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

Comparison of immunological properties of leaf glyoxylate reductase and hydroxypyruvate reductase

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A novel reductase displaying high specificity for glyoxylate and NADPH was purified 3343-fold from spinach leaves. The enzyme was found to be an oligomer of about 125 kDa, composed of four equal subunits of 33 kDa each. A K_m for glyoxylate was about 14-fold lower with NADPH than with NADH (0.085 and 1.10 mM respectively), but the maximal activity, 210 $\mu\text{mol/min}$ per mg of protein, was similar with either cofactor. K_m values for NADPH and NADH were 3 and 150 μM respectively. Optimal rates with either NADPH or NADH were found in the pH range 6.5–7.4. The enzyme also showed some reactivity towards hydroxypyruvate with rates less than 2% of those observed for glyoxylate. Results of immunological studies, using antibodies prepared against either glyoxylate reductase or spinach peroxisomal hydroxypyruvate reductase, suggested substantial differences in molecular structure of the two proteins. The high rates of NADPH(NADH)-glyoxylate reductase in crude leaf extracts of spinach, wheat and soya bean (30–45 $\mu\text{mol/h}$ per mg of chlorophyll) and its strong affinity for glyoxylate suggest that the enzyme may be an important side component of photorespiration *in vivo*. In leaves of nitrogen-fixing legumes, this reductase may also be involved in ureide breakdown, utilizing the glyoxylate produced during allantoin metabolism.

INTRODUCTION

Most glyoxylate formed in leaves comes from the glycolate pathway (photorespiration) and is due to the activity of glycolate oxidase from peroxisomes (Tolbert, 1973; Schnarrenberger & Fock, 1976; Tolbert, 1980; Halliwell, 1984). The glyoxylate derived from glycolate may take part in each of the following reactions: (a) transamination to glycine in peroxisomes (Kisaki & Tolbert, 1969; Betsche, 1983; Yu *et al.*, 1984), (b) decarboxylation by H_2O_2 in peroxisomes and/or chloroplasts (Zelitch, 1971, 1972; Halliwell, 1984), and (c) re-reduction to glycolate by the non-specific reaction of peroxisomal HPR (Tolbert *et al.*, 1970; Kohn & Warren, 1970; Tolbert, 1973) and/or by the NADPH-specific GR in chloroplasts (Zelitch & Gotto, 1962; Thompson & Whittingham, 1967; Tolbert *et al.*, 1970; Zelitch, 1978; Mulligan *et al.*, 1983).

The reduction of glyoxylate to glycolate was at one time considered to be the major reaction catalysed by HPR (Zelitch, 1955). In the early literature, HPR was even referred to as GR (Zelitch, 1955; Kohn & Warren, 1970; Kohn *et al.*, 1970). The evidence that this enzyme does not function as a GR, but, instead, is rather specific for hydroxypyruvate came from studies by Tolbert *et al.* (1970) and Kohn & Warren (1970), who showed that the K_m for glyoxylate of this enzyme (approx. 20 mM) was too high to permit considerable reduction of glyoxylate by peroxisomes *in vivo*. Zelitch & Gotto (1962) had found

a new reductase, apparently specific for both glyoxylate and NADPH and showing only negligible activity with hydroxypyruvate and/or NADH. This reductase could be separated from HPR by differential precipitation with $(\text{NH}_4)_2\text{SO}_4$ and further purified by acid treatment, followed by precipitation with ethanol (Zelitch & Gotto, 1962). The NADPH- and glyoxylate-dependent activity of the spinach enzyme was localized almost exclusively in chloroplasts (Thompson & Whittingham, 1967; Tolbert *et al.*, 1970; Zelitch, 1971), whereas for pea leaves most of the activity was found in the cytosol (Kolesnikov *et al.*, 1973). The activity of chloroplast NADPH-GR was very low [3–6 $\mu\text{mol/h}$ per mg of chlorophyll (Lawyer *et al.*, 1983; Mulligan *et al.*, 1983)] and, as pointed out by Tolbert (1973, 1980), it could not account for rates of glycolate biosynthesis in leaves or cause any considerable decrease in the reducing potential (NADPH/NADP⁺) of chloroplasts in the light.

