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Tyrosine 8 contributes to catalysis but is not required for activity of rat liver glutathione S-transferase, 1-1

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Abstract

Reaction of rat liver glutathione S-transferase, isozyme 1-1, with 4-(fluorosulfonyl)benzoic acid (4-FSB), a xenobiotic substrate analogue, results in a time-dependent inactivation of the enzyme to a final value of 35% of its original activity when assayed at pH 6.5 with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The rate of inactivation exhibits a nonlinear dependence on the concentration of 4-FSB from 0.25 mM to 9 mM, characterized by a K_I of 0.78 mM and k_{max} of 0.011 min^{-1}. S-Hexylglutathione or the xenobiotic substrate analogue, 2,4-dinitrophenol, protects against inactivation of the enzyme by 4-FSB, whereas S-methylglutathione has little effect on the reaction. These experiments indicate that reaction occurs within the active site of the enzyme, probably in the binding site of the xenobiotic substrate, close to the glutathione binding site. Incorporation of [3,5-^{3}H]-4-FSB into the enzyme in the absence and presence of S-hexylglutathione suggests that modification of one residue is responsible for the partial loss of enzyme activity. Tyr 8 and Cys 17 are shown to be the reaction targets of 4-FSB, but only Tyr 8 is protected against 4-FSB by S-hexylglutathione. DTT regenerates cysteine from the reaction product of cysteine and 4-FSB, but does not reactivate the enzyme. These results show that modification of Tyr 8 by 4-FSB causes the partial inactivation of the enzyme. The Michaelis constants for various substrates are not changed by the modification of the enzyme. The pH dependence of the enzyme-catalyzed reaction of glutathione with CDNB for the modified enzyme, as compared with the native enzyme, reveals an increase of about 0.9 in the apparent pK_a, which has been interpreted as representing the ionization of enzyme-bound glutathione; however, this pK_a of about 7.4 for modified enzyme remains far below the pK of 9.1 for the -SH of free glutathione. Previously, it was considered that Tyr 8 was essential for GST catalysis. In contrast, we conclude that Tyr 8 facilitates the ionization of the thiol group of glutathione bound to glutathione S-transferase, but is not required for enzyme activity.

Keywords: affinity labeling; 4-(fluorosulfonyl)benzoic acid; glutathione S-transferase

Glutathione S-transferases (EC 2.5.1.18) are a family of isozymes (grouped into several classes) that are involved in the metabolism of both endogenous and xenobiotic compounds. They catalyze the conjugation reaction of the thiol group of glutathione with electrophilic substrates (Mannervik, 1985; Pickett & Lu, 1989). The isozymes can exist as either homo- or heterodimers, with each subunit having glutathione and xenobiotic binding sites that can accommodate diverse hydrophobic substrates (Mannervik & Danielson, 1988). The three-dimensional structures of representatives of the α class (Sinning et al., 1993), μ class (Ji et al., 1992; Raghunathan et al., 1994), π class (Reinemer et al., 1991, 1992; Garcia-Saez et al., 1994), and σ class (Ji et al., 1995) have been determined from X-ray crystallography. The tyrosine residue near the N-terminal end of the enzyme is conserved in all known mammalian cytosolic glutathione S-transferases and is considered to be essential for catalysis (Wilce & Parker, 1994). It has been postulated that the hydroxyl group of its side chain participates in a hydrogen bond with the thiolate anion of the enzyme-bound glutathione, thereby lowering the pK of the bound glutathione and facilitating the nucleophilic attack of the thiol anion on the xenobiotic substrate (Stenberg et al., 1991b; Liu et al., 1992; Wang et al., 1992; Sinning et al., 1993).

Isozyme 1-1 from rat liver is a member of gene class α, which includes subunits 1, 2, 8, and 10 (Armstrong, 1987; Mannervik & Danielson, 1988). The sequence of this enzyme has been de-

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Abbreviations: 4-FSB, 4-(fluorosulfonyl)benzoic acid; CDNB, 1-chloro-2,4-dinitrobenzene; PTH, phenylthiohydantoin; DMF, *N,N*-dimethylformamide; mBBr, monobromobimane; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; GST 1-1, glutathione S-transferase, isozyme 1-1.

termined (Lai et al., 1984; Pickett et al., 1984), and it is closely related to the human GST 1-1, the crystal structure of which has been determined (Sinning et al., 1993).

4-(Fluorosulfonyl)benzoic acid was used previously in this laboratory as an affinity label for rat liver GST 4-4 of the μ class (Barycki & Colman, 1993). Tyr 115 was identified as an amino acid contributing to xenobiotic substrate binding for that isozyme. However, GST 1-1 does not have a tyrosine in the region corresponding to Tyr 115 of GST 4-4. The fluoride of 4-FSB can be displaced by nucleophilic attack by several amino acids, including Cys, Tyr, His, and Lys (Colman, 1990). In this paper, we show that 4-FSB produces a partially active enzyme by reaction with Tyr 8 of rat liver GST 1-1. Examination of the kinetic characteristics of the modified compared with the native enzyme clarifies the role of Tyr 8 in this mammalian enzyme, indicating that, although it contributes to catalysis by facilitating the ionization of enyzme-bound glutathione, Tyr 8 is not required for enzymatic activity.

Results

Reaction of 4-FSB with GST 1-1

4-FSB was incubated with 0.4 mg/mL GST 1-1 at 25 °C in 0.1 M potassium phosphate buffer, pH 7.5. The enzyme activity, as measured by the conjugation of glutathione with CDNB, decreased as a function of time. Under the same conditions, in the absence of 4-FSB, control enzyme showed no significant loss of activity. Figure 1 illustrates the time-dependent activity loss of the enzyme in the presence of 5 mM 4-FSB. The enzyme activity did not decrease appreciably after 210 min of incubation. Various concentrations of 4-FSB (0.25–9 mM) were used to examine the concentration dependence of the reaction. In each case, enzyme activity reached maximal inactivation, which was 35% of original activity. The half life for the spontaneous hydrolysis of 4-FSB has been determined to be 23 h under these conditions (Barycki & Colman, 1993). Because the reaction time with enzyme is relatively short in comparison to the half life of 4-FSB, it was concluded that the inactivation of enzyme by 4-FSB results in a partially active enzyme that retains 35% of the original enzyme activity. The k_{obs} for inactivation was calculated from the slope of $\ln[(E_t - E_\infty)/(E_0 - E_\infty)]$ versus time, in which E_t is the enzyme activity at time t, E_0 is the enzyme activity at time 0, and E_∞ is equal to 0.35 E_0, the average limiting residual activity.

