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Inactivation of renal γ-glutamyl transferase by 6-diazo-5-oxo-L-norleucylglycine, an inactive precursor of affinity-labeling reagent

(glutathione metabolism/renal brush border membrane/membrane-bound enzyme/peptidases/prodrugs)

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ABSTRACT *In vitro* experiments showed that 6-diazo-5-oxo-L-norleucylglycine, a dipeptide analog of L-glutaminylglycine, inactivates γ-glutamyl transferase bound to renal brush border membrane vesicles but does not inactivate the purified transferase. The rate of inactivation of the membrane-bound enzyme decreased markedly in the presence of dipeptides, such as L-leucylglycine and L-alanylglycine, or in the presence of *o*-phenanthroline, an inhibitor of renal peptidases. The presence of L-cysteinylglycine *S*-acetyldextran polymer (M_r 500,000), which does not permeate membranes, protected the membrane-bound transferase from inactivation by 6-diazo-5-oxo-L-norleucylglycine. This and other findings suggest that the norleucylglycine derivative was hydrolyzed by peptidase(s) bound to the outer surface of the brush border membranes and that the 6-diazo-5-oxo-L-norleucine thus released acts as an affinity-labeling reagent for the membrane-bound transferase. Similar effects were observed *in vivo*. Intravenous administration of 6-diazo-5-oxo-L-norleucylglycine to mice resulted in a marked decrease in renal transferase activity. Mice thus pretreated with 6-diazo-5-oxo-L-norleucylglycine, but not an untreated group, excreted significant amounts of *S*-carbamido[^{14}C]methylglutathione in their urine within 30 min of intravenous administration of this compound. This finding suggests that the renal transferase was involved in the hydrolysis of the glutathione *S*-conjugate in the glomerular filtrate *in vivo* and that the administered 6-diazo-5-oxo-L-norleucylglycine underwent hydrolysis peptidase(s)-catalyzed to liberate 6-diazo-5-oxo-L-norleucine that reacted with the membrane-bound γ-glutamyl transferase.

γ-Glutamyl transferase catalyzes the transfer of the γ-glutamyl group of glutathione to a wide variety of amino acids and peptides (1–3). Initially, it was proposed that this enzyme participates in the transport of these acceptors via the γ-glutamyl cycle (3–5). However, it has since been found that the active site of the enzyme is located exclusively on the outer surface of cell membranes (6–9) and that the predominant role of the enzyme is to catalyze the hydrolysis of γ-glutamyl linkage of glutathione, especially at physiological pH values (10, 11). Thus, the true physiological function of γ-glutamyl transferase remains to be studied.

Previous work (12) has shown that the cellular uptake of intact glutathione is a slow process and that extracellular glutathione is largely taken up after cleavage into its constituent amino acids by the cooperative action of γ-glutamyl transferase and some peptidase(s). Because these hydrolytic enzymes are abundant on the outer surface of renal brush border membranes (8, 9, 13, 14, *), such a coordinated process of hydrolysis and transport for glutathione and related compounds would occur also in the kidney (15, 16, †). Thus, elucidation of the physiological function of renal γ-glutamyl transferase requires the development of an efficient way to inactivate this enzyme, especially *in vivo*. Previous studies (17, 18) have shown that γ-glutamyl transferase is inactivated by the affinity-labeling reagent, 6-diazo-5-oxo-L-norleucine (DON), a glutamine analog. However, DON is not suitable for specifically inactivating the transferase *in vivo*, because L-amino acids are readily taken up by many tissues, especially liver (19) and, hence, it will not accumulate in the kidney in an amount sufficient for inactivating the renal transferase (20). We have now found that 6-diazo-5-oxo-L-norleucylglycine (DON-Gly), an L-glutaminylglycine analog, may be useful for inactivating the renal transferase *in vivo*.

This paper describes the effect of DON-Gly on renal transferase activity *in vitro* and *in vivo* and also the effect of affinity labeling of the renal transferase on the metabolic fate of a glutathione *S*-conjugate *in vivo*.

