Vol. 112, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 29, 1983 Pages 564-570

THE BINDING MECHANISM OF GLUTATHIONE AND THE ANTI-TUMOR DRUG L-(αS,5S)-α-AMINO-3-CHLORO-4,5-DIHYDRO-5-ISOXAZOLEACETIC ACID (AT-125;NSC-163501) TO γ-GLUTAMYLTRANSFERASE*

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Received February 22, 1983

SUMMARY: The glutathione-protein binding interactions of rat renal γ-glutamyltransferase (γGT) were studied by examining the effect of phenylglyoxal (PGO), a chemical modifying agent for arginyl residues. PGO inactivation of γGT followed pseudo-first order kinetics and the rate was dependent upon the concentration of PGO. Glutathione (GSH) protected the enzyme from inactivation by PGO. The anti-tumor drug L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (AT-125) inactivated purified γGT. The inactivation capability of AT-125 was abolished by esterification of the carboxyl moiety and was regained upon incubation of AT-125 methyl ester with a carboxyl esterase. AT-125 and glutathione may bind to γGT via the electrostatic interaction of their respective carboxyl group(s) and an arginyl residue at the active site.

Rat renal γ-glutamyltransferase (E.C.2.3.2.2) is an enzyme localized in the brush border membrane (1,2) which catalyzes the transfer of γ-glutamyl groups from a number of donors to a variety of acceptors. Although the physiological functions of the enzyme are not understood completely, both hydrolysis and transfer reactions proceed through the formation of a covalent γ-glutamyl-enzyme intermediate (3). In the course of our attempts to clarify the nature of the extremely tight binding of glutathione (GSH) to γ-glutamyltransferase, K_m=5.7 μM (4), we report here the inactivation of this enzyme by phenylglyoxal, an arginyl-specific chemical modifier (5). GSH substantially protected γ-glutamyltransferase from phenylglyoxal inactivation. The anti-tumor drug and glutamine analog L-(αS,5S)-α-amino-3-chloro-4,5-

* The abbreviations used are: AT-125, L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; GSH, glutathione.

0006-291X/83 $1.50

dihydro-5-isoxazoleacetic acid (AT-125) also inactivated γ-glutamyltransferase. We report that the carboxyl group of AT-125 is responsible for specific binding to γ-glutamyltransferase and our data support the suggestion that GSH and AT-125 bind to the enzyme via an arginyl residue at the active site.

Materials and Methods

γ-Glutamyltransferase was solubilized from rat renal membranes by treatment with papain and purified to homogeneity as previously described (6). The transpeptidation specific activity, when assayed at pH 8.0 with 2.5 mM L-γ-glutamyl-p-nitroaniline (Sigma Chemical Co., St. Louis, MO) as the donor and 20 mM glycylglycine (Sigma) as the acceptor (7), was 406 $\mu mol \cdot min^{-1} mg^{-1}$ at 25° C. The assay for hydrolytic activity was initiated by the addition of enzyme into a cuvette containing 2.5 mM γ-glutamyl-p-nitroaniline and the exclusion of glycylglycine as acceptor. AT-125 (generously provided by the National Cancer Institute) was esterified by diazomethane in freshly distilled dioxane treated to eliminate peroxides. To a solution of 6.10 mg (34 μmol) of AT-125 in 2 drops distilled water (10 ml round bottom) was added a dioxane solution containing freshly prepared diazomethane (8). The diazomethane in dioxane solution was added dropwise to the first permanent green coloration. Distilled water was added (0.3 ml) and the solution lyophilized to remove the solvents. Thin layer chromatographic analysis [silica gel; tetrahydrofuran: acetone:water (5:3:2)] with ninhydrin visualization showed a spot at R_f=0.79 and no spot corresponding to AT-125 (R_f=0.57). Positive identification of AT-125 methyl ester was made by mass spectrum analysis by fast atom bombardment quadrupole mass spectroscopy (9,10). The methyl ester of AT-125 showed a base parent ion 14 mass units greater than the parent AT-125 and retained the characteristic $^{35}Cl/^{37}Cl$ pattern representative of a mono-chlorine-containing compound.

A typical inactivation experiment with phenylglyoxal consisted of the following components added in this sequence in a total volume of 0.4 ml: 160 μl bicarbonate buffer (250 mM), pH 7.9; 200 μl γ-glutamyltransferase (14

μg/ml) in distilled water; and 40 μl of phenylglyoxal (Sigma, recrystallized twice from water prior to use) (variable concentration) which was prepared in 250 mM bicarbonate buffer, pH 7.90. Aliquots (50 μl) were withdrawn at various time points and assayed for either hydrolytic or transpeptidation activity as described above. In the protection experiments, glutathione (GSH; 0.5 mM) and an equimolar amount of dithiothreitol were introduced prior to the addition of phenylglyoxal.

