Photoaffinity labelling of the active site of the rat glutathione transferases 3–3 and 1–1 and human glutathione transferase A1–1

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The glutathione transferases (GSTs) form a group of enzymes responsible for a wide range of molecular detoxications. The photoaffinity label *S*-(2-nitro-4-azidophenyl)glutathione was used to study the hydrophobic region of the active site of the rat liver GST 1–1 and 2–2 isoenzymes (class Alpha) as well as the rat class-Mu GST 3–3. Photoaffinity labelling was carried out using a version of *S*-(2-nitro-4-azidophenyl)glutathione tritiated in the arylazido ring. The labelling occurred with higher levels of radioisotope incorporation for the Mu than the Alpha families. Taking rat GST 3–3, 1.18 (± 0.05) mol of radiolabel from *S*-(2-nitro-4-azidophenyl)glutathione was incorporated per mol of dimeric enzyme, which could be blocked by the presence of the strong competitive inhibitor, *S*-tritylglutathione ($K_i = 1.4 \times 10^{-7}$ M). Radiolabelling of the protein paralleled the loss of enzyme activity. Photoaffinity labelling by tritiated *S*-(2-nitro-4-azidophenyl)glutathione on a preparative scale (in the presence

and absence of *S*-tritylglutathione) followed by tryptic digestion and purification of the labelled peptides indicated that GST 3–3 was specifically photolabelled; the labelled peptides were sequenced. Similarly, preparative photoaffinity labelling by *S*-(2-nitro-4-azidophenyl)glutathione of the rat liver 1–1 isoenzyme, the human GST A1–1 and the human-rat chimaeric GST, H1R1/1, was carried out with subsequent sequencing of radiolabelled h.p.l.c.-purified tryptic peptides. The results were interpreted by means of molecular-graphics analysis to locate photoaffinity-labelled peptides using the X-ray-crystallographic co-ordinates of rat GST 3–3 and human GST A1–1. The molecular-graphical analysis indicated that the labelled peptides are located within the immediate vicinity of the region occupied by *S*-substituted glutathione derivatives bound in the active-site cavity of the GSTs investigated.

INTRODUCTION

Glutathione transferases (GSTs) form a widespread group of enzymes whose role is to conjugate GSH to various electrophiles, most commonly as part of detoxication via the mercapturic pathway [1–5]. They have also been implicated in cellular resistance to cytotoxic drugs [6,7] and are involved in the inflammatory response by their contribution to the synthesis of leukotriene C_4 [8].

The reaction catalysed is nucleophilic attack of GSH on an electrophile. The molecules of electrophilic substrates are frequently hydrophobic and can be quite large. There have been considerable efforts recently to understand the molecular structure of the binding sites for the two substrates in the GST-catalysed reaction, the G-site and the H-site [9]. These attempts have included site-directed mutagenesis [10–15] and construction of chimaeric protein subunits [16], as well as chemical variation of the GSH structure [17,18] and physicochemical [19] methods. Affinity labelling has been used [20] to covalently modify rat liver GST 4–4 in the region of the active site. GSH-based photoaffinity labels [21] have been used to inhibit GSTs [22] and to covalently label the H-site for sequencing in the case of rat liver GST 1–1 [23]. The recent availability [24] of the X-ray crystal structure atomic co-ordinates of rat liver GST 3–3 along with the construction of chimaeric human–rat class-Alpha GST cDNA and the corresponding proteins [16] prompts us to report a detailed study to detect contributions to the hydrophobic cavity of the H-site on the GSTs.

Photoaffinity labelling represents a useful approach to identifying hydrophobic sites, as the nitrene generated from an aryl azide is relatively undemanding in its choice of chemical target structure during the labelling process. Consequently, we have applied photoaffinity labelling using tritiated

Abbreviations used: FNPA, 4-fluoro-3-nitrophenyl azide; [^3H]FNPA, 4-fluoro-3-nitro[2,6- ^3H]phenyl azide; GST, glutathione transferase; CDNB, 1-chloro-2,4-dinitrobenzene.

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S-(2-nitro-4-azidophenyl)glutathione to study the active site of GSTs by using novel competitive inhibitors to protect the active site, namely *S*-tritylglutathione and *S*-[3-(1-adamantyl)-oxomethyl]glutathione.

MATERIALS AND METHODS

4-Fluoro-3-nitro[2,6-³H]phenyl azide was obtained from Amersham International, Amersham, Bucks., U.K. Trypsin (Tos-Phe-CH₂Cl-treated), GSH, 1-chloro-2,4-dinitrobenzene (CDNB) and other biochemical reagents were from Sigma Chemical Co., Poole, Dorset, U.K. Hydroxyapatite was from Bio-Rad Laboratories. H.p.l.c. was performed using an IBM PC AT-controlled Gilson gradient system with a Gilson 116 u.v. detector and interfaced with a Gilson FC-203 fraction collector. F.p.l.c. was performed using a Pharmacia f.p.l.c. system. All h.p.l.c. and f.p.l.c. buffers were filtered through 0.45 µm filters (Millipore) and, where possible, sterilized through 0.22 µm filters (Gelman Science).

