Inhibition of *Escherichia coli* Glutamine Synthetase by α- and γ-Substituted Phosphinothricins

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ABSTRACT: We have investigated the inhibition of *Escherichia coli* glutamine synthetase (GS) with α- and γ-substituted analogues of phosphinothricin [L-2-amino-4-(hydroxymethylphosphinyl)butanoic acid (PPT)], a naturally occurring inhibitor of GS. These compounds display inhibition of bacterial GS that is competitive vs L-glutamate, with K_i values in the low micromolar range. At concentrations greater than K_i the phosphinothricins caused time-dependent loss of enzyme activity, while dilution after enzyme inactivation resulted in recovery of enzyme activity. ATP was required for inactivation; the nonhydrolyzable ATP analogue AMP-PCP failed to support inhibition of GS by the phosphinothricins. The binding of these inhibitors to the enzyme was also characterized by measurement of changes in protein fluorescence, which provided similar inactivation rate constants k_1 and k_2 for the entire series of compounds. Rate constants k_{off} for recovery were also determined by fluorescence measurement and were comparable for both PPT and the γ-hydroxylated analogue GHPPT and significantly greater for the α- and γ-alkyl-substituted compounds. Electron paramagnetic resonance spectra provided information on the interaction of the phosphinothricins with the manganese form of the enzyme in the absence of ATP, and significant binding was observed for PPT and GHPPT. ^{31}P NMR experiments confirmed that enzyme inactivation is accompanied by hydrolysis of ATP, although phosphorylated phosphinothricins could not be detected in solution. The kinetic behavior of these compounds is consistent with a mechanism involving inhibitor phosphorylation, followed by release from the active site and simultaneous hydrolysis to form P_i and free inhibitor.

We have recently (Logusch et al., 1989) described the inhibition of ovine brain glutamine synthetase (GS; EC 6.3.1.2) by novel α- and γ-substituted analogues of the naturally occurring GS inhibitor phosphinothricin [L-2-amino-4-(hydroxymethylphosphinyl)butanoic acid (PPT)]. The inhibition of mammalian GS was consistent with a mechanistic scheme involving initial phosphorylation and partial inhibitor dissociation, followed by slower release of remaining bound inhibitor. These compounds displayed inhibition competitive with glutamate, as well as time-dependent enzyme inactivation and reactivation. ATP-dependent loss of GS activity was observed during incubation with each inhibitor, followed by slow recovery. Introduction of substituents at the α- and γ-positions of phosphinothricin caused weaker initial binding of inhibitor as well as faster enzyme reactivation. Since identical catalytic reaction mechanisms have been proposed for GS from both eukaryotes and prokaryotes (Weisbrod & Meister, 1973; Maurizi et al., 1987), we were interested in examining the interaction of bacterial GS with the α- and γ-substituted phosphinothricins.

This paper presents a mechanistic investigation of the inhibition of *Escherichia coli* GS by the substituted phosphinothricins. Kinetic methods have been employed to characterize substrate competition and enzyme inactivation and reactivation. Enzyme conformational changes accompanying inhibition have been examined with fluorescence measurements and electron paramagnetic resonance (EPR) spectroscopy. The postulated dependence of GS inhibition on phosphorylation by ATP was supported by the failure of the nonhydrolyzable analogue AMP-PCP to support any interaction of inhibitors with the enzyme, and the stoichiometry of inhibitor binding to the enzyme was studied through the use of [γ-^{32}P]ATP. GS-mediated inhibitor phosphorylation has been further investigated by ^{31}P NMR spectroscopy for substituted phosphinothricins and for a phosphonic acid analogue. While the overall inhibition process appears to be similar for both eukaryotic and prokaryotic enzymes, subtle interspecies differences were observed in the interaction of the phosphinothricins with GS from different sources. The implications of these findings will be discussed.

Materials and Methods

Chemicals. All inorganic, organic, and biochemical reagents were purchased from Sigma Chemical Co., with the exception of tetralithium adenylyl methylenediphosphonate (AMP-PCP), which was purchased from Boehringer Mannheim Biochemicals. Glutamine synthetase was prepared from *E. coli* cells grown in a nitrogen-limiting medium and was isolated by using precipitation with Zn^{2+} (Miller et al., 1974). 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 *E. coli* GS were carried out in a standard 50 mM Hepes buffer (pH 7.5) containing 15 mM $MgCl_2$ and 100 mM KCl unless otherwise stated. Purified enzyme was stored at 4 °C as a precipitate in saturated $(NH_4)_2SO_4$ containing 15 mM $MgCl_2$. Stock solutions of GS were prepared by dialysis of the precipitated enzyme at 4 °C against standard buffer or by desalting in 10-unit aliquots on 6 mL Sephadex G-25 columns (Logusch et al., 1989). The concentration and adenylylation state of the enzyme were measured spectrophotometrically (Stadtman & Ginsburg, 1974). The adenylylation state was determined to be 3.2.

(*a*) *Phosphate Release* (*Shapiro & Stadtman, 1970*). GS activity was determined at 37 °C in a 200-μL reaction volume containing 6 mM NaATP, 50 mM ammonium chloride, and sodium L-glutamate (concentration varied as needed). Re-

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actions were initiated by the addition of enzyme (1.5–10 μg) 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.

(*b*) *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 (1.5–10 μg). 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, and the resulting reaction rates were corrected for any change occurring in the absence of added ammonium chloride (control). The inhibitors employed in this study did not inhibit the coupling enzymes in control reactions which included ADP as a substrate and excluded GS.

