Biochem. J. (1991) 276, 125–127 (Printed in Great Britain)



# Oxalate as a potent and selective inhibitor of spinach (*Spinacia oleracea*) leaf NADPH-dependent hydroxypyruvate reductase

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Purified spinach (*Spinacia oleracea*) NADPH-preferring hydroxypyruvate reductase (HPR-2) was potently and selectively inhibited by oxalate, an end product of metabolism in plants. Both hydroxypyruvate- and glyoxylate-dependent rates of the HPR-2 enzyme were affected. Oxalate acted as an uncompetitive inhibitor of the enzyme, with $K_i$ values of 7 and 36 $\mu$M for the NADPH/hydroxypyruvate and NADPH/glyoxylate pairs of reactants respectively. Oxalate, at millimolar levels, caused less than 10% inhibition of purified spinach NADH-preferring HPR (HPR-1) and had no effect on purified spinach NADPH-preferring glyoxylate-specific reductase (GR-1). The inhibition of spinach HPR-2 by oxalate is by far the strongest for any known inhibitor of leaf HPR and GR activities. In photosynthetic tissues, oxalate could potentially act as a primary regulator of extraperoxisomal metabolism of hydroxypyruvate and glyoxylate.

## INTRODUCTION

In leaves, the reduction of hydroxypyruvate and/or glyoxylate is catalysed by at least three distinct enzymes: NADH-preferring hydroxypyruvate reductase (HPR-1), NADPH-preferring hydroxypyruvate reductase (HPR-2) and NADPH-preferring glyoxylate reductase (GR-1). Both HPR-1 and HPR-2 can use either hydroxypyruvate or glyoxylate as substrates [1–3], whereas GR-1 is specific for glyoxylate [4]. Leaf HPR-1 is localized exclusively in the peroxisomes [2,5], whereas HPR-2 and GR-1 are predominantly located in the cytosol [6,7].

Studies on HPR-2 and GR-1 activities in leaf extracts and partially purified preparations can be hampered by a non-specific NADPH- and/or glyoxylate-dependent rate of the HPR-1 enzyme [8]. For barley (*Hordeum vulgare*) plants, this potential problem has been overcome by the isolation of a mutant lacking the HPR-1 protein [9]. The mutant has proved to be of value in the quantification of non-specific contributions of HPR-1 to the NADPH-dependent HPR (NADPH-HPR) and NAD(P)H-dependent GR [NAD(P)H-GR] rates in wild-type plants, and it allowed a fairly unrestricted study of HPR-2 activities [10]. Another, more feasible, approach to distinguish between the reductases is to use inhibitors specific for a given enzyme [10].

In the present study we have shown that purified spinach HPR-2 is potently and selectively inhibited by oxalate, a well known end product of carbon metabolism [11,12]. A possible regulatory role for oxalate in the hydroxypyruvate and glyoxylate metabolism in leaves is discussed.

## MATERIALS AND METHODS

### Purified enzymes and reagents

Spinach leaf HPR-2 and GR-1 were purified as previously described [3,4]. The enzymes were stored in 50% (v/v) glycerol at −80 °C. Purified spinach HPR-1 (commercial names 'glyoxylate reductase' or 'glycerate dehydrogenase') and oxalate (ammonium salt) were from Sigma. Only enzymically reduced NADPH from Sigma (catalogue no. N-6505) was used in assays of NADPH-HPR and NADPH-GR activities.

### Enzymic assays and other methods

Enzymes were assayed spectrophotometrically at 340 nm (21 °C) in a 1.0 ml volume. Reaction mixtures contain 100 mM-Mops, pH 7.1, 0.2 mM-NAD(P)H and, unless otherwise indicated, 0.5 mM-hydroxypyruvate (HPR assay) or 1 mM-glyoxylate (GR assay). For assays of the oxidation of glyoxylate, 1.0 mM-$NADP^+$ or 0.5 mM-$NAD^+$ was substituted as a cofactor, and glyoxylate was kept at 5 mM. All assays were started with enzyme. Control assays containing all the components of the reaction except hydroxypyruvate or glyoxylate were run to correct for non-specific oxidation of NAD(P)H. A unit of activity was defined as the amount of enzyme required to oxidize 1 $\mu$mol of NAD(P)H/min.

