γ-GLUTAMYLTRANSFERASE IN KIDNEY BRUSH BORDER MEMBRANES

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1. Introduction

γ-Glutamyltransferase catalyses the transfer of the γ-glutamyl moiety of glutathione to a variety of α amino acids. The enzyme is widely distributed in animal tissues, being most active in kidney [1]. It was demonstrated with histochemical methods that hydrolytic activity against γ-glutamyl-peptides is mainly concentrated in the kidney brush border [2]. The function of the enzyme in the formation of mercapturic acids is established [3] and the enzyme may be a part of a general detoxification mechanism in mammals using glutathione [3]. Orlowski and Meister [4] recently proposed a theory for amino acid transport in mammalian kidney in which a membrane bound γ-glutamyltransferase could function in the transport process of amino acids. A sequence of enzymatic reactions can be put into a cycle, called γ-glutamyl cycle, in which an amino acid enters via a transpeptidation process and leaves by the hydrolytic activity of the transpeptidase or the action of a γ-glutamylcyclotransferase [4]. The reaction products cysteinylglycine and pyrrolidone carboxylic acid can enter the cycle again to form glutathione, energy being provided by ATP. We have studied uptake of various amino acids in isolated kidney brush border membranes [5] and were interested in enzymatic reactions which could function in a transport process.

However, besides some histochemical data, no biochemical data are available about the enzyme in brush border fractions. This study was undertaken to determine if γ-glutamyltransferase could be demonstrated in the isolated kidney brush borders and to determine if the enzyme is as highly specific for brush border structures as has been demonstrated for alkaline phosphatase and maltase [6].

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2. Materials and methods

2.1. *Materials*

L-γ-glutamyl-p-nitroanilide was from Sigman, p-nitrophenylthymidilate (sodium salt) was from Calbiochem, p-nitrophenylphosphate was from Sigma, γ-L-glutamyl–glycyl–glycine was a gift from Cyclo.

2.2. *Methods*

2.2.1. Preparation of brush border of rat kidney

Male Sprague-Dawley rats were used for the preparation of kidney brush border. The preparation followed the procedure as described by Wilfong and Neville [6].

2.2.2. Enzyme determinations

5′Nucleotidase was determined according to Michell and Hawthorne [7]. Alkaline phosphatase was determined as previously described [6]. Phosphodiesterase I was determined with *p*-nitrophenyl thymidilate [8]. Maltase was determined with the method of Dahlquist [9], using maltase-free glucose oxidase. Leucinaminopeptidase was tested with leucine-*p*-nitroanilide [10].

γ-Glutamyltransferase was determined with γ-glutamyl-p-nitroanilide in the following two reaction mixtures. Reaction mixture A (this enzymatic activity will be from now on referred to as the unstimulated activity): 20 μl 0.2 M $MgCl_2$, 40 μl γ-glutamyl-nitro-

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anilide (10 mM), 20 µl H_2O, 100 µl 0.2 M Tris-HCl buffer pH 8.8 and 20 µl enzyme. And reaction mixture B: 20 µl 0.2 M $MgCl_2$, 40 µl γ-glutamyl-p-nitroanilide (10 mM), 20 µl 0.04 M glycylglycine (titrated with NaOH to pH 8.2), 100 µl 0.2 M Tris buffer, pH 8.2 and 20 µl enzyme.

It should be noted that the substrate is soluble only if heated before adding to the reaction mixture. The reaction is started by addition of enzyme and stopped after 15, 30, or 60 sec with the addition of 0.1 ml 1.5 M acetic acid and p-nitroaniline was measured by absorption at 410 nm (Orlowski and Meister [12]). Protein was determined with the Lowry method [11]. Chromatography of reaction products was performed on thin layer cellulose plates using the solvent systems described by Orlowski and Meister [12], Leibach and Binkley [13] and n-butanol:water: pyridine:acetic acid solvent (30:30:30:10). Peptides were visualized with a ninhydrin spray (Sigma).

3. Results

3.1. *Reaction characteristics*

Preliminary experiments indicated that kidney brush border preparations were highly active in liberating *p*-nitroaniline from γ-L-glutamyl-*p*-nitroanilide. The reaction was linear up to 2 min and linear with increasing amounts of brush border.

