Active Site of Intestinal γ -Glutamyltransferase Faces Luminal Side of Brush Border Membrane

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Summary Purified γ -glutamyltransferase from hog small intestine was competitively inhibited by glutathione *in vitro* when L- γ -glutamyl-*p*-nitroanilide was used as a substrate. An S-acetyldextran derivative of glutathione inhibited the enzyme as well as glutathione, although dextran had no inhibitory effect. γ -Glutamyltransferase in the rat small intestine could utilize *in situ* L- γ -glutamyl-*p*-nitroanilide circulated in the lumen, and was inhibited by the impermeable derivative of glutathione which was on the luminal side. These data suggested that the active site of intestinal γ -glutamyltransferase faced the luminal side of the brush border membrane.

Key Words active site of γ -glutamyltransferase, inhibition by S-acetyldextran derivative of glutathione, small intestine

Many investigations (1-6) have been carried out to examine the participation of γ -glutamyltransferase [EC 2.3.2.2] in amino acid transport since 1970, at which time Orłowski and Meister proposed the γ -glutamyl cycle and suggested the possibility of the enzyme taking part in renal reabsorption of amino acids (7). However, this has recently become questionable. In 1975, Young *et al.* reported that γ -glutamyltransferase did not participate in amino acid transport in rabbit erythrocyte (8). In the following year, Pellefigue *et al.* reported that normal amino acid uptake by cultured human fibroblast did not require γ -glutamyltransferase (9). Inoue *et al.* demonstrated the lack of functional correlation of the catalytic activity of the enzyme with amino acid transport in rat ascites tumor cell LY-5 (10), and Horiuchi *et al.* proved by using an impermeable polymer derivative of glutathione that the active site of γ -glutamyltransferase of rat renal brush border membrane faced the luminal side of the renal tubules and could not utilize intracellular glutathione (11). It was also demonstrated recently in the human pancreatic carcinoma cell line that cellular transport of the glutamine analog acivicin

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did not require γ -glutamyltransferase (12).

γ -Glutamyltransferase also exists in the small intestine of various animals (5, 13–28), but its enzymatic properties have not been elucidated clearly because of the difficulty of extraction from the organ (22, 27). As the small intestine is the most important organ for amino acid absorption, many investigations have been done on whether the enzyme participates in amino acid absorption in the small intestine. In 1976, Garvey *et al.* correlated the localization and characteristics of intestinal γ -glutamyltransferase with the features of intestinal amino acid absorption, and supported its possible role in amino acid transport (22), although contradictory data had been reported in erythrocyte (8) and fibroblast (9). Since then, participation of γ -glutamyltransferase in intestinal amino acid absorption had been thought to be possible until Berteloot *et al.* reported that digestion of intestinal brush border membrane vesicles with papain resulted in nearly complete removal of γ -glutamyltransferase, but increased specific activity of leucine uptake, indicating the non-involvement of the enzyme in amino acid transport (25). In 1981, we reported some properties of γ -glutamyltransferase from hog small intestine, but were not able to find a close relationship between the substrate (γ -glutamyl acceptor) specificity of the enzyme and amino acid transport systems in the small intestine (27).

This study shows that intestinal γ -glutamyltransferase has the active site facing the luminal side of the brush border membrane *in situ*, and can utilize glutathione in the intestinal lumen, indicating the irrelevance of the enzyme to intestinal amino acid absorption.

