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Selective inhibition of γ-glutamyl-cycle enzymes by substrate analogs

(glutathione/glutamate and γ-glutamyl amino acid analogs/methionine sulfoximine/γ-glutamyl transpeptidase and cyclotransferase/5-oxoprolinase)

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ABSTRACT **Substrate analogs have been obtained that selectively inhibit the reactions of the γ-glutamyl cycle or that are susceptible to only limited metabolism by the cycle. Thus, glutathione synthesis may be inhibited and analogs of glutathione may be synthesized that do not participate in transpeptidation. Specific inhibitors of γ-glutamylcyclotransferase and 5-oxoprolinase have been obtained. The findings offer new approaches to the *in vivo* study of the cycle and also to the design of more specifically directed analogs of inhibitors such as methionine sulfoximine and 6-diazo-5-oxonorleucine.**

The enzymic reactions of the γ-glutamyl cycle [which accounts for the synthesis and degradation of glutathione (Fig. 1) (1)] include synthesis of the γ-glutamyl peptide bond, transfer of the γ-glutamyl group to an acceptor, cyclization of the γ-glutamyl group to form 5-oxoproline, and energy-dependent decyclization of 5-oxoproline to glutamate. In an approach to further understanding of the function of the cycle, we have sought specific inhibitors of its individual reactions and also substrate analogs that would function in some but not all of the reactions. Such goals might be difficult to achieve because all of these reactions involve the γ-carboxyl group of glutamate, and a given substrate analog might therefore be expected to interact with more than one enzyme. However, the several enzymic reaction pathways are clearly different and are presumably associated with differences in the enzyme-bound conformations of the glutamate carbon chain at the active sites of the several enzymes.

In the present work, we have attempted to exploit such expected differences among the enzymes by manipulation of substrate structure.* We have found that suitable modification of the glutamyl moiety of the substrates yields the desired results: effective and specific inhibitors or nonmetabolizable analogs at each of the steps of the γ-glutamyl cycle. The present findings thus offer several potentially new approaches to the study of the cycle *in vivo* and also provide information relevant to further investigations on the mechanisms of action of these metabolically related enzymes. The data reported here may be applied to the modification of potent (but relatively nonspecific) inhibitors such as methionine sulfoximine, azaserine, and 6-diazo-5-oxonorleucine so as to achieve one-enzyme specificity. The findings also make possible improved assay procedures for γ-glutamyl-cysteine synthetase, glutathione synthetase, and γ-glutamylcyclotransferase in which the substrates and products are not degraded by other enzymes present in unpurified tissue extracts.

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EXPERIMENTAL

Materials. The various compounds were obtained or synthesized as follows: *N*-methyl-L-glutamic acid (Vega-Fox), α-methyl-DL-glutamic acid and L-α-aminobutyric acid (Sigma), D-glutamic acid (K and K), *threo*-β-hydroxyglutamic acid (Nutritional Biochemical Corp.), *threo*-γ-hydroxy-L-glutamic acid (Calbiochem), β-methylglutamic acid (3), γ-methylglutamic acid (4), β-aminoglutarate (β-glutamate) and β-aminoadipate (5), and L-imidazolidone-4-carboxylate (6). DL-Aminomethylsuccinic acid was prepared from dimethylitaconate by the general procedure of Feuer and Swartz (5).

D-γ-Glutamyl-L-α-aminobutyric acid, γ-(γ-methyl)glutamyl-L-α-aminobutyric acid, and β-aminoglutaryl-L-α-aminobutyric acid were prepared via the corresponding *N*-phthaloyl glutamic anhydride analogs as described (7). L-γ-(*N*-Methyl)glutamyl-L-α-aminobutyric acid, L-γ-(α-methyl)-glutamyl-L-α-aminobutyric acid, γ-(β-methyl)glutamyl-L-α-aminobutyric acid, and β-aminoglutaryl-L-α-aminobutyric acid were prepared enzymatically with γ-glutamyl-cysteine synthetase by using phospho*enol*pyruvate and pyruvate kinase as an ATP regenerating system. 5-Oxoproline analogs were prepared from the appropriate amino acid as described (8).