MATERIALS AND METHODS

Materials. Glutathione, L-γ-glutamyl-*p*-nitroanilide, and L-leucyl-*p*-nitroanilide were purchased from Sigma. L-Leucylglycine, L-alanylglycine, and aminopeptidase M from porcine kidney were obtained from the Protein Research Foundation (Osaka, Japan). *o*-Phenanthroline and *N*-acetyl-L-cysteine were obtained from Nakarai Chemical (Kyoto, Japan). Iodo[2-^{14}C]acetamide was purchased from Amersham (15.8 Ci/mol; 1 Ci = 3.7×10^{10} becquerels). Other reagents used were of analytical grade.

Enzyme Samples. γ-Glutamyl transferase was purified from rat kidney as described (8). Renal brush border membrane vesicles were purified from male Wister rats as described (8, 14). The purified brush border membrane vesicles showed a 15-fold increase in the specific activity for γ-glutamyl transferase as compared with that in homogenate and were shown by electron microscopic observation to be composed of closed vesicles. The membrane samples used contained 5 units of transferase activity and 0.3 unit of aminopeptidase activity per mg of protein.

Assay for Enzymes. γ-Glutamyl transferase activity was determined as described (7) by the method of Orlowski and Meister (21). Aminopeptidase activity was determined by the method of Pfleiderer (22).

Syntheses. DON-Gly was synthesized from *N*-trifluoroacetyl-L-glutaminylglycine-5-chloride-1-ethyl ester and diazomethane according to the procedure described for the synthesis of DON (23). Because the aliphatic diazoketone was very unstable, crystallization of DON-Gly was not performed. However, the synthetic compound showed an absorption spectrum characteristic of an aliphatic diazoketone (24) and gave a single ninhydrin-positive spot on silica gel thin layer chromatography

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Abbreviations: DON, 6-diazo-5-oxo-L-norleucine; DON-Gly, 6-diazo-5-oxo-L-norleucylglycine.

* Tsao, B. & Curthoys, N. P. (1979) *Eleventh International Congress of Biochemistry*, Toronto, Ontario, p. 355.

† Inoue, M., Okajima, K., Ito, K., Horiuchi, S. & Morino, Y. (1979) *Eleventh International Congress of Biochemistry*, Toronto, Ontario, p. 470.

in 1-butanol/acetic acid/water (4:1:1). Enzymatic hydrolysis of this compound with aminopeptidase M, followed by separation of the products by silica gel thin-layer chromatography in the butanol/acetic acid/water solvent system gave two ninhydrin-positive spots having R_F values corresponding to those of glycine and DON (17), respectively. Glutathione S-acetyldextran polymer was synthesized from reduced glutathione and bromoacetyldextran polymer (M_r 500,000) as described (7). L-Cysteinylglycine S-acetyldextran polymer was prepared by treating the glutathione S-acetyldextran polymer with highly purified γ-glutamyl transferase. After hydrolysis in 6 M HCl for 20 hr at 110°C in a sealed evacuated tube, the preparation showed ninhydrin-positive spots having R_F values corresponding to those of glycine and S-carboxymethyl cysteine, respectively, on silica gel thin layer chromatography in chloroform/methanol/ammonia (2:2:1). S-Carbamido[^{14}C]methyl derivatives of glutathione and N-acetyl-L-cysteine were synthesized from iodo[2-^{14}C]acetamide by reaction with reduced glutathione or N-acetylcysteine. The specific activity of these S-conjugates was 1.9×10^4 cpm/μmol.

Inactivation of γ-Glutamyl Transferase. Incubation of the enzyme with DON-Gly was carried out in reaction mixtures containing, in a final volume of 1 ml, various amounts of purified or membrane-bound enzyme, 50 mM potassium phosphate buffer (pH 7.4), 1 M sodium maleate, various concentrations of DON-Gly, and other substances as indicated for individual experiments. Reaction was started by addition of DON-Gly. Incubations were carried out at 25°C. At various time intervals, aliquots were withdrawn from the reaction mixtures, and the remaining enzyme activity was determined as described (7).

