

Inhibition of Plant Glutamine Synthetases by Substituted Phosphinothricins

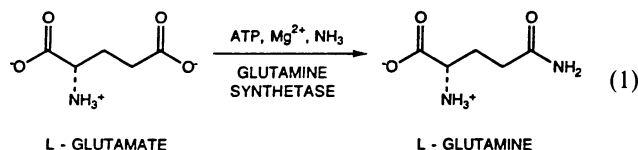
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ABSTRACT

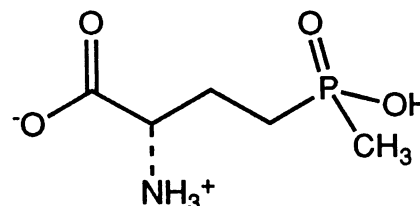
Glutamine synthetase (GS) utilizes various substituted glutamic acids as substrates. We have used this information to design herbicidal α - and γ -substituted analogs of phosphinothricin (L-2-amino-4-(hydroxymethylphosphinyl)butanoic acid, PPT), a naturally occurring GS inhibitor and a potent herbicide. The substituted phosphinothricins inhibit cytosolic sorghum GS₁ and chloroplastic GS₂ competitively versus L-glutamate, with K_i values in the low micromolar range. At higher concentrations, these inhibitors inactivate glutamine synthetase, while dilution restores activity through enzyme-inhibitor dissociation. Herbicidal phosphinothricins exhibit low K_i values and slow enzyme turnover, as described by reactivation characteristics. Both the GS₁ and GS₂ isoforms of plant glutamine synthetase are similarly inhibited by the phosphinothricins, consistent with the broad-spectrum herbicidal activity observed for PPT itself as well as other active compounds in this series.

GS¹ (EC 6.3.1.2) catalyzes a reaction of central importance in plant metabolism, the conversion of L-glutamate to L-glutamine (Eq. 1). Leaf cells contain two GS isoforms in differing proportions in different plant species (11). Both enzymes are octamers of catalytic subunits weighing from 42,000 D to 48,000 D; GS₁ is localized in the cytosol, and GS₂ is found in mature chloroplasts. These isoforms can be separated by ion-exchange chromatography and differ somewhat in kinetic and physical properties (9).



PPT (Scheme 1) is a naturally occurring GS inhibitor developed by Hoechst A.-G. as the broad-spectrum post-mergence herbicide glufosinate (14). Exposure to PPT causes accumulation of ammonia in plant tissues and may also block recycling of carbon from the photorespiratory pathway to the Calvin cycle (13, 16, 17). Kinetic inhibition studies of plant

¹ Abbreviations: GS, glutamine synthetase; GR₅₀, 50% growth reduction; PPT, phosphinothricin; AEPPT, α -ethylphosphinothricin; AMPPT, α -methylphosphinothricin; CHPPT, cyclohexanephosphinothricin; GHPPT, γ -hydroxyphosphinothricin; GMPPT, γ -methylphosphinothricin; μ , multiple of K_i required for reactivation suppression of sorghum GS.



1 PPT

SCHEME 1

GS have been described for PPT, and studies with radiolabeled PPT have demonstrated inactivation of all eight GS subunits (9, 10). PPT inhibits both GS₁ and GS₂ from many plant sources to a similar extent, based on measured inhibitor K_i values (1).

We have recently described a series of α - and γ -substituted phosphinothricins designed by analogy with similarly substituted glutamic acid substrates of the mammalian enzyme (7). The substrate specificity of plant GS is less well known (4), but we anticipated that the plant enzyme would also accommodate variations in the structure of both substrates and inhibitors. We describe here the ability of substituted phosphinothricins to inhibit cytosolic GS₁ from etiolated sorghum seedlings and chloroplastic GS₂ from spinach leaves. These compounds display a range of enzyme inhibitory properties which parallel their observed phytotoxicity. The tolerance of plant glutamine synthetases for modified inhibitors offers new possibilities for GS-targeted herbicide design. In addition, phosphinothricin analogues of differing potency may find use as physiological probes of glutamine synthetase function.