γ-Glutamyl-cysteine synthetase (9) and 5-oxoprolinase (8) were purified from rat kidney. Rat kidney γ-glutamyl transpeptidase and sheep brain γ-glutamylcyclotransferase were prepared by Suresh Tate and Vaira Wellner, respectively, of this laboratory. Glutathione synthetase was purified from rat kidney by a method to be described.

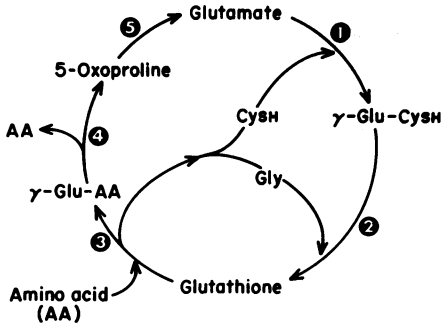
Methods. The various enzyme activities were assayed as described in the tables. In the γ-glutamyl-cysteine synthetase, glutathione synthetase, and γ-glutamyl transpeptidase assays ^{14}C-labeled amino acids were separated from γ-glutamyl ^{14}C-labeled amino acids on small (0.5 × 7 cm) columns of Dowex 1 (acetate). Enzyme reactions were stopped by adding 100-μl aliquots to 1 or 2 ml of 20 mM acetic acid; the solutions obtained were then added to small Dowex 1 columns. ^{14}C-Labeled amino acids were washed through with 4–8 ml of 20 mM acetic acid, and γ-glutamyl amino acids were eluted with 4 ml of 1.5 M ammonium acetate.

RESULTS

Glutathione synthesis

The enzymes that catalyze the two-step synthesis of glutathione from glutamate, cysteine, and glycine (γ-glutamyl-cysteine and glutathione synthetases) also catalyze the synthesis of the naturally occurring analogs, ophthalmic and norophthalmic acids (10–12), compounds in which the cysteine moiety is replaced

* A preliminary account of some of these findings has appeared (2).



Substrate Analogs	ENZYMES ❶ γ-Glu-CysH Synthetase	❷ GSH Synthetase	❸ γ-Glu Trans-peptidase	❹ γ-Glu Cyclo-transferase	❺ 5-Oxoprolinase
D-Glu	0,↓	+	+	0,↓	0
α-Methyl-Glu	(+)	+	+	0	0
N-Methyl-Glu	+	+	0	0	0
β-Glu	+	+	0	0,↓	
β-Methyl-Glu	(+),↓	+,↓	0	+	0,↓
γ-Methyl-Glu	0,↓	+	0	0	(+),↓

FIG. 1. Interaction of glutamate and glutamyl analogs with enzymes of the γ-glutamyl cycle. CysH, cysteine; GSH, glutathione. Symbols are as follows: ↓, >50% inhibition; +, >10% as active as L-Glu; (+), 1–10% as active as L-Glu; and 0, <1% as active as L-Glu.

by α-aminobutyrate and alanine, respectively. In many of the studies that have been carried out on these enzymes, including those reported here, L-α-aminobutyrate has been used in place of L-cysteine to avoid complications associated with the spontaneous oxidation of cysteine and γ-glutamyl-cysteine.

The activity of glutathione synthetase toward a series of γ-glutamyl-α-aminobutyrate analogs is given in Table 1 together with a summary of data previously obtained (9) on γ-glutamyl-cysteine synthetase. It is notable that glutathione synthetase exhibited much broader specificity toward substrates in which the glutamyl group was modified than did γ-glutamyl-cysteine synthetase. Particularly striking was the finding that glutathione synthetase was active toward D-γ-glutamyl-L-α-aminobutyrate. In contrast, although D-glutamate is activated by γ-glutamyl-cysteine synthetase (9), it was not used for peptide formation. Thus, the glutathione synthesis system is protected *in vivo* against introduction of a D-γ-glutamyl residue only at the first step of its biosynthesis.

