Kinetics and Regulation of the NAD(P)H-Dependent Glyoxylate-Specific Reductase from Spinach Leaves

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Kinetic mechanism of purified spinach leaf NAD(P)H glyoxylate reductase (GR-1) was studied using either NADPH and NADH as alternative substrates with glyoxylate. The mechanism was elucidated from substrate kinetic patterns using NADH as a cofactor rather than NADPH. With NADPH varied *versus* glyoxylate, and with NADPH and glyoxylate varied at a constant ratio, the patterns obtained on double reciprocal plots appeared to be consistent with a ping-pong mechanism; however, kinetic patterns with NADH conclusively ruled out the ping-pong reaction in favour of the sequential addition of the reactants. Product inhibition studies with glycolate and NADP have suggested either that NADPH binds to the enzyme before glyoxylate or that the addition of substrates is a random one. Studies with active group modifiers suggested an involvement of histidine, serine and cysteine residues in GR-1 activity. Salts had little or no effect on the activity of the enzyme, with the exception of cyanide, which had an apparent K_i of *ca.* 2 mM. Studies with several metabolites used as possible effectors of GR-1 activity have suggested that the enzyme is modulated only by substrate availability *in vivo*. The apparent insensitivity of GR-1 to metabolic effectors is consistent with the proposed role of the enzyme in detoxifying glyoxylate which may act as a potent inhibitor of photosynthetic processes in plant tissues.

Introduction

In leaves of higher plants there are at least three reductases which can utilize glyoxylate as a substrate. The cytosolic NADPH-preferring glyoxylate reductase (GR-1) is specific for glyoxylate, whereas two other reductases (peroxisomal HPR-1 and cytosolic HPR-2) utilize glyoxylate less efficiently than hydroxypyruvate as a physiological substrate (Kleczkowski *et al.*, 1986, 1990; Givan and Kleczkowski, 1992). The reductases are believed to be closely involved in the glycolate pathway (oxidative photosynthetic carbon cycle), which results in photorespiration. The pathway, which starts with phosphoglycolate formation by ribulose-1,5-bisphosphate oxygenase, involves several cell compartments and is the main route for metabolism of two-carbon and some three-carbon compounds and for nitrogen cycling during photosynthesis (Tolbert *et al.*, 1970; Husic *et al.*, 1987; Givan *et al.*, 1988).

GR-1 is the primary form of a glyoxylate-reducing activity in leaf extracts of many plants, and is characterized by its low K_m (glyoxylate) of less than 0.1 mM (Kleczkowski *et al.*, 1986, 1988, 1990). The GR-1 enzyme appears similar in many respects to a spinach glyoxylate reductase, partially purified over 30 years ago and described as being specific for NADPH as a cofactor (Zelitch and Gotto, 1962). In our previous study (Kleczkowski *et al.*, 1986), using several affinity chromatography columns, we have failed to detect any NADPH-specific glyoxylate reductase in spinach. The GR-1 is also similar to a reductase in *Chlamydomonas reinhardtii*, which preferentially uses NADPH as a cofactor and does not react with hydroxypyruvate (Husic and Tolbert, 1987).

Nothing is known about the kinetic mechanism of the GR-1 reaction. This is in contrast to several reports on kinetics and regulation of HPR-1, which can react non-specifically with glyoxylate, and which has been shown to perform a sequential mechanism (Kohn and Warren, 1970), consistent with both substrates binding to the enzyme before release of any product. Both GR-1 and HPR-1 are

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recognized by antibodies raised against purified GR-1 (although with a different degree of specificity), suggesting common epitopes for the two proteins (Kleczkowski *et al.*, 1986). Surprisingly, preliminary studies on GR-1, using NADPH and glyoxylate as substrates, suggested a ping-pong kinetic mechanism (see present paper), calling for a release of a product before both substrates are bound to the enzyme. This apparently fundamental difference in kinetic mechanism of two closely related enzymes provided stimulus for a more detailed kinetic analysis of GR-1. In the present paper, kinetic properties of spinach GR-1 are evaluated with respect to the kinetic mechanism of the enzyme and to its possible metabolic regulation *in vivo*.

