GR-1. In preliminary studies, we observed that both tartronate and P-hydroxypyruvate selectively inhibited HPR-2 activities from partially purified preparations of maize; thus, the specific inhibition of barley HPR-2 by tartronate and P-hydroxypyruvate may reflect a general property of plant HPRs and GRs. Given the selective effect of acetohydroxamate on GR-1 rates (11, 16), the three reductases could, at least theoretically, be differentiated on the basis of the effect of the inhibitors alone.

CONCLUSIONS

Isolation of the LaPr 88/29 mutant of barley, lacking HPR-1, provided a unique opportunity for an unrestricted study of related HPR and GR enzymes in leaf extracts. It allowed for the development of improved assays of crude leaf or partially purified HPR-2 and GR-1 enzymes, minimizing nonspecific contributions of HPR-1 reactions. Activities of HPR-1, HPR-2 and GR-1 could be quantitated in leaf extracts or partially purified preparations by means of: (a) comparison of HPR and GR activities in wild-type and LaPr 88/29, (b) using specific inhibitors of HPR-2 (tartronate and P-hydroxypyruvate) and GR-1 (acetohydroxamate), and (c) substrate kinetics. Immunological, kinetic and inhibition studies demonstrated that properties of barley leaf HPR-1, HPR-2 and GR-1 were identical, or similar, to those reported for analogous purified enzymes from spinach and other species. Kinetic characteristics of the reductases, such as relatively high activity and low K_m values, are consistent with the involvement of these enzymes in photorespiratory metabolism in barley.

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