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Substrate specificity of rat liver glutathione *S*-transferase isoenzymes for a series of glutathione analogues, modified at the γ-glutamyl moiety

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The substrate specificity of purified rat liver glutathione *S*-transferases (GSTs) for a series of γ-glutamyl-modified GSH analogues was investigated. GST isoenzyme 3–3 catalysed the conjugation of 1-chloro-2,4-dinitrobenzene with six out of the nine analogues. α-L-Glu-L-Cys-Gly and α-D-Glu-L-Cys-Gly showed catalytic efficiencies of 40% and 130% that of GSH respectively. The GSH analogue with an α-D-glutamyl moiety appeared to be a highly isoenzyme-3–3-specific co-substrate: $k_{cat.}/K_m$ with GST isoenzyme 4–4 was only about 5% that with GST isoenzyme 3–3, and no enzymic activity was detectable with GST isoenzymes 1–1 and 2–2. GST isoenzyme 4–4 showed some resemblance to GST 3–3: five out of nine co-substrate analogues were accepted by this second isoenzyme of the Mu multigene family. Isoenzymes 1–1 and 2–2, of the Alpha multigene family, accepted only two alternative co-substrates, which indicates that their GSH-binding site is much more specific.

INTRODUCTION

One of the major detoxification reactions of xenobiotics in the living organism is conjugation with the ubiquitous tripeptide GSH. This reaction is enzymically catalysed by glutathione *S*-transferases (GSTs), which are found in virtually every prokaryotic and eukaryotic cell system. A number of GST isoenzymes have been identified, with different but overlapping substrate specificities (Mannervik, 1985). In the active site of every subunit of a dimeric GST isoenzyme two distinct regions have been defined: a rather unspecific H-site, capable of binding a variety of hydrophobic substrates, and a G-site where GSH can be accommodated. The latter is generally thought to be highly specific (see e.g., Kamisaka *et al.*, 1975). Studies on GSTs have not yet resulted in elucidation of the precise mechanism of catalysis nor in a spatial image of the active centre involved. The abundance of closely related isoenzymes, the observed microheterogeneity of the subunits (Satoh *et al.*, 1985; Tu & Reddy, 1985) and the absence of an accessable prosthetic group have complicated studies in this field. In order to study the enzyme active site and the catalysis at the molecular level, the one part all GSTs have in common, the GSH-binding site, seems to be a valid target. Studies, so far, were hampered by the lack of readily available series of GSH analogues as probes for the G-site in GSTs.

γ-L-Glu-L-Cys-β-Ala, also called 'homoglutathione', has been mentioned to be a good alternative co-substrate for rat liver isoenzymes 3–3 and 3–4, without giving further details (Habig *et al.*, 1974). No GSH analogues with an activity comparable with that of GSH itself have been reported since. The 'end-group-modified', so-called, retro-inverso isomer of GSH, L-2,4-diaminobutyrate-N^4-(D-cysteinyl)maleic acid, turned out to be a poor substrate for the rat liver GST isoenzymes 3–3 and 4–4, in spite of its close resemblance to GSH (Chen *et al.*, 1986). Other peptides, such as L-Cys-Gly, γ-L-Glu-Cys and γ-L-Glu-γ-L-Glu-L-Cys-Gly, and other thiols, such as 2-mercaptoethanol, *N*-acetyl-L-cysteine and L-cysteine, are in general poorly or not at all accepted by the GSTs as substitutes for GSH (Habig *et al.*, 1974; Sugimoto *et al.*, 1985; Abbot *et al.*, 1986). Quite recently it has been shown that GST-4–4-catalysed 2-mercaptoethanol conjugation with 1-chloro-2,4-dinitrobenzene can be increased by addition of the co-substrate derivative *S*-methyl-GSH (Principato *et al.*, 1988). Non-thiol-containing analogues, such as dethio-GSH (γ-L-Glu-L-Ala-Gly) and the oxy analogue (γ-L-Glu-L-Ser-Gly), were shown to be good competitive inhibitors for some GSTs at the GSH-binding site (Chen *et al.*, 1985).