MATERIALS AND METHODS

Partial purification of γ -glutamyltransferase from hog small intestine. The mucosa of hog small intestine was homogenized with 10 mM Tris·HCl buffer, pH 8.5, containing 250 mM sucrose, 140 mM NaCl, and 4 mM KCl, and was centrifuged at $100,000 \times g$ at 0°C for 30 min. The pellet was suspended in 10 mM Tris·HCl buffer, pH 8.5, treated with bromelain (1 mg/10 mg protein) according to the method of Tate and Meister (29), and centrifuged at $100,000 \times g$ at 0°C for 30 min. To the supernatant, solid ammonium sulfate was added to give 70% saturation, followed by centrifugation at $100,000 \times g$ at 0°C for 20 min. The supernatant was dialyzed against 10 mM Tris·HCl buffer, pH 8.5. An aqueous solution of streptomycin sulfate was added to the dialyzate (1.5 mg streptomycin sulfate/mg protein), and centrifuged at $15,000 \times g$ at 0°C for 30 min. Solid ammonium sulfate was added to the supernatant to give 95% saturation. The precipitate was dissolved in 10 mM Tris·HCl buffer, pH 8.5, and dialyzed against the same buffer. The dialyzate was further purified by a series of column chromatography with Sephadex G-200, DEAE-cellulose, AH-Sepharose 4B, and Cu-coupled Sepharose 6B. The final preparation could release $23.5 \mu\text{mol } p\text{-nitroaniline/min} \cdot \text{mg protein}$ when incubated with 5 mM L- γ -glutamyl-*p*-nitroanilide in 10 mM Tris·HCl buffer, pH 8.5, at 37°C .

Fig. 1. An apparatus for determination of intestinal γ -glutamyltransferase activity *in situ* by a circulation method. 1, a sample pot; 2, circulated substrate solution; 3, an incubator; 4, a pump; 5, silicon tubes; 6, the small intestine of a rat anesthetized with ethyl ether.

Synthesis of S-acetyldextran derivative of glutathione. Dextran was bromoacetylated by the method of Robbins and Schneerson (30), and conjugated with glutathione by the method described by Inoue *et al.* (10).

Assay of γ -glutamyltransferase in vitro. γ -Glutamyltransferase activity was determined *in vitro* at 37°C using L- γ -glutamyl-*p*-nitroanilide as a substrate in 1 ml of 10 mM Tris·HCl buffer, pH 8.5, in the presence or absence of glutathione, an S-acetyldextran derivative of glutathione or dextran. The enzyme reaction was terminated by addition of 2 ml of 1.5N acetic acid, and the amount of *p*-nitroaniline released was calculated from the absorbance at 410 nm.

Assay of γ -glutamyltransferase in situ. Albino rats of the Wistar strain with body weights between 200 and 300 g were fasted for 1 day before the experiments. The small intestine of a rat anesthetized with ethyl ether was cut at the top of the duodenum and the bottom of the ileum, washed with 10 mM Tris·HCl buffer, pH 8.5, and connected through silicon tubes to a sample pot containing 40 ml of substrate solution. Five mM L- γ -glutamyl-*p*-nitroanilide in 10 mM Tris·HCl buffer, pH 8.5, was circulated as substrate solution with or without glutathione, an S-acetyldextran derivative of glutathione, and dextran at the rate of 1.8 ml/min by a Perista pump. Absorbance at 410 nm of substrate solution in the sample pot was determined at 5 min intervals (Fig. 1).

Reagents. Streptomycin sulfate and bromelain were purchased from Meiji Confectionary Co., Ltd. and Wako Pure Chemical Industry Ltd., respectively. L- γ -Glutamyl-*p*-nitroanilide was obtained from Nakarai Chemicals Ltd.

RESULTS

Inhibition of purified γ -glutamyltransferase in vitro by glutathione and its polymer derivative

Enzymatic release of *p*-nitroaniline was inhibited by glutathione when purified γ -glutamyltransferase and L- γ -glutamyl-*p*-nitroanilide were incubated *in vitro* (Fig. 2). The Lineweaver-Burk plots did not show a series of straight lines fanning out from the same ordinate intercept. They became parabolic with the increase in glutathione concentration.

Five and 10 mg/ml of the *S*-acetyldextran derivative of glutathione inhibited the enzyme as well as 0.2 and 0.4 mM glutathione respectively although dextran had no inhibitory effect (Fig. 3). But 20 mg/ml of the dextran derivative was more inhibitory than 0.8 mM glutathione. The higher concentration of the dextran derivative was more inhibitory than glutathione.