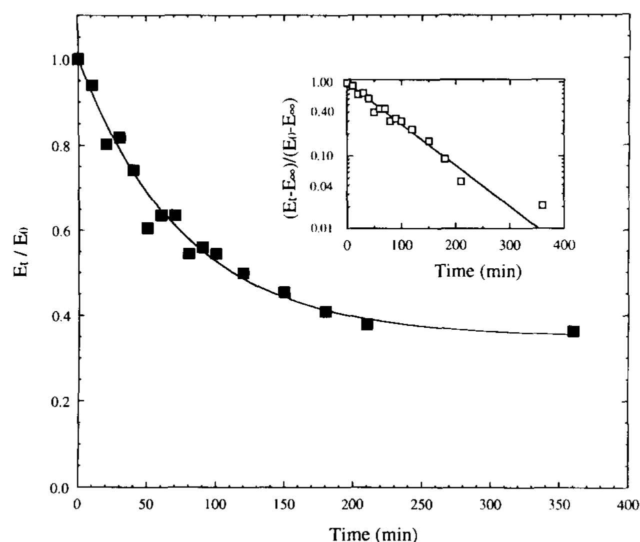

Fig. 1. Inactivation of GST 1-1 by 4-FSB. Rat liver GST 1-1 (0.4 mg/mL) was incubated with 5 mM 4-FSB in 0.1 M phosphate buffer, pH 7.5, at 25 °C. Residual activity, E_t/E_0, was measured using CDNB and glutathione as substrates, as described in Materials and Methods. Inset: k_{obs} for the reaction was determined from the slope of $\ln[(E_t - E_\infty)/(E_0 - E_\infty)]$ versus time, where E_0 and E_t are the enzyme activity at time 0 and time t, respectively, and E_∞ is the enzyme activity at the end of reaction, which is 0.35 E_0. In this case, k_{obs} was found to be 0.011 min^{-1}.

Concentration dependence of k_{obs}

Various concentrations of 4-FSB (0.25–9.0 mM) were incubated with GST 1-1 under the same conditions as in Figure 1, and k_{obs} was determined. Figure 2 shows the concentration dependence of the inactivation rate constant on 4-FSB. Above 5 mM 4-FSB, k_{obs} no longer increased with an increase in the 4-FSB concentration. This "saturation behavior" is typical of an affinity label in which an enzyme–reagent complex is formed before the irreversible inactivation of enzyme occurs. This behavior can be described by the equation: $1/k_{obs} = 1/k_{max} + K_I/k_{max}(1/[4\text{-FSB}])$, where K_I is the apparent dissociation constant of the enzyme-reagent complex. A double reciprocal plot of $1/k_{obs}$ versus 1/[4-FSB], shown in the inset of Figure 2, yields values of 0.78 mM and 0.011 min^{-1} for K_I and k_{max}, respectively.

Effect of substrate analogues on the rate of inactivation of GST 1-1 by 4-FSB

Various substrate analogues, at concentrations at least 5 times their reported K_m or K_I, were included in the reaction mixture, and the rates of inactivation of the enzyme by 5 mM 4-FSB were

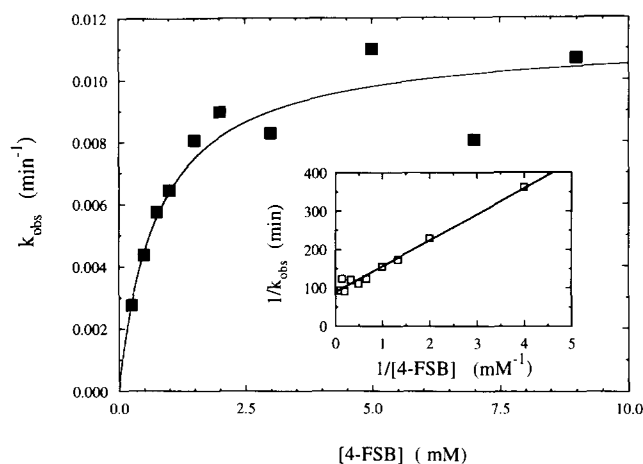

Fig. 2. Concentration dependence of the k_{obs} for the inactivation of GST 1-1 by 4-FSB. Enzyme was incubated with various concentrations of 4-FSB under the same conditions as in Figure 1. At each concentration of 4-FSB, k_{obs} was calculated as exemplified by the inset of Figure 1, with E_∞ = 0.35 E_0. Inset: A double-reciprocal plot of $1/k_{obs}$ versus 1/[4-FSB] yields values of 0.78 mM and 0.011 min^{-1} for K_I and k_{max}, respectively.

measured. The results, shown in Table 1, indicate that neither S-methylglutathione nor Δ^5-androstene-3,17-dione (alone or in combination) affords significant protection against enzyme inactivation, suggesting that 4-FSB does not bind to either the glutathione binding site or the steroid binding site. The compound 2,4-dinitrophenol, an analogue of the xenobiotic substrate CDNB yields substantial protection and S-hexylglutathione or S-(*p*-nitrobenzyl)glutathione provides complete protection. These results indicate that 4-FSB binds to the active site of the enzyme, probably within a xenobiotic binding site, but close to the glutathione site.

Incorporation of 4-FSB into GST 1-1

Glutathione S-transferase was incubated with 5 mM [3,5-^{3}H]-4-FSB for 210 min, at which time 94% of maximum inactivation was reached and 2.8 mol reagent/mol enzyme subunit was incorporated (Table 2). DTT is known to decompose the product of reaction of cysteine with fluorosulfonylbenzoic acid, with subsequent regeneration of cysteine (Likos & Colman, 1981). To evaluate whether cysteine was involved in the modification of GST, half of the modified enzyme was treated with 10 mM DTT for 30 min. This treatment had no effect on enzymatic activity, but reduced the incorporation to 1.84 mol 4-FSB/mol enzyme subunit. This result suggests that about 1 mol 4-FSB/subunit reacts with cysteine, but this reaction does not contribute to the inactivation of the enzyme.

Substrate analogues were included in the reaction mixture to test their ability to prevent 4-FSB incorporation as well as to protect against inactivation. In the presence of 5 mM S-hexylglutathione, the enzyme retained full activity and reagent incorporation was reduced by one mol/mol enzyme subunit both in the absence and in the presence of DTT (Table 2). Comparison of incorporation data with and without S-hexylglutathione protection indicates that approximately 1 mol 4-FSB/mol enzyme subunit is responsible for the loss of the enzyme activity. In the presence of 10 mM 2,4-dinitrophenol, after 210 min of incubation, the activity loss was reduced to 23% of the maximum inactivation and the incorporation was decreased by 0.82 mol 4-FSB/mol subunit in the absence of DTT, consistent with the 23% of maximum inactivation of the enzyme (Table 2).

Table 1. *Effect of substrate or substrate analogues on the rate constant for inactivation by 5.0 mM 4-FSB*[a]

Ligand	k_{+L}/k_{-L}
None	1.00
S-methylglutathione (5.0 mM)	0.94
Δ^5-androstene-3,17-dione (0.3 mM)	1.04
S-methylglutathione (5.0 mM) + Δ^5-androstene-3,17-dione (0.3 mM)	0.95
2,4-dinitrophenol (10.0 mM)	0.15
S-(*p*-nitrobenzyl)glutathione (5.0 mM)	0
S-hexylglutathione (5.0 mM)	0

[a] GST 1-1 (0.4 mg/mL) was incubated in 0.1 M potassium phosphate buffer, pH 7.5, at 25 °C with 5.0 mM reagent in the absence and presence of ligands. Rate constants were calculated by the method illustrated in Figure 1, inset.

