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Serine–borate complex as a transition-state inhibitor of γ-glutamyl transpeptidase

(glycoprotein/membrane/azaserine/6-diazo-5-oxonorleucine/optical specificity)

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ABSTRACT **γ-Glutamyl transpeptidase, a membrane-bound enzyme, functions in the γ-glutamyl cycle to catalyze utilization of glutathione. It has been postulated that the amino-acid-stimulated utilization of glutathione by γ-glutamyl transpeptidase reflects an aspect of amino acid translocation. As one approach to the effective *in vivo* inhibition of this enzyme, the inhibition of the enzyme by L-serine in the presence of borate buffers [Revel, J. P. & Ball, E. G. (1959) *J. Biol. Chem.* 234, 577–582] was reinvestigated. Inhibition by L-serine, D-serine, and α-methyl-DL-serine in the presence of borate is competitive with respect to γ-glutamyl substrate and such inhibition is parallel to the activity of transpeptidase toward L-γ-glutamyl, D-γ-glutamyl, and L-γ-(α-methyl)glutamyl derivatives. L-Serine and borate effectively protect against inactivation of the enzyme by the γ-glutamyl analogs, 6-diazo-5-oxonorleucine and azaserine, which bind to the γ-glutamyl site of the enzyme. These studies, kinetic investigations, equilibrium dialysis experiments, and other data support the view that inhibition is produced by formation of serine–borate complex which binds at the γ-glutamyl binding site of the light subunit of γ-glutamyl transpeptidase. The data indicate that serine–borate complex is a transition state inhibitor of γ-glutamyl transpeptidase.**

γ-Glutamyl transpeptidase is a membrane-bound enzyme that functions in the degradation of glutathione. This enzyme thus catalyzes the first step in the utilization of glutathione by the γ-glutamyl cycle, a metabolic pathway that accounts for the synthesis and degradation of glutathione, and which has been postulated to be one of the systems that mediates the translocation of amino acids across cell membranes (1). To explore further the physiological function of this system, we have carried out studies on the structural and catalytic properties of this enzyme and on its specific inhibition. In the course of this work, we reinvestigated the interesting observation by Revel and Ball (2) that L-serine inhibits γ-glutamyl transpeptidase in the presence of borate buffer but not with other types of buffers, and that borate alone did not inhibit the enzyme.

γ-Glutamyl transpeptidase catalyzes the transfer of the γ-glutamyl moiety of glutathione and other γ-glutamyl compounds to a variety of amino acid and dipeptide acceptors; it also catalyzes the hydrolysis of glutathione and γ-glutamyl compounds (3, 4). Studies in this laboratory have shown that highly purified rat kidney γ-glutamyl transpeptidase (molecular weight 68,000) is composed of two glycopeptide subunits of molecular weight 46,000 and 22,000, respectively (5). The postulate that a covalent γ-glutamyl enzyme intermediate is formed during catalysis (3, 6) is supported by the observations that the γ-glutamyl analogs, 6-diazo-5-oxo-L-norleucine and L-azaserine, inactivate the enzyme by reacting specifically and stoichiometrically to the γ-glutamyl site of the enzyme. The γ-glutamyl site is located on the light subunit (7).

Preparations of γ-glutamyl transpeptidases from many sources are strongly inhibited by L-serine in the presence of borate. Such inhibition has been reported to be competitive with respect to γ-glutamyl substrate, and it was suggested that a serine–borate complex is formed which may bind to the active site of the enzyme by interacting with a carbohydrate residue of the enzyme (8).

In the present studies, we have reinvestigated the inhibition of γ-glutamyl transpeptidase by L-serine plus borate. The findings reported here indicate that L-serine and its analogs bind to the γ-glutamyl site of the enzyme by linkages involving the α-amino and α-carboxyl groups. Such binding is enhanced by complex formation between the serine hydroxyl group, borate, and a hydroxyl group at the active center of the enzyme, which is probably a seryl or threonyl residue. We propose that this complex mimics the tetrahedral intermediate or transition state expected to be formed in the normal catalytic reaction, and thus that serine–borate complex is an inhibitory transition-state analog.

