Inhibition of glutathione *S*-transferase 3-3 by glutathione derivatives that bind covalently to the active site

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In all, 13 GSH derivatives have been synthesized and tested for their potency to inhibit glutathione *S*-transferase (GST) 3-3. All of these derivatives contained a reactive group that could potentially react with the enzyme active site. Best results were obtained with the phenylthiosulphonate derivative of GSH, GSSO₂Ph. Preincubation of GST 3-3 with a 100 μ M concentration of this inhibitor resulted in a time-dependent loss of activity: after 30 min at pH 6.5 and 25 °C, 51 % of the activity was lost. At more alkaline pH, the activity is more rapidly inhibited: at pH 8.0 the 90 %-inhibition level is already reached after 10 min preincubation. Separation of enzyme and excess unbound GSSO₂Ph after preincubation by gel-filtration chromatography did not result in a reappearance of enzyme activity. If 100 μ M-GSH was added to the preincubation mixture at pH 7.4, inhibition was almost completely prevented. Addition of *S*-(hexyl)glutathione (20 μ M) could delay the inhibition but, ultimately, not prevent it. The inhibited enzyme could be re-activated by addition of 10 mM-2-mercaptoethanol: 60 min after this thiol was added, the inhibited GST 3-3 activity was back to the control level. GSH at the same concentration could not re-activate the enzyme. On the basis of these results, on the known reactivity of thiosulphonate compounds, and on current knowledge about the amino acid residues involved in GST catalysis, a covalent modification of an active-site cysteine residue by mixed-disulphide formation between enzyme and the co-substrate GSH is postulated. Information on the synthesis and characterization of the GSH derivatives is given in Supplementary Publication SUP 50166 (5 pages) which has been deposited at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in Biochem. J. (1991) 273, 5.

INTRODUCTION

The biotransformation of xenobiotics that contain an electrophilic centre often leads to conjugation with GSH. These conjugation reactions are catalysed by isoenzymes of the glutathione *S*-transferase (GST) family. Recently, the properties and functions of the GSTs have been reviewed (Ketterer & Mulder, 1990; Hayes *et al.*, 1990). A complete description of the three-dimensional structure of the active site of a GST isoenzyme is not yet available. Although catalytically active crystals of several isoenzymes of GST have been obtained, only limited progress has been reported since (Sesay *et al.*, 1987; Schäffer *et al.*, 1988; Armstrong *et al.*, 1990). Kinetic studies with purified GST isoenzymes have revealed some general characteristics about the order of substrate binding and the absence of covalent enzyme–substrate interactions as deduced, for instance, from the stereochemistry of the reaction (Jacobson *et al.*, 1977; Cobb *et al.*, 1983; Mangold & Abdel-Monem, 1983; Ivanetich & Goold, 1989). Chemical modification with group-specific reagents has implicated a histidine (Awasthi *et al.*, 1987; Mannervik & Danielson, 1988), an arginine (Mannervik *et al.*, 1978; Schasteen *et al.*, 1983) and a cysteine residue (Carne *et al.*, 1979; van Ommen *et al.*, 1989) in substrate binding and catalysis. Covalent binding of such reagents led to irreversible inhibition of the enzyme. The relative position of these amino acids in the active site of GSTs remains to be established. Matters are complicated by the observed conformational changes that take place after substrate and ligand binding (Van der Jagt *et al.*, 1983; Pricipato *et al.*, 1988).

Studies with γ -glutamyl-, cysteinyl- and glycyl-modified GSH analogues have resulted in a description of the binding interactions of GSH in the GSH-binding site (G-site) in GSTs (Adang *et al.*, 1988b, 1990). The model thus obtained has been used as a starting point for the development of GST inhibitors, directed towards the G-site, which were designed to be resistant to degradation by γ -glutamyl transpeptidase (Adang *et al.*, 1991). The latter enzyme is responsible for the first step in the degradation of GSH and GSH conjugates (Ketterer & Mulder, 1990). The dipeptide γ -L-glutamyl-D-amino adipic acid was shown to be an efficient competitive inhibitor of GST isoenzymes belonging to the Mu multigene family.

