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Biochemistry

Affinity labeling of γ-glutamyl transpeptidase and location of the γ-glutamyl binding site on the light subunit

(6-diazo-5-oxo-norleucine/azaserine/5-diazo-4-oxo-norvaline/5-chloro-4-oxo-norvaline/enzyme subunits)

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ABSTRACT **γ-Glutamyl transpeptidase, which consists of two nonidentical subunits, is rapidly inactivated with respect to its transpeptidase and hydrolase activities by the γ-glutamyl analogs 6-diazo-5-oxo-L-norleucine and L-azaserine. Inactivation, which is prevented by γ-glutamyl substrates (but not by acceptor substrates), is accelerated by maleate, which was previously shown to enhance utilization of glutamine by transpeptidase. 6-Diazo-5-oxo-norleucine reacts specifically, covalently, and stoichiometrically at the γ-glutamyl site of the enzyme, which was localized through studies with 6-diazo-5-oxo-[^{14}C]norleucine to the light subunits of both the transpeptidase of rat kidney (which has subunits of molecular weights 22,000 and 46,000) and the transpeptidase of human kidney (which has subunits of molecular weights 22,000 and 62,000). The findings, which indicate that these enzymes have similar γ-glutamyl binding subunits, are relevant to the structure-function relationships of this membrane-bound enzyme and its physiological role.**

γ-Glutamyl transpeptidase, an enzyme that interacts with glutathione and other γ-glutamyl compounds, is found in the plasma membranes of cells that are intimately involved in rapid absorptive and secretory processes (1). Its proposed role in transmembrane transport is consistent with its potential for transferring the γ-glutamyl moiety of intracellular glutathione to a wide range of extracellular amino acids and peptides (2–4). Highly purified rat kidney γ-glutamyl transpeptidase is a glycoprotein (molecular weight, 68,000) composed of two subunits having molecular weights of 46,000 and 22,000, respectively (5). The enzyme also catalyzes the transfer of the γ-glutamyl moiety to water as well as to hydroxylamine (6, 7). Maleate enhances hydrolysis and γ-glutamylhydroxamate formation, simultaneously inhibiting transpeptidation (6, 7). Thus, the utilization of L-glutamine by the transpeptidase (which occurs at a very low rate compared to that of glutathione), especially its hydrolysis, is increased about 10-fold by maleate. These findings and kinetic studies support the hypothesis that the reaction mechanism involves a γ-glutamyl-enzyme intermediate (6, 8). Consideration of the differences between the subunit compositions and acceptor specificities of transpeptidases obtained from various mammalian kidneys led to the speculation that the γ-glutamyl binding site is located on the light subunit (5). It seems evident that data on the locations of the γ-glutamyl donor and acceptor sites on the enzyme subunits and on the orientation of the enzyme in the plasma membrane are crucial to an understanding of the enzyme's physiological function.

The studies reported here, in which covalent binding of certain γ-glutamyl analogs to the enzyme has been demonstrated, support the hypothesis that the reaction mechanism involves intermediate γ-glutamyl enzyme formation. The present data demonstrate that the γ-glutamyl binding site is located on the light subunit of the enzyme and suggest that these and other γ-glutamyl analogs may be useful in probing the orientations and conformations of the substrates at the active site.*

Abbreviations: DON, 6-diazo-5-oxo-L-norleucine; DONV, 5-diazo-4-oxo-L-norvaline; CONV, 5-chloro-4-oxo-L-norvaline (L-2-amino-4-oxo-5-chloropentanoic acid); $NaDodSO_4$, sodium dodecyl sulfate.

EXPERIMENTAL

Materials. L-γ-Glutamyl-*p*-nitroanilide, *S*-methylglutathione, and glycylglycine (GlyGly) were obtained from Sigma. L-Azaserine (*O*-diazoacetyl-L-serine) was purchased from Calbiochem. The chloroketone, 5-diazo-4-oxo-L-norvaline (CONV), was prepared as described (10, 11). We are very grateful to Dr. R. E. Handschumacher for a gift of 6-diazo-5-oxo-L-norleucine (DON) and 5-diazo-4-oxo-L-norvaline (DONV), and to Dr. John S. Holcenberg for a gift of [^{14}C]DON (C-6 labeled; 400 cpm/nmol). γ-Glutamyl transpeptidase was purified from rat kidney as described (7) [specific activity, 1100 μmol of *p*-nitroaniline formed/min per mg of protein; assayed with γ-glutamyl-*p*-nitroanilide and GlyGly (8)].