Enzyme Inhibition. Determinations of inhibition constants (K_i) according to the Lineweaver–Burk method were made as described previously (Logusch et al., 1989). For K_i determinations according to the Dixon method, GS reaction velocities (V) were measured at 37 °C over a range of inhibitor concentrations, at three or more substrate concentrations. Plots of reciprocal velocity vs inhibitor concentration were generated by using a least-squares analysis, and K_i values were determined from the x coordinate (inhibitor concentration) of the intersection point of such plots. In cases where points corresponding to low substrate and high inhibitor concentration deviated from linearity because of enzyme inactivation, such points were deleted before least-squares analysis.

Enzyme Inactivation. Enzyme aliquots (0.01 unit) were incubated at 37 °C in 25 μL with 6 mM NaATP and inhibitor (concentration varied as needed). At each desired time point an aliquot was diluted with 975 μL of a solution containing the components necessary for the coupled assay, as well as sodium L-glutamate (40 mM) and ammonium chloride (50 mM), preincubated for 5 min at 37 °C. After vortexing, the resulting mixture was immediately transferred to a cuvette, and absorbance was monitored continuously for 10 s, and an average activity was calculated as a percent of control (uninhibited) activity. For incubations with tetralithium AMP-PCP, ATP was excluded from the reaction mixture and AMP-PCP was included (6 mM), and subsequent assay dilutions contained ATP. Separate control experiments showed that GS was not inhibited in the presence of both ATP and AMP-PCP, indicating that reagent Li^+ was not inhibitory under these conditions.

Inactivation Stoichiometry. Incubations were performed in 1-mL reaction volumes containing 200 μM GS, 400 μM [γ-^{32}P]ATP (885 cpm/nmol), and 1 mM inhibitor in standard buffer. After 30 min at 37 °C, the reaction mixture was applied to 3-mL gel permeation columns and centrifuged according to the method of Penefsky (1979). The eluant was counted for radioactivity and analyzed spectrophotometrically at 260 nm for adenine. Alternatively, the incubation mixture was centrifuged at 150000*g* for 1 h, and the supernatant was analyzed for nucleotide concentration and radioactivity.

Enzyme Reactivation. Enzyme (0.01 unit) was incubated for 20 min at 37 °C in 25 μL with 6 mM NaATP and inhibitor (concentration varied as needed). The incubation mixture was then diluted to 1.0 mL and assayed continuously as described under enzyme inactivation.

Fluorescence Measurements. Fluorescence experiments were carried out at 25 °C in 1-cm quartz cuvettes on a Perkin-Elmer MPF-44B fluorescence spectrophotometer. An excitation wavelength of 300 nm was employed to excite only tryptophan residues, and emission was detected at 336 nm.

Fluorescence titrations were performed by adding titrant solutions containing inhibitors and 5 μM GS to initial enzyme–ligand complexes, followed by mixing, so as to obtain final reagent concentrations as follows: enzyme, 5 μM; substituted phosphinothricin, 500 μM; MSO (when used in place of PPT analogues), 1 mM; NaATP, 2 mM; tetralithium AMP-PCP (when used in place of ATP), 1.3 mM.

Determination of rates of fluorescence change during GS inactivation (k_1 and k_2) was performed by adding enzyme–ligand complexes to solutions of inhibitor, followed by mixing, so as to obtain final concentrations as follows: (reagents) enzyme, 5 μM; NaATP, 2 mM; (inhibitors) PPT, 200 μM; GHPPT, 250 μM; AMPPT, 500 μM; GMPPT, 500 μM; CHPPT, 500 μM; MSO, 2 mM. Rate constants were calculated from semilogarithmic plots of fluorescence change ($F_\infty - F_t$) vs time, where F_∞ is the calculated fluorescence at infinite time and F_t is fluorescence at time t.

Determination of rates of fluorescence change during GS reactivation (k_{off}) was performed by first incubating the enzyme, 100 μM, with PPT analogue, 100 μM, and ATP, 200 μM, at 37 °C for 90 min or longer, followed by aliquoting 20 μL of the incubation mixture into 1.98 mL of 12 mM ADP and mixing.

EPR Measurements. The apoenzyme used for EPR experiments was obtained by dialysis of the magnesium-containing enzyme against 10 mM EDTA and standard buffer, followed by exhaustive dialysis against standard buffer. The enzyme solution was concentrated on an Amicon concentrator and determined by spectrophotometric measurement. Solutions of $MnCl_2$ were prepared immediately before use and were not corrected for concentration. EPR spectra were recorded at 26 °C on a Varian E-12 spectrometer at 35 GHz with an E-266 cavity, with data collection and reduction on a Telek S-100 280A computer and CalData D/A converter. Quartz sample tubes (0.2 mm i.d.) were prepared with enzyme concentrations of 800 μM in standard buffer containing 0.7 mM $MnCl_2$ and 10 mM inhibitor and were allowed to stand for 10 min before spectra were taken.

NMR Measurements. ^{31}P NMR spectra were recorded at ambient temperature on a Bruker WM-360 spectrometer at 145.8 MHz with broad-band proton decoupling at 6.4 kHz. Samples of the enzyme (10–40 mg) were incubated for 5–12 h at ambient temperature in standard buffer with varied but equal concentrations of inhibitor and NaATP. Carbon tetrachloride was added to each sample, and the mixture was centrifuged at 10000*g* to remove precipitated enzyme. EDTA was added to the aqueous phase to a concentration of 40 mM, and the resulting sample was diluted with an equal volume of D_2O and transferred to a 10-mm NMR tube.