Rat GST isoenzymes were isolated from rat liver (male Wistar rats induced with sodium phenobarbitone) by a modification of the method of Simons and Vander Jagt [25] followed by hydroxyapatite chromatography. The subunits were identified by reversed-phase h.p.l.c. [26] and by enzyme assay at 25.0 °C against a range of substrates, namely CDNB, 1,2-dichloro-4-nitrobenzene and *trans*-4-phenyl-3-buten-2-one [27]. The isolated homodimeric isoenzymes were shown to be homogeneous by the above techniques as well as by SDS/PAGE.

Human class-Alpha subunit hA1 [28] (for nomenclature see [29]) and the human-rat chimaeric subunit H1R1/1 [16] (derived from human subunit A1 and rat subunit 1) are recombinant proteins expressed in *Escherichia coli* and were purified by a

single-step affinity-chromatography method using *S*-hexylglutathione-Sepharose.

Kinetic and inhibition data were analysed by least-squares non-linear regression analysis using the Enzfitter program (distributed by Elsevier Biosoft). Most kinetic points were determined in duplicate. Detailed kinetic studies were carried out with careful checks for enzyme and substrate stability over the course of the experiment. Inhibition type for this two-substrate GST system was determined by varying the concentrations of the inhibitor and one of the substrates while holding that of the other substrate in great excess over its K_m value. Inhibition type was diagnosed on the basis of the patterns produced by three plotting routines (Lineweaver-Burk, $1/v_0$ versus $1/[S_0]$; Dixon, $1/v_0$ versus $[I]$; Cornish-Bowden, $[S_0]/v$ versus $[I]$) with K_i being calculated from the appropriate plot.

Synthesis and isolation of *S*-(4-azido-2-nitro[3,5-³H]phenyl)glutathione

S-(4-Azido-2-nitrophenyl)glutathione was synthesized as described [21] and had m.p. 197–200 °C (lit. 198–200 °C, [21]) along with satisfactory elemental and spectroscopic (¹H n.m.r., u.v., i.r.) analyses. The synthesis of the radioactive version was very similar to that used for the non-radioactive form, except that it had to be carried out on a much smaller scale. Consequently, different purification procedures were needed. To a solution of GSH (50 µl; 0.52 µmol in 0.1 M NH₄HCO₃, pH 9.5) in a Reacti-vial, 4-fluoro-3-nitro[2,6-³H]phenyl azide was added (75 µl; 1.44 nmol; 75 µCi) followed by 4-fluoro-3-nitrophenyl azide (25 µl; 0.51 µmol). The mixture was allowed to stir (ambient temperature; 24 h) before separation by h.p.l.c., using a 30 nm Dynamax Microsorb C18 column (25 cm × 4.6 mm) (Rainin Instrument Co., Woburn, MA, U.S.A.). The solvents were water with 0.06% (v/v) trifluoroacetic acid (solvent A) and acetonitrile with 0.04% (v/v) trifluoroacetic acid (solvent B). The sample was injected at 10% (v/v) solvent B and a linear gradient run from 10 to 60% solvent B (40 min) with a flow rate of 1.0 ml/min and detection at 260 nm.

Even at the low percentage of trifluoroacetic acid used, the mobile phase was extremely acidic (pH ≈ 2), and, should a non-acidic separation protocol be required, one based on a water/methanol solvent system and the same C18 column can be used. In this case the reaction sample should be injected at 0% (v/v) methanol and the compounds separated on a linear gradient of 0–70% (v/v) methanol over 35 min at a flow rate of 1 ml/min.

The reaction conditions described above gave a 51% yield of radiolabel {*S*-(4-azido-2-nitro[3,5-³H]phenyl)glutathione} with specific radioactivity 6.8×10^{13} c.p.m./mol. Figure 1 shows the elution and radioactivity profiles for the purification step. Fractions containing *S*-(2-nitro-4-azidophenyl)glutathione were pooled and shown by h.p.l.c. to be pure. Thus the radiolabelled produce was eluted as a single peak, as measured by u.v. absorbance and radioactivity counting, and was coeluted with unlabelled *S*-(4-azido-2-nitrophenyl)glutathione on spiking the h.p.l.c. sample. Fractions (retention time 31 min) containing unchanged starting material, 4-fluoro-3-nitro[2,6-³H]phenyl azide, were pooled, and the starting material was recovered by evaporation of solvent.

Figure 1 Purification of *S*-(4-azido-2-nitro[3,5-³H]phenyl)glutathione from the synthesis mixture by h.p.l.c.

The sample was applied to a Dynamax 'Microsorb' C18 column (4.6 m × 25 cm) in solvent A (0.06% trifluoroacetic acid in water) and eluted with a gradient of 10–60% solvent B (0.04% trifluoroacetic acid in acetonitrile). Elution was monitored at 260 nm (a), and 1 ml fractions were collected, of which 5 µl samples were taken for liquid-scintillation counting (b).