Table 2. *Incorporation of [3,5-^{3}H]-4-FSB into enzyme*[a]

Additions	Incorporation (mol of [3,5-^{3}H]-4-FSB/mol of subunit) Without DTT	With DTT[b]	% Maximum inactivation
None	2.80	1.84	94%
10.0 mM 2,4-Dinitrophenol	1.98	0.83	23%
5.0 mM S-hexylglutathione	1.73	0.76	0

[a] Determined after 210 min of incubation of GST with 5 mM [3,5-^{3}H]-4-FSB.
[b] DTT treatment does not reactivate the enzyme.

Separation of thermolysin digest of modified GST

GST (0.4 mg/mL) was incubated with 5 mM of [3,5-3H]-4-FSB for 210 min in the absence or presence of 5 mM S-hexylglutathione, after which both samples were treated with DTT. Unreacted 4-FSB was removed by gel filtration and the enzyme was digested with thermolysin. The resulting peptides were separated by C_{18} reverse-phase HPLC, as shown in Figure 3. The distribution of radioactivity in the thermolysin digest of inactivated modified enzyme is shown in Figure 3A. A single large radioactive peak at 23% acetonitrile is observed. This peak was reduced substantially in the digest of enzyme modified when S-hexylglutathione was included in the reaction mixture (Fig. 3B). Modification of the peptide eluting at 23% acetonitrile clearly correlates with inactivation of the enzyme. Additional small peaks of radioactivity were observed in the digests of protected and unprotected enzyme. These small peaks may collectively account for the additional 0.8 mol of nonspecifically incorporated reagent that are unrelated to the inactivation of the enzyme.

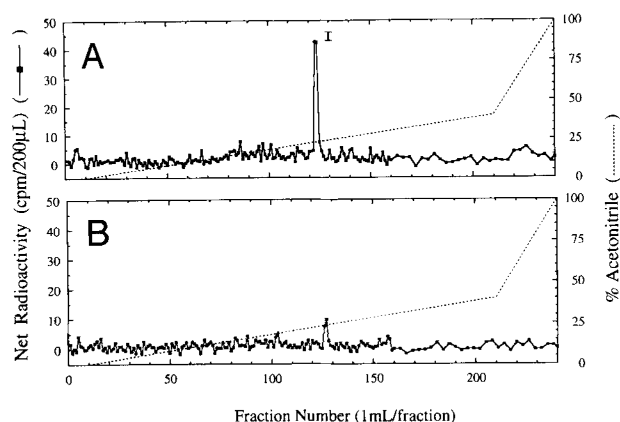

Fig. 3. Fractionation by HPLC of thermolysin digest of [3,5-^{3}H]-4-FSB-modified GST 1-1. Peptides from the thermolysin digest of the 5 mM 4-FSB-modified GST 1-1, treated with DTT, were isolated by HPLC using a C_{18} column equilibrated with 0.1% trifluoroacetic acid and an acetonitrile gradient, as described in Materials and Methods. **A:** Distribution of radioactivity in the digest after a 210-min incubation with [3,5-^{3}H]-4-FSB in the absence of S-hexylglutathione. **B:** Distribution of radioactivity in the digest of active enzyme modified in the presence of 5 mM S-hexylglutathione.

When DTT treatment was omitted prior to digestion, the same major Peak I was observed, along with a radioactive peak at the void volume, corresponding to the hydrolyzed 4-FSB; we did not isolate an additional radioactively labeled peptide. These observations indicate that this additional covalent linkage between the enzyme and 4-FSB was not stable under these conditions.

Identification of Peak I

When subjected to gas phase amino acid sequencing (Table 3), peak I (Fig. 3A) was found to yield the sequence of a single pure peptide: Val-Leu-His-X. The PTH-modified amino acid residue in cycle 4 did not have the same retention time as that of the usual PTH-amino acid derivatives; however, the retention time did correspond to that of the PTH derivative of O-(4-carboxyphenylsulfonyl)tyrosine (Saradambal et al., 1981). Radioactivity was detected in cycle 4 of the peptide sequence run. The sequence shown in Table 3 uniquely corresponds to the tetrapeptide (Val 5-Leu 6-His 7-Tyr 8) of the known sequence of GST 1-1 (Lai et al., 1984; Pickett et al., 1984). We conclude that Tyr 8 is the target for reaction with 4-FSB and its modification is responsible for the loss of enzyme activity.

Relationship between modification of Tyr 8 and loss of enzyme activity

GST was incubated with 5 mM [3,5-^{3}H]-4-FSB in the absence or presence of S-hexylglutathione and the reagent incorporation was determined as a function of time and of percent maximum inactivation either with or without DTT treatment. The moles of tyrosine residue modified were calculated from the difference in incorporation between unprotected and protected enzymes either following DTT treatment or without DTT treatment. Figure 4 shows the linear relationship between mol tyrosine modified/subunit and percent maximum inactivation. Extrapolation to 100% of maximum inactivation yields 1.13 mol tyrosine modified/mol enzyme subunit.

Identification of Cys peptide modified

The decreased reagent incorporation after treatment with DTT (Table 2) suggested that one of the two cysteines of the enzyme was modified by 4-FSB; however, we could not isolate a cysteinyl peptide linked to the sulfonylbenzoic acid moiety. These observations indicated that the bond between the sulfhydryl of cysteine and the sulfonylbenzoic acid had limited stability, as has been noted previously (Likos & Colman, 1981). In order to identify the cysteine target of 4-FSB, either the unmodified control or modified enzyme was exposed to radioactive NEM. It was reasoned that both cysteines should be labeled by NEM in the control enzyme, whereas in the modified enzyme, only cysteines that had not reacted with 4-FSB would be available to react with NEM. Thus, the cysteine reaction site of 4-FSB would be indicated by a decreased reaction with NEM in the 4-FSB-modified enzyme compared with the control enzyme. The [1-^{14}C]NEM-peptides from thermolysin digests of control and 4-FSB-modified GST were purified by HPLC as described in Materials and Methods. In the control enzyme, the ratio of radioactivity contributed by the NEM-Cys 17 to that of NEM-Cys 111 is 0.7:1.0 (data not shown), indicating that the two free cysteines are almost equally labeled by NEM. In contrast, in the 4-FSB-modified enzyme, the ratio of NEM-Cys 17 to NEM-Cys 111 is decreased to 0.02:1.0. We conclude that, in this sample, Cys 17 is unavailable to NEM and is therefore the cysteine residue modified by 4-FSB.

When 4-FSB-modified enzyme was incubated with DTT prior to [^{14}C]NEM treatment, the distribution of radioactivity in the HPLC pattern of its proteolytic digest was the same as that of control enzyme. This experiment demonstrates that the product of 4-FSB and Cys 17 is the one decomposed by DTT treatment.