MATERIALS AND METHODS

Chemicals

All inorganic, organic, and biochemical reagents were purchased from Sigma Chemical Co. The inhibitors employed in this study were racemic mixtures synthesized as described previously: D,L-phosphinothricin and α - and γ -substituted D,L-phosphinothricins (6, 15).

Plant Material

Seedlings of sorghum (*Sorghum vulgare* L. var Dekalb 59E) were maintained in shallow metal containers filled with com-

mercial vermiculite in a dark growth chamber at 26°C, with daily watering for 14 d after planting, and were processed immediately upon removal into the light. Mature spinach leaves (*Spinacia oleracea* L.) were obtained via shipment to a local retailer at 4°C and were processed immediately upon receipt.

Purification of GS₁ from Etiolated Sorghum Seedlings

A purification procedure was adapted from reported methods (5). All operations were carried out at 4°C. Protein concentrations were determined by the method of Bradford (2). Etiolated sorghum leaf tissue (50 g) was ground to a powder in liquid nitrogen and was then stirred into 250 mL of a standard buffer containing 100 mM Tris-HCl (pH 7.6), 1 mM MgCl₂, and 12.5 mM 2-mercaptoethanol. Polyvinylpyrrolidone (20 g) was added to adsorb phenolics, and the resulting mixture was homogenized with a Polytron homogenizer three times for 1 min before being filtered through cheesecloth. The filtrate was centrifuged at 27,000g for 20 min, and the resulting supernatant was collected, and ammonium sulfate was added to 80% saturation at 0°C with stirring to precipitate protein. This mixture was centrifuged at 22,000g for 10 min, and the resulting pellet was resuspended in 15 mL of standard buffer. This preparation was loaded onto a Sephadex G-25 column (2.5 cm × 17 cm) equilibrated with standard buffer. Fractions were assayed for GS activity by the hydroxamate assay. Fractions containing GS were combined to give a total of approximately 6000 units (1 unit = 1 μmol/min at 30°C) which also contained some phosphatase activity. This preparation was loaded onto a DEAE-Sephacel column (5 cm × 16 cm) equilibrated with standard buffer and eluted with 500 mL of a linear NaCl gradient (0–0.4 M) at 0.6 mL/min. Fractions (10 mL) were assayed for both GS and phosphatase activity, giving a single GS peak eluting at 0.15 M NaCl, with most of the phosphatase activity eluting afterward. This separation reduced phosphatase contamination to a level that permitted kinetic determinations with the GS phosphate assay. Fractions containing GS were combined and concentrated to 10 mL on an Amicon XM-300 membrane. After desalting on a Sephadex G-25 column (2.5 cm × 17 cm) equilibrated with standard buffer, the final preparation was found to contain 500 units of GS activity with a specific activity of 15 units/mg protein. The preparation was stable for several months when stored at –20°C in standard buffer containing 40% ethylene glycol.

Purification of GS₂ from Mature Spinach Leaves

A purification procedure was adapted from reported methods (3). All operations were carried out at 4°C. Spinach leaf tissue (100 g) was ground to a powder in liquid nitrogen and was then stirred into 500 mL of a standard buffer containing 50 mM imidazole-HCl (pH 8.0), 20 mM MgSO₄, 1 mM dithiothreitol, and 10 mM 2-mercaptoethanol. The resulting mixture was homogenized with a Polytron homogenizer three times for 1 min before being filtered through cheesecloth. The filtrate was treated with polyvinylpyrrolidone (20 g) and was centrifuged at 27,000g for 20 min. The resulting supernatant was loaded onto a DEAE cellulose column (5 cm ×