In the present study we report the identification and purification of an apparently novel isoenzyme of spinach GR, which can utilize both NADPH and NADH as cofactors. Activity of this GR in crude leaf extracts is about one order of magnitude higher than that demonstrated for chloroplast NADH-GR. Immunological properties of leaf NADPH(NADH)-GR and HPR are compared by using antibodies prepared against each of these proteins. A preliminary communication describing some of the data presented in this paper has been reported (Kleczkowski *et al.*, 1986).

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

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MATERIALS AND METHODS

Plant material

Spinach (*Spinacia oleracea* L.) leaves were purchased from a local grocery store. Seedlings of maize (*Zea mays* L.), pea (*Pisum sativum* L.), rye (*Secale cereale* L.), soya bean (*Glycine max* L.) and wheat (*Triticum aestivum* L.) were grown as previously described (Kleczkowski & Randall, 1985).

Reagents

NADH and NADPH were purchased from P-L Biochemicals. Purified spinach leaf HPR (other names: 'glycerate dehydrogenase' or 'glyoxylate reductase') was obtained from Sigma. Affinity matrices of agarose-hexane-adenosine 2',5'-bisphosphate type 2 (AG2',5'ADP-2), agarose-hexane-diadenosine pentaphosphate type 4 (AGdApP-4), agarose-hexane-AMP type 3 (AGAMP-3), agarose-hexane-ATP type 3 (AGATP-3), agarose-hexane-NAD⁺ type 3 (AGNAD-3), agarose-hexane-NAD⁺ type 3 (AGNADP-3) and agarose-hexane-NADP⁺ type 4 (AGNADP-4) were 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, 1 mM-benzamidine, 1 mM-6-aminohexanoic acid, 5 µM-leupeptin and 14 mM-2-mercaptoethanol. Sephadex G-75SF and affinity columns were equilibrated with buffer A, containing 20 mM-Mops, pH 7.1, and 14 mM-2-mercaptoethanol. All buffers were degassed before use.

Purification of spinach leaf hydroxypyruvate reductase

Commercially available HPR from spinach leaves (Sigma) was further purified by gel filtration on a Sephadex G-75SF column (2.5 cm × 68 cm) under conditions identical with those used for GR (see the Results section). HPR fraction eluted from the gel-filtration column contained an essentially homogeneous enzyme, as tested by silver staining of polyacrylamide gel after SDS/polyacrylamide-gel electrophoresis. The subunit size of HPR on SDS gels was about 41 kDa.

Isolation of crude glyoxylate reductase

Crude leaf GR from spinach and other plants was 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. For the purpose of SDS/polyacrylamide-gel electrophoresis, samples of crude extracts were kept frozen at –20 °C.

Immunological studies

Antibodies against purified spinach GR were obtained by injecting a New Zealand White male rabbit with a dose of the homogeneous GR (40 µg) mixed (1:1) with Freund's complete adjuvant (Cappel Laboratories). Injections were done subcutaneously along the spine of the rabbit. Blood was collected 3 weeks after the immunization. Antibodies against spinach leaf HPR

were prepared by injecting a second rabbit with 200 µg of the purified enzyme mixed with Freund's complete adjuvant (1:1). Blood was collected 4 weeks after the immunization. The sera containing antibodies against GR and HPR were centrifuged at 20000 g for 20 min and the supernatants were frozen at –20 °C. The serum containing antibodies against HPR was further purified by chromatography on a protein A column.

'Western' blotting was performed by a slight modification of the procedure of Towbin *et al.* (1979), as described by Kleczkowski (1985). An alkaline phosphatase-labelled goat anti-rabbit IgG (Sigma) was used as a second antibody. Specific antigen-antibody complexes on nitrocellulose were detected by using Fast Red (Sigma) as a substrate for the phosphatase.