Results

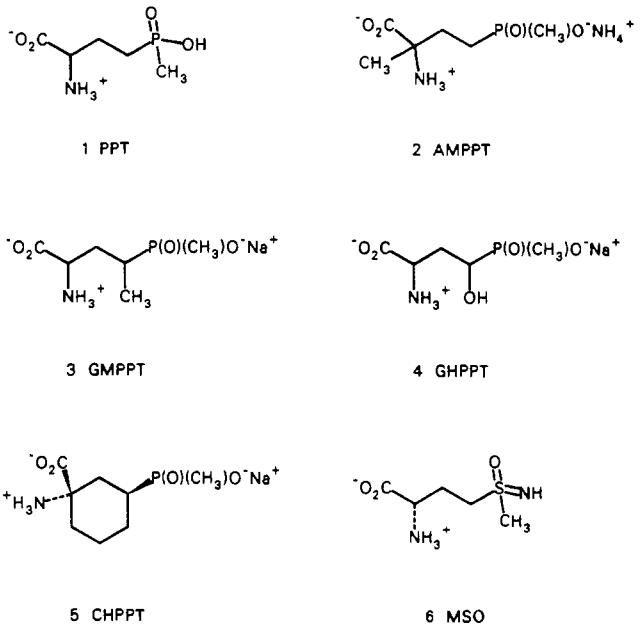
Competitive Inhibition. The phosphate release assay was

Table I: Measured and Corrected K_i Values for Inhibition of *E. coli* GS by MSO and the Phosphinothricins

inhibitor	K_i (μM)	corr K_i[a] (μM)	inhibitor	K_i (μM)	corr K_i[a] (μM)
L-MSO (**6**)	2.0	1.0[b]	D,L-GMPPT (**3**)	25	6.3[c]
D,L-PPT (**1**)	1.1	0.6	D,L-GHPPT (**4**)	1.6	0.8
D,L-AMPPT (**2**)	5.7	2.9	D,L-CHPPT (**5**)	24	12

[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.

used for K_i determinations with the following compounds, as described previously (Logusch et al., 1989): D,L-phosphinothricin (**1**, PPT); D,L-α-methylphosphinothricin (**2**, AMPPT); D,L-γ-methylphosphinothricin (**3**, GMPPT); D,L-γ-hydroxyphosphinothricin (**4**, GHPPT); D,L-cyclohexanephosphinothricin (**5**, CHPPT).



Determination of K_i values was performed by using either the Lineweaver–Burk or Dixon method, and the results are presented in Table I. The K_i value determined for diastereomerically mixed L-methionine (*R,S*)-sulfoximine (**6**, MSO) in the phosphate release assay was 2.0 μM, in agreement with the value of 0.96 μM previously reported for the single isomer L-methionine *S*-sulfoximine (Wedler et al., 1982). The K_i value measured for PPT in the phosphate release assay was 1.1 μM, while a value of 1.3 μM was obtained by using the coupled assay. Corrected K_i values are also given in Table I, taking into account the enantiomeric and stereoisomeric characteristics of the substituted phosphinothricins (Logusch et al., 1989).

The α- and γ-substituted phosphinothricins all displayed K_i values in the low micromolar range, with PPT being the most potent inhibitor. The tight binding of the phosphinothricins to *E. coli* GS is consistent with the inhibition observed for MSO, which displayed an inhibitor K_i value 2 orders of magnitude smaller than that observed with ovine brain GS (2.0 vs 210 μM; Logusch et al., 1989). Since substrate K_m values for α- and γ-substituted glutamates have not been reported for the bacterial enzyme, no correlation of substrate and inhibitor kinetic properties is possible at this time.

Enzyme Inactivation. Inactivation experiments were performed by incubating individual aliquots of *E. coli* GS with each inhibitor at a concentration of $5K_i$, together with ATP and $MgCl_2$. The results are illustrated in Figure 1. Phosphinothricin and MSO both produced complete inactivation within 5 min. The inactivation of bacterial GS by PPT was first order throughout, in agreement with previous results (Colanduoni & Villafranca, 1986), while GS inactivation by MSO displayed a significant decrease in rate with time, consistent with previous observations for the inactivation with MSO of bacterial GS (Rhee et al., 1981) and mammalian GS (Logusch et al., 1989). Initial rates of inactivation were calculated for both PPT and MSO by using measurements taken within the first 60 s, which adhered to first-order kinetics. On the basis of these data, methionine sulfoximine and phosphinothricin displayed $t_{1/2}$ values of 48 and 26 s, respectively, and K'_{inact} values of 1.44×10^{-2} and 2.67×10^{-2} s^{-1}, respectively. The latter value is in agreement with the previously reported rate constant for inactivation of 2.5×10^{-2} s^{-1} (Colanduoni & Villafranca, 1986). None of the substituted phosphinothricins produced complete inactivation of *E. coli* GS (Figure 1). However, kinetic rate data could be obtained from direct measurement of changes in enzyme fluorescence (vide infra). The inactivation process for all compounds was dependent on the presence of ATP in the incubation mixture, since no decrease in GS activity was noted in the absence of ATP. No inhibition or inactivation was observed when the nonhydrolyzable ATP analogue tetralithium adenylyl methylenediphosphonate (AMP-PCP) was substituted for ATP in incubations.

