Affinity Labeling of Glutathione *S*-Transferase, Isozyme 4-4, by 4-(Fluorosulfonyl)benzoic Acid Reveals Tyr[115] To Be an Important Determinant of Xenobiotic Substrate Specificity†

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ABSTRACT: Incubation of 4-(fluorosulfonyl)benzoic acid (4-FSB), a xenobiotic substrate analogue, with the 4-4 isozyme of rat liver glutathione *S*-transferase at pH 7.5 and 25 °C results in a time-dependent inactivation of the enzyme. The rate of inactivation exhibits a nonlinear dependence on 4-FSB concentration from 0.50 to 7.85 mM, with k_{max} = 0.082 min^{-1} and a K_I of 1.95 mM. Nearly 1 mol of reagent/mol of enzyme subunit is incorporated when the enzyme is maximally inactivated. Protection against incorporation and inactivation is provided by bromosulfophthalein, a competitive inhibitor with respect to the hydrophobic substrate, 1-chloro-2,4-dinitrobenzene (CDNB), suggesting that the reaction occurs in the binding site of the xenobiotic substrate. Fractionation by high-performance liquid chromatography of a tryptic digest of inactivated enzyme yields a single, modified, 14-residue peptide containing Tyr[115] as the altered amino acid. Modified and control enzymes have comparable affinities for glutathione, as indicated by fluorescence titration. In contrast, as distinguished from the control enzyme, modified enzyme does not adsorb to a column of an agarose-linked Cibacron Blue derivative, indicating that it has lost its ability to bind a hydrophobic substrate analogue. These results are supported by kinetic characteristics of modified and control enzymes: upon modification of the enzyme with 4-FSB, the apparent K_m for glutathione is unchanged, while the apparent K_m for CDNB increases dramatically from 193 to 1690 μM. When the reaction of 4-FSB with enzyme is monitored, the final percent residual activity is found to be dependent on the substrate used in the assay: 11% for CDNB, 20% for ethacrynic acid, 2.5% for *trans*-stilbene oxide, and 2% for *trans*-4-phenyl-3-butene-2-one. Analysis of the kinetics of modified enzyme suggests that Tyr[115] of glutathione *S*-transferase, isozyme 4-4, contributes to xenobiotic substrate binding and, when certain types of substrates are employed, is involved in catalysis.

Glutathione *S*-transferases (EC 2.5.1.18) constitute a family of dimeric isozymes involved in the detoxification of both xenobiotics and endogenous compounds. Each subunit of the enzyme contains a glutathione-binding site and a hydrophobic substrate-binding site that has been shown to accommodate a diverse range of substrates (Mannervik & Danielson, 1988), such as 1-chloro-2,4-dinitrobenzene (CDNB),[1] ethacrynic acid (EA), *trans*-4-phenyl-3-buten-2-one (*t*PBO), and *trans*-stilbene oxide (*t*SO) (Figure 1, structures II–V). The isozymes, which may exist as homo- or heterodimers, catalyze a nucleophilic attack by the thiol group of glutathione on the hydrophobic substrate, resulting in either a substitution or an addition product.

The glutathione *S*-transferases are generally grouped into at least five distinct gene families, on the basis of primary sequence similarity, immunological reactivity, isoelectric point, inhibition properties, and substrate specificity (Rushmore & Pickett, 1993). Rat liver glutathione *S*-transferase, isozyme 4-4, is a member of the μ gene class that encodes subunit types 3, 4, 6, 9, and 11 (Mannervik & Danielson, 1988; Armstrong, 1987, 1991). The 4-4 isozyme has been purified to homogeneity, and its amino acid sequence has been determined (Ding *et al.*, 1986; Alin *et al.*, 1986). It is very closely related to the 3-3 isozyme, with 78% identity plus 9% similarity in sequence based on the ALIGN program (PC Gene, Intelligenetics).