8 cm) equilibrated with standard buffer and was eluted with 1 L of a linear NaCl gradient (0–0.35 M) at 0.6 mL/min. Fractions (10 mL) were assayed for GS activity by the hydroxamate assay. GS activity eluted as a single peak at 0.19 M NaCl. Fractions containing GS were combined to give approximately 2000 units (1 unit = 1 μmol/min at 30°C) which also contained some phosphatase activity. This preparation was placed in an Amicon filtering cell (500 mL) fitted with a Whatman GFA filter. A suspension of hydroxyapatite in standard buffer containing 0.5 M NaCl was added at a 1:1 ratio, and the mixture was stirred for 30 min. This suppression was slurried onto a gravity column that was washed with standard buffer containing 50 mM sodium arsenate, and the eluant was assayed for phosphatase activity. When no further phosphatase activity was detected, GS was eluted in 10-mL fractions with a 250 mM sodium arsenate wash (100 mL). Fractions containing GS activity were combined and concentrated on an Amicon XM-300 membrane to a volume of 10 mL. After desalting on a Sephadex G-25 column (2.5 cm × 17 cm) equilibrated with standard buffer, the final preparation was found to contain 60 units of GS activity, with phosphatase activity reduced to a level that permitted kinetic determinations with the GS phosphate assay. The enzyme prepared in this way had a specific activity of 13 units/mg protein and was stable for 2 months when stored at –20°C in standard buffer containing 40% ethylene glycol.

Enzyme Assay

All assays of sorghum GS₁ were carried out in a standard 100 mM Tris-HCl buffer (pH 7.6) containing 50 mM MgCl₂, 60 mM KCl, 1 mM 2-mercaptoethanol, and 0.1 mM sodium EDTA unless otherwise stated. All assays of spinach GS₂ were carried out in a standard 50 mM imidazole-HCl buffer (pH 8.0) containing 50 mM MgCl₂, 60 mM KCl, 1 mM 2-mercaptoethanol, and 0.2 mM sodium EDTA unless otherwise stated. Assay conditions were adapted from those reported previously (7). The coupled continuous assay method was used for enzyme inhibition kinetics in order to observe time-dependent changes in GS activity.

Coupled assay determinations were made at 30°C in a 1-mL reaction solution containing 6 mM sodium ATP, 50 mM ammonium chloride, 1 mM phosphoenolpyruvate, 0.4 mM NADH, lactic dehydrogenase (rabbit muscle, 40 units), pyruvate kinase (rabbit muscle, 20 units), and sodium L-glutamate (concentration varied as needed). Reactions were initiated by the addition of enzyme (0.01 units). Product formation was measured continuously by the absorbance change at 340 nm caused by the decrease in NADH concentration. For assays involving GS incubation with inhibitors, reactions were initiated by addition of ammonium chloride to assay solutions already containing enzyme.

Enzyme Inhibition

Determinations of inhibitor dissociation constants (K_i) for sorghum GS₁ were made according to the Lineweaver-Burk method as described previously (7). Spinach GS₂ K_i determinations were made according to the Dixon method, because of greater observed variation in enzyme reaction velocity

measurements. Reaction velocities (V) were measured over a range of inhibitor concentrations, at three or more substrate concentrations. Plots of reciprocal velocity *versus* inhibitor concentration were generated by a least squares analysis, and K_i values were determined from the x -coordinate (inhibitor concentration) of the intersection point of such plots.

Enzyme Inactivation and Reactivation

Inactivation experiments were performed by incubating enzyme (0.01 unit) with 6 mM sodium ATP and inhibitor (at a concentration of $5 \times K_i$) in a volume of 20 to 50 μ L of standard buffer for various periods. Recovery of GS activity following initial binding of an inhibitor at saturating concentrations was characterized by reactivation plots. The inactivation reaction mixture was diluted as needed with a reaction solution containing 50 mM sodium L-glutamate, 50 mM ammonium chloride, and the reagents necessary for the coupled assay, as well as any other desired components. Activity was then assayed continuously and was plotted as a percentage of control (uninhibited) activity *versus* time. Reactivation rate constants k_{react} were determined from a least squares analysis of semilogarithmic plots of activity *versus* time.

