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Interaction of rat glutathione *S*-transferases 7–7 and 8–8 with γ-glutamyl- or glycyl-modified glutathione analogues

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Analogues of GSH in which either the γ-glutamyl or the glycyl moiety is modified were synthesized and tested as both substrates for and inhibitors of glutathione *S*-transferases (GSTs) 7–7 and 8–8. Acceptor substrates for GST 7–7 were 1-chloro-2,4-dinitrobenzene (CDNB) and ethacrynic acid (ETA) and for GST 8–8 CDNB, ETA and 4-hydroxynon-*trans*-2-enal (HNE). The relative ability of each combination of enzyme and GSH analogue to catalyse the conjugation of all acceptor substrates was similar with the exception of the combination of GST 7–7 and γ-L-Glu-L-Cys-L-Asp, which used CDNB but not ETA as acceptor substrate. In general, GST 7–7 was better than GST 8–8 in utilizing these analogues as substrates, and glycyl analogues were better than γ-glutamyl analogues as both substrates and inhibitors. These results are compared with those obtained earlier with GSH analogues and GST isoenzymes 1–1, 2–2, 3–3 and 4–4 [Adang, Brussee, Meyer, Coles, Ketterer, van der Gen & Mulder (1988) Biochem. J. **255**, 721–724] and the implications with respect to the nature of their active sites are discussed.

INTRODUCTION

Cytosolic glutathione *S*-transferases (GSTs) are dimeric isoenzymes concerned with the detoxication of compounds that either are themselves electrophilic or become so on metabolism. In the rat 11 subunits have been characterized, given a numerical nomenclature and shown to be products of three multigene families. Subunits 1, 2, 8 and 10 are members of the Alpha multigene family, subunits 3, 4, 6, 9 and 11 of the Mu multigene family and subunit 7 the only member of the Pi family that is known to be expressed (Ketterer *et al.*, 1988; Mannervik & Danielson, 1988; Kispert *et al.*, 1989).

Recently a series of GSH analogues modified at the γ-glutamyl moiety was tested for substrate activity with homodimers of the four major subunits expressed in the liver, namely GST 1–1, 2–2, 3–3 and 4–4, with the intention of probing the GSH-binding site in a systematic manner (Adang *et al.*, 1988*b*). In these experiments 1-chloro-2,4-dinitrobenzene (CDNB) was used as the acceptor substrate. A big difference in the specificity between the Alpha and Mu isoenzymes tested was observed. GSTs 1–1 and 2–2 of the Alpha family accepted only two out of the nine analogues as co-substrates, whereas six out of nine γ-glutamyl-modified GSH analogues were accepted by GST isoenzyme 3–3 and, of these, five were also accepted by GST 4–4, showing the GSH-binding site of these Mu-family enzymes to be much less specific than those of the Alpha-family enzymes. GST 3–3 was unique in this study in its ability to accept α-D-Glu-L-Cys-Gly.

The present paper describes an extension of these studies. Two further isoenzymes from the rat are examined, and some new analogues, including some modified at the glycyl moiety, have been added to the study and also two acceptor substrates in addition to CDNB. One of these isoenzymes is GST 7–7, which represents the third GST multigene family, namely the Pi family. This isoenzyme is not normally present in the rat liver but is characteristic of the preneoplastic liver. Good acceptor substrates for this isoenzyme are CDNB and its usual marker substrate ethacrynic acid (ETA) (Ketterer *et al.*, 1988).

The other isoenzyme is GST 8–8, which is a member of the Alpha family and present in small amounts in the liver. Acceptor substrates for this enzyme are CDNB, 4-hydroxynon-*trans*-2-enal (HNE) and ETA. GST 8–8 is remarkable for its very high activity with HNE.

It is shown that GST 8–8 has the most stringent requirements of all isoenzymes so far studied, having appreciable activity only with GSH. GST 7–7, on the other hand, displays a selectivity for γ-glutamyl analogues similar to that previously observed with GSTs 1–1 and 2–2. Glycyl analogues were accepted more generally than γ-glutamyl analogues as substrates for GST 7–7, but were not accepted at all by GST 8–8. These glycyl analogues were inhibitors for GST 7–7 and especially for GST 8–8.

