AFFINITY LABELING OF γ-GLUTAMYL TRANSPEPTIDASE BY GLUTAMINE ANTAGONISTS

Effects on the γ-glutamyl transfer and proteinase activities

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1. Introduction

γ-Glutamyl transpeptidase, a membrane-bound enzyme, exhibits a number of catalytic activities: transfer of the γ-glutamyl group of glutathione and other γ-glutamyl compounds to amino acids, peptides, and to water [1,2]; a proteinase activity inherent to its light subunit [3]. The rat kidney enzyme is a heterodimer (M_r 68 000) of glycosylated subunits (heavy (H) and light (L) subunits, M_r 46 000 and 22 000, respectively) [4,5]. The reaction mechanism for the utilization of γ-glutamyl compounds involves an intermediate formation of a covalent γ-glutamyl–enzyme intermediate [6–9]. γ-Glutamyl analogs, such as DON, abolish the γ-glutamyl transfer and proteinase activities of the enzyme by formation of a covalent adduct at the γ-glutamyl site [3,6]. The residue alkylated by DON, and thus presumably the site at which γ-glutamylation also occurs, is located on the L subunit.

The anti-tumor drug, AT-125 [10], a glutamine antagonist which inactivates a number of glutamine amidotransferases and glutaminases [11,12], inhibits the γ-glutamyl transpeptidase activity of kidney cells [13] and a human pancreatic carcinoma cell line [14]. We report here the potent inhibition of purified rat kidney transpeptidase by AT-125. The inactivation is accompanied by stoichiometric and covalent attachment of AT-125 to a residue in the γ-glutamyl binding domain of the enzyme. This residue, as in the case of DON, is located on the L subunit. The inhibition of the γ-glutamyl transfer activity by AT-125 is qualitatively similar to that observed with DON, although AT-125 is 20-fold more potent than DON. Interestingly, however, AT-125 does not inactivate the proteinase activity of the L subunit whereas DON does.

Abbreviations: DON, 6-diazo-5-oxo-L-norleucine; AT-125, L-(α*S*, 5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; DTT, dithiothreitol; SDS–PAGE, polyacrylamide gel electrophoresis in presence of sodium dodecylsulfate

2. Materials and methods

L-γ-Glutamyl-*p*-nitroanilide, glycylglycine and DTT were obtained from Sigma. Urea (ultra pure grade) was purchased from Schwarz/Mann. AT-125 and [2-^{3}H]AT-125 (23 μCi/μmol) were generous gifts from Dr L. J. Hanka of Upjohn, Kalamazoo MI. Stock [^{3}H]AT-125 was mixed with unlabeled AT-125 to give 3820 cpm/nmol. Rat kidney γ-glutamyl transpeptidase was purified as in [15] (spec. act. 750 μmol *p*-nitroaniline released . min^{-1} . mg protein^{-1} when assayed with 1 mM L-γ-glutamyl-*p*-nitroanilide and 20 mM Gly–Gly [3]).

The effect of preincubation of transpeptidase with DON and AT-125 on its activity was studied essentially as described for DON [6]. Affinity labeling of the enzyme with [^{3}H]AT-125 was carried out as follows: transpeptidase (1 mg/ml 0.05 M sodium phosphate buffer (pH 7.5)) was incubated at 37°C for 1 h with 0.05 mM [^{3}H]AT-125. Following extensive dialysis at 4°C against 0.01 M Tris–HCl buffer (pH 8), the samples were analyzed for protein [16], residual transpeptidase activity, and bound radioactivity. Subunit localization of bound [^{3}H]AT-125 was achieved by subjecting the labeled enzyme to SDS–PAGE (8%

gels, 0.1% SDS [4]). The subunits were localized by staining a gel with Coomassie blue R-250. A duplicate gel was sliced into 2 mm sections; each slice was incubated in a glass scintillation vial for 12 h at 37°C with 10 ml toluene scintillation medium containing 0.6% PPO, 0.0075% POPOP, and 3% Protosol (New England Nuclear). The samples were then counted in a liquid scintillation counter.

The proteinase activity of the light subunit of transpeptidase [3] was determined as follows: transpeptidase (10–15 μg) was incubated at 37°C in a solution (0.1 ml) containing 5 mM Tris–HCl (pH 8), 6 M urea and 5 mM DTT. The reaction was terminated by addition of SDS (final conc. 1.5% (w/v)) and the samples placed in a boiling water bath for 5 min. The samples were then subjected to SDS–PAGE (10% gels, 0.1% SDS) and the gels stained with Coomassie blue R-250. The destained gels were scanned at 575 nm in a Corning 740 gel scanner; the relative amounts of the H and L subunits were determined by comparison with a scan obtained from an equivalent amount of enzyme subjected to SDS–PAGE without prior urea treatment. The proteinase activity is expressed as the percentage of H subunit degraded.