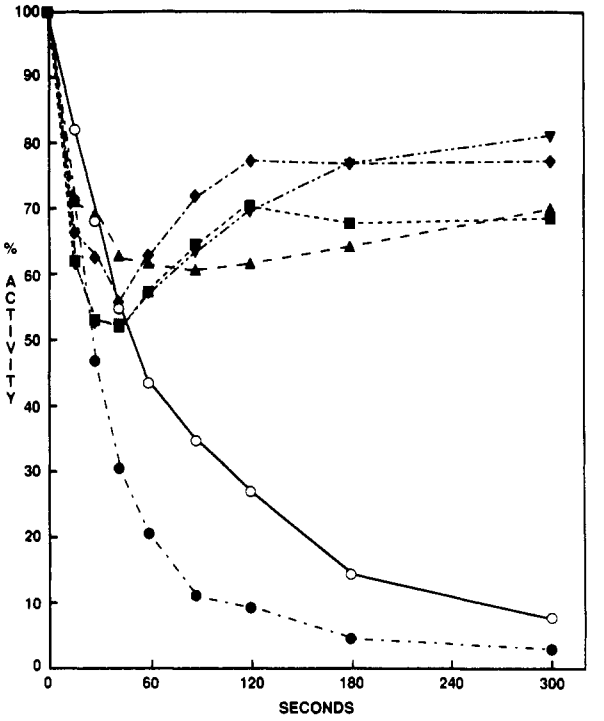


FIGURE 1: Inactivation of glutamine synthetase by phosphinothricin analogues. The enzyme was incubated with each inhibitor at a concentration of 5 K_i, and enzyme activity at each time point was determined by coupled assay after 40-fold dilution. (○) MSO; (■) AMPPT; (◆) GMPPT; (●) PPT; (▲) GHPPT; (▼) CHPPT.

Enzyme Reactivation. Reactivation of GS following inactivation by the phosphinothricins has been previously reported for the ovine brain enzyme (Logusch et al., 1989). *E. coli* GS was inactivated in a similar manner and was assayed after 40-fold dilution (Figure 2). Under these conditions no recovery was seen in the activity of bacterial GS inactivated with methionine sulfoximine, whereas slight but measurable recovery of enzyme activity was observed after inactivation with phosphinothricin, particularly at longer times (30-min time point of Figure 2). Considerable recovery was observed for

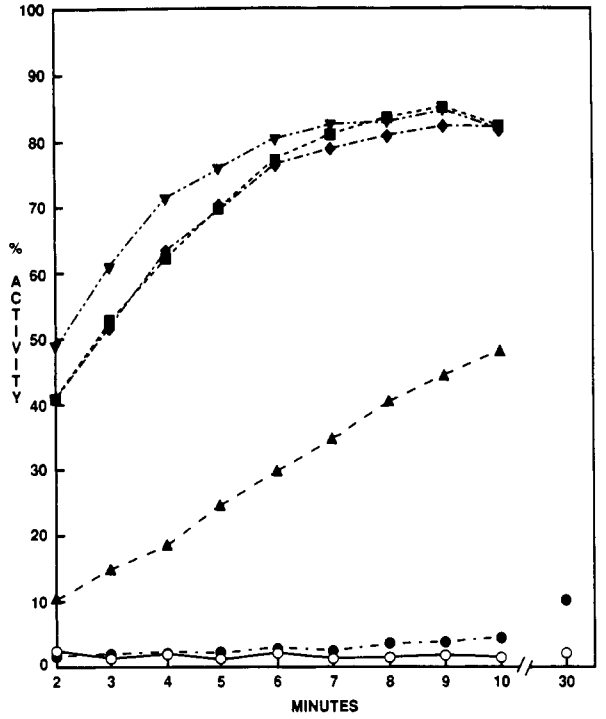


FIGURE 2: Recovery of glutamine synthetase activity after inactivation with phosphinothricin analogues. The enzyme was incubated with each inhibitor at a concentration of $5K_i$, and enzyme activity was measured continuously via coupled assay after 40-fold dilution. Final time point shows activity measured for MSO and PPT 30 min after dilution. (○) MSO; (■) AMPPT; (◆) GMPPT; (●) PPT; (▲) GHPPT; (▼) CHPPT.

Table II: Rate Constants for Inactivation (k_1, k_2) and Reactivation (k_{off}) of *E. coli* GS by MSO and the Phosphinothricins As Determined by Fluorescence Perturbation

inhibitor	k_1 (s^{-1})	k_2 (s^{-1})	k_{off} (s^{-1})
L-MSO (**6**)	0.1	0.8×10^{-2}	
D,L-PPT (**1**)	0.5	3×10^{-2}	
D,L-AMPPT (**2**)	0.2	4×10^{-2}	2×10^{-2}
D,L-GMPPT (**3**)	0.3	4×10^{-2}	2×10^{-2}
D,L-GHPPT (**4**)	0.3	3×10^{-2}	0.4×10^{-2}
D,L-CHPPT (**5**)	0.2	4×10^{-2}	2×10^{-2}

GHPPT, while the most dramatic reversal of inactivation was displayed by the α- and γ-alkylated phosphinothricins. The rapid reactivation observed after incubation with the latter compounds is in contrast to the behavior of ovine brain GS, for which no recovery was seen in the case of AMPPT.