MATERIALS AND METHODS

Materials

CDNB and anhydrous trifluoroacetic acid were purchased from Merck (Darmstadt, Germany); *NN'*-dicyclo-

Abbreviations used: GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; ETA, ethacrynic acid; HNE, 4-hydroxynon-*trans*-2-enal; 4-Abu, 4-aminobutyric acid; Boc, t-butyloxycarbonyl; Bzl, benzyl; Cbz, benzyloxycarbonyl; DCHA salt, dicyclohexylamine salt; PTS salt, toluene-*p*-sulphonic acid salt.

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hexylcarbodi-imide was obtained from Fluka (Buchs, Switzerland). ETA was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). GSH was obtained from Janssen Chimica (Beerse, Belgium). 4-Hydroxynon-*trans*-2-enal was generously given by Dr. H. Esterbauer (Universität Graz, Graz, Austria).

Synthesis of peptides

The γ-glutamyl-modified GSH analogues (compounds 2–7) were synthesized as described by Adang *et al.* (1988*a*). The six glycyl-modified GSH analogues (compounds 8–13) were prepared by following essentially the same procedure.

β-Ala-OBzl PTS salt, 4-Abu-OBzl PTS salt and L-Ala-OBzl PTS salt were prepared as described by Zervas *et al.* (1957). After two or more crystallizations from methanol/diethyl ether yields were between 80 and 85%. L-Phenylglycine and aminoethane did not require protection before coupling with cysteine. L-Asp-di-OBzl PTS salt was synthesized according to the procedure of Determann *et al.* (1962). Two crystallizations from ethanol/diethyl ether afforded the diprotected L-aspartic acid in 90% yield. Each of these six glycyl substitutes was coupled to *N*-t-Boc-*S*-Bzl-L-Cys according to the procedure of König & Geiger (1970) as adapted by Adang *et al.* (1988*a*). Protected dipeptides were crystallized from ethyl acetate/light petroleum (b.p. 40–60 °C) to give yields between 75 and 85%. Removal of the t-Boc protective group at the *N*-terminus was performed as described by Adang *et al.* (1988*a*).

Coupling of dipeptides, deprotected at their *N*-terminus, to protected L-glutamic acid gave the fully protected tripeptides. *N*-Cbz-α-OBzl-L-Glu DCHA salt was sequentially synthesized as described by Adang *et al.* (1988*a*) and coupled to the Cys-Xaa dipeptides by the active ester method (dicyclohexylcarbodi-imide/1-hydroxybenzotriazole). The protected tripeptides were crystallized in the same way as the dipeptides. In general, good yields (78–87%) of colourless crystalline material were obtained.

Deprotection of the glycyl-modified tripeptides (compounds 8–13) was carried out in a single step using Na in liquid NH_3 (Adang *et al.*, 1988*a*). After completion of the reaction, NH_4Cl was added until the blue colour disappeared, NH_3 was then evaporated off under a stream of dry N_2 and the residue was desiccated over conc. H_2SO_4 overnight. The residue was dissolved in 150 ml of ice-cold degassed distilled water, Dowex 50W X4 (H^+ form) was quickly added to lower the salt concentration and the pH was adjusted to 7–7.5. Ion-exchange material was filtered off, and the solution was concentrated to 25–30 ml and applied to a Dowex 1 X2 resin (100–200 mesh; Cl^- form) column (30 cm × 3 cm). After the peptide had bound, the column was washed with 5 column volumes of water and the product was eluted with a linear gradient of formic acid (0–2 M). The ninhydrin-positive fractions were pooled (50–60 ml) and, if formic acid was present, it was largely removed by adding Dowex 1 X1 (100–200 mesh; OH^- form). Freeze-drying removed the water and traces of formic acid and afforded the deprotected tripeptides as white fluffy compounds, often static and hygroscopic, in overall yields of 40–55%.