3. Results

Preincubation of the enzyme with AT-125 results in irreversible inactivation of transpeptidase (fig.1, curve 1). Addition of L-serine plus borate (a combination which forms a complex that mimics the transition state γ-glutamyl–enzyme intermediate [7]) protects against inactivation by AT-125 (fig.1, curve 2) whereas maleate (which stimulates hydrolysis of γ-glutamyl substrates [8,17]) enhances the rate of inactivation ~8-fold (fig.1, curve 3). Acceptor substrates (Gly–Gly and L-methionine, 10 mM each) have no effect on inactivation rate. These results, which indicate that AT-125 inactivates transpeptidase by binding to the γ-glutamyl site of the enzyme, are qualitatively similar to those previously reported for DON-mediated inactivation of transpeptidase (see [6]; also fig.1, curves 4,5). However, at equimolar levels, AT-125 is ~20-fold more effective as an inhibitor than DON. Plot of the time required for 50% inactivation against the reciprocal of AT-125 concentration [18] yields an app. K_i value for AT-125 of 0.6 mM. The app. K_i value for DON, similarly derived, is 2 mM [6].

Complete inactivation of transpeptidase with [^{3}H]-AT-125 is accompanied with the covalent binding of close to 1 mol ^{3}H-labeled compound/mol enzyme (table 1), and maximal binding is not affected by the presence of maleate. L-Serine plus borate decreases the extent of inactivation and the amount of binding of AT-125. Prior treatment of the enzyme with DON prevents subsequent binding of [^{3}H]AT-125. SDS–PAGE of the [^{3}H]AT-125-labeled enzyme reveals that covalent attachment of the [^{3}H]derivative occurs exclusively to the L subunit (not shown), as shown in [6] for DON.

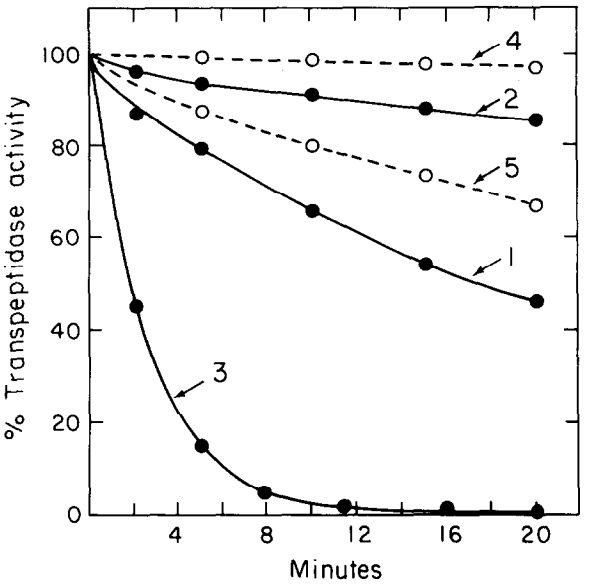

Fig.1. Effect of AT-125 and DON on the activity of rat kidney γ-glutamyl transpeptidase. The enzyme (5 μg) was incubated at 37°C in 0.2 ml 0.05 M phosphate buffer (pH 7.5) containing 0.05 mM either AT-125 (curves 1–3) or DON (curves 4,5) and other compounds as indicated below: (1) AT-125; (2) AT-125 plus 5 mM each of L-serine and sodium borate; (3) AT-125 plus 50 mM maleate; (4) DON; (5) DON plus 50 mM maleate. Aliquots (2 μl) were assayed for residual transpeptidase activity with 1 mM L-γ-glutamyl-*p*-nitroanilide and 20 mM Gly–Gly.

Treatment of transpeptidase with dissociating agents (e.g., urea) unmasks a proteinase activity inherent to the L subunit which preferentially and rapidly degrades the H subunit (fig.2B) [3]. Prior treatment of the enzyme with DON abolishes both the transpeptidase activity and the proteinase activity (fig.2D); presence of serine plus borate during DON treatment prevents loss of not only the transpeptidase activity but also of the proteinase activity (fig.2E). Surprisingly, however, AT-125, which like DON inactivates the

Table 1
Binding of [^{3}H]AT-125 to γ-glutamyl transpeptidase

Expt.	Additions	Incubation (min)	Bound AT-125 (mol/mol enzyme)	Residual transpeptidase activity (%)
1	None	20	0.43	48
2	None	60	0.92	2.6
3	Maleate	20	0.93	<0.1
4	L-Serine + borate	20	0.12	85
5	DON-treated	60	0.01	<0.1