In the preceding paper (Adang *et al.*, 1988) the synthesis of a series of GSH analogues, systematically modified in the γ-glutamyl moiety, is described. Also, the nucleophilic reactivity of these compounds towards 1-chloro-2,4-dinitrobenzene was determined (Adang *et al.*, 1988). The substantial rate differences that were observed led to the assumption that intramolecular interactions of the γ-glutamyl side chain with the cysteinyl thiol group of GSH were acting to decrease the pK_a of the thiol group of the GSH analogues. In the present paper we report on the propensity of these γ-glutamyl-modified GSH analogues to act as a co-substrate in the GST-mediated

Abbreviation used: GST, glutathione *S*-transferase.

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reaction with 1-chloro-2,4-dinitrobenzene, utilizing the major purified homodimeric GST isoenzymes available from rat liver, i.e. isoenzymes 1–1, 2–2, 3–3 and 4–4.

MATERIALS AND METHODS

Chemicals

1-Chloro-2,4-dinitrobenzene was obtained from Merck (Darmstadt, Germany). GSH was obtained from Janssen Chimica (Beersse, Belgium).

Synthesis of peptides

The synthesis of the nine γ-glutamyl-modified GSH analogues used in the present study is described in the preceding paper (Adang *et al.*, 1988). Solutions were prepared fresh every day, and argon flushing was applied before solutions were stored on ice.

GSH-transferase isoenzyme purification

Rat liver GST isoenzymes 1–1, 2–2, 3–3 and 4–4 were prepared from rat liver soluble supernatant fraction as reported previously (Beale *et al.*, 1983).

Enzyme kinetics

The activity of the GST isoenzymes was determined by measuring the initial rate of the enzyme-catalysed conjugation of GSH with 1-chloro-2,4-dinitrobenzene at 25 °C and pH 6.5 as described by Habig *et al.* (1974). This initial rate was determined spectrophotometrically by monitoring the increase in absorbance at 340 nm with time. A Cary 219 double-beam spectrophotometer was used for this purpose. Correction was made for the non-enzymic conjugation by recording the increase in A_{340} without addition of enzyme. The activity is expressed as μmol/min per mg of protein. This method was also used to determine the kinetic parameters for the GSH analogues.

Implicit in the subsequent discussions is the assumption that GSH analogue conjugates with 1-chloro-2,4-dinitrobenzene have the same molar absorption coefficient at 340 nm as the GSH–1-chloro-2,4-dinitrobenzene conjugate itself. Protein concentration was determined by the procedure of Lowry *et al.* (1951), with bovine serum albumin as standard.

Kinetic data were analysed by using the direct-plot method (Eisenthal & Cornish-Bowden, 1978). From this plot K_m (mM) and maximal velocity, $V_{max.}$ (μmol/min per mg), for GSH and nine GSH analogues were determined at 1 mM-1-chloro-2,4-dinitrobenzene.

RESULTS AND DISCUSSION

The ability of each GSH analogue to replace the naturally occurring co-substrate GSH was tested with 1-chloro-2,4-dinitrobenzene as acceptor substrate. Conjugation was measured at 1 mM-1-chloro-2,4-dinitrobenzene and concentrations of the co-substrate varying between 0.1 and 2 mM for the more efficient GSH analogues and between 0.5 and 10 mM for the less efficient. Table 1 shows the kinetic parameters determined for the major purified soluble GST isoenzymes of rat liver. Isoenzyme 3–3 was active with six out of nine GSH analogues. This isoenzyme appears to be the least specific with regard to the binding of GSH. The findings with this form are discussed first in general terms.