Urine Samples. Urine samples collected from 10 mice over a period of 30 min after intravenous administration of S-carbamido[^{14}C]methylglutathione (0.1 mmol kg^{-1}) were separated by paper chromatography on Whatman 3MM paper in 1-butanol/acetic acid/water (60:15:25). The chromatogram was stained with ninhydrin, and its radioactivity was assayed by using a radiochromatogram scanner (Packard model 7201). The R_F values for the S-carbamidomethyl derivatives of glutathione and N-acetyl-L-cysteine were 0.33 and 0.43, respectively.

RESULTS

***In Vitro* Reactivity of DON-Gly.** The effects of DON-Gly on purified and membrane-bound γ-glutamyl transferase preparations are compared in Fig. 1. Incubation of renal brush border membranes with DON-Gly resulted in a marked decrease in the activity of the enzyme. Inactivation was very slow during the first 5 min of incubation but then accelerated. DON-Gly did not inactivate the purified enzyme preparation. Both enzyme samples were rapidly inactivated by DON without any initial lag phase, and the inactivation followed typical pseudo-first-order kinetics as described (7, 17). Serine–borate complex, a transition-state inhibitor of the transferase (25, 26), largely protected the enzyme from inactivation. Because brush border membranes possess bound peptidases in addition to γ-glutamyl transferase (13, 14), this finding was interpreted as showing that DON was released from DON-Gly by the action of some membrane-bound peptidase(s) and then acted as the affinity-labeling reagent for the transferase. To test this hypothesis, the membrane-bound transferase was incubated with DON-Gly in the presence of various reagents. The rate of inactivation decreased significantly in the presence of either L-leucylglycine or L-alanylglycine, which should compete with DON-Gly for some peptidase(s) (Fig. 2). Furthermore, *o*-phenanthroline, a well-known inhibitor of metal-requiring peptidases (27), markedly protected the transferase from inactivation. Thus, peptidase activity in the membrane preparation appears to be required for

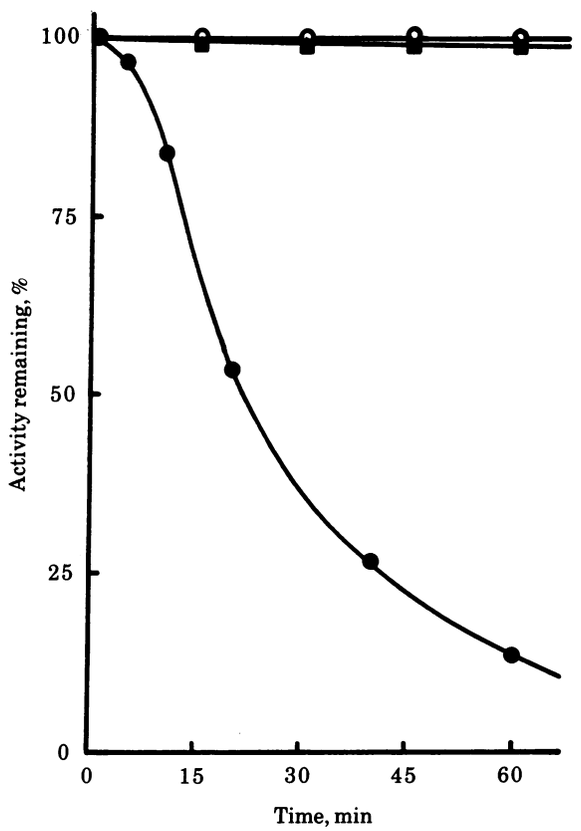

FIG. 1. Inactivation of membrane-bound γ-glutamyl transferase by DON-Gly. Reaction mixtures contained, in a final volume of 1 ml, 6.5 units of purified or membrane-bound γ-glutamyl transferase, 6 mM DON-Gly, 50 mM potassium phosphate buffer (pH 7.4), and 1 M sodium maleate. ●, Membrane-bound transferase; ○, purified renal transferase; ■, membrane-bound transferase plus 10 mM L-serine–borate complex.

inactivation of membrane-bound γ-glutamyl transferase by DON-Gly. Thus, as the membrane concentration increased, the rate of inactivation increased and the length of the initial lag period decreased (data not shown). These findings suggest that the rate of cleavage of DON-Gly by the membranous peptidase(s) and, hence, the amount of DON accumulated, determine the rate of the enzymic process leading to inactivation of γ-glutamyl transferase.