The enzyme was treated with 0.05 mM [^{3}H]AT-125 in the absence or presence of 50 mM maleate or 5 mM each of L-serine and borate and then extensively dialyzed (section 2). Aliquots were then analyzed for protein, ^{3}H and residual transpeptidase activity. In expt. 5, the enzyme was first inactivated with DON as in fig.2 and then treated with [^{3}H]-AT-125

transpeptidase activity by covalently interacting with a residue in the γ-glutamyl binding site, had no effect on the proteinase activity (fig.2C). Since the binding of AT-125 and DON to the enzyme is mutually exclusive, prior incubation with AT-125 (<0.1% residual transpeptidase activity) prevented the inactivation of the proteinase activity by subsequent DON treatment (fig.2F). The rate of degradation of the H subunit of [^{3}H]AT-125-treated transpeptidase, incubated in 6 M urea in presence of DTT (31, 56, 69 and 76% in 15, 30, 45 and 60 min, respectively), was similar to that observed with untreated enzyme. It is of interest however, that the radioactivity associated with the L chain (due to bound [^{3}H]AT-125) did not diminish during incubation of the enzyme in urea indicating that the affinity label was not lost and reinforcing our prior observations that incubation of the enzyme in urea results in preferential degradation of the H subunit.

4. Discussion

The potent inhibition of the γ-glutamyl transfer activity of γ-glutamyl transpeptidase by AT-125, an anti-tumor glutamine antagonist, is shown to be due to its covalent attachment to a residue in the γ-glutamyl binding site of the enzyme. The effects of AT-125 resemble in several respects the actions of DON [6], another glutamine analog. Thus, inactivation by both is prevented by competitive inhibitors

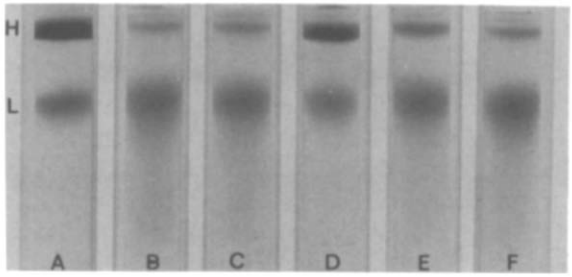

Fig.2. Effect of AT-125 and DON on the proteinase activity of γ-glutamyl transpeptidase. The enzyme (0.5 mg) was incubated at 37°C in 0.3 ml 0.05 M phosphate buffer (pH 7.5) containing either 0.25 mM AT-125 or 2.5 mM DON and, where indicated, 5 mM L-serine plus 10 mM sodium borate. The samples were dialyzed extensively against 10 mM Tris–HCl buffer (pH 8) and then analyzed for protein and residual transpeptidase activity. The proteinase activity (as indicated by the decrease in H subunit) of the treated and untreated enzyme samples (12 μg each) was determined by incubating in a solution containing 6 M urea and 5 mM DTT at 37°C for 30 min (section 2), following which the samples were subjected to SDS–PAGE (10% gels; 0.1% SDS). The gels shown are as follows (treatment of the enzyme prior to proteinase assay is indicated and the residual transpeptidase activities (%) are shown in parentheses): (A) native enzyme applied directly to the gels; (B) proteinase activity of untreated enzyme; (C) AT-125-treated (0.1%); (D) DON-treated (0.1%); (E) DON treatment in presence of L-serine plus borate (73%); (F) AT-125 followed by DON (<0.1%). H and L represent the heavy and light subunits of transpeptidase, respectivelv.

of γ-glutamyl substrates and enhanced markedly by maleate (a modulator of γ-glutamyl transfer activities [8,17]). Both inhibitors are covalently attached to residues located on the L subunit of the enzyme. AT-125 is, however, 20-fold more potent than DON and also exhibits greater apparent affinity for the enzyme as reflected by the respective K_i values.

Despite these similarities between the actions of AT-125 and DON there is a marked contrast in their effects on the proteinase activity of the L subunit. The proteinase activity, not seen in the native enzyme, is revealed upon treatment of the enzyme with protein dissociating agents (e.g., urea, SDS) [3]. The H subunit is preferentially degraded. Prior treatment of the native enzyme with DON inhibits the proteinase activity whereas AT-125 treatment has no effect on this activity. Thus, although both analogs inhibit γ-glutamyl transfer activities of the native enzyme by binding to the γ-glutamyl site, is appears that they covalently modify different residues in this site. Previous studies, based on the stability of the enzyme–

DON adduct, suggest that a hydroxyl residue of the enzyme is alkylated by DON [7]. Preliminary studies with the AT-125–enzyme adduct indicate that a group other than a hydroxyl group may be involved in the covalent attachment of AT-125 (S. J. G., unpublished). Further studies are required to elucidate the involvement of the various active center residues in the catalytic activities exhibited by transpeptidase.

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