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Substrate Variability as a Factor in Enzyme Inhibitor Design: Inhibition of Ovine Brain Glutamine Synthetase by α- and γ-Substituted Phosphinothricins

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ABSTRACT: Ovine brain glutamine synthetase (GS) utilizes various substituted glutamic acids as substrates. We have used this information to design α- and γ-substituted analogues of phosphinothricin [L-2-amino-4-(hydroxymethylphosphinyl)butanoic acid], a naturally occurring inhibitor of GS. These compounds display competitive inhibition of GS, and a correlation between the inhibitor K_i values and the K_m/V_{max} values of the analogously substituted glutamates supports the hypothesis that the phosphinothricins participate in transition-state analogue inhibition of GS. At concentrations greater than K_i these inhibitors caused biphasic time-dependent loss of enzyme activity, with initial pseudo-first-order behavior; k'_{inact} parameters were determined for several compounds and were similar to the 2.1×10^{-2} s^{-1} value measured for PPT. Dilution after GS inactivation caused a non-first-order recovery of activity. Reactivation kinetics were insensitive to inhibitor and ADP concentrations over wide ranges, although very high postdilution concentrations of inhibitor suppressed reactivation. The burst activity level, β, as well as the concentration of inhibitor required to suppress reactivation to this level, μ, expressed as a multiple of the K_i value, was characteristic for each compound in the phosphinothricin series. Increasing substitution of the phosphinothricin parent structure caused an increase in K_i values as well as in the inactivation/reactivation parameters. The kinetic behavior of these inhibitors is consistent with a mechanistic scheme involving initial phosphorylation and rapid partial inhibitor dissociation, followed by slow release of remaining bound inhibitor.

Glutamine synthetase [GS; L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2] is a ubiquitous enzyme in both prokaryotes (Stadtman & Ginsburg, 1974; Rhee et al., 1985) and eukaryotes (Meister, 1974, 1985) and catalyzes a central reaction in nitrogen metabolism, the conversion of glutamate to glutamine (eq 1). Prokaryotic GS consists of 12 identical catalytic subunits weighing approximately 45 kDa, while the eukaryotic enzyme is an octomer. The crystal structure of dodecameric GS from *Salmonella typhimurium* has recently been determined to a resolution of 3.5 Å (Almassy et al., 1986;

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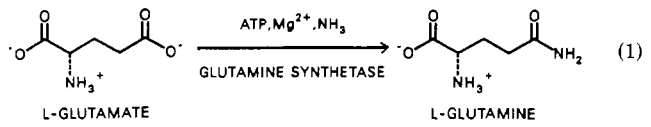
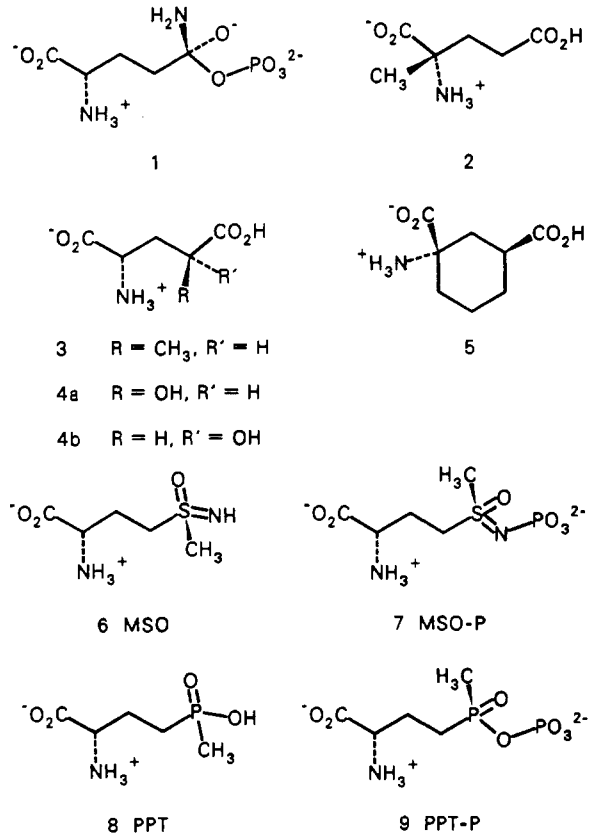


(1)

Janson et al., 1986) and displays an exceptionally complex architecture characterized by unique subunit interactions. The mechanistic features of GS catalysis are common to both prokaryotic and eukaryotic enzymes (Weisbrod & Meister, 1973; Maurizi et al., 1987) and have been correlated with primary structure in considerable detail (Pinkofsky et al., 1984; Colanduoni & Villafranca, 1985; Colanduoni et al., 1987). Substrate binding is ordered, with product release following the formation of a high-energy, tetrahedral intermediate **1** (Meek & Villafranca, 1980). The latter species arises by addition of ammonia to an initially formed γ-glutamyl phosphate intermediate, in a step accompanied by a deep-seated enzyme conformational change (Chung et al., 1984).

Besides L-glutamic acid, variously substituted glutamic acids also serve as efficient substrates of ovine brain GS (Meister, 1968). Of particular importance are the α- and γ-substituted analogues **2**–**5**, all of which reportedly display substrate K_m values comparable to that of glutamic acid (Meister, 1969). The correlation between substrate efficacy of a congeneric series of substrates and the potency of analogously substituted inhibitors has been advanced as a fundamental thermodynamic criterion for transition-state analogue inhibition (Bartlett & Marlowe, 1983, 1987). The wide range of permissible structure variation in GS substrates suggested to us a study of the inhibitory properties of analogously substituted GS transition-state analogue inhibitors.