RESULTS

Photoirradiation of solutions was effected by means of an Applied Photophysics Quantum Yield Photoreactor (model 2001) with a 250 W medium-pressure mercury lamp as previously described [21,30]. Rat GST 3–3 (6 µM active sites) was irradiated in the

Figure 2 (a) Time course for inactivation of rat GST 3-3 by *S*-(4-azido-2-nitrophenyl)glutathione and (b) protection of rat GST 3-3 against *S*-(4-azido-2-nitrophenyl)glutathione by *S*-tritylglutathione

(a) The loss of enzyme activity (6 μ M enzyme subunits) was measured under the following conditions: (A) enzyme plus photolabel (5.6 μ M) with photolysis, (B) enzyme alone with photolysis and (C) enzyme with photolabel in the dark. All points are means of three experimental measurements. (b) GST 3-3 (7.1 μ M) was irradiated with light of wavelength greater than 305 nm under the following conditions: (A) enzyme alone, (B) enzyme plus *S*-tritylglutathione (2.1 μ M) and (C) enzyme plus photolabel (0.7 μ M) and *S*-tritylglutathione (2.1 μ M). All points are means of triplicate measurements.

presence of *S*-(4-azido-2-nitrophenyl)glutathione (5.6 μ M) (λ for excitation > 305 nm) in phosphate buffer (0.1 M KH_2PO_4 , pH 6.8) at 4 $^\circ\text{C}$, and the loss of enzyme activity towards CDNB was measured as a function of irradiation time (Figure 2a) relative to both unirradiated enzyme and enzyme plus photolabel incubated in the dark. The enzyme did not denature significantly within the first 5 min of photolysis. Figure 2(a) also shows that there was no time-dependent loss of enzyme activity when rat GST 3-3 was mixed with the photoaffinity label in the dark. Thus irreversible covalent inhibition arises only from photolysis and not without photoactivation. Irradiation of rat GST 3-3 with *S*-(4-azido-2-nitrophenyl)glutathione led to approximately 50% loss of activity after 5 min. The concentrations of protein and photolabel were approximately equimolar to minimize non-specific covalent labelling.

Protection of GST 3-3 against photoaffinity labelling by *S*-tritylglutathione

To assess non-specific labelling and inactivation of rat GST 3-3 by *S*-(4-azido-2-nitrophenyl)glutathione, photolysis was carried out with the active site 'protected' by the strong competitive inhibitor *S*-tritylglutathione [K_i $1.37(\pm 0.20) \times 10^{-7}$ M]. The

effect of *S*-tritylglutathione itself on enzyme activity during photolysis was also measured and the amount of enzyme inactivation from non-specific labelling (Figure 2b) was shown to be only 5%. Figure 2(b) also shows that there was no time-dependent loss of enzyme activity when GST 3-3 was photolysed with *S*-tritylglutathione alone. Similar results were obtained for the other GSTs investigated.

Relationship between enzyme activity and incorporation of radioactivity

Samples of rat GST 3-3 were photolysed with various concentrations of the radioactive photoaffinity label (standard conditions; 10 min; λ > 305 nm) and assayed for remaining enzyme activity towards CDNB. The covalently labelled radioactive protein formed was isolated by reversed-phase h.p.l.c. to determine the amount of radioactivity incorporated. Increasing the concentration of radiolabel with respect to protein concentration (Figure 3) led to greater levels of covalent labelling, as measured by the amount of radioactive incorporation into the protein and loss of enzyme activity. However, this was not a linear relationship and half-maximal labelling occurred at a radiolabel/protein ratio of 1.6:1. Even at higher affinity label/protein ratios, enzyme activity was only reduced to ~43% of the original. It was calculated that $1.18 (\pm 0.05)$ mol of radioactive *S*-(4-azido-2-nitrophenyl)glutathione bound to and inhibited 1 mol of rat GST 3-3 (dimeric form), i.e. about 59% of the potentially available active sites were labelled, as there are two active sites per dimer. From the data used to construct Figure 3 a plot of mol of photolabel incorporated versus mol of (dimeric) enzyme inactivated has a slope of 0.59 over the whole range of ligand/enzyme ratios used. This may indicate that a feature such as half-the-sites reactivity is active, i.e. that labelling the active site on one subunit inactivates the active site on the other subunit of the dimer. However, we do not feel that the present data allow us to discuss this further at this stage.

Time-dependent photolysis of rat GST 2-2

A time course was determined for the photoaffinity labelling of the rat class-Alpha isoenzyme, GST 2-2, with *S*-(4-azido-2-nitrophenyl)glutathione in molar excess over the protein, as the photoaffinity label is a weaker competitive inhibitor towards the

Figure 3 Relationship between enzyme activity and incorporation of radioactivity at different radiolabel/protein ratios

Rat GST 3-3 (100 μ l; 3.72 μ M in subunits) was photolysed with various concentrations of radiolabel for 10 min with light radiation > 305 nm.