Inhibition of intestinal γ -glutamyltransferase in situ by glutathione and its polymer derivative

When L- γ -glutamyl-*p*-nitroanilide was circulated *in situ* with or without glutathione, *S*-acetyldextran derivative, and glycylglycine, release of *p*-nitroaniline from the synthetic substrate linearly increased with time after 15 min circulation although, at the early time, *p*-nitroaniline was released parabolically (Fig. 4). Therefore we expressed enzymatic activities *in situ* as slopes of these lines after 15 min. When 1 mM L- γ -glutamyl-*p*-nitroanilide was circulated, the enzyme activity was 16.6 pmol/min. Addition of 5 mM glycylglycine activated the enzyme activity

Fig. 2. Inhibition of *p*-nitroaniline release from L- γ -glutamyl-*p*-nitroanilide by glutathione. Activity of purified γ -glutamyltransferase from hog small intestine was determined using L- γ -glutamyl-*p*-nitroanilide (γ -GPNA) as a substrate in 100 mM Tris·HCl buffer, pH 8.5. Concentrations of glutathione were 0 (a), 0.1 (b), 0.2 (c), and 0.3 (d) mM.

Fig. 3. Effects of glutathione, *S*-acetyldextran derivative of glutathione, and dextran on purified γ -glutamyltransferase from hog small intestine. One mM *L*- γ -glutamyl-*p*-nitroanilide was used as a substrate. Symbols denote: ○, glutathione; ●, *S*-acetyldextran derivative of glutathione; and ■, dextran, respectively.

Fig. 4. γ -Glutamyltransferase activity *in situ* determined by a circulation method. Symbols denote: ○, 1 mM *L*- γ -glutamyl-*p*-nitroanilide; □, 1 mM *L*- γ -glutamyl-*p*-nitroanilide plus 5 mM glycylglycine; ■, 1 mM *L*- γ -glutamyl-*p*-nitroanilide plus 0.63 mM glutathione; ●, 1 mM *L*- γ -glutamyl-*p*-nitroanilide plus 15 mg/ml *S*-acetyldextran derivative of glutathione. All values are means of 3 to 5 experiments. Standard deviations of the means were 10 to 20% of these values. There were statistically significant differences among all cases ($p < 0.01$) except that there was no difference between the case of 1 mM *L*- γ -glutamyl-*p*-nitroanilide (○) and that of 1 mM *L*- γ -glutamyl-*p*-nitroanilide plus 0.63 mM glutathione (■).

1.72-fold (28.6 pmol/min) although the peptide activated the enzyme 2.5-fold *in vitro*. Fifteen mg/ml of the dextran derivative of glutathione effectively inhibited the enzyme (8.5 pmol/min). But 0.6 mM glutathione did not inhibit the enzyme, although both 15 mg/ml of the dextran derivative and 0.6 mM glutathione inhibited the enzyme about 55% *in vitro* (Fig. 3). Release of *p*-nitroaniline at the rate of 5.8 pmol/min was observed in the sample pot when circulation was stopped after 40 min circulation of 1 mM L- γ -glutamyl-*p*-nitroanilide. This might express the activity of the enzyme in the fragments which were torn off from the intestinal mucosa during circulation of the substrate solution.