By the use of Ellman's (1959) reagent the free thiol content was determined to be greater than 95% in each case. The deprotected tripeptide preparations moved as single spots on t.l.c. (Adang *et al.*, 1988*a*). All products were analysed with ^{1}H-n.m.r. and noise-decoupled ^{13}C-n.m.r. spectroscopy (Table 1).

Purification of rat GST isoenzymes

GST 7–7 was purified from rat kidney cytosol by affinity chromatography on GSH–agarose (Vander Jagt *et al.*, 1985) followed by hydroxyapatite chromatography (Meyer *et al.*, 1985). GST 8–8 was purified from liver cytosol by specific elution from GSH–agarose with GSSG (Meyer *et al.*, 1989), the contaminating GST 1–1 being removed by hydroxyapatite chromatography. The purified enzymes were checked for purity and quantified by reverse-phase h.p.l.c. by the method of Ostlund Farrants *et al.* (1987) modified in accordance with Kispert *et al.* (1989). Finally, the purified isoenzymes were dialysed against 0.1 mM-potassium phosphate buffer, pH 6.5, before assay with GSH analogues.

Enzyme assays and kinetics

The specific activities of GST isoenzymes 7–7 and 8–8 were determined by measuring the initial rate of the enzyme-catalysed conjugation of GSH or GSH analogues with CDNB, ETA or HNE at 25 °C and pH 6.5. The initial rates for all substrates were detected spectrophotometrically by measuring an increase in absorbance at a specific wavelength (product formation), except for HNE, where a decrease was monitored (substrate disappearance). Assays with CDNB and ETA were performed as described by Habig *et al.* (1974), and those with HNE by the method of Ålin *et al.* (1985) with minor modifications. The substrate concentrations in the CDNB assay were 1 mM-CDNB and 1 mM-GSH or analogue, in the ETA assay 0.17 mM-ETA and 0.20 mM-GSH or analogue and in the HNE assay 0.05 mM-HNE and 0.50 mM-GSH or analogue. Corrections were made for non-enzymic conjugation by recording the initial rates without addition of enzyme.

Kinetic studies were performed with those GSH analogues that exhibited high activities. The concentration of the acceptor substrate was held constant and the concentration of GSH or the GSH analogues was varied. The kinetic data were analysed by the direct-plot method (Eisenthal & Cornish-Bowden, 1978). The $V_{max.}$ and the K_m values for GSH and GSH analogues were determined from this plot.

Inhibition

To test for inhibitory effects, GSH analogues were added to the standard enzyme assay at a concentration equimolar with respect to that of the natural co-substrate GSH. All co-substrate analogues were tested this way with GST isoenzymes 7–7 and 8–8 and the percentage inhibition was determined for those analogues that showed no appreciable enzyme activity.

RESULTS

GST isoenzyme 7–7

The GST-catalysed conjugation of GSH was determined with CDNB, a general substrate for GSTs, and ETA, a marker substrate for GST isoenzyme 7–7 (see Table 2). It should be noted that with GSH the specific activity for CDNB is 5 times that for ETA and a similar ratio is seen with analogues that are acceptors. When GST 7–7 was assayed with CDNB, five out of six γ-glutamyl-modified GSH analogues showed some activity.

Table 1. ^{1}H n.m.r. and noise-decoupled ^{13}C n.m.r. (200 MHz and 50 MHz respectively): chemical shifts and coupling constants of the glycyl analogues of GSH

^{1}H n.m.r.