EXPERIMENTAL

L-γ-Glutamyl-*p*-nitroanilide, α-methyl-DL-serine, β-phenyl-L-serine, L-serylglycine, glycyl-L-serine, and glycylglycine were purchased from Sigma. L-γ-Glutamyl-L-serine was synthesized in this laboratory by Ralph Stephani. L-[*U*-^{14}C]Serine (135 Ci/mol) was a product of New England Nuclear Corporation. γ-Glutamyl transpeptidase was purified from rat kidney as described (9) [specific activity, 900 μmol of *p*-nitroaniline formed per min per mg of protein; assayed with L-γ-glutamyl-*p*-nitroanilide (2.5 mM) and GlyGly (20 mM)].

The binding of L-[^{14}C]serine to γ-glutamyl transpeptidase was measured by equilibrium dialysis as described by Myer and Schellman (10), with Lucite cells (The Chemical Rubber Co., Cleveland, OH) having a total capacity of 2 ml. The cell was divided into two equal compartments by a semipermeable membrane (3 cm in diameter) cut from cellulose dialysis tubing. The enzyme, 0.48 mg in 0.2 ml of 0.1 M Tris·HCl, pH 8.0/20 mM sodium borate, was dialyzed for 6–8 hr at 25° against 0.2 ml of the Tris borate buffer containing 10–50 nmol of L-[^{14}C]serine. Samples were withdrawn from each compartment of the cell for the determination of [^{14}C]serine.

The mixture of glycosidases used to remove the carbohydrate moieties from transpeptidase was the generous gift of Gil Ashwell (National Institute of Arthritis, Metabolism, and Digestive Diseases, Bethesda, MD). The preparation (from *Diplococcus pneumoniae*, type 1) contained β-galactosidase (4.6 units/ml), *N*-acetylglucosaminidase (10.4 units/ml), neuraminidase (2.1 units/ml), and an undetermined amount of endoglycosidase. α-Mannosidase (from jack bean; 18 units/ml) was purchased from Sigma.

γ-Glutamyl transpeptidase was treated with glycosidases as follows. A reaction mixture containing (per ml) 1.1 mg of transpeptidase, 10 μl of glycosidase preparation, 10 μl of α-mannosidase, and 0.1 M Na_2HPO_4 (adjusted to pH 6.5 with

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citric acid) was incubated at 25° for 16 hr. The transpeptidase was precipitated by adding 4 vol of acetone; the pellet was dissolved in 1 ml of 10 mM Tris·HCl (pH 8.0) and dialyzed against several volumes of this buffer at 4°. Residual carbohydrate was determined as described (9).

L-Aza[^{14}C]serine (labeled at C-6; 4.4 Ci/mol) was synthesized in Standish Hartman's laboratory (Boston University). The labeled compound was diluted with unlabeled azaserine to give a specific activity of 1450 cpm/nmol. Rat kidney transpeptidase was treated with aza[^{14}C]serine as described (7), resulting in covalent binding of 0.85 mol/mol of enzyme and >95% inactivation.

RESULTS

Table 1 shows the effect of L-serine and related compounds on the activity of transpeptidase in the presence and absence of 10 mM sodium borate. Both L-serine and D-serine are potent inhibitors in the presence of borate. Borate alone, as shown previously (2), has no effect on the activity. α-Methyl-DL-serine and L-homoserine also inhibit in the presence of borate, although less effectively than L-serine. Compounds with substitutions on the β-carbon atom of L-serine (e.g., L-threonine and β-phenyl-L-serine) do not inhibit in the presence of borate. β-Hydroxy-L-aspartate (*threo-* and *erythro*-derivatives), L-cysteine, and L-alanine were also ineffective. Of interest is the finding that dipeptides containing L-serine (e.g., L-serylglycine and glycyl-L-serine), although excellent acceptor substrates, do not affect the activity in the presence of borate. L-γ-Glutamyl-L-serine presumably inhibits by virtue of its competition with γ-glutamyl-*p*-nitroanilide as a γ-glutamyl donor substrate; inhibition by this compound is not significantly enhanced by borate.