We now report on a second class of GST inhibitors which inhibit the GSTs irreversibly by covalent modification of a residue in the active site. The cysteinyl thiol group of GSH is involved in the bond-breaking and bond-forming process in the catalytic site of the GSTs and is covalently modified to form a thioether. Presumably, certain parts of amino acid residues in the active site facilitate this process. By necessity, they have to be located in close proximity to the GSH thiol group. In order to find a GST inhibitor that covalently blocks the active site, we therefore synthesized GSH derivatives with a reactive group at the C β -SH position. In most of these derivatives the cysteine sulphur atom was highly activated towards attack by nucleophiles through the presence of a good leaving group at sulphur. It was anticipated that inhibitors with the GS-R structure still would possess affinity for the active site because of their similarity to the GSH conjugates. There are strong indications that a nitrogen-containing group or a thiol in the active site is involved in

Abbreviations used: ACC, 1-aminocyclopropane-1-carboxylic acid; CDNB, 1-chloro-2,4-dinitrobenzene; GS-Me: *S*-(methyl)glutathione; GSSO₂Ph, *S*-(phenylsulphonyl)glutathione; GST, glutathione *S*-transferase; TFA, trifluoroacetic acid; Bu^t, *t*-butyl; Me, methyl; Hcy homocysteine; Boc, butoxycarbonyl.

catalysis by activating the thiol group of GSH. It was anticipated that such a group in the enzyme close to, or at, the catalytic site could be attacked by a reactive group in a GSH derivative. We have developed a GST inhibitor targeted towards the G-site, equipped with a thiosulphonate group, capable of inhibiting GST 3-3 quantitatively by reacting with a nearby nucleophilic site. Its mode of action is strongly suggestive of a reaction with an essential amino acid residue in the active site of the enzyme, most likely with a cysteine through its thiol group.

MATERIALS AND METHODS

Materials

1-Chloro-2,4-dinitrobenzene (CDNB) and anhydrous trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). GSH and *o*-nitrosulphenyl chloride were obtained from Janssen Chimica (Beerse, Belgium); di-*t*-butyl pyrocarbonate was from Aldrich (Brussels, Belgium). β -Chloro-L-alanine was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals, including the amino acids, were of reagent grade; dimethylformamide was of analytical purity; all other solvents were distilled before use.

Purification of GST 3-3

Rat liver GST 3-3 was kindly provided by Professor B. Ketterer and Dr. D. J. Meyer of the Department of Biochemistry, Middlesex Hospital Medical School, London W1P 6DB, U.K.; it was purified as described by Beale *et al.* (1983).

Inhibition of GST 3-3 activity

The inhibitory potency of the synthetic GSH derivatives was measured with purified GST 3-3 (final concn. 0.72 μ g/ml) using the standard assay for GST with CDNB as substrate (Habig *et al.*, 1974). Initially, the inhibition was determined without preincubation, at 25 °C in 0.1 M-potassium phosphate buffer, pH 6.5, with 200 μ M-GSH, 200 μ M-CDNB and four different concentrations of inhibitor (0, 25, 100 and 400 μ M). Solutions of the inhibitors were prepared in water, except for compounds 6, 10 and 11 (see Table 1 below), which were dissolved in the buffer described above, because of their limited water-solubility. The effect of preincubating the enzyme with the inhibitor (without the other substrates) to determine the time-dependency of inhibition was performed as follows. Freshly diluted GST 3-3 (7.2 μ g/ml) was incubated with 100 μ M-inhibitor at 25 °C in the buffer described above (final vol. 700 μ l). At regular time intervals, a 100 μ l sample was taken and assayed as described above at (final concns.) 200 μ M-GSH, 200 μ M-CDNB, 0.72 μ g of GST 3-3/ml and 10 μ M-inhibitor in a total volume of 1000 μ l. Preincubation lasted up to 30 min. Control mixtures without inhibitor were preincubated similarly to establish the control GST 3-3 activity under the conditions employed. Enzyme dilutions were only stable for 30 min without 2-mercaptoethanol if they were freshly prepared. Identical preincubations, including controls, were performed at pH 7.4 and pH 8.0 to determine the pH-dependency of inhibition. The effect of GSH (100 μ M) and *S*-(hexyl)-glutathione (20 μ M) on the preincubation was established in identical experiments by preincubation for 20 min at pH 7.4. Corrections were made for the presence of 10 μ M-GSH and for 2 μ M-*S*-(hexyl)glutathione (final concns.) in the assay mixture. Re-activation of GST activity with 10 mM-2-mercaptoethanol and 10 mM-GSH was determined after preincubation without a protective factor [GSH, *S*-(hexyl)glutathione] at pH 7.4. The inhibition after preincubation was 90%; the thiol was added, and samples were taken over 60 min to measure the GST activity.