Material and Methods

Reagents

Spinach (*Spinacia oleracea* L.) leaf GR-1 was purified as described in (Kleczkowski *et al.*, 1986). The enzyme was homogeneous, as determined by SDS-electrophoresis of purified GR-1, and by immunoblots of the purified protein using anti-spinach-GR-1 IgG raised in a rabbit (Kleczkowski *et al.*, 1986). Cofactors were from P-L Biochemicals.

Enzyme assay

A 1.0 ml assay mixture contained 100 mM 3-(N-morpholino)propanesulfonic acid (Mops) (pH 7.1) and appropriate concentrations of NADPH (or NADH) and glyoxylate, as indicated in legends to Figures and Tables. Reactions were initiated by the addition of glyoxylate, and the oxidation of NAD(P)H 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 NAD(P)H. For assays of NADH-dependent activity of GR-1, the enzyme was desalted on a small Sephadex G-25 column to remove NADPH present after affinity purification of the enzyme (Kleczkowski *et al.*, 1986). One unit of GR-1 activity was defined as amount of the enzyme required to oxidize one mole NAD(P)H per min under assay conditions.

Kinetic studies

The K_m values for substrates of GR-1 were calculated from replots of v^{-1} intercepts of the double reciprocal plots obtained during substrate kinetics studies (Segel, 1975). For product inhibition studies, assays were carried out by varying either NADPH or glyoxylate at a fixed level of a second substrate at several fixed concentrations of either NADP or glycolate. The K_{ii} and K_{is} values were determined as described by (Segel, 1975).

Results and Discussion

Substrate kinetics

Substrate kinetics of GR-1 were initially carried out by determination of the rate of NADPH oxidation in the reaction mixtures containing varying concentrations of glyoxylate or NADPH, with the other substrate kept at several fixed concentrations. The data were represented as double reciprocal plots. With either NADPH or glyoxylate as a varied substrate, the lines drawn through experimental points appeared to be parallel (Fig. 1). In each case, replots of v^{-1} intercepts *versus* re-
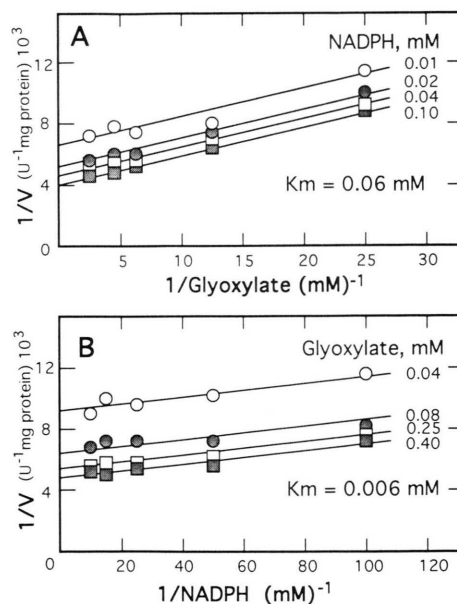

Fig. 1. Substrate kinetics of spinach GR-1 with glyoxylate and NADPH. **(A)** glyoxylate varied (0.04 - 0.4 mM) at indicated fixed concentrations of NADPH. **(B)** NADPH varied (0.01 - 0.1 mM) at indicated fixed concentrations of glyoxylate.

ciprocal of either glyoxylate or NADPH concentration were linear (data not shown). The K_m values for glyoxylate and NADPH determined from these plots were 59 and 6 μM, respectively. Varying NADPH and glyoxylate in a constant ratio *versus* activity of GR-1 (Fig. 2) resulted in a straight line; however, at high concentration of
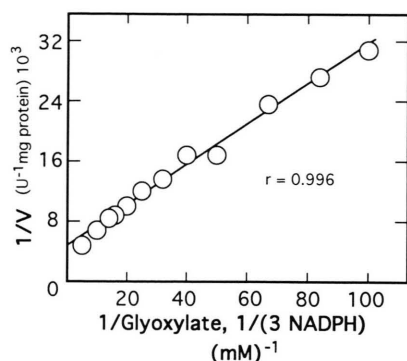