Mutagenesis and X-ray crystallography of the 3-3 isozyme of rat liver glutathione *S*-transferase, complexed with glutathione, have helped implicate several amino acid residues in catalysis and binding of glutathione (Ji *et al.*, 1992; Rushmore & Pickett, 1993). However, little is known about which residues contribute to the binding of the hydrophobic substrate or how these residues determine the substrate specificities of the various isozymes of glutathione *S*-transferase.

Previously in this laboratory, *S*-(4-bromo-2,3-dioxobutyl)-glutathione (*S*-BDB-G) was synthesized and shown to be an affinity label for glutathione *S*-transferase, isozyme 4-4, which labeled Tyr[115] while inactivating the enzyme (Katusz & Colman, 1991). However, the role of Tyr[115] was not clearly established. Protection against modification was afforded by *S*-alkylglutathione derivatives, with effectiveness as protectants increasing with length of the alkyl chain. It was postulated that Tyr[115] was important, not for glutathione binding but instead for facilitating the binding of the non-glutathione substrate by hydrophobic interactions.

4-(Fluorosulfonyl)benzoic acid, 4-FSB (Figure 1, structure I), which is a reactive analogue of the hydrophobic substrate 1-chloro-2,4-dinitrobenzene and is structurally related to other

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[1] Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; EA, ethacrynic acid; *t*PBO, *trans*-4-phenyl-3-buten-2-one; *t*SO, *trans*-stilbene oxide; *S*-BDB-G, *S*-(4-bromo-2,3-dioxobutyl)glutathione; 4-FSB, 4-(fluorosulfonyl)benzoic acid; HPLC, high-performance liquid chromatography; DMF, *N*,*N*-dimethylformamide; NEM, *N*-ethylmaleimide; *S*-HG, *S*-hexylglutathione; BSP, bromosulfophthalein; PTH, phenylthiohydantoin; CPS-Tyr, *O*-[(4-carboxyphenyl)sulfonyl]tyrosine; GSH, reduced glutathione.

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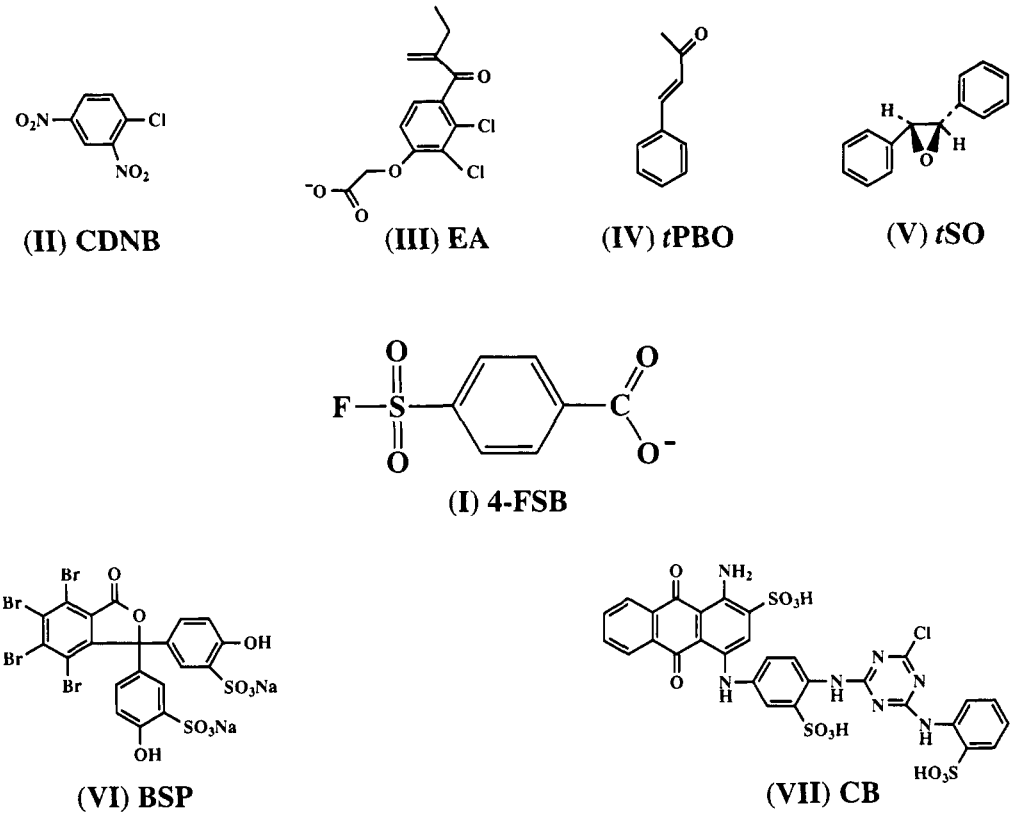

