Chemical modification of GSH transferase P1-1 confirms the presence of Arg-13, Lys-44 and one carboxylate group in the GSH-binding domain of the active site

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GSH transferase P1-1 (GSTP1-1) was modified with group-specific reagents. Kinetic experiments demonstrated that inactivation of GSTP1-1 occurred upon reaction of one arginine residue per subunit with diacetyl, one lysine residue per subunit with 2,4,6-trinitrobenzene sulphonate, or one carboxylate group per subunit with 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide. All three inactivation reactions were inhibited by compounds known to bind at the GSH site of the enzyme but were unaffected by the electrophile 1-chloro-2,4-dinitrobenzene. N-terminal sequence analysis showed that Arg-13 was modified by diacetyl and that this modification was inhibited by GSH. Arg-11 was not modified. The lysine residue modified by 2,4,6-

trinitrobenzene sulphonate and protected by *S*-octylglutathione was identified as Lys-44 by sequencing of tryptic peptides. The findings are in agreement with the involvement of Arg-13 and Lys-44 in binding of GSH, as determined from the crystal structure [Reinemer, Dirr, Ladenstein, Huber, Lo Bello, Frederici and Parker (1992) *J. Mol. Biol.* **227**, 214–226]. The present data also implicate a single carboxylate in GSH binding, consistent with the involvement of Asp-98 of subunit B determined from the crystallographic study. The GSH-binding determinants of GSTP1-1 are compared using sequence similarity with those of GSTs of Alpha, Mu and Theta classes.

INTRODUCTION

GSH S-transferases (GSTs) are associated with the detoxication of a variety of reactive compounds, including electrophiles and hydroperoxides (Coles and Ketterer, 1990; Ketterer and Coles, 1991), and they are thought to contribute to intrinsic and acquired cellular resistance to xenobiotics such as anti-cancer drugs, herbicides and insecticides (Hayes and Wolf, 1988). A detailed knowledge of the structure of the active site of GSTs should allow the design of inhibitors which can overcome resistance. Previous studies of the active site of rat GSTs using analogues modified in the glutamine or glycine moiety of GSH indicated that the glutamine group was the most important determinant of GSH binding in the Alpha, Mu and Pi class enzymes, and that the Mu class showed the least specificity (Adang et al., 1988, 1989). More recently, the three-dimensional structures of porcine GSTP1-1 (Reinemer et al., 1991) and human GSTP1-1 (Reinemer et al., 1992) have been determined. The structural analysis revealed residues Arg-13, Trp-38, Lys-44, Gln-51, Leu-52, Gln-64, Ser-65 and Asp-98 (from the neighbouring subunit) to be involved in the recognition and binding of glutathione. In the present work, human GSTP1-1 is chemically modified and the modified residues which are responsible for activity loss are determined and correlated with the crystallographic analysis.

MATERIALS AND METHODS

Purification of GSTP1-1

GSTP1-1 was purified from fresh human placenta or *post-*

mortem kidney by successive GSH affinity, hydroxyapatite and anion-exchange chromatography as described in the accompanying paper (Meyer et al., 1993). The enzyme was stored at -20°C in 2 mM dithiothreitol, 10% (v/v) glycerol, 0.05 M NaCl and 20 mM Tris/Cl, pH 7.6, before use and, upon thawing, was rapidly transferred into the appropriate buffer for modification by gel filtration using Sephadex G-25 at 4°C .

Enzyme activity

The standard assay for GSTP1-1 activity was based on the reaction of 1-chloro-2,4-dinitrobenzene (CDNB) with GSH (Habig et al., 1974). A stock solution of CDNB was prepared in 40% ethanol/water. The reaction mixture contained 1 mM CDNB and 1 mM GSH in 0.1 M sodium phosphate, pH 6.5, at 25°C ; 1 unit of activity is defined as $1\ \mu\text{mol}\cdot\text{min}^{-1}$.

Modification by diacetyl

The kinetics of modification of GSTP1-1 by diacetyl were determined by incubation of $0.14\ \mu\text{M}$ enzyme in buffer A (2 mM EDTA and 50 mM sodium borate, pH 8.5) with various concentrations of diacetyl at 25°C . Aliquots were removed at 5 min intervals for determination of activity. Protection from inactivation was sought by preincubating GSTP1-1 for 5 min in buffer A alone or with 10, 100 or 500 μM GSH, with 1, 10 or 100 μM *S*-n-octylglutathione, *S*-n-hexylglutathione or bilirubin, or with 0.5 mM CDNB. Diacetyl (50 mM) was added to each reaction mixture (an equal volume of buffer A was added to controls) and aliquots were removed at 5 min intervals for determination of

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide; GST, glutathione S-transferase (EC 2.5.1.18); TNBS 2,4,6-trinitrobenzene sulphonate.