Fluorescence Measurements. Fluorescence titration experiments were performed to measure directly the binding of the substituted phosphinothricins to glutamine synthetase and to various complexes of the enzyme. Addition of saturating concentrations of these inhibitors to native GS produced essentially no change in observed fluorescence. In addition, no change in fluorescence was seen upon addition of inhibitors to a preincubated complex of GS and AMP-PCP. However, addition of inhibitors to the enzyme–ATP complex produced a rapid fluorescence change.

Kinetic characterization of the GS inactivation process was obtained by adding a large saturating excess of inhibitor to the enzyme–ATP complex and monitoring the resulting increase in tryptophan fluorescence. Semilogarithmic plots of fluorescence change vs time were biphasic and permitted the calculation of two rate constants k_1 and k_2, which are given in Table II. The initial rate constants k_1 are significantly greater than the inactivation rate constants obtained from assays of enzyme activity, while the secondary values k_2 are comparable to assay-derived values. The rate constants observed for the entire series of phosphinothricins are similar and are slightly greater than the values for MSO.

Table III: EPR Line Widths Measured for the Binding of the Phosphinothricins to *E. coli* GS[a,b]

complex	line width, *G* (35 GHz)
GS-Mn^{2+}	17.5
GS-Mn^{2+}-D,L-PPT (**1**)	11
GS-Mn^{2+}-D,L-AMPPT (**2**)	20
GS-Mn^{2+}-D,L-GMPPT (**3**)	19
GS-Mn^{2+}-D,L-GHPPT (**4**)	11.5
GS-Mn^{2+}-D,L-CHPPT (**5**)	18

[a] Measured at 35 GHz. [b] Using the Mn^{2+} enzyme.

The observation of two distinct rate constants for the inactivation process is consistent with a mechanistic scheme based on an earlier proposal (Colanduoni & Villafranca, 1986). According to this scheme, inactivation is initiated by reversible binding, characterized by the inhibition constant, K_i, followed by irreversible steps of inhibitor phosphorylation (k_1) and enzyme conformational change (k_2), as illustrated in

$$E + I \overset{K_i}{\rightleftharpoons} E{\cdot}I \xrightarrow{k_1} E{\cdot}I{-}P \xrightarrow{k_2} E^{*}{\cdot}I{-}P \qquad (1)$$

The recovery of GS activity after dilution has been previously attributed to release of the phosphorylated inhibitor I–P from the enzyme active site or to hydrolysis of I–P at the active site followed by dissociation of inhibitor (Logusch et al., 1989). The rate of recovery for *E. coli* GS could be measured directly by following the decrease in protein tryptophan fluorescence. The enzyme was incubated for 90 min with inhibitor and ATP and was diluted 100-fold with standard buffer containing 12 mM ADP. Initial rate constants k_{off} were determined from semilogarithmic plots as described earlier for GS inactivation and are presented in Table II. Fluorescence changes at extended times were non first order. The off-rates for α- and γ-alkyl-substituted phosphinothricins are similar and are significantly higher than the off-rate observed for γ-hydroxyphosphinothricin. These results are consistent with the slower recovery in GS activity seen with the latter analogue (Figure 2).

EPR Measurements. An EPR investigation of the binding of methionine sulfoximine to *E. coli* GS has recently been described (Eads et al., 1988). That study demonstrated a direct interaction of the imine nitrogen of L-methionine *S*-sulfoximine with Mn^{2+} at the tight (n_1) metal binding site of GS. A similar direct interaction with Mn^{2+} was not observable for D,L-phosphinothricin, due to very weak Mn–O–P coupling. However, it appeared to us that an EPR study of inhibitor binding to the enzyme active site could provide information on the extent of interaction of GS with the α- and γ-substituted phosphinothricins.

Since the difference between the binding constants of Mn^{2+} at the n_1 and n_2 sites of GS is at least 100-fold in the presence of transition-state analogues such as MSO and PPT, selective binding of Mn^{2+} at the n_1 site can be achieved by using a slight excess concentration of GS over $MnCl_2$. As illustrated in Figure 3, the EPR spectrum of native GS-Mn^{2+} at 35 GHz displays peaks of moderate line width due to the relatively asymmetric ligand field and easy access of solvent (Villafranca et al., 1976). The binding of PPT to GS-Mn^{2+} causes a dramatic change in the EPR spectrum, increasing the peak intensities and decreasing the line width (Figure 3), reflecting increased solvent exclusion and greater ligand field symmetry. The EPR spectra of GS complexes with the substituted phosphinothricins fall between these two extremes, and the spectral shapes and line widths provide an indication of differences in the interaction of these analogues with Mn^{2+} at

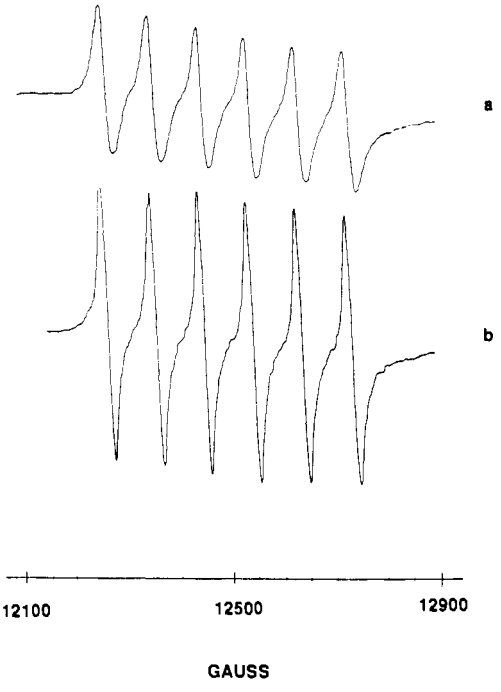


FIGURE 3: EPR spectra (35 GHz) of Mn^{2+} bound to native GS (a) and in the presence of phosphinothricin (b). Solutions contained 800 μM enzyme (subunit concentration) and 700 μM Mn^{2+}, as well as 10 mM PPT (solution b).