FIGURE 1: Selected substrates and inhibitors of glutathione S-transferase, isozyme 4-4: 4-(fluorosulfonyl)benzoic acid, 4-FSB (I); 1-chloro-2,4-dinitrobenzene, CDNB (II); ethacrynic acid, EA, (III); *trans*-4-phenyl-3-buten-2-one, *t*PBO, (IV); *trans*-stilbene oxide, *t*SO, (V); bromosulfophthal ein, BSP (VI); Cibacron Blue, CB (VII).

substrates (Figure 1, structures II–V) and inhibitors (Figure 1, structures VI and VII) of the enzyme, has been chosen to further investigate the hydrophobic substrate binding site of the 4-4 isozyme. Fluoride can be displaced from the fluorosulfonyl group by nucleophilic attack by several amino acids, including Cys, Tyr, His, and Lys (Colman, 1990). In this paper, we demonstrate that 4-FSB reacts specifically with Tyr[115] of glutathione S-transferase, isozyme 4-4, and present evidence implicating Tyr[115] as important in the binding of the non-glutathione substrate and as an active participant in catalysis. A preliminary version of this work has been presented (Barycki & Colman, 1993).

EXPERIMENTAL PROCEDURES

Materials. Frozen Sprague-Dawley rat livers were purchased from Pel Freez Biologicals. Glutathione, S-hexylglutathione, S-methylglutathione, S-(nitrobenzyl)glutathione, S-hexylglutathione–sepharose, 4-(fluorosulfonyl)benzoic acid, ethacrynic acid, *trans*-stilbene oxide, bromosulfophthalein, 2,4-dinitrophenol, Sephadex G-50-80, and N-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin were all obtained from Sigma Chemical Co. 1-Chloro-2,4-dinitrobenzene, 4-sulfobenzoic acid, *trans*-4-phenyl-3-buten-2-one, and *p*-aminobenzoic acid were purchased from Aldrich Chemical Co. S-(4-Bromo-2,3-dioxobutyl)glutathione was prepared by R. M. Katusz, as described by Katusz and Colman (1991). O-[(4-Carboxyphenyl)sulfonyl]tyrosine was prepared as described by Saradambal *et al.* (1981). Merck provided the Silica Gel 60 TLC plates with fluorescent indicator, and Whatman Biosystems supplied the DEAE-cellulose. Protein Assay Dye Reagent Concentrate and hydroxylapatite (Bio-Gel HT) were supplied by Bio-Rad Laboratories. Urea (ultrapure) was from Schwartz/Mann Biotech, Liquiscint was from National Diagnostics, and Matrex Gel Blue-A was provided by Amicon. [3,5-^{3}H]-4-Aminobenzoic acid was purchased from Moravek Biochemicals, Inc. All other chemicals used were reagent grade.

Enzyme Preparation. The 4-4 isozyme of glutathione S-transferase was purified from Sprague-Dawley rat livers by the method of Chen *et al.* (1988) which uses column chromatography on DEAE-cellulose, S-hexylglutathione–Sepharose, and hydroxylapatite. Values of ϵ_{270nm} = 36 700 M^{-1} cm^{-1} (Graminski *et al.*, 1989) and M_r of 26 500 per subunit (Mannervik *et al.*, 1985) were employed to determine the enzyme concentration. The purity of the enzyme was determined by HPLC using a C_4 reverse-phase column (Vydac 214TP) by a modification of the method of Benson *et al.* (1989) as described by Katusz and Colman (1991); the resultant enzyme preparation was determined to be greater than 98% pure. Its identity as the 4-4 isozyme was confirmed by amino-terminal sequencing through the first 19 amino acid residues by comparison with the known primary structure of the 4-4 isozyme (Alin *et al.*, 1986; Ding *et al.*, 1986).