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activity and calculation of pseudo-first-order rate constants (k_i) for the modification reaction.

To identify the arginine residue inactivated by diacetyl, GSTP1-1 (4 μ M in buffer A) was incubated with 10 mM diacetyl in the presence or absence of 0.7 mM GSH for 30 min at 25 °C. Aliquots were removed and activity towards CDNB was measured. The remainder of the samples were re-purified by reverse-phase h.p.l.c. using a Brownlee Aquapore RP 300 (30 mm $\times$ 2.1 mm) C8 column. Elution was with a gradient of 5–60% acetonitrile in 0.06% trifluoroacetic acid at 0.13 ml $\cdot$ min $^{-1}$ over 30 min. The protein was then subjected to automated N-terminal sequencing.

Modification by 2,4,6-trinitrobenzene sulphonate (TNBS)

The modification of GSTP1-1 by TNBS was carried out in the dark at 22 °C in buffer B (2 mM EDTA and 0.1 M sodium phosphate, pH 8.0). To determine the number of lysine residues essential for activity by kinetic measurements, GSTP1-1 was treated with 0, 25, 50, 100 or 400 μ M TNBS. Aliquots were removed at 5 min intervals for measurement of activity towards CDNB and derivation of k_i . Alternatively, GSTP1-1 was treated with similar concentrations of TNBS for 45 min. Activity was then assayed and the number of lysine residues modified was determined from the increase in A_{420} ($\epsilon = 6.25 \text{ M}^{-1} \cdot \text{cm}^{-1}$; Hollenberg et al., 1971).

To examine which portion of the enzyme active site was affected by TNBS, GSTP1-1 (0.5 μ M in buffer B) was pre-incubated for 5 min alone, with 1, 10 or 100 μ M *S*-n-octylglutathione, with 1, 10 or 100 μ M bilirubin, or with 0.5 mM CDNB at 22 °C. TNBS (50 μ M) was then added and the incubation continued. Aliquots were removed and activity determined at 5 min intervals.

To identify the modified residue(s) essential for activity, GSTP1-1 was incubated with 25 μ M TNBS in the presence or absence of *S*-octylglutathione and the A_{420} was recorded at various intervals. After 45 min the reaction was essentially complete and the activity of aliquots towards CDNB was measured and compared with that of control samples lacking TNBS. The two TNBS-treated samples of GSTP1-1 were then repurified by reverse-phase h.p.l.c. as described above for the diacetyl-treated enzyme. The protein was collected and the acetonitrile removed with a stream of argon at 30 °C. One-fifth volume of 1.0 M ammonium bicarbonate, pH 8.4, was added followed by TPCK-treated trypsin (0.05 μ g/ μ g of GST) and the mixture was incubated at 25 °C for 20 h in the dark. Tryptic peptides were then separated by reverse-phase h.p.l.c. as described above, except that elution was with a gradient of 2–45% over 60 min and was monitored at both 214 and 346 nm. Peptides apparently containing a trinitrophenyl modification or protected from modification by *S*-octylglutathione were sequenced.

Modification of 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDAC)

The importance of carboxylate groups in the activity of GSTP1-1 was investigated by reaction with EDAC. A stock solution of EDAC was freshly prepared in ethanol. GSTP1-1 (0.27 μ M) in buffer C (10 mM diaminopropane/HCl, pH 4.8) was incubated at 25 °C with 10, 20, 50 or 100 mM EDAC (final ethanol concentration 4%, v/v). Aliquots were removed for determination of activity at 5 min intervals, and the pseudo-first-order rate constants for inactivation were determined. Protection from

inactivation by EDAC was sought by repeating the experiment in the presence or absence of various concentrations of GSH, *S*-octylglutathione, *S*-hexylglutathione, bilirubin or CDNB as described above for diacetyl modification.

Structural modelling

The data obtained regarding the involvement of chemically modified residues in the activity of GSTP1-1 were analysed by visual inspection of the structural model of GSTP1-1 (Reinemer et al., 1992).

RESULTS

Modification of arginine

Modification of the guanidino group of arginine residues was

Figure 1 Kinetics of inactivation of GSTP1-1 by diacetyl

(a) The rate of inactivation of GSTP1-1 was determined by incubation at 25 °C in buffer A with 0 (●), 20 mM (○), 50 mM (■) or 100 mM (▲) diacetyl. At intervals aliquots were removed and GST activity towards CDNB was determined. V_0 is the activity at $t = 0$ and V_t is the activity at the end of the incubation. (b) Determination of order of inactivation.