Methods. The effect of preincubating the transpeptidase with various analogs on its activity was determined as follows. The enzyme (2.5 μg) was incubated at 37° in a solution (50 μl) containing 0.1 M sodium phosphate buffer (pH 7.5) and the analog (0.2–2 mM). Aliquots (2 μl) were withdrawn at intervals and mixed with 1 ml of assay solution containing 0.1 M Tris·HCl buffer (pH 8), 1 mM γ-glutamyl-*p*-nitroanilide, and 20 mM GlyGly; the rate of *p*-nitroaniline formation was recorded at 410 nm.

Binding of [^{14}C]DON to transpeptidase was studied by the following general procedure. The enzyme (191 μg) was incubated at 37° in a solution (250 μl) containing 0.1 M sodium phosphate buffer (pH 7.5) and 1 mM [^{14}C]DON. The reactions were terminated by adding 250 μl of 0.1 M L-glutamine at 0°. The solutions were then dialyzed at 4° against several changes of 5 mM sodium phosphate buffer (pH 7.5). Aliquots of the enzyme solution were then analyzed for protein (12), radioactivity, and transpeptidase activity. The covalently bound [^{14}C]DON derivative was located on the enzyme subunits by lyophilizing the labeled enzyme and redissolving it in 100 μl of water. The enzyme was dissociated into subunits and their sodium dodecyl sulfate ($NaDodSO_4$) complexes were prepared; electrophoresis was carried out in 8% polyacrylamide gels containing 0.1% $NaDodSO_4$ (gel volume, 2 ml; length, 6 cm) as described (5). After electrophoresis, the gel was sliced into 2-mm sections; each section was cut into smaller pieces and shaken for 24 hr at 25° with 0.5 ml of 0.1% $NaDodSO_4$. Aliquots (0.3 ml) of the extracts were mixed with 10 ml of Bray's scintillation medium (13) and the radioactivity was determined. A duplicate gel containing the ^{14}C-labeled enzyme was electrophoresed and stained for protein with Coomassie brilliant

*A preliminary account of this work has appeared (9).

Table 1. Effect of L-γ-glutamyl analogs on γ-glutamyl transpeptidase activity

Analog	% Inactivation of transpeptidase[a]		Activity of analogs as γ-glutamyl acceptors[b]
	No maleate	With maleate (50 mM)	
L-DON	22	82	84
L-Azaserine	7	28	—
L-DONV	0	0	45
L-CONV	0	0	42

[a] Enzyme was incubated for 3 min with 1 mM of the analog as described under *Experimental*. Aliquots were assayed for residual transpeptidase activity.

[b] Transpeptidase activity was determined in a solution (1 ml) containing 0.25 mM γ-glutamyl-*p*-nitroanilide, 5 mM of the analog (or L-glutamine), and 100 ng of the enzyme (added last). The initial rate of release of *p*-nitroaniline was followed at 410 nm. The activities given are relative to that found with L-glutamine (100). Under these conditions, the relative activity with 5 mM L-asparagine was 49.

blue R250 to locate the heavy and the light subunits. The transpeptidase-catalyzed formation of γ-glutamylhydroxamate from L-glutamine and NH_2OH was measured as described (7).