Table 1 Photoaffinity labelling of the major rat liver GSTs

GSTs were photoaffinity-labelled by incubation for 5 min in the presence of *S*-(2-nitro-4-azido[³H]phenyl)glutathione using light of $\lambda > 305$ nm in phosphate buffer, pH 6.8, at 4 °C with a 5% molar excess of photoaffinity label over GST active-site concentration. Where indicated, the active site was blocked using 35.4 μ M *S*-tritylglutathione. After photolysis, samples were assayed for activity towards CDNB and covalently labelled radioactive protein isolated by h.p.l.c., and the amount of radioactivity incorporated was calculated. Values are the means $\pm$ S.E.M. of at least two determinations, except where no S.E.M. is given. The percentage of protein radiolabelled was calculated from liquid-scintillation counting of the protein fractions. Complete 100% binding was the number of mol of enzyme present multiplied by the specific radioactivity of the radiolabel, assuming a stoichiometric binding of radiolabel of 1 mol of label/mol of protein based on the molecular mass of the individual subunits. ND, not determined.

Isoenzyme	Class	Protein labelled in the absence of protection (%)	Protein labelled with active site blocked (%)
1-1	Alpha	9.97 ($\pm$ 1.72)	2.6
2-2	Alpha	5.89 ($\pm$ 0.72)	ND
3-3	Mu	25.9 ($\pm$ 1.8)	2.75 ($\pm$ 0.85)
4-4	Mu	22.8 ($\pm$ 1.7)	3.63 ($\pm$ 0.81)

Alpha family of isoenzymes than the Mu family. Even after 20 min and a molar excess of photoaffinity label/protein of 4.5:1, enzyme activity was only reduced by 30% compared with approx. 50% for rat isoenzyme 3-3 under less forcing conditions. Other preliminary studies with rat GSTs 1-1 and 4-4 showed that these isoenzymes are less amenable to photoaffinity labelling by *S*-(4-azido-2-nitrophenyl)glutathione than rat 3-3 isoenzyme.

Table 1 shows the results of photoaffinity labelling for the four isoenzymes 1-1, 2-2, 3-3 and 4-4 and the effect of using an *S*-substituted GSH analogue as inhibitor to measure non-specific binding. Under standard photolysis conditions, the Mu-class isoenzymes are more susceptible to affinity labelling (Table 1). Approx. 23-26% of the Mu-class protein is radioactively labelled compared with 6-10% for the Alpha class (note that these comparisons are based on mol of label incorporated per subunit in contrast with Figure 2 for which the stoichiometry calculation is expressed per dimer, the functional form of these enzymes). Blocking the GSH-binding site with GSH analogues allowed the best protective agent to be chosen and the amount of non-specific binding to be measured. Use of *S*-tritylglutathione (35.4 μ M), the strongest inhibitor used, showed the amount of non-specific binding to rat GST 3-3 to be 2.75%. *S*-Hexylglutathione (42 μ M) and *S*-[3-(1-adamantyl)oxomethyl]glutathione (44 μ M) were not as effective as *S*-tritylglutathione at blocking the active site and showed correspondingly higher levels of radiolabel bound non-specifically after photoirradiation (respectively 8.1 and 7.0% for rat GST 3-3). The K_i value of *S*-[3-(1-adamantyl)oxomethyl]glutathione was determined to be 6.40×10^{-5} M with rat 3-3 GST. GSH was not used to block its own binding site because it is very efficient at reducing arylazides such as *S*-(4-azido-2-nitrophenyl)glutathione to their corresponding amines, thus neutralizing them as photoaffinity labels [31], and in addition thiols are excellent scavengers of nitrenes.

Preparative photoaffinity labelling

Rat GST 3-3

Rat GST 3-3 was photolabelled with equimolar *S*-(4-azido-2-nitro[³,⁵-³H]phenyl)glutathione under conditions optimized as described above. To measure non-specific covalent labelling,

Figure 4 Tryptic peptide map of radiolabelled rat GST 3-3 using *S*-(4-azido-2-nitrophenyl)glutathione

Peptides were separated by reversed-phase h.p.l.c. (conditions described in the text) and monitored at (a) 215 nm. Portions (100 μ l) were taken from the 1 ml fractions and radioactivity was measured by liquid-scintillation counting (b).

GST 3-3 was also photoirradiated in the presence of a 3-fold molar excess of *S*-tritylglutathione to block competitively the GSH-binding site. Radiolabelled protein was separated from unbound photolabel and its photoproducts by gel chromatography. Fractions containing radioactive GST 3-3 protein were pooled, the volume reduced by vacuum dialysis to approx. 0.8 ml, trypsin (1 mg/ml of 0.1 M NH_4CO_3 , pH 8.5) added to give a GST/trypsin ratio of 100:1 and the sample incubated overnight (16 h) at 37 °C. The digested sample was then injected directly on to a reversed-phase C18 column for peptide mapping, with liquid-scintillation counting to identify radiolabelled fractions. The profile (Figure 4) showed four reproducible major radioactive peaks (labelled 1-4) produced by specific labelling (comparison with *S*-tritylglutathione). The acetonitrile contents of the fractions containing the radioactive peptides were lowered by addition of water (0.5 ml) to permit direct sample re-injection on to the reversed-phase column for further purification. Each radioactive peptide was repurified by the same h.p.l.c. procedure. Figure 5 shows the reisolation of peptide 4. All of these peptides (1-4) were well resolved, except for peptide 2, which was a shoulder on a broad peak. However, all retained their radioactivity on re-injection and separation.