In order to separate excess inhibitor from enzyme after preincubation, gel-filtration column chromatography was used. A 0.5 cm $\times$ 10 cm Bio-Gel P6-DG desalting column was equilibrated with the assay buffer. The inhibition was assessed after enzyme and excess inhibitor that had not reacted had been separated on this column. Control incubation mixtures were treated similarly. Incubation with *S*-(hexyl)glutathione (a reversible inhibitor) and subsequent removal of *S*-(hexyl)glutathione on this column completely restored enzyme activity under conditions identical with those used for the inhibitors in this study.

The inhibition with mixed disulphides and the thiosulphonate derivative of GSH is very sensitive to thiols present in the enzyme solution (e.g. GSH, 2-mercaptoethanol). It is essential that thiols are not present in this solution, since then only low inhibition will be obtained. Desalting or dialysis of the enzyme preparation should be applied before incubating the enzyme with inhibitor if thiols are present in the stock solution of the GST enzymes.

Analytical methods

Optical rotations were measured on a Perkin-Elmer 141 polarimeter at 20 °C. U.v. assays were recorded on a Cary 219 double-beam spectrometer kept at a constant temperature of 25 °C. ^1H -n.m.r. (200 MHz) and ^{13}C -n.m.r. (50 MHz) spectra were obtained from a JEOL JMN-FX 200 spectrometer in $^2\text{H}_2\text{O}$ unless otherwise stated. Chemical shifts (δ) are given in p.p.m. relative to an internal H_2O ($^1\text{H}^2\text{HO}$) signal or an external 3-(trimethylsilyl)propionic acid signal. In view of the fact that all synthetic compounds described in this investigation have a γ -L-Glu-Xaa-Gly structure, only those n.m.r. chemical shifts belonging to the central amino acid (Xaa) are given here. The chemical shifts of the two adjacent amino acids were compared with previous studies and showed only low variations with Xaa (York *et al.*, 1987; Adang *et al.*, 1988a, 1990).

Synthesis of GSH derivatives

Information on the synthesis and characterization of the GSH derivatives is given in Supplementary Publication SUP 50166 (5 pages), which has been deposited with the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in Biochem. J. (1991) 273, 5.

γ -L-Glu-[*S*-(phenylsulphonyl)-L-Cys]-Gly, $2\text{H}_2\text{O}$ (GSSO₂Ph) (11)

The thiosulphonate-derivatized GSH was prepared by a method similar to that described by Hart (1985). After recrystallization from water, the yield was 79% of the dihydrate; m.p. 175–176 °C (decomp.), $[\alpha]_{\text{D}} -26.9^\circ$ [c 1, water (pH 8)/methanol 1:1 (v/v), ^1H n.m.r. { $^2\text{H}_2\text{O}$, NaO ^2H (p ^2H 8), [^2H]methanol}: 3.38 and 3.50 (ddd, 2H, J_{AB} 14.7 Hz, J_{AX} 5.1 Hz, J_{BX} 7.2 Hz, $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{S}$), 4.70 (dd, 1H, J_{AX} 5.1 Hz, J_{BX} 7.5 Hz, $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{S}$), 7.51, 7.68, 7.76, 7.98 (m, 5H, $\text{SSO}_2\text{C}_6\text{H}_5$) ^{13}C : 37.41 ($\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{S}$), 53.14 ($\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{S}$), 124.42, 127.78, 129.80, 130.79, 135.81, 143.87 ($\text{SSO}_2\text{C}_6\text{H}_5$).