Fig. 2. Substrate kinetics of spinach GR-1 using a constant ratio of substrates of the enzyme. Assay concentrations of glyoxylate and NADPH were kept at a 3:1 ratio. The ranges of concentrations of glyoxylate and NADPH were 0.01 - 0.2 mM and 0.0033 - 0.067 mM, respectively.
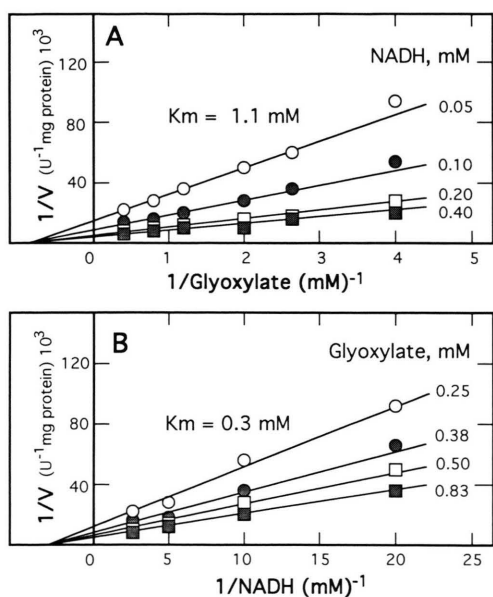
both substrates a slight curvature of the plot appeared.

Substrate kinetics of GR-1 were also studied using NADH rather than NADPH as a cofactor. When either glyoxylate or NADH were varied at several fixed concentrations of the second substrate, the lines of the double reciprocal plots converged to the left of the ordinate axis (Fig. 3). This was in contrast to kinetics of GR-1 with NADPH and glyoxylate, where the plots were parallel (Fig. 1). Replots of v^{-1} intercepts and of slopes were linear for either of the substrates of GR-1 (data not shown). The K_m values for glyoxylate and NADH were 1.1 and 0.3 mM, respectively.

Product inhibition

The double reciprocal plots were also used to represent the data obtained during product inhibition studies with glycolate and NADP (Figs. 4 and 5). NADP was a clear competitive inhibitor *versus* NADPH, while other product inhibition patterns appeared to be uncompetitive or mixed. The competitive pattern has indicated that both

Fig. 3. Substrate kinetics of spinach GR-1 with glyoxylate and NADH. **(A)** glyoxylate varied (0.25 - 2.5 mM) at indicated fixed concentrations of NADH. **(B)** NADH varied (0.05 - 0.4 mM) at indicated fixed concentrations of glyoxylate.
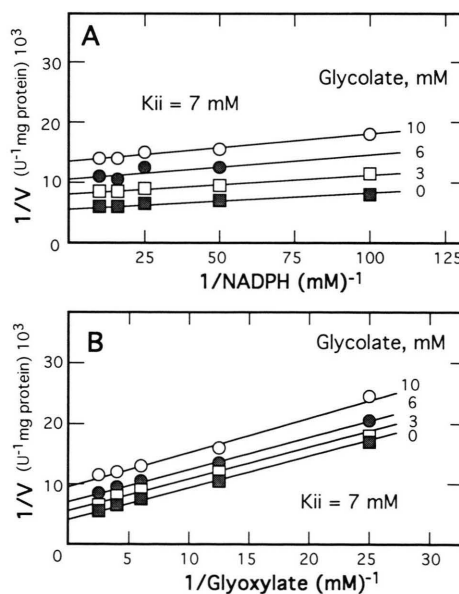