In a separate experiment, S-hexylglutathione was included in the reaction mixture when enzyme was modified by 4-FSB, and this active, modified enzyme was subsequently incubated with [^{14}C]NEM. The resultant [^{14}C]NEM radioactive HPLC pattern was the same as that of the inactive 4-FSB-modified enzyme (data not shown), indicating that S-hexylglutathione does not protect Cys 17 from modification by 4-FSB.

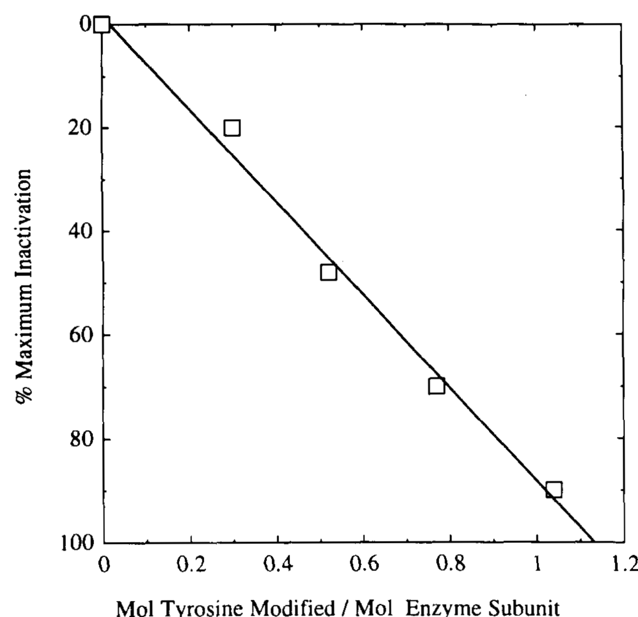

Fig. 4. Percent maximal inactivation as a function of mol modified tyrosine/enzyme subunit. Completely modified enzyme retains 35% of activity when CDNB and glutathione are used as substrates; the activity loss is expressed as percent maximal inactivation $[(E_t - E_\infty)/(E_0 - E_\infty)] \times 100\%$, where $E_\infty = 0.35\ E_0$. Calculation of mol modified tyrosine/mol subunit is described in Results.

Table 3. *Sequence of labeled peptide from the thermolysin digest of 5 mM [3,5-^{3}H]-4-FSB-modified GST 1-1*

Cycle	Amino acid	pmol
1	Val	673
2	Leu	648
3	His	242
4	X[a]	205

[a] A novel peak appeared in cycle 4 with a retention time distinct from that of standard PTH-amino acids. The amount of X was determined from the radioactivity recovered from peptide sequencing.

Comparison of kinetic constants of native and modified enzymes

The catalytic activity of the modified enzyme was investigated using various substrates, as summarized in Table 4. The $K_{m\text{-}app}$ for glutathione, CDNB, mBBr, and Δ^5-androstene-3,17-dione do not change appreciably upon modification by 4-FSB. V_{max} of modified enzyme is about 34% of that of native enzyme when CDNB and glutathione are used as substrates, suggesting that the loss in the enzyme activity in the modified enzyme is due to the reduced $k_{cat\text{-}app}$ rather than to the change in the K_m value. With mBBr as the xenobiotic substrate, $k_{cat\text{-}app}$ of modified enzyme is 30% of that of the native enzyme. The kinetic constants with Δ^5-androstene-3,17-dione, shown in Table 4 for native and modified enzymes, were determined under the standard conditions at pH 8.5 described by Benson et al. (1977); the $k_{cat\text{-}app}$ of modified enzyme was 67% of that of native enzyme. Because the catalytic activity of the enzyme is pH dependent (see below), $k_{cat\text{-}app}$ of native and modified enzymes were measured again at pH 6.5 using a saturating concentration of Δ^5-androstene-3,17-dione (350 μM). At this lower pH, $k_{cat\text{-}app}$ of modified enzyme is about 37% of that of the native enzyme, suggesting that enzymatic activity of modified enzyme in comparison to that of the native enzyme decreases to a similar extent with each of the three different substrates, when assayed at pH 6.5.

pH Profile of the enzyme activity

The activity for native and modified enzyme, with CDNB and glutathione as substrates, was determined from pH 5.2 to 10.1 under conditions similar to those used by Wang et al. (1992), Huskey et al. (1991), and Graminski et al. (1989) (i.e., at saturating concentrations of glutathione and nonsaturating concentrations of CDNB). It was found that "pK_{a1}" of modified enzyme is similar to that of unmodified enzyme, whereas there is a small shift of "pK_{a2}" toward high pH for modified enyzme compared with control enzyme (data not shown). A more detailed analysis of the pH dependence of the kinetic constants was conducted from pH 5.55 to 7.91. Using a CDNB concentration of 1.6 mM, the K_m for glutathione at pH 5.50 was measured as 190 μM and 290 μM for unmodified and modified enzymes, respectively; whereas at pH 6.5, the K_m for glutathione was 260 μM for both unmodified and modified enzymes; and at pH 7.93, the K_m for glutathione was 110 μM and 100 μM for unmodified and modified enzymes, respectively. Therefore, the concentration of glutathione was maintained at 3.0 mM, at least 10-fold higher than the K_m for glutathione at any pH in the range of 5.5–7.9. At each pH used, the concentration of CDNB was varied, and V_{max} and K_m were calculated by least-squares fit of the data to the Michaelis-Menten equation $v_i = V_{max}/(1 + K_m/[S])$. Figure 5 shows the pH dependence of k_{cat}/K_m^{CDNB} for control and modified enzymes. The data can be used to estimate maximum k_{cat}/K_m^{CDNB} values, independent of pH, of $(7.0 \pm 0.4) \times 10^4$ and $(5.9 \pm 0.5) \times 10^4$ M^{-1} s^{-1} for the control and modified enzymes, respectively. The data indicate an apparent pK_a value of 6.45 ± 0.10 for control enzyme, compared with 7.36 ± 0.09 for modified enzyme. Thus, the apparent pK_a of modified enzyme is increased by approximately 0.9 pH unit compared with that of control enzyme. This pK_a has been interpreted as representing the ionization constant of enzyme-bound glutathione (Liu et al., 1992). This result suggests that modification of the enzyme increases somewhat the pK of enzyme-bound glutathione; it is notable, however, that the pK for the modified enzyme is still far below the pK of 9.13 for the -SH of free glutathione (data not shown).

Table 4. *Apparent kinetic constants for several substrates of modified and control GST 1-1*

Substrate	$K_{m\text{-}app}$ (μM)	$V_{max\text{-}app}$ (μmol min^{-1}[mg enzyme]$^{-1}$)	$k_{cat\text{-}app}$[a] (s^{-1})	$(k_{cat}/K_m)_{app}$ (μM^{-1} s^{-1})
CDNB[b]				
Control enzyme	860	73	31	0.036
Modified enzyme	820	20	8	0.0099
Glutathione[c]				
Control enzyme	260	58	25	0.094
Modified enzyme	260	23	10	0.037
mBBr[d]				
Control enzyme	29	95	40	1.38
Modified enzyme	33	29	12	0.36
Glutathione[e]				
Control enzyme	150	72	31	0.21
Modified enzyme	200	24	10	0.052
Δ^5-Androstene-3,17-dione[f]				
Control enzyme	26	4.8	2.0	0.079
Modified enzyme	24	3.2	1.4	0.057

[a] $k_{cat\text{-}app}$ is defined as moles of substrates converted by 1 mol of enzyme in 1 s.
[b] 2.5 mM glutathione present.
[c] 1.0 mM CDNB present.
[d] 2.4 mM glutathione present.
[e] 100 μM mBBr present.
[f] 100 μM glutathione and 100 μM DTT present at pH 8.5.