Previous inhibitor studies have provided considerable mechanistic understanding of GS catalysis (Gass & Meister, 1970; Gibbs et al., 1984). The first reported inhibitor capable of inactivating GS was L-methionine (*S*)-sulfoximine (**6**, MSO), which undergoes enzyme-mediated sulfoximine phosphorylation, forming MSO phosphate (**7**, MSO-P). MSO-P functions as a transition-state analogue of reaction intermediate **1** (Maurizi & Ginsburg, 1982), binding tightly to GS with a stoichiometry of one molecule of inhibitor per enzyme subunit (Ronzio et al., 1969; Maurizi et al., 1986). Phosphinothricin [**8**, [L-2-amino-4-(hydroxymethylphosphinyl)butanoic acid, acid, PPT]] is a more recently discovered inhibitor of GS. PPT is produced by various streptomycete species as a component of excreted antibacterial peptides (Seto, 1986) and is a potent inhibitor of GS from all sources, as well as an effective broad-spectrum herbicide (Ridley & McNally, 1985; Acaster & Weitzman, 1985). GS inactivation by PPT is thought to involve a phosphorylated phosphinate species PPT-P (**9**) (Colanduoni & Villafranca, 1986). We have been engaged in the design of analogues of PPT, with the aim of obtaining a better understanding of its interaction with GS. We anticipated that a series of PPT analogues bearing α- and γ-substituents by analogy with GS substrates of types **2**–**5** might display a range of properties paralleling the substrate efficacy of analogous glutamic acids. This paper presents a mechanistic investigation of the inhibition of ovine brain GS with α- and γ-substituted phosphinothricins. Various kinetic methods have been used to evaluate the properties of substituted phosphinothricins, each of which displays inhibition competitive with glutamate, as well as time-dependent enzyme inactivation and reactivation. The synthesis of the inhibitors examined in this study has been reported in detail (Logusch, 1986; Walker et al., 1987; Logusch et al., 1988).

Materials and Methods

Chemicals. All inorganic, organic, and biochemical reagents were purchased from Sigma Chemical Co. Ovine brain glutamine synthetase was purchased from Sigma as a lyophilized powder prepared from a phosphate buffer solution (Rowe et al., 1970). The inhibitors employed in this study were racemic mixtures synthesized as described previously: D,L-phosphinothricin (Logusch, 1986) and α- and γ-substituted D,L-phosphinothricins (Walker et al., 1987; Logusch et al., 1988).

Enzyme Assay. All assays of ovine brain GS were carried out in a standard 50 mM imidazole–HCl buffer (pH 7.1) containing 50 mM $MgCl_2$, 60 mM KCl, 1 mM 2-mercaptoethanol, and 0.1 mM NaEDTA, unless otherwise stated. Enzyme stock solutions were prepared by dissolving 10-unit aliquots of commercial lyophilysate in standard buffer, followed by desalting on 6 mL Sephadex G-25 columns to remove phosphate (Christopherson et al., 1979). Stock solutions were assayed both for phosphate content and GS activity by using the phosphate release assay (1 unit = 1 μmol of phosphate formed per minute). Protein concentrations were determined by the method of Bradford (Bradford, 1976). Specific activity of such desalted GS ranged from 20 to 25 units/mg of protein, as compared with 12 units reported previously (Rowe et al., 1970). SDS–PAGE gel analysis of this material gave a predominant band corresponding to a molecular weight of approximately 45 000. Only very low levels of ATPase activity were observed with such preparations. Stock solutions were diluted with ethylene glycol to a final glycol concentration of 40% and stored at −20 °C for months without detectable loss of activity.

(*a*) *γ-Glutamylhydroxamate Synthesis (Weisbrod & Meister, 1973).* GS activity was determined at 37 °C in a 1-mL reaction volume containing 6 mM NaATP, 100 mM hydroxylamine, and sodium L-glutamate (concentration varied as needed). Reactions were initiated by the addition of enzyme (0.03 unit), and were allowed to proceed 5–15 min before the addition of 1.5 mL of an aqueous stop solution prepared in

100-mL lots from 5.5 g of ferric chloride hexahydrate, 2.0 g of trichloroacetic acid, and 2.1 mL of 12 N HCl. Product formation was determined from the absorbance of the ferric complex at 550 nm, on the basis of a standard curve generated over a range of 0.1–1.0 μmol.

(*b*) *Phosphate Release* (*Shapiro & Stadtman, 1970*). GS activity was determined at 37 °C in a 200-μL reaction volume contained 6 mM NaATP, 50 mM ammonium chloride, and sodium L-glutamate (concentration varied as needed). Reactions were initiated by the addition of enzyme (0.003 unit) and were allowed to proceed 5–15 min before the addition of 1.0 mL of aqueous stop solution of 1% ammonium molybdate, 0.034% malachite green, and 0.04% NP-40 surfactant, followed after 1 min by 200 μL of 34% aqueous sodium citrate. Phosphate formation was determined from the absorbance at 660 nm of the malachite green–phosphomolybdate complex, on the basis of a standard curve generated over a range of 1–30 nmol of tripotassium phosphate (Lanzetta et al., 1979). For assays involving GS incubation with inhibitors, reactions were initiated by the addition of ammonium chloride to assay solutions already containing enzyme.

(*c*) *Coupled Assay* (*Kingdon et al., 1968*). GS activity was determined at 37 °C in a 1-mL reaction solution containing 6 mM NaATP, 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 unit). 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. Inhibition constants (K_i) were determined by the Lineweaver–Burk method, utilizing data obtained from either the phosphate release or coupled assays. Reaction velocities (V) were measured at 37 °C over a range of L-glutamate concentrations (1.5–20 mM) without inhibitor present and at three or more inhibitor concentrations. Reciprocal plots of velocity vs substrate concentration were generated, and apparent K_m values were determined from the x intercept of a line fitted to the data by using a least-squares analysis. In cases where points at lower substrate concentrations deviated from linearity because of enzyme inactivation, such points were deleted before least-squares analysis. K_i values were obtained from $K_i = (K_m)[I]/(K_m - K_{m(app)})$ and were determined at three or more inhibitor concentrations. The final value was reported as an average of those determinations for which the V_{max} deviated from the control by less than 20%.

Enzyme Inactivation. Time-dependent inactivation of GS was demonstrated by incubating enzyme (0.003 unit) at 37 °C with 6 mM NaATP and inhibitor (concentration varied as needed) in the absence of sodium L-glutamate and ammonium chloride. At specific time points, 8-μL aliquots were removed and diluted to 400 μL with standard buffer containing 6 mM NaATP and 50 mM sodium L-glutamate. Duplicate 180-μL aliquots of the resulting solution were then removed, and reaction was initiated by the addition of 20 μL of 500 mM ammonium chloride. GS activity was determined by the phosphate release assay. For each condition, activity was calculated as a percent of control (uninhibited) activity and was plotted vs time.