Fig. 4. Kinetics of glycolate inhibition of spinach GR-1. **(A)** NADPH varied (0.01 - 0.1 mM) at indicated fixed concentrations of glycolate. Glyoxylate was fixed at 1 mM. **(B)** Glyoxylate varied (0.04 - 0.4 mM) at indicated fixed concentrations of glycolate. NADPH was fixed at 0.2 mM.
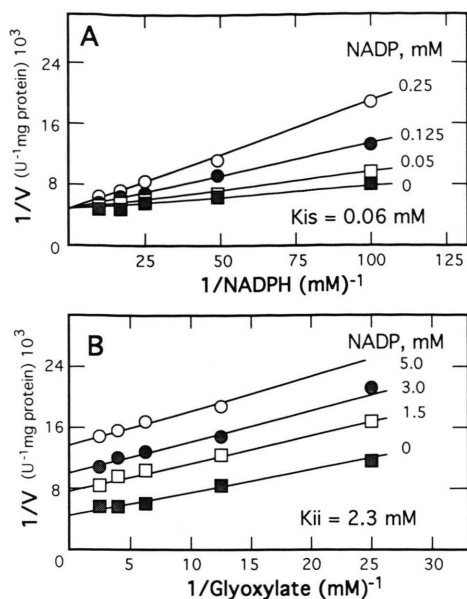
Fig. 5. Kinetics of NADP inhibition of spinach GR-1. **(A)** NADPH varied (0.01 - 0.10 mM) at indicated fixed concentrations of NADP. Glyoxylate was fixed at 1 mM. **(B)** Glyoxylate varied (0.04 - 0.4 mM) at indicated fixed concentrations of NADP. NADPH was fixed at 0.2 mM.

NADPH and NADP bind to the same form of the enzyme. Replots of slopes (NADP inhibition *versus* NADPH) and v^{-1} intercepts (all other product inhibition patterns) gave straight lines (data not shown), allowing for calculation of K_{is} (determined from slopes) and K_{ii} (determined from interecepts) inhibition constants (Segel 1975). NADP was a much stronger inhibitor than glycolate, as reflected by lower K_i values, regardless of the varied substrate (Figs. 4 and 5).

Kinetic mechanism

When kinetics of GR-1 were analyzed by varying either NADPH or glyoxylate concentrations, a parallel set of lines was obtained by double reciprocal plots of activity *versus* substrate concentration (Fig. 1). The parallel patterns obtained during substrate kinetics studies are routinely assumed to represent a ping-pong kinetic mechanism, which calls for a release of a product before all substrates are bound to the enzyme (Segel, 1975). The ping pong mechanism may also be indicated by an apparently straight line on double reciprocal plots where concentration of substrates,

kept at a constant ratio, is plotted against the determined enzymatic activity (Fig. 2). Double reciprocal plots of GR-1 activity with NADH and glyoxylate (Fig. 3), however, indicated that a change in the concentration of any fixed substrate alters both the intercepts and slopes of the lines drawn through experimental points. This pattern is consistent only with a sequential kinetic mechanism (Segel, 1975), *i.e.* that both glyoxylate and NADH have to combine with the enzyme before any product can be released. This apparent discrepancy between kinetic patterns obtained with alternative substrates of GR-1 can be rationalized by considering the theoretical basis for an alternative substrate protocol for segregating between sequential and ping-pong mechanisms, as presented by Webb *et al.* (1976). Using the nomenclature of Cleland (1963), the most general form of the rate equation for bireactant mechanisms is:

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB), \quad \text{(a)}$$

where A and B are substrate concentrations; v, initial velocity; V, maximal velocity; K_a, K_b and K_{ia}, kinetic constants. Eqn. (a) can be expressed in a reciprocal form in terms of ϕs, as defined by Dalziel (1957):