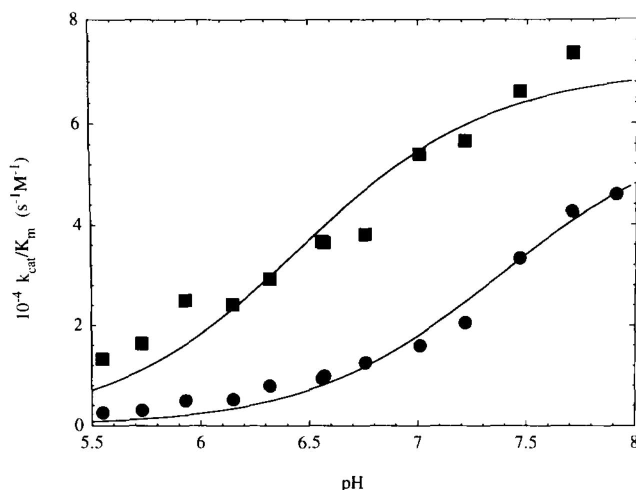

Fig. 5. pH profiles of $(k_{cat}/K_m^{CDNB})_o$. V_{max} and K_m^{CDNB} were determined at each pH by a least-squares fit of the initial rate of the conjugation reaction of CDNB and 3.0 mM glutathione to the Michaelis–Menten equation $v_i = V_{max}/(1 + K_m/[\text{CDNB}])$. $(k_{cat}/K_m^{CDNB})_o$ was calculated for both control enzyme (■) and modified enzyme (●), as described in the text, and is given by the ordinate.

Discussion

4-FSB behaves as an affinity label in its reaction with rat GST 1-1. The rate of inactivation is dependent in a nonlinear fashion on the concentration of 4-FSB, and S-hexylglutathione both prevents inactivation and reduces the incorporation of the reagent by approximately 1 mol 4-FSB/mol enzyme subunit as measured either with or without DTT treatment. These results suggest that modification of a single amino acid residue is responsible for the inactivation of the enzyme, and that this reaction product is stable in the presence of DTT.

Protection studies indicate that the 4-FSB reaction site responsible for loss of enzyme activity is located within the xenobiotic substrate binding site, yet close to the glutathione binding site. Nearly complete protection against inactivation is provided by the xenobiotic substrate analogue, 2,4-dinitrophenol or by S-hexylglutathione, but not by the considerably smaller S-methylglutathione. It is notable that Δ^5-androstene-3,17-dione does not prevent the loss of enzyme activity associated with modification by 4-FSB, even at concentrations well above its K_m value, suggesting that the steroid binding site may be distinct from the classically defined xenobiotic binding site. We have previously presented evidence supporting the existence of more than one xenobiotic substrate site for other glutathione S-transferase isoenzymes (Barycki & Colman, 1993; Hu & Colman, 1995).

The peptide containing the DTT-stable, covalently linked, radioactive 4-FSB was isolated and found to contain residues 5–8 of the known sequence of isoenzyme 1-1 (Lai et al., 1984; Pickett et al., 1984), and the amino acid residue modified by 4-FSB was determined to be Tyr 8. The glutathione analogue, S-hexylglutathione, which prevents loss of enzyme activity, dramatically reduces the appearance of this radioactively labeled peptide. The other major target of 4-FSB, Cys 17, is not associated with loss of enzyme activity.

The Michaelis constants of the modified enzyme for all substrates tested show no appreciable difference from those of native enzyme (Table 4). These results are consistent with those from mutagenesis studies, confirming that Tyr 8 does not participate in substrate binding (Wang et al., 1992). Several previous site-directed mutagenesis studies of mammalian glutathione S-transferases (Stenberg et al., 1991a; Kolm et al., 1992; Liu et al., 1992; Wang et al., 1992) have demonstrated that mutation of the tyrosine in the N-terminal region results in an enzyme with about 0.3–2% of the activity of the wild-type enzyme toward CDNB, suggesting that this tyrosine is the crucial amino acid involved in the ionization of glutathione. We were surprised initially that modification of Tyr 8 by 4-FSB resulted in a partially active enzyme with as much as 35% residual activity (determined at pH 6.5 using CDNB, Δ^5-androstene-3,17-dione, or mBBr as the xenobiotic substrate) instead of in a completely inactive enzyme.

Tyr 8 is conserved in all known cytosolic glutathione S-transferases, not only from mammals, but also from other species including bacteria. Results of X-ray crystallographic studies on several glutathione S-transferases (Reinemer et al., 1991, 1992; Ji et al., 1992; Sinning et al., 1993; Garcia-Saez et al., 1994; Raghunathan et al., 1994) have shown that Tyr 8 is spatially located in the vicinity of the thiol group of the enzyme-bound glutathione. These studies have led to the postulate that this tyrosine residue is essential for the enzymatic mechanism by stabilizing the thiolate anion of the enzyme-bound glutathione, although it has been noted that positively charged Arg is nearby and may also contribute to stabilizing the thiolate (Stenberg et al., 1991a; Sinning et al., 1993; Wang et al., 1993).

We examined the pH dependence of k_{cat}/K_m^{CDNB} (at saturating glutathione concentration) for modified and native enzymes in order to determine whether modification by 4-FSB affected the ability of the enzyme to stabilize the thiolate anion (Fig. 5). The apparent pK_a of the modified enzyme, when it is determined at saturating glutathione concentration, has been interpreted to represent the pK of the enzyme-bound glutathione (Liu et al., 1992). An increase of about 0.9 pK unit, as we have observed for the 4-FSB-modified enzyme compared with native enzyme, indicates that Tyr 8 does have a role in facilitating the ionization of the thiol group of glutathione. However, this difference is not as large as the 1.8 pK unit observed for the Tyr to Phe or for the Tyr to Thr, mutants of the same isozyme (Wang et al., 1992). The maximum k_{cat}/K_m^{CDNB} for the 4-FSB-modified enzyme (independent of pH) is approximately 84% of that of the native enzyme, suggesting that the increase in apparent pK_a accounts for a large part of the activity loss. The ~16% decrease in the maximum value of k_{cat}/K_m^{CDNB} for the 4-FSB-modified enzyme is quite different from the >20-fold decrease observed when Phe or Thr was substituted for the N-terminal region Tyr. (Because Arg 15 was retained in all of these enzymes [Wang et al., 1992], it cannot account for the difference between the 4-FSB-modified and mutant enzymes.) The discrepancy between the chemically modified and mutant enzymes implies that other substituents of the 4-FSB-modified enzyme may fulfill a role in catalysis normally carried out by the unmodified tyrosine.