Enzymatic Hydrolysis of DON-Gly on Outer Surface of Membranes. To test whether or not the enzymatic hydrolysis of DON-Gly occurred on the outer surface of the brush border membrane vesicles, the following experiment was performed. The L-cysteinylglycine derivative of acetyldextran polymer is incapable of permeating membranes and therefore the peptide bond of the cysteinylglycine moiety should be accessible only to peptidases located on the outer surface of membrane vesicles. The presence of this dipeptide derivative significantly retarded the inactivation of membrane-bound transferase by DON-Gly (Table 1), probably by competition for the peptidase(s) located on the outer surface of the membrane vesicles. It is also likely that the peptidase(s) hydrolyzed the DON-Gly to generate the affinity-labeling reagent, DON. Aminopeptidase M, one of the most potent and abundant renal enzymes acting on oligopeptides (13), is an outside-oriented membranous metalloenzyme that is easily solubilized by limited proteolysis of the membrane (13). The possibility that this enzyme might be responsible for the hydrolytic cleavage of DON-Gly was tested by incubating purified γ-glutamyl transferase with DON-Gly in the presence of purified aminopeptidase; the progress curve for the inacti-

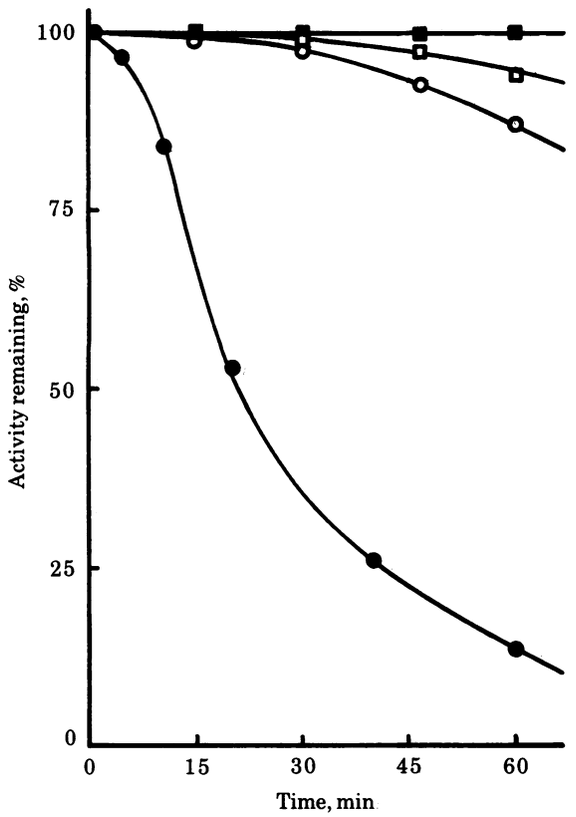

FIG. 2. Effect of various reagents on inactivation of renal transferase. Reaction mixtures contained 6.5 units of membrane-bound γ-glutamyl transferase and 6 mM DON-Gly in the presence or absence of various reagents. ●, Control; ○, plus 50 mM L-leucylglycine; □, plus 50 mM L-alanylglycine; ■, plus 10 mM *o*-phenanthroline.

vation of the transferase was similar to that observed with the membrane preparations (Fig. 3). Also, the presence of serine–borate complex markedly protected the transferase from inactivation. These observations support the view that DON-Gly is hydrolyzed by a peptidase to give DON, which acts as an affinity-labeling reagent for the transferase on the outer surface of brush border membrane vesicles.

