Subcellular Location of NADPH-Dependent Hydroxypyruvate Reductase Activity in Leaf Protoplasts of *Pisum sativum* L. and Its Role in Photorespiratory Metabolism[1]

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ABSTRACT

Protoplasts purified from pea (*Pisum sativum* L.) leaves were lysed and fractionated to assess the subcellular distribution of NADPH-dependent hydroxypyruvate reductase (NADPH-HPR) activity. Rate-zonal centrifugation and sucrose-gradient experiments demonstrated that most (about 70%) of the NADPH-HPR activity was located in the supernatant or cytosol fraction. Detectable, but relatively minor activities were associated with the chloroplast fraction (up to 10% on a chlorophyll basis when compared to the lysate) and with peroxisomes. The minor NADPH-HPR activity in the peroxisomes could be fully accounted for by the secondary NADPH-dependent activity of NADH-dependent HPR. The subcellular distribution of NADPH-HPR followed closely that previously determined for NADPH-dependent glyoxylate reductase (NADPH-GR), an enzyme localized predominantly in the cytosol of pea leaf protoplasts (CV Givan *et al.* 1988 J Plant Physiol 132: 593–599). Low activities of both NADPH-HPR and NADPH-GR were also found in purified chloroplasts prepared by mechanical homogenization of *Pisum* and *Spinacia* leaves. In pea and spinach chloroplasts, rates of both NADPH-HPR and NADPH-GR were lower than the activity of the NADH-dependent GR. The results are discussed in relation to a possible role for NADPH-HPR in the oxidative carbon pathway of photorespiration. Both NADPH-HPR and the GRs could function as auxiliary reactions to photorespiration, utilizing hydroxypyruvate and/or glyoxylate 'leaked' or otherwise exported from peroxisomes. NADPH-HPR function might be especially significant under conditions of limiting NADH supply to peroxisomes, with extraperoxisomal reduced pyridine nucleotide acting as the reductant.

Hydroxypyruvate is a primary metabolite in the photorespiratory carbon pathway. Hydroxypyruvate formation in leaf cells occurs primarily in peroxisomes as a result of the transamination of serine by serine-glyoxylate aminotransferase (8). Serine-glyoxylate aminotransferase is accompanied by the peroxisomal

NADH-dependent HPR,[3] and glycerate is therefore readily generated from serine in the peroxisomes. These two peroxisomal reactions constitute an integral part of the photorespiratory cycle (C_2 or glycolate/glycerate pathway) which is initiated by P-glycolate formation from RuBP and, after a number of enzymic steps, leads to glycerate-3-P synthesis from the glycerate ultimately derived from P-glycolate (8, 15).

Peroxisomal hydroxypyruvate reductase shows some nonspecific activity with both NADPH-hydroxypyruvate and NADH/NADPH-glyoxylate (23). However, because of low substrate affinities, these secondary reactions are very unlikely to be of any physiological significance (8, 11, 23). Recently we have identified and purified to homogeneity a novel isoenzyme of HPR that uses NADPH preferentially to NADH and shows significant reactivity with glyoxylate (5–23%) (12). The two HPRs differed substantially in their molecular structures (different M_r values for either native or SDS-dissociated enzymes; lack of common antigenic determinants), and could be almost completely resolved from one another by ammonium-sulfate fractionation. The novel NADPH-HPR enzyme has been proposed (12) to be localized outside the peroxisomes, physically distinct from the peroxisomal NADH/NADPH-HPR. This earlier proposal was inferential; it was based principally on information derived from an earlier study by Tolbert *et al.* (23) who clearly demonstrated that peroxisomes contained only a single form of the NADH/NADPH-HPR, as determined by isoelectric focusing of spinach peroxisome preparations.

In this study we investigated the subcellular distribution of NADPH/NADH-HPR in pea leaf protoplast lysates and in leaf extracts of both pea and spinach. We report that the bulk of the NADPH-dependent HPR activity is localized in the cytosol, with minor activity detected also in the chloroplast fractions.