Determination of protein was performed by using the Bio-Rad Protein Assay.

## RESULTS

Purified spinach HPR-2 was found to be potently inhibited by oxalate (Table 1). Both NADPH and NADH-dependent activities with hydroxypyruvate and glyoxylate were affected. Oxalate at 2 mM caused over 90% inhibition of NAD(P)H-HPR and NADPH-GR reactions and 72% inhibition of the NADH-GR activity. Tartronate or phosphohydroxypyruvate, compounds previously reported to inhibit barley HPR-2 [10], were less effective than oxalate, causing 30–55 and 44–71% inhibition of spinach HPR-2 activities respectively (Table 1).

More details on the nature of oxalate inhibition of spinach HPR-2 were obtained from kinetic studies of the NADPH-HPR and NADPH-GR reactions by using Dixon plots (Fig. 1). With varying hydroxypyruvate or glyoxylate, the inhibition patterns appeared parallel, indicating that oxalate binds to the enzyme–substrate form of HPR-2 rather than to the free enzyme

Abbreviations used: GR, glyoxylate reductase; GR-1, NADPH-preferring GR; HPR, hydroxypyruvate reductase; HPR-1, NADH-preferring HPR; HPR-2, NADPH-preferring HPR.

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**Table 1. Effect of tartronate (TA), phosphohydroxypyruvate (PHA) and oxalate (OA) on the activities of purified spinach leaf HPR-2**

The inhibitor concentration was 2 mM in each case. Values in parentheses are 'uninhibited' activities (%).

| Reaction | Activity (units/mg of protein) | | | |
|---|---|---|---|---|
| | Control | +TA | +PHA | +OA |
| NADPH-HPR | 260 (100) | 116 (45) | 75 (29) | 5 (2) |
| NADH-HPR | 86 (100) | 47 (55) | 27 (31) | 6 (7) |
| NADPH-GR | 103 (100) | 72 (70) | 58 (56) | 9 (9) |
| NADH-GR | 18 (100) | 9 (50) | 7 (39) | 5 (28) |

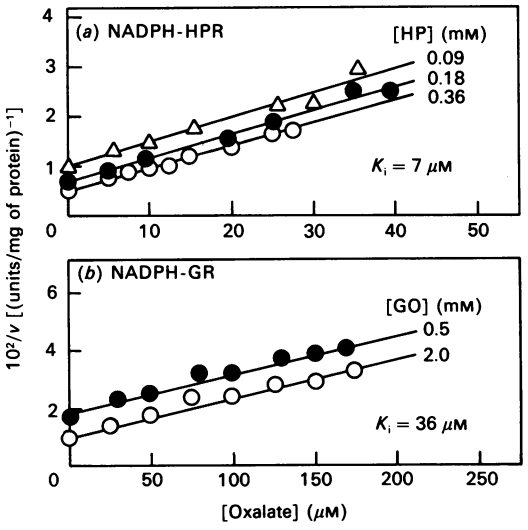


**Fig. 1. Kinetics of the oxalate inhibition of NADPH-HPR and NADPH-GR reactions of purified HPR-2 from spinach leaves**

(*a*) NADPH-HPR; (*b*) NADPH-GR. The indicated true $K_i$ values [13] were calculated from plots of apparent $K_i$ versus reciprocal of hydroxypyruvate (HP) or glyoxylate (GO) concentration.

[13]. For the NADPH-HPR and NADPH-GR reactions, the true $K_i$ values [13] for oxalate, calculated from plots of apparent $K_i$ versus reciprocal of hydroxypyruvate or glyoxylate concentration, were 7 and 36 μM respectively (Fig. 1).