Glycyl-modified GSH analogue	$C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$	$C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$	$C_{(\alpha)}HC_{(\beta)}H_2SH$	$^-O_2CC_{(\alpha)}H(NH_3^+)C_{(\beta)}H_2$	$C_{(\alpha)}HC_{(\beta)}H_2SH$	CONH–*R*
8. -β-Ala	2.10 (2H, q, J = 7.3 Hz)	2.49 (4H, m, overlap with $C_{(\alpha)}H_2C_{(\beta)}H_2CO_2^-$)	2.85 (2H, d, J = 6.8 Hz)	3.74 (1H, t, J = 6.3 Hz)	4.38 (1H, t, J = 6.3 Hz)	$C_{(\alpha)}H_2C_{(\beta)}H_2CO_2^-$ 2.49 (4H, m, overlap with $C_{(\gamma)}H_2CO$; $C_{(\alpha)}H_2C_{(\beta)}H_2CO_2^-$) 3.44 (2H, m, $C_{(\alpha)}H_2C_{(\beta)}H_2CO_2^-$)
9. -4-Abu	2.16 (2H, q, J = 7.2 Hz)	2.56 (2H, t, J = 7.2 Hz)	2.92 (2H, d, J = 6.7 Hz)	3.78 (1H, t, J = 6.3 Hz)	4.45 (1H, t, J = 6.7 Hz)	$C_{(\alpha)}H_2C_{(\beta)}H_2C_{(\gamma)}H_2CO_2^-$ 1.80 (2H, qui, J = 7.3 Hz; $C_{(\beta)}H_2$) 2.31 (2H, t, J = 7.6 Hz; $C_{(\alpha)}H_2$) 3.26 (2H, t, J = 6.7 Hz; $C_{(\gamma)}H_2$)
10. -L-Ala	2.14 (2H, q, J = 7.2 Hz)	2.50 (2H, m)	2.90 (2H, m)	3.78 (1H, t, J = 6.3 Hz)	4.50 (1H, t, J = 6.2 Hz)	$CH(CH_3)CO_2^-$ 1.38 [3H, d, J = 7.2 Hz; $CH(CH_3)CO_2^-$] 4.28 [1H, q, J = 7.4 Hz; $CH(CH_3)CO_2^-$]
11. -L-Ph-Gly	2.18 (2H, q, J = 6.9 Hz)	2.55 (2H, m)	2.95 (2H, d, J = 6.5 Hz)	3.72 (1H, m)	4.45 [2H, m, overlap with $CH(C_6H_5)CO_2^-$]	$CH(C_6H_5)CO_2^-$ 4.45 [2H, m, overlap with $C_{(\alpha)}HC_{(\beta)}H_2SH$; $CH(C_6H_5)CO_2^-$] 7.37 [5H, m, $CH(C_6H_5)CO_2^-$]
12. -L-Asp	2.17 (2H, q, J = 7.1 Hz)	2.54 (2H, dt, J_{ax} = 7.7 Hz, J_{bx} = 7.5 Hz)	2.93 [4H, m, overlap with $CH(CH_2CO_2^-)CO_2^-$]	3.90 (1H, t, J = 6.4 Hz)	4.50 (1H, t, J = 6.2 Hz)	$CH(CH_2CO_2^-)CO_2^-$ 2.93 [4H, m, overlap with $C_{(\alpha)}HC_{(\beta)}H_2SH$; $CH(CH_2CO_2^-)CO_2^-$] 4.14 [1H, t, J = 6.3 Hz; $CH(CH_2CO_2^-)CO_2^-$]
13. -NHC_2H_5	2.15 (2H, q, J = 7.3 Hz)	2.52 (2H, m)	2.95 (2H, m)	3.77 (1H, t, J = 6.4 Hz)	4.42 (1H, t, J = 6.4 Hz)	CH_2CH_3 1.11 (3H, t, J = 7.2 Hz; CH_3) 3.23 (2H, q, J = 7.2 Hz; CH_2)

^{13}C n.m.r.