MATERIALS AND METHODS

Protoplast Preparation and Fractionation. Protoplasts were isolated and purified from leaves of 10- to 14-d-old pea (*Pisum sativum* L.) plants according to a modification of methods described elsewhere (17, 24), as detailed by Givan *et al.* (6). The purified protoplasts were resuspended in 6 to 7 mL of a buffer containing 20 mM tricine (pH 7.6), 500 mM sucrose, 1 mM 2-mercaptoethanol, and 1 mg/mL bovine serum albumen. The

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[3] Abbreviations: HPR, hydroxypyruvate reductase; GR, glyoxylate reductase; P-glycolate P'ase, phosphoglycolate phosphatase; RuBP, ribulose-1,5-bisphosphate; MOPS, 3-(N-morpholino)propanesulfonic acid.

protoplast preparation was virtually devoid of free chloroplasts as determined microscopically.

Protoplasts were lysed by one or two passages through 15 or 20 μm pore-size nylon bolting cloth covering the aperture of a 5 mL hypodermic syringe. The resulting lysate (about 4 mL) was then loaded onto either (a) a discontinuous Percoll gradient consisting of layers of 45, 30, and 10% (v/v) Percoll in 0.5 M sucrose, 20 mM tricine (pH 7.6) or (b) a sucrose density gradient, consisting of 4 mL of 60% (w/w) sucrose overlain by 10 mL of a linear gradient of 60 to 42% sucrose, a 4 mL layer of 42% sucrose, and finally by 13 mL of a linear gradient of 42 to 30% sucrose. All sucrose solutions were buffered with 50 mM Hepes (pH 7.4). For lysates spun through Percoll, centrifugation was carried out in a Sorvall SS34 rotor for 40 min at 12,000g. For the sucrose gradients, centrifugation was done using a Beckman SW 28 rotor for 5 min at 3,370g and then 10 min at 13,200g. This is a rate-zonal (*i.e.* not isopycnic) gradient centrifugation method in which the smaller peroxisomes and mitochondria are recovered as bands running behind the larger intact chloroplasts after a 15 min spin (16). Following centrifugation, 1.5 ml fractions were eluted from the top of the gradient by upward displacement with 65% sucrose.

Purification of Chloroplasts. Chloroplasts were isolated from mechanically homogenized leaves of *Pisum sativum* and *Spinacia oleracea*. Leaves were ground by means of a few short bursts in a Polytron homogenizer in buffer containing 20 mM tricine (pH 7.6) and 500 mM sucrose. The crude homogenate was filtered twice through Miracloth and spun for 45 s at 1100g in a Sorvall SS34 rotor. The chloroplast-enriched pellet was resuspended in grinding buffer and resedimented as before and again similarly resuspended and resedimented. After a further resuspension, the washed chloroplasts were layered over a layer of 40% (v/v) Percoll in grinding buffer and centrifugaled for 5 min at 1100g. The resulting pellet was washed at least once in 50 mM Hepes, 400 mM sucrose buffer (pH 7.4) to yield the final purified Percoll-free chloroplast suspension. The majority of the chloroplasts were judged to be intact by microscopic examination (shiny appearance under phase contrast), and this was confirmed by a relatively high retention of a stromal marker, P-glycolate P'ase (*cf.* Table II).

