DIFFERENTIAL EFFECT OF AT-125 ON RAT RENAL GLUTAMINASE ACTIVITIES

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1. Introduction

Rat kidney contains two distinct glutaminase activities; a mitochondrial phosphate-dependent glutaminase and a phosphate-independent, but maleate-stimulated glutaminase [1]. The latter reaction is an activity of the brush border membrane-associated γ-glutamyltranspeptidase [2,3]. Assays specific for each glutaminase activity were devised by taking advantage of their differences in activators and pH optima [4]. However, rat renal glutaminase activity has frequently been quantitated using conditions where the relative contribution of the two activities is uncertain. The availability of an inhibitor specific for one of the two glutaminase activities would greatly facilitate the assessment of the specificity of various assay conditions.

AT-125 [L-(α*S*, 5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid] is a glutamine antagonist which inactivates various glutamine amidotransferases [5] and glutaminases [6]. Rat renal γ-glutamyltranspeptidase is also rapidly and irreversibly inactivated by low concentrations of AT-125 [7,8]. In contrast, the phosphate-dependent glutaminase activity was found to be completely resistant to inactivation by AT-125. Therefore, this inhibitor can be used to determine the specificity of various in vitro glutaminase assays and to establish the in vivo contribution of γ-glutamyltranspeptidase to rat renal ammonia excretion.

2. Materials and methods

White male rats (100–200 g) were obtained from Zivic Miller and were maintained on Purina Rat Chow. AT-125 was kindly provided by Dr Ruth Davis of the National Cancer Institute.

Phosphate-dependent glutaminase was purified from acidotic rats [9] and stored as a 1–2 mg/ml suspension in 10 mM sodium borate, 100 mM potassium phosphate, 100 mM potassium pyrophosphate buffer (pH 8.6). Before use in various experiments, the enzyme was dialyzed against two 1–1 changes of 10 mM Tris–acetate, 1 mM dithiothreitol buffer (pH 8.6). Glutaminase activity was determined by using glutamate dehydrogenase to quantitate glutamate formation [10].

γ-Glutamyltranspeptidase was purified following its solubilization with papain [11]. The transpeptidase activity was determined using either γ-glutamyl-*p*-nitroanilide [3] or as phosphate-independent glutaminase activity [4]. Glutaminase activity was also determined in crude homogenates using either 50 mM Tris–HCl, 10 mM glutamine and 50 mM sodium maleate (pH 8.0) [3] or 10 mM Hepes, 2 mM glutamine (pH 7.4) as the initial reaction mixture. After incubating for 10 min at 37°C the reaction was stopped by heating to 90°C for 2 min and the glutamate produced was then quantitated using glutamate dehydrogenase [10]. Protein was determined by the method of Lowry [12]. Plasma glutamine [13] and urinary ammonia [14] concentrations were determined as described.

3. Results

When purified γ-glutamyltranspeptidase is incubated at 25°C with 0.1 mM AT-125, its activity decreases with first order kinetics and a $t_{1/2}$ of 7 min. The inactivated transpeptidase failed to utilize either γ-glutamyl-*p*-nitroaniline or glutamine as a substrate. In contrast, as shown in table 1, incubation of purified phosphate-dependent glutaminase for 30 min at 25°C with up to 10 mM AT-125 results in no loss of activity.

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Table 1
Effect of AT-125 on the activity of purified rat renal phosphate-dependent glutaminase

Samples	$-P_i$	$+P_i$
	(μmol . min^{-1} . ml^{-1})	
Control	11.4	14.1
+ 0.3 mM AT-125	11.7	14.2
+10 mM AT-125	11.2	14.1

Aliquots of purified phosphate-dependent glutaminase containing 10 μg protein were diluted into 100 μl 10 mM Tris–HCl buffer (pH 8.6) containing either no phosphate ($-P_i$) or 150 mM potassium phosphate ($+P_i$) and the indicated concentrations of AT-125. After incubating at 25°C for 30 min, the samples were assayed for phosphate-dependent glutaminase activity

Since inactivation of phosphate-dependent glutaminase with 6-diazo-5-oxo-L-norleucine occurs only in the presence of phosphate [15], the incubations with AT-125 were repeated in the presence of sufficient phosphate to promote complete conversion of the glutaminase to its active dimeric conformation. Even under these conditons, the glutaminase was completely resistant to inactivation by AT-125.

Because of the selective inhibition of γ-glutamyltranspeptidase by AT-125 this agent should provide an effective criterion of the specificity of assays which quantitate glutamine hydrolysis activity in crude homogenates of kidney tissue. As shown in table 2, preincubation of a crude homogenate of normal kidney tissue with AT-125 results in complete inactivation of the transpeptidase activity determined with either γ-glutamyl-*p*-nitroanilide or the assay for phosphate-independent glutaminase activity. However, ~30% of both the glutaminase activity measured at pH 7.4 in the absence of exogenous activator and the maleate-stimulated glutaminase activity measured at pH 8.0 are resistant to inactivation by AT-125. Therefore, activities in addition to γ-glutamyltranspeptidase contribute significantly to the total glutaminase activity measured under the latter two conditions.