RESULTS

Effect of DON on Activity of γ-Glutamyl Transpeptidase. Addition of 5 mM DON, azaserine, DONV, or CONV to the complete assay solution containing 2.5 mM γ-glutamyl-*p*-nitroanilide and 20 mM GlyGly did not affect the initial rate of transpeptidase-catalyzed release of *p*-nitroaniline. Indeed, as shown in Table 1, DON, DONV, and CONV, like glutamine and other amino acids, served as γ-glutamyl acceptors. However, preincubation of the enzyme with DON and azaserine (but not with DONV and CONV) led to irreversible inactivation of the transpeptidase (Table 1; Fig. 1, curve 1). Addition of *S*-methylglutathione and L-glutamine during preincubation with DON protected effectively against inactivation (Fig. 1, curves 2 and 3). However, GlyGly and L-methionine (good

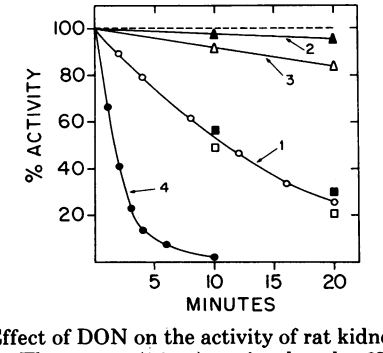

FIG. 1. Effect of DON on the activity of rat kidney γ-glutamyl transpeptidase. The enzyme (2.5 μg) was incubated at 37° in a solution (50 μl) containing 0.1 M sodium phosphate buffer (pH 7.5), 1 mM DON, and other compounds as indicated below. Aliquots (2 μl) were assayed for the residual transpeptidase activity as described under *Experimental*. Curve 1, 1 mM DON; curve 2, 1 mM DON plus 10 mM *S*-methylglutathione; curve 3, 1 mM DON plus 10 mM L-glutamine; curve 4, 1 mM DON plus 50 mM maleate. (□) 1 mM DON plus 10 mM GlyGly; (■) 1 mM DON plus 10 mM L-methionine. The broken line gives the activity of the enzyme incubated in phosphate buffer (control).

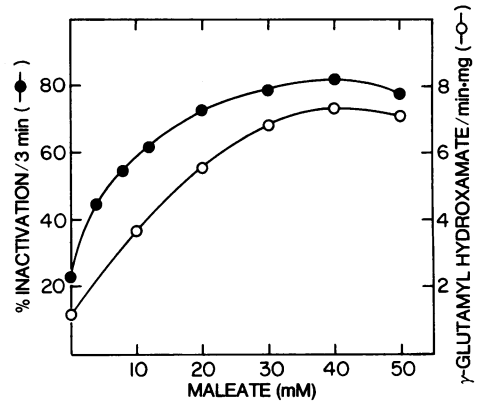

FIG. 2. Effect of maleate on the rate of inactivation by DON and on γ-glutamylhydroxamate-forming activity. (●) Transpeptidase (2.5 μg) was incubated with 1 mM DON and maleate as described in the legend of Fig. 1; transpeptidase activity was determined after 3 min of incubation. (○) Rate of formation of γ-glutamylhydroxamate from L-glutamine and NH_2OH.

γ-glutamyl acceptors) did not affect the rate of inactivation. These results suggest that DON binds to and reacts with the γ-glutamyl binding site of the enzyme under these conditions. This interpretation is supported by the finding that maleate increases the rate of inactivation severalfold (Fig. 1, curve 4). Maleate is known to enhance the utilization of L-glutamine by transpeptidase (6, 7). The effect of maleate on glutamine hydrolysis is much greater than on transpeptidation (i.e., transfer of the γ-glutamyl moiety of glutamine to glutamine to form γ-glutamyl-glutamine). With other γ-glutamyl substrates (e.g., *S*-methylglutathione), maleate enhances hydrolysis and inhibits transpeptidation.

Reaction of DON with the γ-glutamyl site of the enzyme is further supported by the parallelism between the effect of maleate concentration on the rate of inactivation by DON and on the formation of γ-glutamyl hydroxamate from L-glutamine and NH_2OH (Fig. 2). Fig. 3 shows the effect of DON concentration on the loss of enzyme activity in the absence and presence of maleate. Plots of inactivation half-time ($T_{1/2}$) against the reciprocal of inhibitor concentration (14) yield apparent K_I values for DON of 2 and 1.5 mM in the absence and presence of maleate, respectively. The inactivation half-time at saturating DON concentration is decreased about 8-fold by the presence of maleate.