γ -L-Glu-(L-Hcy- S^+ -Me,Bu t)-Gly $\cdot \text{Cl}^-$ (4)

The tripeptide γ -L-Glu-L-Met-Gly (synthesized as described in Supplementary Publication SUP 50166) was converted into its methyl-*t*-butylsulphonium salt similarly to the corresponding cysteine compound, 2. Purification (at 4 °C) followed by conversion into the chloride form, resulted in a greater-than-95% yield of 4. Owing to its instability above 0 °C, no m.p. and $[\alpha]_{\text{D}}$ could be obtained, ^1H n.m.r.: 1.55 and 1.56 [ss, 9H, $\text{S}^+(\text{C}_4\text{H}_9)\text{CH}_3$], 2.20 (m, 2H, $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{S}^+$, overlap with γ -Glu- C_βH_2), 2.83 and 2.84 (ss, 3H, $\text{S}^+(\text{C}_4\text{H}_9)\text{CH}_3$), 3.21–3.36 (m, 2H, $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{S}^+$), 4.60 (m, 1H, $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{C}_\gamma\text{H}_2\text{S}^+$) (this

sulphonium compound has an additional chiral centre at the sulphur atom, which explains the double peaks where singlets are expected; ratio 1.3:1); ^{13}C n.m.r.: even if cooling was applied, the time required to obtain a spectrum (3 h) was sufficient for substantial dissociation of the sulphonium compound to occur; results not shown.

γ -L-Glu-(S-acetyl-L-Cys)-Gly·HCl (5)

The commercially available disulphide of GSH, GSSG, was converted into bis-*N*-t-Boc-GSSG by using the procedure for blocking the amine group of amino acids described by Moroder *et al.* (1976), followed by quantitative reduction with 1.1 equiv. of dithioerythritol in 10% (w/v) KHCO_3 solution for 1 h at room temperature (Cleland, 1964), to give an 80% yield of *N*-t-Boc-GSH [m.p. 70–71 °C, $[\alpha]_D -13.3^\circ$ (*c* 1, dimethylformamide)]. *N*-t-Boc-S-acetyl-GSH was prepared by acylation of 2.5 mmol of *N*-t-Boc-GSH in 30 ml of water and 10 ml of dioxan at pH 9 under argon at 4 °C, with 15 mmol of acetic anhydride (in portions). After 3 h of pH adjustments and testing for remaining free thiol with Ellman's reagent the acylation was completed. The pH was adjusted to 1–1.5 and the product was extracted with ethyl acetate, concentrated, and dissolved in 20 ml of TFA/dichloromethane (1:1, v/v). This resulted in removal of the t-Boc protective group; during work-up, a 90% yield of the TFA salt of S-acetyl-GSH (5) crystallized out. With a batch of Dowex 1X2 (Cl^-) ion-exchange resin added to a solution of S-acetyl-GSH (TFA salt) in water, the TFA salt was converted into the chloride salt. TFA itself is an inhibitor of GST activity (A. E. P. Adang, unpublished work); (hydrochloride salt): m.p. 82–83 °C, $[\alpha]_D -12.8^\circ$ (*c* 1, water); ^1H n.m.r.: 2.38 [s, 3H, $\text{SC}(\text{O})\text{CH}_3$], 3.20 and 3.43 (ddd, 2H, $J_{\text{AB}} 14.3\text{ Hz}$, $J_{\text{AX}}, 5.1\text{ Hz}$, $J_{\text{BX}} 7.7\text{ Hz}$, $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{S}$), 4.62 (dd, 1H, $J_{\text{AX}} 5.3\text{ Hz}$, $J_{\text{BX}} 7.5\text{ Hz}$, $\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{S}$); ^{13}C n.m.r.: 30.51 [$\text{SC}(\text{O})\text{CH}_3$], 30.60 ($\text{C}_\alpha\text{HC}_\beta\text{H}_2\text{S}$), 53.47 ($\text{C}_\beta\text{HC}_\gamma\text{H}_2\text{S}$).