GST form 3–3

If GSH is replaced by an analogue missing one of the two functional groups in the γ-glutamyl moiety, the catalytic efficiency ($k_{cat.}/K_m$) is dramatically decreased. In the case of decarboxy-GSH (3) no activity can be detected, and for deamino-GSH (2) it falls to 6% of the catalytic efficiency found for GSH. This suggests that the α-carboxy group is essential for binding of GSH to GST 3–3. The amino function of the γ-L-glutamic acid residue may interact with the cysteinyl thiol group to lower its pK_a in the same way as described for the non-enzymic nucleophilic reactivity (Adang *et al.*, 1988). In addition, the NH_3^+ terminus might also be involved in the binding of GSH to the active centre. The neighbouring anionic

Table 1. Kinetic parameters for the major purified forms of rat liver GST with GSH and nine GSH analogues

Enzyme activity was measured at 340 nm at 25 °C at pH 6.5 with 1 mM-1-chloro-2,4-dinitrobenzene as electrophilic substrate. – means no net detectable enzyme activity at co-substrate concentrations exceeding 1 mM. Abbreviations: Glr, glutaric acid; 4-Abu, 4-aminobutyric acid.

	Isoenzyme 1–1		Isoenzyme 2–2		Isoenzyme 3–3		Isoenzyme 4–4	
Co-substrate	K_m (mM)	$V_{max.}$ (μmol/min per mg)	K_m (mM)	$V_{max.}$ (μmol/min per mg)	K_m (mM)	$V_{max.}$ (μmol/min per mg)	K_m (mM)	$V_{max.}$ (μmol/min per mg)
(1) γ-L-Glu-L-Cys-Gly	0.2	18	0.4	5.8	0.3	29	0.3	15
(2) Glr-L-Cys-Gly	2.8	3.6	10	4.2	1.4	8.8	1.6	3.2
(3) 4-Abu-L-Cys-Gly	–	–	–	–	–	–	–	–
(4) γ-D-Glu-L-Cys-Gly	–	–	–	–	2.9	3.8	–	–
(5) α-L-Glu-L-Cys-Gly	0.5	8.6	2.5	3.6	0.5	18	1.4	11
(6) α-D-Glu-L-Cys-Gly	–	–	–	–	0.5	63	1.3	7.8
(7) β-L-Asp-L-Cys-Gly	–	–	–	–	–	–	–	–
(8) β-D-Asp-L-Cys-Gly	–	–	–	–	0.8	3.9	1.6	0.8
(9) α-L-Asp-L-Cys-Gly	–	–	–	–	–	–	–	–
(10) α-D-Asp-L-Cys-Gly	–	–	–	–	2.3	11	1.8	0.5

carboxylate group most probably interacts with amino acid side chains of opposite charge in the G-site.

The γ-D-glutamyl-containing analogue (4) showed only a small capacity to replace the natural co-substrate, and none with the other isoenzymes. This suggests a highly stereospecific recognition pattern for the co-substrate by these isoenzymes. In the γ-D-glutamic acid derivative the two ionic groups are probably no longer in optimal juxtaposition to opposite charges in the active centre, but, in contrast, have to reorientate in order to avoid juxtaposition to ionized groups in the protein with identical charge, leading to repulsion rather than attraction. In addition, the relatively low chemical reactivity due to altered intramolecular interactions compared with GSH (Adang *et al.*, 1988), may be reflected in its rather low maximum rate ($V_{max.}$).

In tripeptides (5) and (6) L- and D-glutamic acid were coupled to cysteine by the α-carboxylic acid group to establish an α-peptide bond. Remarkably, these two GSH analogues proved to be good substrates for GST 3–3, at 1 mM concentration, their specific activities being approx. 50% and 200% that of GSH. The affinity of transferase 3–3 for α-L-glutamyl- and α-D-glutamyl-modified GSH appeared to be identical, their K_m values being 0.5 mM for both tripeptides, which is only slightly higher than the K_m value for GSH, namely 0.3 mM. The maximum rate with α-D-glutamyl-modified GSH was 3.5 times higher than the $V_{max.}$ with α-L-glutamyl-modified GSH as co-substrate and 2 times higher than that with GSH (Table 1). A possible explanation for these findings is that the α-D-glutamylcysteinyl moiety is stereochemically more similar to the γ-L-glutamylcysteinyl residue in GSH than the analogue with an α-L-glutamyl residue (5): the $-NH_3^+$ function of glutamic acid in GSH and α-D-Glu-L-Cys-Gly is orientated in the same direction whereas in the α-L-glutamyl-peptide (5) the $-NH_3^+$ orientation is opposite to these structures. It may be noted that the isomer with the highest chemical reactivity (the α-D-isomer, 161% relative to GSH; Adang *et al.*, 1988) also shows the highest activity in association with the enzyme.