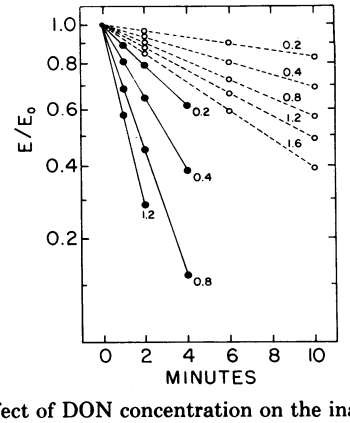

FIG. 3. Effect of DON concentration on the inactivation of γ-glutamyl transpeptidase in the absence (○) and in the presence (●) of 50 mM maleate. The concentrations (mM) of DON are given in the figure. Ordinate: ratio of the observed activity (E) to that of the uninhibited enzyme (E_0).

Effect of Other Analogs on Activity of γ-Glutamyl Transpeptidase. Table 1 shows the effect of preincubation of transpeptidase with DON and related compounds. DON was about twice as effective as L-azaserine. Longer incubation (60 min) with azaserine led to complete inactivation. The 5-carbon compounds, DONV and CONV, had no effect in the absence or presence of maleate, even with preincubation periods of 1 hr.

Binding of [^{14}C]DON to γ-Glutamyl Transpeptidase. Results of experiments in which the labeling of the enzyme with [^{14}C]DON was determined under various conditions are shown in Table 2. Complete inactivation of the enzyme is associated with the covalent binding of close to 1 mol of the ^{14}C-labeled derivative per mol of enzyme, and maximal binding is not affected by the presence of maleate. L-Glutamine decreases the extent of inactivation and also the amount of binding of the ^{14}C-labeled derivative.

It is noteworthy that the loss of transpeptidase activity of [^{14}C]DON-treated enzyme paralleled the decrease in its activity in forming γ-glutamylhydroxamate from L-glutamine and NH_2OH both in the absence and presence of maleate. These findings are consistent with the view that DON does indeed react with the γ-glutamyl binding site.

Location of Enzyme-Bound [^{14}C]DON. After incubation of the enzyme with [^{14}]DON as described in Table 2, the labeled enzyme was subjected to NaDodSO$_4$–gel electrophoresis; the gels were cut into slices and their radioactivity was determined as described under *Experimental*. The results of a representative experiment (Table 2, Exp. 4) are given in Fig. 4. About 92% of the recovered radioactivity was associated with the light subunit. Similar results were obtained with enzyme treated with [^{14}C]DON in the absence of maleate (Table 2; Exp. 2).

It is of interest that purified human kidney γ-glutamyl transpeptidase (which has a molecular weight of 84,000 and is composed of two subunits having molecular weights of 62,000 and 22,000, respectively) was also inactivated by preincubation with DON and azaserine, but not with DONV and CONV. Complete inactivation of this enzyme was associated with the binding of 0.94 mol of [^{14}C]DON per mol of enzyme, and virtually all of the protein-bound label was found in the light subunit (S. S. Tate and M. E. Ross, unpublished data).

Table 2. Binding of [^{14}C]DON to γ-glutamyl transpeptidase

Exp. no.	Other additions	Incubation time (min)	Moles of DON bound per mol of enzyme[a]	Percent of initial transpeptidase activity
1	None	15	0.41(0.77)[b]	47
2	None	30	0.76(0.90)	15
3	Maleate	15	0.83(0.88)	6
4	Maleate	30	0.89(0.91)	2
5	L-Glutamine	15	0.23(1.04)	78

[a] The enzyme was treated with 1 mM [^{14}C]DON in the absence or presence of 50 mM sodium maleate or 20 mM L-glutamine and then extensively dialyzed (see *Experimental*). Aliquots were then analyzed for protein, ^{14}C, and residual transpeptidase activity.

[b] The values in parentheses are calculated for DON binding to 100% inactivated enzyme, assuming a linear relationship between loss of activity and the extent of DON binding.