***In Vivo* Effects of DON-Gly.** Experiments with mice showed that DON-Gly is also effective as an affinity-labeling reagent for renal transferase *in vivo* (Table 2). The renal transferase activity was inhibited ≈50% by administration of DON-Gly—this effect seemed to be specific for the renal enzyme; i.e., no significant change in hepatic transferase activity was observed. (The specific activity of the hepatic transferase in both the control and DON-Gly-pretreated mice was 1.0 ± 0.12 milliunit per mg protein.)

Table 1. Effect of cysteinylglycine *S*-acetyldextran polymer on inactivation of membrane-bound γ-glutamyl transferase by DON-Gly

Incubation time, min	Activity remaining, units per ml	
	Presence	Absence
0	5.0	5.0
30	3.8	4.9
60	2.4	3.5
90	1.5	2.6

Incubation mixtures contained, in a final volume of 1 ml, 5 units of membrane-bound transferase, 3 mM DON-Gly, 50 mM potassium phosphate buffer (pH 7.4), and 1 M sodium maleate. Incubation was carried out in the presence or absence of cysteinylglycine *S*-conjugate (30 mg/ml).

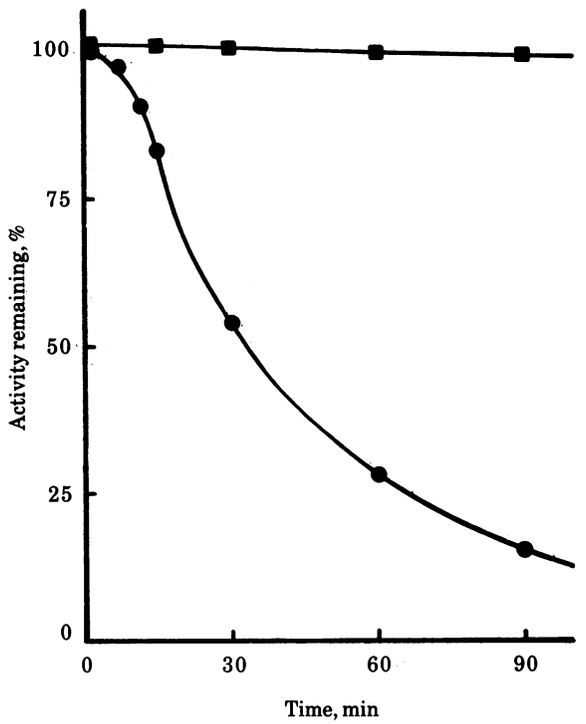

FIG. 3. Inactivation of purified transferase in presence of pure aminopeptidase M. Reaction mixtures contained, in a final volume of 1 ml, 1 M sodium maleate and 8 mM DON-Gly. ●, Control; ■, plus 10 mM L-serine–borate complex.

Previous studies (12, †) using ascites tumor cells have suggested that the uptake of extracellular glutathione is facilitated by its hydrolysis into component amino acids by membranous γ-glutamyl transferase and some peptidases. To test this possibility *in vivo*, the metabolic fate of *S*-carbamido[^{14}C]methylglutathione was studied by using mice whose renal transferase activity had been depressed by administration of DON-Gly. During the 30 min-period after intravenous administration, a significant amount of radioactivity (≈20% of the injected dose)

Table 2. Effect of DON-Gly on renal transferase activity and metabolic fate of *S*-carbamido[^{14}C]methylglutathione administered *in vivo*

	Control mice	DON-Gly-treated mice
Renal γ-glutamyl transferase, units per mg of protein	1.46 ± 0.23	0.72 ± 0.26
Radioactivity administered, cpm/20 g of mouse	3.8×10^4	3.8×10^4
Urinary radioactivity, cpm (% of administered)		
As *S*-carbamidomethylglutathione	0	7290 (19.2%)
As *S*-carbamidomethyl-*N*-acetylcysteine	650 (1.7%)	380 (1.0%)