The fact that GSH analogues (5) and (6) are highly active in 1-chloro-2,4-dinitrobenzene conjugation catalysed by isoenzyme 3–3, in combination with the proposed requirement of a CO_2^- group and an NH_3^+ group, suggests that an NH_3^+ group α to the amide bond is able to compensate for the loss of interaction of the γ-glutamyl zwitterionic NH_3^+ group of GSH. The carboxylic group is still available for the binding in the co-substrate analogue. Apparently both the α-L- and α-D-glutamate derivatives are able to establish such an interaction, although the α-D-glutamyl-containing analogue with its higher catalytic efficiency has a more favourable configuration.

In addition, folding of the γ-glutamyl moiety in the enzyme active site could play a role. A recent n.m.r. study on the behaviour of GSH in solution led to the conclusion that the GSH structure is not extended, as it apparently is in the crystalline state, but rather is folded so as to orient the amine and carboxylate groups of the γ-glutamate moiety towards the carbonyl and amino residues of the peptide bond between γ-glutamic acid and cysteine respectively (York *et al.*, 1987). X-ray-diffraction analyses of other GSH-dependent enzymes with GSH bound in the active site on the other hand have revealed an extended tripeptide structure (Karplus & Schultz, 1987; Epp *et al.*, 1983). Since no such studies have been carried out with GST isoenzymes so far, such information is not available at present. However, crystalline GST isoenzyme 3–3 has been obtained recently and crystallographic data may be forthcoming in the near future (Sesay *et al.*, 1987).

If GSH is bound to the enzyme hydrophilic G-site with a folded γ-glutamyl moiety, analogous to the folding of GSH in water, this might explain the activities of analogues (5) and (6): the carboxylate and possibly the amino function could well be oriented by the enzyme in such a way that the intramolecular interactions, including the electronic effects on the thiol function, are comparable with those in GSH.

In the last four analogues presented in Table 1, the γ-glutamyl moiety has been replaced by aspartate. These analogues show very low activity, if any, with GST isoenzyme 3–3. Interestingly, α-D-Asp-L-Cys-Gly has a higher catalytic efficiency than its α-L-aspartyl analogue, just as was found for the two α-glutamyl analogues. Comparing the α-D-glutamyl with the α-D-aspartyl analogue and the α-L-glutamyl with the α-L-aspartyl analogue it becomes clear that shortening the peptide backbone by one methylene unit results in a pronounced decrease of the catalytic efficiencies of the co-substrates.

GST isoenzymes 1–1, 2–2 and 4–4

Comparison of results obtained for isoenzyme 3–3 with those obtained with isoenzymes 1–1, 2–2 and 4–4 leads to interesting differences in substrate recognition and catalysis. Co-substrate (6), with an α-D-glutamyl moiety, appears to be an isoenzyme-3–3-specific GSH analogue. Both isoenzyme 1–1 and isoenzyme 2–2 are inactive towards analogue (6), and the $k_{cat.}/K_m$ for GST isoenzyme 4–4 is only about 5% compared with that for GST isoenzyme 3–3 (Fig. 1). This co-substrate analogue has a higher catalytic efficiency than GSH for isoenzyme 3–3. In contrast with the pronounced isoenzyme specificity of analogue (6) stands the behaviour of GSH analogue (5), which is a co-substrate for all isoenzymes tested. Catalytic efficiencies are lower than with GSH, and variations in $k_{cat.}/K_m$ show a variation with isoenzyme similar to those shown by GSH (see Fig. 1).