DISCUSSION

Glutathione must bind to the active site of γ -glutamyltransferase, competing with L- γ -glutamyl-*p*-nitroanilide and consequently inhibit the release of *p*-nitroaniline from the synthetic substrate, because the tripeptide is a natural substrate for the enzyme. However, the Lineweaver-Burk plots did not give a series of straight lines crossing the same ordinate intercept (Fig. 2) when L- γ -glutamyl-*p*-nitroanilide was used as a substrate and glutathione as an apparent inhibitor. It is well known that γ -glutamyltransferase catalyzes three reactions (transpeptidation, autotranspeptidation, and hydrolysis) depending upon the species of the acceptor (31). In all cases, the formation of a glutamyl enzyme is supposed as an intermediate, according to a ping-pong bi-bi mechanism (31). If the enzyme catalyzes hydrolysis of L- γ -glutamyl-*p*-nitroanilide (A) and glutathione (B) under the experimental conditions, the reaction velocity v that is measured as release of *p*-nitroaniline is expressed as the equation

$$v = \frac{V_{\max}^h [A]}{K_a \{1 + ([B]/K_b)\} + [A]} \quad (1)$$

where $V_{\max}^h$ is the maximal velocity of the hydrolytic reaction of L- γ -glutamyl-*p*-nitroanilide, and K_a and K_b are Michaelis constants for A and B respectively. But the Lineweaver-Burk plots of Eq. 1 must give a series of straight lines crossing at the same ordinate intercept not to agree with the experimental result (Fig. 2). It was therefore speculated that the main reaction under the experimental conditions was not hydrolysis, but autotranspeptidation. The speculation was supported by the fact that the Michaelis constant for L- γ -glutamyl-*p*-nitroanilide acting as an acceptor was lower than that for the synthetic substrate acting as a donor (27).

When γ -glutamyltransferase catalyzes autotranspeptidation mainly using L- γ -glutamyl-*p*-nitroanilide (A) as a substrate, and glutathione (B) binds to the donor site of the enzyme competing with the synthetic substrate, the reaction velocity v that is determined as release of *p*-nitroaniline is expressed as the equation

$$v = \frac{V_{\max}^{\text{aut}} [A]^2}{(K_m^{\text{aut}} K_{ia}^{\text{aut}} / K_{ib}^{\text{aut}}) [B] + K_m^{\text{aut}} \{1 + ([B]/K_{ib}^{\text{aut}})\} [A] + [A]^2} \quad (2)$$

where V_m^{aut} is the maximal velocity of autotranspeptidation, K_m^{aut} is the Michaelis constant for A as previously reported (27), and K_{ia} and K_{ib} are inhibitory constants which are newly defined. According to Eq. 2, plots of $1/v$ versus $1/[A]$ must give a series of parabolic curves crossing the vertical axis at $1/V_m^{\text{aut}}$ to agree with the experimental results (Fig. 2). In other words, the results shown in Fig. 2 supported the thought that glutathione bound to the donor site of the enzyme competing with L- γ -glutamyl-*p*-nitroanilide.

If glutathione residues in the dextran derivative could inhibit the enzyme as well as free glutathione molecules, it was speculated that 1 g of the derivative contained 40 μ mol glutathione residues because 10 mg/ml of the derivative inhibited the enzyme as well as 0.4 mM glutathione (Fig. 3). But the difference in degree of inhibition at high concentration suggested that glutathione and its dextran derivative did not have exactly the same inhibitory effect on the enzyme.

Figure 4 proves that intestinal γ -glutamyltransferase *in situ* can utilize the circulated substrate in the lumen, and was inhibited by the *S*-acetyldextran derivative of glutathione which was on the luminal side. As the polymer derivative was thought to be impermeable (11), the result strongly suggested that the active site of intestinal γ -glutamyltransferase faced the luminal side. According to the hypothesis for amino acid transport proposed by Orłowski and Meister (2, 7), γ -glutamyltransferase must utilize intracellular glutathione; *i.e.*, the active site of the enzyme must face the intracellular side. Considering the results along with other investigation that removal of γ -glutamyltransferase by papain from intestinal brush border membrane vesicles did not decrease leucine uptake (25), and the substrate specificity of the intestinal enzyme could not be correlated closely with features of intestinal amino acid absorption (27), it is reasonable to conclude that γ -glutamyltransferase is irrelevant to amino acid absorption in the small intestine as well as in erythrocytes (8), fibroblasts (9), ascites tumor cells (10), and renal brush border membrane (11).

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