Glyoxylate reductase assay

A 1.0 ml assay mixture contained, unless otherwise indicated, 100 mM-Mops, pH 7.1, 0.2 mM-NADPH, 2.5 mM-glyoxylate and various amounts of GR. For assays of NADH-dependent activity, NADH was kept at 0.2 mM. Reactions were initiated by addition of glyoxylate, and the oxidation of NADPH or NADH was monitored spectrophotometrically at 340 nm (25 °C). Control assays containing all the components of the reaction but glyoxylate were performed to correct for non-specific oxidation of NADPH (NADH). Before assay, crude leaf extracts were desalted on a Sephadex G-25 column. For assays of NADH-dependent activity of purified GR, the enzyme was desalted (on Sephadex G-25) to remove NADPH present after elution from the AGdApP-4 column. One unit of GR activity was defined as amount of the enzyme required to oxidize 1 µmol of NADPH/min under conditions of the assay.

Assay of hydroxypyruvate-dependent activity

The reaction mixture (1.0 ml) contained 100 mM-Mops, pH 7.1, 0.2 mM-NADH (or -NADPH), 2.5 mM-hydroxypyruvate and various amounts of enzyme. Reaction was initiated by addition of hydroxypyruvate. Other conditions were as described for the GR assay.

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 Bio-Rad.

RESULTS

Purification of glyoxylate reductase

About 350 g of spinach leaves were homogenized in a Waring blender for 20 s in the presence of 350 ml of 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 (NH₄)₂SO₄ was added to the supernatant to 49% of saturation, and the suspension was equilibrated by stirring for 30 min and centrifuged at 12000 g for 10 min. The resulting supernatant was further adjusted to 60% of saturation with solid (NH₄)₂SO₄, equilibrated for 30 min

Table 1. Summary of purification of spinach leaf NADPH(NADH)-glyoxylate reductase

Procedure	Sp. activity (units/mg of protein)	Total activity (units)	Yield (%)	Purification (fold)
Crude extract	0.063 (0.139)*	93.4	100	1
(NH ₄) ₂ SO ₄ (49–60%)	0.710 (0.723)*	80.4	86	11
AG2'5'ADP-2	23.5	58.1	62	373
Sephadex G-75SF	132.4	26.8	29	2102
AGdApP-4	210.6	26.1	28	3343

* Assay contained 25 mM-glyoxylate.

and centrifuged at 12000 *g* for 10 min. The pellet was dissolved in buffer A and concentrated/desalted with the same buffer by using a Diaflo PM10 filter (Amicon). After centrifugation to remove denatured proteins, GR was applied on to an AG2'5'ADP-2 affinity column (1.5 cm × 3.5 cm) equilibrated in buffer A. After extensive washing with buffer A, the enzyme was eluted with 0.2 mM-NADPH in the same buffer. GR was concentrated by using a Diaflo PM10 filter and applied (3 ml) on to a Sephadex G-75SF column (2.5 cm × 68 cm) operating at a flow rate of 15 ml/h. After gel filtration, GR was chromatographed on an AGdApP-4 affinity column (0.8 cm × 2.0 cm), equilibrated in buffer A, and eluted with 0.2 mM-NADPH in buffer A. The purified enzyme was relatively stable when stored with 50% glycerol and retained about 60% of its activity after 2 months at –20 °C. Results of the purification procedure of GR are summarized in Table 1.

Spinach leaf GR was purified 3343-fold with a yield of 28%. Both (NH₄)₂SO₄ fractionation and chromatography on AG2'5'ADP-2 affinity column were found to be the crucial and most effective purification steps employed. By combining these two procedures, a 370-fold purification of GR could be accomplished in less than 8 h after homogenization. The (NH₄)₂SO₄ treatment, besides being very effective as a first purification step for GR, removed about 80–90% of HPR activity (measured with NADH and hydroxypyruvate), which was precipitated at low (NH₄)₂SO₄ concentration (0–49%). Similar results were previously reported by Zelitch & Gotto (1962). HPR has been known to utilize both NADPH and glyoxylate as non-specific substrates (Tolbert *et al.*, 1970; Kohn & Warren, 1970) and theoretically could interfere with the assay for GR. Since the *K_m* of HPR for glyoxylate is very high [about 20 mM (Tolbert *et al.*, 1970; Kohn & Warren, 1970; Titus *et al.*, 1983)], these two activities could be differentiated by using high and low concentrations of glyoxylate in the GR assay employed. Although for crude extracts the activity with 25 mM-glyoxylate was about 120% higher than the rate with 2.5 mM-glyoxylate, there was virtually no difference between these activities after (NH₄)₂SO₄ treatment (Table 1). This suggested that the rate observed for crude enzyme with 25 mM-glyoxylate was a sum of at least two activities, most probably GR and HPR, which could be resolved by (NH₄)₂SO₄ fractionation.