A typical inactivation experiment with AT-125 consisted of the following components added in this sequence in a total volume of 0.4 ml: 160 μl phosphate buffer (0.05M), pH 7.50; 200 μl γ-glutamyltransferase (14 μg/ml) and 40 μl AT-125 (1mM), both in phosphate buffer (0.05 M), pH 7.50. Aliquots (50 μl) were removed at various time points and assayed for either transpeptidase or hydrolysis activities as described above. AT-125 methyl ester experiments were identical to those previously described except after 15 min. 15 units of carboxyl esterase (Sigma Chem. Co.; 10 μl of 1500 units/ml) were added and aliquots withdrawn and assayed as described above. All experiments were initiated by addition of AT-125 or its methyl ester and incubation was carried out at 25° C. The percent activity remaining at any given time was calculated relative to zero time activity.

Results and Discussion

Evidence that an arginyl residue was essential for γ-glutamyltransferase activity was provided by phenylglyoxal, a chemical modifying reagent specific for arginyl residues (5). In figure 1 is shown a plot of the log of the percentage transpeptidase activity remaining vs. incubation time with varying concentrations of phenylglyoxal. The inactivation followed pseudo-first order kinetics with respect to enzymatic activity remaining and the rate of inactivation was dependent upon the concentration of phenylglyoxal. Similar results were obtained when assaying the hydrolysis activity of γ-glutamyltransferase in the presence of varying concentrations of phenylglyoxal (Figure 2).

The role of the arginyl residue(s) on γ-glutamyltransferase essential for catalytic activity was discerned from protection experiments with GSH (Table

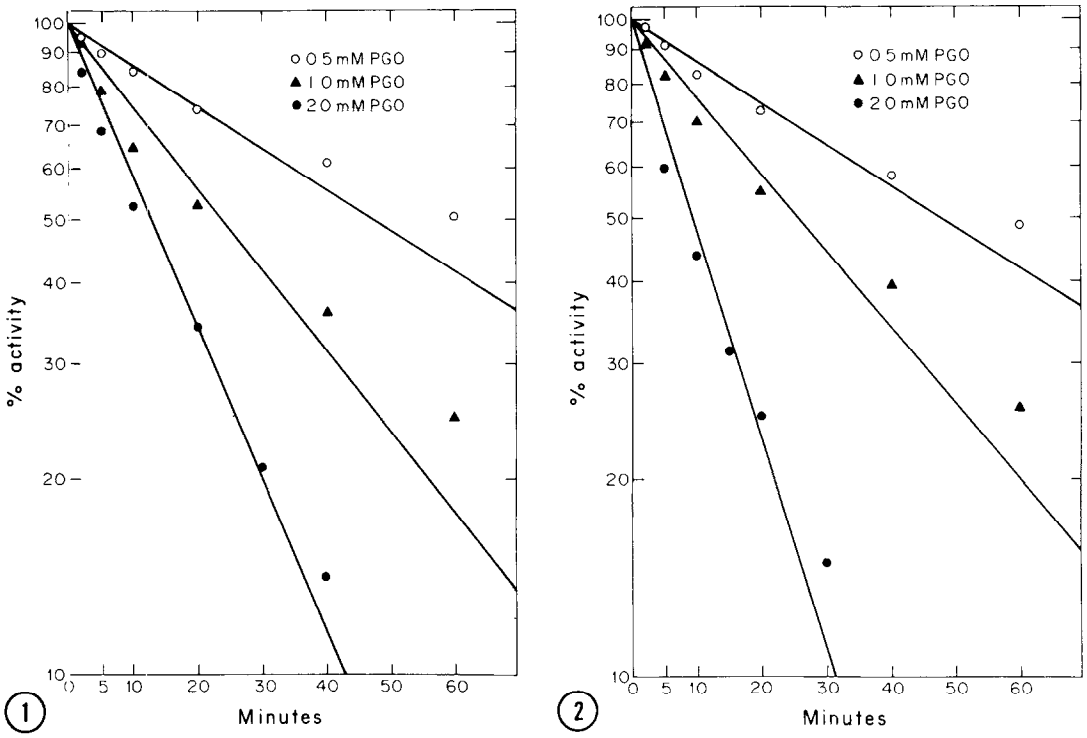

Figure 1: Effect of varying concentrations of phenylglyoxal on the transpeptidase activity of γ-glutamyltransferase. γ-Glutamyltransferase was incubated with 0.5 mM (∘), 1.0 mM (▲) or 2.0 mM (•) phenylglyoxal. The hydrolysis activity of γ-glutamyltransferase was determined as described in the Materials and Methods section.