Suppression of GS activity recovery was demonstrated via a modified reactivation protocol in which high concentrations of inhibitor were included in the incubation mixture, so as to produce final concentrations of inhibitor after dilution in excess of the K_i value. Activity was then assayed continuously and was plotted as a percentage of control (uninhibited) activity *versus* time.

Phytotoxicity Evaluation

Herbicidal compounds displayed foliar (postemergence) activity. The dicot test species were: cocklebur (*Xanthium pennsylvanicum*), wild buckwheat (*Polygonum convolvulus*), morning glory (*Ipomoea lacunosa*), hemp sesbania (*Sesbania exaltata*), common lambsquarters (*Chenopodium album*), Pennsylvania smartweed (*Polygonum pennsylvanicum*), and velvetleaf (*Abutilon theophrasti*). The monocot test species were: proso millet (*Panicum miliaceum*), downy brome (*Bromus tectorum*), barnyardgrass (*Echinochloa crusgalli*), large crabgrass (*Digitaria sanguinalis*), and green foxtail (*Setaria viridis*). Test plants were grown in a greenhouse under conditions of natural lighting and high humidity. Seeds were germinated in perforated-bottom aluminum pans filled with sterilized silt loam topsoil and watered daily from below. Pans containing 2-week-old test plants were individually removed to a spray chamber and were sprayed by means of an atomizer with water solutions of the test compounds containing 0.40% by volume of nonionic surfactant. The spray solutions were formulated with each test compound so as to provide several application rates ranging from 5.0 to 0.5 kg per hectare. The pans were returned to the greenhouse and were watered daily from below. Injury to the test plants was rated at 14 d after spraying, as compared with randomly placed controls receiving spray solution without test compound, according to a scale which rated injury as a percentage of growth reduction ranging from 0% (no effect) to 100% (kill). A proprietary Monsanto software package was used to combine the injury

and rate titration data to yield calculated GR_{50} values for each tested compound, which represent the applied rate calculated to produce a 50% growth reduction, expressed in terms of moles/hectare and averaged over the species tested.

RESULTS AND DISCUSSION

Enzyme Characterization

End-point assays measuring phosphate release or glutamate hydroxamate formation were used to monitor GS purification and assess enzyme stability. The coupled biosynthetic ADP assay was used for continuous measurement of GS inhibition by the phosphinothricins.

Etiolated sorghum leaves served as a convenient source of cytosolic GS_1 (5). Crude sorghum GS_1 contained phosphatase activity, but the ion-exchange separation conventionally employed to separate GS isoforms also reduced phosphatase activity significantly. Sorghum GS_1 prepared in this way gave a substrate K_m for L-glutamate of 4.6 mM. This value is comparable to those reported in the literature for other GS_1 sources (1).

Mature spinach leaves served as a convenient source of chloroplastic GS_2 (3). Ion-exchange chromatography failed to remove phosphatase activity, since both GS_2 and phosphatase activities eluted later in a NaCl gradient than did GS_1 . However, differential elution from hydroxyapatite with sodium arsenate reduced phosphatase activity significantly. Spinach GS_2 prepared in this way gave a substrate K_m for L-glutamate of 9.1 mM, similar to the value of 6.7 mM reported previously for spinach (3) and within the range of values published for other GS_2 sources (1).