Pseudo-first-order initial rate constants of inactivation (k'_{inact}) were determined by using the method of Mildvan (Mildvan & Leigh, 1964). Plots of GS activity vs time were generated under the conditions described above, except that sodium L-glutamate was present in the incubation mixtures at concentrations of 2.5, 5, 10, 20, 50, and 100 mM, in addition to inhibitor ($10K_i$). $T_{1/2}$ values were determined from a semilogarithmic treatment of the data from each curve and were converted to rate constants according to the equation $k_{obs} = 0.693/T_{1/2}$. A least-squares-fitted plot of $1/k_{obs}$ vs [L-glutamate] was linear with a y intercept of $1/k'_{inact}$. The validity of each determination was evaluated by adherence of inactivation curves to first-order kinetics, i.e., linear semilogarithmic plots. Only early time points generally satisfied this criterion, and remaining points were not utilized in the determination of k_{obs}.

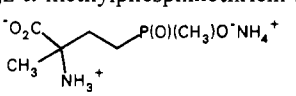
Enzyme Reactivation. Recovery of GS activity following initial binding of an inhibitor at saturating concentrations was characterized by reactivation plots. Enzyme (0.01 unit) was incubated at 37 °C with 6 mM NaATP and inhibitor (concentration varied as needed) in a volume of 20–50 μL of standard buffer for various periods. The incubation 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 percent of control (uninhibited) activity vs time. For longer term reactivation studies, dilution was made in the absence of ammonium chloride, and the reaction was initiated at times later than 30 min by the addition of 50 μL of 1 M ammonium chloride.

Enzyme Reactivation/Suppression. Suppression of GS activity recovery was demonstrated by use of 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 percent of control (uninhibited) activity vs time.

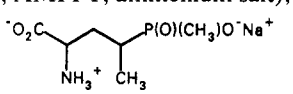
Results

Competitive Inhibition. Substrate K_m determinations for commercial ovine brain GS were made from Lineweaver–Burk plots utilizing phosphate release and coupled assay data. L-Glutamate gave K_m values of 2.6 and 2.7 mM with these methods, in agreement with published values determined under comparable conditions (2.5 mM; Rowe et al., 1970). Competitive inhibition experiments using the phosphate release and coupled assay methods yielded K_i values for L-methionine (*R*,*S*)-sulfoximine of 0.21 and 0.23 mM, respectively, also in agreement with published values (0.21 mM; Wedler et al., 1980).

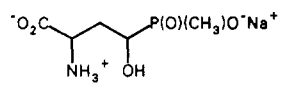
The phosphate release assay was used for K_i determinations with the following compounds: D,L-phosphinothricin (**8**, PPT); D,L-α-methylphosphinothricin (**10**, AMPPT, ammonium salt);



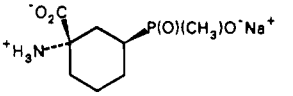
10 AMPPT



11 GMPPT



12 GHPPT



13 CHPPT

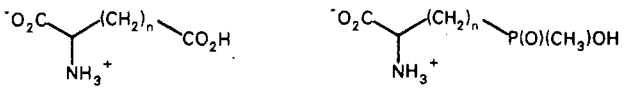
D,L-γ-methylphosphinothricin (**11**, GMPPT, sodium salt); D,L-γ-hydroxyphosphinothricin (**12**, GHPPT, sodium salt); D,L-cyclohexanephosphinothricin (**13**, CHPPT, sodium salt). As discussed elsewhere, GMPPT consisted of an unassigned

Table I: Measured and Corrected K_i Values for Inhibition of Ovine Brain GS by MSO and the Phosphinothricins

inhibitor	K_i (μM)	corrected K_i[a] (μM)
L-MSO (**6**)	210	105[b]
D,L-PPT (**8**)	28	14
D,L-AMPPT (**10**)	137	69
D,L-GMPPT (**11**)	347	87[c]
D,L-GHPPT (**12**)	47	24
D,L-CHPPT (**13**)	109	55
D,L-(CH_2)PPT (**15a**)	*d*	*d*

[a] Assuming inhibition by L enantiomer only. [b] Assuming inhibition by sulfoximine *S* isomer only. [c] Assuming 1:1 mixture of γ-methyl isomers and inhibition by threo L isomer only. [d] No inhibition was observed.

57:43 mixture of γ-methyl diastereomers (Logusch et al, 1988) of which only the threo isomer was expected to show inhibitory activity, on the basis of the reported substrate activity of only the threo glutamate isomer **3** (Kagan & Meister, 1966). GHPPT consisted of an unassigned 56:44 mixture of γ-hydroxyl diastereomers (Walker et al., 1987). Both isomers were expected to be inhibitory since the diastereomeric γ-hydroxyglutamic acids **4a** and **4b** were both reported to be efficient substrates of ovine brain GS, the threo isomer **4a** possessing a lower K_m value than that of L-glutamic acid (Kagan & Meister, 1966). Since both optical isomers of 2-aminoadipic acid (**14a**) were reported to be weak substrates of GS (Wellner



14a n = 3 15a n = 3

14b n = 1 15b n = 1

et al., 1966), we also tested the ability of D,L-homophosphinothricin (**15a**) to inhibit the enzyme (Logusch et al., 1988). It should be noted that aspartic acid (**14b**) is not a substrate for GS (Khedouri & Meister, 1965) and that the corresponding PPT analogue D,L-norphosphinothricin (**15b**) is not an inhibitor of the enzyme (Soroka & Mastalerz, 1976).

Double-reciprocal plots of GS velocity vs glutamate concentration showed hyperbolic deviation from linearity at higher concentrations of PPT, indicating inhibitor-associated loss of enzyme activity analogous to that reported for L-methionine (*S*)-sulfoximine (Ronzio et al., 1969) and for PPT inhibition of glutamine synthetase from plant sources (Leason et al., 1982; Ridley & McNally, 1985). This effect could be minimized by selection of PPT and L-glutamate concentrations that permitted the observation of competitive inhibition. K_i values determined in this way for the phosphinothricins are presented in Table I, with the exception of D,L-homophosphinothricin (**15a**), for which GS inhibition was not observed. In principle, all listed K_i values can be corrected to account for the presence of inactive D amino acid enantiomers, since D-phosphinothricin is inactive as a GS inhibitor (Zeiss, 1987), consistent with the very low or unobservable substrate efficacy of α- and γ-substituted D-glutamic acid analogues (Meister, 1969). The value for GMPPT can be additionally corrected for the inactive erythro γ-methyl isomer, assuming a 1:1 presence of both isomers. The methylphosphinic acid moiety common to all phosphinothricins is symmetric when ionized at pH 7.1 and does not contribute to further stereoisomeric differentiation of these inhibitors. Both the experimentally measured and the adjusted K_i values are given in Table I.