Figure 2: Effect of varying concentrations of phenylglyoxal on the hydrolysis activity of γ-glutamyltransferase. γ-Glutamyltransferase was incubated with 0.5mM (∘), 1.0 mM (▲) or 2.0 mM (•) phenylglyoxal. The transpeptidase activity of γ-glutamyltransferase was determined as described in the Materials and Methods section.

1). Inclusion of GSH with γ-glutamyltransferase prior to treatment with phenylglyoxal provided substantial protection from inactivation (Table 1).

AT-125 has been shown to rapidly inactivate γ-glutamyltransferase from human pancreatic carcinoma cells (11). This drug also inactivates γ-glutamyltransferase activity in isolated rat kidney cells as measured with GSH as a substrate (12). A time course for the inactivation of purified rat renal enzyme with 100 μM AT-125 is shown in Figure 3. The inactivation of both hydrolysis and transpeptidation activities was rapid and followed pseudo-first order kinetics with respect to the remaining enzymatic activity. The methyl ester of AT-125 did not inactivate either catalytic activity of γ-glutamyltransferase (Figure 3). Further evidence for the importance of the free

Table 1

Glutathione Protection of γ-Glutamyltransferase from Inactivation by Phenylglyoxal (1.0 mM)

Protecting agent[a]	Concentration (mM)	Hydrolytic residual activity (%)[b]	Transpeptidase residual activity (%)
None	---	25	26
Dithiothreitol (DTT)	0.5	29	28
Glutathione + DTT	0.5	79	61

[a] The standard incubation mixture consisted of phenylglyoxal (1.0 mM); bicarbonate buffer, pH 7.90 (125 mM); and the γ-glutamyltransferase preparation in a total of 0.4 ml. The protecting agent glutathione (0.5 mM) was added with an equimolar amount of dithiothreitol prior to addition of phenylglyoxal. The incubation was carried out for 60 minutes at 25°C and aliquots were removed and assayed for specific activities as described in the Materials and Methods section.

[b] The specific activity (hydrolytic or transpeptidation) remaining was calculated relative to the respective zero time activity.

carboxyl moiety in binding AT-125 to the enzyme was provided by the addition of a carboxyl esterase to the mixture containing AT-125 methyl ester and γ-glutamyltransferase (Figure 3). Following addition of the esterase, the γ-glutamyltransferase activities (hydrolysis and transpeptidase) were inactivated at rates comparable to that shown by the parent compound. This evidence suggests that AT-125 binds to γ-glutamyltransferase via its carboxyl moiety.

Substrates containing a glycyl moiety serve as γ-glutamyl acceptors to γ-glutamyltransferase (7,13). We have recently shown the importance of the glycyl carboxyl moiety of GSH in binding to an arginyl residue at the active site of yeast glyoxalase I (14). The protection of γ-glutamyltransferase from phenylglyoxal inactivation by GSH described here provides evidence that electrostatic interactions (carboxyl moiety to arginyl residue) may be important in the binding of GSH to many enzymes. AT-125 is known to inactivate a variety of bacterial and mammalian amidotransferases (15-17). It would appear from the results reported here that AT-125 binds to γ-glutamyltransferase through its carboxyl moiety. We propose that this binding mechanism may be a general phenomenon for enzymes that are inactivated by AT-125. We further suggest that the respective carboxyl moieties of GSH and AT-125 may be respon-

