INTERACTION OF THE ANTITUMOR DRUG, L-(αS, 5S)-α-AMINO-3-CHLORO-4,5-DIHYDRO-5-ISOXAZOLEACETIC ACID (AT-125) WITH RENAL BRUSH BORDER MEMBRANES

Specific labeling of γ-glutamyl transpeptidase

Elena M. KOZAK and Suresh S. TATE
Department of Biochemistry, Cornell University Medical College, New York, NY 10021, USA

Received 4 November 1980

1. Introduction

γ-Glutamyl transpeptidase initiates degradation of glutathione by transfering the γ-glutamyl moiety to acceptors such as amino acids, dipeptides, and water [1]. The enzyme is concentrated in cell membranes which function primarily in transport processes [1–3]. Highest activity is found in kidney where the enzyme is localized on the lumenal surface of proximal tubular brush borders [2]. Proposed physiological functions of this enzyme include mediation of amino acid and peptide transport [1,4], a role in detoxification processes which lead to mercapturic acids [1,3], renal ammoniagenesis [5,6], and generation of bioactive derivatives such as leukotrienes [7]. Several compounds which inhibit transpeptidase [3] have been employed in attempts to elucidate its role [4]. Many of these compounds, however, may also affect proteins other than transpeptidase when used in whole cells, tissues and experimental animals. Here, we describe the interaction of rat renal brush border membranes with the glutamine antagonist, AT-125, an anti-tumor drug which inactivates a number of glutamine amidotransferases and glutaminases [8,9] as well as γ-glutamyl transpeptidase [10,11]. Inactivation of membrane-bound transpeptidase by AT-125 is accompanied by covalent binding of the inhibitor to the membranes in amounts that approximate the value expected from the enzyme content of these membranes. Various approaches indicate that, in brush border membranes, transpeptidase is most likely the only protein modified by AT-125 thus providing a useful in vitro experimental system to probe the enzyme's proposed functions. We also describe a relatively rapid and efficient method for the purification of kidney transpeptidase.

2. Materials and methods

L-γ-Glutamyl-*p*-nitroanilide, glycylglycine, papain (18 units/mg), and D-mannitol were purchased from Sigma. AT-125 and [2-^{3}H]AT-125 (23 μCi/μmole) were generous gifts from Dr L. J. Hanka of Upjohn, Kalamazoo MI. Anti-rat kidney γ-glutamyl transpeptidase IgC was purified as in [2].

Brush border membranes from the kidneys of male Sprague-Dawley rats were prepared according to [12]. The activities of marker enzymes were determined as in [13]. γ-Glutamyl transpeptidase activity was assayed in presence of 1 mM L-γ-glutamyl-*p*-nitroanilide, 20 mM Gly–Gly, and 0.1 M Tris–HCl (pH 8.0); its activity is expressed as μmol *p*-nitroaniline released/min (units). Protein was determined as in [14].

Treatment of the brush border membranes with [^{3}H]AT-125 was carried out as follows: the membranes (total protein, 11.9 mg; 293 units transpeptidase) were suspended in 0.5 ml 0.01 M phosphate buffer (pH 7.4) containing 0.15 M NaCl (phosphate–NaCl) and incubated at 37°C with [^{3}H]AT-125 (final conc. 0.1 mM; 3820 cpm/nmol obtained by dilution of stock [^{3}H]AT-125 with unlabeled AT-125). After 20 min incubation (~14% residual transpeptidase activity), 1 ml phosphate–NaCl was added and the mixture quickly cooled to 4°C. The membranes were pelleted by centrifugation at 35 000 × *g* then suspended in 1.5 ml phosphate–NaCl; this washing process was repeated thrice. Labeling of the membranes

is expressed as nmol [^{3}H]AT-125 bound/mg total protein.

Brush border membranes (final conc. ~10 mg protein/ml) were treated with papain as in [2]. [^{3}H]AT-125-Labeled membranes were similarly treated. The membranes were then separated by centrifugation at 35 000 × *g* for 20 min and the pellet resuspended in phosphate–NaCl. The suspension and the supernatant were assayed for transpeptidase and other marker enzyme activities, and in the case of AT-125-treated membranes, for radioactivity. The proteins solubilized by papain were fractionated on a Sephadex G-150 column (1.5 × 90 cm), equilibrated and eluted with 0.05 M Tris–HCl buffer (pH 8).

