Purification and characterization of a novel NADPH(NADH)-dependent hydroxypyruvate reductase from spinach leaves

Comparison of immunological properties of leaf hydroxypyruvate reductases

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A novel hydroxypyruvate reductase preferring NADPH to NADH as a cofactor was purified over 1500-fold from spinach leaf extracts. The enzyme was an oligomer of about 70 kDa, composed of two subunits of 38 kDa each. The K_m for hydroxypyruvate (with NADPH) was about 0.8 mM in the pH range 5.5–6.5, and 0.3 mM at pH 8.2. The V_{max} was highest in the pH range 5.5–6.5 and decreased by about 65% at pH 8.2. Above pH 6.0, the enzyme was prone to a strong substrate inhibition by hydroxypyruvate. The reductase could use glyoxylate as an alternative substrate, with rates up to one-quarter of those with hydroxypyruvate. This glyoxylate-dependent activity preferred NADPH to NADH as a cofactor. Rabbit antibodies prepared against NADPH(NADH)-hydroxypyruvate reductase were highly specific for this enzyme and did not cross-react with peroxisomal NADH(NADPH)-dependent hydroxypyruvate reductase, as found by Western immunoblots of proteins from leaf extracts of spinach, pea and wheat. Antibodies raised against purified NADH(NADPH)-hydroxypyruvate reductase were also highly specific, recognizing only their own antigen. To our knowledge, this is the first report in the literature of the occurrence of NADPH(NADH)-hydroxypyruvate reductase in leaves, and the first to provide immunological comparison of leaf hydroxypyruvate reductases. Because of the relatively high rates of the novel reductase in leaf extracts (at least 20 μ mol/h per mg of chlorophyll), this enzyme might be an important side-component of the glycolate pathway (photorespiration), possibly utilizing hydroxypyruvate 'leaked' from peroxisomes, and thus contributing to the glycerate pool derived from glycolate. Because of the glyoxylate-dependent activity, the enzyme may also contribute to glycolate formation in leaves.

INTRODUCTION

Most hydroxypyruvate in leaves comes from the glycolate pathway (photorespiration) and is due to the activity of a serine-dependent glyoxylate transaminase localized exclusively in peroxisomes (Tolbert, 1980; Noguchi & Hayashi, 1980; Nakamura & Tolbert, 1983). Hydroxypyruvate serves as a substrate for NADH-(NADPH)-HPR, which is also localized in peroxisomes and is closely coupled to the transaminase activity, allowing a net conversion of serine into D-glycerate (Schmitt & Edwards, 1983; Yu & Huang, 1986). The reductase is one of the most extensively studied enzymes of the glycolate pathway, and has been purified and characterized from leaves of several species (see, e.g., Zelitch, 1955; Sallach, 1966; Titus *et al.*, 1983; Anderson & Emes, 1985). The enzyme preferentially utilizes NADH rather than NADPH as a cofactor and can non-specifically react with glyoxylate (Kohn & Warren, 1970; Tolbert *et al.*, 1970). This GR activity of NADH-(NADPH)-HPR probably has no significance *in vivo*, because of the high, non-physiological, K_m for glyoxylate of the enzyme (about 5–20 mM; Tolbert *et al.*, 1970; Titus *et al.*, 1983; Kleczkowski *et al.*, 1987a).

It has usually been assumed that the NADH-(NADPH)-dependent HPR is the only form of HPR in leaves. During enzyme distribution studies, most HPR

activity was found in peroxisomes (Tolbert *et al.*, 1970; Schmitt & Edwards, 1983), and only low, if any, rates were detected in the cytosol and chloroplast fractions. The extra-peroxisomal HPR has been considered to be the result of cross-contamination problems inherent in cell fractionation methods.

In the present study, we report the identification, purification and some characteristics of an apparently novel isoenzyme of leaf HPR which utilizes NADPH in preference to NADH as a cofactor. Immunological properties of leaf NADPH(NADH)-HPR and NADH-(NADPH)-HPR are compared by using antibodies prepared against each of these proteins. A preliminary communication summarizing some of the data presented in this paper has been reported (Kleczkowski *et al.*, 1987a).