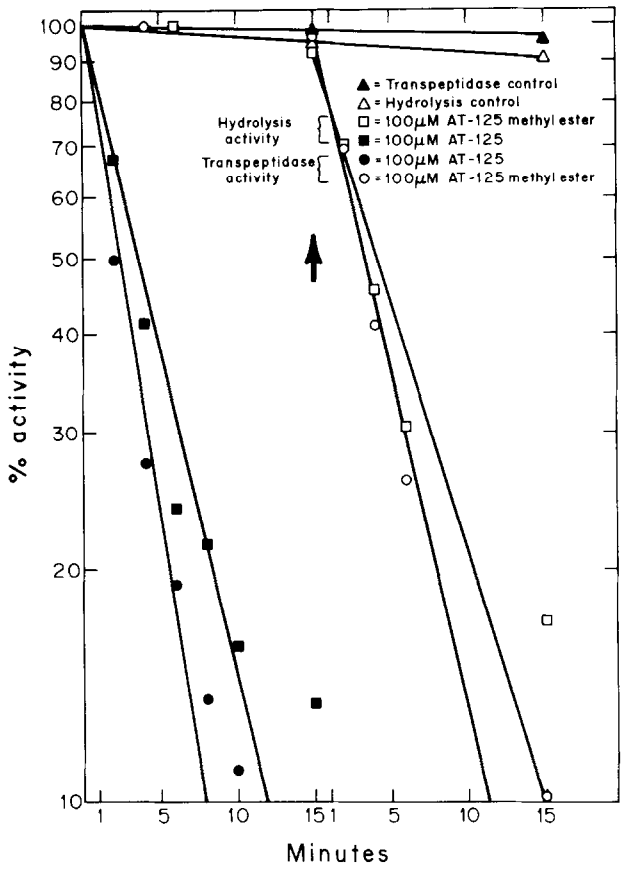

Figure 3: Effect of 100 μM AT-125 or AT-125 methyl ester on γ-glutamyltransferase activities (hydrolysis and transpeptidation) as a function of incubation time. γ-Glutamyltransferase was incubated with either 100 μM AT-125 (■ = hydrolysis activity; ∘ = transpeptidase activity) or 100 μM AT-125 methyl ester (□ = hydrolysis activity; 0 = transpeptidase activity). The arrow indicates the addition of 15 units of carboxyl esterase to the AT-125 methyl ester experiments. The controls were: Δ = hydrolysis activity; ▲ = transpeptidase activity. The hydrolysis and transpeptidation activities were determined as described in the Materials and Methods section.

sible for binding of these substrates to γ-glutamyltransferase via the electrostatic interaction with the proposed arginyl residue present at the active site.

Acknowledgements: The authors gratefully acknowledge the excellent technical assistance of Brian Arbogast in obtaining the FAB mass spectra. This investigation was supported by grants from the National Institutes of Health [(ES-00040) C.S.S., D.J.R.] and the National Institute of Arthritis and Metabolic Diseases [(AM-26012) N.P.C.].

References

1. George, S.G., Kenny, A.J. (1973) Biochem J. 134, 43-57.
2. Glossman, H., Neville, D.M. (1972) FEBS Lett. 19, 340-344.
3. Elce, J.S. and Broxmeyer, B. (1976) Biochem. J. 153, 223-232.

4. McIntyre, T.M. and Curthoys, N.P. (1979) J. Biol. Chem. 254, 6499-6504.
5. Takahashi, K. (1968) J. Biol. Chem. 243, 6171-6179.
6. Hughey, R.P. and Curthoys, N.P. (1976) J. Biol. Chem. 251, 7863-7870.
7. Tate, S.S. and Meister, A. (1974) J. Biol. Chem. 249, 7593-7602.
8. Fales, H.M., Jaouni, T.M. and Babashak, J.F. (1973) Anal. Chem. 45(13), 2302-2303.
9. Surman, D.J. and Vickerman, J.C. (1981) J.C.S. Chem. Commun., 324-325.
10. Barber, M., Bardoli, R.S., Sedgwick, R.D. and Tyler, A.N. (1981) J.C.S. Chem. Commun. 325-327.
11. Allen, L., Meck, R. and Yunis, A. (1980) Res. Commun. Chem. Pathol. Pharm. 27, 175-182.
12. Reed, D.J., Ellis, W.W., and Meck, R.A. (1980) Biochem. Biophys. Res. Commun. 94, 1273-1277.
13. Thompson, G.A. and Meister, A. (1977) J. Biol. Chem. 252(19), 6792-6798.
14. Schasteen, C.S. and Reed, D.J. (1983) Biochim. Biophys. Acta, 742, 419-425.
15. Cooney, D.A., Jayaram, H.N., Ryan, J.A. and Bono, V.H. (1974) Cancer Chemother. Rep. 58, 793-802.
16. Jayaram, H.N., Cooney, D.A., Ryan, J.A., Neil, G. Dion, R.L. and Bono, V.H. (1975) Cancer Chemother. Rep. 59, 481-491.
17. Tso, J.Y., Bower, S.G. and Zalkin, H. (1980) J. Biol. Chem. 255(14), 6734-6738.