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The glutathione-binding site in glutathione *S*-transferases

Investigation of the cysteinyl, glycyl and γ-glutamyl domains

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The GSH-binding site of glutathione *S*-transferase (GST) isoenzymes was studied by investigating their substrate-specificity for three series of GSH analogues; further, a model of the interactions of GSH with the G-site is proposed. Twelve glycyl-modified GSH analogues, four ester derivatives of GSH and three cysteinyl-modified GSH analogues were synthesized and tested with purified forms of rat liver GST (1–1, 2–2, 3–3 and 4–4). The glycyl analogues exhibited spontaneous chemical reaction rates with 1-chloro-2,4-dinitrobenzene comparable with the GSH rate. In contrast, the enzymic rates ($V_{max.}$) differed greatly, from less than 1 up to 140 μmol/min per mg; apparently, a reaction mechanism is followed that is very sensitive to substitutions at the glycyl domain. No correlation exists between the chemical rates and $V_{max.}$ values for the analogues. Analogues of GSH in which L-cysteine was replaced by D-cysteine, L-homocysteine or L-penicillamine showed little or no capacity to replace GSH as co-substrate for the GSTs. GSH monomethyl and monoethyl esters showed $V_{max.}$ values greater than the $V_{max.}$ measured with GSH: the $V_{max.}$ for the monoethyl ester of GSH and GST 3–3 was 5-fold that for GSH. The data obtained in this and previous studies [Adang, Brussee, Meyer, Coles, Ketterer, van der Gen & Mulder (1988) Biochem. J. **255**, 721–724; Adang, Meyer, Brussee, van der Gen, Ketterer & Mulder (1989) Biochem. J. **264**, 759–764] allow a model of the interactions of GSH in the G-site in GSTs to be postulated. The γ-glutamyl site is the main binding determinant: the α-carboxylate group is obligatory, whereas shifting of the amino group and shortening of the peptide backbone only decreased $k_{cat.}/K_m$. Furthermore, the GSTs appear to be very critical with respect to a correct orientation of the thiol group of the GSH analogue. The glycyl site is the least restrictive domain in the G-site of GSTs: amino acid analogues all showed K_m values between 0.2 and 0.6 mM (that for GSH is 0.2–0.3 mM), but large differences in $V_{max.}$ exist. The glycyl carboxylate group is not essential for substrate recognition, since decarboxy analogues and ester derivatives showed high activities. The possible mechanisms for an increased $V_{max.}$ in some analogues are briefly discussed.

INTRODUCTION

The glutathione *S*-transferase (GST) isoenzyme families are present in high concentration in the cytosol of many tissues in mammalian species and catalyse a wide spectrum of reactions. They express broad but overlapping substrate-specificities that enable the conversion of many electrophilic substrates, provided that they are sufficiently hydrophobic to bind to the hydrophobic site (H-site) in the catalytic centre of the GSTs (Jakoby, 1978; Armstrong, 1987; Mannervik & Danielson, 1988; Ketterer *et al.*, 1988). In addition to exogenous compounds, endogenous electrophilic substrates have recently been identified: hydroxyalkenals such as 4-hydroxynon-2-enal, which are end-products of lipid peroxidation, were shown to be efficient substrates for certain forms of GST, especially rat liver GST 8–8 (Ålin *et al.*, 1985).

To understand the catalytic mechanism of the GSTs, information is required about the site where the co-substrate GSH binds, the G-site. Until recently, little was known about this G-site. On the basis of the limited data available and supported by similar results on GSH specificity in other GSH-dependent enzymes it was suggested that the G-site probably was very specific towards the co-substrate GSH. Thiols such as *N*-Ac-L-Cys, L-cysteine, 2-mercaptoethanol and the tetrapeptide γ-L-Glu-γ-L-Glu-L-Cys-Gly are not accepted as co-substrate replacements for GSH (Habig *et al.*, 1974; Abbott *et al.*, 1986). *N*-Ac-GSH and the 'retro-inverso' isomer of GSH had only very low $k_{cat.}/K_m$ values compared with GSH (Chen *et al.*, 1986, 1988). The dipeptide γ-L-Glu-L-Cys could replace GSH to some extent, but the K_m value was one order of magnitude higher than that of GSH, and $V_{max.}$ was approximately half that of GSH (Sugimoto *et al.*, 1985). Only the plant homologue γ-L-Glu-L-Cys-β-Ala was reported in a brief statement to show an activity comparable with that of GSH with two class Mu GST isoenzymes (Habig *et al.*, 1974).

Recently we reported on a series of GSH analogues in which the γ-glutamyl moiety was varied. Differences in the spontaneous non-enzyme-catalysed reaction rate with the substrate 1-chloro-2,4-dinitrobenzene (CDNB) were interpreted in terms of intramolecular interactions in the peptide structure that might facilitate deprotonation of the thiol group of GSH (Adang *et al.*, 1988*a*). GSTs 1–1, 2–2, 3–3 and 4–4 were tested with the γ-glutamyl-modified GSH analogues and the substrate CDNB. The Mu multigene family especially accepted various modifications in the co-substrate structure, whereas the Alpha family was more restrictive. α-L-Glutamyl- and α-D-glutamyl-modified GSH analogues were efficient co-substrate analogues. Surprisingly, the α-D-glutamyl analogue was highly GST-3-3-specific, with a catalytic efficiency exceeding that of the natural co-substrate (Adang *et al.*, 1988*b*).

Abbreviations used: GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; Ac, acetyl; Ph, phenyl; Bzl, benzyl; t-Boc, t-butyloxycarbonyl; Cbz, benzyloxycarbonyl; NPS, *o*-nitrosulphenyl; DCHA salt, dicyclohexylamine salt; PTS, toluene-*p*-sulphonic acid salt; 4-Abu, 4-aminobutyric acid; Hcy, homocysteine; Pen, penicillamine.

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In a separate study, GST 7–7 and especially GST 8–8 were shown to be more restrictive in accepting alternative co-substrates. Furthermore, the relative rates for the various GSH analogues showed big differences if, instead of CDNB, other acceptor substrates were used (Adang *et al.*, 1989).

In the present paper we report on series of cysteinyl- and glycyl-modified GSH analogues towards GSTs 1–1, 2–2, 3–3 and 4–4. A model of the G-site, based on these and previously obtained results, is presented.

MATERIALS AND METHODS

Materials

The same rat liver GST isoenzyme 1–1, 2–2, 3–3 and 4–4 preparations were used as in previous studies (Adang *et al.*, 1988*b*), supplied by Professor B. Ketterer and Dr. D. J. Meyer (Department of Biochemistry, University College and Middlesex Hospital Medical School, London, U.K.) and described by Beale *et al.* (1983). 1-Chloro-2,4-dinitrobenzene (CDNB) was obtained from Merck (Darmstadt, Germany). GSH was obtained from Janssen Chimica (Beerse, Belgium).

Synthesis of glycyl-modified GSH analogues

Melting points are given of fluffy salt-free material unless the salt form is indicated. Optical rotations were measured with a Perkin–Elmer 141 polarimeter at 20 °C unless otherwise stated. N.m.r. spectra were obtained in ^2H_2O, except where noted otherwise, at 200 MHz for ^1H n.m.r. and at 50 MHz for ^{13}C n.m.r. with a JEOL JMN-FX 200 spectrometer. Chemical shifts (δ) are given in p.p.m. relative to an external standard (3-trimethylsilylpropionic acid) (pD 3.5 unless one or more carboxy groups are blocked or omitted).