The α- and γ-substituted PPT analogues all displayed K_i values in the micromolar range, approximately 2 orders of magnitude smaller than the reported substrate K_m values of the corresponding glutamic acid analogues (Table II). PPT was the most potent competitive inhibitor (28 μM). The methyl group attached to the terminal phosphinic acid functionality thus contributes significantly to binding at the active site of GS. We were also interested in determining the extent of correlation between the K_i and K_m/V_{max} values in this series. To provide a standard for alternative substrate activity, we evaluated commercially available D,L-α-methylglutamic acid as a GS substrate, using the coupled assay. A K_m of 7.0 mM and a $V_{max(rel)}$ of 75 were determined, in agreement with previously reported values of 6.7 mM and 75, respectively (based on an assigned $V_{max(rel)}$ of 100 for L-glutamate; Kagan & Meister, 1966).

Table II: Reported K_m and V_{max} Values for Glutamic Acid Substrates of Ovine Brain GS (Meister, 1969)

substrate	K_m (mM)	$V_{max(rel)}$	log K_m/V_{max}
L-GLU	3.9	100	−1.41
L-AMGLU (**2**)	6.7	75	−1.05
L-(*t*)GMGLU (**3**)	3.6	27	−0.88
L-(*t*)GHGLU (**4a**)	2.4	100	−1.62
L-(*e*)GHGLU (**4b**)	5.6	81	−1.16
L-CHGLU[a] (**5**)	5.1	29	−0.75

[a] Assuming substrate activity for L enantiomer only.

Bartlett has described a mathematically derivable proportionality between substrate K_m/V_{max} and inhibitor K_i values for a congeneric series of structurally analogous enzyme substrates and transition-state analogue inhibitors (eq 2)

$$K_i \propto K_m/V_{max} \quad (2)$$

(Bartlett & Marlowe, 1983, 1987). A least-squares-fitted logarithmic plot of the values derived for the α- and γ-substituted glutamic acids and phosphinothricins (Tables I and II) is illustrated in Figure 1. A resonable fit of the data was obtained, considering the approximations made to account for uncertain diasteromeric assignments of the γ-substituted PPT analogues. This result supports the hypothesis that the substituted phosphinothricins participate in transition-state analogue inhibition of GS.

Enzyme Inactivation. Time-dependent inactivation of GS has been well documented for MSO (Manning et al., 1969; Weisbrod & Meister, 1973; Rhee et al., 1981) and has been reported for PPT (Colanduoni & Villafranca, 1986; Manderscheid & Wild, 1986). Such inactivation was also observed for the phosphinothricin analogues described in this study, as illustrated in Figure 2. Methionine sulfoximine caused complete inactivation of the enzyme, in agreement with previous studies of the ovine brain enzyme (Ronzio & Meister, 1968). For the phosphinothricins, the greatest degree of enzyme inactivation was observed for AMPPT, while significantly less inactivation was caused by phosphinothricin. The remaining three analogues caused decreased loss of enzyme activity. No inactivation was observed for either MSO or the substituted phosphinothricins in the absence of ATP, or upon substitution of ADP for ATP, suggesting a similar mechanistic requirement for phosphorylation of both types of inhibitor. Semilogarithmic plots of GS inactivation by these compounds display a decrease in rate with time, as illustrated in Figure 3 for MSO, PPT, and AMPPT.

The inactivation curves plotted in Figure 2 suggest a similar initial rate of inactivation for this group of inhibitors. The method of Mildvan (Mildvan & Leigh, 1964) was therefore employed to obtain the kinetic parameter k'_{inact}, the pseudo-first-order rate constant of inactivation (eq 3). The rate of

$$-d[E]/dt = k'_{inact}[E] \quad (3)$$

$$1/k'_{obs} = 1/k'_{inact} + [S]/K_D \quad (4)$$

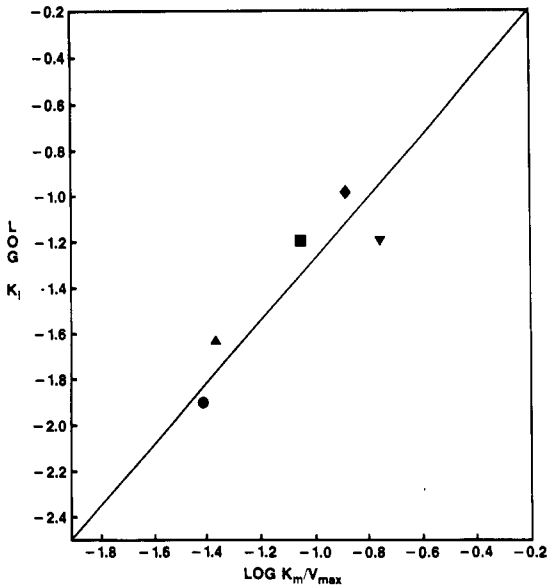


FIGURE 1: Transition-state-analogue correlation of glutamate and phosphinothricin analogues. Values for K_i were obtained from Lineweaver–Burk plots of reaction velocity vs glutamate concentration at three or more inhibitor concentrations and are corrected to account for the presence of the inactive D enantiomer of phosphinothricin analogues (Table I). K_m and V_{max} values for glutamate analogues have been published previously (Table II). (●) PPT; (▲) GHPPT; (▼) CHPPT; (■) AMPPT; (◆) GMPPT.