$$1/v = \phi_o + \phi_A/A + \phi_B/B + \phi_{AB}/AB. \quad \text{(b)}$$

The ϕ_{AB}/AB term is characteristic for a sequential mechanism, and imparts the convergence of the substrate kinetic patterns on double reciprocal plots. This term is missing from the equation for a ping-pong mechanism and thus a set of parallel lines is obtained rather than a convergent pattern. However, for some enzymes exhibiting a sequential mechanism, the ϕ_{AB}/AB term is small relative to the other terms in the second equation, and the lines may appear so nearly parallel that it is difficult to distinguish between the two alternative kinetic mechanism. This obstacle may be largely overcome by using an alternative substrate, if possible. The rationale is that in a ping-pong mechanism the slope equals ϕ_A or ϕ_B and is independent of the nature of the other substrate. For a sequential mechanism, the slope is ($\phi_A + \phi_{AB}/B$) or ($\phi_B + \phi_{AB}/A$), which is a function of the second substrate, and rarely will the dependence be negligible for an alternative substrate (Rudolph and Fromm, 1983; Purich, 1983).

The ϕ_{AB} constant is also critical for the determination of a kinetic mechanism by means of varying both substrates at a constant ratio (Fig. 2). The rationale is that if substrates A and B are kept at a constant ratio, then A = a(B), where a is a constant. Substitution of this relationship into Eqn. (b) for a sequential mechanism gives:

$$1/v = \phi_o + \phi_A/A + a\phi_B/A + a\phi_{AB}/(A)^2. \quad (c)$$

When $1/v$ is plotted versus $1/A$, the substrate squared term makes the plot nonlinear. The term $a\phi_{AB}/(A)^2$ is missing in the equation for a ping-pong mechanism and the plot should be linear. However, for a sequential mechanism, if ϕ_{AB} is very small compared to other terms in Eqn. (c), the equation will reduce to that characteristic of a ping pong reaction and the curvature will not be apparent (Rudolph and Fromm, 1983; Purich, 1983). Thus, results of both Figs. 1 and 2 are not necessarily in conflict with the data in Fig. 3, and they can be reconciled assuming that the ϕ_{AB} constant is small compared to other terms in Eqns. (b) and (c). In fact, the small curvature observed at high concentration of both substrates (Fig. 2) does suggest a nonlinear response, even though overall data fit well ($r = 0.996$) to a straight line.

Mammalian hexokinase and *Escherichia coli* acetate kinase were once considered to exhibit the properties of a ping-pong mechanism, because parallel patterns were observed on double reciprocal plots when D-glucose and Mg-ATP (hexokinase) and acetate phosphate and Mg-ADP (acetate kinase) served as substrates (Purich *et al.*, 1973). However, with D-fructose (hexokinase) and propionyl phosphate (acetate kinase) the patterns were convergent, which is consistent solely with the sequential mechanism (Webb *et al.*, 1976; Purich *et al.*, 1973). As pointed out by Webb *et al.* (1976), the principal advantage of an alternative substrate protocol is that the mechanism should always be the same for closely related substrates. Depending on the substrate employed, the slope effect (characteristic of the sequential mechanism) may be as apparent as that observed with NADH for spinach GR-1 (Fig. 3) or the results might be ambiguous, as with NADPH as a cofactor (Fig. 1). Based on substrate kinetics with NADH, our data conclusively ruled out the ping-pong system as a possible mechanism for spinach leaf GR-1.

Assuming that the sequential addition of substrates is the only viable mechanism for GR-1, the kinetic patterns obtained during product inhibition studies (Figs. 4 and 5) are consitent either with NADPH binding as the first substrate to a free form of the enzyme or with a random addition of both substrates. In the latter case, formation of abortive enzyme-substrate-product complexes (Segel, 1975) is necessary to account for three uncompetitive (or mixed) product inhibition patterns determined in the present study (Figs. 4 and 5). More studies are required, involving dead-end inhibitors and ligand-binding approaches, to further distinguish between the ordered and random mechanisms proposed for spinach GR-1.