Enzyme Assays. Both HPRs and GRs were assayed spectrophotometrically by monitoring NADPH or NADH oxidation at 340 nm (18°C). Prior to assays, 50 to 100 μL aliquots of extracts or organelles were diluted with 1.0 mL of 10 mM Mops (pH 7.1) and incubated (18°C) for 3 to 5 min. The mixtures were then centrifuged (10,000 g for 5 min) to remove membrane fragments, and transferred quantitatively to assay cuvettes. Assays for HPRs contained, in 2.0 mL, 100 mM Mes (pH 6.5), 0.2 mM NADPH or NADH, 1 mM hydroxypyruvate, and aliquots of HPR. Assays for GRs contained, in 2.0 mL, 100 mM MOPS (pH 7.1), 0.2 mM NADPH or NADH, 1 mM glyoxylate, and aliquots of GR. Reactions were initiated by hydroxypyruvate (HPRs) or glyoxylate (GRs). Rates of NADPH-HPR from lysed protoplasts, leaf homogenates, and from supernatants obtained following centrifugation through sucrose gradients (top of gradients) and through a Percoll gradient were frequently biphasic, showing an initial 'burst' of activity (15–30 s) which was then followed by a slower, linear rate. Only the latter was taken for calculations of NADPH-HPR activity, the burst most probably belonging to nonspecific activity of peroxisomal NADH(NADPH)-HPR (12). P-Glycolate P'ase, catalase and NAD$^+$-isocitrate dehydrogenase were assayed as described by Randall and Givan (17). One unit of either HPR or GR activity was defined as amount of the enzyme required to oxidize 1 μmol NADPH or NADH per min under conditions of assays. One unit of P-glycolate P'ase was taken as 1 μmol inorganic phosphate produced/min.

Other Methods. Chl determination was done according to Arnon (2).

RESULTS

Following centrifugation of a pea-protoplast lysate through a Percoll gradient (Table I), about 70% of the NADPH-HPR activity remained in the supernatant fraction. This result can be compared with the 26% for P-glycolate P'ase (chloroplast stroma marker) and the 34 and 35% of the catalase and NADH-HPR, respectively (peroxisomal markers) in the supernatant. The residual catalase and P-glycolate P'ase in the supernatant are attributable to breakage of peroxisomes and chloroplasts. About half the NADH-dependent GR activity was found in the supernatant. The fraction of NADPH-HPR activity remaining in the supernatant was very similar to that of NADPH-dependent GR activity, associated predominantly with the cytosol of pea leaf protoplasts (6).

Sucrose-gradient fractionation of the lysed protoplasts (Fig. 1) indicated minor organelle-associated NADPH-HPR activity (Fig. 1B) was associated with intact chloroplasts (*cf* P-glycolate phosphatase peak, Fig. 1A) and, to some extent, with the peroxisomes (catalase peak, Fig. 1A). There was virtually no NADPH-HPR activity in the mitochondria (fractions No. 6 and 7 as per NAD-dependent isocitrate dehydrogenase, data not shown). Under conditions of our assays (1 mM hydroxypyruvate and 0.2 mM NADH or NADPH) a ratio of NADPH-HPR to NADH-HPR in the peroxisomes was about 0.02 to 0.03 (Fig. 1B) and was the same as the ratio with purified spinach-peroxisomal NADH/NADPH-HPR (data not shown). In chloroplasts, however, a ratio of about 0.08 was obtained (fractions 15 and 16, Fig. 1B), which suggested the presence of a reductase different from peroxisomal NADH/NADPH-HPR. Nevertheless, the clear majority of the NADPH-dependent activity of HPR in the protoplast lysate was cytosolic, being predominantly located at the top of the gradient (fractions 1–3, Fig. 1B). Although some peroxisomes have sedimented with the intact chloroplasts (*cf.* catalase, Fig. 1A), the minimal amount of NADPH-HPR in the main peroxisomal peak (Fig. 1B, fractions 8–11) means that any peroxisomal contribution to NADPH-HPR activity in the intact chloroplast peak is negligible.

The presence of distinct, although very minor, chloroplastic activity of NADPH-dependent HPR was also confirmed in chloroplasts purified from mechanically ground pea leaf homogenates (Table II), where the ratio of NADPH-HPR to NADH-HPR activities exceeded 0.40. These chloroplasts were mostly intact

Table I. *Hydroxypyruvate- and Glyoxylate-Reductase Activities in the Supernatant following Centrifugation of Pea Protoplast Lysate through Percoll (see "Materials and Methods")*

Values in brackets refer to results of a second experiment. Chl concentration in the lysate in the first and second experiments was 0.287 and 0.130 mg $\times$ ml^{-1}, respectively.

