Interaction of Glutathione Analogues with *Hydra attenuata* γ -Glutamyltransferase

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γ -Glutamyltransferase activity was studied in extracts of the cnidarian *Hydra attenuata*. The binding of γ -glutamyl peptide analogues to the enzyme was studied by observing their effects on heat denaturation and their inhibition of *p*-nitroaniline release from γ -glutamyl *p*-nitroanilide. Neither position-1 analogues, in which the γ -glutamyl moiety was changed to a β -aspartyl (β -Asp-Abu-Gly) or an α -glutamyl (Glu-Abu-Gly) linkage, nor glutamate protected the enzyme against inactivation at 58°C. GSH (reduced glutathione), γ -Glu-Abu-Gly and γ -Glu-Met on the other hand did prevent heat denaturation. GSH and analogues of GSH were competitive inhibitors of *p*-nitroaniline release, but those analogues in which glycine was replaced by 2-aminoisobutyrate, phenylalanine, leucine or tyrosine had K_i values that were approximately five times those of analogues with the cysteine residue replaced.

γ -Glutamyltransferase (EC 2.3.2.2), first discovered by Hanes *et al.* (1950), has been a subject of study in many organisms [see Meister (1974) for a review]. More recently, knowledge of this enzyme has been extended to another phylum by reports on γ -glutamyltransferase activity in two species of the fresh-water cnidarian hydra (Danner *et al.*, 1976; Tate & Meister, 1976).

The enzyme catalyses the reversible transfer of the γ -glutamyl moiety of a peptide to water, an amino acid or another peptide (Fodor *et al.*, 1953). The natural substrate is thought to be GSH (γ -Glu-Cys-Gly) (Orlowski & Meister, 1970), but other γ -glutamyl peptides also serve as glutamyl donors (Hanes *et al.*, 1952; Orlowski & Meister, 1965; Tate & Meister, 1974). We focused our attention on GSH and on the structural characteristics required by the peptide for a favourable interaction with the enzyme. Previous

Abbreviations used: GSH, reduced glutathione; GSMe, *S*-methylglutathione; MeCys, *S*-methylcysteine; Abu, 2-aminobutyrate; Aib, 2-aminoisobutyrate; Nva, norvaline; pGlu, pyroglutamate (5-oxopyrrolidine-2-carboxylic acid); Cle, cycloleucine; γ -Abu, γ -aminobutyrate.

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studies with GSH analogues in which cysteine (position 2) was either altered or replaced showed that the thiol group was not a requirement of the transferase substrates (Tate & Meister, 1974, 1976). There were no reports on GSH analogues in which glycine (position 3) was replaced by other amino acids until the one describing activity in *Hydra attenuata* (Danner *et al.*, 1976) appeared. The data from these experiments suggested that analogues containing leucine or tyrosine in position 3 bound less well to the enzyme compared with those containing glycine in that position, but the data did not provide a rationale for the apparent decrease in binding.

The present studies were undertaken to evaluate kinetically the effect of modifications of position 2 or position 3 in γ -glutamyl peptides on the *H. attenuata* γ -glutamyltransferase activity and to test analogues not reported before. We made further use of a position-2 analogue, γ -Glu-Abu-Gly, by altering the γ -glutamyl linkage (position 1) and assessing the ability of the resulting peptides to protect *H. attenuata* γ -glutamyltransferase against heat inactivation.

Experimental

Amino acids, GSH, GSMe, γ -glutamyl *p*-nitroanilide, pig kidney γ -glutamyltransferase and rifampicin were purchased from Sigma Chemical Corp. (St. Louis, MO, U.S.A.). γ -Glu-Met was purchased from Bachem (Marina del Rey, CA, U.S.A.) and γ -Glu-Leu from Vega-Fox Biochemicals (Tucson, AZ, U.S.A.). Peptides not purchased from commer-

cial sources were synthesized as will be described elsewhere. All other reagents were of Reagent Grade.