RESULTS

Inhibition by various GSH derivatives

Four classes of GSH derivatives were synthesized, of the type γ -L-Glu-Xaa-Gly, i.e. with modifications only at the central amino acid. These derivatives were tested for their inhibitory properties with GST isoenzyme 3-3 without preincubation, in a GST assay mixture containing 200 μM -GSH and CDNB as acceptor substrate (Table 1). At 25 μM -inhibitor, no inhibition was measured with any of the derivatives (activity > 95% of control). Various sulphonium derivatives containing dialkylated thiol moieties ($\text{GS}^+-\text{RR}'$), and therefore possessing a positively charged sulphur atom, were tested. In derivatives 1 and 2 (Table 1) a cysteinyl thiol is dialkylated. Sulphonium derivatives 3 and 4 contain a longer carbon side chain, since they are derived from the peptide γ -L-Glu-L-HCy-Gly. Only at a concentration of 400 μM did the sulphonium derivatives show appreciable inhibition, compound 3 being the most efficient.

Thioesters derived from GSH [$\text{GS}-\text{C}(=\text{O})-\text{R}$] were capable of inhibiting GST 3-3 at much lower concentrations. At 400 μM , the thioesters showed moderate to high inhibition of GST 3-3.

The mixed disulphides (9 and 10) ($\text{GS}-\text{SR}$) also showed considerable inhibitory potency with GST 3-3. The mixed disulphide with the *o*-nitrothiophenol moiety attached to GSH showed hardly any increase in inhibition if the concentration was raised from 100 to 400 μM . However, since this compound and also free *o*-nitrothiophenol have absorbance maxima at 370 nm, the absorbance of the CDNB/GSH assay at 340 nm is affected.

Of the three potential inhibitors that make up the last group, each possesses an electrophilic centre of a different nature: a thiosulphonate, a chloride and a cyclopropyl group respectively.

The thiosulphonate derivative (GSSO_2Ph) (11) proved to be an efficient inhibitor: at 400 μM , the activity had almost completely disappeared. By contrast, replacement of L-Cys by β -Cl-L-Ala (12) did not lead to significant inhibition of GST 3-3, although it is nearly isosteric with GSH. Finally, when 1-aminocyclopropane-1-carboxylic acid (ACC) was substituted for the central amino acid in GSH, this derivative (13) showed only a modest inhibition of 39% at the highest concentration.

Table 1. Inhibition of GST 3-3 with GSH derivatives

Inhibition was measured at three different concentrations as percentage inhibition compared with the uninhibited activity; at 25 μM , inhibition was between 0 and 5% (results are means $\pm$ s.d., *n* = 3).

GSH derivative	Concn. (μM) ...	Inhibition (%)	
		100	400
Sulphonium derivatives of GSH			
1 γ -L-Glu[S ⁺ -(Me) ₂ -L-Cys]-Gly		0	22 $\pm$ 2
2 γ -L-Glu-[S ⁺ -(Me,Bu ^t)-L-Cys]-Gly		5 $\pm$ 1	37 $\pm$ 2
3 γ -L-[S ⁺ -(Me) ₂ -L-Hcy]-Gly		33 $\pm$ 3	60 $\pm$ 3
4 γ -L-Glu-[S ⁺ -(Me,Bu ^t)-L-Hcy]-Gly		11 $\pm$ 2	18 $\pm$ 2
Thioesters of GSH			
5 S-(Acetyl)GSH		18 $\pm$ 2	68 $\pm$ 3
6 S-(Benzoyl)GSH		25 $\pm$ 2	74 $\pm$ 3
7 S-(2-Chloroacetyl)GSH		21 $\pm$ 2	57 $\pm$ 2
8 S-(Oxalyl)GSH		12 $\pm$ 2	27 $\pm$ 2
Mixed disulphides of GSH			
9 S-(Ethylthio)GSH		48 $\pm$ 3	77 $\pm$ 2
10 S-(<i>o</i> -Nitrophenylthio)GSH		47 $\pm$ 2	52 $\pm$ 3
Miscellaneous			
11 S-(Phenylsulphonyl)GSH		41 $\pm$ 2	> 98
12 γ -L-Glu-(β -Cl-L-Ala)-Gly		0	17 $\pm$ 2
13 γ -L-Glu-ACC-Gly		24 $\pm$ 2	39 $\pm$ 4