DISCUSSION

The findings demonstrate coincident active-site labeling and inactivation of γ-glutamyl transpeptidase. Inactivation is prevented by γ-glutamyl donors but not by acceptor substrates, and the rate of inactivation is increased about 8-fold by maleate. The enhancement by maleate of the transpeptidase-catalyzed hydrolysis of γ-glutamyl compounds (6, 7) is presumably due to increased accessibility of water to the γ-glutamyl-enzyme intermediate. DON and azaserine appear to bind to the enzyme sites that normally bind the α-amino and α-carboxyl moieties of γ-glutamyl donors; the conformation and orientation of the analogs must be such as to facilitate attack of a nucleophilic group of the enzyme on the ω-carbon atom of the analog. It seems significant that analogs that have chains of only five carbon atoms (DONV and CONV) do not inhibit the transpeptidase, whereas those with longer chains (DON and azaserine) do. γ-Glutamyl transpeptidase exhibits high affinity for both L- and D-γ-glutamyl donors (15), as does glutamine synthetase (16, 17). Studies on the mapping of the active site of glutamine synthetase showed that L-glutamate binds to this enzyme in an extended conformation, and it thus seems possible that the γ-glutamyl donor substrate has a similar conformation at the active site of the transpeptidase. The identity of the enzyme group that interacts with DON and azaserine is not yet known. Rat kidney transpeptidase activity is not affected by preincubation with *p*-chloromercuribenzoate or 5,5′-dithio(2-nitrobenzoate) either in the presence or absence of maleate, suggesting that a sulfhydryl group may not be involved; however, a "buried" SH-group cannot be excluded. (In all cases thus far studied, the inhibition of glutamine amidotransferases by DON, azaserine, and CONV has been shown to involve interaction with a specific enzyme sulfhydryl group.)

The localization of the DON-binding site on the light subunit of transpeptidase indicates that the γ-glutamyl binding site is on this subunit. It is significant that this site is located on the light subunits of both the rat and the human kidney transpeptidases. These enzymes have light subunits of similar size (molecular weight, 22,000), but differ with respect to the molecular weights of the heavy subunits (Table 3). That these transpeptidases have similar specificity towards γ-glutamyl substrates but exhibit significant differences in amino acid acceptor specificities and in response to maleate (ref. 5; S. S.

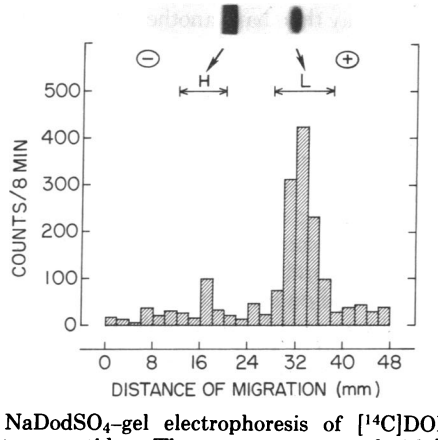

FIG. 4. NaDodSO$_4$–gel electrophoresis of [^{14}C]DON-labeled γ-glutamyl transpeptidase. The enzyme was treated with [^{14}C]DON as described in Table 2, Exp. 4; 40 μg of the labeled enzyme was subjected to gel electrophoresis (8% polyacrylamide gel) in the presence of 0.1% NaDodSO$_4$. The radioactivity was determined on 0.3-ml aliquots (see *Experimental*). A duplicate gel was stained for protein with Coomassie brilliant blue R250 to locate the subunits (H and L refer to the heavy and light subunits, respectively). The total radioactivity applied to the gel was 1470 counts/8 min. The recovery was 1240 counts/8 min (84%); 92% of the protein-bound radioactivity was found in fractions corresponding to the light subunit.

Table 3. Effect of γ-glutamyl analogs on enzymes that catalyze reactions involving the γ-carboxyl group of glutamate