Glycyl analogues γ-L-Glu-L-Cys-L-Asp (3), γ-L-Glu-L-Cys-L-(Ph-)Gly (4), γ-L-Glu-L-Cys-L-Ala (8), γ-L-Glu-L-Cys-β-Ala (10), γ-L-Glu-L-Cys-4-Abu (11) and γ-L-Glu-L-Cys-NH-C_2H_5 (12) were synthesized as described by Adang *et al.* (1989). Compounds γ-L-Glu-L-Cys-L-Val (2), γ-L-Glu-L-Cys-L-Phe (5), γ-L-Glu-L-Cys-L-Lys (6), γ-L-Glu-L-Cys-L-His (7), γ-L-Glu-L-Cys-D-Ala (9) and γ-L-Glu-L-Cys-NH-CH_2CF_3 (13) were prepared by following essentially the same procedure; L-Val-OBzl PTS salt, L-Phe-OBzl PTS salt and D-Ala-OBzl PTS salt were prepared as described by Zervas *et al.* (1957). Two or more crystallizations from methanol/diethyl ether resulted in yields between 80 and 90% [L-Val-OBzl PTS salt, m.p. 155–156 °C and $[\alpha]_D$ −2.6° (*c* 2 in methanol); L-Phe-OBzl PTS salt, m.p. 162–164 °C and $[\alpha]_D$ +7.3° (*c* 2 in dimethylformamide); D-Ala-OBzl PTS salt, m.p. 116–118 °C and $[\alpha]_D$ +5.4° (*c* 4 in methanol)]. N^{ϵ}-Cbz-L-Lys was prepared by the method of Kjer & Larsen (1961); after recrystallization from water crystals were obtained in 70% yield, m.p. 240–242 °C and $[\alpha]_D$ +13.8° (*c* 1.4 in 0.1 M-HCl). N^{ϵ}-Cbz-L-Lys-OBzl PTS salt was obtained in 76% yield after recrystallization from ether/light petroleum (b.p. 40–60 °C) by using the method of Abe *et al.* (1967), m.p. 112–115 °C and $[\alpha]_D$ −5.3 (*c* 2 in dimethylformamide). N^{im}-Bzl-L-His was synthesized by the method of du Vigneaud & Behrens (1937); recrystallization from 70% ethanol yielded 68% of crystalline material, m.p. 242–244 °C and $[\alpha]_D$ +19.6° (*c* 1 in 2 M-HCl). N^{im}-Bzl-L-His-OBzl di-PTS salt was prepared by the method of Theodoropoulos & Fölsch (1958); recrystallization from propan-2-ol/diethyl ether resulted in white crystals in 75% yield, m.p. 176–178 °C and $[\alpha]_D$ +8.2° (*c* 1.5 in dimethylformamide). 1-Amino-2,2,2-trifluoroethane did not require protection before coupling to L-cysteine and subsequently to L-glutamic acid in the synthesis of γ-L-Glu-L-Cys-NH-CH_2CF_3.

The *C*-protected glycine substitutes were coupled to *N*-t-Boc-*S*-Bzl-L-Cys by using the procedure of König & Geiger (1970) as adapted by Adang *et al.* (1988*a*). Yields between 75 and 85% of crystallized dipeptides [from ethyl acetate/light petroleum (b.p. 40–60 °C)] were obtained. Quantitative removal of the t-butyloxycarbonyl protective group at the *N*-terminus (Adang *et al.*, 1988*a*) resulted in trifluoroacetate salts of the *N*-terminal-deprotected dipeptides. Coupling of dipeptides to *N*-Cbz-α-OBzl-L-Glu DCHA salt (synthesized as described by Adang *et al.*, 1988*a*) was performed by using the active ester method in which *NN'*-dicyclohexylcarbodi-imide and 1-hydroxybenzotriazole were the coupling reagents (König & Geiger, 1970; Adang *et al.*, 1988*a*). Protected tripeptides [*N*-Cbz-α-OBzl-L-Glu-(*S*-Bzl-)L-Cys-Xaa-OBzl] were obtained in good yields, 80–90% after crystallization from ethyl acetate/light petroleum (b.p. 40–60 °C). Deprotection in a single step (Na/liquid NH_3) and purification on Dowex 1-X2 (Cl^- form) was performed as previously described (Adang *et al.*, 1989). Freeze-drying afforded deprotected tripeptides as white fluffy compounds in overall yields of 45–60% (in general approx. 0.5 g of free peptide was obtained unless otherwise specified).

Determination of the free thiol content of the six glycyl-modified GSH analogues showed it to be greater than 95% in each case (Ellman, 1959). The peptides moved as single spots on t.l.c. (Adang *et al.*, 1988*a*). Melting points (decomp.) and optical rotation were respectively: γ-L-Glu-L-Cys-L-Val (2), m.p. 124 °C and $[\alpha]_D$ −31.3° (*c* 1 in water); γ-L-Glu-L-Cys-L-Phe (5), m.p. 143–145 °C and $[\alpha]_D$ −15.4° (*c* 1 in water); γ-L-Glu-L-Cys-L-Lys (6), m.p. 185–186 °C and $[\alpha]_D$ −33.7° (*c* 1 in water); γ-L-Glu-L-Cys-L-His (7), m.p. 92–94 °C and $[\alpha]_D$ −3.8° (*c* 1 in water); γ-L-Glu-L-Cys-D-Ala (9), m.p. 110–112 °C and $[\alpha]_D$ −16.5° (*c* 1 in water); γ-L-Glu-L-Cys-NH-CH_2CF_3 (13), m.p. 164 °C and $[\alpha]_D$ −35.2° (*c* 1 in water). ^1H-n.m.r. and ^{13}C-n.m.r. data on these six compounds are given in Table 1.

Esterification of GSH

Synthesis of GSH monoesters, with the glycyl carboxylate being esterified, was performed similarly to the preparation of the γ-half-ester of L-glutamic acid (Bergmann & Zervas, 1933) Monomethyl, monoethyl and monobutyl esters were obtained in 1 h at 4 °C if this procedure is followed (monobutyl ester formation required a co-solvent, dioxan). T.l.c. analysis [with propan-1-ol/acetic acid/water (16:3:5, by vol.) (Anderson *et al.*, 1985)] cleanly distinguished between GSH, monoester and contaminating diester. Purification was carried out by chromatography on a Dowex 1-X2 (100–200 mesh; Cl^- form) anion-exchanger. The product was dissolved in 10 ml of water, pH 7, and applied to the column. A typical elution showed that non-esterified GSH was bound to the column, the corresponding diester was eluted in the void volume and the required monoester was eluted after a delay in pure form. The procedure for GSH diethyl ester was essentially the same, only the amount of HCl gas was increased and the reaction was continued until only diester was present, as shown by t.l.c. analysis (see above). Yields were generally higher than 90%; 2 g of ester was obtained in one reaction; Ellman's (1959) test showed the free thiol content to be greater than 95%; m.p., optical rotation, ^1H-n.m.r. and ^{13}C-n.m.r. ester chemical shifts were: GSH monomethyl ester (14), m.p. 99–101 °C, $[\alpha]_D$ −13.3° (*c* 1 in water), ^1H n.m.r. δ (p.p.m.) 3.76 (3H, s) and ^{13}C n.m.r. δ (p.p.m.) 53.26; GSH monoethyl ester (15), m.p. 168.5–170 °C, $[\alpha]_D$ −26.4° (*c* 1 in water), ^1H n.m.r. δ (p.p.m.) 1.29 (3H, t, J = 7.2 Hz) and 4.24 (2H, q, J = 7.2 Hz) and ^{13}C n.m.r. δ (p.p.m.) 14.19 and 63.48; GSH monobutyl ester (16), m.p. 188–190 °C (decomp.), $[\alpha]_D$ −17.6° (*c* 1 in water), ^1H n.m.r. δ (p.p.m.) 0.95 (3H, t, J = 7.2 Hz, $C_{(\delta)}H_3$), 1.44 (2H, six, J = 7.6 Hz, $C_{(\gamma)}H_2$), 1.68 (2H, m, $C_{(\beta)}H_2$) and 4.23 (2H, t, J = 6.6 Hz,