Table 2. Time-dependent inhibition of GST 3-3 with four classes of GSH derivatives

The percentage loss of activity was calculated after preincubation of enzyme and 100 μM -GSH derivative for 15 and 30 min, followed by a GST assay in which the final concentration of GSH derivative was 10 μM (which is not inhibitory by itself) (results are means $\pm$ s.d., *n* = 3). The compounds 1–13 have the names given in Table 1.

Inhibitor	Preincubation period (min) ...	Loss of activity (%)	
		15	30
Sulphonium derivatives of GSH			
1		14±2	20±2
2		15±1	17±1
3		5±1	9±1
4		23±1	39±1
Thioesters of GSH			
5		19±2	32±2
6		0	0
7		0	7±1
8		0	0
Mixed disulphides of GSH			
9			0
10			10±2
Miscellaneous			
11		35±2	51±2
12		0	0
13		0	0

Fig. 1. pH-dependent loss of activity of GST isoenzyme 3-3 during a preincubation with GSSO₂Ph

Fig. 2. Protective effect of GSH and *S*-(hexyl)GSH against loss of activity of GST isoenzyme 3-3 by GSSO₂Ph at pH 7.4

Time- and pH-dependent loss of activity

In order to determine whether the extent of inhibition is increased by preincubation with the inhibitor (without GSH), GST 3-3 was preincubated with a 100 μ M concentration of the GSH derivatives 1-13 at 25 $^{\circ}$ C at pH 6.5 for a 30 min period. Control experiments were performed without inhibitor. At selected time intervals a sample was taken and assayed for remaining GST activity. The final concentration of the GSH derivative in this activity assay was 10 μ M, which (without preincubation) did not result in any inhibition in the assay. Table 2 shows the loss of activity after 15 min and 30 min preincubation. All four sulphonium derivatives (1-4) showed loss of activity after 30 min preincubation, ranging from 9 to 39%. Of the other derivatives, the acetyl thioester of GSH (5) and the thio-sulphonate derivative (GSSO₂Ph) (11) caused appreciable loss of activity. Preliminary results also showed a time-dependent decrease of the activity of rat GST isoenzymes 1-1, 2-2 and 4-4 with GSSO₂Ph.

To evaluate further the mode of inhibition, the preincubation was also performed at pH 7.4 with the three most active compounds: 4, 5 and 11. The remaining GST activity was assayed at the standard pH of 6.5. For the sulphonium derivative (4) and the acetyl thioester of GSH (5) preincubation at pH 7.4 resulted in loss of inhibitory potency: after 30 min at pH 7.4 the enzyme activity is nearly identical with that without inhibitor (less than 5% loss of activity). However, the thiosulphonate derivative (11) caused a strongly increased loss of activity: 90% ($\pm$ 3) of the GST 3-3 activity is lost during preincubation at pH 7.4. Fig. 1 shows the time- and pH-dependence of this inhibition of GST 3-3 by the thiosulphonate derivative GSSO₂Ph. At pH 8.0 the loss of activity occurs even more rapidly than at pH 7.4: after 10 min the activity is already decreased to 10% of the original value, and 10 min later no measurable activity remains. Neither addition of 1 mM-GSH to the completely inhibited enzyme, nor dilution, could restore enzyme activity.