It is also notable that β-methylglutamate and γ-methylglutamate were essentially inactive as substrates for γ-glutamyl-cysteine synthetase; indeed, they inhibited this enzyme markedly. Whereas the mechanism of γ-glutamyl-cysteine synthetase resembles that of glutamine synthetase in a number of respects (12, 13), the glutamate binding sites of these enzymes seem to differ significantly. Thus, *N*-methylglutamate was not a substrate of glutamine synthetase,† whereas glutamine synthetase was active toward β-methylglutamate and γ-methylglutamate.

Enzymes that utilize glutathione

γ-Glutamyl transpeptidase was significantly active with analogs containing D-glutamyl and α-methylglutamyl residues but not with those containing *N*-methylglutamyl, β-glutamyl, β-methylglutamyl, or γ-methylglutamyl moieties (Table 2). The corresponding analogs of glutathione would be expected to show similar substrate activity with transpeptidase. Previous studies in this laboratory showed that D-γ-glutamyl-*p*-nitroanilide is a highly active substrate of γ-glutamyl transpeptidase (14, 15); because D-amino acids are not acceptor substrates (16, 17), D-γ-glutamyl-*p*-nitroanilide may be used in a convenient assay procedure that precludes autotranspeptidation (15, 18, 19). Similarly, α-substituted amino acids are not acceptors of the γ-glutamyl group (17) and therefore autotranspeptidation does not occur with α-methyl-L-glutamyl-α-aminobutyrate (20). The ability of γ-glutamyl transpeptidase to interact with D- and L-γ-glutamyl substrates is reminiscent of findings with glutamine synthetase that showed that both D- and L-glutamate and α-methyl-L-glutamate bind to the enzyme in an extended conformation (21, 22). However, the substrate–enzyme interactions involved in glutamine synthetase and γ-glutamyl transpeptidase are clearly different because β-aminoglutaryl-L-α-aminobutyrate, β-methylglutamyl-L-α-aminobutyrate, and γ-methylglutamyl-L-α-aminobutyrate are neither substrates nor inhibitors of the transpeptidase whereas the corresponding free glutamate analogs (β-glutamate, β-methylglutamate, and γ-methylglutamate) are substrates of glutamine synthetase (22).

In contrast to the results observed with γ-glutamyl transpeptidase, only one of the analogs examined here, γ-(β-methyl)glutamyl-L-α-aminobutyrate, was a substrate of γ-glutamylcyclotransferase. The fact that the glutamyl derivatives with modifications at the α-carbon atom (i.e., D-glutamyl and α-methylglutamyl) do not react presumably reflects the need in the cyclotransferase reaction to bring the α-carbon atom quite close to the catalytic center of the active site. The requirements for proper size and alignment in this region would be expected to be stringent.

β-Aminoglutaryl-L-α-aminobutyrate was not expected to be a substrate of γ-glutamylcyclotransferase because its reaction would require the formation of a 4-member ring. This analog is, however, an extremely potent inhibitor of the enzyme. As shown in Table 2, β-aminoglutaryl-L-α-aminobutyrate almost completely inhibited the reaction of L-γ-glutamyl-L-α-aminobutyrate at an equimolar concentration. In a similar experiment in which the concentration of inhibitor was reduced 10-fold, the inhibition was 67%. Whereas the inhibition is not accompanied by covalent attachment of the inhibitor, the inhibition is sufficiently strong to be useful *in vivo*. Preliminary experiments have shown that the inhibitor is well transported.

5-Oxoprolinase

Table 3 summarizes data on the interaction of 5-oxoprolinase with several 5-oxoproline analogs. As with the cyclotransferase reaction, the tolerance toward substitution by methyl groups was very limited. 5-Oxo-D-proline, *N*-methyl-5-oxoproline, and 2-methyl-5-oxoproline were neither substrates nor inhibitors. 4-Methyl-5-oxoproline was slightly but significantly active as a substrate; this compound and 3-methyl-5-oxoproline were potent inhibitors. Previous studies showed that the enzyme is active toward 2-piperidone-6-carboxylate and the 3- and 4-hydroxy derivatives of 5-oxo-L-proline (8). L-2-Imidazolidone-4-carboxylate is a potent competitive inhibitor *in vitro* (8, 23), and the effect of this compound has also been demonstrated *in vivo* (6, 23). It is of interest that several of the compounds studied here, which are not decyclized by the enzyme, nevertheless induce ATP cleavage (8, 24, 25).