MATERIALS AND METHODS

Plant material

Spinach (*Spinacia oleracea* L.) leaves were purchased from a local grocery store. Seedlings of pea (*Pisum sativum* L.) and wheat (*Triticum aestivum* L.) were grown as previously described (Kleczkowski & Randall, 1985).

Abbreviations used: GR, glyoxylate reductase; HPR, hydroxypyruvate reductase.

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Reagents

NADPH and NADH were purchased from P-L Biochemicals. Purified spinach leaf NADH-(NADPH)-HPR (other names 'glycerate dehydrogenase' or 'glyoxylate reductase') was obtained from Sigma. Green A dye-ligand matrix was from Amicon. An affinity matrix of agarose-hexane-adenosine 2',5'-bisphosphate type 2 (AG2',5'ADP-2) was from P-L Biochemicals.

Buffers

All purification procedures were performed at 0–4 °C. Extraction medium contained 40 mM-Tricine, pH 7.8, 2 mM-MgCl₂, 1 mM-EDTA, 14 mM-2-mercaptoethanol, 1 mM-benzamidine, 1 mM-6-aminohexanoic acid and 5 μM-leupeptin. DEAE-cellulose, Sephadex G-75SF, Green A and AG2',5'ADP-2 columns were equilibrated with buffer A, containing 20 mM-Mops, pH 7.1, and 14 mM-2-mercaptoethanol. All buffers were degassed before use.

Preparation of crude leaf extracts for SDS/polyacrylamide-gel electrophoresis

Crude leaf HPRs from spinach, pea and wheat were isolated in a grinding buffer containing 40 mM-Tricine, pH 7.8, 2 mM-MgCl₂ and 1 mM-EDTA. Samples of leaves (7–15 g) were cut into 4–6 mm-wide slices and homogenized for 15 s in a Waring blender in 50 ml of the grinding medium (at 0–4 °C). All homogenates were squeezed through four layers of cheesecloth and one layer of Miracloth, and centrifuged at 10000 *g* for 10 min. Samples of supernatants were kept frozen at –20 °C.

Immunological studies

Antibodies against spinach NADPH(NADH)-HPR were obtained by injecting a New Zealand White male rabbit with a dose of the homogeneous enzyme (120 μg) mixed (1:1) with Freund's complete adjuvant (Cappel Laboratories). Injections were done subcutaneously along the spine of the rabbit. Blood was collected 6 weeks after the immunization. Serum was stored in batches at –20 °C. Antibodies against spinach NADH-(NADPH)-HPR were prepared as previously described (Kleczkowski *et al.*, 1986).

Western blotting was performed by a slight modification of the procedure of Towbin *et al.* (1979), as described by Kleczkowski (1985). The modifications included the use of an alkaline phosphatase-labelled goat anti-rabbit IgG (Sigma) as a linking antibody (instead of radioactively labelled or fluorescein- or peroxidase-conjugated secondary antibodies as described in the original method) and the use of Fast Red (Sigma) as a substrate for the phosphatase to detect antigen-antibody complex(es) on nitrocellulose.

Assay of enzymic activities

NADPH(NADH)-HPR and NADH(NADPH)-HPR were assayed spectrophotometrically, NADPH/NADH oxidation being monitored at 340 nm (25 °C). A 1.0 ml assay mixture contained, unless otherwise indicated, 100 mM-Mes, pH 6.5, 0.2 mM-NADPH or -NADH, 1 mM-hydroxypyruvate or -glyoxylate and various amounts of HPR. Reactions were initiated by addition of hydroxypyruvate or glyoxylate. Control assays containing all the components of the reaction but hydroxypyruvate or glyoxylate were performed to correct for

non-specific oxidation of NADPH or NADH. For assays of NADH-dependent activity of purified NADPH(NADH)-HPR, the enzyme was desalted (on Sephadex G-25) to remove NADPH present after elution from the AG2',5'ADP-2 column. One unit of HPR activity was defined as the amount of enzyme required to oxidize 1 μmol of NADPH or NADH/min under the conditions of the assay.

Rates of NADPH(NADH)-GR were determined as previously described (Kleczkowski *et al.*, 1986, 1987b). The assay mixture contained, in 1.0 ml, 100 mM-Mops, pH 7.1, 0.2 mM-NADPH or -NADH, 1 mM-glyoxylate, and various amount of GR. Reaction was initiated by addition of glyoxylate. Other conditions were as described for the HPR assay.