Figure 2 Effect of ligands on the inactivation of GSTP1-1 by diacetyl

GSTP1-1 (0.14 μ M in buffer A) was incubated for 5 min at 25 °C alone (●) or with 0.1 mM GSH (○), 10 μ M S-hexylglutathione (▲), 1 μ M S-octylglutathione (■), 0.5 mM CDNB (△) or 0.1 mM bilirubin (□). Diacetyl (50 mM) was added and aliquots were removed at intervals for determination of activity towards CDNB.

Table 1 Sequence analysis of arginine modification by diacetyl

GSTP1-1 (4 μ M in buffer A) was incubated at 25 °C with 10 mM diacetyl in the presence or absence of 0.7 mM GSH for 30 min. Activity towards CDNB was compared with that of a control incubated in buffer A without diacetyl. The remainder of the samples were repurified by reverse-phase h.p.l.c. and subjected to N-terminal sequence analysis. The yield of arginines 11, 13 and 18 in diacetyl-treated samples were compared with those of the control sample and are considered to be accurate to within 15%.

Sample	Activity (%)	Unmodified residues (%)		
		Arg-11	Arg-13	Arg-18
GSTP1-1 (control)	100	100	100	100
GSTP1-1 + diacetyl	4.5 $\pm$ 1.1	100	0	25
GSTP1-1 + diacetyl + GSH	106.0 $\pm$ 7.0	100	80	67

studied using the specific reagent diacetyl. The kinetics of inactivation of GSTP1-1 by several concentrations of diacetyl were determined (Figure 1a). The order of enzyme inactivation was determined according to Hollenberg et al. (1971) by plotting the $\log_{10}$ of the pseudo-first-order rate constant versus the $\log_{10}$ of the diacetyl concentration (Figure 1b). This showed that inactivation occurred with the modification of only 1.00 ($r > 0.99$) arginine residue. The inactivation was inhibited by GSH, S-hexylglutathione and S-octylglutathione, but CDNB was without a significant effect (Figure 2), indicating that the inactivation is associated with the function of the GSH-binding

portion of the active site. The rate of inactivation by diacetyl was enhanced by bilirubin.

Since one arginine residue (Arg-13) had been implicated in the binding of GSH from the crystal structure of GSTP1-1 (Reinemer et al., 1992), the modification of this residue in the presence and absence of GSH was investigated by N-terminal sequencing. This procedure also allowed comparison of the modification of Arg-13 with that of Arg-11 and Arg-18. The results (Table 1) show that Arg-13 is completely modified in diacetyl-inactivated GSTP1-1 and that both Arg-13 and activity are substantially protected by the presence of 0.7 mM GSH. Arg-11, by contrast, was unaffected by diacetyl, while Arg-18 was partially derivatized (somewhat less so in the presence of GSH). Diacetyl-modified arginine was not observed in the sequencer chromatograms, presumably because it is too hydrophobic to elute during the monitoring period.

Modification of lysine

Modification of primary amino groups of lysine residues in GSTP1-1 was studied with TNBS. Kinetic data were obtained of the inactivation with several concentrations of TNBS and pseudo-first-order rate constants were calculated as in the case of modification by diacetyl (see above). A plot of $\log k_i$ versus $\log[\text{TNBS}]$ yielded $n = 0.95$ ($r > 0.99$), indicating that inactivation occurs as a result of modification of a single residue. Titration experiments in which both inactivation and lysine modification were measured (results not shown) showed that 10 of the 12 lysine residues could be modified by TNBS at high concentrations. Analysis of the latter data confirmed that modification of only one lysine residue was correlated with activity loss. Spectral analysis (Hollenberg et al., 1971) showed that cysteinyl thiol groups were not modified under these conditions.

TNBS-induced inactivation was inhibited by low concentrations of S-octylglutathione but not by CDNB, and was enhanced by bilirubin (Figure 3).