Enzyme: source (ref.)	Inactivation by γ-glutamyl analogs[a]				Subunit molecular weights
	DON	Azaserine	CONV	DONV	
γ-Glutamyl transpeptidase					
Rat kidney (this work)	Yes*	Yes	No	No[b]	46,000; 22,000*
Human kidney (Tate & Ross, unpublished data)	Yes*	Yes	No	No	62,000; 22,000*
γ-Glutamylcysteine synthetase					
Rat kidney (20)	No[c]	No[c]	Yes*	No[c]	74,000*; 24,000
Glutamine synthetase					
Ovine brain[d]	No	No	No	No	49,000
Carbamyl phosphate synthetase					
Escherichia coli (10, 11)	Yes	Yes	Yes*	No[e]	130,000; 42,000*
5-Phosphoribosyl-1-pyrophosphate amidotransferase					
Chicken liver (22, 23)	Yes*	Yes		No	50,000*
Formylglycinamide ribonucleotide amidotransferase					
Chicken liver (21, 24)	Yes	Yes*	Yes	No	133,000*
Salmonella typhimurium (21, 24)	Yes	Yes*	Yes	No	135,000*
CTP synthetase					
Escherichia coli (25, 26)	Yes*	Yes	Yes	No	50,000*
Asparagine synthetase (glutamine-dependent)					
Mouse pancreas and leukemia cells (27, 28)	Yes	Yes	Yes		
Anthranilate synthase					
S. typhimurium (29)	Yes*	Yes	Yes		64,000; 63,000*
Serratia marcescens (29)	Yes*				60,000; 21,000*

[a] Asterisks identify the labeled compound used in studying inhibition and binding to the enzyme, and the subunit in which the label was found.
[b] DONV was not degraded when incubated with the enzyme.
[c] L.-A. Yeh, unpublished observations in this laboratory.
[d] S. S. Tate, unpublished observations in this laboratory.
[e] S. G. Powers, unpublished observations in this laboratory.

Tate and M. E. Ross, unpublished data) may indicate that both the acceptor and maleate sites are located on the heavy subunits of these enzymes. However, both the γ-glutamyl site and the acceptor site may well be located on the light subunit, and the heavy subunit may then have another function associated with integration of the enzyme in the plasma membrane. It is known that the γ-glutamyl transpeptidase activities of lymphoid cells and renal tubular cells are accessible to externally supplied γ-glutamyl donor (4, 18). However, glutathione is found predominantly intracellularly. It seems notable that administration of glycylglycine to rats leads to a marked decrease in renal glutathione, a finding considered to be mediated by the action of γ-glutamyl transpeptidase (19). It is evident that further studies on the subunit structure and function of γ-glutamyl transpeptidase and on the nature of its orientation within the plasma membrane are of considerable importance to a detailed understanding of the physiological role of this enzyme.

The present studies and recent findings on γ-glutamylcysteine synthetase (20) have extended the usefulness and applicability of the class of active site directed reagents used here. Previously such compounds have been used in studies on enzymes that catalyze utilization of the amide N of glutamine (Table 3). Most of the glutamine amidotransferases are inhibited with respect to their glutamine-dependent functions by DON, azaserine, and CONV; in the several cases studied, DONV did not inhibit. Several but not all of the glutamine amidotransferases have separate glutamine-binding subunits (21). Many of these enzymes, like the transpeptidase, exhibit hydrolase (glutaminase) activity. However, in contrast to the reaction catalyzed by γ-glutamyl transpeptidase, the putative γ-glutamyl-enzyme intermediates formed by the glutamine amidotransferases undergo only hydrolysis. A γ-glutamyl-enzyme intermediate is also conceivable in the reaction catalyzed by γ-glutamylcysteine synthetase; this would be consistent with the finding that this enzyme is inhibited by CONV, which binds to its glutamate site (20). Table 3 summarizes data on the properties of several representative enzymes that catalyze reactions involving the synthesis and utilization of glutamine and other γ-glutamyl compounds. There are some interesting differences among these enzymes with respect to inhibition by azaserine, DON, CONV, and DONV. Thus, the glutamine amidotransferases are typically inhibited by all of these reagents except DONV, whereas only azaserine and DON inhibit γ-glutamyl transpeptidase and CONV is the only reagent that inhibits γ-glutamylcysteine synthetase. The latter enzyme and glutamine synthetase appear to catalyze closely analogous reactions involving intermediate formation of γ-glutamyl phosphate; however, thus far there is no convincing evidence for inhibition of glutamine synthetase by any of these reagents. The structural features of these enzymes that account for these findings are undoubtedly relevant to their mechanisms of action and perhaps also to the evolutionary considerations involved in the development of these enzymes that interact with glutamate and γ-glutamyl compounds.

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