Studies with amino acid reagents

Results obtained with functional group inhibitors (Table I) have suggested that neither arginine, lysine nor tyrosine participate in substrate binding or catalysis. Inhibition by diethylcarbonate, PMSF and thiol reagents implied the existence of histidine, serine and cysteine residues at, or nearby, the active site of the enzyme, although the inhibitory effects were seen at rather high concentrations of the reagents. As the enzyme had not been dialysed after final purification step (elution from an affinity column with NADPH (Kleczkowski *et al.*, 1986)), a possibility can not be excluded that a small amount of NADPH present with the enzyme during incubation with amino acid modifiers protected against an effective binding of the reagent(s) to the active site. With respect to inhibition

Table I. Effect of site-specific modifiers on the activity of spinach leaf GR-1. Incubation mixtures contained an aliquot of purified GR-1, 100 mM Mops (pH 7.1), and a modifier. After 1 min incubation, reactions were initiated by addition of 0.05 mM NADPH and 2.5 mM glyoxylate.

Reagent	Target	Activity (%)
Control		100
Diacetyl (1 mM)	arginine	100
Diethylpyrocarbonate (1 mM)	histidine	67
PMSF (1 mM)	serine	70
Pyridoxal phosphate (0.1 mM)	lysine	100
Tetranitromethane (0.1 mM)	tyrosine	100
N-Ethylmaleimide (1 mM)	cysteine	100
Iodoacetate (1 mM)	cysteine	72
2-Mercaptoethanol (5 mM)	cysteine	63
Dithiothreitol (5 mM)	cysteine	49

of GR-1 activity by thiol reagents (Table I), it is not clear whether the inhibition reflects modification of the protein itself rather than resulting from a spontaneous formation of glyoxylate-thiol adducts, which would deplete glyoxylate concentration in the assay (Hamilton, 1985).

Despite a considerable number of studies on kinetics of glyoxylate and hydroxypyruvate reductases, both from plant and animal tissues, very little is known about amino acid groups involved in substrate-binding or catalysis. Several critical cysteine residues were previously demonstrated for spinach HPR-1 enzyme (Warren and Kohn, 1970). Inhibition by thiol reagents was also shown for glyoxylate reductase isozymes from yeast (Tochikura *et al.*, 1979; Fukuda *et al.*, 1980). Other than the role for cysteine residues, to my knowledge, no other amino acids have been implicated in the functioning of glyoxylate and/or hydroxypyruvate reductases from any source. The identification of histidine and serine residues as those of importance, in addition to cysteine(s), for functioning of spinach GR-1 (Table I) should provide necessary background for further structure/ function studies on this and other glyoxylate-reducing enzymes.

Effect of salts

Salts, with the exception of cyanide anions, had no appreciable effect on activity of GR-1. At a 20 mM concentration, the salts had either no effect (sulfate) or caused only 7-16% inhibition (chloride, carbonate, nitrate, nitrite) or 17% activation (phosphate) of GR-1 activity (data not shown). This is in an apparent contrast to a pronounced effect of salts (several fold inhibition or activation) on other glyoxylate and hydroxypyruvate reductases from a variety of tissues (*e.g.* [Zelitch, 1955; Kohn and Warren, 1970; Coderch *et al.*, 1979]). Cyanide, at 2 and 20 mM, caused 46 and 92% inhibition, respectively, of GR-1 activity (data not shown). Although the inhibition was much stronger than that of other salts, it is unlikely to be of physiological significance due to low (micromolar) concentrations of cyanide in plant tissues (Solomonson, 1981).

Metabolic regulation

Several intermediates of the glycolate pathway and some other compounds of primary metab-

olism were tested with respect to their possible regulatory effect on GR-1 activity (Table II). The data were obtained using low concentration of NADPH and glyoxylate to detect possible competitive inhibitors of GR-1 [*e.g.* NADP (Fig. 5)], which would otherwise be ineffective at saturating levels of both substrates. Besides NADP, the enzyme was significantly inhibited (over 25%) by ATP, glycolate, α-ketoglutarate and pyruvate. Other compounds had either no effect or caused only a slight change in enzymatic activity (Table II). It seems important to point out that GR-1 activity was not affected by 5 mM oxalate, which is a potent inhibitor of spinach HPR-2 enzyme (K_i of few μM) (Kleczkowski *et al.*, 1991). Both oxalate and acetohydroxamate [a selective inhibitor of GR-1 activity (Kleczkowski *et al.*, 1987)] can be regarded as useful tools to distinguish glyoxylate-dependent rates of GR-1, HPR-1 and HPR-2 in crude and partially purified preparations (Kleczkowski *et al.*, 1991, 1992).