Table 1. ^{1}H-n.m.r. and ^{13}C-n.m.r. chemical shifts of six glycyl-modified GSH analogues

^{1}H n.m.r.

Glycyl-modified GSH analogue	$C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$	$C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$	$C_{(\alpha)}HC_{(\beta)}H_2SH$	$C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$	$C_{(\alpha)}HC_{(\beta)}H_2SH$	CONH-*R*
2. -L-Val	2.16 [3H, m, overlap with $C_{(\alpha)}HC_{(\beta)}H(CH_3)_2CO_2^-$]	2.53 (2H, m)	2.82 and 2.91 (2H, ddd, J_{ab} = 14.1 Hz, J_{ax} = 6.9 Hz, J_{bx} = 5.7 Hz)	3.99 (1H, t, J = 6.4 Hz)	4.51 (1H, t, J = 6.7 Hz)	$C_{(\alpha)}H[C_{(\beta)}H(CH_3)_2]CO_2^-$ 0.91 (3H, d, J = 6.7 Hz, CH_3) 0.92 (3H, d, J = 6.7 Hz, CH_3) 2.15 (3H, m, overlap with $C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$, $C_{(\beta)}H$) 4.24 (1H, m, $C_{(\alpha)}H$)
5 -L-Phe	2.07 (2H, m)	2.40 (2H, m)	2.91 and 3.18 (2H, ddd, J_{ab} = 14.0 Hz, J_{ax} = 8.9 Hz, J_{bx} = 5.3 Hz)	3.73 (1H, m)	4.56 (1H, m)	$C_{(\alpha)}H(C_{(\beta)}H_2C_6H_5)CO_2^-$ 2.58 (2H, m, $C_{(\beta)}H_2$) 4.34 (1H, t, J = 7.2 Hz, $C_{(\alpha)}H$) 7.25 (5H, m, C_6H_5)
6. -L-Lys	2.16 (2H, m)	2.54 (2H, m)	3.01 (4H, m, overlap with $C_{(\epsilon)}H_2NH_2$)	3.81 (1H, t, J = 6.3 Hz)	4.53 (1H, t, J = 6.9 Hz)	$C_{(\alpha)}H([CH_2]_4NH_2)CO_2^-$ 1.41 (2H, m, $C_{(\beta)}H_2$) 1.69 (4H, m, $C_{(\gamma)}H_2$ and $C_{(\delta)}H_2$) 3.01 (4H, m, overlap with $C_{(\alpha)}HC_{(\beta)}H_2SH$, $C_{(\epsilon)}H_2NH_2$) 4.16 (1H, m, $C_{(\alpha)}H$)
7. -L-His	2.15 (2H, m)	2.51 (2H, m)	2.82 and 2.91 (2H, ddd, J_{ab} = 14.0 Hz, J_{ax} = 6.9 Hz, J_{bx} = 6.5 Hz)	3.81 (1H, t, J = 6.4 Hz)	4.51 (1H, t, J = 6.7 Hz)	$C_{(\alpha)}H[C_{(\beta)}H_2(C_3H_3N_2)]CO_2^-$ 3.12 and 3.31 (2H, ddd, J_{ab} = 15.4 Hz, J_{ax} = 8.5 Hz, J_{bx} = 5.0 Hz, $C_{(\beta)}H_2$) 4.13 (1H, m, $C_{(\alpha)}H$) 7.28 and 8.60 (2 × 1H, 2 × s, $C_3H_3N_2$)
9. -D-Ala	2.21 (2H, m)	2.57 (2H, m)	2.91 (2H, d, J = 6.7 Hz)	4.01 (1H, m)	4.52 (1H, t, J = 6.3 Hz)	$C_{(\alpha)}H(CH_3)CO_2^-$ 1.43 (3H, d, J = 7.2 Hz, CH_3) 4.38 (1H, q, J = 7.2 Hz, $C_{(\alpha)}H$)
13. -NH-CH_2CF_3	2.25 (2H, q, J = 7.2 Hz)	2.64 (2H, dt, J_{ax} = 7.9 Hz, J_{bx} = 7.5 Hz)	3.04 (2H, dd, J_{ax} = 0.8 Hz, $J_{a'x}$ = 0.8 Hz, $J_{aa'}$ = 15.4 Hz)	3.88 (1H, t, J = 6.3 Hz)	4.60 (1H, t, J = 6.2 Hz)	CH_2CF_3 4.07 (2H, dq, J = 9.3 Hz, J = 9.2 Hz)

^{13}C n.m.r.

Glycyl-modified GSH alalogue	$C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$	$C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$	$C_{(\alpha)}HC_{(\beta)}H_2SH$	$C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$	$C_{(\alpha)}HC_{(\beta)}H_2SH$	CO_2^-, CONH	CONH-*R*
2. -L-Val	26.48	31.74	26.10	53.41	56.47	172.89, 173.01, 175.14, 175.84	$C_{(\alpha)}H(C_{(\beta)}H[CH_3]_2)CO_2^-$: 18.10 and 19.21 ($CH_3$), 30.72 ($C_{(\beta)}H$), 59.36 ($C_{(\alpha)}H$)
5. -L-Phe	26.92	32.03	26.31	54.66	56.50	169.77, 172.31 173.74, 175.61	$C_{(\alpha)}H(C_{(\beta)}H_2C_6H_5)CO_2^-$: 37.67 ($C_{(\beta)}H_2$), 55.48 ($C_{(\alpha)}H$), 127.84, 129.47, 130.14, 137.65 (C_6H_5)
6. -L-Lys	27.06	31.97	26.86	54.75	56.59	169.80, 172.69 175.20, 177.13	$C_{(\alpha)}H([CH_2]_4NH_2)CO_2^-$: 22.89, 30.09, 31.04, 40.09 (CH_2), 50.81 ($C_{(\alpha)}H$)
7. -L-His	27.01	31.94	25.99	54.81	56.62	172.22, 174.70 175.38, 176.46	$C_{(\alpha)}H[C_{(\beta)}H_2(C_3H_3N_2)]CO_2^-$: 27.89 ($C_{(\beta)}H_2$), 53.58 ($C_{(\alpha)}H$), 99.25, 117.71, 134.03 (C_3H_3)
9. -D-Ala	27.14	32.32	26.22	54.90	56.53	171.90, 174.18 175, 40, 176.23	$C_{(\alpha)}H(CH_3)CO_2^-$: 18.02 ($CH_3$), 51.33 ($C_{(\alpha)}H$)
13. -NH-CH_2CF_3	27.16	32.30	26.19	55.19	57.03	171.99, 173.92 175.90	CH_2CF_3: 40.32, 41.03, 41.73, 42.48 (q, CH_2CF_3)

$C_{(\alpha)}H_2$) and ^{13}C n.m.r. δ (p.p.m.) 13.90 (CH_3), 19.51, 30.90 and 66.93 ($3 \times CH_2$); GSH diethyl ester hydrochloride (17), m.p. 73–74 °C, $[\alpha]_D$ −40.1° (*c* 1 in water), ^1H n.m.r. δ (p.p.m.) 1.25 (6H, m) and 4.31 (4H, m) and ^{13}C n.m.r. δ (p.p.m.) 13.87 ($2 \times CH_3$), 63.13 and 63.25 ($2 \times CH_2$).