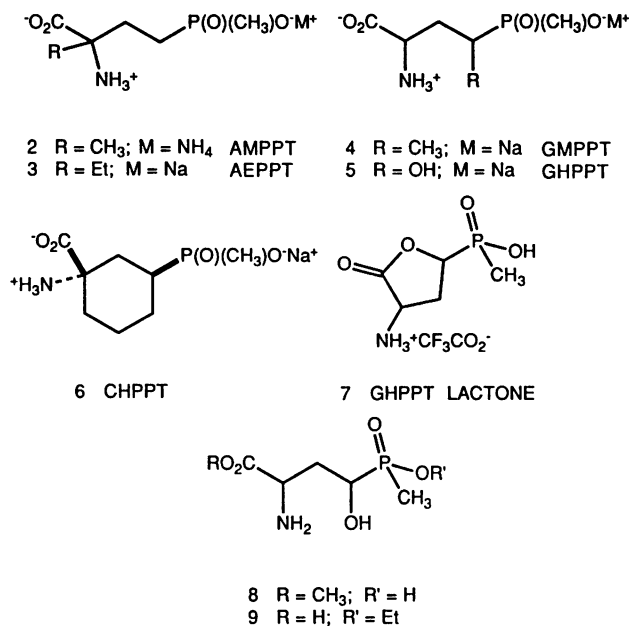
Competitive Inhibition

As we have shown elsewhere (7), GS inhibition by the phosphinothricins involves a sequence of initial inhibitor binding followed by rapid phosphorylation, which results in binding of phosphorylated inhibitor and ADP (Eq. 2).



The inactivated enzyme complex subsequently undergoes dissociation via hydrolytic release of inhibitor, ADP, and inorganic phosphate (8). Initial binding of the phosphinothricins to GS was demonstrated to be competitive *versus* glutamate, allowing determinations of K_i at low concentrations of inhibitor relative to glutamate, *i.e.* where the rate of enzyme inactivation is negligible. K_i values measured in this way represent dissociation constants of the phosphinothricins prior to phosphorylation and inactivation. The variable stability of inactivated enzyme-inhibitor complexes can be evaluated from GS reactivation kinetics, as discussed in the following section.

K_i values were determined for the following compounds: PPT, AMPPT (ammonium salt) (Scheme 2), AEPPT (sodium salt) (Scheme 3), GMPPT (sodium salt) (Scheme 4), GHPPT (sodium salt) (Scheme 5), CHPPT (sodium salt) (Scheme 6). The γ -substituted phosphinothricins GMPPT and GHPPT were obtained as diastereomeric mixtures at the γ -position; only one of the four diastereomers of D,L-GMPPT was ex-



SCHEMES 2-9

pected to be active as a GS inhibitor (7). In addition to these compounds, several intermediates related to GHPPT were examined for their biological properties. These included the γ -lactone (Scheme 7), the methyl carboxylate ester (Scheme 8), and the ethyl phosphinate ester (Scheme 9) (15).

K_i values for sorghum GS₁ listed in Table I are corrected for the inactivity of the D enantiomer in each racemic inhibitor mixture (7). The value for GMPPT was also corrected for the presence of the inactive *erythro* γ -methyl diastereomer. D,L-PPT displayed the lowest K_i value of 4.0 μ M, similar to K_i values reported for D,L-PPT inhibition of GS₁ from other plant sources (1, 12). GHPPT was only slightly weaker in competitive binding *versus* glutamate. Alkyl substitution at the α - and γ -positions resulted in weaker initial binding to the enzyme. The GHPPT γ -lactone, while showing potent binding, was found to be in equilibrium with GHPPT and kinetic evaluation was therefore not pursued further. Finally,

the esters (Schemes 8 and 9) were inactive as inhibitors of the sorghum enzyme, demonstrating that charged acidic groups are required for GS binding.

K_i values for spinach GS₂ are listed in corrected form in Table I. The lowest value of 1.5 μ M was observed for D,L-PPT, similar to values reported for D,L-PPT inhibition of GS₂ from other plant sources (1, 12). The overall pattern of inhibitor binding was similar to that of the sorghum enzyme.

Inactivation and Reactivation of Sorghum GS₁ and Spinach GS₂

As we have shown previously (7, 8), the substituted PPTs inactivate mammalian and bacterial GS. Similar observations have been reported for plant GS and PPT (9, 10). Inactivation of sorghum GS₁ by the substituted PPTs required the presence of ATP in the incubation mixture, consistent with the hypothesis that inhibitor phosphorylation accompanies inactivation (7). Plant GS inactivation rate constants for the PPT analogs could not be determined by enzyme assay due to rapid competing reactivation, a phenomenon previously observed for bacterial GS (8).