In order to assess the above possibility, a homologous protein structure for the rat GST 1-1 was constructed, based on the crystal structure of human GST 1-1 (Sinning et al., 1993). Comparison of the primary structure of these two enzymes reveals that they are closely related, having 76% identity and 11% sim-

ilarity, based on the BESTFIT program of the Genetics Computer Group package (from the University of Wisconsin). Thus, the human enzyme provides an excellent basis for constructing a model of the rat 1-1 enzyme by homology modeling. Examination of the model for the rat enzyme reveals only minor changes in comparison with the human enzyme structure: the two structures have nearly identical topologies (data not shown). Furthermore, modification of Tyr 8 and Cys 17 by 4-FSB and subsequent energy minimization suggest that the overall structure of rat GST 1-1 does not change appreciably upon modification (Fig. 6). A notable exception, however, is the movement of the hydroxyl group of Tyr 8, by nearly 2 Å, away from the thiol group of glutathione in order to accommodate the sulfonylbenzoic acid moiety. It is also noteworthy that, in the native enzyme, Cys 17 appears to be inaccessible to modification because it is located deep within the interior of the protein. Our experimental evidence demonstrates that Cys 17 is a reaction site for 4-FSB; hence, the enzyme must "breathe" while in solution, allowing access to this "buried" residue.

Molecular modeling suggests that a 4-FSB moiety linked to Tyr 8 would not perturb the binding of glutathione or CDNB (Fig. 7), consistent with our K_m determinations (Table 4). Comparison of the location of glutathione in the modified and native rat GST 1-1 models shows little or no perturbation of the carbon backbone of the tripeptide and only a small displacement (0.3 Å) in the position of the thiol group. Similarly, the position of CDNB is not very different from that occupied by the benzene ring of the S-benzylglutathione, which is the substrate analogue crystallized with the human isoenzyme. However, the precise position of CDNB is difficult to predict; several slightly different orientations are possible, all having similar energies and being located in the same vicinity within the binding pocket. We have illustrated an orientation in which C1 of CDNB is located about 2.9 Å away from the sulfur atom of glutathione (Fig. 7).

Fig. 6. Overlay of rat GST 1-1 and 4-FSB-modified rat GST 1-1. The models, based on the crystal structure of human GST 1-1, were built with InsightII and Discover 3 from Biosym Technology, Inc., running on a Silicon Graphics Indigo 2 work station. The native structure is colored pink and the 4-FSB-modified enzyme is yellow. Side chains of the two major sites of reaction, Tyr 8 and Cys 17, are shown before and after modification with 4-FSB.

It is generally considered that a hydrogen bond exists between the hydroxyl group of Tyr 8 and the sulfur of glutathione that stabilizes the thiolate anion (Liu et al., 1992; Wang et al., 1992). In our model, although the hydroxyl group of Tyr 8 is no longer available for hydrogen bonding with the thiolate of the enzyme-bound glutathione, an oxygen atom from the sulfonyl group of the 4-FSB moiety linked to Tyr 8 is only 2.9 Å away from the sulfur atom of glutathione (Fig. 7). This distance is within the favorable range for regular hydrogen bond formation (Cleland & Kreevoy, 1994). The sulfonyl oxygen might act as a proton acceptor in a hydrogen bond formed with the thiol group, thereby promoting the ionization of the glutathione's -SH. In this manner, the sulfonyl oxygen would be functioning as a general base to facilitate the removal of the proton of glutathione, thus contributing to the catalytic reaction in an alternative role from that generally assigned to Tyr 8.

Recently, site-directed mutagenesis has been performed on the counterpart of the N-terminal region tyrosine residue in a bacterial (i.e., *Escherichia coli*) glutathione S-transferase (Nishida et al., 1994). In this enzyme, many of the residues deemed important for the enzyme structure and activity in mammalian cytosolic glutathione S-transferase were conserved. However, replacement of the N-terminal region Tyr by Phe resulted in a completely active mutant enzyme when tested with CDNB as substrate, leading to the conclusion that Tyr 8 is not essential in that bacterial enzyme. Our present paper represents the first demonstration that the free -OH of Tyr 8 is not required in a mammalian glutathione S-transferase.

In summary, 4-FSB reacts with active site residue Tyr 8 of rat liver GST 1-1, resulting in modified enzyme that retains substantial activity toward CDNB, mBBr, and Δ^5-androstene-3,17-dione. We postulate that, in this modified enzyme, the sulfonyl oxygen of the derivatized Tyr 8 residue may serve as a general base to facilitate the removal of the proton from glutathione. Thus, Tyr 8 in the native mammalian enzyme is important in terms of its ability to facilitate the ionization of the thiol group of the enzyme-bound glutathione, but it is not unique in its ability to carry out this function.

Materials and methods

Materials

[3,5-^{3}H]-4-(Fluorosulfonyl)benzoic acid was synthesized as described previously (Barycki & Colman, 1993) from [3,5-^{3}H]-4-aminobenzoic acid by the method of Esch and Allison (1978). *p*-Aminobenzoic acid was converted to 4-(chlorosulfonyl)benzoic acid, from which chloride was displaced by fluoride, yielding [3,5-3H]-4-FSB. The specific radioactivity of the product was 1.95×10^{11} cpm/mol when $\epsilon_{232nm} = 10{,}550\ M^{-1}\ cm^{-1}$ was used for 4-FSB.

Frozen Sprague-Dawley rat livers were purchased from Pel Freez Biologicals; Rogers, Arkansas; Glutathione, S-hexylglutathione, S-methylglutathione, S-(nitrobenzyl)glutathione, S-hexylglutathione-Sepharose, 4-FSB, 2,4-dinitrophenol, Sephadex G-50, and thermolysin were purchased from Sigma Chemical Co., St. Louis, Missouri; CDNB, 4-sulfobenzoic acid, and *p*-aminobenzoic acid were supplied by Aldrich Chemical Co., Milwaukee, Wisconsin; Merck, Darmstadt, West Germany; provided the Silica Gel 60 TLC plates with fluorescent indicator.