Laboratory-reared specimens of *Hydra attenuata* were cultured and treated with rifampicin as described by Danner *et al.* (1976) and then homogenized (Teflon/glass) in 10mM-Tris/HCl, pH9, containing 10mM-MgCl₂. After a 3 min centrifugation at 1000g the supernatant was either dialysed against the extraction buffer at 4°C for 18–24 h (fraction S₁) or it was centrifuged at 12000g for 20–30 min and the sediment was resuspended in the homogenization medium (fraction P₂).

γ -Glutamyltransferase activity was assayed by the method of Orlowski & Meister (1965). Gly-Gly was added to the reaction to increase the sensitivity of the assay. The dipeptide was chosen because, of the compounds tested, it was the best activator of the *H. attenuata* γ -glutamyltransferase activity (Table 1). The reaction conditions used in the subsequent experiments were as follows. The assay solution (0.5 ml) contained 2.5 mM- γ -glutamyl *p*-nitroanilide, 100 mM-Tris/HCl, pH9, 10 mM-MgCl₂, 20 mM-Gly-Gly and extract protein. Protein was measured by the method of Lowry *et al.* (1951). After 20 min at 38°C, 0.45 ml of 1 M-acetic acid was added, followed by 0.05 ml of 2% sodium dodecyl sulphate. The *p*-nitroaniline formed was determined by measuring *A*₄₁₀, and corrections for no-enzyme and no-substrate controls were applied. Enzyme units are expressed as nmol of *p*-nitroaniline formed per min and specific activity as units per mg of protein. When the pig kidney enzyme was assayed, a 5 min incubation period was used.

Heat-denaturation experiments were carried out as described by Orlowski & Meister (1965).

T.l.c. was carried out on plastic-backed cellulose sheets (Eastman-Kodak, Rochester, NY, U.S.A.) and the chromatograms were developed with butan-1-ol/pyridine/water (1:1:1, by vol.) (Orlowski & Meister, 1965). α -Amino groups were detected with cadmium/ninhydrin reagent (Niederweiser, 1972), and u.v.-absorbing material was detected by long-wave u.v. irradiation.

Results

Heat inactivation

When *H. attenuata* γ -glutamyltransferase activity was incubated at 58°C, 82% of the activity was lost in 15 min. In the presence of 6.7 mM-GSH only 31% of the activity was lost under the same conditions. γ -Glu-Abu-Gly, a transferase substrate (Tate & Meister, 1974), also prevented the inactivation. Such protection was not afforded by tripeptides in which the γ -glutamyl moiety was replaced by either the α -glutamyl or β -aspartyl residue (Fig. 1). In the

Fig. 1. Effect of the γ -glutamyl bond on the activation of *H. attenuata* γ -glutamyltransferase activity at 58°C. The P₂ fraction (0.36 mg of protein) was incubated with a peptide (6.7 mM). At the indicated times, samples were withdrawn and assayed as described in the Experimental section. $\circ$, γ -Glu-Abu-Gly; $\bullet$, Glu-Abu-Gly; $\square$, β -Asp-Abu-Gly.

experiment described in Fig. 1 the cysteine residue of glutathione was replaced by 2-aminobutyrate in order to eliminate problems associated with oxidation of the thiol group.

Protection against heat inactivation did not require the presence of the position-3 amino acid residue (Fig. 2). γ -Glu-Met (6.7 mM) protected against inactivation as did the tripeptides. On the other hand, the component amino acids glutamate (20 mM) and methionine (20 mM) were ineffective. [Glutamate is a weak inhibitor of the transferase and methionine is an acceptor (Table 1).] Like methionine, the dipeptide Gly-Gly (20 mM) did not prevent the inactivation at 58°C. In the experiments described in Figs. 1 and 2, the samples that were used for the enzyme assay contained peptides or amino acids that were at a final concentration too low to affect the rate of release of *p*-nitroaniline from γ -glutamyl *p*-nitroanilide (see below).