Five out of the nine GSH analogues tested were, to some measure, accepted by GST isoenzyme 4–4, like GST isoenzyme 3–3 belonging to the Mu multigene family. The $k_{cat.}/K_m$ values for GST isoenzyme 4–4 for all GSH analogues are considerably less than for GST isoenzyme 3–3. However, it should be remembered that the value for GST isoenzyme 4–4 is also lower for the natural co-substrate GSH.

In contrast, isoenzymes of the Alpha multigene family, 1–1 and 2–2, do not accept any γ-glutamyl modifications except deamino-GSH (2) and the 'true' GSH analogue (5) with the α-L-glutamyl moiety. Therefore GST isoenzymes 1–1 and 2–2 are much more specific towards alternative co-substrates than are those of the Mu multigene family.

The results presented here reveal clear differences in the GSH-binding sites between the Mu and Alpha multigene families.

Inhibition

The analogues were also tested for their ability to act as inhibitors of GST isoenzymes. They were added at 1 mM concentrations to enzyme incubation mixtures

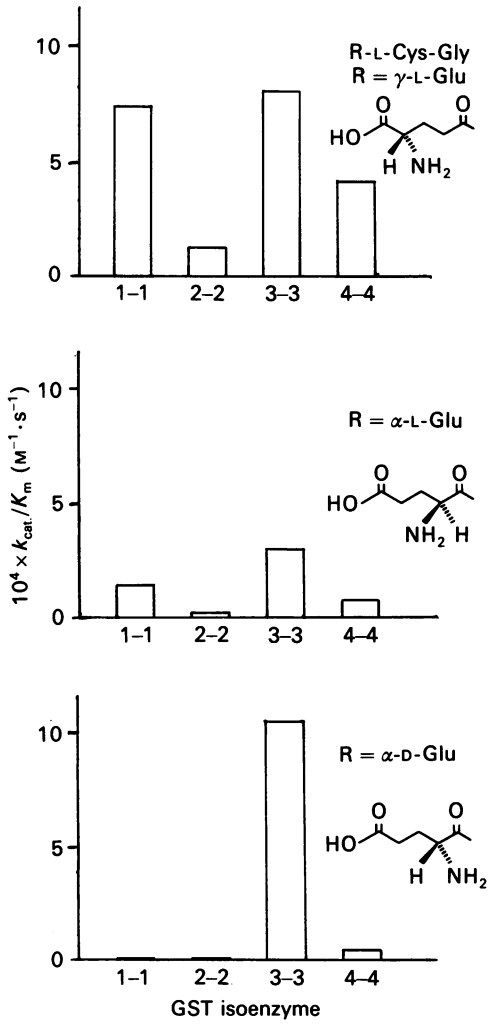

Fig. 1. Catalytic efficiencies ($k_{cat.}/K_m$) of GSH and two modified GSH analogues, (5) and (6), with four major cytosolic GST isoenzymes

already containing 1 mM-1-chloro-2,4-dinitrobenzene and 1 mM-GSH. No inhibition occurred at 1 mM concentration of each of the nine analogues tested. The specific activities for the GST isoenzymes under investigation did not differ significantly from activities obtained from incubations without the addition of one of the GSH analogues.

Therefore it may be concluded that analogues of GSH with modifications at the γ-glutamyl site appear to act solely as substrate analogues with no significant inhibitory properties in the GST-catalysed conjugation of 1-chloro-2,4-dinitrobenzene and GSH.

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CONCLUSIONS

The present paper shows that the requirements for binding of the co-substrate to the G-site of some major GST isoenzymes are less strict than was commonly believed. Six out of a total of nine GSH analogues tested showed appreciable activity when tested with GST isoenzyme 3-3. Two members of this series, the L-α-glutamyl- and D-α-glutamyl-containing analogues (5) and (6), even afforded an enzymic activity similar to that of the natural co-substrate GSH. Isoenzyme 4-4 showed a dependence on co-substrate structure similar to that of isoenzyme 3-3 but at a lower level of activity. Isoenzymes 1-1 and 2-2, on the other hand, showed a higher specificity, displaying a significant activity only with deamino-GSH (2) and with the α-L-glutamyl derivative (5).

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