Rat GST 1-1

The alpha-class family rat GST 1-1 was photoaffinity-labelled with radiolabelled *S*-(4-azido-2-nitrophenyl)glutathione in the absence and presence of *S*-tritylglutathione. A portion of radiolabelled *S*-(4-azido-2-nitrophenyl)glutathione (20 μ l; 14 nmol) was added to rat GST 1-1 (0.5 ml; 13.8 nmol) and the mixture left to equilibrate in the dark at 4 °C for 5 min before photolysis. A parallel protection experiment was carried out in the presence

Figure 5 Purification of fraction no. 26 (radioactive peak no. 4) from the tryptic digest of photoaffinity-labelled rat GST 3-3

Peptides (a) were detected at 215 nm. Portions (100 μ l) taken from the 1 ml fractions were also counted for radioactivity (b).

Table 2 Competitive inhibition constants for the inhibition of rat liver GSTs by the photoaffinity label (*S*-(4-azido-2-nitrophenyl)glutathione

Values of K_i were determined from the Dixon plot ($1/v$ versus $[I_0]$) at pH 6.8 in 0.1 M phosphate buffer with the variable substrates indicated. Results are means $\pm$ S.E.M. for determinations.

Isoenzyme	$10^5 \times K_i$ (M)	
	GSH as variable substrate	CDNB as variable substrate
1-1	2.46 ($\pm$ 0.53)	2.20 ($\pm$ 0.21)
2-2	1.42 ($\pm$ 0.47)	2.46 ($\pm$ 0.15)
3-3	0.868 ($\pm$ 0.077)	3.02 ($\pm$ 0.41)
4-4	1.03 ($\pm$ 0.14)	2.90 ($\pm$ 0.19)

of inhibitor by adding photoaffinity label (20 μ l; 14 nmol) to a mixture of rat GST 1-1 (0.5 ml; 13.8 nmol) and *S*-tritylglutathione (20 μ l; 58.3 nmol), and the incubation mixture again left for 5 min at 4 $^{\circ}$ C in the dark before photoactivation. The ratio GST 1-1/photolabel/protector was 1.00:1.02:4.23. Both samples were photolysed for 10 min ($\lambda > 305$ nm). Covalently radiolabelled rat GST 1-1 was separated from unchanged *S*-(4-azido-2-nitrophenyl)glutathione and its photoproducts by reversed-phase h.p.l.c. [26]. Fractions (1.5 ml) were collected and from these, 50 μ l portions were counted for radioactivity. Photolysis of rat GST 1-1 under these conditions resulted in 10% of the protein being covalently radiolabelled. In the presence of the inhibitor *S*-tritylglutathione to block the active site, approx. 2.5% of the protein was non-specifically radiolabelled.

Figure 6 Tryptic peptide map of radiolabelled rat GST 1-1 by reversed-phase h.p.l.c. (conditions as described in the text)

Peptides were monitored at (a) 215 nm. Portions (100 μ l) taken from the 1 ml reactions were counted for radioactivity. (b) Radioactivity profile for the tryptic peptides of radiolabelled rat GST 1-1; —, radioactivity profile for the tryptic peptide formed after photolysis in the presence of *S*-tritylglutathione.

Fractions containing radioactive protein were concentrated and digested with trypsin, and radioactive peptides were purified by h.p.l.c. as described above.

Three radioactive peaks (labelled 1, 2 and 3) were produced when rat GST 1-1 was photolysed with *S*-(4-azido-2-nitro[3,5- 3 H]phenyl)glutathione. When the enzyme was photolysed in the presence of both radiolabelled *S*-(2-nitro-4-azidophenyl)glutathione and *S*-tritylglutathione, the radioactive counts were very much reduced and no radioactive h.p.l.c. peaks were clearly identifiable against the background radiation (see Figure 6).

Human GST A1-1 and human-rat chimaeric GST H1R1/1

Photoaffinity labelling of the human class-Alpha GST 1-1 and the chimaeric human-rat GST H1R1/1 [16] was carried out essentially as described above.

To minimize non-specific labelling, the molar ratio of label to active sites was 0.9:1 and typically 0.6–1.2 mg of protein was used in a labelling experiment. The photoaffinity-labelled protein was separated from unlabelled protein, free label and photoproducts by gel filtration. Before tryptic digestion, the protein was denatured by addition of 8 M urea to a final concentration of 2 M to facilitate digestion. The digested protein was filtered, applied directly to a 25 cm reversed-phase Dynamax C18 column and eluted with a water/acetonitrile gradient (0–45% over 90 min, and finally 45–100% in 20 min) at a flow rate of 1 ml/min. Fractions were collected and assayed for radioactivity.

Radioactive fractions were freeze-dried (not completely) to eliminate the acetonitrile, re-injected on to the same column and eluted by a gradient increasing 0.5% per min. Radioactive fractions were identified and stored at -20°C until further analysis.