Fig. 2. Effect of γ -Glu-Met, Glu and Met on the inactivation of *H. attenuata* γ -glutamyltransferase activity at 58°C

The P_2 fraction (0.28 mg of protein) was incubated with γ -Glu-Met, Met or Glu at 58°C. At the indicated times, samples were withdrawn and assayed as described in the Experimental section. O, 6.7 mM- γ -Glu-Met; ▲, 20 mM-methionine; ●, 20 mM-glutamate.

Table 1. Effect of Gly-Gly and various amino acids on *H. attenuata* γ -glutamyltransferase activity

The reaction mixture (0.5 ml) contained 2.5 mM- γ -glutamyl *p*-nitroanilide, 100 mM-Tris/HCl, pH 9, 10 mM-MgCl₂, 20 mM-amino acid or Gly-Gly and extract protein. After 20 min at 38°C, the reaction was stopped and the amount of *p*-nitroaniline formed was determined by the procedure described in the Experimental section. A relative activity of 1.00 corresponds to a specific activity of 35 nmol of *p*-nitroaniline formed/min per mg of protein.

Addition (20 mM)	Relative activity
None	1.00
Gly-Gly	3.20
Met	1.89
Phe	1.57
Tyr	1.56
Leu	1.47
Arg	1.41
Ile	1.26
Val	1.20
Thr	1.17
γ -Abu	1.15
His	1.09
Gly	1.08
Pro	1.05
Abu	1.02
Cle	0.98
pGlu	0.91
Ser	0.89
Gln	0.88
Aib	0.75
Glu	0.73
Ala	0.62

Effects of GSH analogues on *H. attenuata* γ -glutamyltransferase activity

In a previous report we described the effect of a number of GSH analogues on the release of *p*-nitroaniline from γ -glutamyl *p*-nitroanilide, by using *H. attenuata* transferase activity prepared in phosphate buffer (Danner *et al.*, 1976). In the experiments reported here, we investigated newly synthesized peptides by using the S_1 fraction prepared in the Tris/Mg²⁺ buffer (see the Experimental section) to eliminate possible interference by phosphate (Tate & Meister, 1974). The new peptides were γ -Glu-Ala-Gly, γ -Glu-Aib-Gly, γ -Glu-Nva-Gly, γ -Glu-Phe-Gly, γ -Glu-Cle-Gly, γ -Glu-Val-Gly, γ -Glu-Leu-Gly, γ -Glu-Abu-Aib and γ -Glu-Nva-Phe.

The assay was as described in the Experimental section, with the addition of 4 mM-tripeptide. The data have been expressed as the percentage inhibition of *p*-nitroaniline release by a 4 mM-peptide analogue relative to the amount formed in the absence of

peptide. The peptides that were previously tested (Danner *et al.*, 1976) were assayed again to make a direct comparison.

The data of Table 2 (row 1) describe the results obtained with position-2-modified peptides, i.e. GSH analogues in which cysteine was replaced by another amino acid. In the case of GSH a separate measurement (Beutler *et al.*, 1963) showed that, under the conditions of the transferase assay, 95% of the GSH remained in the reduced state. All peptides tested inhibited the release of *p*-nitroaniline. GSH and γ -Glu-Gly-Gly were good inhibitors, whereas γ -Glu-Val-Gly and γ -Glu-Leu-Gly were relatively poor inhibitors of *p*-nitroaniline release.

To determine if position-2 analogues could function as glutamyl donors, the reaction products formed after a 3 h incubation of peptide with methionine in the presence of the *H. attenuata* S_1 fraction were analysed by t.l.c. The peptides tested were GSH, γ -Glu-Gly-Gly, γ -Glu-Ala-Gly, γ -Glu-Aib-Gly, γ -Glu-Abu-Gly, GSMe and γ -Glu-Leu-Gly.

When methionine was present, incubated mixtures including any of these peptides contained a component with the same mobility as γ -Glu-Met. Hence these peptides were probably functioning as γ -glutamyl donors.

γ -Glutamyl peptides may also function as acceptors (Orlowski & Meister, 1965). We have assumed, however, that the presence of an excess of Gly-Gly, itself an efficient acceptor, precluded the expression of such activity.