DON-Gly was administered to mice intravenously (2.5 μmol/kg) 10 min after intraperitoneal administration of sodium hippurate (20 mmol/kg), a potent stimulator of inactivation of transferase by DON (31). After 4 hr, *S*-carbamido[^{14}C]methylglutathione (0.1 mmol/kg) was administered intravenously and mice were placed in metabolic chambers. Urine samples were collected from 10 mice during the 30 min-period after administration of the glutathione *S*-conjugate. Mice were killed 30 min after administration of labeled glutathione *S*-conjugate, and renal transferase activity was determined. DON-Gly was replaced by 0.15 M sodium chloride (0.2 ml) for the control experiments.

appeared in the urine collected from animals pretreated with DON-Gly but only a very small amount (1.7% of the injected dose) was excreted in the urine of control mice. Paper chromatographic analysis of the urine samples from DON-Gly-pretreated mice showed that about 95% of the urinary radioactivity could be accounted for as unchanged glutathione S-conjugate and that the remaining 5% could be accounted for as N-acetyl-S-carbamidomethylcysteine. Most of radioactivity in the urine samples from control animals could also be accounted for as N-acetyl-S-carbamidomethylcysteine (data not shown).

DISCUSSION

We have shown that γ-glutamyl transferase bound to renal brush border membranes is inactivated by DON-Gly both *in vitro* and *in vivo*. This inert dipeptide analog must be hydrolyzed to produce DON, a well-known affinity-labeling reagent for the transferase (7, 8, 17, 18), before the inactivation of the transferase. The renal brush border membrane contains many peptidases on its lumenal surface (13) that could liberate DON. Aminopeptidase is one such enzyme, as shown here. A renal dipeptidase is also abundant (28), and preliminary experiments showed that this dipeptidase is also located on the outer surface of brush border membranes and can hydrolyze DON-Gly (data not shown). Other membranous peptidases of broad substrate specificity may also contribute to the cleavage of DON-Gly. As shown in Fig. 4, the initial lag in the inactivation of γ-glutamyl transferase by DON-Gly can be explained by assuming that hydrolysis of the dipeptide is slow so that time is required for the accumulation of DON. This mode of reaction resembles that of the so-called prodrugs (29), which undergo transformation into reactive forms through cellular metabolism.

We have also shown that a 50% decrease in the renal transferase activity in mice leads to overflow of the ingested glutathione S-conjugate into urine. This situation mimics an inborn deficiency of γ-glutamyl transferase that is accompanied by a marked glutathionemia and glutathionuria (30). Glutathionuria is also induced by administration of γ-glutamyl-(*o*-carboxy)-phenylhydrazide (31), a competitive inhibitor of the transferase (32, 33).

Previously, 6-diazo-5-oxo-D-norleucine, a D-glutamine analog, was shown to be effective in inactivating γ-glutamyl transferase *in vitro*. This compound (the D form of DON) should react specifically with γ-glutamyl transferase located on the outer surface of cell membranes, because its cellular transport rate is very low (20). Furthermore, few, if any, mammalian enzymes react with D-glutamine, whereas the L-isomer reacts with many intracellular glutamine amidotransferases (34–36). A preliminary experiment showed that this D form of DON also inactivates the renal transferase *in vivo*. Comparison of the metabolic perturbations induced by DON-Gly, the D form of DON, and γ-glutamyl-(*o*-carboxy)-phenylhydrazide would thus be of interest.

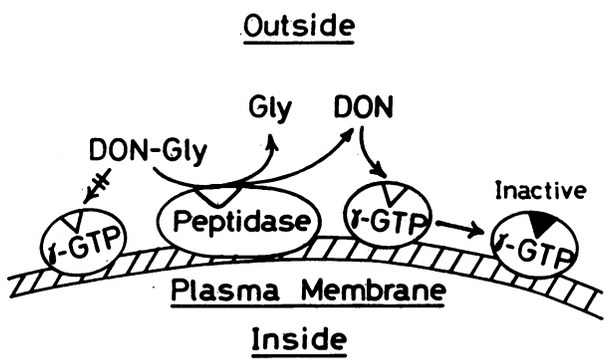

FIG. 4. Mechanism of action of DON-Gly on renal brush border membranes. γ-GTP, γ-glutamyl transferase.

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