Table IV: Stoichiometry of γ-^{32}P Labeling of *E. coli* GS by MSO and the Phosphinothricins[a]

inhibitor	^{32}P equiv	ADP equiv
L-MSO (**6**)	11.1	10.9
D,L-PPT (**1**)	10.2	9.9
D,L-AMPPT (**2**)	9.9	9.2
D,L-GMPPT (**3**)	9.9	9.5
D,L-GHPPT (**4**)	10.7	9.9
D,L-CHPPT (**5**)	9.9	9.5

[a] Determined after centrifugation of inactivation mixture at 150000*g* for 1 h.

the n_1 site. Quantitative line-width values for the phosphinothricins are presented in Table III.

Stoichiometry of Inhibitor Binding to GS. Since the inactivation process is presumed to involve phosphorylation of the inhibitor, we considered that the use of [γ-^{32}P]ATP as a substrate could provide a direct measurement of the binding of $^{32}P_i$ and ADP to the enzyme after incubation with the phosphinothricins. Inactivation experiments were performed with inhibitors and [γ-^{32}P]ATP; after incubations of 30 min, the reaction mixtures were centrifuged through Sephadex G-25 columns (Penefsky, 1979), and the eluant was analyzed for ^{32}P and adenine. Under these conditions retention of radiolabel and ADP was observed only for MSO and PPT, implying that the binding of the substituted phosphinothricins in a GS inactivation complex was too weak to observe by this method.

An alternative approach to stoichiometry measurement involved ultracentrifugation of the inactivation reaction mixture. Inactivations were performed as described before; the incubation mixtures were contrifuged at 150000*g* for 1 h, and the supernatants were analyzed for radioactivity and adenine concentration. It was found that ATP alone does not bind tightly to GS under these conditions, since no [γ-^{32}P]ATP was bound to the enzyme when inhibitors were withheld from incubation mixtures. As illustrated in Table IV, inactivation of GS with MSO and PPT results in labeling of between 10 and 11 enzyme subunits. Enzyme inactivation with the substituted phosphinothricins resulted in a comparable degree of

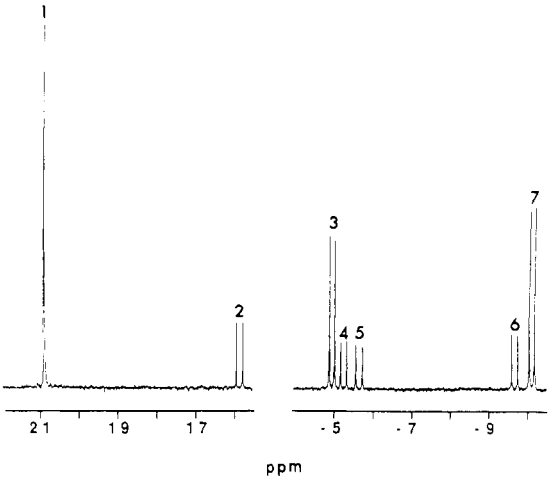
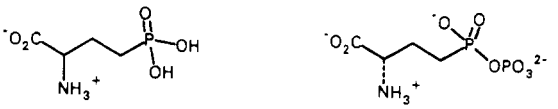


FIGURE 4: ^{31}P NMR spectrum (145.8 MHz) of GS reaction with D,L-APB. 40 mg of enzyme was incubated for 12 h with 100 mM ATP and 100 mM inhibitor, followed by precipitation with carbon tetrachloride and centrifugation. ^{31}P resonances identified as follows (shifts in ppm downfield from H_3PO_4; coupling constants in Hz): (1) APB (20.89, uncoupled); (2) α-APB–P (15.92; 24.32); (3) γ-ATP (−4.91; 19.50); (4) β-ADP (−5.22; 21.76); (5) β-APB–P (−5.62; 24.41); (6) α-ADP (−9.64; 21.77); (7) α-ATP (−10.08; 19.27).

labeling. This result indicates that essentially all subunits of the enzyme can be occupied by inhibitors and ATP under saturating conditions, although binding of phosphorylated inhibitor and ADP cannot be distinguished from binding of unchanged inhibitor and ATP by this method.