Enzyme	Activity		
	Lysate	Supernatant	% Activity in supernatant[a] (avg of two experiments)
	nmol $\times$ min^{-1} $\times$ ml^{-1}		
NADPH-HPR	96 (28)	74 (19)	72.5
NADH-HPR	1498 (672)	415 (285)	35
NADPH-GR	242 (96)	192 (64)	73
NADH-GR	106 (70)	ND[b] (37)	53
P-Glycolate P'ase	5740 (3800)	1490 (1040)	26.5
Catalase[c]	6.4 (2.64)	2.18 (0.86)	33.5

[a] Average of two experiments, % of total lysate activity. [b] Not determined. [c] Catalase activity expressed as ΔA $\times$ min^{-1} $\times$ ml^{-1}.
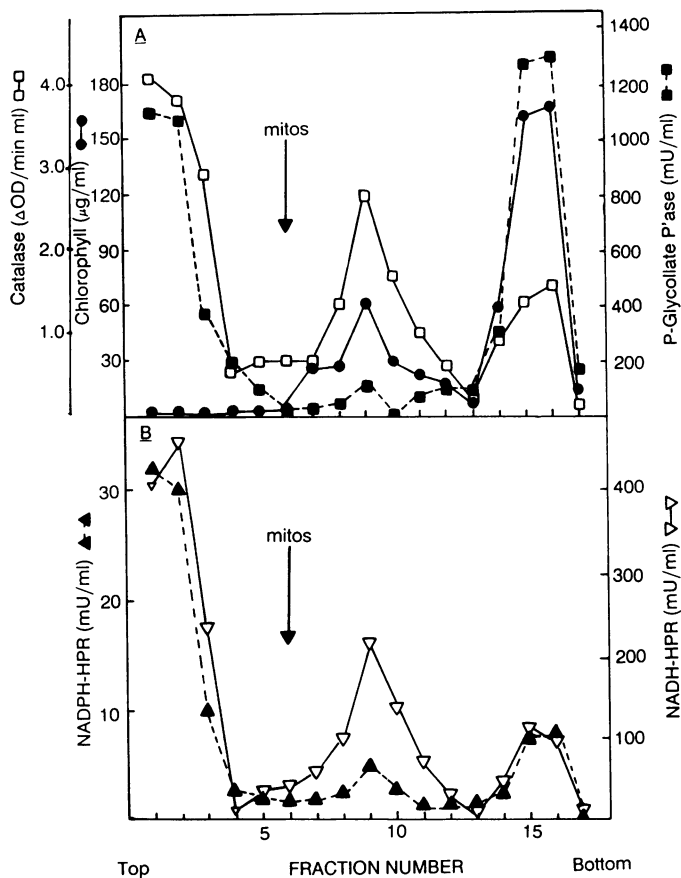
FIG. 1. Sucrose gradient fractionation of pea leaf protoplast lysate. Details of the fractionation are provided in "Materials and Methods."

Table II. *Nucleotide Specificity of Hydroxypyruvate and Glyoxylate Reductases in Chloroplasts Isolated and Purified from Homogenates of Pea and Spinach Leaves*