Synthesis of cysteinyl-modified GSH analogues

γ-L-Glu-D-Cys-Gly (18). *N*-t-Boc-*S*-Bzl-D-Cys was prepared similarly to the procedure used for *N*-t-Boc-*S*-Bzl-L-Cys (Adang *et al.*, 1988*a*); the yield was 72%, m.p. 87–88 °C, $[\alpha]_D$ +40.1° (*c* 1 in acetic acid). Coupling to Gly-OBzl PTS salt, deprotecting the *N*-terminus and coupling to *N*-Cbz-α-OBzl-L-Glu DCHA salt, complete deprotection and purification [in this case on Dowex 1-X2 (100–200 mesh; Cl^- form; 0–2 M-formic acid)] were all performed as described previously (Adang *et al.*, 1988*a*). Overall yield was 46% (0.3 g); t.l.c. analysis (see above) showed one spot; Ellman's (1959) test showed the thiol content to be greater than 95%; the compound had m.p. 189–190 °C (decomp.), $[\alpha]_D$ +38.2° (*c* 1 in water), ^1H n.m.r. δ (p.p.m.) 2.23 (2H, m, $C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$), 2.58 (2H, m, $C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2$-CO), 2.85 (2H, d, $J = 6.3$ Hz, $C_{(\alpha)}HC_{(\beta)}H_2SH$), 4.03 2H, s, $NHCH_2CO_2^-$), 4.19 (1H, t, $J = 6.6$ Hz, $C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$), 4.43 (1H, t, $J = 6.3$ Hz, $C_{(\alpha)}HC_{(\beta)}H_2SH$) and ^{13}C n.m.r. δ (p.p.m.) 24.88 ($C_{(\alpha)}HC_{(\beta)}H_2SH$), 25.55 ($C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$), 31.57 ($C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$), 41.29 ($NHCH_2CO_2^-$), 51.95 ($C_{(\alpha)}H$-$C_{(\beta)}H_2C_{(\gamma)}H_2CO$), 53.06 ($C_{(\alpha)}HC_{(\beta)}H_2SH$), 169.36, 170.59, 171.67 and 171.81 (CO_2^-, CONH).

γ-L-Glu-L-Hcy-Gly (19). *S*-Bzl-L-Hcy was prepared from L-methionine in liquid NH_3 with addition of Na, and *S*-demethylation resulted in the formation of L-homocysteine. Addition *in situ* of 1 equiv. of benzyl bromide (du Vigneaud & Patterson, 1934) and purification on Dowex 50W-X4 (H^+ form) lead to *S*-Bzl-L-Hcy in 70% yield, m.p. 240–242 °C and $[\alpha]_D$ +23.5 (*c* 1 in 1 M-HCl). The preparation of *N*-t-Boc-*S*-Bzl-L-Hcy was done similarly to the procedure used for *N*-t-Boc-*S*-Bzl-L-Cys (Adang *et al.*, 1988*a*). Coupling to Gly-*O*Bzl PTS salt, *N*-deprotection, coupling to *N*-Cbz-α-*O*Bzl-L-Glu DCHA salt, Na/liquid NH_3 deprotection and purification were all performed as described previously (Adang *et al.*, 1988*a*). Overall yield was 45% (0.6 g); t.l.c. analysis (see above) showed one spot; the thiol content was greater than 95%; the compound had m.p. 119–120 °C, $[\alpha]_D$ −54.5° (*c* 1 in water), ^1H n.m.r. δ (p.p.m.) 1.90–2.23 (4H, m, $C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$ and $C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2$-SH), 2.45–2.73 (4H, m, $C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$ and $C_{(\alpha)}HC_{(\beta)}H_2$-$C_{(\gamma)}H_2SH$), 3.60 (2H, s, $NHCH_2CO_2^-$), 3.83 (2H, m, $C_{(\alpha)}HC_{(\beta)}H_2$-$C_{(\gamma)}H_2CO$ and $C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2SH$) and ^{13}C n.m.r. δ (p.p.m.), 20.93 ($C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2SH$), 26.32 ($C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2SH$), 31.65 ($C_{(\alpha)}HC_{(\beta)}C_{(\gamma)}H_2CO$), 35.14 ($C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$), 42.06 ($NHCH_2CO_2^-$), 52.32 and 52.38 ($C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$ and $C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2SH$), 172.01, 175.11, 175.74 and 176.20 (CO_2H, CONH).

γ-L-Glu-L-Pen-Gly (20). *S*-NPS-L-Pen was prepared from L-penicillamine and *o*-nitrosulphenyl chloride in 99% formic acid (Fontana *et al.*, 1968). *N*-t-Boc-*S*-NPS-L-Pen was synthesized with the use of 1.1 equiv. of di-t-butyl pyrocarbonate in dimethylformamide and 2 equiv. of triethylamine; after 10 min at room temperature the reaction was acidified, extracted with ethyl acetate, dried and crystallized by addition of light petroleum (b.p. 40–60 °C) in 83% yield, m.p. 133.5–135 °C and $[\alpha]_D$ −5.3° (*c* 1 in acetic acid). Coupling to Gly-*O*Bzl PTS salt, *N*-deprotection, coupling to *N*-Cbz-α-*O*Bzl-L-Glu DCHA salt, deprotection of all protective groups in one step and purification was performed as described previously (Adang *et al.*, 1988*a*). Total yield (deprotection and purification) was 40% (0.5 g); t.l.c. (see above) gave one spot; Ellman's (1959) test gave, even for L-penicillamine itself, severe underestimations of free thiol content; the compound had m.p. 200–201 °C (decomp.), $[\alpha]_D$ −6.7° (*c* 1 in water), ^1H n.m.r. δ (p.p.m.) 1.42 [3H, s, $C_{(\alpha)}HC_{(\beta)}(CH_3)_2SH$], 1.48 [3H, s, $C_{(\alpha)}HC_{(\beta)}(CH_3)_2SH$], 2.13 (2H, q, $J = 6.8$ Hz, $C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$), 2.50 (2H, m, $C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$), 3.74 (1H, t, $J = 7.0$ Hz, $C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$), 3.78 (2H, s, $HNCH_2CO_2^-$), 4.44 [1H, s, $C_{(\alpha)}HC_{(\beta)}(CH_3)_2SH$] and ^{13}C n.m.r. δ (p.p.m.) 23.77 and 26.83 [$C_{(\alpha)}HC_{(\beta)}(CH_3)_2SH$], 27.19 ($C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$), 32.24 ($C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$), 43.82 ($NHCH_2CO_2^-$), 54.98 ($C_{(\alpha)}HC_{(\beta)}H_2C_{(\gamma)}H_2CO$), 61.26 [$C_{(\alpha)}H$-$C_{(\beta)}(CH_3)_2SH$], 171.40, 174.79, 175.31, 176.22, 176.66 [CO_2^-, CONH, $C_{(\alpha)}HC_{(\beta)}(CH_3)_2SH$].