Glycyl-modified GSH analogue	$C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$	$C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$	$C_{(\alpha)}HC_{(\beta)}H_2SH$	$^-O_2CC_{(\alpha)}H(NH_3^+)C_{(\beta)}H_2$	$C_{(\alpha)}HC_{(\beta)}H_2SH$	CO_2^-, CONH	CONH–*R*
8. -β-Ala	26.86	31.96	26.04	53.72	56.70	172.65, 175.54 176.01, 177.47	$C_{(\alpha)}H_2C_{(\beta)}H_2CO_2^-$: 34.71, 36.23
9. -4-Abu	26.95	32.09	26.10	54.96	56.94	172.87, 174.76, 175.73,	$C_{(\alpha)}H_2C_{(\beta)}H_2C_{(\gamma)}H_2CO_2^-$: 25.55, 34.11, 39.86
10. -L-Ala	27.04	32.18	26.31	54.87	56.41	172.25, 174.16, 175.64, 176.21	$CH(CH_3)CO_2^-$: 17.52, 50.60
11. -L-Ph-Gly	26.86	32.17	26.04	53.90	56.64	172–173	$CH(C_6H_5)CO_2^-$: 54.97, 128.06, 128.20, 129.49
12. -L-Asp	26.69	31.94	26.08	54.08	56.47	172.57, 174.97, 175.26, 175.43, 175.52	$CH(CH_2CO_2^-)CO_2^-$: 36.53, 50.34
13. -NHC_2H_5	26.81	32.13	26.09	54.03	56.53	172.18, 174.31, 175.03	CH_2CH_3 18.09, 25.72

Table 2. Specific activities of rat kidney GST 7–7 and rat liver GST 8–8 with two series of GSH analogues, glutamyl-modified and glycyl-modified respectively, for the substrates CDNB and ETA for GST 7–7 and the substrates CDNB, ETA and HNE for GST 8–8

Abbreviation: N.D., no detectable activity.

	Specific activity of GST 7–7 (μmol/min per mg)		Specific activity of GST 8–8 (μmol/min per mg)		
Co-substrate	CDNB	ETA	CDNB	ETA	HNE
1. GSH	9.8	2.0	6.0	4.1	150
γ-Glutamyl-modified GSH analogues					
2. Glutaryl-	0.3	N.D.	0.1	N.D.	N.D.
3. γ-D-Glu-	0.2	N.D.	N.D.	0.2	N.D.
4. α-L-Glu-	2.0	0.3	0.3	0.5	3
5. α-D-Glu	1.6	N.D.	0.3	N.D.	N.D.
6. α-L-Asp-	N.D.	N.D.	N.D.	N.D.	N.D.
7. α-D-Asp-	0.2	N.D.	0.1	N.D.	N.D.
Glycyl-modified GSH analogues					
8. -β-Ala	4.6	1.1	0.4	1.1	7
9. -4-Abu	0.5	0.2	N.D.	< 0.1	< 1
10. -L-Ala	0.9	N.D.	0.2	0.6	< 1
11. -L-Ph-Gly	3.0	0.4	0.2	1.5	4
12. -L-Asp	6.2	N.D.	0.1	0.3	3
13. $-NHC_2H_5$	1.4	0.5	0.1	0.6	< 1

Table 3. Inhibition of rat kidney GST 7–7 and rat liver GST 8–8 with two series of GSH analogues, glutamyl-modified and glycyl-modified respectively, for the substrates CDNB and ETA for GST 7–7 and CDNB, ETA and HNE for GST 8–8

With CDNB as substrate the concentrations of CDNB, GSH and inhibitor were all 1 mM. With ETA as substrate the concentration of ETA was 0.17 mM and those of GSH and inhibitor were 0.2 mM. With HNE as substrate the HNE concentration was 50 μM and those of GSH and inhibitor were 500 μM. Abbreviations: N.D., no detectable inhibition; *, enzyme activity too high, no inhibition given.

	Inhibition of GST 7–7 activity (%)		Inhibition of GST 8–8 activity (%)		
Inhibitor	CDNB	ETA	CDNB	ETA	HNE
γ-Glutamyl-modified GSH analogues					
2. Glutaryl-	N.D.	N.D.	31	N.D.	N.D.
3. γ-D-Glu-	N.D.	N.D.	23	N.D.	20
4. α-L-Glu	23	N.D.	80	N.D.	81
5. α-D-Glu-	N.D.	N.D.	< 5	N.D.	< 5
6. α-L-Asp-	N.D.	N.D.	< 5	N.D.	< 5
7. α-D-Asp-	N.D.	N.D.	< 5	N.D.	< 5
Glycyl-modified GSH analogues					
8. -β-Ala	*	*	74	22	61
9. -4-Abu	54	N.D.	62	N.D.	50
10. -L-Ala	30	57	32	N.D.	19
11. -L-Ph-Gly	34	32	31	12	42
12. -L-Asp	*	N.D.	21	N.D.	31
13. $-NHC_2H_5$	59	N.D.	63	N.D.	66