When GS is inactivated with the different substituted PPTs and then allowed to recover activity after high dilution, substantial variability is observed in reactivation (7). The recovery of enzyme activity occurs via inhibitor dissociation accompanied by hydrolysis (Eq. 2) (8). Obtaining dissociation constants for the phosphorylated inhibitors is difficult because the reactivation process is not a reversible dissociation, but instead involves hydrolytic turnover with concomitant release of phosphate and unchanged inhibitor (8). However, reactivation rate constants k_{react} can be determined under conditions of high dilution, where further inactivation is minimized. If one makes the reasonable assumption that inactivation rates, *i.e.* phosphorylation rates, are similar across the series of inhibitors (7), then determination of k_{react} values provides a good relative indicator of phosphorylated inhibitor binding.

For reactivation studies, sorghum GS₁ was incubated for 15 min with each inhibitor at a concentration of $5 \times K_i$, and activity was measured continuously after a 20-fold dilution. As illustrated in Figure 1, all compounds displayed measurable reactivation after dilution, although the lowest level was

Table I. K_i , k_{react} , and GR_{50} Values for the Phosphinotricins

Inhibitor ^a		Sorghum GS ₁		Spinach GS ₂		μ^b	GR_{50}^c
		$K_i^{d,e}$	k_{react}^f	$K_i^{d,e}$	k_{react}^f		
D,L-PPT	1	4.0 \pm 0.5	0.48	1.5 \pm 0.1	0.60	1	0.11
D,L-AMPPT	2	12 \pm 1	0.78	8.0 \pm 2.6	1.00	1	0.28
D,L-AEPPT	3	11 \pm 2	2.58	16 \pm 5	5.20	5	5.0
D,L-GMPPT ^g	4	17 \pm 1	4.22	123 \pm 15	9.47	10	>20
D,L-GHPPT	5	7.0 \pm 0.8	2.95	6.0 \pm 1.7	4.26	5	1.3
D,L-CHPPT	6	10 \pm 2	3.43	36 \pm 3	10.0	10	>20
C-ester	8						8.6
P-ester	9						

^a Assuming inhibition by L enantiomer only. ^b μ , Multiple of K_i required for reactivation suppression of sorghum GS₁. ^c GR_{50} Values calculated in mol/ha. ^d K_i values calculated in μ M. ^e Error values based on standard deviation of three replicate K_i determinations. ^f k_{react} Values calculated in 10^{-4} s⁻¹. ^g Assuming 1:1 mixture of γ -methyl isomers and inhibition by *threo* L isomer only.

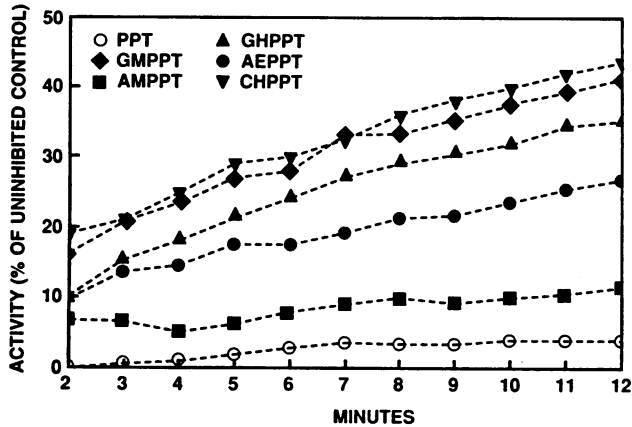


Figure 1. Recovery of sorghum GS₁ activity after incubation with each inhibitor at $5 \times K_i$, followed by 20-fold dilution into continuous assay buffer.