Kinetic analysis

All kinetic data were collected at 25 °C as previously described with CDNB as the electrophilic substrate (Adang *et al.*, 1988*b*). The data were analysed by using the direct-linear-plot method (Eisenthal & Cornish-Bowden, 1978). K_m (mM) and $V_{max.}$ (μmol/min per mg) values for GSH and the GSH analogues were determined from this plot. Inhibition of the GSH analogues was measured as previously described (Adang *et al.*, 1989). None of the glycyl-modified GSH analogues nor the ester derivatives was inhibitory at a concentration of 1 mM (1 mM-CDNB and 1 mM-GSH).

RESULTS

Chemical reactivity of the GSH analogues towards CDNB

The rates of the spontaneous non-catalysed reaction of cysteinyl-, glycyl- and γ-glutamyl-modified GSH analogues with CDNB are shown in Table 2. The cysteinyl-modified analogues exhibited rates considerably lower than the reaction rate between GSH and CDNB. In contrast, most of the 14 analogues with a modification at the glycine moiety showed rates comparable with the GSH rate.

GST activity with cysteinyl-modified analogues

The GSTs 1–1, 2–2, 3–3 and 4–4 were tested for their ability

Table 2. Chemical reactivities of GSH analogues as measured with CDNB as the electrophile

Rates are expressed as percentages of the GSH rate, which was 0.88 nmol of product/min per ml.

Co-substrate	Reactivity (%)
1. GSH	100
Glycyl-modified GSH analogues	
2. -L-Val	82
3. -L-Asp	94
4. -L-Ph-Gly	140
5. -L-Phe	97
6. -L-Lys	90
7. -L-His	163
8. -L-Ala	107
9. -D-Ala	103
10. -β-Ala	98
11. -4-Abu	102
12. -NH-C_2H_5	101
13. -NH-CH_2CF_3	104
Ester derivatives of GSH	
14. Monomethyl	122
15. Monoethyl	120
16. Monobutyl	110
17. Diethyl	108
Cysteinyl-modified GSH analogues	
18. -D-Cys-	20
19. -L-Hcy-	23
20. -L-Pen-	< 5

Table 3. Inhibition of cysteinyl-modified GSH analogues

Inhibition was measured at 1 mM-CDNB, 1 mM-GSH and 1 mM cysteinyl-modified GSH analogue in a standard GST assay (see the Materials and methods section).

Cysteinyl-modified GSH analogue	GST isoenzyme...	Inhibition (% of GSH activity) 1–1	2–2	3–3	4–4
18. -D-Cys-		0	0	0	0
19. -L-Hcy-		56	35	30	31
20. -L-Pen-		15	49	0	0

to accept cysteinyl-modified GSH analogues. None of these analogues could replace the natural co-substrate: only the D-cysteine analogue showed a measurable activity with only GST 3–3: 0.5 μmol conjugated/min per mg of protein. The L-homocysteine analogue of GSH was inhibitory (at 1 mM) towards the GST isoenzymes (at a GSH concentration of 1 mM). The L-penicillamine analogue seemed only to inhibit the Alpha family (Table 3).

GST activity with glycyl-modified analogues

A total of 16 analogues in which the glycine moiety was replaced by another group were tested with the isoenzymes. A series of seven α-L-amino acid substitutions for glycine was used for an initial characterization (Table 4). Six out of these seven analogues, representatives of different classes of amino acids, were accepted by the GSTs, the L-lysine analogue being the only exception. Furthermore, the L-histidine analogue and, surprisingly, the L-valine analogue exhibited very low maximum rates. The phenylglycine analogue was most efficiently used by GST 1–1, and the L-aspartic acid analogue was somewhat selective for GST 2–2. All α-L-amino acid analogues exhibited K_m values similar to that of GSH itself (0.2–0.6 mM), but the $V_{max.}$ values differed markedly. There appears to exist a tendency for analogues with large side chains (R) to possess lower maximum rates, but the results also indicate that the binding site for GSH is not very restrictive in the glycyl domain: the glycine residue can be replaced by very different groups without affecting the K_m as long as it meets the requirements –HN–CHR–CO_2H.

One α-D-amino acid was used: the D-alanine analogue is the diastereoisomer of γ-L-Glu-L-Cys-L-Ala. As it turned out, the D-alanine and L-alanine analogues have the same K_m as GSH, but

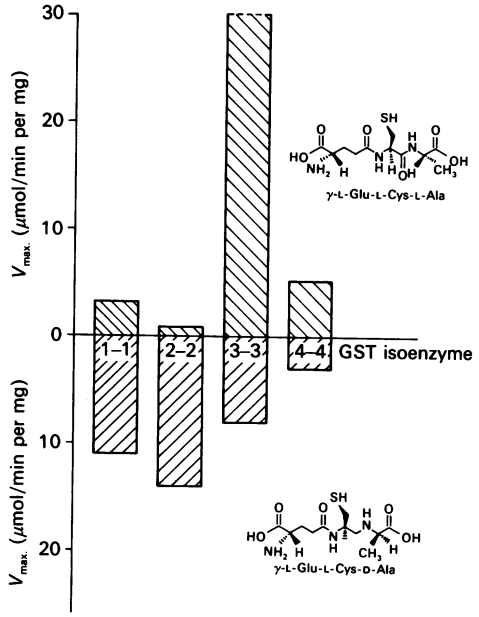

Fig. 1. Stereospecificity of GSH conjugation with L-alanine and D-alanine instead of glycine in the co-substrate structure, measured with GST isoenzymes 1–1, 2–2, 3–3 and 4–4

Table 4. Kinetics of glycyl-modified GSH analogues

Co-substrate	GST isoenzyme... 1–1 K_m (mM)	1–1 $V_{max.}$ (μmol/min per mg)	2–2 K_m (mM)	2–2 $V_{max.}$ (μmol/min per mg)	3–3 K_m (mM)	3–3 $V_{max.}$ (μmol/min per mg)	4–4 K_m (mM)	4–4 $V_{max.}$ (μmol/min per mg)
1. GSH	0.2	18	0.3	14	0.3	29	0.3	15
Glycyl-modified GSH analogues								
2. -L-Val	0.6	1.3	0.6	0.4	0.4	1.8	0.4	0.4
3. -L-Asp	0.5	7.4	0.5	16	0.3	8.1	0.3	2.5
4. -L-Ph-Gly	0.2	8.3	0.4	1.1	0.3	6.1	0.4	0.7
5. -L-Phe	0.3	5.0	0.6	4.5	0.4	9.6	0.4	4.2
6. -L-Lys	N.D.*	N.D.*	N.D.*	N.D.*	N.D.*	N.D.*	N.D.*	N.D.*
7. -L-His	N.D.*	N.D.*	N.D.*	N.D.*	0.4	2.0	0.5	0.5
8. -L-Ala	0.4	3.3	0.4	0.8	0.4	38	0.2	5.1
9. -D-Ala	0.2	11	0.3	14	0.2	8.5	0.3	3.1
10. -β-Ala	0.4	23	0.6	8.9	0.4	104	0.3	27
11. -4-Abu	0.3	6.5	0.4	2.1	0.3	28	0.3	4.0
Decarboxy analogues of GSH								
12. -NH-C_2H_5	1.2	8.3	1.4	3.3	1.9	20	1.0	4.6
13. -NH-CH_2CF_3	1.7	14	3	4.5	1.5	6.7	2.1	3.6
Ester derivatives of GSH								
14. Methyl	0.9	30	1.2	5.7	0.9	25	1	10
15. Ethyl	1.5	29	1.7	8.0	1.4	140	1.5	25