Characterization of labelled sites

Reversible inhibition of rat GSTs by photoaffinity label I was determined with both GSH and CDNB as the variable substrate in turn. The results, summarized in Table 2, show that *S*-(2-nitro-4-azidophenyl)glutathione is a linear competitive inhibitor of all four homodimeric rat GSTs, with respect to either GSH or CDNB as variable substrate. The K_i values did not vary greatly from isoenzyme to isoenzyme (ranging only from 9 to $25\ \mu\text{M}$ versus GSH).

The radiolabelled tryptic peptides isolated as described were found to have the amino acid sequences described below.

Rat GST 3-3

Peptides 1-4 were sequenced by automated Edman degradation. Peptide 1 showed the N-terminal sequence FEK which comes from the sequence MQLIMLCYNPD¹¹⁹FEK. No sequence could be obtained for peptide 2. Peptide 3 showed sequences from three peptides not resolved by h.p.l.c., namely (a) ITQ-SNA⁷⁵IMR, (b) ²⁰²YLSTPIFSK and (c) ⁴³SQWLNEK, with the Edman-detected residues underlined. Peptide 4 had the sequence ¹¹GLTHPIR, with Edman-detected residues underlined. In this sequence cycle, the penultimate isoleucine residue had the retention time not of isoleucine but of asparagine, perhaps indicating it as the site of photolabelling. In all cases, the first amino acid identified represents the first cycle of Edman degradation; thus peptides 1 and 3b appear to be the result of anomalous tryptic cleavage.

Rat GST 1-1

The radioactive peptides (1, 2 and 3) were further purified and subjected to amino acid sequencing to give: peptide 1, ¹⁸⁸ISS-LPNV; peptide 2, ³⁴LIQSPEDLE; peptide 3, ¹³²YLPAFE.

Human GST A1-1 and human-rat chimaeric GST H1R1/1

Nine peptides were identified in the radioactive fractions, of which five were reproducibly found in several experiments. The peptides are listed below as they appear in the primary sequence, using the residue numbering based on the cDNA-derived sequence, with the Edman-detected residues underlined. Peptides identified in only one experiment are shown in parentheses.

(⁷LHYFNAR, hA1)

(¹⁴GRMEST, hA1)

³⁴FIK, hA1 and H1R1/1

⁷⁸YNLYGK, hA1 and H1R1/1

(¹²¹LALIK, H1R1/1)

¹³⁰NRYFPFAFEK, H1R1/1

²⁰⁵KPPMDEK, hA1

²⁰⁵KPAMDAK, H1R1/1

²¹²SLEEAR, hA1

DISCUSSION

In Hoesch and Boyer's study [23] of rat GST 1-1 using *S*-azidophenacylglutathione ($\text{GSCH}_2\text{COC}_6\text{H}_4\text{N}_3$), the proteolytic

peptides detected and sequenced were found to correspond to residues 212-218 (using the numbering of Hoesch and Boyer). These authors [23] also reported that residues 91-110 and 206-218 were labelled for the rat GST 2-2 isoenzyme.

In Figure 7 we have aligned all of the protein tryptic peptides detected by photoaffinity labelling of GSTs using *S*-(4-azido-2-nitrophenyl)glutathione and sequenced in the present study and include the results of Hoesch and Boyer [23] for comparison.

Considering rat GST 3-3 first, it was specifically photoaffinity-labelled at five reproducible sites, located in the tryptic peptides corresponding to the regions of sequence: 11-17 (residues 16 and 17 were not detected by the sequencer protocol), 43-49 (with 45-49 undetected), 69-77 (with 69-74 undetected), 108-121 (with 108-118 undetected) and 202-210 (with 205-210 undetected). The lack of detection during sequencing of the last few (C-terminally located) residues of a short peptide is unsurprising and may not be significant. However, when the N-terminally originating Edman sequence data can only be obtained for the C-terminal residues (e.g. fragments 108-121 and 69-77), it is possible that the photolabelled site lies in the sequenator-undetected region of the tryptic peptide concerned. In considering the location of the arylazide moiety of *S*-(2-nitro-4-azidophenyl)glutathione in its complex with GST 3-3, we have therefore used the whole of the detected tryptic peptide. In Figure 8(a) we have mapped these five detected tryptic peptides on to the X-ray co-ordinates of rat GST 3-3 in complex with *S*-(2,4-dinitrophenyl)glutathione. From the X-ray data it is clear that GSH binds to GST 3-3 towards the bottom of a large pocket. Considering the highlighted (photolabelled) sites on Figure 8(a), it is clear that all of the five labelled tryptic peptides lie within reasonable access of the general region of *S*-(2-nitro-4-azidophenyl)glutathione when bound. Some of the peptides lie at the back of the substrate cavity ('behind' the ligand) and the others lie on the walls leading down to the GSH-binding site. It is likely that those peptides lying at the 'bottom' of the cavity are labelled by the photolabel in its EI complex. The others may well have been labelled by glutathionylarylnitrene as it diffused out of the cavity ($\text{EI} \rightarrow \text{E} + \text{I}$). The peptide fragments ¹¹GLTHPIR, ¹⁻⁸MQLIMLCYNPDFEK and ²⁰²YLSTPIFSK contain residues

Figure 7 Positions of radiolabelled tryptic peptides, detected by photoaffinity labelling of GSTs using *S*-(4-azido-2-nitrophenyl)glutathione, on the sequence backbone

The alignment is as described by Mannervik and Danielson [1] with gaps inserted in the lines representing the GSTs to reflect the alignment. R3, rat subunit 3; R1, rat subunit 1; H1R1, human-rat chimaeric subunit [16]; hA1, human class- α subunit; *, radiolabelled peptide from photoaffinity labelling of rat 1-1 with *S*-(*p*-azidophenacyl)glutathione by Hoesch and Boyer [23]; ----, section of rat subunit 1 replaced by human class- α subunit.