The enzymatic activity was markedly stimulated by magnesium ions and with the dipeptide glycylglycine (table 1).

The pH dependence of the reaction was studied and different pH optima were found in the presence or the absence of glycylglycine (fig. 1).

The pH optimum in the presence of glycylglycine was 8.2 and without glycylglycine 8.8 at 37°. Consequently, we measured the activity of the glycylglycine stimulated activity at pH 8.2 and the activity of the unstimulated activity at pH 8.8.

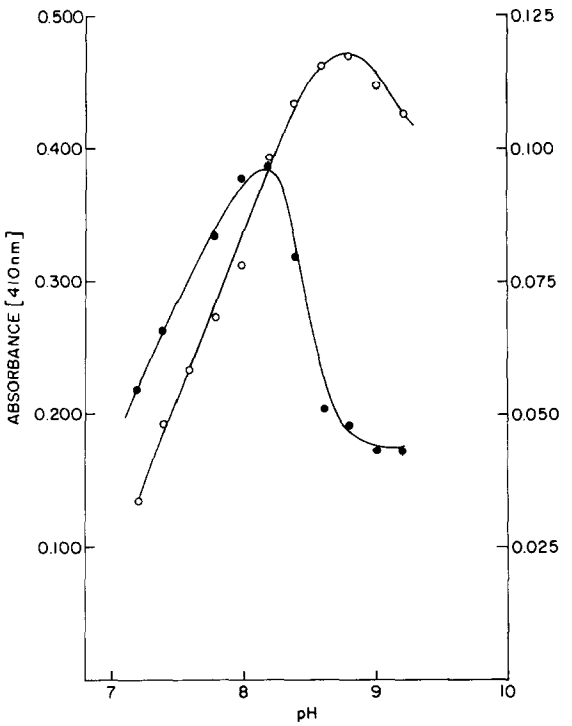

Fig. 1. pH profile of *p*-nitroaniline formation by rat kidney brush border (14.5 µg protein). Closed circles and left ordinate: activity in the presence of 4 mM glycylglycine. Open circles and right ordinate: activity without glycylglycine. Incubation time was 1 min, 0.1 M Tris-HCl buffer, 20 mM $MgCl_2$, 2 mM γ-glutamylparanitroanilide. Temperature 37°.

3.2. *Identification of the enzymatic activity as transpeptidase activity*

Though it is established that *p*-nitroaniline is liberated from γ-L-glutamyl-*p*-nitroaniline by kidney

Table 1
Stimulation of p-nitroaniline formation from γ-glutamyl-p-nitroanilide.

pH	No addition	20 mM $MgCl_2$	4 mM glycylglycine	+20 mM $MgCl_2$ 4 mM glycylglycine
8.2	1.0	2.0 ± 0.4	6.5 ± 2	8.2 ± 2
8.8	1.0	1.8 ± 0.6	3.3 ± 1	3.5 ± 1

The values are mean values from three different brush border preparations. Activity without addition = 1.

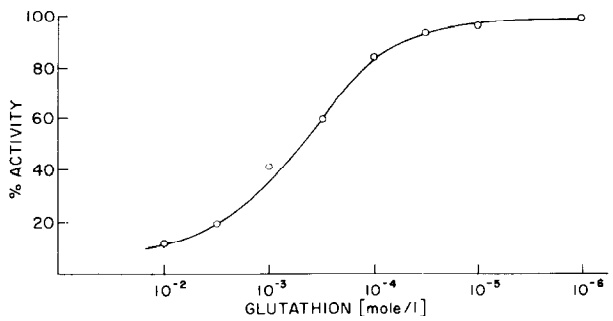

Fig. 2. Inhibition of γ-glutamyltransferase activity by reduced glutathione in rat kidney brush border. Experimental conditions: 0.1 M Tris-HCl buffer pH 8.2, 20 mM $MgCl_2$, 4 mM glycylglycine, 2 mM γ-glutamylparanitroanilide. Temperature 37°. Activity is expressed in percent of control values without inhibitor present.

brush border preparations, this is not evidence enough to demonstrate transpeptidase activity since a hydrolyzing activity (γ-L-glutamyl-*p*-nitroanilidase) could also liberate *p*-nitroaniline from the substrate.