* Not detectable.

their $V_{max.}$ profiles towards the isoenzymes differ sharply (Fig. 1). The L-alanine analogue shows a preference for the Mu family with high values for GST 3–3 in particular, and very low catalytic efficiencies ($k_{cat.}/K_m$) for GST 1–1 and 2–2. The D-alanine analogue, however, prefers the Alpha class of isoenzymes, and the analogue showed $V_{max.}$ values and catalytic efficiencies that approached the GSH values for GST 1–1 and 2–2.

When the amino group was moved further away from the carboxy group, as in β-alanine and 4-aminobutyric acid, analogues were very efficiently utilized by the GSTs: these analogues showed K_m values almost identical with that of GSH (Table 4). The β-alanine analogue expressed an even higher $V_{max.}$ than GSH with three out of four GST isoenzymes. GST 3–3 especially showed a very high rate towards the β-alanine analogue: its $V_{max.}$ was 4 times that of GSH. For the 4-aminobutyric acid substitution the $V_{max.}$ parameters for the isoenzymes are lower than with GSH except for GST 3–3, for which the kinetic parameters are identical with the GSH parameters.

GST activity with decarboxylated glycyl analogues

To explore the glycyl domain further, two decarboxy analogues were tested, with aminoethane and aminotrifluoroethane instead of glycine. All GSTs accepted these modifications (Table 4). The GSTs had K_m values one order of magnitude higher than for the amino acid analogues. $V_{max.}$ values varied more, but were always lower than those of GSH.

GST activity with ester derivatives of GSH

Derivatives of GSH esterified at the glycyl carboxy group with methanol or ethanol also showed K_m values one order of magnitude higher than GSH (Table 4). Their $V_{max.}$ values approach and sometimes even surpass those found for the natural co-substrate GSH; the highest rate (5 times that of GSH) was measured for the ethyl ester, in conjunction with GST 3–3. In contrast, the butyl ester showed only low activity with isoenzyme 3–3 (specific activity of 3.1 μmol/min per mg for GST 3–3) as compared with the methyl ester and ethyl ester.

GSH diethyl ester, in which both the glycyl carboxy group and the γ-glutamyl α-carboxy group were esterified, was inactive in the CDNB assay with the GSTs, in spite of the fact that the analogue with the monoethyl ester at the glycyl residue showed the highest activity so far recorded for a GSH analogue.

DISCUSSION

Chemical reactivity of the analogues

The three classes of GSH analogues (in each of which one of the three amino acid residues was replaced) showed marked differences in chemical reaction rates with CDNB. In a previous study on a series of γ-glutamyl-modified GSH analogues we postulated that a transient interaction between the glutamyl $^+NH_3$ group and the thiol group could result in an increase in the acidity of the GSH thiol group. For steric reasons this intramolecular rate-enhancing effect will strongly depend on the position of the $^+NH_3$ group relative to the thiol group in the GSH analogue, as indeed was observed for the glutamyl analogues. In the cysteinyl-modified GSH analogues the position of the thiol group relative to the $^+NH_3$ group is different from the situation in GSH: for the L-homocysteine, L-penicillamine and D-cysteine analogues the presumed interaction within the tripeptide structure is less favourable, as can be appreciated from molecular models. This most probably is the cause for their low chemical rates. Extending the cysteinyl side chain by a methylene group moves the thiol group away from the γ-glutamyl amino group. The rate for the D-cysteine analogue, comparable (as has to be expected) with that of its enantiomer γ-D-Glu-L-Cys-Gly (26%; Adang *et al.*, 1988*a*), shows that permutation of one of the asymmetric centres distorts the intramolecular activation of the thiol group. Substitution with L-penicillamine most probably resulted in steric hindrance around the reaction site. The two additional methyl groups at the β-position will not only interfere with the intramolecular interactions but also may make it more difficult for the thiol group to engage in the nucleophilic aromatic substitution at the CDNB aromatic ring: the L-penicillamine amino acid itself is already less reactive in reacting with CDNB than is L-cysteine (20% of the L-cysteine rate; A. E. P. Adang & J. Brussee, unpublished work). Rates measured with the glycyl analogues showed only minor deviations from the GSH rate. The glycyl residue, most probably, is not involved in significant interactions with the thiol group of GSH.

There is no correlation at all between chemical reaction rates and $V_{max.}$ values of the enzyme-catalysed rates for the analogues used. Clearly, therefore, the efficiency of enzymic conjugation is not determined by the reactivity of the thiol group as present in freely dissolved GSH analogues. Interestingly, for enzymic catalysis a reaction mechanism is followed that is very sensitive for substitutions at the glycyl domain.

Cysteinyl-modified GSH analogues and GST activity

The proper alignment of the thiol group in the cysteinyl domain in GSH appears to be extremely critical in view of the almost complete loss of activity if L-cysteine is replaced by D-cysteine, L-homocysteine or L-penicillamine. It cannot be established whether K_m or $V_{max.}$ is primarily affected. The lack of inhibition by the D-cysteine analogue suggests a major loss of binding affinity if the thiol group is not oriented as in the L-cysteinyl residue of GSH. Graminski *et al.* (1989) determined the K_D for GSH and the K_i of a number of peptide analogues of GSH in which L-cysteine was replaced towards GST 4–4. The K value was dependent on the electronegativity of the group at the $C_{(\beta)}$ atom of the central amino acid. For a tripeptide to be recognized by GST 4–4 an electronegative atom at $C_{(\beta)}$ of the central α-L-amino acid is required. This may indicate that GSH also has a binding interaction at the thiol group with the enzyme.

Glycyl-modified GSH analogues and GST activity

Out of 14 glycyl analogues 13 were accepted by most of the GST isoenzymes. Their kinetic parameters showed that the isoenzymes had a low selectivity for the exact dimensions of the *C*-terminal amino acid. This low specificity suggests that space is available near the glycyl subsite that can accommodate larger R groups. The distance between the amino group and the carboxylate group can be extended by at least two methylene groups without affecting the K_m for the co-substrate (the β-alanine and 4-aminobutyric acid analogues), although the maximum rates are influenced by these chain elongations.