Kinetic studies, in which GlyGly was held constant at 20 mM and γ-glutamyl-*p*-nitroanilide was varied from 0.1 to 1.5 mM show that, in the presence of borate, L-serine, D-serine, and α-methyl-DL-serine are competitive inhibitors with respect to the γ-glutamyl substrate (Fig. 1*A*); the respective K_i values for these compounds were 0.02, 0.17, and 0.31 mM (Table 2). That these compounds interact with the γ-glutamyl binding site of the enzyme is further supported by the striking parallelism between their effectiveness as inhibitors in the presence of borate and the activity of transpeptidase towards the γ-glutamyl-L-α-aminobutyrate analogs derived from L-glutamate, D-glutamate, and α-methyl-L-glutamate (Table 2). It is notable that γ-(β-methyl)glutamyl-L-α-aminobutyrate and L-threonine are inactive as a substrate and as an inhibitor, respectively. Of interest also is the parallelism between the K_m values for hydrolysis of L- and D-γ-glutamyl-*p*-nitroanilide [5 and 31 μM, respectively (12)] and the K_i values for L- and D-serine in the presence of borate (0.02 and 0.17 mM, respectively). Furthermore, inactivation of the enzyme by the γ-glutamyl analogs, 6-diazo-5-oxo-L-norleucine and L-azaserine, is effectively prevented by both L-serine plus borate.

Table 1. Effect of serine and related compounds on the activity of γ-glutamyl transpeptidase in the presence and absence of borate

Compound added (mM)	Relative activity − Borate	Relative activity + Borate
None	[100]	[100]
L-Serine (5.0)	93	2
D-Serine (5.0)	100	12
α-Methyl-DL-serine (5.0)	100	54
L-Homoserine (5.0)	100	58
L-Threonine (5.0)	100	90
β-Phenyl-L-serine (5.0)	100	100
Ethanolamine (10.0)	100	88
L-Serylglycine (5.0)	100	96
Glycyl-L-serine (5.0)	100	97
L-γ-Glutamyl-L-serine (1.0)	88	82

γ-Glutamyl transpeptidase activity was determined in a solution (final volume, 1.0 ml) containing 0.1 M Tris·HCl (pH 8.0), 2.5 mM L-γ-glutamyl-*p*-nitroanilide, and 20 mM GlyGly. The effect of the compounds shown (concentration given in parentheses) was determined in the presence or in the absence of 10 mM sodium borate. Specific activity of the enzyme (100%; ±borate) was 900.

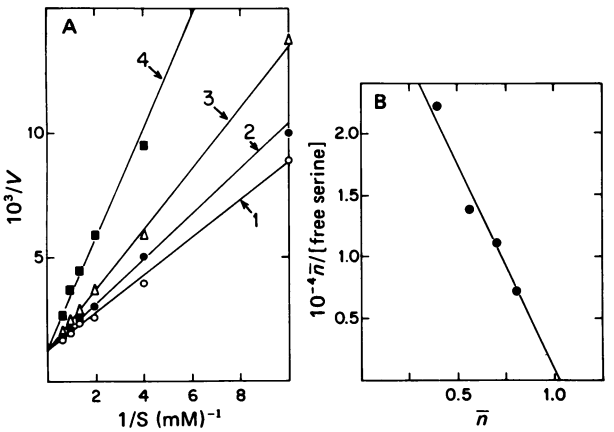

FIG. 1. (*A*) Effect of L-serine, D-serine, and α-methyl-DL-serine on the activity of γ-glutamyl transpeptidase. The reaction mixtures (1.0 ml) contained 0.1 M Tris·HCl (pH 8.0), 10 mM sodium borate, 20 mM GlyGly, and 0.125 μg of the enzyme. L-γ-Glutamyl-*p*-nitroanilide (S) was varied from 0.1 to 1.5 mM in the absence (control, curve 1), or in the presence of 0.3 mM α-methyl-DL-serine (curve 2), 0.1 mM D-serine (curve 3), or 0.05 mM L-serine (curve 4). V = μmol of *p*-nitroaniline released/min per mg of enzyme. (*B*) Binding of L-serine in the presence of borate by γ-glutamyl transpeptidase. Binding of L-[^{14}C]serine in the presence of 20 mM sodium borate was measured by equilibrium dialysis. The Scatchard plot of the data is shown; $\bar{n}$, average moles of L-serine bound per mole of enzyme.

The strong interaction between the enzyme, borate, and L-serine indicated by the kinetic studies was confirmed by binding measurements carried out by equilibrium dialysis (Fig. 1*B*). The dissociation constant for the enzyme–L-serine–borate complex calculated from these data was 0.03 mM; this is in good agreement with the K_i value for L-serine plus borate obtained by kinetic measurements (Table 2). The data given in Fig. 1*B* extrapolate to a maximum of 1 mol of L-serine bound per mol of enzyme. Of interest is the finding that enzyme inactivated by preincubation with diazo-oxonorleucine, as described (7), followed by removal of excess diazo-oxonorleucine by dialysis, did not bind L-serine in the presence of borate under the conditions of the experiments described in Fig. 1*B*. No binding of L-serine to the untreated enzyme could be demonstrated under these conditions in the absence of borate.