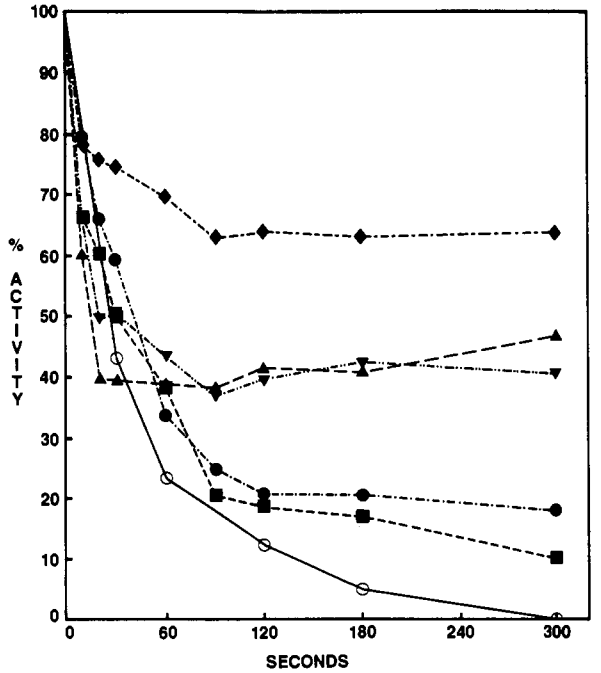


FIGURE 2: Inactivation of glutamine synthetase by phosphinothricin analogues. The enzyme was incubated with each inhibitor at a concentration of $10K_i$, and enzyme activity at each time point was assayed by phosphate release after 50-fold dilution. (○) MSO; (■) AMPPT; (◆) GMPPT; (●) PPT; (▲) GHPPT; (▼) CHPPT.

enzyme inactivation was varied by the addition of L-glutamate, and semilogarithmic plots of GS activity vs time were used to calculate $T_{1/2}$ values and k'_{obs}. A linear graph of $1/k'_{obs}$ vs glutamate concentration provided an x intercept of $-K_D$ and a y intercept of $1/k'_{inact}$, where K_D is the enzyme–substrate dissociation constant (eq 4). First-order inactivation kinetics, i.e., linear semilogarithmic plots, were observed at early time points for MSO and the substituted phosphinothricins, with the exception of GMPPT, for which insufficient time points adhered to linear kinetics as a result of less effective enzyme

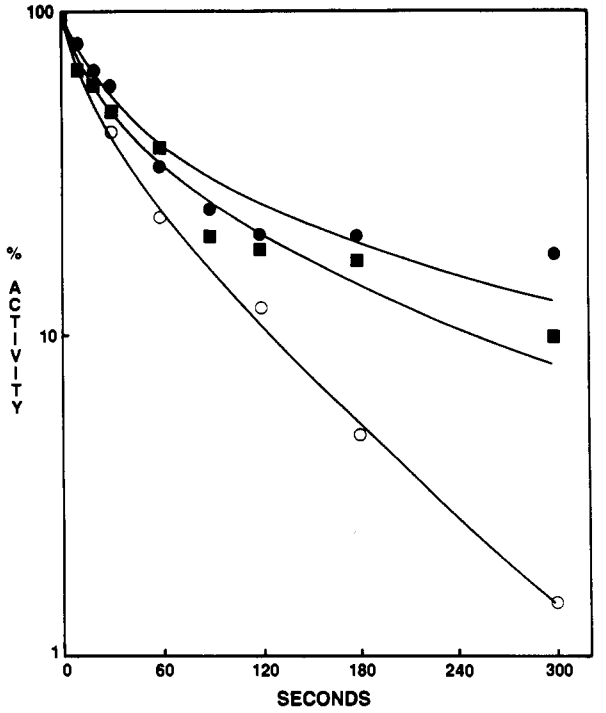


FIGURE 3: Semilogarithmic plot of glutamine synthetase inactivation. Values from Figure 2 are plotted semilogarithmically for inhibitors that continued to show a progressive loss of enzyme activity after 1 min of incubation. (○) MSO; (■) AMPPT; (●) PPT.

Table III: Pseudo-First-Order Rate Constants k'_{inact} for Inactivation of Ovine Brain GS by MSO and the Phosphinothricins

inhibitor	k'_{inact} (10^{-2} s^{-1})
L-MSO (**6**)	2.5
D,L-PPT (**8**)	2.1
D,L-AMPPT (**10**)	2.2
D,L-GHPPT (**12**)	2.9
D,L-CHPPT (**13**)	2.3

inactivation. Table III lists the calculated values for MSO and the phosphinothricin series. These compounds displayed essentially identical initial rate constants k'_{inact}. If inhibitor phosphorylation is required for GS inactivation (Colanduoni & Villafranca, 1986), then these results suggest that such inhibitors undergo phosphorylation at the same rate. This interpretation is applicable only for early subunit inactivation events, since subsequent inactivation departs from first-order behavior.

Enzyme Reactivation. Residual GS activity observed after incubation with PPT and its analogues can in principle be explained by the functioning of stable, partially inactivated GS octamers incapable of further inhibitor binding or by the establishment of a binding equilibrium between GS and inhibitor. An end-point assay such as the phosphate method is most informative only for a complete inactivator such as MSO (Rhee et al., 1980; Kim & Rhee, 1987). We therefore examined the non-steady-state inhibition kinetics of the phosphinothricins using the coupled assay method. GS was incubated for 15 min with ATP, $MgCl_2$, and a $5K_i$ concentration of inhibitor and was assayed almost immediately after 50-fold dilution (Figure 4); an initial lag phase in the activity of the coupled assay's enzyme components was observed 1–2 min after dilution. Under these conditions MSO caused essentially complete inactivation, with no subsequent recovery. A similar degree of inactivation, followed by very slight recovery, was seen for the α-methyl-substituted analogue AMPPT. Phosphinothricin itself showed significant recovery of activity,