NMR Experiments. Inhibitor phosphorylation has been strongly implicated in GS inactivation, and ^{31}P NMR experiments were performed in an attempt to obtain direct evidence for this process. We first examined the interaction of *E. coli* GS with the glutamate phosphonate analogue **7** [D,L-



7 APB 8 APB-P

2-amino-4-phosphonobutyric acid (APB)]. APB has been previously reported as a weak competitive inhibitor of GS, with a K_i value of 1.3 mM for the rat liver enzyme (Lejczak et al., 1981). For ovine brain GS we have previously determined that D,L-APB displays an inhibitor K_i value of 0.32 mM vs L-glutamate and, when incubated alone with GS, causes ATP turnover with a V_{max} equivalent to 5% of the value for L-glutamate (Logusch and McDonald, unpublished results). On the other hand, no time-dependent inactivation of GS by APB was observed. Substrate characteristics are also displayed by APB for *E. coli* GS (substrate k_{cat} 1.3% of the glutamate value and a K_m of 88 mM; Schineller, 1989). These results imply that L-APB undergoes phosphorylation in a manner analogous to L-glutamate, with subsequent release from the enzyme. Such phosphorylation was demonstrated spectroscopically by incubation of GS with 100 mM ATP and 100 mM APB, followed by precipitation with carbon tetrachloride. A detectable accumulation of APB–phosphate **8** in solution was observed, as illustrated by the ^{31}P NMR spectrum shown in Figure 4. Characteristic doublets at 15.92 and −5.62 ppm were observed for the phosphate–phosphonate anhydride of **8**, arising from P–P coupling through the anhydride oxygen linkage.

Because of enzyme inactivation, PPT and the substituted phosphinothricins cause an extremely slow turnover of ATP,

making it difficult to observe directly any accumulation of phosphorus species in solution. Furthermore, the extreme peak broadening exhibited by phosphorus compounds bound to the enzyme prevents their direct observation at the active site. GS inactivation experiments were therefore carried out on a scale sufficient to allow ^{31}P NMR measurement after the release of bound inhibitor from the enzyme was induced. GS was inactivated with the phosphinothricins and then reactivated by treatment with 50 mM acetate, 1 M KCl, and 0.40 M $(NH_4)_2SO_4$ (Maurizi & Ginsburg, 1982a; Colanduoni & Villafranca, 1986). ^{31}P NMR spectra of such reaction mixtures after precipitation, centrifugation, and addition of 10 mM EDTA showed the presence of only inhibitor, P_i, ATP, and ADP. No doublet was detectable in the phosphinate chemical shift region around 40 ppm, indicating the absence of an anhydride with phosphate.

Discussion

Inhibitor K_i values for the substituted phosphinothricins were considerably lower for glutamine synthetase from *E. coli* than for the ovine brain enzyme (Logusch et al., 1989). The K_i value for methionine sulfoximine was also lower in the case of bacterial GS and was comparable to the bacterial K_i value measured for PPT. These results suggest that the active site of the *E. coli* enzyme is better able to accommodate tetrahedral mimics of glutamate in reversible binding. The order of K_i values differs somewhat for both enzymes, although the γ-hydroxyl substituent in GHPPT appears to be generally well tolerated, since K_i values for PPT and GHPPT differ in both cases by a factor of less than 2. EPR studies of the interaction of the phosphinothricins with the Mn^{2+} enzyme in the absence of ATP supported the kinetic data and indicated a close similarity in the binding of PPT and GHPPT to *E. coli* GS.

Inactivation experiments indicate that all the phosphinothricin analogues inactivate *E. coli* GS at about the same initial rate, despite considerable differences in K_i values. In addition, measurement of tryptophan fluorescence perturbation allowed direct observation of enzyme conformational changes during inactivation. It was possible to determine two distinct rate constants for the inactivation process (Table II), which we suggest may represent the phosphorylation reaction (k_1), followed by a subsequent rate-limiting enzyme conformational change (k_2), as illustrated in eq 1. These steps are both considerably slower than glutamate phosphorylation and ammonia addition in the biosynthetic reaction (Meek et al., 1982). The dependence of enzyme inactivation on phosphate transfer from ATP was confirmed by assay and fluorescence measurements and by the failure of AMP-PCP to support any inhibition of the enzyme. The observation that the entire series of phosphinothricins inactivate *E. coli* GS at comparable rates suggests that the enzyme active site is relatively insensitive to inhibitor substitution patterns during phosphorylation.

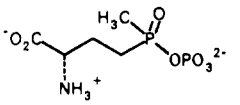
Reactivation experiments assaying enzyme activity after inactivation and dilution showed clear differences in the rate and level of *E. coli* GS recovery, depending on the substitution pattern of the inhibitor used. Only MSO caused complete and irreversible inactivation, similar to the behavior previously observed for the ovine brain enzyme (Logusch et al., 1989). Of the phosphinothricins, PPT caused the greatest level of inactivation, although significant recovery of activity was seen at longer times. The hydroxyl-substituted GHPPT was less effective in inactivation, although recovery was slow and kinetically complex. The alkyl-substituted analogues recovered rapidly and at similar rates. The early phase of the recovery process could be observed directly by changes in protein fluorescence. The initial rate constants k_{off} measured in this way were consistent with the observation of comparable rates of GS recovery for the alkyl-substituted analogues, while a slower rate of fluorescence change was seen for the hydroxyl-substituted GHPPT, reflecting the slower GS reactivation seen for this inhibitor.

Correlation of postdilution GS activity with phosphorylation stoichiometry suggests that essentially all enzyme subunits are occupied by inhibitor during inactivation. Centrifugation after inactivation with all the phosphinothricins resulted in an essentially equimolar association of enzyme, inhibitor, and nucleotide. However, gel filtration experiments indicated that inhibitor binding was qualitatively much greater for PPT and MSO than for any of the other inhibitors, since only these remained bound to the enzyme together with the label from [γ-^{32}P]ATP. These results suggest that all subunits of the enzyme can bind both inhibitor and nucleotide under saturating conditions, although the binding of phosphorylated inhibitor and ADP cannot be distinguished from the binding of unphosphorylated inhibitor and ATP. The varying levels of enzyme recovery observed after inactivation with different inhibitors may reflect combinations of both modes of binding or weaker binding of phosphorylated inhibitor to some subunits, depending on the degree of inhibitor substitution.