DISCUSSION

Data on the interaction of substrate analogs with the five enzymes of the γ-glutamyl cycle that catalyze reactions involving

† V. P. Wellner, unpublished studies in this laboratory.

Table 1. Activity of glutathione synthesis enzymes toward substrate analogs

Substrate or analog	γ-Glutamyl-cysteine synthetase* Relative activity	% inhibition	Glutathione synthetase† Relative activity	% inhibition
L-Glutamate	[100]		[100]	
D-Glutamate	1.2	59	51	19
β-Aminoglutarate	17.6	6	13	8
N-Methyl-L-glutamate	23.5	31	62	3
α-Methyl-DL-glutamate	5.9	38	38	15
β-Methyl-DL-glutamate‡	1.6	56	64	59
γ-Methyl-DL-glutamate§	<0.5	95	20	12

* From ref. 9; based on P_i formation. No peptide was formed with D-glutamate.

† Activity was determined by measurement of the incorporation of [^{14}C]glycine into tripeptide in the presence of γ-glutamyl (or analog) L-α-aminobutyrate in 1-ml reaction mixtures containing 150 mM imidazole·HCl (pH 7.2), 100 mM KCl, 10 mM [^{14}C]glycine, 5 mM peptide (10 mM for γ-methyl derivative), 10 mM ATP, 10 mM phospho*enol*pyruvate, 20 mM $MgCl_2$, 0.5 mg of bovine serum albumin, and 2 international units of pyruvate kinase and glutathione synthetase. After 10–60 min, aliquots were assayed for [^{14}C]tripeptide formation (see *Experimental*). Inhibition of L-γ-glutamyl-L-α-aminobutyrylglycine synthesis was determined in 250-μl reaction mixtures containing 100 mM imidazole·HCl (pH 7.2), 100 mM KCl, 10 mM ATP, 10 mM phospho*enol*pyruvate, 20 mM $MgCl_2$, 10 mM L-γ-glutamyl-L-α-aminobutyrate, 10 mM peptide analog (20 mM for γ-methyl derivative), 10 mM [^{14}C]glycine, 0.5 mg of bovine serum albumin, and 2 international units of pyruvate kinase and glutathione synthetase. After 15 and 30 min, aliquots were assayed for [^{14}C]tripeptide formation.

‡ γ-Glutamyl-cysteine synthetase was tested with a mixture of four isomers; the product of this enzymatic reaction was used for studies on glutathione synthetase.

§ Mixture of four diastereoisomers.

glutamate or glutamate derivatives are summarized in Fig. 1. It would be expected that the analogs studied here would be transported into cells and, indeed, this is supported by preliminary studies on a number of them. It is thus possible to consider several *in vivo* approaches in which selective inhibition of specific reactions might be achieved. Glutathione biosynthesis is normally controlled by feedback inhibition of γ-glutamyl-cysteine synthetase by glutathione and probably also by the tissue concentrations of cysteine (12, 13, 26). Depression of tissue levels of glutathione has been observed after administration of D-glutamate (27). The present data indicate that glutathione biosynthesis may be inhibited by administration of β-methylglutamate and γ-methylglutamate.

γ-Glutamyl-cysteine synthetase, like glutamine synthetase (1, 13, 28, 29), is inhibited by methionine sulfoximine, an effect due to phosphorylation of this analog and its binding to the active site of the enzyme as methionine sulfoximine phosphate (29, 30). Administration of methionine sulfoximine to animals leads to markedly decreased levels of glutathione in the kidney and liver (31). The convulsant activity of methionine sulfoximine is probably due to inhibition of glutamine synthetase or γ-glutamyl-cysteine synthetase or both (32, 33). The possibility that the convulsant activity is due to metabolic products derived from methionine sulfoximine via the corresponding α-imino or α-keto derivatives (34) is excluded by recent work in this laboratory in which α-methylmethionine sulfoximine was prepared (O. W. Griffith and A. Meister, unpublished data). As expected from the present (Fig. 1) and previous (28, 29)