Source/Enzyme	Activity		% in Chloroplasts
	Crude homogenate	Chloroplasts	
	$\mu mol \cdot h^{-1} \cdot mg\ Chl^{-1}$		
Pisum sativum			
NADPH-HPR	18.1	1.5	8
NADH-HPR	206.0	3.5	2
NADPH-GR	38.4	3.6	9
NADH-GR	23.7	6.2	26
P-Glycolate P'ase	915	631	69
Spinacia oleracea			
NADPH-HPR	28.5	2.0	7
NADH-HPR	316	4.4	1
NADPH-GR	31.6	6.7	21
NADH-GR	18.0	8.1	45
P-Glycolate P'ase	1866	1267	68

as determined by a relatively high retention of P-glycolate P'ase and contained low levels of NADH-HPR on a Chl basis compared to the crude homogenate. A ratio of over 0.40 was also found for chloroplasts purified from pea protoplasts (chloroplasts 84% intact, data not shown). The higher ratio of NADPH-HPR:NADH-HPR in the purified chloroplasts (0.40) compared to the sucrose-gradient chloroplast fraction (0.08) is due to a more effective removal of peroxisomes by the mechanical homogenization and purification procedure. In both species, however, the actual magnitude of the NADPH-HPR activity was

about 10% (on a Chl basis) of the rates determined for crude leaf extracts or protoplast lysates, indicating a very largely extra-chloroplastic localization of this reductase. In several experiments, the range of NADPH-HPR activity in pea chloroplasts purified from ground leaf homogenates was 1.1 to 2.3 $\mu mol \cdot h^{-1} \cdot mg^{-1}$ Chl. These rates were always considerably lower than those of the NADPH-GR and especially of the NADH-GR in the chloroplasts. This very low chloroplastic HPR activity nevertheless did show 'latency' as previously demonstrated for GR (6) (data not shown). Supporting data were obtained from chloroplasts purified from pea protoplasts in which similar amounts of NADPH-HPR were detected (not shown).

Low NADPH-HPR activity was also found in chloroplasts purified from mechanically ground homogenates of spinach leaves (Table II). In general, the rates of the chloroplastic HPRs and GRs in spinach were slightly higher than in pea chloroplasts, but the relative ratios of activities were rather similar in the two tissues. In spinach, the activity of chloroplastic NADPH-GR was about twice as high as in pea chloroplasts, but was still consistently lower than the corresponding rates of chloroplastic NADH-GR activity.

DISCUSSION

The results presented here, obtained both from centrifuging pea protoplast lysates on rate-zonal sucrose gradients and through Percoll (Table I; Fig. 1), indicate that the bulk of leaf NADPH-HPR activity is located in the cytosol. This activity probably belongs to NADPH/NADH-HPR, a newly discovered enzyme recently purified from spinach leaf extracts (12). The discovery of a hydroxypyruvate-utilizing reductase located in the cytosol necessitates reconsideration of the generally accepted view that the HPR activity of plant cells is totally confined to the peroxisomal compartment (18, 19, 21, 23). Most previous HPR-localization studies have examined either the distribution of the NADH-dependent rates in fractionated leaf preparations (21) or have involved the use of specific antibodies against NADH/NADPH-HPR, the well-characterized peroxisomal enzyme (18). These approaches are very likely to have overlooked the presence of NADPH/NADH-HPR for two reasons. First, its *NADH-dependent rate* in leaf extracts is only a few percent of that of the peroxisomal NADH/NADPH-HPR; second, the two enzymes lack common antigenic determinants in their molecular structure, as previously demonstrated by means of immunoblotting analyses (12).

Since hydroxypyruvate in leaves is largely generated within the peroxisomes (22), where a highly active NADH/NADPH-HPR is also present and able to convert the hydroxypyruvate to D-glycerate, the role played by the extraperoxisomal enzyme is not immediately obvious. The problems requiring consideration are the source(s) of (a) hydroxypyruvate and (b) the reducing power required for the conversion of hydroxypyruvate to glycerate in the cytosol.