To identify the modified essential lysine residue, a more concentrated sample of GSTP1-1 was treated with a low concentration of TNBS in the presence or absence of S-octylglutathione and the activity compared with that of controls lacking TNBS. In the absence of S-octylglutathione, the enzyme was completely inactivated and approx. 1.3 mol of lysine residues per subunit were modified. S-Octylglutathione prevented 90–100% of both activity loss and lysine modification. The modification of lysine should result in loss of a trypsin-sensitive site. Thus tryptic peptide chromatograms of the TNBS-treated samples yielded an extra peak compared with those of the S-octylglutathione-protected sample having a relatively high A_{246} (arrowed), presumably due to a trinitrophenyl group (Figure 4). This peptide had a sequence EEVTVETWQEGSL-AS[C]LY, corresponding to residues 30–49 of GSTP1-1. No peak was obtained in the sequencer chromatogram at the position of Lys-42. It is concluded that Lys-42 is modified by TNBS concomitant with inactivation of the GSH site of GSTP1-1. As predicted, in the presence of S-octylglutathione additional peaks were obtained in the tryptic digest of the TNBS-treated protein (dotted trace in Figure 4). One of these, (arrowed) gave the sequence EEVTVETWQEGSLK(end), confirming that modification of Lys-42 was prevented by S-octylglutathione.

Modification of carboxylate

GSTP1-1 was also treated with EDAC, which reacts with carboxylic acids and may thus modify aspartate and glutamate residues and the C-terminal residue (glutamine). As with diacetyl

Figure 3 Effect of ligands on the inactivation of GSTP1-1 by TNBS

GSTP1-1 (0.5 μ M in buffer B) was incubated for 5 min alone (●), or with 1 μ M *S*-octylglutathione (■), 0.5 mM CDNB (▽) or 0.1 mM bilirubin (□). TNBS (50 μ M) was added and aliquots were removed at intervals for determination of activity towards CDNB.

Figure 4 Tryptic peptides of TNBS-treated GSTP1-1

GSTP1-1 was treated with TNBS in the presence or absence of *S*-octylglutathione and digested with trypsin, and peptides were separated by reverse-phase h.p.l.c. all as described in the text. The continuous trace shows A_{214} for the sample treated with TNBS in the absence of *S*-octylglutathione (scale 0–0.2). The dashed trace shows the A_{346} (scale 0–0.05). The A_{214} of the sample treated with TNBS in the presence of GS-OCT is shown as the dotted trace.

and TNBS (above), a kinetic analysis of the inactivation of GSTP1-1 with various concentrations of EDAC was carried out. A plot of $\log k_i$ versus $\log[\text{EDAC}]$ yielded $n = 1.00$ ($r > 0.99$),

Figure 5 Effect of ligands on the inactivation of GSTP1-1 by EDAC

GSTP1-1 (0.27 μ M in buffer C) was incubated for 5 min alone (●) or with 1 μ M *S*-hexylglutathione (▲), 1 μ M *S*-octylglutathione (■), 0.5 mM GSH (○), 0.5 mM CDNB (△) or 0.1 mM bilirubin (□). EDAC (50 mM) was added and aliquots were removed at intervals for determination of activity towards CDNB.

indicating that a single carboxylate group was essential for activity. The inactivation was inhibited by compounds binding at the GSH-binding site (Figure 5). In view of the potential of EDAC-modified groups for further reaction with side-chain amino groups which would result in cross-linking, identification of the carboxylate group essential for activity by sequencing was not considered feasible.

DISCUSSION

The loss of activity after modification of Arg-13 of GSTP1-1 by diacetyl and its protection by GSH confirms the important role of this residue in activity, as predicted both from a crystallographic study (Reinemer et al., 1992) in which Arg-13 was found to be adjacent to the γ -glutamyl carboxylate group of *S*-hexylglutathione, and from a study of the activity of a GSTP1-1 mutant protein having Ala substituted for Arg-13 (Widersten et al., 1992; Manoharan et al., 1992). Relative to protection from diacetyl modification by GSH, the alkyl glutathiones protected poorly. Surprisingly, *S*-octylglutathione, which has a lower K_i than *S*-hexylglutathione, was the least effective. There are two additional arginine residues in the neighbourhood of the active site, namely Arg-11 and Arg-18. While Arg-18 was also modified by diacetyl, no modification of Arg-11 was detected. Arg-18 is situated apart from the active site in helix αA , with its side chain pointing to the surface of the molecule. Since it should be at least partly accessible, modification is easily explained. The partial protection of this residue from diacetyl modification by GSH may signify that it is associated with a conformational change.