The α-L-glutamyl and α-D-glutamyl analogues were most effective, conjugating CDNB with approx. 20% of the activity obtained with GSH. Glycyl-modified GSH analogues were more efficient in replacing GSH: β-alanine, L-phenylglycine and L-aspartic acid substitutions for glycine resulted in co-substrate analogues with activities between 30 and 70% of that of GSH.

When identical experiments were performed with ETA as substrate, all the γ-glutamyl-modified GSH analogues showed very low activities, only the α-L-glutamyl analogue having significant activity. As with assays with CDNB, glycyl analogues were more active than γ-glutamyl analogues. However, the analogue with L-aspartic acid behaved very differently towards the two acceptor substrates: with CDNB, activity was more than 60% of that obtained with GSH, but no activity at all was detected with ETA.

With respect to inhibition, the capacities of the γ-

Table 4. Kinetic parameters of rat kidney GST 7–7 with GSH and the most active co-substrate analogues (see Table 2) determined with fixed concentrations of CDNB and ETA

The concentration of CDNB was held constant at 1 mM while the GSH or analogue concentration was varied between 0.05 and 1.5 mM to determine the K_m for GSH or an analogue and the $V_{max.}$ at fixed CDNB concentration. The same applied for ETA kinetics: the ETA concentration was fixed at 0.17 mM and the GSH concentration was varied between 0.02 and 0.4 mM or the analogue concentration was varied between 0.05 and 1.5 mM.

Co-substrate: GSH or glycyl- or α-glutamyl-modified analogue	Electrophilic substrate...	CDNB		ETA	
		K_m (mM)	$V_{max.}$ (μmol/min per mg)	K_m (mM)	$V_{max.}$ (μmol/min per mg)
GSH		0.3	14.9	0.05	2.6
-β-Ala		0.7	7.7	0.3	2.4
-L-Ph-Gly		0.4	4.8	0.8	2.9
α-L-Glu-		0.7	4.2	0.4	1
-L-Asp		0.3	10.9	N.D.	N.D.

glutamyl analogues were very different from those of the glycyl analogues (see Table 3). The former inhibited neither the conjugation of CDNB nor that of ETA. In contrast, four out of six glycyl-modified analogues were substantial inhibitors of the CDNB conjugation and two out of six inhibited ETA conjugation.

Analogues that showed appreciable activity in the enzyme assay have not been included in Table 3 because of the complexity of the kinetics involved.

K_m and $V_{max.}$ values for the most active GSH analogues for GST isoenzyme 7–7 were determined (Table 4). With CDNB as acceptor substrate a comparison between GSH and the analogues showed that modifications mainly lead to a lower $V_{max.}$. The K_m is less affected. In contrast, with ETA as acceptor substrate the K_m is more sensitive to substitution of amino acid residues and may reach values 6-fold higher than that for GSH; the L-aspartyl modified glycyl analogue was not accepted at all.

GST isoenzyme 8–8

GST 8–8 was tested with three different acceptor substrates and was found to be highly specific for GSH (Table 2). All analogues had very low activities with one exception: conjugation of ETA with the L-phenylglycyl analogue was 37% the rate obtained with GSH.

Analogues inhibited conjugation of both CDNB and HNE to a similar extent. Little inhibition of conjugation of ETA was observed. For all acceptor substrates the concentration of GSH (and inhibitor) was 3–4 times the K_m value for GSH as measured, for instance, with GST 7–7 (Table 4). Thus the difference in inhibition for the three acceptor substrates is not due to different $[S]/K_m$ ratios for GSH in relation to the inhibitor concentration. The γ-glutamyl-modified analogues were all poor inhibitors with the exception of the α-L-glutamyl analogue, which showed a high level of inhibition (80%) with CDNB and HNE. On the other hand all glycyl-modified GSH analogues were inhibitory with CDNB and HNE.