If it is assumed that differences in K_m value reflect only differences in binding affinity, the results suggest that the glycine carboxylate group is involved in substrate binding, possibly by a salt bridge between the negatively charged terminal carboxylate group and a protonated group in the active site: the K_m for analogues that lack this group, or in which it is blocked by esterification, is an order of magnitude higher than that for the carboxylate-containing analogues. Apparently, in the analogues with additional methylene groups between the carboxylate group and the amino group, as in the β-alanine and 4-aminobutyric acid analogues, the *C*-terminus can fold in the binding site in order to optimize the CO_2^- group–enzyme interaction. The results with the two decarboxy analogues show kinetic parameters similar to those of the dipeptide γ-L-Glu-L-Cys (Sugimoto *et al.*, 1985), namely a K_m between 1 and 2 mM and $V_{max.}$ values lower than that of GSH. Omission of the *C*-terminal carboxylate group

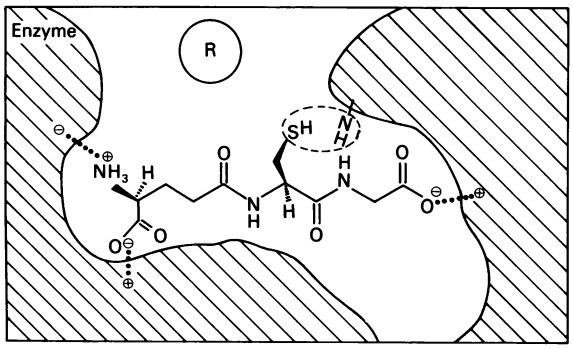

Fig. 2. GSH–enzyme interactions and a model of the G-site in GSTs

in the co-substrate structure thus affected both K_m and $V_{max.}$ as compared with GSH, but this carboxylate group is not obligatory (as is the carboxylate group at the γ-glutamyl residue). The diethyl ester of GSH showed no activity at all, in contrast with the monoethyl ester at the glycyl carboxylate group of GSH, which showed a high catalytic efficiency. Again, this confirms the absolute requirement for the γ-glutamyl carboxylate group.

There appears to be an interaction between the esterified carboxylate group and the enzyme that increases the $V_{max.}$ despite the loss of a possible salt bridge (see below). Other enzymes that utilize the GSH moiety as substrate, namely γ-glutamyltranspeptidase and glutathione reductase, were, very briefly, reported to have very low or no affinity for GSH monoesters (Anderson *et al.*, 1985).

Model of the G-site of GST (Fig. 2)

From the above results and a previous paper on γ-glutamyl modifications in GSH (Adang *et al.*, 1988*b*), the following model of the interactions between GST and the co-substrate GSH can be derived. Since the dipeptide Cys-Gly is not a substrate for GST isoenzymes, whereas the γ-Glu-Cys dipeptide is accepted to some extent by GSTs (Sugimoto *et al.*, 1985), the γ-glutamyl moiety is the major binding determinant that allows the thiol group to align properly at the reaction site. The most important recognition site is the carboxylate group of the γ-glutamyl side chain, which is obligatory: this is the only functional group in the GSH molecule (besides, obviously, the thiol group) of which deletion leads to complete loss of activity. In agreement with this, the diethyl ester of GSH also was inactive, as was γ-L-Glu-γ-L-Glu-L-Cys-Gly (Abbott *et al.*, 1986). In the Mu family, especially in GST 3–3, the carboxylate group need not be that of a γ-glutamyl residue, but can also be provided by an α-L-glutamyl or α-D-glutamyl residue. Omission of the amino group of the glutamyl residue resulted in an analogue retaining considerable enzyme activity, especially in the Mu type isoenzymes: for GST 3–3 a K_m of 1.4 mM and a $V_{max.}$ of 8.8 μmol/min per mg were found, and with GST 4–4 a K_m of 1.6 mM and a $V_{max.}$ of 3.2 μmol/min per mg. Shortening the peptide backbone by one methylene group resulted in pronounced decreases of catalytic efficiencies of the co-substrates with various aspartyl substitutions for the γ-L-glutamyl residue. Because the 'retro-inverso isomer' of GSH [N^4-(malonyl-D-cysteinyl)-L-2,4-diaminobutyrate], in which the direction of the peptide bonds has been reversed, was found to be a very poor substrate, the conclusion was drawn that either or both peptide bonds may be involved in a substrate–enzyme interaction (Chen *et al.*, 1986). Because K_m and $V_{max.}$ values for the γ-Glu-Cys dipeptide and the decarboxy tripeptide analogues (see Table 4 and Sugimoto *et al.*, 1985) are very similar, the Cys–Gly peptide bonds seems not to be involved in binding or catalysis.

None of the cysteinyl-modified GSH analogues was used by the GST isoenzymes, except for a very low activity for D-Cys with GST 3–3, in agreement with its enantiomer, γ-D-Glu-L-Cys-Gly (see Adang *et al.*, 1988*b*). The GSTs therefore appear to be very critical with respect to a correct position of the thiol group for conjugation. However, they are not selective towards the group at the $C_{(\beta)}$ atom of the central amino acid: a hydroxy or carboxylate group instead of the thiol group leads to high-affinity inhibitors (Chen *et al.*, 1985; Graminski *et al.*, 1989).

The glycyl subsite is the least restrictive domain in the GSTs. Amino acids with different R groups all seem to fit in this G-subsite, their K_m values being almost identical with that of GSH (Table 4). The variation in the maximum rates suggests an effect of the R group on enzyme catalysis that affects the catalytic efficiency of the enzyme. If it is assumed that the K_m is purely a binding parameter and the $V_{max.}$ is purely a kinetic parameter, two interactions between the glycyl carboxylate group and the enzyme can be postulated. First, the negative charge of the carboxylate group clearly is involved in binding. The second very intriguing effect is the increase of $V_{max.}$, which is possible especially for the Mu family, but also occurs for isoenzyme 1–1 (with the monoesters of GSH). How can the catalytic efficiency of the enzyme be increased above that for GSH itself? One possibility is that the thiol group of GSH is most probably activated by interaction with a positively charged amino group in the active site; e.g. a histidine and an arginine residue have both been shown to be essential for enzyme activity (Awasthi *et al.*, 1987; Schasteen *et al.*, 1983). If this activation is not yet maximal, because, for example, the distance between the thiol group and NH_3^+ is not yet optimal for maximal interaction, a small change in the quaternary structure of the enzyme at the active site could result in an enhanced reactivity of the thiol group, with a subsequent increased reaction rate. If this were the mechanism then certain substitutions at the glycyl position might activate more than GSH or less than GSH, resulting in higher or lower $V_{max.}$ values. The observed effect on $V_{max.}$ is also highly stereoselective: replacement of glycine by D-alanine and L-alanine resulted in identical binding parameters (K_m), but the maximum rates varied in a very interesting way (Fig. 1), i.e. there was a shift from Mu class preference to Alpha class if L-alanine rather than D-alanine replaces glycine. Alternative explanations are that, once bound at the active site, the thiol group of the analogue might be more directly influenced by the group replacing glycine, resulting in a change in pK_a. The chemical reactivities, however, do not suggest a direct effect, because they are very similar. Finally, the release of the formed conjugate from binding might be more rapid for analogues with, for example, the ethyl ester analogue.

A crystallographic study of the GSH-binding site of another GSH-dependent enzyme, GSH reductase, has revealed binding interactions between GSH and the enzyme that also seem to depend on the side chains rather than on interactions with the peptide backbone; the carboxylate group and amino group of the γ-glutamyl residue are the most important groups. The cysteine sulphur atom interacts at van der Waals distance with a cysteine residue and a histidine residue in the enzyme. Furthermore, the glycine carboxylate group interacts with an arginine residue, which induces a conformational change. These binding characteristics resemble the GSH interactions in the GST enzymes (Douglas, 1987; Karplus *et al.*, 1989).