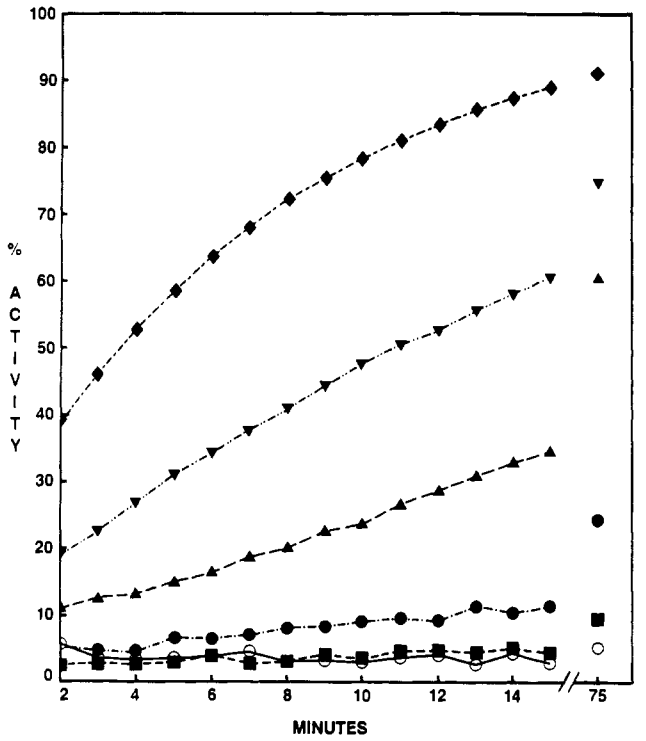


FIGURE 4: Recovery of glutamine synthetase activity after inactivation with phosphinothricin analogues. The enzyme was incubated with each inhibitor at a concentration of $5K_i$, and enzyme activity was measured continuously via coupled assay after 50-fold dilution. Final time point shows activity measured 75 min after dilution. (○) MSO; (■) AMPPT; (◆) GMPPT; (●) PPT; (▲) GHPPT; (▼) CHPPT.

particularly after 1 h. The other substituted analogues showed dramatic reversal of inhibition with time. When these results are compared with the phosphate release data (Figure 2), it is evident that the earlier curves reflect both the inactivation process and activity recovery during the end-point assay. Reactivation behavior was essentially independent of incubation time; identical recovery curves were obtained after incubations of 5 and 20 min, as well as 1 and 3 h, indicating that saturation of the enzyme with inhibitor was rapidly achieved under these conditions.

Reactivation was dependent on the presence of ATP in the incubation mixture. Incubation of enzyme and inhibitor in the absence of ATP, followed by dilution into a buffer containing ATP, resulted in recovery curves identical with those illustrated in Figure 4. This result indicated that the rate of release of bound unphosphorylated inhibitor by the enzyme was considerably slower than the rate of subsequent ATP binding and inactivation and suggests the absence of a strict requirement in the order of binding of inhibitor and ATP. When GS was incubated with each compound and diluted in the absence of ATP, followed by addition of ATP after 2 min, no decrease of enzyme activity was noted relative to uninhibited controls, suggesting a rapid release of unphosphorylated inhibitor as compared with the putative phosphorylated form.

The detection of residual GS activity after inactivation with PPT and its analogues led us to consider whether the enzyme would be susceptible to competitive inactivation by MSO, even in the presence of saturating concentrations of a phosphinate inhibitor. As described earlier, only MSO inactivates ovine brain GS fully. To examine the possibility of competitive inactivation, GS was incubated as before with a $10K_i$ concentration of CHPPT, and after 30 min, MSO was added at a concentration of $10K_i$. Aliquots were periodically withdrawn and assayed for activity after a 50-fold dilution. A parallel control dilution was performed at each time point by using aliquots from a GS incubation containing CHPPT but not MSO. Little difference in recovery was noted after 1 min of incubation with MSO, but within 5 min a progressive inactivation of GS was apparent, which was essentially complete after 180 min of incubation with MSO. Despite its inability to inactivate GS completely, CHPPT competes well with MSO for enzyme binding, prolonging the time required for complete GS inactivation by more than 3 h.

The results of mixed inactivation are consistent with a hypothetical reaction scheme involving inhibitor phosphorylation and release. It is possible that only partial subunit inactivation by inhibitor occurs, allowing immediate access of MSO to catalytically competent enzyme active sites. Alternatively, all sites may be initially occupied by CHPPT, but with sufficient inhibitor turnover occurring in the incubation mixture to permit subsequent enzyme inactivation by MSO. It is unclear whether complete displacement of CHPPT by MSO occurs in this experiment, although total inactivation of mammalian brain GS by MSO has been shown to proceed with binding of 1 equiv of MSO/subunit (Ronzio et al., 1969; Maurizi et al., 1986). Dissociation of phosphorylated inhibitor should be accompanied by ADP formation. However, the actual rate of turnover of ATP under these incubation conditions is very low. No ADP production was detected after 3 h of GS incubation with $10K_i$ of CHPPT, under coupled assay conditions with a detection limit of 0.02% of the ADP produced in the biosynthetic reaction.

Suppression of Enzyme Reactivation. The non-first-order behavior of reactivation curves contrasts with the first-order recovery of *Escherichia coli* GS activity observed after inactivation with MSO followed by acidification to pH 4.1 (Maurizi & Ginsburg, 1982). This may be a consequence of cooperative subunit interactions during inhibitor release. Alternatively, the recovery behavior may reflect the establishment of a new binding equilibrium after dilution. In the latter case, it should be possible to affect the reactivation behavior by varying significantly the concentration of inhibitor in the incubation mixture and in the final reactivation solution.

The enzyme was incubated for 15 min with ATP and $5K_i$ of CHPPT and was diluted 50-, 100-, and 200-fold, producing final inhibitor solution concentrations of 0.25-, 0.10-, 0.05-, and $0.025K_i$; the same experiment was also performed for GMPPT. No difference was noted in reactivation curves, even at extended times. The enzyme was also incubated for 30 min with ATP and CHPPT at varying concentrations of 5-, 10-, 50-, and $100K_i$, prior to 50-fold dilution, producing final inhibitor solution concentrations of 0.1-, 0.2-, 1.0-, and $2.0K_i$. Again, no difference was noted in reactivation curves. Thus, reactivation behavior was insensitive to final inhibitor concentrations over 2 orders of magnitude. The effect of varied ADP concentration on GS reactivation was investigated by incubation of the enzyme with ATP and $5K_i$ of GHPPT or AMPPT, followed by 50-fold dilution into a solution containing 50 mM $MgCl_2$ and ADP at concentrations of 0.5, 1.0, and 2.5 mM. No difference was noted in reactivation behavior, as compared with dilution in the absence of added ADP. It should be noted that rapid reinactivation of the *E. coli* enzyme by MSO-P released at pH 4.1 has been reported at solution ADP concentrations as low as 0.2 μM (Maurizi & Ginsburg, 1982). Taken together, these experiments suggest that an essentially irreversible dissociation of phosphinothricins from GS occurs upon dilution. If the reactivation mechanism involves irreversible inhibitor dissociation, then it should be possible to suppress GS recovery by including inhibitor in the final dilution mixture, so that competing reactivation and