Detergent extract of brush border membranes was made by suspending the membrane pellet (1 mg protein) in 0.25 ml phosphate–NaCl containing 1% Triton X-100 (w/v). The suspension was thoroughly mixed by several passages through a 100 μl Hamilton syringe fitted with a 22-gauge needle and then centrifuged at 35 000 × *g* for 30 min. This procedure solubilized 100% of γ-glutamyl transpeptidase. For immunoprecipitation, an aliquot of the extract was treated with anti-transpeptidase IgG, incubated 1 h at 25°C and then 18 h at 4°C. The precipitate was removed by centrifugation and the supernatant assayed for residual transpeptidase activity.

The procedures for SDS–PAGE (8% gels; 0.1% SDS) and for localization of the bound radioactivity in transpeptidase subunits have been described [11,15].

3. Results

Incubation of brush border membranes with AT-125 results in rapid loss of transpeptidase activity. The resultant binding of AT-125 to the membranes (table 1) approximated the value expected from the units of transpeptidase contained in the untreated membranes, assuming that the membrane-bound enzyme exhibits the same activity (units/mol) as the purified rat kidney transpeptidase (M_r 68 000; activity = 51 units/nmol enzyme [11]). Inactivation of purified enzyme by AT-125 is accompanied by covalent attachment of 1 mol inhibitor/mol enzyme [11]. Incubation of the membranes with AT-125 in the presence of L-serine and borate (a combination known to serve as competitive inhibitor of transpeptidase [16]) prevents loss of transpeptidase activity and, to a large extent, binding of AT-125 (table 1). Immunoprecipitation of the enzyme from Triton X-100 extract of labeled membranes indicates that most of the bound radioactivity is associated with transpeptidase.

Papain treatment of AT-125 labeled membranes results in solubilization of 97% of the residual transpeptidase activity and 95% of membrane-associated radioactivity (table 1). About 14% of alkaline phos-

Table 1
Binding of [^{3}H]AT-125 to renal brush border membranes

A. Binding and effect on transpeptidase activity

Expt.	Pretreatment of membranes[a]	Residual transpeptidase activity (%)	Bound [^{3}H]AT-125 (nmol/mg membrane protein)
1.	None	100 (24.6)[b]	–
2.	[^{3}H]AT-125	13.7	0.48 (0.42)[c]
3.	[^{3}H]AT-125 + L-serine + borate	99.4	0.07

B. Experiments with [^{3}H]AT-125 treated membranes (from expt. 2 in A)

Expt.	Treatment of [^{3}H]AT-125 labeled membranes[d]	Transpeptidase activity in supernatant (%)	^{3}H (%) in supernatant
1.	Papain	97.4	95.4
2.	Immunoprecipitation of transpeptidase	<0.1	9.0

[a] Membranes were treated with 0.1 mM [^{3}H]AT-125 at 37°C for 20 min either in absence or in presence of L-serine (5 mM) plus sodium borate (10 mM) (section 2)

[b] Transpeptidase activity in membranes (units/mg protein)

[c] Binding of [^{3}H]AT-125 expected from the transpeptidase content of membranes (after making an allowance for the percentage inactivation after AT-125 treatment), assuming that binding occurs only to transpeptidase. Longer incubation of membranes with AT-125 results in complete inactivation of transpeptidase and the binding increases proportionately

[d] Papain treatment of membranes and immunoprecipitation of transpeptidase from Triton X-100 extract of the membranes were carried out as in section 2. The residual transpeptidase activity and ^{3}H in supernatants, after centrifugation to remove membranes and immunoprecipitate, were determined and expressed as percentage of the values prior to these treatments