Purity and *M_r*

The final enzyme preparation exhibited a single band on silver-stained SDS/polyacrylamide-gel electrophoresis

(Fig. 1). Purity of GR was further confirmed by Western-blot studies using antibodies raised against this enzyme (see Fig. 5). The position of GR on the SDS/polyacrylamide gel corresponded to a value of

Fig. 1. SDS/polyacrylamide-gel electrophoresis of purified NADPH(NADH)-glyoxylate reductase

GR 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) and (c), 1.5 and 3.0 µg of GR respectively; (b) 10 µg of standard proteins. Standard protein kit (Bio-Rad) contained: phosphorylase *b* (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soya-bean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

Fig. 2. Substrate kinetics of NADPH(NADH)-glyoxylate reductase

Conditions of assays were as described in the Materials and methods section.

33 kDa, while chromatography of native GR on a calibrated Sephadex G-75SF column gave a value of 125 kDa. These data suggest that GR is an oligomer composed of four equal-sized subunits each of 33 kDa.

Substrate specificity

GR was found to be highly specific for glyoxylate and NADPH as substrates. Although hydroxypyruvate could substitute as a substrate, the NADPH- or NADH-dependent rates were less than 2% of those found with glyoxylate. GR showed no activity with 2-oxobutyrate, 2-oxoglutarate, 4-methyl-2-oxopentanoate, 3-methyl-2-oxobutyrate, oxaloacetate or pyruvate. All these compounds were tested at 2.5 mM concentration with either NADPH and NADH as possible cofactors. GR could utilize both NADPH and NADH as substrates with glyoxylate; however, K_m for NADH was 50-fold higher than for NADPH (150 and 3 μ M respectively), strongly suggesting that NADPH was the preferred substrate. The K_m for glyoxylate was 0.085 and 1.1 mM with NADPH and NADH respectively, and V_{max} with NADPH was about 60% higher than with NADH (Fig. 2). However, since NADH was kept fixed at non-saturating concentration (0.2 mM), it seemed plausible to assume that V_{max} was similar for both cofactors.

pH optimum

Optimal activity of GR was found in the pH range about 6.5–7.4, regardless of whether NADPH or NADH served as a second substrate (Fig. 3). In the pH range 5.3–9.3, 2.5 mM-glyoxylate was sufficient to saturate GR when NADPH was used as a cofactor. On the other hand, when NADH served as cofactor, optimal rates at more acidic pH (below 7.0) required a higher concentration of glyoxylate; this suggested that the K_m for

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

The buffer used for assays of GR activity was 33 mM-Tricine/33 mM-Mops/33 mM-Mes. This mixture was adjusted to the appropriate pH with 10 M-NaOH. $\circ$, Δ , 2.5 mM-glyoxylate; $\bullet$, $\blacktriangle$, 25 mM-glyoxylate.

glyoxylate increased at lower pH values. A pK_a value calculated from the descending slope of the pH-activity curve was about 8.4 for both NADPH- and NADH-dependent activity, suggesting possible involvement of an ionizable imidazole group of histidine in catalysis.

Immunological comparison of leaf glyoxylate reductase and hydroxypyruvate reductase

Since both GR and HPR could utilize the same compounds as substrates (glyoxylate/hydroxypyruvate, NADPH/NADH), although with a different degree of specificity, it seemed possible that this common property might somehow reflect similarities in molecular structure of the two proteins. We tested this hypothesis by Western-blot studies, with antibodies prepared against homogeneous preparations of each of these enzymes.