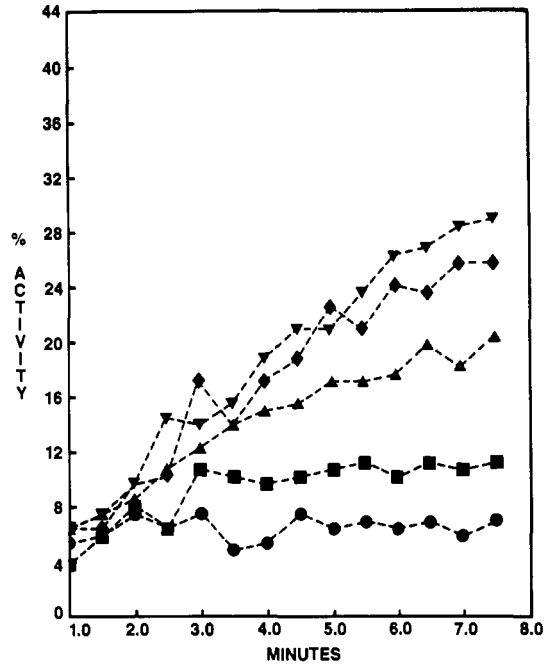


FIGURE 5: Reactivation–suppression of glutamine synthetase activity for CHPPT. The enzyme was incubated with CHPPT at a concentration of $10K_i$, and activity was measured continuously after 50-fold dilution into assay buffer containing the indicated concentrations of CHPPT. (●) $10K_i$; (▲) $2K_i$; (▼) $0.5K_i$; (■) $5K_i$; (◆) $1K_i$.

inactivation may occur simultaneously. Progressive suppression of the reactivation rate should occur with increasing inhibitor concentration, eventually leading to a steady state in which the rates of the two processes are the same. The level of GS activity measured at this point would correspond to the activity available immediately upon dilution in previous inactivation experiments.

The enzyme was incubated for 15 min with ATP and varying concentrations of CHPPT chosen so as to produce final inhibitor concentrations ranging from $0.5K_i$ to $10K_i$ after 50-fold dilution. As illustrated in Figure 5, complete suppression of reactivation occurred at an inhibitor concentration of $10K_i$. Similar behavior was exhibited by the other phosphinothricins, with the exception of PPT and AMPPT, for which GS exhibited insufficient recovery to permit suppression to be measured. Two parameters can be defined from these results: β, the enzyme activity measured at a steady state in reactivation and inactivation, expressed as a fraction of the control (uninhibited) activity, and μ, the inhibitor concentration necessary to achieve this steady state, expressed as a multiple of K_i. Table IV lists these parameters for each of the phosphinothricins. It is evident that besides producing an increase in the K_i value, greater substitution of the phosphinothricin parent structure causes the values of the inactivation/reactivation parameters to increase as well. These changes presumably reflect a poorer ability to compete with L-glutamate for initial binding to GS, as well as a weaker affinity of the enzyme for the inhibitor in the phosphorylated state.

Discussion

Inhibitor K_i values for the substituted phosphinothricins (Table I) were found to correlate with Meister's reported K_m/V_{max} values for the analogously substituted glutamic acids (Figure 1). Enzyme inhibitors functioning as transition-state analogues are expected, on thermodynamic grounds, to exhibit such a correlation with the kinetic properties of the corresponding enzyme substrates (Bartlett & Marlowe, 1983). Although a basic assumption of the Bartlett correlation is that

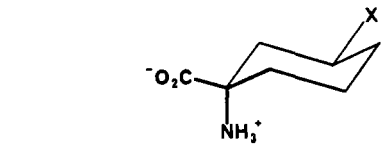
Table IV: Reactivation Parameters for Inhibition of Ovine Brain GS by the Phosphinothricins

inhibitor	K_i (μM)	β^a	μ^b
D,L-PPT (**8**)	14		1
D,L-AMPPT (**10**)	69		1
D,L-GMPPT (**11**)	87	0.08	20
D,L-GHPPT (**12**)	24	0.04	20
D,L-CHPPT (**13**)	55	0.07	10

[a] GS activity measured at steady state in reactivation and inactivation as a fraction of control (uninhibited) activity. [b] Inhibitor concentration necessary to achieve steady state expressed as a multiple of inhibitor K_i value.

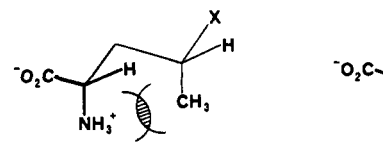
introduced substrate variations not change the energetics of the transformation at the substrate reaction center, a condition most likely met by structural variations at some distance away, our results support the hypothesis that the substituted phosphinothricins participate in transition-state analogue inhibition of GS.

In parallel with substrate data, inhibitor α-alkyl substitution appears to be better tolerated by the enzyme than γ-alkyl substitution. Connection of the α- and γ-substituents in a cyclohexane ring causes a decrease in the measured K_i value relative to both acylic analogues. This effect appears to have a conformational basis, since CHPPT presents its functional groups to the enzyme in a locked, fully staggered array **16b**, whereas acyclic analogues must adopt such a conformation from among many energetically similar possibilities. This interpretation is supported by the failure of *erythro*-γ-methylglutamate (**17a**) to serve as a substrate, in contrast to