Mutational replacement of the equivalent residue (Arg-20) in GSTA1-1 by Ala similarly resulted in altered activity (Stenberg et al., 1991). In contrast, it is not clear why Arg-11 is found

Figure 6 Sequence relationships of GSH binding determinants among different GST classes

The GSH binding determinants of GSTP1-1 (**bold** type) are compared for sequence similarity with GSTs of other classes. Partial sequences for rat GST subunits 2, 4, 5 and 12 are taken respectively from Telakowski-Hopkins et al. (1985), Rothkopf et al. (1986), Pemble and Taylor (1992) and Ogura et al. (1991).

unmodified, since it is situated in the element connecting strand β 1 and helix α A with its side chain lying between the side chain of Tyr-196 and the backbone of residues 196–201 and pointing towards the surface. Modification of GSTP1-1 by EDAC indicated that only one carboxylate was essential for activity and was involved with the function of the GSH-binding site. According to mutagenesis studies (Widersten et al., 1992; Manoharan et al., 1992) and crystallography (Reinemer et al., 1991, 1992), this is concluded to be that of Asp-98 in the neighbouring subunit, which interacts with the glutamyl amino group of GSH.

Up to 10 lysine residues were modifiable with TNBS in the absence of urea. However, only one (Lys-44) was essential for activity and was associated with the GSH-binding site. These data are in agreement with the crystallographic (Reinemer et al., 1991, 1992) and mutagenesis (Widersten et al., 1992; Manoharan et al., 1992) studies which determined Lys-44 as the bonding partner of the glycine carboxylate of GSH. Since only Lys-44 was modified by TNBS at low concentrations it is likely that TNBS is preferentially concentrated in the vicinity of the active site.

Recent studies of the inactivation of GSTP1-1 by pyridoxal 5'-phosphate (Lo Bello et al., 1992) indicated that Lys-127 is associated with the GSH-binding site. Reference to the crystal structure of GSTP1-1 shows Lys-127 to be 14 Å (1.4 nm) from the glycine carboxylate. Therefore the behaviour of pyridoxal 5'-phosphate remains to be clarified.

The stimulation by bilirubin of modification of components of the GSH-binding site (Arg-13, Lys-44 and Asp-98 in the neighbouring subunit) is consistent with its mechanism of inhibition of activity, classified as homotropic positive co-operative (Xia and Chen, 1989). Thus, in the presence of bilirubin, the enzyme undergoes a conformational change such that the GSH-binding site is more accessible to either GSH or a GSH-site modifier.

The analysis of inactivation by chemical modification is limited in its specificity when compared with studies of the activity of site-specific mutants. However, one aspect of chemical modification should continue to be a useful adjunct to other structural studies, namely the determination of the number of a particular active group which is essential to activity.

In view of the sequence similarity existing between the four classes of soluble GSTs, it is possible to relate the GSH binding determinants Arg-13, Lys-44 and Asp-98 studied herein to those of the other enzymes. Examples are shown in Figure 6, which also shows the motif Gln-Leu-Pro (residues 51–53) containing the important hydrogen bonding interactions of Gln-51 (side chain N with the glutamyl carbonyl and the glycine carboxylate of glutathione) and Leu-52 (carbonyl O and amide N with the amide N and carbonyl O of the cysteine) (Reinemer et al., 1992). It is seen that the Alpha-class GSTs appear to contain all the important GSH-binding residues. Thus Arg-13, Lys-44, Gln-51 and Asp-98 of GSTP1 correspond to Arg-14, Arg-44, Gln-53 and

Asp-100 of rat GST2. In contrast, the Mu-class GSTs, typified by rat GST4, lack the 'Arg-13' determinant, which is considered to interact with the glutamyl carboxylate, but the other determinants are represented by Lys-49, Asn-58 and Asp-105. Lack of 'Arg-13' is consistent with the studies of Adang et al. (1988, 1989), which showed that Mu-class GSTs have a relatively low specificity compared with Alpha- and Pi-class enzymes for substrate analogues of GSH modified in the glutamyl moiety. The Theta-class GSTs are evolutionarily more ancient and diverse (Pemble and Taylor, 1992). Examples such as rat GST5 and GST12 contain an Arg-14 which may be a glutamyl-carboxylate determinant as in the Alpha- and Pi-class enzymes, and Lys-51 is likely to be the Theta equivalent of Gln-51 of GSTP1. The Theta-class enzymes clearly differ from the other three classes in the region of the glycine-carboxylate binding determinant (Lys-44 in GSTP1), with no basic residue occurring close to that position. It is likely, therefore, that interaction with the glycine carboxylate is altered or missing in this class of GST. Another difference is that the glutamyl-amino determinant (Asp-98 in GSTP1), although present in the Theta GST12 as Asp-103, is absent from GST5 and other Theta-class enzymes (not shown). The absence of one or more GSH-binding determinants in GST5-5 is consistent with its much higher K_m for GSH compared with GSTs of the other classes (Fjellstedt et al., 1973; D. J. Meyer, unpublished work).

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