Separation of the preincubated enzyme and excess inhibitor on a desalting column did not result in reappearance of enzyme activity in the protein fraction.

The inhibition by GSSO₂Ph by preincubation at pH 7.4 (20 min preincubation) could be almost completely prevented if 100 μ M-GSH was present in the preincubation mixture (Fig. 2). *S*-(Hexyl)GSH (20 μ M) also was protective: the inhibition is decreased from 80 to 55% after 20 min, but cannot be prevented ultimately (Fig. 2). Corrections were made for the additional GSH or *S*-(hexyl)GSH in the GST assay by appropriate controls.

Re-activation of enzyme activity after GSSO₂Ph inhibition

After preincubation with GSSO₂Ph at pH 7.4 had resulted in a 90% loss of enzyme activity, the enzyme was further incubated with a large excess of 2-mercaptoethanol (10 mM). At regular time intervals samples were taken to assay for GST activity (at pH 6.5). The enzyme activity showed an increase in time such that, after 60 min, the enzyme was completely re-activated: its activity was identical with that after preincubation without inhibitor. The re-activation of GST by 2-mercaptoethanol could be accelerated if the enzyme was separated from excess inhibitor on a Bio-Gel P-6DG column before the addition of the thiol. When the experiment was done with 10 mM-GSH instead of 2-mercaptoethanol, no re-activation was observed.

Stability of GSSO₂Ph

To rule out the possibility that not GSSO₂Ph itself, but rather a degradation product, could be the ultimate inhibitor, its stability was investigated. ¹H-n.m.r. and ¹³C-attached-proton-test-n.m.r. spectra of GSSO₂Ph (100 mg in 1 ml of ²H₂O) were taken at pH 8.0. This was repeated every 20 min. No change was observed in the ¹H and ¹³C spectra over a 2 h time span. If any intra- or inter-molecular reactions of GSSO₂Ph had occurred, this would have been visible in changes in the n.m.r. spectrum: the β -methylene signals of cysteine are extremely sensitive to electronic effects of the group attached to sulphur, which in this case is the -SO₂Ph moiety.

Only 5% of a 100 μ M-solution of GSH had reacted with the inhibitor GSSO₂Ph (100 μ M) after 30 min at pH 7.4 to form the disulphide (GSSG) as was measured with Ellman's (1959) reagent.

DISCUSSION

The general structure of the GSH derivatives that have been tested for their inhibitory potency can be denoted as G-S-R, in which the S-R bond is susceptible to nucleophilic attack. Depending on the nature of the R group, this attack can be directed towards the sulphur atom or towards the R group itself. Furthermore, the R group determines whether the inhibitor will be able to attack groups in the active site: sulphur nucleophiles, nitrogen nucleophiles or both. This can be explained by the hard and soft principle in nucleophilicity. Ideally, the inhibitor should not be too reactive by itself; rather the GSH moiety should target it to the G-site, where a covalent-bond-forming reaction should take place as a consequence of the propinquity of a nucleophilic moiety in the side chain of one of the amino acid residues that constitute the active site.

The most promising compound is certainly the phenyl thio-sulphonate derivative of GSH, GSSO₂Ph (11). Alkylalkane thiosulphonates react non-destructively with proteins under mild conditions, especially with free thiol groups, which can be quantitatively converted into mixed disulphides (Bruce & Kenyon, 1985). Since the thiosulphonate moiety is highly sus-

Scheme 1. Mechanism of the covalent modification of GST isoenzyme 3-3 by GSSO_2Ph

ceptible to nucleophilic attack by sulphur (Field & Ravichandran, 1979), it was expected that a GSH thiosulphonate derivative possessing an appropriate hydrophobic group could be successfully targeted to an essential cysteine side chain in the active site of GST 3-3. Indeed, GSSO_2Ph proved to be an effective inhibitor. It is not unlikely that the hydrophobic site in GST 3-3 is involved in non-covalent binding of the inhibitor by interacting with the phenyl moiety. The inhibition can be increased by preincubating the enzyme with the inhibitor. This is clearly due to irreversible inhibition: after preincubation, the enzyme activity could not be restored. Separation of enzyme protein and excess inhibitor that had not reacted, on a Bio-Gel P-6DG column, did not result in reappearance of enzyme activity. The inhibition increases at higher pH, which is consistent with the presence of a nucleophile in the enzyme that becomes available after deprotonation thus attacking the inhibitor at higher pH values.