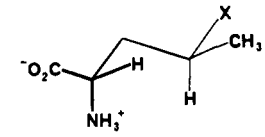
16a X = CO_2^-

16b X = $P(CH_3)O_2^-$



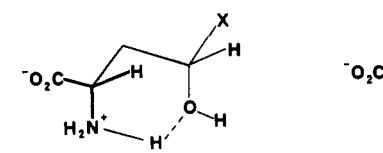
17a X = CO_2^-

17b X = $P(CH_3)O_2^-$



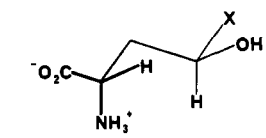
18a X = CO_2^-

18b X = $P(CH_3)O_2^-$



19a X = CO_2^-

19b X = $P(CH_3)O_2^-$



20a X = CO_2^-

20b X = $P(CH_3)O_2^-$

the reported substrate properties of the threo isomer **18a**. In the former case a severe 1,3 steric interaction prevents adoption of the staggered conformation. On the other hand, both γ-hydroxy isomers appear to be well accommodated by the enzyme, since the K_i value of the GHPPT diastereomeric mixture is only slightly higher than that measured for PPT. The staggered conformation of the erythro isomers **19a,b** is likely to be stabilized by hydrogen bonding between the amine and hydroxyl substituents. The exceptional substrate capability of the threo isomer **20a** may reflect the stabilizing effect of a hydrogen-bonding interaction between the γ-hydroxyl group

and the enzyme itself. The enzyme's accommodation of substrate diversity does not extend to the relative distance that must be adopted by the terminal acid functionalities. Loss or introduction of a single methylene unit causes a complete loss of activity, as demonstrated by the failure of D,L-homophosphinothricin (**15a**) to inhibit GS (Table I).

Both L-glutamate and L-phosphinothricin possess terminal acid functionalities that are ionized to the same extent at pH 7.1 and which are capable of undergoing phosphorylation in the same fashion. The inhibitor K_i values may thus be indicative of the ability of the phosphinothricins to compete with glutamate for active-site binding prior to phosphorylation. In each case phosphorylation of the terminal acid group results in an essentially irreversible reaction, i.e., formation of glutamine (Meek & Villafranca, 1980) or formation of the phosphorylated inhibitor I-P. The low inhibitor K_i values observed for the phosphinothricins suggest that the enzyme is able to interact in a favorable way with the *P*-methyl substituent.

Experiments designed to explore the time-dependent behavior of the phosphinothricins provide important additional information concerning their ability to inhibit ovine brain GS. Enzyme inactivation for these compounds was non-first-order overall, as has been observed for MSO inactivation of dodecameric GS from *E. coli* (Rhee et al., 1981; Maurizi & Ginsburg, 1982) and octameric GS from *Saccharomyces cerevisiae* (Kim & Rhee, 1987). This could be due to homologous subunit interactions that progressively decrease the affinity of remaining subunits for inhibitor as inactivation proceeds. However since the rate of inactivation of GS can depend on ATP concentration in the incubation mixture (Maurizi & Ginsburg, 1982), this point requires further study. Initial linearity in logarithmic plots of inactivation permitted the calculation of k'_{inact} values for both MSO and the phosphinothricins. Essentially identical values were observed for these inhibitors, indicating a similar early interaction of the different inhibitors with the octameric enzyme and suggesting that initial enzyme subunits which bind inhibitor are relatively insensitive to structural variation within the inhibitor backbone and even at the terminal group undergoing phosphorylation. Such an interpretation is suggested by kinetic studies showing that addition of ammonia to enzyme-bound γ-glutamyl phosphate, i.e., the formation of reaction intermediate **1**, is considerably slower than the phosphorylation of L-glutamate (Meek et al., 1982). The inactivation behavior of the phosphinothricins does not appear to influence K_i values significantly, since GHPPT displays a considerably smaller K_i than AMPPT, yet is a demonstrably poorer inactivator of the enzyme. Several substituted phosphinothricins displayed smaller K_i values than MSO, yet were incapable of completely inactivating GS.

Reactivation and reactivation/suppression experiments indicate that only phosphinothricins with minimal substitution are capable of producing complete inactivation, presumably as a consequence of phosphorylation and binding of I–P to most if not all subunits of the enzyme. These results suggest that the conformation eventually adopted by subunits binding I–P is highly sensitive to inhibitor substitution, perhaps with a cumulative effect arising from subunit–subunit interactions. Highly substituted inhibitors may also bind to all subunits, either without phosphorylation at some active sites or with formation of loosely bound I–P at these sites. In either case, dilution causes a rapid dissociation of inhibitor from these subunits, manifested as the "burst" activity. Inactivation of GS by MSO under mixed incubation conditions reflects the ready access of MSO to such loosely blocked active sites. The potent, essentially irreversible inactivation of GS by the α-methyl-substituted AMPPT suggests a highly favorable interaction of the enzyme in this conformation with the α-methyl group, enhancing the binding of the corresponding phosphorylated I–P as compared with PPT itself. By comparison, the inactivation behavior of GHPPT is relatively ineffective, considering the low K_i value for this inhibitor. Such a result indicates that the γ-hydroxyl group contributes favorably to initial inhibitor binding to the enzyme, but destabilizes the binding of I–P after phosphorylation has occurred.

The apparent irreversibility of GS reactivation after incubation with the phosphinothricins, and the absence of an equilibrium after inhibitor dissociation, are likely to be the result of hydrolysis of I–P. Mixed phosphinate–phosphate anhydrides of this type should hydrolyze more readily than the relatively stable MSO-P, which can be isolated (Ronzio et al., 1969; Maurizi & Ginsburg, 1982), but which is considerably less stable in solution than when bound to GS (Ginsburg et al., 1987). Because of such hydrolysis of I–P, either in solution or within the enzyme active site itself, the course of GS inhibition by the phosphinothricins is best depicted as

$$E + I \rightleftharpoons E{\cdot}I \rightarrow E{\cdot}I\text{–}P \rightarrow E + I + P \qquad (5)$$

The kinetic behavior of the phosphinothricins makes it possible to distinguish the effects of substitution on different aspects of the inhibition process, as measured by K_i determinations or observation of inactivation/reactivation. The experimental parameters β and μ provide a simple characterization of the latter processes and can be used in evaluating the overall effect of these inhibitors on the enzyme. The phosphinothricins display a continuum of kinetic properties that are a function of substitution pattern and in which the unsubstituted parent structure of PPT may not always ensure maximal interaction with the enzyme. The interaction of the phosphinothricins with ovine brain GS raises interesting questions about the stoichiometry of inhibitor binding and the involvement of the presumed phosphorylated inhibitor I–P in a mechanistic scheme such as eq 5. Studies aimed at elucidating these questions are in progress and will be reported in due course.

Acknowledgments

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