Table 2 (row 2) describes the inhibition of *p*-nitroaniline release by position-3 analogues, i.e. GSH analogues in which glycine was replaced by another amino acid. In these studies cysteine was replaced by either 2-aminobutyrate or norvaline to eliminate the problems associated with thiol oxidation. Peptides containing these substitutions were good inhibitors of *p*-nitroaniline release (row 1). The inhibition of *p*-nitroaniline release by peptides containing position-3 residue substitutions was much less than the inhibition caused by GSH, γ -Glu-Abu-Gly, γ -Glu-Nva-Gly or the corresponding position-2 analogues.

Double-reciprocal plots (Dixon & Webb, 1964) were constructed to characterize the type of inhibition of *p*-nitroaniline release caused by several of the GSH tripeptide analogues. Inhibition by GSMe was competitive, as shown in Fig. 3. In addition GSH, γ -Glu-Aib-Gly, γ -Glu-Abu-Aib, γ -Glu-Tyr-Gly, γ -Glu-Abu-Tyr, γ -Glu-Abu-Leu and γ -Glu-Nva-Phe

Fig. 3. Inhibition of *p*-nitroaniline release by GSMe. Assays were made with the S_1 fraction. v is in terms of nmol of *p*-nitroaniline/min, and the concentration of γ -glutamyl *p*-nitroanilide is in mM. O, No additions, ●, 1 mM-GSMe; Δ, 4 mM-GSMe.

Table 3. Competitive inhibition constants of GSH analogues with *H. attenuata* γ -glutamyltransferase. K_i values were calculated from double-reciprocal plots obtained in the presence and absence of the inhibitor. Assays were conducted as for Fig. 3 with the S_1 fraction.

Analogue	K_i (mM)
GSH (reference)	0.21
Position-2 modifications	
γ -Glu-Aib-Gly	0.21
GSMe	0.26
γ -Glu-Tyr-Gly	0.35
Position-3 modifications	
γ -Glu-Abu-Tyr	1.1
γ -Glu-Nva-Phe	1.2
γ -Glu-Abu-Aib	1.3
γ -Glu-Abu-Leu	1.5

Table 2. Effects of GSH analogues on the release of *p*-nitroaniline catalysed by *H. attenuata* γ -glutamyltransferase activity

The S_1 fraction was used in the presence of 20 mM-Gly-Gly. In the absence of added GSH or GSH analogue, the specific activity was 111 nmol of *p*-nitroaniline formed/min per mg of protein.

Substitution (4 mM)	Percentage inhibition	
	Cys replaced	Gly replaced
None (GSH)	63.6	
Gly	64.5	
Ala	55.3	*
Cys (Me)	54.3	*
Aib	53.5	16.3†
Abu	47.5	*
Tyr	47.0	19.5†
Nva	46.8	*
Phe	43.3	16.0‡
Cle	42.9	*
Val	40.6	*
Leu	32.3	8.62†

* Not tested.

† Cys was replaced by Abu.

‡ Cys was replaced by Nva.

were also competitive inhibitors of *p*-nitroaniline release. The inhibition constants of the various analogues are given in Table 3. The K_i values of the position-3-modified peptides were about five times those of the position-2 analogues.

That the effects of the peptide analogues on *H. attenuata* γ -glutamyltransferase activity were not due to non-specific interfering proteins was determined by comparing measurements carried out with pig kidney γ -glutamyltransferase. The pig enzyme was chosen because it had been well characterized (Orlowski & Meister, 1965) and can be obtained commercially with a specific activity sufficiently high that the contribution of the hydra homogenate to the

total activity should be negligible even with a several-fold excess of hydra protein. Neither the activity of the pig kidney enzyme (4 μ g of protein) nor the inhibition of *p*-nitroaniline release by 4mM-GSH or -GSMe was significantly altered by the presence of the *H. attenuata* homogenate (28 μ g of protein). Also, after a 20min incubation in 100mM-Tris/HCl, pH9, and 10mM-MgCl₂ at 37°C (assay conditions for *H. attenuata* transferase activity) of pig kidney enzyme and hydra homogenate, the activity of the former (with Gly-Gly as acceptor) did not decrease. Such results suggest that the activity we have been observing with the *H. attenuata* extract was not changed during the assay. Confirming our results with hydra transferase activity, the pig kidney transferase-catalysed release of *p*-nitroaniline was inhibited less by 4mM- γ -Glu-Abu-Aib (a position-3 analogue) than it was by the same concentration of γ -Glu-Aib-Gly, γ -Glu-Abu-Gly (position-2 analogues) or GSH (6%, 50%, 66% and 68% respectively).