Reverse reactions of NADPH(NADH)-HPR were assayed with the use of 1.0 ml assay mixtures containing 100 mM-Mes, pH 6.5, 1 mM-D-glycerate or -DL-glycerate or -glycollate, 0.2 mM-NADP⁺ or -NAD⁺ and the purified enzyme. The amount of HPR used in these assays corresponded to an activity of at least 30 nmol/min, as determined for the forward reaction (NADPH/hydroxypyruvate).

Analytical methods

Protein and chlorophyll were determined by the Bradford (1976) and Arnon (1949) procedures respectively. Slab-gel electrophoresis was done as described by Laemmli (1970). Protein *M_r* markers for gel filtration were from Boehringer Mannheim. Protein standards for SDS/polyacrylamide-gel electrophoresis were from Sigma.

RESULTS AND DISCUSSION

Identification of NADPH(NADH)-hydroxypyruvate reductase

Crude leaf extracts of spinach contain high activities of both NADPH- and NADH-dependent HPRs, reaching values of 0.1–0.2 μmol/min per mg of protein (NADPH-dependent) and 0.8–1.5 μmol/min per mg of protein (NADH-dependent). These rates have usually been ascribed to a single enzyme, the NADH(NADPH)-dependent HPR from peroxisomes, which uses NADH in preference to NADPH (Tolbert *et al.*, 1970; Kohn & Warren, 1970). The peroxisomal HPR can be precipitated by a low concentration (0–50 % satn.) of (NH₄)₂SO₄ with an almost full recovery of its NADH-dependent activity in the pellet (Zelitch & Gotto, 1962; Kleczkowski *et al.*, 1986). We have repeated these experiments, but, instead of monitoring only NADH-dependent rates, we have also determined the recovery of the NADPH-dependent activity. At low concentration of (NH₄)₂SO₄ (0–45 % saturation), the precipitated NADH- and NADPH-dependent activities of HPR constituted about 85 and 75 % respectively of those in crude extracts. After further fractionation with 45–60 %-satd. (NH₄)₂SO₄, the recovered total activity of NADH-dependent HPR was a mere 3 % of that before the salt treatment (Table 1). However, the recovery of NADPH-dependent activity was 20 %, and its magnitude was about equal to that with NADH. These results have strongly suggested that a significant portion of the NADPH-dependent activity of HPR in leaf extracts belongs to a distinct enzyme which does not co-precipitate with NADH-(NADPH)-HPR.

Table 1. Separation of spinach leaf NADPH(NADH)-hydroxypyruvate reductase and NADH(NADPH)-hydroxypyruvate reductase by $(\text{NH}_4)_2\text{SO}_4$ fractionation

Values in parentheses correspond to the steady rate after the initial 'burst' of activity.

Procedure	Activity with NADPH		Activity with NADH	
	Total activity (units)	Yield (%)	Total activity (units)	Yield (%)
Leaf extract	157 (36)	100 (100)	906	100
$(\text{NH}_4)_2\text{SO}_4$ ppt. (45–60 % satn.)	31 (31)	20 (86)	31.5	3

Table 2. Summary of purification of spinach leaf NADPH(NADH)-hydroxypyruvate reductase

Procedure	(Activity units/mg of protein)	Total activity (units)	Yield (%)	Purification (fold)
Crude extract	0.164 (0.038)*	915 (212)*	100	1
$(\text{NH}_4)_2\text{SO}_4$ (45–60 % satn.)	0.157	218	24	1
DEAE-cellulose	0.340	160	18	2
Green A	27.7	148	16	169
Sephadex G-75SF	78.1	91	10	476
AG2',5'ADP-2	257.0	78	9	1567

* Steady rate after initial 'burst' of activity.