Table 2. Activity of glutathione-utilizing enzymes toward substrate analogs

Substrate or analog*	Transpeptidase† Relative activity	% inhibition	Cyclotransferase‡ Relative activity	% inhibition
L-γ-Glutamyl-L-Aba‡	[100]		[100]	
D-γ-Glutamyl-L-Aba	44	12	0	69
β-Aminoglutaryl-L-Aba	0	0	0	96
L-γ-(*N*-Methyl)glutamyl-L-Aba	0	5	0	12
L-γ-(α-Methyl)glutamyl-L-Aba	24	0	0	16
γ-(β-Methyl)glutamyl-L-Aba	0	0	90	22
γ-(γ-Methyl)glutamyl-L-Aba	3	0	0	12
Glutathione	156	0	0	0

* Aba, α-aminobutyrate.

† Transpeptidase activity was determined in 600-μl reaction mixtures containing 175 mM Tris·HCl (pH 8.0), 10 mM donor analog [20 mM for γ-(γ-methyl)glutamyl-L-Aba], 25 mM L-[^{14}C]methionine, and 1.6 units of transpeptidase. Formation of γ-glutamyl-L-[^{14}C]methionine (or analog) was assayed at 5–30 min (see *Experimental*). Inhibition of transpeptidation between L-γ-glutamyl-L-Aba and L-[^{14}C]methionine was measured in 250-μl reaction mixtures containing 175 mM Tris·HCl (pH 8.0), 10 mM L-γ-glutamyl-L-α-aminobutyrate, 10 mM analog [20 mM for γ-(γ-methyl)glutamyl-L-Aba], 25 mM L-[^{14}C]methionine, and 0.8 unit of transpeptidase. Formation of γ-glutamyl-L-[^{14}C]methionine was assayed at 10–60 min. The product formed presumably includes material derived both from L-γ-glutamyl-L-α-aminobutyrate and from those analogs that are active donors.

‡ Cyclotransferase activity was determined as enzyme-dependent release of L-α-aminobutyrate. The 200-μl reaction mixtures contained 100 mM Tris·HCl (pH 8.0), 10 mM analog, and 10 units of cyclotransferase. After 15–120 min, aliquots were removed, quenched in 5% sulfosalicyclic acid, and analyzed for α-aminobutyrate on a Durrum amino acid analyzer. Cyclotransferase inhibition of 5-oxo-L-[^{14}C]proline formation from L-γ-[^{14}C]glutamyl-L-α-aminobutyrate was measured in 250-μl reaction mixtures containing 100 mM Tris·HCl (pH 8.0), 8 mM L-γ-[^{14}C]glutamyl-L-α-aminobutyrate, 8 mM inhibitor, and 10 units of cyclotransferase. At 45 and 90 min, 100-μl aliquots were removed and quenched with 0.5 ml of 1% trichloroacetic acid. The resulting solution was loaded on columns (0.5 × 3 cm) of Dowex 50W (H^+), followed by 3 ml of H_2O. An aliquot of the total effluent was analyzed for ^{14}C.

Table 3. Activity of 5-oxo-L-prolinase toward substrate analogs*

Analog	Relative activity	% inhibition
5-Oxo-L-proline	[100]	
5-Oxo-D-proline	0	1
N-Methyl-5-oxo-L-proline	0	0
2-Methyl-5-oxo-DL-proline	0	1
3-Methyl-5-oxoproline†	0	88
4-Methyl-5-oxoproline†	4	66
threo-3-Hydroxy-5-oxo-DL-proline	19	1
threo-4-Hydroxy-5-oxo-DL-proline	0.3	0
DL-2-Pyrrolidone-4-carboxylate	0	0
DL-2-Piperidone-6-carboxylate	7	6
DL-5-Carboxymethyl-2-pyrrolidone	0	3
L-Imidazolidone-4-carboxylate	0	86