As another test for interference, a number of peptides (GSH, γ -Glu-Abu-Aib, γ -Glu-Aib-Gly and GSMe) were examined with the *H. attenuata* P₂ fraction (12000g pellet). At a concentration of 4mM-peptide the percentage inhibition was the same as that exhibited when the S₁ fraction was used, indicating that the soluble enzymes of the γ -glutamyl cycle [e.g. cyclotransferase; see Cliffe & Waley (1961)] were not interfering with our experiments.

Effects of γ -Glu-Met and γ -Glu-Leu

The effect of dipeptides on the transferase-catalysed release of *p*-nitroaniline was studied to determine whether or not the glycyl moiety of GSH was necessary for recognition by the *H. attenuata* enzyme. The P₂ fraction (12000g pellet) was used to eliminate

the cyclotransferase activity (Tate & Meister, 1976) that is associated with the soluble fraction (Cliffe & Waley, 1961; Okonkwo *et al.*, 1974). Fig. 4 shows that γ -Glu-Met and γ -Glu-Leu inhibited the release of *p*-nitroaniline from γ -glutamyl *p*-nitroanilide. In addition, the extent of inhibition caused by γ -Glu-Leu is similar to that caused by the same concentration of γ -Glu-Leu-Gly, a position-2 analogue (Table 2).

Discussion

The effects of amino acids and Gly-Gly on the *H. attenuata* γ -glutamyltransferase activity were similar to their effects on enzymes from other sources (Orlowski & Meister, 1965; Tate & Meister, 1974; DeLap *et al.*, 1975; Bodnaryk & Skillings, 1971; Milbauer & Grosswicz, 1965; Mooz & Wigglesworth, 1976). Although reaction products have not been directly identified, analysis by t.l.c. indicates the formation of the expected transferase products with methionine or leucine as acceptor (Danner *et al.*, 1976). The observed stimulation is a reflection of the increased reactivities of Gly-Gly, methionine and other amino acids relative to that of water (Fodor *et al.*, 1953). Differences, however, were observed in the effects of some of the amino acids. Alanine, for example, which was a good activator with the transferases from rat kidney (Tate & Meister, 1974), rat seminal vesicles (DeLap *et al.*, 1975) and housefly (*Musca domestica*) (Bodnaryk & Skillings, 1971), functioned as an inhibitor with the *H. attenuata* transferase activity.

The relative activity of alanine suggests that when this amino acid binds to the acceptor site on the enzyme, the resulting glutamyl acceptor-enzyme complex (Karkowsky *et al.*, 1976; Elce & Broxmeyer 1976) breaks down slowly. Hence the enzyme is hindered in its re-entry into the reaction sequence. Consistent with this proposal is the observation that, when Gly-Gly was the acceptor, the presence of 2-aminobutyrate, 2-aminoisobutyrate, glycine, serine or alanine caused an even greater decrease in the release of *p*-nitroaniline [relative activity = 0.49, 0.61, 0.60, 0.32 and 0.25 respectively with respect to Gly-Gly (J. Danner, unpublished work)].