We have previously reported (Kleczkowski *et al.*, 1987b) that the activity of crude spinach leaf NADPH-dependent HPR proceeds non-linearly in time, being strongly inhibited after 10–15 s after initiation of the reaction with hydroxypyruvate. The newly established rate, which constitutes about 15–25 % of the initial activity, is then linear for at least 1 min until exhaustion of substrates. The decrease of activity was not seen when, before assay, leaf extract was passed through a Sephadex G-25 column [or after $(\text{NH}_4)_2\text{SO}_4$], indicating an involvement of some endogenous low- M_r factor(s) in the inhibition. The biphasic course of assays of crude NADPH-dependent HPR suggests that perhaps two different enzymic activities could be involved, one sensitive to the inhibition and other unaffected by the inhibitor(s). It is tempting to speculate that the former (75–85 % of total activity) is the peroxisomal NADH-(NADPH)-HPR, whereas the latter (15–25 % of total activity) belongs to the new HPR described in the present study. In several attempts (e.g. Table 1) to correlate the magnitude of the inhibitor(s)-unaffected activity of crude NADPH-HPR with that obtained after $(\text{NH}_4)_2\text{SO}_4$ fractionation (45–60 % satn.), the recovery of the partially purified enzyme was consistently very high, ranging from 80 to 107 %. This could suggest that the uninhibited activity of NADPH-dependent HPR represents a true measure of the novel HPR in leaf extracts.

Purification

About 760 g of spinach leaves were homogenized in a Waring blender for 25 s in the presence of 760 ml of the extraction medium. The homogenate was squeezed through four layers of cheesecloth and one layer of Miracloth and centrifuged at 15000 *g* for 15 min. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to 45 % saturation; the suspension was equilibrated by stirring for 45 min, and then centrifuged at 15000 *g* for 15 min. The resulting supernatant was further adjusted to 60 %

saturation with solid $(\text{NH}_4)_2\text{SO}_4$ and then equilibrated for 30 min and centrifuged at 15000 *g* for 25 min. The pellet was dissolved in buffer A and the suspension centrifuged at 15000 *g* for 5 min.

The preparation obtained after the salt treatment was chromatographed on a DEAE-cellulose column (2.5 cm $\times$ 15 cm) with a 550 ml linear gradient of 0–0.5 M-NaCl in buffer A. A peak of NADPH(NADH)-HPR activity was eluted at about 0.10 M-NaCl. The most active fractions were pooled and applied directly on to a Green A column (1.5 cm $\times$ 10 cm). After extensive washing with buffer A, the enzyme was eluted with a step gradient of 0.2 mM-NADPH in buffer A. The eluate was concentrated with a Diaflo PM10 filter (Amicon) and applied (3 ml) on to a Sephadex G-75SF column (2.5 cm $\times$ 68 cm) operating at a flow rate of 15 ml/h (see Fig. 2). After gel filtration, NADPH(NADH)-HPR was chromatographed on an AG2',5'ADP-2 column (0.8 cm $\times$ 2.0 cm) and eluted with a step gradient of 0.2 mM-NADPH in buffer A. The purified protein was stable when stored in 50 % (v/v) glycerol at -20°C . Results of the purification procedure are summarized in Table 2.

Spinach leaf NADPH(NADH)-HPR was purified 1567-fold with a yield of 9 %. The actual yield and the degree of purification of the novel reductase are probably much higher than shown in Table 2, since the NADPH-dependent rate of crude leaf enzyme is strongly overestimated, owing to non-specific activity of peroxisomal NADH(NADPH)-HPR (Table 1). The biggest 'loss' of total activity of NADPH(NADH)-HPR occurred during $(\text{NH}_4)_2\text{SO}_4$ fractionation (Table 2) and was most probably caused by the removal of NADH-(NADPH)-HPR, which precipitates at low salt concentration (Zelitch & Gotto, 1962; Anderson & Emes, 1985; Kleczkowski *et al.*, 1986). With the assumption that 100 % of NADPH(NADH)-HPR is recovered during $(\text{NH}_4)_2\text{SO}_4$ fractionation (see the previous section), the

actual yield could have been as high as 37% and the protein purified over 6700-fold.