DISCUSSION

GSH structure and γ-glutamyl modifications

It is becoming apparent that GST isoenzymes have a wide range of selectivity towards GSH analogues. It was shown previously (Adang *et al.*, 1988*b*) that GSTs 3–3 and 4–4 from the Mu family were very accommodating, accepting glutaryl, α-L-glutamyl, α-D-glutamyl, β-D-aspartyl and α-D-aspartyl GSH analogues as co-substrates. GSTs 1–1 and 2–2 from the Alpha family were much more selective, accepting only glutaryl and α-L-glutamyl analogues. The present paper shows that a third member of the Alpha family, GST 8–8, is very stringent, having appreciable activity only with GSH.

GST 7–7 of the Pi family is also much more selective than those enzymes from the Mu family so far studied. Thus although GSTs 1–1 and 2–2 will utilize the glutaryl and α-L-glutamyl analogues in addition to GSH, GST 7–7 will only use the L- and D-isomers of the α-glutamyl analogues.

A marked variation in the specificity is seen in members of the Alpha family. It will be of interest to determine whether or not similar differences are also found in the Mu family, e.g. with GSTs 6–6 and 11–11.

So far, apart from GSH, the α-L-Glu analogue is acceptable to all GSTs, since it is accepted as substrate by all GST isoenzymes except GST 8–8, and with this isoenzyme it is an inhibitor.

GSH structure and glycyl modifications

Several modifications are accepted as co-substrates by GST isoenzyme 7–7, with rates of 50% or more those of GSH, showing that the glycine domain is less specific than the γ-glutamyl site. This effect is similar with both CDNB and ETA, except in the case of the analogue with L-aspartic acid. Though active with CDNB, this GSH analogue was inactive with ETA. It is possible that the negative carboxylate group of ETA and the β-carboxylate group in the L-aspartyl analogue are involved in electrostatic repulsion.

Some glycyl-modified GSH analogues were inhibitory, but only with CDNB as acceptor substrate. Thus the extent of inhibition of GST 7–7 is strongly dependent on the acceptor substrate. This is reminiscent of data reported by Meyer & Ketterer (1987) on the inhibition of GST isoenzyme 3–3 by linoleic acid: the activity towards DCNB was inhibited by 50% whereas the activity with CDNB was unaffected. It may be that binding of an acceptor substrate induces a conformation change in the enzyme significant enough to alter the catalytic capacity

of GST isoenzyme 7–7. An induced fit might explain the dependence of catalysis on the acceptor substrate. The kinetic results with GST isoenzyme 7–7 also depend on the acceptor substrate used. Apparently, in the conjugation of CDNB (a nucleophilic aromatic substitution) and ETA (a 1,4 Michael addition to an $\alpha\beta$-unsaturated ketone) different interactions with the thiol peptide occur. The differences in K_m for GSH measured with ETA and CDNB might reflect these different modes of action (Table 4).

Almost no changes of the GSH molecule at the glycyl moiety were accepted by GST isoenzyme 8–8 as far as conjugation was concerned. However, these analogues were apparently able to compete with GSH for the G-site in the active centre because they were inhibitory.

Differences between GST 7–7 and GST 8–8

Again, it was shown that peptide analogues of GSH possess affinity for the active site of GST isoenzymes. For GST 7–7 this mainly resulted in catalysis of the conjugation, whereas for GST 8–8 inhibition was the result. Thus GST isoenzymes 7–7 and 8–8 show a different behaviour towards the analogues. Either their GSH-binding sites are different or GSH analogues induce different conformational changes that affect the catalytic efficiency. GST isoenzyme 8–8 is a member of the Alpha multigene family with respect to binding characteristics of GSH. GST isoenzymes 1–1 and 2–2 behaved similarly, as Adang *et al.* (1988*b*) showed. Only α-L-Glu-L-Cys-Gly was able to inhibit GST 8–8 for two of the acceptor substrates.

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