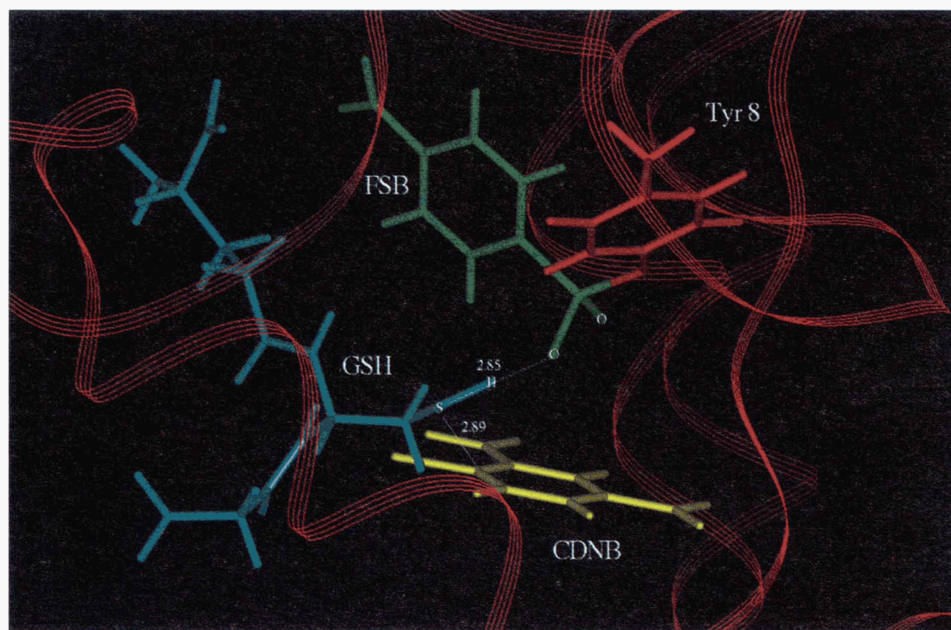

Fig. 7. Proposed orientation of substrates within the active site of 4-FSB-modified GST 1-1. Rat GST 1-1 is illustrated as red ribbons. The side chain of Tyr 8 is shown in red, the sulfonylbenzoic acid moiety in green, CDNB in yellow, and glutathione in blue. In this orientation, the sulfur of glutathione is located 2.89 Å from C1 of CDNB and 2.85 Å from the sulfonyl oxygen of 4-FSB-modified Tyr 8.

Bio-Rad Laboratories, Hercules, California; supplied Protein Assay Dye Reagent Concentrate. Urea was from Schwartz/Mann Biotech, Cleveland, Ohio; and Liquiscint from National Diagnostics, Atlanta, Georgia; PBE-118 and Pharmalyte were from Pharmacia, Uppsala, Sweden; and mBBr from Molecular Probes, Inc., Eugene, Oregon; Δ^5-Androstene-3,17-dione was purchased from Steraloids, Inc., Wilton, New Hampshire; and [3,5-^{3}H]-4-aminobenzoic acid from Moravek Biochemicals, Inc., Brea, California; [1-^{14}C]-*N*-ethylmaleimide was from Schwarz Bioresearch Inc., Orangeburg, New York. All other chemicals used were reagent grade.

Enzyme purification

GST 1-1 was purified from rat livers by modification of the methods of Cobb et al. (1983) and Jensson et al. (1985), which use affinity chromatography and chromatofocusing. Rat livers were homogenized in a Waring blender with 10 mM Tris-HCl, pH 7.8, buffer. The homogenate was centrifuged for 90 min at 16,000 × *g*, and the supernatant was filtered through glass wool. The filtered supernatant was applied to a S-hexylglutathione-Sepharose column equilibrated with 10 mM Tris-HCl, pH 7.8. The column was washed with 10 mM Tris-HCl buffer. The same buffer, with the addition of 0.2 M NaCl, was used to elute GST 1-1. (The column was then washed with 10 mM Tris-HCl plus 2.5 mM S-hexylglutathione to elute the other GST isozymes.) After concentration and dialysis against 10 mM Tris-HCl, pH 7.8, for 18 h, the GST 1-1 pool was re-applied to the S-hexylglutathione-Sepharose column equilibrated with 10 mM Tris-HCl, pH 7.8. This time, after washing with the starting buffer plus 0.2 M NaCl, the 1-1 isoenzyme was eluted by the addition of 2.5 mM S-hexyl-glutathione to the elution buffer. The difference in elution position of the 1-1 isoenzyme in the two chromatographic steps may be explained by its relatively weak affinity for S-hexylglutathione-Sepharose, as shown by Hayes (1988). In addition, a small molecule(s) must be present in the initial homogenate, which further weakens the 1-1 isozyme's affinity for the resin, in order to account for the differential binding of GST 1-1 before and after dialysis. It has been reported that oxidized glutathione selectively elutes the 1-1 and the 8-8 isoenzymes from a glutathione-agarose column (Meyer et al., 1989). Perhaps the presence of oxidized glutathione, reduced glutathione, or some combination of the two in the crude homogenate is responsible for the observed behavior. In any case, this unusual behavior of GST 1-1 provides a convenient method for separating it from most of the other glutathione S-transferases.

The concentrated enzyme obtained from the above affinity chromatography steps was dialyzed against 10 mM Tris-HCl, pH 8.0, and was loaded onto a PBE column equilibrated with 25 mM triethylamine-HCl buffer, pH 10.8. The column was eluted with a 1.25% Pharmalyte solution, pH 8.0. The GST 1-1 pool, which emerged at pH 8.6, was then applied to the S-hexylglutathione-Sepharose column (equilibrated with 10 mM Tris-HCl, pH 7.8) to remove the ampholytes. Once the Pharmalyte peak eluted during the wash with buffer plus 0.2 M NaCl, GST 1-1 was eluted from the column with 10 mM Tris-HCl buffer, pH 7.8, plus 2.5 mM S-hexylglutathione, pH 7.8. The purity of GST 1-1 was determined by HPLC using a reverse-phase C4 column (Vydac214TP) as described previously (Benson et al., 1989). The purity of the enzyme isolated by this procedure was greater than 99%.

Enzyme assay

The enzymatic activity of GST 1-1 during the purification and the incubation with 4-FSB was measured on a Gilford 240 spec-

trophotometer by monitoring the formation of the conjugate of CDNB (1 mM) and glutathione (2.5 mM) at 340 nm ($\Delta\epsilon$ = 9.6 mM^{-1} cm^{-1}) in 0.1 M potassium phosphate buffer, pH 6.5, at 25 °C, according to the method of Habig et al. (1974). All measurements were corrected for the spontaneous nonenzymatic conjugation of glutathione and CDNB.

Inactivation of GST by 4-FSB

GST 1-1 (0.4 mg/mL) was incubated in 0.1 M potassium phosphate buffer, pH 7.5, with 10% DMF at 25 °C with various concentrations of 4-FSB. Aliquots (20 μL) were removed at different incubation times and the residual activity was measured. Substrate analogues were prepared, either by dissolving in DMF or in an appropriate potassium phosphate buffer to maintain the pH, buffer concentration, and the final concentration of DMF in the reaction mixture as in the absence of the ligands. The k_{obs} was calculated from the slope of $\ln[(E_t - E_\infty)/(E_0 - E_\infty)]$ versus time, where E_t and E_0 are the activities at a particular time and zero time, respectively, and E_∞ is the final constant activity.

Incorporation of [3,5-³H]-4-FSB into GST

Enzyme was incubated with 5 mM [3,5-^{3}H]-4-FSB under the conditions described above. At various time points, approximately 500 μL of the reaction mixture was removed and, where indicated, DTT solution was added to give a final concentration of 10 mM. To remove free reagent, the gel centrifugation procedure of Penefsky (1979) was used: the reaction mixture was applied to two successive 5-mL Sephadex G-50 columns, the first of which was equilibrated with 0.1 M potassium phosphate buffer, pH 7.5, and the second with 0.01 M potassium phosphate buffer, pH 7.5. The protein concentrations in the eluates were determined by the Bio-Rad method, which is based on the Bradford dye binding method (Bradford, 1976), using unmodified enzyme as a standard. The subunit molecular weight was taken as 25,500 (Mannervik & Danielson, 1988). The amount of reagent was determined from the radioactivity measured using a Packard Tri-Carb liquid scintillation counter.