A survey of the reaction products was done with thin layer chromatography and the main reaction products formed in the presence of glycylglycine were γ-L-glutamylglycylglycine and *p*-nitroaniline confirming the transpeptidase reaction:

γ-L-glutamyl-*p*-nitroaniline + glycylglycine →
p-nitroaniline + γ-L-glutamylglycylglycine

Without glycylglycine present the main reaction products were γ-L-glutamyl-γ-L-glutamyl-*p*-nitroanilide, γ-L-glutamyl-γ-L-glutamyl-γ-L-glutamyl nitroanilide, glutamic acid and *p*-nitroaniline, again confirming a transpeptidase activity in which γ-L-glutamyl-*p*-nitroanilide can serve as both donor and acceptor for the γ-glutamyl moiety. This establishes the reactions:

2 γ-L-glutamyl-*p*-nitroanilide →
γ-L-glutamyl-γ-L-glutamyl-*p*-nitroanilide +
p-nitroaniline

and

γ-L-glutamyl-γ-L-glutamyl-*p*-nitroanilide +
γ-L-*p*-nitroaniline →
γ-L-glutamyl-γ-L-glutamyl-*p*-nitroanilide +
p-nitroaniline

The appearance of glutamic acid demonstrates that hydrolysis also takes place and the γ-glutamyl residues are transferred on water acting as acceptor. These results are in complete agreement with those obtained by Szewczuk and Baranowski [14] and Orlowski and Meister [12] with the isolated enzyme.

Additional evidence for transpeptidase activity in the presence of glycylglycine should be inhibition of *p*-nitroaniline formation by the natural donor glutathione. Both oxidized and reduced glutathione inhibited the liberation of *p*-nitroaniline. In fig. 2 the inhibition curve for reduced glutathione is shown. 6×10^{-4} M glutathione (reduced) inhibited 50%.

3.3. *Enrichment of γ-glutamyltransferase in kidney brush border preparations*

In order to show enrichment of γ-glutamyltransferase activity in brush borders we followed the activity at each step of the purification procedure. In table 2 a representative experiment is shown, comparing homogenate and final brush border preparation. According to the relative specific activities, the enzymes tested can be divided into two groups: one having relative specific activities in the range of 12–17 and a second with relative specific activities significantly lower. Both the activity of γ-L-glutamyltransferase in the presence of glycylglycine and without glycylglycine belong evidently to the second group.

According to previous results [6], Na^+K^+-stimulated ATPase and adenylcyclase belong also to the second group, having relative specific activities of 1.9 and 2.1, respectively.

4. Discussion

γ-L-glutamyltransferase in the rat kidney has been shown with histochemical methods to be located mainly in the brush border of proximal tubular convoluted tubules, but also in medullar vascular bundles [2] and the Henle loop [1]. We can confirm by our results that enzymatic activity is present in a well characterized brush border preparation. The relative specific activities for both activities (stimulated by glycylglycine and unstimulated enzyme) however, are significantly lower than those for maltase and alkaline phosphatase believed to be primarily located in the brush border. We can confirm that other cell fractions

Table 2
Specific activities and relative specific activities of various enzymes located in the kidney brush border of the rat[1].

Enzyme	Specific activity in homogenate	Specific activity in final brush border preparation	Relative specific activity
Alkaline phosphatase	6.00 ± 0.7	100 ± 4.0	16
5'nucleotidase	2.8 ± 0.2	45 ± 3.0	17
5'nucleotidase corrected[2]	2.5 ± 0.2	40.5 ± 3.0	17
Phosphodiesterase I	2.2 ± 0.6	28 ± 1.5	12
Maltase	4.0 ± 0.5	69 ± 5.8	17
Leucinamino-peptidase	3.4 ± 0.2	14.0 ± 0.3	4
γ-L-glutamyltransferase (unstimulated activity)	18 ± 2	86.0 ± 8	5
γ-L-glutamyl-transferase (stimulated activity)	41 ± 6	215 ± 9	5

The values are obtained by measuring activities for 4 different conc. of homogenate and final brush border preparation and 2 different times of incubation. All experiments were performed at 37° and the values are expressed in μmole/mg protein/hr with the exception of maltase where activity is expressed in μmole glucose formed/mg protein/hr.