When proteins from crude leaf extracts of several species were resolved by SDS/polyacrylamide-gel electrophoresis and then transferred on to nitrocellulose paper, probing with anti-HPR IgG yielded a single major protein band corresponding to the subunit size of HPR (Fig. 4, lanes b–f). Since crude leaf extracts were tested by this approach, the presence of a single band indicated high specificity of the antibodies for HPR. Immunoblots of rye and maize crude extracts (Fig. 4, lanes d and f respectively) contained also a second, much weaker, band which corresponded to a value of about 39 kDa. At present it is unclear whether this finding reflects the presence of two isoenzymes of HPR in rye and maize or is a result of non-specific immunoreaction. There was no recognition between anti-HPR IgG and purified GR run on the same gel (Fig. 4, lane a), which confirmed the results obtained for crude leaf extracts and suggested that the two reductases probably do not share common determinants in their molecular structure. The lack of cross-binding between the antibodies and preparation of purified GR indicated that the low hydroxypyruvate-dependent activity associated with the enzyme did not result from contamination by HPR.

Fig. 4. Immunodetection of leaf hydroxypyruvate reductase after SDS/polyacrylamide-gel electrophoresis

Samples (25–40 μ g) of proteins from crude leaf extracts were loaded on 12.5%-polyacrylamide gel and electrophoresed in the SDS buffer. After electrophoresis, resolved proteins were transferred on to nitrocellulose paper and incubated with the rabbit anti-spinach HPR IgG (1:100 dilution). Formation of the specific antigen-antibody complex(es) was determined as described in the Materials and methods section. Lanes: (a) 1.5 μ g of purified spinach NADPH(NADH)-GR; (b)–(f), crude leaf extracts of spinach (b), pea (c), rye (d), wheat (e) and maize (f).

The high specificity of anti-spinach HPR IgG for HPR allowed comparison of the subunit size of the enzyme from different plants without actually purifying this protein from each species. HPR from spinach and pea (dicotyledonous species) was found to have the same subunit size of 41 kDa, whereas the enzyme from wheat, rye and maize (monocotyledonous species) was slightly larger at 43–44 kDa (Fig. 4). A published (Titus *et al.*, 1983) value of 40.5 ± 1.4 kDa for HPR from cucumber (another dicot) is in fact identical with the subunit size of the spinach and pea enzyme determined in the present study. The M_r value found for spinach HPR is significantly lower than the previously reported estimate of the subunit size of commercially available spinach HPR [46.5 kDa (Kohn *et al.*, 1970)]. This apparent discrepancy could possibly be due to different methods of determination of subunit size of HPR employed in both studies. The possibility that HPR used in the present study is a degraded form of a larger- M_r protein seems rather unlikely, since Western blots of both purified spinach HPR and crude extracts of different species consistently yielded values significantly lower than 46.5 kDa (Fig. 4).

When proteins from crude leaf extract of spinach were separated by SDS/polyacrylamide-gel electrophoresis, antibodies prepared against GR recognized both GR and HPR bands on the immunoblot (Fig. 5, lane a). On the other hand, immunoblotting of purified GR yielded only a single band corresponding to the position of this protein on polyacrylamide gel (Fig. 5, lane b). Similar

Fig. 5. Immunodetection of spinach leaf NADPH(NADH)-glyoxylate reductase after SDS/polyacrylamide-gel electrophoresis

The rabbit anti-spinach GR IgG (1:25 dilution) was used as a probe. Other details were the same as in the legend to Fig. 4. Lanes: (a) 60 μ g of spinach leaf proteins; (b) 0.5 μ g of purified spinach NADPH(NADH)-GR; (c) 15 μ g of purified spinach HPR.

data were obtained for purified HPR (Fig. 5, lane c). Taken together, these results confirmed the purity of GR used as an antigen and indicated that both GR and HPR did in fact share some common determinants in their molecular structure. It seems likely that, for HPR used to immunize a rabbit, these common sites were buried inside the quaternary and/or tertiary structure of the antigen, and were not accessible to the immunogenic apparatus of the animal. It is important to point out that the relative amount of leaf HPR *in vivo* is much larger than that of GR and, at least for spinach, in order to obtain a homogeneous enzyme GR had to be purified more than 3300-fold (Table 1) compared with about 500-fold purification required for HPR from cucumber (Titus *et al.*, 1983) and about 200-fold for the tobacco enzyme (Zelitch, 1955). This indicates that a very weak band found for HPR when crude spinach proteins were probed with anti-GR IgG (Fig. 5, lane a) could even be less detectable had the amount of leaf HPR *in vivo* been comparable with that of GR.