* 5-Oxoprolinase was assayed as enzyme-dependent formation of amino acid. The 1-ml reaction mixtures contained 150 mM Tris·HCl, (pH 7.8), 150 mM KCl, 10 mM analog (20 mM for DL-mixtures, 40 mM for four-isomer mixtures), 10 mM ATP, 5 mM phospho*enol*pyruvate, 15 mM $MgCl_2$, 0.15 mM EDTA, 5 mM dithiothreitol, 10 international units of pyruvate kinase, and 11 units of 5-oxoprolinase. After 30 min, aliquots of the reaction mixtures were analyzed for amino acid on a Durrum amino acid analyzer. Inhibition of 5-oxoprolinase was assayed in 500-μl reaction mixtures containing 100 mM Tris·HCl (pH 7.8), 150 mM KCl, 2 mM 5-oxo-L-[^{14}C]proline, 2 mM 5-oxoproline analog (4 mM for DL-mixtures, 8 mM for four-isomer mixtures), 4 mM ATP, 2 mM phospho*enol*pyruvate, 7.5 mM $MgCl_2$, 0.1 mM EDTA, 5 mM dithiothreitol, 10 international units of pyruvate kinase, and 1 unit of 5-oxo-L-prolinase. After 30 min, the reaction mixtures were analyzed for L-[^{14}C]glutamate as described (8).

† Mixture of four isomers.

findings, α-methylmethionine sulfoximine (which has convulsant activity) inhibits both γ-glutamyl-cysteine synthetase and glutamine synthetase. Additional studies on methionine sulfoximine analogs, based on the glutamate specificities observed here, are now needed with the aim of selectively inhibiting these synthetases.

Glutathione biosynthesis may also be perturbed by administration of *N*-methyl-glutamate or β-glutamate. These glutamate analogs can thus lead to synthesis of glutathione analogs that are not substrates for transpeptidation. It would be of interest to learn whether such modified glutathiones can be used by glutathione reductase or by glutathione *S*-transferases; this type of information might be helpful in elucidating the physiological role of glutathione in relation to specific enzymic phenomena.

Of the several γ-glutamyl-L-α-aminobutyrate analogs that were examined as substrates and inhibitors of glutathione synthetase, all but two (those derived from D-glutamate and γ-methylglutamate) would be expected to be synthesized *in vivo*. It might be possible to introduce *in vivo* a glutathione analog possessing a D-glutamyl moiety or a γ-methylglutamyl moiety by administration of the corresponding COOH-terminal cysteine dipeptide. It would be expected that the D-glutamyl and α-methyl-L-glutamyl modifications of glutathione would be substrates for γ-glutamyl transpeptidase (in contrast to modified glutathiones in which the γ-glutamyl moieties are replaced by *N*-methylglutamyl, β-glutamyl, β-methylglutamyl, or γ-methylglutamyl groups). Although D-γ-glutamyl and α-methylglutamyl derivatives are substrates of the transpeptidase, these compounds are not substrates of γ-glutamylcyclotransferase and in fact D-γ-glutamyl amino acids inhibit this enzyme. Studies with D-γ-glutamyl- or L-α-methyl-glutamyl-L-amino acids might therefore elucidate the extent to which such compounds are hydrolyzed *in vivo* by the action of γ-glutamyl transpeptidase. In a similar manner, γ-(β-methyl)glutamyl-L-α-aminobutyrate may be used to measure *in vivo* cyclotransferase activity without interference by transpeptidase. These studies have also led to the finding of two highly active inhibitors of γ-glutamylcyclotransferase [D-γ-glutamyl-L-α-aminobutyrate and γ-(β-glutamyl)-L-α-aminobutyrate]; preliminary studies indicate that these compounds are transported into cells and they, therefore, may be of value in elucidating the *in vivo* function of γ-glutamylcyclotransferase.

The present and earlier studies thus indicate that the γ-glutamyl cycle may be inhibited with respect to glutathione synthesis (D-glutamate, β-methylglutamate, methionine sulfoximine), transpeptidation [γ-glutamyl hydrazones of α-keto acids (17), azaserine, 6-diazo-5-oxonorleucine (35, 36)], cyclization (D-γ-glutamyl amino acids, β-aminoglutaryl amino acids), and decyclization of 5-oxoproline. These studies have also revealed a number of analogs that are susceptible to only limited metabolism; finally, they suggest logical approaches to the design of new and more specific analogs of azaserine, 6-diazo-5-oxonorleucine, and methionine sulfoximine.

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