Oxalate (at 2 mM) had no effect on the activity of purified spinach GR-1 and caused less than 10% inhibition of purified spinach HPR-1. About 50% inhibition of HPR-1 was observed at 40 mM-oxalate (results not shown), which should be compared with the inhibition constants for HPR-2 (Fig. 1), which are of the order of micromolar.

## DISCUSSION

Oxalate is known to inhibit several enzymes of glyoxylate metabolism in plants, e.g. glycolate oxidase [14], lactate dehydrogenase [15] or malate synthase [16]. However, inhibition constants with oxalate reported for these enzymes were at least two orders of magnitude higher than the $K_i$ values found for HPR-2 in the present study (Fig. 1). Enzymes from animal tissues, where oxalate has been proposed [17] to serve as an important effector of glycolysis and gluconeogenesis, appear to be more susceptible to oxalate inhibition. At its known physiological concentration in animals of 10–100 μM, oxalate has been demonstrated to inhibit glycolate oxidase and lactate dehydrogenase as well as pyruvate kinase, 'malic' enzyme and pyruvate carboxylase (cited in [18]). In plant tissues, the concentration of soluble oxalate is unknown, but it seems likely that its levels there could be comparable with those in animals, especially since, in some plants, oxalate (in a crystal form) is the most abundant component on a dry-weight basis [11,12,19].

With its very low $K_i$ value of few micromoles (Fig. 1), oxalate can potentially function as a primary regulator of HPR-2 activity *in vivo*. Since HPR-2 is localized in the cytosol and, perhaps, in the chloroplasts [6], oxalate may likely regulate the entire extraperoxisomal metabolism of hydroxypyruvate (and, to some extent, glyoxylate) in leaves. However, the significance of such a regulatory mechanism is obscure at present. One possibility is that HPR-2 is involved in an as yet unknown metabolic route for oxalate biosynthesis, with oxalate serving as an end-product inhibitor. Perhaps, under some conditions, HPR-2 itself forms oxalate by oxidation of glyoxylate, but this would be very unusual for an HPR enzyme. It is generally believed that oxalate can be formed from glyoxylate by lactate dehydrogenase, but not by an HPR enzyme activity.We have failed to observe any reduction of $NADP^+$ or $NAD^+$ with glyoxylate for purified spinach HPR-2 (results not shown), which could have been indicative of oxalate production [15]. In addition, purified spinach HPR-2 showed no reactivity with pyruvate [3] and thus cannot be classified as lactate dehydrogenase. Another possibility is that, *in vivo*, the inhibition of HPR-2 by oxalate is 'relieved' by some an as yet unknown mechanism, and the enzyme can function effectively even at a potentially inhibitory oxalate concentration. In a barley mutant lacking the peroxisomal HPR-1 enzyme, HPR-2 does function in the reduction of hydroxypyruvate during photorespiration [9], although at lower rates than expected (on the basis of assays *in vitro* [10]), and thus the potential inhibition by oxalate has to be controlled by some means.

The potent and selective inhibition of spinach HPR-2 by oxalate should be of value for future studies on hydroxypyruvate and glyoxylate metabolism in leaves. Oxalate can be used in assays of HPR-1, HPR-2 and GR-1 activities to quantify the contribution of HPR-2 to a given NAD(P)H-dependent rate with hydroxypyruvate or glyoxylate. Together with acetohydroxamate, which selectively inhibits GR-1 activities [20], these compounds may serve as practical means to discriminate between the three reductases in crude extracts or partially purified preparations.

This research was supported in part by National Science Foundation grants DMB-8506473 (to D.D.R.) and DCB-8816322 (to G.E.E.). This is journal report no. 11298 from the Missouri State Agricultural Experiment Station.

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Received 8 October 1990/2 November 1990; accepted 18 December 1990