Purity and M_r

The final enzyme preparation exhibited a single band on Coomassie-Blue-stained SDS/polyacrylamide gel (Fig. 1). The position of NADPH(NADH)-HPR on the gel corresponded to a size of 38 kDa, whereas chromatography of native enzyme on a calibrated Sephadex G-75SF column yielded a value of 70 kDa. These data suggest that NADPH(NADH)-HPR is an oligomer composed of two subunits of 38 kDa each. The dimeric structure is common for both NADPH(NADH)-HPR and NADH(NADPH)-HPR. The native molecular mass of the latter is about 90–95 kDa (Kohn *et al.*, 1970; Titus *et al.*, 1983; Kleczkowski *et al.*, 1987a), and that of the subunit ranges between 41 and 43 kDa, depending on the plant species (Kleczkowski *et al.*, 1986). In spinach, NADH(NADPH)-HPR has a molecular mass of 41 kDa (Fig. 1).

Fig. 1. SDS/polyacrylamide-gel electrophoresis of purified hydroxypyruvate reductase from spinach leaves

Each protein sample was mixed with an equal volume of a solution of 6 M-urea, 4% 2-mercaptoethanol and 4% SDS, heated at 80 °C for 20 min, and applied to a 12.5%-polyacrylamide gel. Lanes: (a), 2 μ g of NADPH(NADH)-HPR; (b), 10 μ g of standard proteins; (c) 6 μ g of NADH(NADPH)-HPR. The standard protein kit (Sigma) contained: carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase *b* (97.4 kDa), β -galactosidase (116 kDa) and myosin (205 kDa).

Fig. 2. Resolution of spinach NADPH(NADH)-hydroxypyruvate reductase from NADPH(NADH)-glyoxylate reductase after chromatography on a Sephadex G-75SF column

The proteins were partially purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation and by chromatography on DEAE-cellulose and Green A columns, as described in the Results and discussion section. The flow rate through Sephadex G-75SF column was 15 ml/h; 2.9 ml fractions were collected. Assays contained 40 mM-Mops, pH 7.1, 0.2 mM-NADPH or -NADH and 1 mM-hydroxypyruvate or -glyoxylate. ●, NADPH-GR; ○, NADH-GR; ■, NADPH-HPR; □, NADH-HPR.

Although purified NADPH(NADH)-HPR could be judged homogeneous on the basis of a single Coomassie-Blue-stained band on polyacrylamide gel (Fig. 1), the criterion of purity was not satisfied by more sensitive methods. When gels were over-stained with a silver reagent (Bio-Rad), which is about 10 times more sensitive than the Coomassie Blue stain (Merril *et al.*, 1981), two additional very weak bands appeared, at about 33 and 60 kDa (results not shown). The 33 kDa band probably corresponds to trace amounts of NADPH(NADH)-GR (Kleczkowski *et al.*, 1986), which co-purifies with NADPH(NADH)-HPR up to the gel-filtration step (see Fig. 2). The purified preparation contained no NADH(NADPH)-HPR, as found by SDS/polyacrylamide-gel electrophoresis (regardless of the stain used) and by immunoblots using antibodies prepared against this enzyme (see Fig. 6).

Substrate specificity and pH-dependence

NADPH(NADH)-HPR was found to react with both hydroxypyruvate and glyoxylate and to use either NADPH or NADH as a cofactor. However, activity with hydroxypyruvate and NADPH was at least 2-fold higher than with any other pair of reactants. This specificity was not found for crude or partially purified enzyme preparations, because of interfering activities of NADH(NADPH)-HPR and NADPH(NADH)-GR. The latter enzyme prefers NADPH and glyoxylate as substrates and decisively contributes to glyoxylate-dependent rates of reductases in leaf extracts (Kleczkowski *et al.*, 1986, 1987b). Although the interfering activity of NADH(NADPH)-HPR could be removed early during purification by using $(\text{NH}_4)_2\text{SO}_4$ fractionation (Tables 1 and 2), the separation of NADPH(NADH)-HPR from the GR occurred only during gel-filtration chromatography (Fig. 2). Further purification

of NADPH(NADH)-HPR on the AG2',5'ADP-2 column, the last step of purification, did not change the relative magnitude of reactions catalysed by this enzyme. Under assay conditions, the most specific pair of reactants was NADPH/hydroxypyruvate (100% activity), followed by NADH/hydroxypyruvate (about 50%), NADPH/glyoxylate (23%) and NADH/glyoxylate (5%) (Fig. 2).