Separation of thermolysin digest of 4-FSB-modified GST

Modified enzyme was prepared by incubation of 5 mM [3,5-3H]-4-FSB in the reaction mixture with 0.4 mg/mL of GST 1-1 for 210 min. Excess 4-FSB was removed as described previously. Solid urea was added to the effluent of the Sephadex G-50 columns to a final concentration of 2 M. Digestion was conducted at 37 °C using 2.5% (w/w) thermolysin for 1 h. Another 5% (w/w) of thermolysin was added for a second hour. The solution was filtered, with no loss of radioactivity, and was immediately subjected to HPLC on a Varian 5000 LC equipped with a Vydac C_{18} column. The absorbance at 220 nm was monitored. Chromatography System I was used: 0.1% trifluoroacetic acid (solvent A) and acetonitrile containing 0.075% trifluoroacetic acid (solvent B). After elution with solvent A for 10 min, a linear gradient was run to reach 40% solvent B at 210 min, followed by another linear gradient to 100% solvent B at 240 min. The flow rate was 1 mL/min and 1 min fractions were collected. Aliquots (200 μL) were mixed with 5 mL Liquiscint for determination of radioactivity.

In order to identify the cysteine residue modified by 4-FSB, GST 1-1 was incubated at 25 °C in the absence or presence of 5 mM nonradioactive 4-FSB for 210 min as above. In certain experiments, as indicated, S-hexylglutathione was included together with 4-FSB. The reaction mixture was either treated with 10 mM DTT first or applied directly to a 5-mL Sephadex G-50 column equilibrated with 0.1 M potassium phosphate buffer, pH 7.5, to remove free reagents. [1-^{14}C]-NEM was added to the effluent of the column to a final concentration of 5 mM. After the reaction mixture was incubated at 25 °C for 10 min, free [1-^{14}C]-NEM was removed by applying the reaction mixture to another 5-mL Sephadex G-50 column equilibrated with 0.01 M potassium phosphate buffer, pH 7.5. The effluent of the Sephadex G-50 column was digested by thermolysin and was subjected to HPLC as above. The first radioactive peak was lyophilized and subjected to HPLC using a Vydac C_{18} column with Chromatography System II: 20 mM ammonium acetate, pH 6.0, in water (solvent A) and 20 mM ammonium acetate, pH 6.0, in 50% acetonitrile (solvent B). After elution with solvent A for 10 min, a linear gradient was run to reach 40% solvent B at 210 min followed by another linear gradient to 100% solvent B at 240 min. The flow rate was 1 mL/min and 1-min fractions were collected.

Analysis of separated peptides

Amino acid sequences of peptides were determined on an Applied Biosystems gas-phase protein (peptide) sequencer, model 470, equipped with a PTH analyzer, model 120, and a model 900A computer. Typically, 50-1,000-pmol samples of peptide were analyzed.

Kinetic characterization of modified enzyme

Modified enzyme was prepared by reaction with 5 mM 4-FSB for 210 min, as described previously, and the excess reagent was removed by column centrifugation using Sephadex G-50 equilibrated with 0.1 M potassium phosphate buffer, pH 7.5. The conjugate of glutathione and CDNB absorbs at 340 nm with $\Delta\epsilon_{340nm}$ = 9.6 mM^{-1} cm^{-1} (Habig et al., 1974) and the conjugate of glutathione and mBBr has a fluorescence emission maximum at 480 nm, when excited at 395 nm (Hulbert & Yakubu, 1983). The abilities of the modified and control enzymes to catalyze the reaction of glutathione with CDNB and mBBr were, respectively, measured spectrophotometrically or fluorometrically in either 0.1 M potassium phosphate buffer or 0.1 M Pipes, pH 6.5, at 25 °C. The ability of modified and control enzyme to catalyze the isomerization of Δ^5-androstene-3,17-dione to Δ^4-androstene-3,17-dione was determined spectrophotometrically from the change in A_{248nm} ($\Delta\epsilon_{248nm}$ = 16.3 mM^{-1} cm^{-1}) (Benson et al., 1977) in 25 mM Tris/phosphate buffer, pH 8.5, or in 0.1 M potassium phosphate buffer, pH 6.5, at 25 °C in the presence of 0.1 mM glutathione and 0.1 mM DTT. Corrections for the nonenzymatic reactions were made for each of the assays. Kinetic constants were determined by a least-squares fit of the data to the hyperbolic saturation curves of the Michaelis–Menten equation.

pH Dependence of enzymatic activity

The dependence on pH of k_{cat}/K_m^{CDNB} for both modified and control enzymes were determined using 0.1 M MES (from pH 5.55–6.56), 0.1 M PIPES (from pH 6.57–7.91), and 3.0 mM glutathione, a concentration at least 10 times the $K_m^{glutathione}$ over the pH range 5.5–7.95. The concentration of CDNB was varied from 0.10 to 3.0 mM and triplicate determinations of initial velocity were made at each CDNB concentration. The rates of all measured enzymatic reactions were corrected for the corresponding nonenzymatic reactions. Kinetic constants were determined by a least-squares fit of the data to the Michaelis–Menten equation $v_i = V_{max}/(1 + K_m^{CDNB}/[S])$. The pH dependence of k_{cat}/K_m^{CDNB} was determined by a least-squares fit of the data to the equation:

$$(k_{cat}/K_m^{CDNB})_o = (k_{cat}/K_m^{CDNB})_i/(1 + [H^+]/K_a),$$

where $(k_{cat}/K_m^{CDNB})_o$ represents the values observed at a given $[H^+]$, and $(k_{cat}/K_m^{CDNB})_i$ is the maximum value that is independent of pH (Liu et al., 1992).

Molecular modeling

Molecular modeling was conducted using the InsightII software package from Biosym Technologies on an Indigo 2 work station from Silicon Graphics. The atomic coordinates for the human GST 1-1 (1GUH) were obtained from the Brookhaven Protein Data Bank (Sinning et al., 1993). The rat isozyme 1-1 model was constructed by first using the Homology module of the InsightII package to replace residues in the human sequence with those of the rat sequence. In this fashion, various side chains were replaced and positioned in a local energy minimum without disrupting the peptide backbone of the human structure. Once all substitutions were complete, the structure was submitted to the Discover 3 module for energy minimization, using steepest descent and conjugate gradient methods to obtain the optimized rat isozyme 1-1 structure. The 4-FSB-modified enzyme model was assembled by first constructing 4-FSB using the Builder module and then covalently linking 4-FSB to the Cys 17 and Tyr 8 side chains of the rat GST 1-1 model. The structure was then submitted for minimization as described above. CDNB was also constructed using the Builder module and substrate docking studies were conducted using the Docking module, which monitors both van der Waals and electrostatic interactions between the substrate and the enzyme.

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