Concentration of compounds which did significantly inhibit GR-1 activity (ATP, glycolate, α-ketoglutarate and pyruvate) (Table II) was 5 mM, which exceeds their estimated levels in the cytosolic compartment *in vivo*. For instance, for spinach leaf cytosol, α-ketoglutarate was estimated at 0.58-0.70 mM, and ATP - at 1.45-2.55 mM (Heineke *et al.*, 1991). Glycolate levels in leaves

Table II. Effect of selected metabolites on the activity of spinach leaf GR-1. Unless otherwise indicated, concentration of an effector was 5 mM. Glyoxylate and NADPH were kept at 0.10 and 0.008 mM, respectively.

Metabolite	Activity (%)
Control	100
ATP	72
Glyceraldehyde	111
DL-Glycerate	93
Glycine	95
Glycolate	58
DL-Isocitrate	108
α-Ketobutyrate	93
α-Ketoglutarate	43
L-Malate	104
NADP (0.5 mM)	23
NAD (0.5 mM)	107
Oxalacetate	88
Oxalate	101
Phosphoglycolate	96
Pyruvate	67
L-Serine	92

are generally believed to be on the order of micromolar (Husic *et al.,* 1987). Concentration of pyruvate has not, to my knowledge, been determined in leaf cytosol. Total concentration of pyruvate in leaves of maize (a C_4 plant) has been estimated at up to 10 mm (Leegood, 1985), but in that plant pyruvate is involved in the specialized intercellular transport of carbon during C_4- photosynthesis. Plants of a C_3-type (*e.g.* spinach) fix CO_2 directly through Rubisco, and may have several times lower total levels of pyruvate in leaves when compared to C_4 species (Leegood and Caemmerer, 1994). Thus, it appears that the above-mentioned metabolites, even though effectively inhibiting GR-1 under assay conditions (low concentration of substrates and high concentration of effectors), are unlikely to affect GR-1 activity *in vivo.* The same conclusion applies when evaluating a possible regulatory role for NADP. Estimated concentration of NADP in spinach leaf cytosol is *ca.* 0.04 mm (Heineke *et al.,* 1991). In the cytosol of barley leaf protoplasts, a concentration of over 0.1 mm for NADP was determined (Wigge *et al.,* 1993). However, in both spinach and barley, the cytosolic NADPH/ NADP ratio is at *ca.* 1.5 to 4.0. Considering low K_m of GR-1 for NADPH of 6 μm, and the fact that NADP is a competitive inhibitor *versus* NADPH (Fig. 5), it seems that NADP is unlikely to play any significant regulatory role for GR-1 under normal physiological conditions.

Overall, the data suggest that GR-1 is regulated solely by substrate availability. This is perhaps not surprising, considering that glyoxylate is toxic to cell metabolism (Hamilton, 1985; Husic *et al.,* 1987; Givan and Kleczkowski, 1992) and that plants would benefit from keeping levels of glyoxylate as low as possible. GR-1 is well suited to such a role because of its relatively high activities in leaf extracts of many plants (*e.g.* [Kleczkowski *et al.,* 1988, 1992]) and its low K_ms with both glyoxylate and NADPH (Kleczkowski *et al.,* 1986) (Fig. 1). The enzyme can be regarded as a safety device for an efficient elimination of glyoxylate that may arise in the cytosol as an unavoidable product/ intermediate of the glycolate pathway reactions (Kleczkowski *et al.,* 1986; Givan and Kleczkowski, 1992; Heupel and Heldt, 1994).

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