Contribution of NADPH(NADH)-glyoxylate reductase to the overall NADPH- and glyoxylate-dependent activity in leaves

Crude extracts of spinach, wheat and soya bean were found to contain a considerable NADPH- and glyoxylate-dependent activity, ranging from 30 to 45 μ mol/h per mg of chlorophyll (45–70 nmol/min per mg of protein). These rates were about one order of magnitude higher than the activity reported for chloroplast NADPH-GR (Lawyer *et al.*, 1983; Mulligan *et al.*, 1983), which has been considered to be the only isoenzyme of GR in leaves (Thompson & Whittingham, 1967; Tolbert, 1973). It

should be pointed out that comparably high activity of crude NADPH-dependent GR (expressed on a protein basis) were previously reported for leaf extracts of both spinach and tobacco (Zelitch & Gotto, 1962; Zelitch, 1978). A likely explanation of the high rate of NADPH- and glyoxylate-dependent reductase in leaf extracts might have been that most of this activity belonged, in fact, to peroxisomal HPR, which has been known to utilize both NADPH and glyoxylate as non-specific substrates (Tolbert *et al.*, 1970; Kohn & Warren, 1970). On the other hand, at least for spinach, HPR could be almost completely precipitated by a low concentration of $(\text{NH}_4)_2\text{SO}_4$, while in excess of 86% of total activity of GR still remained soluble (Table 1). These data strongly indicated that the assay system employed for GR, containing NADPH and low concentration of glyoxylate (2.5 mM), could accurately measure rates of GR in crude extracts, with negligible interference on the part of non-specific NADPH/glyoxylate-dependent activity of HPR. One the other hand, our assay system was similar to that used by Zelitch & Gotto (1962), who partially purified spinach NADPH-specific GR which showed only negligible activity with NADH and had a pH optimum of about 6.5. The question which remained to be answered was which of these apparently distinct isoenzymes of GR was the prevalent form in leaves.

In an attempt to differentiate between NADPH-specific activity described by Zelitch & Gotto (1962) and the NADPH(NADH)-dependent GR purified in the present study, crude spinach GR was precipitated by 45–60% -satd. $(\text{NH}_4)_2\text{SO}_4$, concentrated/desalted by using a Diaflo PM10 filter and applied on to a set of affinity-chromatography columns (Table 2). Since $(\text{NH}_4)_2\text{SO}_4$ fractionation resulted in a high yield of the NADPH- and glyoxylate-dependent activity (see Table 1), we have assumed that, at this stage of purification, the ratio of activities of both of these isoenzymes should rather closely represent their relative magnitude in crude extracts. Another assumption was that the use of highly specific affinity matrixes would allow separation of both

activities. After application of GR on to affinity-chromatography columns and washing with buffer A, the eluates were assayed for both NADPH- and NADH-dependent activities of GR. Results of this experiment, presented in Table 2, indicated that both activities were closely associated one with another and their ratio rather accurately reflected properties of GR purified in the present study (see Fig. 3). None of the affinity-chromatography steps resulted in significant enrichment of the NADPH-specific activity, which would be expected were the NADPH-specific GR a primary form of GR in leaves. We consider that this evidence strongly suggests that the enzyme purified in the present study is by far the most active form of GR in spinach.

DISCUSSION

During studies with crude or partially purified leaf preparations, it has usually been assumed that the NADH-dependent rates with glyoxylate are the result of non-specific activity of peroxisomal HPR, whereas the glyoxylate- and NADPH-dependent activity has been attributed to NADPH-specific GR from chloroplasts. Only rarely has the possibility of other enzymic activities utilizing the same compounds as substrates been considered (Murray *et al.*, 1973; Betsche, 1981). Data in the present paper indicate that leaves do contain considerable activities of an apparently novel isoenzyme of GR which can utilize either NADPH and NADH as a cofactor and shows some reactivity towards hydroxypyruvate.