Current schemes of the glycolate/glycerate pathway (8) indicate that the NADH required for hydroxypyruvate reduction in the peroxisomes is equimolar to the NADH generated from the oxidation of glycine in the mitochondria. It has been proposed that redox equivalents formed in the mitochondrial matrix can be transferred to the peroxisomes, e.g. by either a malate/aspartate or malate/oxaloacetate shuttle operating across the peroxisomal membrane (5, 9, 19, 25), but doubts have been raised as to the efficiency of both of these proposed mechanisms (4, 19). A possible role for chloroplasts has been postulated to provide an alternative source of redox equivalents for hydroxypyruvate metabolism within peroxisomes (4, 7). The functioning of a chloroplastic source of reductant for the reduction of hydroxypyruvate would permit at least part of the NADH generated via mitochondrial glycine oxidation to remain available for contin-

ued operation of the mitochondrial electron transport system rather than being entirely shuttled to the peroxisomes. Conversion of hydroxypyruvate to glycerate in peroxisomes may be limited by NADH availability (26) and perhaps even more importantly by the apparently high rates of hydroxypyruvate leakage across the peroxisomal membranes, as recently demonstrated for lettuce-peroxisome preparations (1). Extraperoxisomal HPR activity may serve as an important back-up mechanism to ensure a complete reduction of hydroxypyruvate that passes out of the peroxisomes into the cytosol. Glycerate formed by either peroxisomal or extraperoxisomal HPRs can presumably be phosphorylated to 3-phosphoglycerate by the chloroplast-based glycerate kinase (15).

Reducing equivalents formed by both mitochondria and chloroplasts could support extra-peroxisomal HPR activity; e.g. cytosolic nonreversible NADP-dependent glyceraldehyde-3-P dehydrogenase and glucose-6-P dehydrogenase may both generate cytosolic NADPH (10). Because purified spinach NADPH/NADH HPR shows up to 50% activity with NADH compared to NADPH-dependent rates (12) it is also possible that NADH (e.g. supplied by a cytosolic malate dehydrogenase or by NAD-dependent glyceraldehyde-P dehydrogenase) may support the cytosolic reduction of hydroxypyruvate.

Chloroplasts purified from leaf extracts contained low activities of both NADPH-HPR and NADPH-GR (Table II). This contrasts with the chloroplast-associated NADH-dependent GR activity, which constitutes a much higher proportion (about 26 and 45%) of the total NADH-GR in pea and spinach leaves respectively (Table II). Similar data with respect to the distribution of the GRs were recently reported for purified chloroplasts from pea protoplast lysates (6). Future work must establish whether the chloroplast-based activities of both HPRs and GRs belong to four distinct enzymes or are the expression of one or two multi-substrate reductases. The preferential chloroplastic utilization of NADH and glyoxylate as compared to the three other possible pairs of reactants, as shown both by Givan et al. (6) and in the present study, is a new finding and has not been previously demonstrated for any leaf GRs or HPRs in any subcellular compartment. A significant portion of the NADH-glyoxylate-reduction activity in leaves may turn out to be due to a specific chloroplast-associated reductase. Why chloroplasts should contain a GR preferring NADH is at present unclear, although it is already known that chloroplasts contain at least one enzyme that can generate NADH, i.e. pyruvate dehydrogenase complex (3).

The data obtained for nucleotide specificity of chloroplast GRs from spinach (Table II) do not support the previously held conclusion that the spinach leaf NADPH-GR activity is wholly confined to the chloroplasts (20, 23), with most of the NADH-GR being extrachloroplastic (23). The reasons for this apparent discrepancy may be twofold: the use of different organelle-fractionation methods (nonaqueous method used in Ref. 20) and the different GR assay systems employed (e.g. 75 mM glyoxylate, Ref. 23). In physiological terms the use of low glyoxylate levels in GR assays seems to us to be of particular importance in order to minimize a nonspecific contribution of peroxisomal NADH/NADPH HPR activity (13, 14). The minor NADPH-dependent-GR in chloroplasts could perhaps correspond to the originally discovered NADPH-GR, the enzyme partially purified from spinach and tobacco leaf extracts over 25 years ago by Zelitch and Gotto (27).

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