[1] A balance study of the membrane preparation has been published [6].

[2] Isolated alkaline phosphatase hydrolyses nucleotide monophosphates [20]. In order to measure the activity of 5'nucleotidase in the presence of alkaline phosphatase the rate of hydrolysis of the mixed isomer substrate 2'AMP and 3'AMP was subtracted from the rate of hydrolysis of 5'AMP. In addition to this we used specific inhibitors to discriminate between the 2 enzymatic activities: 5 mM cysteine and 5 mM dithiothreitol (15 min preincubation at pH 9.1) inhibited alkaline phosphatase activity 95%, 5'nucleotidase activity only 2.6%. 50 mM fluoride inhibited 5'nucleotidase activity 65% but had no effect on alkaline phosphatase. 1% dodecylsulfate (sodium salt) inhibited 5'nucleotidase activity 96% but stimulated alkaline phosphatase about 25%. 1 mM nickel chloride inhibited 5'nucleotidase activity 55% but had no inhibitory effect on alkaline phosphatase. 20 mM L-homoarginine inhibited alkaline phosphatase 97% but inhibited 5'nucleotidase only 6%.

must contain enzymatic activity, whether or not due to the same enzyme cannot be answered yet. Another possibility, though less likely, is since during the preparation small vesicles are pinched off from microvilli of the brush borders, a slightly higher enrichment of the basilar portion of the brush border takes place [6]. If the enzymatic activity is concentrated in the distal part of the microvilli, loss of activity and less enrichment compared to enzymes located in the basilar part (e.g., alkaline phosphatase and maltase) would result.

The specific activity of the enzyme measured in the presence of glycylglycine with γ-L-glutamyl-*p*-nitroaniline is the highest of all enzymes studied so far in the kidney brush border of the rat. With the natural substrate glutathione the activity is about 5 times higher [12], and the values should be in the 1000 μmole/mg/hr range for the glycylglycine stimulated activity if measured with glutathione. We calculate that about 1.5% of the total protein in the brush border would be γ-glutamyl transferase if the specific activity of the rat enzyme is in the same range as reported for the purified enzyme from hog [12].

Though the enzyme has been purified about 1000-fold from kidney cortex of beef [14] and hog [12], it is not a homogeneous preparation and it was suggested that more than one γ-glutamyltransferase is present in the kidney. The enzyme purified by Orlowski and Meister [12] is a glycoprotein containing 18% neutral sugars, 8% amino sugars and 10% sialic acid, its pH optimum for hydrolysis of *p*-nitroanilide was 8.8 (as was for our kidney brush border preparation of the rat (without glycylglycine present).

Leibach and Binkley, on the contrary [13], purified

a γ-glutamyltransferase from swine kidney cortex which contained no carbohydrate. It had a molecular weight of above 80,000 and was strictly dependent on added acceptor (glycylglycine). The data of Orlowski and Meister [12] taken together with the data of Leibach and Binkley [13] as cited above could suggest the existence of at least two separate transferase enzymes. However, in the case of the hog enzymes a purification step was used which involved proteolysis. Two recent examples of artifactual molecular species obtained by proteolytic cleavage demonstrate the danger of such a step [15, 16], especially since some glycoproteins contain peptide bonds extremely sensitive to proteolytic enzymes [17]. The pH optima observed in the presence of glycylglycine and its absence are close to the pK values of the terminal amino groups of gly–gly (8.25) and of γ-glutamyl derivatives (9.2). (Data from [18]).

The difference seen in the pH activity profile can be due at least in part to the fact that it is the uncharged amino group of the acceptor which is necessary for transpeptidation. We have recently characterized glycoproteins in different cell membranes including the kidney brush border [19] and reported that kidney brush border (rat) contains a complex mixture of at least 10–12 different glycoprotein subunits. Since we calculate that γ-glutamyltransferase represents about 1.5% of the membrane protein in the brush border fraction, it should be observable by Schiff stain after gel electrophoresis [19].

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