The enzyme purified in the present study is the most active isoenzyme of GR in leaves. Since most, if not all, of the NADPH-dependent activity of GR has usually been attributed to chloroplasts (Thompson & Whittingham, 1967; Tolbert *et al.*, 1970; Mulligan *et al.*, 1983), it seems likely that the enzyme could be localized in these organelles. On the other hand, there is a considerable discrepancy between the rates reported for chloroplast NADPH-GR [3–6 $\mu\text{mol/h}$ per mg of chlorophyll (Lawyer *et al.*, 1983; Mulligan *et al.*, 1983)] and the activity of crude leaf GR determined in the present study (30–45 $\mu\text{mol/h}$ per mg of chlorophyll), suggesting that perhaps a significant portion of activity of GR is localized outside chloroplasts. Because of high affinity for glyoxylate (K_m of 0.085 mM), GR could be a very efficient coupling step for any reactions producing glyoxylate. To our knowledge, leaves contain no other enzyme which would be as specific for glyoxylate as GR and at the same time show comparable rates in crude extracts. Since most glyoxylate in leaves comes from glycolate metabolism (Tolbert, 1973; Schnarrenberger & Fock, 1976; Tolbert, 1980; Halliwell, 1984), it seems very likely that NADPH(NADH)-GR might be an important side component of the glycolate pathway, possibly participating in the glycolate/glyoxylate shuttle between chloroplasts and peroxisomes. If GR were localized in the chloroplasts, a shuttle transport of glycolate could provide means to export and oxidize some excess of NADPH produced by these organelles under photorespiratory conditions (Kisaki & Tolbert, 1969; Zelitch, 1971; Schnarrenberger & Fock, 1976). The shuttle would result in no net glycolate synthesis or CO_2 formation, and there would be no net O_2 exchange between glycolate oxidation and photolysis of water. Presence of a considerable activity of NADPH(NADH)-GR in

Table 2. Resolution of NADPH- and NADH-dependent activities of spinach leaf glyoxylate reductase by affinity chromatography

For this experiment, crude leaf GR was partially purified as the 45–60% -satd. $(\text{NH}_4)_2\text{SO}_4$ fraction and then concentrated/desalted with buffer A by using a Diaflo PM10 filter (Amicon). The protein mixture loaded on to each affinity column contained about 490 nmol of NADPH-dependent activity/min and 250 nmol of NADH-dependent GR activity/min. Each column was washed with buffer A and the eluates were assayed for GR activity.

Affinity matrix	Activity (nmol of glyoxylate reduced/min)	
	NADPH-dependent	NADH-dependent
AG2',5'-ADP-2	0	0
AGAMP-3	420	233
AGATP-3	80	35
AGdApP-4	0	0
AGNAD-3	440	185
AGNADP-3	0	0
AGNADP-4	35	19

chloroplasts could also explain an inhibitory effect of glyoxylate on photosynthesis by isolated chloroplasts, as reported by several investigators (Zelitch, 1978; Lawyer *et al.*, 1983; Mulligan *et al.*, 1983). An NADPH-dependent metabolism of glyoxylate in the stroma could decrease the reducing potential (NADPH/NADP⁺) of chloroplasts and, consequently, lead to a fall in the rate of photosynthesis. Unfortunately, the activity *in vitro* of chloroplast GR measured in such experiments was too low to account for the magnitude of glyoxylate-dependent inhibition of photosynthesis observed for isolated chloroplasts (Tolbert *et al.*, 1970; Schnarrenberger & Fock, 1976; Mulligan *et al.*, 1983). Besides its supposed role in the glycolate/glyoxylate shuttle, in some nitrogen-fixing legumes (e.g. soya bean) GR may also be coupled to ureide catabolism in leaves, utilizing glyoxylate produced during allantoin breakdown (Winkler *et al.*, 1985) and directing carbon atoms transported from nodules back to general carbon metabolism via the glycolate pathway.

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