The protective effect of GSH and *S*-(hexyl)glutathione against enzyme inactivation strongly suggests that covalent modification takes place at the G-site of GST 3-3. Re-activation of the inactivated enzyme with a large excess of 2-mercaptoethanol is consistent with a nucleophilic attack on the disulphide bond between enzyme and inhibitor, resulting in regeneration of catalytically active enzyme molecules. The inability of GSH to reduce this disulphide suggests that this larger molecule cannot reach the disulphide bond made by the inhibitor at the active site, whereas the small nucleophile 2-mercaptoethanol can. $\text{p}K_a$ differences cannot explain the observed reactivity differences ($\text{p}K_a$ 9.0 and 9.7 for GSH and 2-mercaptoethanol respectively). GSSO_2Ph reacts only very slowly with GSH under the conditions employed.

In accordance with the known reactivity of thiosulphonate reagents (Bruce & Kenyon, 1985) and the existence of a cysteine residue involved in GST catalysis in the active site (Carne *et al.*, 1979; van Ommen *et al.*, 1989) we postulate that a cysteine residue forms a mixed disulphide with GSSO_2Ph . Thus a covalent S-S bond is formed between the enzyme and its normal co-substrate GSH, which results in complete loss of enzyme activity (Scheme 1).

Sulphonium derivatives of GSH are alkylating compounds susceptible to nucleophilic attack directly at its R groups (R = methyl or *t*-butyl). We used different combinations of R groups, and two sulphur-containing amino acids, cysteine and homocysteine. Only at the highest concentration was appreciable inhibition observed. All four derivatives showed a time-dependent loss of activity of GST during preincubation, which disappeared at a higher pH, due to dissociation of the sulphonium analogues. The reactivity appears not sufficient for a fast and quantitative modification of GST 3-3 activity. More drastic conditions might enhance this process, but the stability of the enzyme and the sulphonium derivatives prevent this. Even under the conditions employed, partial dissociation of sulphonium compounds, especially the methyl-*t*-butyl derivatives, occurred.

^1H -n.m.r. and ^{13}C -n.m.r. spectra of the sulphonium derivatives clearly showed a time-dependent dissociation back to GS-Me. For instance, 25% of a 0.3 M- γ -L-Glu-[S^+ -(Me,Bu^t)-L-Cys]-Gly dissociated at 25 °C in 30 min to GS-Me and *t*-butanol.

Compounds with the general structure, GS-C(=O)-R are quite reactive towards nitrogen nucleophiles. The acyl carbon atom is the most electrophilic site at which a nucleophilic attack is directed, resulting in hydrolysis of the thioester. S→N intramolecular shift of the acyl group to the corresponding *N*-(acyl)GSH is very rapid. This leads to an *N*-acylated product that is not an inhibitor but only a poor substrate for GST (Chen *et al.*, 1988). With the exception of the oxalyl thioester of GSH, all thioesters showed considerable GST inhibition. Only the *S*-(acetyl)GSH thioester showed a limited time-dependent loss of GST activity. At pH 7.4 the inhibition disappeared, probably because the intramolecular acylation prevails at the expense of the desired intermolecular reaction with the enzyme. This again showed that further investigation of the inhibition observed at pH 6.5 was impossible. At pH 6.5 the acetyl thioester of GSH is stable for 30 min at 25 °C.

The use of mixed disulphides did not result in interesting inhibition patterns and neither did the tripeptides with β -Cl-L-Ala or ACC instead of cysteine in the GSH structure.

In conclusion, GSSO_2Ph is an efficient inhibitor. The nature of the inhibition appears to be irreversible by covalently blocking an essential cysteine residue.

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