The observed difference in reactivity suggested that injection of AT-125 may result in selective in vivo inactivation of γ-glutamyltranspeptidase. As shown in table 3, within 1 h after injection of 8–10 mg AT-125/kg body wt, 95% of the total renal γ-glutamyltranspeptidase activity is inactivated. In contrast, the injection of AT-125 had no effect on the total renal phosphate-dependent glutaminase activity. When a set of AT-125-treated rats were made acutely acidotic, the observed increase in ammonia excretion was not significantly different from that observed with non-

Table 3
Selective in vivo inhibition of γ-glutamyltranspeptidase by injection of AT-125

Animals	Phosphate-dependent glutaminase	γ-glutamyl-transpeptidase
	(nmol . min^{-1} . mg^{-1})	
Control	104 ± 5	1230 ± 78
+AT-125	107 ± 6	66 ± 8

Two groups of five 100–120 g rats were injected intraperitoneally with either 0.5 ml saline (control) or 0.5 ml saline containing 1 mg AT-125 (+AT-125). After 1 h, the animals were decapitated and the kidneys excised and homogenized in 8 vol. 0.33 M sucrose, 20 mM Tris–HCl, 0.2 mM EDTA buffer (pH 7.5). The crude homogenates were then assayed for phosphate-dependent glutaminase and γ-glutamyltranspeptidase activities and for protein. The values reported are the mean ± SE

Table 2
Use of AT-125 to determine the specificity of glutaminase assays applied to crude homogenates of normal rat kidney

Homogenate	γ-Glutamyl-transpeptidase activity	Glutaminase activity (nmol . min^{-1} . mg^{-1})		
		PIG	Gln (pH 7.4)	Gln (pH 8.0) +maleate
Control	1320 ± 230	70 ± 4	3.1 ± 0.3	83 ± 3
+AT-125	<10	<1	0.9 ± 0.2	23 ± 1

Aliquots of three crude homogenates were incubated for 30 min at 25°C in the presence or absence of 1 mM AT-125 and then assayed for γ-glutamyltranspeptidase activity using γ-glutamyl-*p*-nitroanilide and for glutaminase activity using the conditions described for phosphate-independent glutaminase (PIG) or the conditions in section 2. The values reported are the mean ± SE

Table 4
Effect of in vivo inhibition of γ-glutamyltranspeptidase on renal adaptation to acute acidosis

Animals	Plasma [Glutamine] (mM)	Urine (pH)	Urine $[NH_4^+]$ (μmol/100 g . 4 h)	γ-Glutamyltranspeptidase act. (nmol/min^{-1} . mg^{-1})
Normal	0.469 ± 0.023	7.4 ± 0.27	34 ± 7	1230 ± 80
Acute acidosis	0.696 ± 0.013	5.7 ± 0.02	185 ± 16	1120 ± 40
+AT-125	0.648 ± 0.044	5.6 ± 0.08	209 ± 28	77 ± 6

Two groups of six 100–120 g rats were injected with saline or AT-125 as in table 3. After 1 h, the animals were stomach-loaded with 20 μmol NH_4Cl/kg and retained in metabolism cages for 4 h to permit collection of urine. After this, the rats were anesthetized and arterial blood and the kidneys were withdrawn for glutamine and γ-glutamyltranspeptidase measurements, respectively. The normal controls received only the saline injection. The values reported are the mean ± SE

inhibited, but acutely acidotic control animals (table 4). In addition, injection of AT-125 had no effect on the observed increase in plasma glutamine concentration or the acidification of the urine that are associated with onset of acute acidosis. However, the total transpeptidase activity in the AT-125 treated rats was only 7% of that observed in the controls.

4. Discussion

The data reported in this study indicate that AT-125 can be used to inactivate γ-glutamyltranspeptidase without effecting the phosphate-dependent glutaminase activity. It is thus possible to use this inhibitor to determine the relative contribution of the two activities to glutamine metabolism observed in crude homogenates of rat kidney tissue. The inhibitor should be equally effective in experiments using kidney slices or isolated renal tubules. Because of this specificity, AT-125 will also produce in vivo a selective inhibition of γ-glutamyltranspeptidase.

The observation that the activity measured in crude homogenates under the conditions originally developed to quantitate phosphate-independent glutaminase activity [4] is completely inhibited by AT-125 indicates that this assay is specific for the glutaminase activity due to γ-glutamyltranspeptidase. Therefore, even though the activity observed under these conditions is less than maximal, the high degree of specificity offered by these conditions make them more suitable for detecting the presence of glutaminase activity which is due to γ-glutamyltranspeptidase. The lack of specificity observed with maleate at pH 8.0 is probably due to the fact that maleate will substitute for phosphate in activating the phosphate-dependent glutaminase [4].

On the basis of reports that γ-glutamyltranspeptidase activity is increased in response to chronic metabolic acidosis [16,17], it has been proposed that this enzyme may contribute to renal ammonia synthesis. We have confirmed that the specific activity of γ-glutamyltranspeptidase, assayed with γ-glutamyl-*p*-nitroanilide, is slightly, but significantly increased (1.3-fold) during prolonged acidosis. This increased activity is retained throughout a 10-fold purification of brush border membrane vesicles. However, we found that the specific activity of alkaline phosphatase is also increased slightly in both crude homogenates and isolated brush border membranes derived from acidotic rats. Therefore, this adaptation may result from the generalized renal hypertrophy which occurs in response to chronic acidosis [18]. The observed adaptation in transpeptidase activity is very small in comparison to the 20-fold increase in phosphate-dependent glutaminase activity which occurs in the proximal convoluted tubules during chronic acidosis [4]. The results reported here indicate that γ-glutamyltranspeptidase activity is not essential to elicit the adaptive increase in ammonia synthesis observed with onset of acidosis.

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