Figure 8 Plots of C_{α} co-ordinates for one monomer of (a) rat GST 3-3 in complex with *S*-(2,4-dinitrophenyl)glutathione [32] and (b) human GST A1-1 in complex with *S*-benzylglutathione [33]

The location of the GSH derivative is shown as a bold framework; the GSH-binding site lies at the bottom of a deep cavity. The photolabelled peptides are indicated by dashes along the protein main chain with the sequences appropriate to them given nearby.

Leu-12, Met-108, Tyr-116, Phe-208 and Ser-209 which contribute to the electrophilic substrate-binding site, the H-site [9]. The remaining two labelled peptides include amino acid residues involved in the binding of GSH; Trp-45 and Lys-49 in peptide 43 SQWLNEK are hydrogen-bonded or salt-linked to the glycine carboxylate, and Gln-71 and Ser-72 in 69 ITQSNAIMR are co-ordinated to the γ -glutamyl part of the GSH molecule [32].

Let us now consider the class-Alpha isoenzymes rat GST 1-1, human GST A1-1 and the corresponding chimaeric GST H1R1/1. The labelling pattern can be interpreted by reference to the topology of the three-dimensional GST structure now available for human GST A1-1 with the competitive inhibitor *S*-benzylglutathione in the active site [33]. Figure 8(b) schematically shows the location of the five peptides reproducibly labelled in the experiments with GST A1-1 and the chimaeric GST H1R1/1. Peptide 34 LIQ or 34 FIK was labelled in subunits r1, hA1 as well as in chimaera H1R1/1. This region corresponds to the loop containing the second β strand (β 2) and the second α -helix (α 2), which is a loop assigned to the active-site structure. Peptide 205 KPPMD (in subunit hA1, one experiment) and 205 KPAMDA (in subunit H1R1/1), like peptide 212 SLEEAR (in subunit hA1), are located in the C-terminal helix (α 9) known to contribute to the active site. In addition, peptide 7 LHYFNAR (found in one experiment for subunit hA1) contains the active-site tyrosine (Tyr-9) and the immediately following residues, known to be at or close to the active site.

Peptide 79 YNLYGK (labelled in subunits hA1 and H1R1/1) is in the hinge region between the two structural domains, which is at a considerable distance from the active-site region. The consistent labelling of this segment may signify a second binding site distinct from the active site of the enzyme. Evidence for such a secondary binding site has been obtained from equilibrium-binding experiments by other investigators [34-36].

Peptide 121 LALIK (labelled only in one experiment in subunit H1R1) appears to be located in α -helix 5. Peptide 130 NRYFPFAFEK, like the overlapping peptide from subunit r1 peptide

132 YLPFAFEK, is part of helix 5 and contributes to the active site in the other subunit of the dimeric enzyme molecule [33].

Finally, peptide 188 ISSLPNVK (in subunit r1) represents a segment preceding the C-terminal region which has been shown to contribute to the active site. It is not clear whether this peptide really contributes to the active site.

In summary, in the class-Alpha enzymes several peptides that with certainty have been identified in regions contributing to the active site are now shown to be photoaffinity-labelled. In other cases, as represented by peptide 79 YNLYGK, labelling suggests binding sites in addition to the active site itself. In the chimaeric subunit (H1R1/1) the fusion point between the human A1 subunit and the rat subunit 1 is at His-143. Thus the chimaera would be expected to have a labelling pattern similar to human subunit A1 in the segment preceding His-143 and a labelling pattern similar to rat subunit 1 in the segment after this residue. However, certain differences in the labelling pattern of the C-terminus are noted in comparing rat subunit 1, human subunit A1 and the chimaeric subunit. This difference may reflect differences in the folding of the C-terminal segment against the remainder of the subunit in the three distinct subunits. A detailed understanding of the significance of these differences has to await identification of the exact site of the photoaffinity labelling. Nevertheless, the results indicate the importance of the C-terminal segment of GST for the active-site structure.