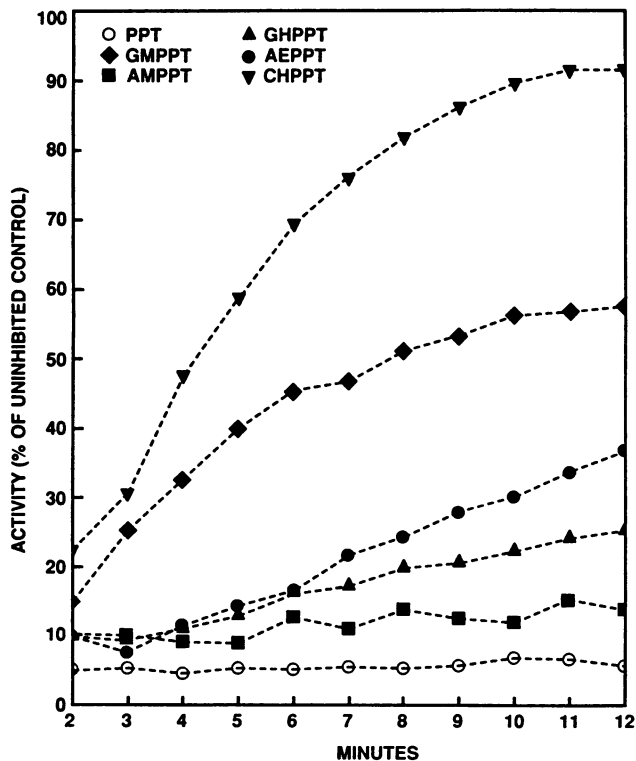


Figure 2. Recovery of spinach GS₂ activity under same conditions as sorghum GS₁ (Fig. 1).

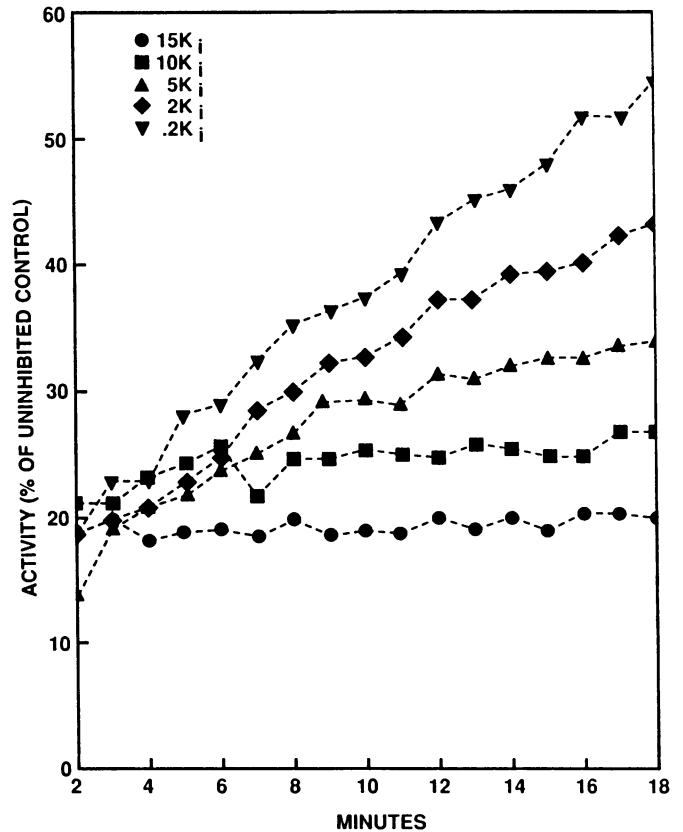


Figure 3. Reactivation suppression of sorghum GS₁ for GMPPT 4, after incubation at $10 \times K_i$, followed by 50-fold dilution into continuous assay buffer containing 4.

seen for PPT itself. Reactivation curves were identical after incubation times as short as 5 min and were insensitive to various concentrations of ATP and ADP in the dilution buffer as well as to different levels of dilution. These observations are consistent with an irreversible dissociation of inhibitor from the enzyme active site (8). The rate constants k_{react} for sorghum GS₁ reactivation are listed in Table I.