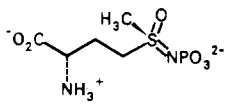
While available evidence supports inhibitor phosphorylation as a requirement for inactivation of GS by the phosphinothricins, efforts to observe phosphorylated inhibitors directly

$$E + I \overset{K_i}{\rightleftharpoons} E{\cdot}I \xrightarrow{k_1} E{\cdot}I{-}P \xrightarrow{k_2} E^*{\cdot}I{-}P \xrightarrow{k_{off}} E + I + P \quad (2)$$

by ^{31}P NMR were unsuccessful. Attempts to dissociate PPT and its analogues from the enzyme caused the release of inhibitor and P_i into solution, as observed spectroscopically. However, such experiments were successful in the case of APB **7**, allowing the detection of free APB–phosphate **8**. This result indicates that the corresponding PPT–phosphate **9**, as well as its analogues, is hydrolytically unstable even at neutral pH. A mechanistic scheme describing the observed kinetic behavior of the phosphinothricins would therefore be as shown in eq 2. It is unclear whether a rate-limiting hydrolysis occurs



9 PPT-P



10 MSO-P

directly at the active site and triggers inhibitor dissociation and enzyme reactivation or whether inhibitors dissociate from the active site in the form **9**, with hydrolysis occurring afterward. It should be noted that MSO–P **10**, the product of GS-mediated phosphorylation of MSO, is considerably less stable in solution than when bound to the enzyme (Ginsburg et al., 1987). In any event, the rate of GS recovery depends on the ability of the enzyme to accommodate substitution in bound intermediates of the type **9**, with PPT displaying the greatest degree of binding apparently as a consequence of its minimal substitution. The crystal structure of *Salmonella typhimurium* GS (Almassy et al., 1986), currently undergoing refinement, may eventually provide sufficient information to rationalize these binding differences in structural terms.

In conclusion, we have shown that the α- and γ-substituted phosphinothricins function as inhibitors of *E. coli* glutamine synthetase with varying degrees of effectiveness. The kinetic properties of the phosphinothricins can be rationalized as a function of their substitution pattern, but in qualitative terms all such inhibitors interact with GS in a similar way, causing enzyme inactivation at comparable rates, followed by enzyme

recovery at differing rates. Phosphorylation of inhibitor accompanies inactivation, and hydrolysis of the resulting inhibitor phosphate accompanies reactivation. By comparison, the extremely slow reactivation of GS inactivated with MSO-P suggests that the free energy of binding of the latter is significantly lower than for PPT-P (Ginsburg et al., 1987). The stabilization of GS quaternary structure that accompanies MSO-P binding (Maurizi & Ginsburg, 1982b) may be a function of the greater hydrolytic stability of the sulfoximine phosphate moiety at the enzyme active site. Additional studies exploring the difference in binding of MSO and PPT-based inhibitors are currently in progress.

Registry No. **1**, 53369-07-6; **2**, 115730-43-3; **3**, 119617-93-5; **4**, 119617-94-6; **5**, 119567-65-6; **6**, 121249-46-5; GS, 9023-70-5.

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A Protein Isolated from *Brucella abortus* Is a Cu-Zn Superoxide Dismutase†

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ABSTRACT: *Brucella abortus* contains a protein that elicits an antigenic response in cattle previously exposed to the organism. The amino acid sequence of the recombinant form of this antigenic protein was determined by gas-phase sequencing of the pyridylethylated protein and its peptides obtained by digestion with cyanogen bromide (CNBr), clostripain, and *Staphylococcus aureus* V8 protease. The *Brucella* protein demonstrated 53.6% identity with the Cu-Zn superoxide dismutase (SOD) from *Photobacterium leiognathi*. Residues essential for metal coordination and enzymatic activity and cysteines required for the formation of the intrasubunit disulfide bridge of Cu-Zn SOD were conserved in the *Brucella* protein. The *Brucella* protein also exhibited SOD activity that was inhibited by cyanide, which is characteristic of a Cu-Zn SOD. *Brucella abortus* Cu-Zn SOD is the second prokaryotic Cu-Zn SOD to be sequenced, and the fifth found in prokaryotes. The high degree of conservation between *Photobacterium* and *Brucella* Cu-Zn SOD supports the hypothesis of a separately evolved prokaryotic and eukaryotic Cu-Zn SOD gene.

Brucella abortus contains a group of immunogenic cell surface and periplasmic proteins which have potential value as a vaccine or as a diagnostic reagent for the prevention and diagnosis of bovine brucellosis (Tabatabai & Deyoe, 1984a,b; Tabatabai et al., 1989). One of the proteins with an apparent molecular weight of 20K, as estimated from sodium dodecyl sulfate-polyacrylamide gel electrophoresis under denaturing conditions, was purified as previously described (Tabatabai et al., 1989). The present study reports the complete amino acid sequence of the recombinant form of this protein from *B. abortus*. Through homology searches and enzyme activity studies, we have determined that the protein from *B. abortus*

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