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REFERENCES

- 1 Mannervik, B. and Danielson, U. H. (1988) *CRC Crit. Rev. Biochem.* **23**, 283-337
- 2 Boyer, T. D. (1988) *Hepatology* **9**, 486-496
- 3 Pickett, C. B. and Lu, A. Y. H. (1989) *Annu. Rev. Biochem.* **58**, 734-764

- 4 Coles, B. and Ketterer, B. (1990) *CRC Crit. Rev. Biochem.* **50**, 6449–6454
- 5 Armstrong, R. N. (1991) *Chem. Res. Toxicol.* **4**, 131–140
- 6 Waxman, D. J. (1990) *Cancer Res.* **50**, 6449–6454
- 7 Hayes, J. D., Pickett, C. B. and Mantle, T. J. (1990) *Glutathione S-transferases and Drug Resistance*, Taylor and Francis, London
- 8 Soberman, R. J. and Austen, K. F. (1989) *Adv. Prostaglandin Thromboxane Leukotriene Res.* **19**, 21–25
- 9 Mannervik, B., Guthenberg, C., Jakobson, I. and Warholm, M. (1978) in *Conjugation Reactions in Drug Biotransformation* (Aitio, A., ed.), pp. 101–110, Elsevier/North-Holland, Amsterdam
- 10 Stenberg, G., Board, P. G., Carlberg, I. and Mannervik, B. (1991) *Biochem. J.* **274**, 549–555
- 11 Stenberg, G., Board, P. G. and Mannervik, B. (1991) *FEBS Lett.* **293**, 153–155
- 12 Kolm, R. H., Sroga, G. E. and Mannervik, B. (1992) *Biochem. J.* **285**, 537–540
- 13 Widersten, M., Kolm, R. H., Björnstedt, R. and Mannervik, B. (1992) *Biochem. J.* **285**, 377–381
- 14 Liu, S., Zhang, P., Ji, X., Johnson, W. W., Gilliland, G. L. and Armstrong, R. N. (1992) *J. Biol. Chem.* **267**, 4296–4299
- 15 Zhang, P., Graminski, G. F. and Armstrong, R. N. (1991) *J. Biol. Chem.* **266**, 19475–19479
- 16 Björnstedt, R., Widersten, M., Board, P. G. and Mannervik, B. (1992) *Biochem. J.* **282**, 505–510
- 17 Adang, A. E. P., Brussee, J., Meyer, D. J., Coles, B., Ketterer, B., Van der Gen, A. and Mulder, G. J. (1988) *Biochem. J.* **255**, 721–724
- 18 Chen, W.-J., Graminski, G. F. and Armstrong, R. N. (1988) *Biochemistry* **27**, 647–654
- 19 Caccuri, A. M., Polizio, F., Piemonte, F., Tagliatesta, P., Federici, G. and Desideri, A. (1992) *Biochem. Biophys. Acta* **1122**, 265–268
- 20 Katusz, R. M. and Colman, R. F. (1991) *Biochemistry* **30**, 11230–11238
- 21 D'Silva, C., Seddon, A. P. and Douglas, K. T. (1981) *J. Chem. Soc. Perkin Trans. II*, 3029–3033
- 22 Seddon, A. P., Bunni, M. A. and Douglas, K. T. (1980) *Biochem. Biophys. Res. Commun.* **95**, 446–452
- 23 Hoesch, R. M. and Boyer, T. D. (1989) *J. Biol. Chem.* **264**, 17712–17717
- 24 Ji, X., Zhang, P., Armstrong, R. N. and Gilliland, G. L. (1992) *Biochemistry* **31**, 10169–10184
- 25 Simons, P. C. and Vander Jagt, D. L. (1977) *Anal. Biochem.* **82**, 334–341
- 26 Ostlund Farrants, A.-K., Meyer, D. J., Coles, B., Southan, C., Aitken, A., Johnson, P. J. and Ketterer, B. (1987) *Biochem. J.* **245**, 423–428
- 27 Habig, W. H., Pabst, M. J. and Jakoby, W. B. (1974) *J. Biol. Chem.* **249**, 7130–7139
- 28 Board, P. G. and Pierce, K. (1987) *Biochem. J.* **248**, 937–941
- 29 Mannervik, B., Awasthi, Y. C., Board, P. G., Hayes, J. D., Di Ilio, C., Ketterer, B., Listowsky, I., Morgenstern, R., Muramatsu, M., Pearson, W. R., Pickett, C. B., Sato, K., Widersten, M. and Wolf, C. R. (1992) *Biochem. J.* **282**, 305–306
- 30 Zimmerman, H. E. (1971) *Mol. Photochem.* **3**, 281–292
- 31 Staros, J. V., Bayley, H., Standring, D. N. and Knowles, J. R. (1978) *Biochem. Biophys. Res. Commun.* **80**, 568–572
- 32 Ji, X., Armstrong, R. N. and Gilliland, G. L. (1993) *Biochemistry* **32**, 12949–12954
- 33 Sinning, I., Kleywegt, G. J., Cowan, S. W., Reinemer, P., Dirr, H. W., Huber, R., Gilliland, G. L., Armstrong, R. N., Ji, X., Board, P. G., Olin, B., Mannervik, B. and Jones, T. A. (1993) *J. Mol. Biol.* **232**, 192–212
- 34 Bhargava, M. M., Listowsky, I. and Arias, I. M. (1978) *J. Biol. Chem.* **253**, 4112–4115
- 35 Jakobson, I., Warholm, M. and Mannervik, B. (1979) *J. Biol. Chem.* **254**, 4085–4089
- 36 Vander Jagt, D. L., Hunsaker, L. A., Garcia, K. B. and Royer, R. E. (1985) *J. Biol. Chem.* **260**, 11603–11610