Both the hydroxypyruvate- and glyoxylate-dependent activities of the enzyme were not inhibited by 2 mM-acetohydroxamate, and analogue of glycine, which had been shown to inhibit spinach NADPH(NADH)-GR strongly (K_i 0.3 mM) and which had no effect on spinach NADH(NADPH)-HPR (Kleczkowski *et al.*, 1987b). This indicated that the trace amount of the GR associated with purified NADPH(NADH)-HPR, as found on gels over-stained with silver and by immunoblotting (see Fig. 5), does not contribute to any significant degree to the glyoxylate-dependent activities of the HPR. Thus the reactivity with glyoxylate seems to represent an intrinsic property of NADPH(NADH)-HPR, similarly to NADH(NADPH)-HPR (Kohn & Warren, 1970). The insensitivity of both NADPH(NADH)-HPR and NADH(NADPH)-HPR to acetohydroxamate suggests that this compound might be used to differentiate the glyoxylate-dependent activities of HPRs from those of the GR in leaf extracts.

Purified NADPH(NADH)-HPR had no activity with pyruvate, oxaloacetate, 2-oxobutyrate or 2-oxoglutarate (all tested at 2.5 mM), regardless of whether NADPH or NADH was used as a cofactor. The enzyme catalyses an

Fig. 3. pH optimum of NADPH(NADH)-hydroxypyruvate reductase

The buffer used for assays of HPR activity was 33 mM-Tricine/33 mM-Mops/33 mM-Mes. This mixture was adjusted to the appropriate pH with 10 M-NaOH. The concentration of NADPH or NADH in the assay was 0.2 mM. ●, NADPH + 6 mM-hydroxypyruvate; ○, NADPH + 1 mM-hydroxypyruvate; ▲, NADH + 6 mM-hydroxypyruvate; △, NADH + 1 mM-hydroxypyruvate.

Fig. 4. pH-dependent changes in V_{max} and K_m values of NADPH(NADH)-hydroxypyruvate reductase

For each pH value shown, rates of the enzyme were assayed at nine different concentrations of hydroxypyruvate (0.24, 0.30, 0.40, 0.60, 1.2, 2.0, 3.0, 6.0 and 10.0 mM), and Lineweaver/Burk reciprocal plots were constructed to determine V_{max} (○) and K_m (□) values. Other conditions were the same as in the legend to Fig. 3.

apparently irreversible reaction, since it had no activity with D-glycerate, DL-glycerate or glycolate with NADP⁺ or NAD⁺ as cofactor. This is in contrast with NADH(NADPH)-HPR, which catalyses fully reversible reactions (Kohn & Warren, 1970; Gaudillere *et al.*, 1983).

The apparent K_m value for hydroxypyruvate was 0.8 mM at pH 6.5. The NADPH-dependent activity of the enzyme was prone to a strong substrate inhibition by hydroxypyruvate, which was especially evident above pH 6.0 in the assay (Fig. 3). Because of this complex substrate kinetics, optimal activity of NADPH(NADH)-HPR did not correspond to V_{max} values determined by graphical methods (reciprocal plots). The highest rates were seen at about 1–2 mM-hydroxypyruvate, regardless of the assay pH (results not shown). The V_{max} was constant in the pH range 5.5–6.5, and declined by about 65% at pH 8.2 (Fig. 4). The pH-dependent changes in K_m values for hydroxypyruvate followed the same pattern as those of V_{max} (Fig. 4), indicating that a K_m/V_{max} ratio is constant over the pH range 5.5–8.2. The substrate inhibition by hydroxypyruvate was previously observed for the peroxisomal NADH(NADPH)-HPR from both spinach and *Lemna* leaf extracts (Anderson & Emes, 1985).

Immunological comparison of leaf hydroxypyruvate reductases

The Western-blot procedure is a very sensitive analytical tool, allowing detection of nanogram amounts of a given protein, provided that specific antibodies are available (Towbin *et al.*, 1979). The method may also allow determination of the M_r of a protein, regardless of its purity (see, e.g., Kleczkowski *et al.*, 1986). In the present study, we used immunoblots to compare immunological properties of NADPH(NADH)-HPR and NADH(NADPH)-HPR from spinach leaves, and to probe for possible other isoforms of HPR in crude leaf extracts. Because the two reductases utilize the same substrates, although with a different degree of specificity, it seemed possible that this common property may somehow reflect similarities in molecular structure of these and other leaf HPRs.