Differences between GST isoenzymes

Active-site mapping of the G-site with a series of γ-glutamyl-modified GSH analogues has shown a more restrictive behaviour towards the analogues of the Alpha class compared with the Mu class of GST isoenzymes (Adang *et al.*, 1988*b*). This is now

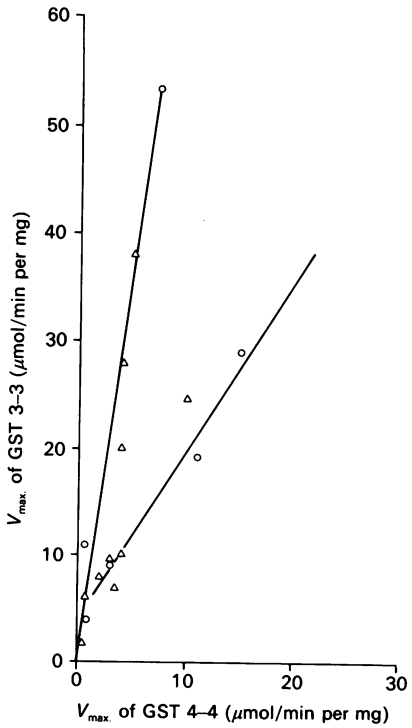

Fig. 3. Correlation between $V_{max.}$ of GST 4–4 and $V_{max.}$ of GST 3–3 with γ-glutamyl-modified (○) and glycyl-modified (△) GSH analogues

confirmed by the results obtained with the glycyl-modified GSH analogues. Interestingly, L-alanine and D-alanine analogues showed opposite effects in acceptance by the Alpha class and the Mu class of GST isoenzymes. Different G-sites for the different classes of isoenzymes is the most likely explanation. GST 3–3 especially accepts many variations, suggesting that its G-site is the least specific one. Even within one class of isoenzymes differences exist. In the Mu class GST 3–3 is more accommodating for the synthetic GSH analogues than is GST 4–4, α-D-glutamic acid instead of γ-L-glutamic acid results in an analogue highly specific for GST 3–3 (Adang *et al.*, 1988*b*). Also, glycyl-modified analogues with high maximum rates such as the β-alanine analogue and the monoethyl ester are especially active with GST 3–3. Fig. 3 shows the correlation between $V_{max.}$ values for GST 3–3 and GST 4–4 tested with the GSH analogues described in this and a previous paper (Adang *et al.*, 1988*b*). It appears as if there are two sets of correlating analogues, one with a $V_{max.}$ (GSH 3–3)/$V_{max.}$ (GSH 4–4) ratio of approx. 10:1 and a set of analogues with a ratio of about 2:1. Whether this is a fortuitous correlation or a causal one due to different GSH interactions in slightly different G-sites of these two isoenzymes remains to be established.

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REFERENCES

Abbott, W. A., Griffith, O. W. & Meister, A. (1986) J. Biol. Chem. **261**, 13657–13661
Abe, O., Takiguchi, H., Ohno, M., Makisumi, S. & Izumiya, N. (1967) Bull. Chem. Soc. Jpn. **40**, 1945–1951
Adang, A. E. P., Duindam, A. J. G., Brussee, J., Mulder, G. J. & van der Gen, A. (1988*a*) Biochem. J. **255**, 715–720
Adang, A. E. P., Brussee, J., Meyer, D. J., Coles, B., Ketterer, B., van der Gen, A. & Mulder, G. J. (1988*b*) Biochem. J. **255**, 721–724
Adang, A. E. P., Meyer, D. J., Brussee, J., van der Gen, A., Ketterer, B. & Mulder, G. J. (1989) Biochem. J. **264**, 759–764
Ålin, P., Danielson, U. H. & Mannervik, B. (1985) FEBS Lett. **179**, 267–269
Anderson, M. E., Powrie, F., Puri, R. N. & Meister, A. (1985) Arch. Biochem. Biophys. **239**, 538–548
Armstrong, R. N. (1987) CRC Crit. Rev. Biochem. **22**, 39–88
Awasthi, Y. C., Bhatnagar, A. & Singh, S. V. (1987) Biochem. Biophys. Res. Commun. **143**, 965–970
Beale, D., Meyer, D. J., Taylor, J. B. & Ketterer, B. (1983) Eur. J. Biochem. **137**, 125–129
Bergmann, M. & Zervas, L. (1933) Hoppe-Seyler's Z. Physiol. Chem. **221**, 51–54
Chen, W. J., Boehlert, C. C., Rider, K. & Armstrong, R. N. (1985) Biochem. Biophys. Res. Commun. **128**, 233–240
Chen, W. J., Lee, D. Y. & Armstrong, R. N. (1986) J. Org. Chem. **51**, 2848–2850
Chen, W. J., Graminski, G. F. & Armstrong, R. N. (1988) Biochemistry **27**, 647–654
Douglas, K. T. (1987) Adv. Enzymol. Relat. Areas Mol. Biol. **59**, 103–167
du Vigneaud, V. & Behrens, O. K. (1937) J. Biol. Chem. **117**, 27–36
du Vigneaud, V. & Patterson, W. I. (1934) J. Biol. Chem. **109**, 97–103
Eisenthal, R. & Cornish-Bowden, A. (1978) Biochem. J. **139**, 715–730
Ellman, G. L. (1959) Arch. Biochem. Biophys. **82**, 70–77
Fontana, A., Scoffone, E. & Benassi, C. A. (1968) Biochemistry **7**, 980–986
Graminski, G. F., Kubo, Y. & Armstrong, R. N. (1989) Biochemistry **238**, 3562–3568
Habig, W. H., Pabst, M. J. & Jacoby, W. B. (1974) J. Biol. Chem. **249**, 7130–7139
Jakoby, W. B. (1978) Adv. Enzymol. Relat. Areas Mol. Biol. **46**, 383–414
Karplus, P. A., Pai, E. F. & Schulz, G. E. (1989) Eur. J. Biochem. **178**, 693–703
Ketterer, B., Meyer, D. J. & Clark, A. G. (1988) in Glutathione Conjugation, Mechanisms and Biological Significance (Sies, H. & Ketterer, B., eds.), pp. 73–135, Academic Press, London
Kjer, A. & Larsen, P. O. (1961) Acta Chem. Scand. **15**, 750–759
König, W. & Geiger, R. A. (1970) Chem. Ber. **103**, 788–798
Mannervik, B. & Danielson, U. H. (1988) CRC Crit. Rev. Biochem. **23**, 281–334
Schasteen, C. S., Krivak, B. M. & Reed, D. J. (1983) Fed. Proc. Fed. Am. Soc. Exp. Biol. **42**, 2036
Sugimoto, M., Kuhlenkamp, J., Ookhtens, M., Aw, T. Y., Reeve, J. & Kaplowitz, N. (1985) Biochem. Pharmacol. **34**, 3643–3647
Theodoropoulos, D. & Fölsch, G. (1958) Acta Chem. Scand. **12**, 1955–1964
Zervas, L., Winitz, M. & Greenstein, J. P. (1957) J. Org. Chem. **22**, 1515–1521