The inhibition by serine plus borate is a readily reversible process; thus, the activity is restored by dilution or by dialysis. Prolonged incubation of transpeptidase with L-serine and borate (2 mM and 10 mM, respectively; 18 hr at 37°), followed by analysis of the mixture on a Durrum model D500 amino acid analyzer, did not reveal a new product, nor did it show disappearance of serine.

The possibility that a carbohydrate residue of transpeptidase may be involved in the binding of serine and borate was investigated by comparing the properties of native enzyme with the enzyme that was treated with a mixture of glycosidases as described above. Such treatment resulted in complete removal of sialic acid and the removal of about 75% of the aminohexose and hexose residues. The enzyme activity, as well as the sus-

Table 2. Activity of transpeptidase towards various γ-glutamyl derivatives and its inhibition by serine and related compounds in presence of borate

Substrate*	Relative activity	Inhibitor†	% inhibition	K_i, mM
L-γ-Glutamyl-L-Aba	[100]	L-Serine	82	0.02
D-γ-Glutamyl-L-Aba	44	D-Serine	43	0.17
L-γ-(α-Methyl)glutamyl-L-Aba	24	α-Methyl-DL-serine	9	0.31
γ-(β-Methyl)glutamyl-L-Aba	0	L-Threonine	0	—

* Data from Griffith and Meister (11). L-Aba, L-α-aminobutyrate.

† Present work. Measurements of transpeptidase activity were made with 0.5 mM L-γ-glutamyl-*p*-nitroanilide, 20 mM GlyGly, 10 mM sodium borate, and 50 mM Tris·HCl (pH 8.0). The inhibitor, when present, was at a concentration of 0.2 mM.

ceptibility of transpeptidase to serine–borate inhibition, remained unaffected after such treatment.

The nature of the amino acid residues at the γ-glutamyl site was explored by the following studies on aza[^{14}C]serine-labeled enzyme. Hydrolysis of the labeled enzyme with 6 M HCl at 110° for 24 hr did not yield labeled carboxymethylcysteine, carboxymethylhistidine, or carboxymethyllysine (hydrolysate was analyzed on a Durrum model D500 amino acid analyzer). However, such treatment, as well as treatment with 1 M HCl at 100° for 1 hr, released all of the bound radioactivity. No release of radioactivity was observed after treatment of the labeled enzyme with 1 M NaOH at 37° for 2 hr or with 1 M NH_2OH at 37° for 2 hr.

DISCUSSION

The data indicate that L-serine and its analogs inhibit γ-glutamyl transpeptidase in the presence of borate by interacting with the γ-glutamyl binding site of the enzyme. This conclusion is based on the following findings: (*i*) inhibition by L-serine, D-serine, and α-methyl-DL-serine in the presence of borate is competitive with respect to the γ-glutamyl donor substrate; (*ii*) L-serine plus borate effectively protect against inactivation of the enzyme by the γ-glutamyl analog 6-diazo-5-oxo-L-norleucine; (*iii*) the effectiveness of L-serine, D-serine, and α-methyl-DL-serine as inhibitors in the presence of borate is strikingly parallel to the activity of the transpeptidase toward the L-γ-glutamyl, D-γ-glutamyl, and L-γ-(α-methyl)glutamyl derivatives of L-α-aminobutyrate. Thus, the inhibition produced by L-serine, D-serine, and α-methyl-DL-serine in the presence of borate is consistent with the broad optical and steric specificity exhibited at the γ-glutamyl binding site of the enzyme (11, 12). The acceptor site, on the other hand, shows a much more strict steric requirement in that D-amino acids and α-methyl amino acids do not serve as acceptors of the γ-glutamyl group (6). Furthermore, dipeptides containing L-serine (e.g., L-serylglycine and glycine-L-serine), which are excellent acceptor substrates, do not inhibit in the presence of borate, indicating that the acceptor site is not involved in the binding of inhibitor.