When proteins from wheat, pea and spinach leaf

Fig. 5. Immunoblots of proteins from crude leaf extracts probed with antibodies against spinach NADPH(NADH)-hydroxypyruvate reductase

Samples (25–40 μ g) of proteins from crude leaf extracts were loaded on 12.5%-polyacrylamide gel and electrophoresed in the SDS-containing buffer. After electrophoresis, resolved proteins were transferred on to nitrocellulose paper and incubated with the rabbit anti-[spinach NADPH(NADH)-HPR] IgG (1:50 dilution). Formation of the specific antigen-antibody complex(es) was determined as described in the Materials and methods section. Lanes: (a), (b), (c), crude leaf extracts of wheat, pea and spinach respectively; (d), 0.2 μ g of NADPH(NADH)-HPR; (e) 10 μ g of NADPH(NADH)-HPR.

extracts were resolved by SDS/polyacrylamide-gel electrophoresis and then transferred to nitrocellulose paper, probing with anti-[NADPH(NADH)-HPR] IgG yielded a single major protein band (of 38 kDa), corresponding to the subunit size of NADPH(NADH)-HPR (Fig. 5, lanes a, b, c). Immunoblots of spinach leaf proteins (Fig. 5, lane c) contained also five minor bands, of 33, 35, 36, 60 and 67 kDa respectively. The 33 and 60 kDa proteins had been detected in preparations of purified NADPH(NADH)-HPR (on silver-stained SDS/polyacrylamide gels) before immunization of the rabbit, and thus their occurrence on the immunoblot could be due to immuno-

reaction with their own antibodies. This was partially confirmed by presence of the trace 33 and 60 kDa bands on the immunoblot of purified NADPH(NADH)-HPR (Fig. 5, lane d). Minor bands were also seen for pea (31 and 35 kDa; Fig. 5, lane b) and wheat (41 kDa; Fig. 5, lane a) leaf proteins.

The antibodies against NADPH(NADH)-HPR failed to cross-react with NADH(NADPH)-HPR from leaf extracts, but they did bind to the purified spinach enzyme, yielding a trace band at 41 kDa (Fig. 5, lane e). This binding was most likely enforced by the large amount of purified NADH(NADPH)-HPR loaded on the SDS gel before transfer on to nitrocellulose (10 μ g of purified enzyme, 30 μ g of crude leaf proteins). The very weak immunoreaction with NADH(NADPH)-HPR indicates only minor common determinants in structure of the two HPRs under study. The immunoblot of purified NADH(NADPH)-HPR (Fig. 5, lane e) also yielded a second weak band corresponding to NADPH(NADH)-HPR. This indicates that preparations of the former enzyme contain a trace amount of the novel reductase.

It should be pointed out that the minor bands seen in Fig. 5 developed only after prolonged (> 20 s) incubation of the immunoblot with Fast Red (the substrate for phosphatase linked to secondary goat antibodies), whereas there was an almost instant (1–2 s) development of the 38 kDa band corresponding to NADPH(NADH)-HPR. This fact alone indicates high specificity of the antibodies.

At present it is unclear whether the minor bands observed on immunoblots of crude leaf proteins (Fig. 5, lanes a, b, c) represent yet unknown forms of HPR, or are the result of some non-specific immunoreaction. The lack of immunoreaction with crude leaf NADH(NADPH)-HPR, which in spinach, pea and wheat is characterized by a subunit of 41, 41 and 43 kDa respectively (Kleczkowski *et al.*, 1986), suggests that the antibodies are highly specific, and thus the detected proteins must indeed very closely resemble the structure of NADPH(NADH)-HPR. The possibility that the bands of 35 and 36 kDa (spinach) as well as 31 and 35 kDa (pea) arose by limited proteolysis of NADPH(NADH)-HPR during extraction of leaf proteins also can not be excluded.