The greatest degree of reactivation was observed for GMPPT and CHPPT. Alkyl substitution at the α -position was better tolerated by the enzyme as illustrated by the recovery curves for AEPPT and AMPPT. The recovery curve for the γ -hydroxy analog GHPPT showed an intermediate degree of enzyme reactivation. PPT itself produced the most sustained level of enzyme inactivation.

Spinach GS₂ also displayed recovery of activity after incubation with the PPTs and dilution. Figure 2 illustrates the reactivation curves observed for spinach GS₂ after 20-fold dilution of incubation mixtures containing $5 \times K_i$ of each inhibitor. The rate constants k_{react} for spinach GS₂ reactivation are listed in Table I. Spinach GS₂ generally displayed more rapid reactivation than did the sorghum enzyme, particularly with the alkyl-substituted analogs.

Reactivation Suppression of Sorghum Glutamine Synthetase

We previously reported that increasing inhibitor concentrations in the dilution mixture cause a progressive suppression

of GS recovery via competing simultaneous reactivation and inactivation (7). At some inhibitor concentration, the rates of the two processes are the same. All the substituted PPTs exhibited reactivation suppression of sorghum GS₁, as illustrated in Figure 3 for GMPPT. The solution concentration required for suppression is characteristic for each PPT analog and provides a nonsteady-state measurement of the inhibitor level required to saturate the enzyme active site. These concentrations are listed in Table I, expressed as μ .

Phytotoxicity of the Substituted PPTs

The following compounds showed herbicidal activity: PPT, AMPPT, AEPPT, GHPPT, GHPPT lactone, and the methyl carboxylate ester (Scheme 8). Phytotoxic symptoms included generalized chlorosis within 48 h followed by desiccation and were similar for monocot and dicot species. Both GMPPT and CHPPT caused moderate interveinal chlorosis in most species within 48 h after spraying at 5.0 kg/ha, but plants recovered within 14 d. The ethyl phosphinate ester (Scheme 9) showed no phytotoxic effects. The composite GR₅₀ values for PPT analogs are listed in Table I. Dicot species were more sensitive to the PPTs than monocot species, probably reflecting differences in uptake between the two plant groups rather than differential enzyme susceptibility (1, 12). In a given plant species, the polar, charged PPTs should translocate and reach their target in similar concentrations. Variable inhibitor phytotoxicity should thus reflect differences in enzyme inhibition. As illustrated in Table I, herbicidal PPTs possess a combination of low K_i , k_{react} , and μ values, indicative of tight binding to GS at all stages of the inactivation process. A special case is the phytotoxicity of the methyl carboxylate ester (Scheme 8), which suggests conversion in plants to GHPPT. The lack of herbicidal activity for the ethyl phosphinate ester suggests the absence of plant esterase activity capable of hydrolyzing this moiety.

In conclusion, we have shown that a variety of α - and γ -substituted PPTs potently inhibit the GS₁ and GS₂ isoforms of plant glutamine synthetase. The effectiveness of GS inhibition by the PPTs depends both on the potency of initial inhibitor binding (K_i) and on the stability of the inactivated enzyme-inhibitor complex, as exhibited by reactivation kinetics (k_{react} and μ). Successful design of herbicidal PPT analogs requires maximizing inhibitor binding and minimizing reactivation. Our results demonstrate that plant glutamine synthetases exhibit a tolerance for modified inhibitors which is similar to that shown by the mammalian and bacterial enzymes, opening the way to previously unexplored structural modifications of PPT. This characteristic also offers considerable promise for the design of other novel herbicides which undergo GS-mediated phosphorylation and binding at the enzyme active site. Furthermore, the availability of modified

PPT analogs may provide a better understanding of the effects of glutamine synthetase blockage in plants, particularly the relative importance of ammonia accumulation and glutamine deprivation.

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