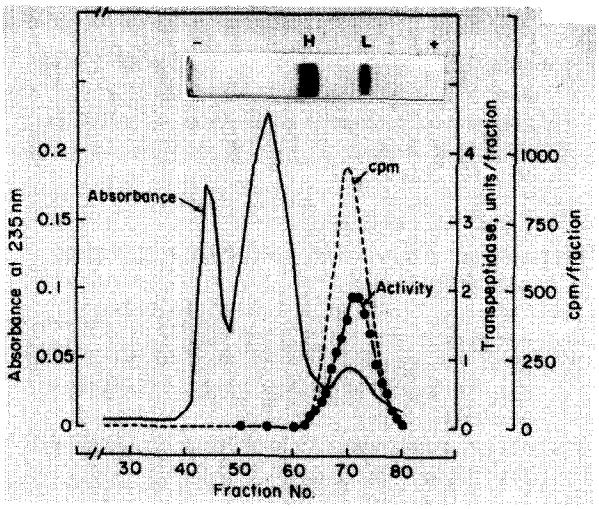

Fig.1. Gel filtration of proteins solubilized by papain from [^{3}H]AT-125 labeled brush border membranes. Membranes were treated with [^{3}H]AT-125 as in table 1A, expt. 2 (13.7% residual transpeptidase activity). The labeled membranes (6 mg membrane protein) were treated with papain and the supernatant (containing 97.4% of residual transpeptidase activity and 95.4% of radioactivity (see table 1B) was chromatographed on a Sephadex G-150 column (1.5 × 90 cm; 1.3 ml/fraction). Aliquots were taken for activity and ^{3}H determinations. Fractions 66–76 were pooled and concentrated by pressure dialysis and subjected to SDS–PAGE (inset; H and L represent the heavy and light subunits of transpeptidase, respectively). Aminopeptidase (assayed with L-leucyl-*p*-nitroanilide) elutes in fractions 50–60. Similar enzyme activity and protein profiles were obtained when papain supernatant from untreated membranes was chromatographed in the same manner.

phatase and 98% of aminopeptidase (assayed with leucyl-*p*-nitroanilide [17]) were also solubilized. Incubation of untreated membranes with papain causes similar release of three membrane-bound activities. Gel-filtration of proteins solubilized by papain shows that radioactivity (bound [^{3}H]AT-125) and transpeptidase activity elute in the same fractions (fig.1). These fractions were pooled, concentrated, and shown to contain 0.98 mol AT-125 bound/68 000 g protein. SDS–PAGE revealed exclusively the bands corresponding to the heavy and light subunits of transpeptidase, M_r 46 000 and 22 000, respectively (fig.1, inset) [15]. All of the bound radioactivity was associated with the light subunit in accord with studies which showed that the γ-glutamyl binding site is located on this subunit [11,18].

The gel filtration experiment in fig.1 indicates that highly purified transpeptidase can be isolated with relative ease by solubilization of the membrane-bound enzyme with papain followed by gel filtration. The method was adapted to a larger scale purification of the enzyme; the results are summarized in table 2. The supernatant obtained after papain treatment of the brush border membranes was treated with $(NH_4)_2SO_4$ to 90% of saturation; the precipitate obtained by centrifugation (18 000 × *g* for 30 min) was dissolved in 3 ml 0.05 M Tris–HCl buffer (pH 8) and the solution chromatographed on a Sephadex G-150 column (2.5 cm × 100 cm). Fractions containing transpeptidase were pooled, dialyzed against 100 vol. water, lyophilized, and the residue dissolved in water. About 300-fold purification with over 50% yield can be achieved.

Table 2
Purification of rat kidney γ-glutamyl transpeptidase[a]

Step	Vol. (ml)	Total protein (mg)	Transpeptidase activity[b]		
			Total (units)	Specific (units/mg)	Yield (%)
1. Homogenate	441	3704	9920	2.7	[100]
2. Brush border membranes	29.5	264	6140	23.3	62
3. Papain treatment followed by chromatography on Sephadex G-150	2.7	6.5	5150	792	52

[a] From 29.4 g rat kidney. Steps 1 and 2 were carried out as in [12]

[b] Transpeptidase activity was determined with 1 mM L-γ-glutamyl-*p*-nitroanilide and 20 mM glycylglycine

4. Discussion

These results indicate that, in the renal brush border membranes, as far as our methods allow us to discriminate, possibly the only protein irreversibly modified by AT-125 is γ-glutamyl transpeptidase. The brush border membranes isolated as in [12] form intact, right-side-out vesicles and retain their transport properties. The specificity of the interaction of AT-125 with these membrane vesicles and the potent inhibition of transpeptidase should provide an excellent probe in studies directed towards elucidating the manner in which this enzyme functions in brush bor-

der membranes. It should be noted that animals treated with AT-125 (and other inhibitors of transpeptidase [4,19]) excrete larger than normal amounts of glutathione in their urine.

We noted that the only protein, solubilized from the surface of the renal brush border vesicles and exhibiting M_r ~68 000, is γ-glutamyl transpeptidase. This has enabled us to devise a method to purify the enzyme relatively rapidly and with much higher yields than the cumbersome isolation procedures in [5,20,21].

Acknowledgement

This work was supported in part by National Institutes of Health grant GM 25152.

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