To compare directly the position of both NADPH(NADH)-HPR and NADH(NADPH)-HPR on immunoblots of crude leaf proteins, we used a mixture of antibodies prepared against each of these two proteins (Fig. 6). Antibodies against NADH(NADPH)-HPR had previously been shown (Kleczkowski *et al.*, 1986) to react monospecifically with this enzyme, as determined for immunoblots of spinach, pea and wheat, and thus in the present study they labelled only the bands corresponding to 41 kDa (spinach, pea) and 43 kDa (wheat). The use of the mixture of antibodies directed against the two HPRs of known molecular mass was necessary to determine accurately the molecular masses of the minor bands.

Role of NADPH(NADH)-hydroxypyruvate reductase in leaves

The metabolic role of a given enzyme can be seriously considered only when subcellular localization of the protein is established. There is no information on localization of the NADPH/hydroxypyruvate-dependent activity within leaf cells, and thus the function of

Fig. 6. Immunoblots of proteins from crude leaf extracts probed with a mixture of antibodies against spinach NADPH(NADH)-hydroxypyruvate reductase and spinach NADH(NADPH)-hydroxypyruvate reductase

Antibodies against NADPH(NADH)-HPR (1:50 dilution) and against NADH(NADPH)-HPR (1:100 dilution) were mixed and used to probe the transferred proteins. Other details were the same as in the legend to Fig. 5. Lanes: (a), (b), (c), crude leaf extracts of wheat, pea and spinach respectively; (d), 0.2 μ g of NADPH(NADH)-HPR; (e) 0.2 μ g of NADH(NADPH)-HPR.

NADPH(NADH)-HPR can be discussed only in purely speculative terms. The enzyme is most probably localized outside the peroxisomes; these organelles are thought to contain only one form of HPR (preferring NADH), as found by isoelectrofocusing of crude peroxisomal preparations from spinach leaves (Tolbert *et al.*, 1970). Since hydroxypyruvate is considered to be primarily, if not solely, produced and utilized within the peroxisomes (Tolbert, 1973, 1980), the role for an extra-peroxisomal HPR is not apparent. However, peroxisomal membrane does not constitute an absolute barrier for hydroxypyruvate (Liang & Huang, 1983; Anderson & Butt, 1986), and some 'overflow' of hydroxypyruvate to the cytosol might be expected, especially at times when the

glycollate pathway operates at its full rate, e.g. at high light intensity (Tolbert, 1973). It seems important to point out that the utilization of hydroxypyruvate in intact peroxisomes strongly depends on the supply of NADH, which can be provided either directly through the peroxisomal membrane, or indirectly by a malate/oxaloacetate shuttle (Schmitt & Edwards, 1983; Yu & Huang, 1986). The supply of NADH has been reported to be the actual rate-limiting step of the serine-to-glycerate conversion in peroxisomes (Yu & Huang, 1986) and may affect the utilization/leakage of hydroxypyruvate within/from these organelles.

If NADPH(NADH)-HPR were localized in the cytosol, the hydroxypyruvate leaked from peroxisomes could be immediately metabolized to glycerate and the latter converted into 3-phosphoglycerate by chloroplast glycerate kinase, the terminal enzyme of the photorespiratory cycle (Kleczkowski & Randall, 1983). Thus NADPH(NADH)-HPR could have the same function as peroxisomal NADH(NADPH)-HPR, i.e. recovering carbon skeletons originating from glycollate, and, together with glycerate kinase, restoring to the biosynthetic pathways the 'lost' carbon resulting from oxygenation of ribulose 1,5-bisphosphate, the first reaction of the glycollate pathway.

Of some importance *in vivo* might also be a glyoxylate-dependent activity of NADPH(NADH)-HPR, provided that the enzyme is localized apart from NADPH(NADH)-GR, which shows very strong affinity for glyoxylate (K_m 0.084 mM) and is the most active NADPH/glyoxylate-utilizing reductase in leaves (Kleczkowski *et al.*, 1986, 1987b). These two enzymes, perhaps together with peroxisomal NADH(NADPH)-HPR and the NADPH-specific GR described by Zelitch & Gatto (1962), can contribute to glycollate production from glyoxylate and must be considered in studies of glycollate metabolism.

Note added in proof (received 2 December 1987)

Givan *et al.* (1987) have recently reported that both NADPH(NADH)-HPR and NADPH(NADH)-GR are predominantly localized in the cytosol of pea protoplasts.

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