Our studies on the heat inactivation of *H. attenuata* γ -glutamyltransferase activity confirm the results obtained with the enzyme from other sources (Orlowski & Meister, 1965; Tanaguchi, 1974; Zelazo & Orlowski, 1976). In addition, we have shown that protection was not due to an oxidation product of GSH because we used the non-oxidizable analogue, γ -Glu-Abu-Gly, a known transferase substrate (Tate & Meister, 1974). We have also shown for the first time that protection against heat denaturation requires the presence of the γ -glutamyl moiety. It does not, however, require a third amino acid residue. Whereas γ -glutamyl donors such as GSH, γ -Glu-

Fig. 4. Inhibition of *p*-nitroaniline release by γ -glutamyl dipeptides

Assays were made with the P₂ fraction. ●, γ -Glu-Leu; ○, γ -Glu-Met.

Abu-Gly and γ -Glu-Met protect against heat inactivation, acceptors such as methionine and Gly-Gly do not. Although glutamine was not tested in the present heat-denaturation experiments, Orlowski & Meister (1965) showed that it protected the pig enzyme against heat inactivation. The data suggest that either donors and acceptors bind at different sites on the enzyme or donors occupy at least two sites, one of which is shared by acceptors. Thus the filling of the Cys-Gly site of GSH by an acceptor (e.g. Gly-Gly) does not protect against heat inactivation. The data also suggest that the γ -glutamyl amide bond is a necessary structure for such protection.

The position-2-modified peptides used in the studies on inhibition of *p*-nitroaniline release could be functioning as alternative substrates or 'dead-end' inhibitors. The analysis of reaction products suggests that they are acting as substrates, and kinetic analysis shows that they are competing with γ -glutamyl *p*-nitroanilide for the same site on the enzyme.

The present study not only confirms previous reports (Tate & Meister, 1974, 1976) that showed that the thiol group of GSH was not required for binding to γ -glutamyltransferase, it also provides a rationale for the decrease in inhibition of *p*-nitroaniline release by position-3-modified analogues, which we reported previously (Danner *et al.*, 1976). Analysis of Table 3 reveals that analogues containing 2-aminoisobutyrate leucine, tyrosine or phenylalanine in position 3 have inhibition constants nearly five times greater than similar analogues with glycine as the third residue. The increased K_i values are not due to the presence of second-residue amino acids other than cysteine, because γ -Glu-Abu-Gly and γ -Glu-Nva-Gly inhibited *p*-nitroaniline release about three times more at the same concentration than did comparable analogues with glycine replaced. This observation is consistent with the data from rat kidney transferase (Tate & Meister, 1974), which showed that γ -Glu-Abu-Gly is an excellent substrate.

The decreased inhibition of *p*-nitroaniline release and increased K_i values of analogues in which glycine was replaced by other amino acids suggest that the glycine residue of GSH fulfills a steric requirement, i.e. its small size allows the peptide to fit correctly on the enzyme. The data of Fig. 4 reveal that the glycine residue is unnecessary, since dipeptides inhibited product release, results that are consistent with the data of Tate & Meister (1974) on dipeptide γ -glutamine donors. The direct implication is that the presence of 2-aminoisobutyrate, leucine, tyrosine or phenylalanine in position 3 may interfere with the proper alignment of peptide and enzyme required for tighter binding.

The S_1 fraction used for the peptide studies contains other enzymes of the γ -glutamyl cycle (Tate & Meister, 1976). We excluded the possibility that such

activities could be responsible for our observations by determining that when a particulate fraction (12000g pellet) was used, the inhibition of *p*-nitroaniline release by a number of analogues was the same as with the S_1 fraction. In addition, the results obtained with the pig kidney transferase confirmed those obtained with the activity from *H. attenuata*.

The results of our studies allow two general conclusions. (1) The γ -glutamyl linkage of GSH is required for protection against heat inactivation as it is for binding (Kinoshita & Ball, 1953; Danner *et al.*, 1976). Although the mechanism for such protection has not been established, the data suggest that peptides that protect against heat denaturation bind to the γ -glutamyl site. (2) By determining the inhibition constants of the position-2 and position-3 peptides we were able to characterize the inhibition of *p*-nitroaniline release. The presence of large amino acid residues at position 3 results in peptides with affinities for the enzyme that are decreased by five times compared with glycine-containing peptides, suggesting that large side chains at the C-terminus interfere with tight binding.

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