Thompson and Meister (4) showed that amino acids such as glycine, L-alanine, D-alanine, and L-serine inhibit the transpeptidase at high concentrations in the absence of borate by virtue of their interaction with the α-carboxyl and α-amino binding regions of the γ-glutamyl site. The K_i value for L-serine is about 10 mM, which is in contrast with the K_i value of 0.02 mM for L-serine in the presence of borate. The high affinity of the enzyme for L-serine in the presence of borate is undoubtedly enhanced by complex formation between its hydroxyl group, borate, and a group on the enzyme at or near the γ-glutamyl binding site (see Fig. 2). Since borate forms reversible complexes with vicinal hydroxyl groups, the most likely site for complex formation between the enzyme, borate, and L-serine appears to be a hydroxyl group at the active center. This would be consistent with the observations described above on the properties of the aza[^{14}C]serine-labeled enzyme, which seem to exclude linkages involving an enzyme amino, imidazole nitrogen, sulfhydryl, or carboxyl group. The properties of the labeled transpeptidase thus resemble those of 5-diazo-4-oxo-L-norvaline-labeled asparaginase from *Escherichia coli* (13) and 6-diazo-5-oxo-L-norleucine-labeled glutaminases from *Acinetobacter* and *Pseudomonas* (14), which involve active site seryl and threonyl residues, respectively. We therefore

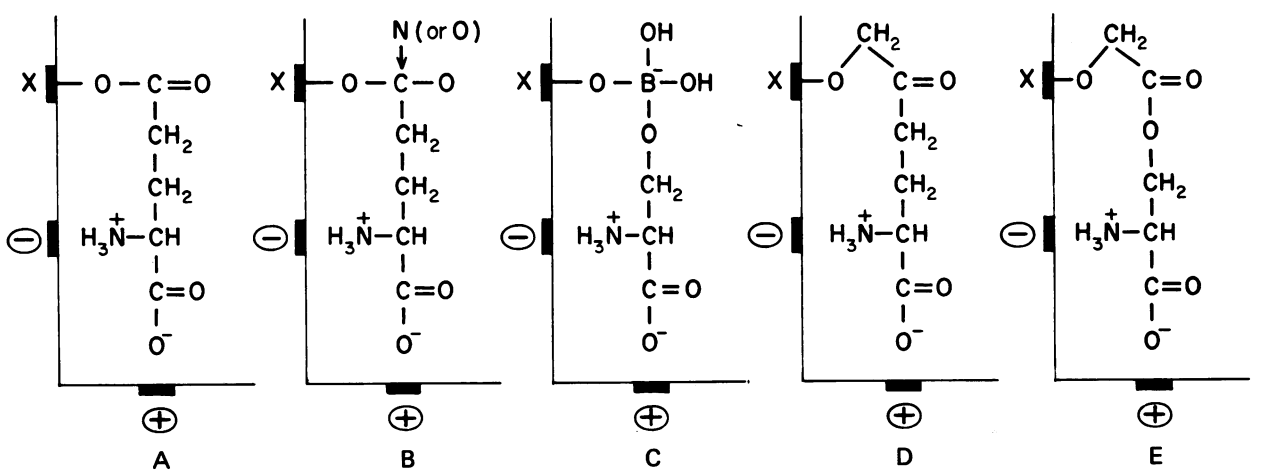

FIG. 2. Schematic representation of the active center of γ-glutamyl transpeptidase depicting the γ-glutamyl-enzyme intermediate (*A*), the expected tetrahedral transition state intermediate that would be formed during the transfer of the enzyme-bound γ-glutamyl moiety to the α-amino group of acceptor, or to water (*B*), the proposed enzyme–L-serine–borate complex (*C*), and the covalent derivatives obtained by treatment of the enzyme with the γ-glutamyl analogs 6-diazo-5-oxo-L-norleucine (*D*) and L-azaserine (*E*). The amino acid residue, X, at the active center appears to be either a seryl or a threonyl residue.

tentatively propose the structures given in Fig. 2, in which the γ-glutamyl and borate moieties are linked to the enzyme by ester bonds. The serine–borate complex closely resembles the expected tetrahedral transition state intermediate that would be formed during the transfer of the enzyme-bound γ-glutamyl moiety to the α-amino group of the acceptor, or to water (Fig. 2*B*); such similarity would be consistent with the high affinity of the enzyme for serine plus borate. The possibility that γ-glutamylation of the enzyme occurs via another aminoacyl side chain and not through the hydroxyl groups that appear to be involved in the binding of serine–borate and azaserine must also be considered and requires further investigation.

It is of interest that alkyl and aryl boronic acids have been shown to be transition state analogs for serine proteases because they can form tetrahedral adducts with the hydroxyl group of the serine residue at the catalytic site of these enzymes (15–18).

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