Synthesis of [3,5-^{3}H]-4-(Fluorosulfonyl)benzoic Acid. [3,5-^{3}H]-4-FSB was synthesized from [3,5-^{3}H]-4-aminobenzoic acid as described by Esch and Allison (1978). The synthesis involves conversion of the *p*-aminobenzoic acid to the corresponding sulfonyl chloride, 4-(chlorosulfonyl)benzoic acid, followed by the displacement of chloride by fluoride, to give the product, 4-(fluorosulfonyl)benzoic acid. The synthesis was monitored by silica TLC using a solvent system of 2-butanone/acetone/water (65:20:15), as well as by observing characteristic shifts in the ultraviolet spectra. *p*-Aminobenzoic acid has R_f = 0.90 and λ_{max} = 266 nm; 4-(chlorosulfonyl)-benzoic acid has R_f = 0.28 and λ_{max} = 230 nm, as compared to a R_f = 0.80 and λ_{max} = 226 nm for 4-FSB.

The purity of the product was determined by HPLC on a Varian 5000 LC equipped with a Vydac C_{18} column (1 × 25

cm) and a UV-100 detector. The solvent system used was 0.1% trifluoroacetic acid (solvent A) and acetonitrile containing 0.075% trifluoroacetic acid (solvent B). The column was equilibrated with solvent A containing 15% solvent B. After elution for 20 min, a linear gradient was run to 40% solvent B at 120 min, followed by successive linear gradients in solvent B to 50% at 140 min and 100% B at 160 min. The flow rate was 1 mL/min. The effluent was monitored continuously for absorbance at 232 nm, and 1-mL fractions were collected. Aliquots of fractions were mixed with 5 mL of Liquiscint, and the samples were monitored for radioactivity using a Packard Tri-Carb liquid scintillation counter, Model 1500. 4-FSB eluted at 37% solvent B and 4-sulfobenzoic acid eluted in the void volume as judged by comparison with commercial 4-FSB and 4-sulfobenzoic acid standards. The synthesized compound was determined to be >98% pure by absorbance at 232 nm and distribution of radioactivity. The specific radioactivity was calculated to be 5.11×10^{11} cpm/mol by using $\epsilon_{232nm} = 10\,550\ M^{-1}\ cm^{-1}$ for 4-FSB, which was determined in 0.1 M potassium phosphate buffer, pH 7.5.

Determination of the Decomposition Rate of 4-(Fluorosulfonyl)benzoic Acid. The rate of decomposition of 4-FSB was determined by monitoring the release of fluoride by means of a fluoride electrode (Orion). A standard curve was determined for the dependence of electrode potential on fluoride concentration. The fluoride concentration was measured over a 66-h period in a solution of 1 mM 4-FSB in 0.1 M potassium phosphate buffer, pH 7.5, devoid of enzyme. The 4-FSB was found to decompose at a rate of $0.030\ h^{-1}$ ($t_{1/2}$ = 23 h). The possibility of enzyme-catalyzed 4-FSB decomposition was also evaluated by the inclusion of glutathione *S*-transferase, isozyme 4-4 (0.4 mg/mL), in the reaction mixture. No significant change in the rate of decomposition of 4-FSB was observed.

Enzymatic Assays. Generally, enzymatic activity was measured using a Gilford Model 240 spectrophotometer by monitoring the formation of the conjugate of glutathione (2.5 mM) and 1-chloro-2,4-dinitrobenzene (1 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). In the measurement of the enzyme-catalyzed rate, a correction was always made for the spontaneous nonenzymatic reaction rate between glutathione and 1-chloro-2,4-dinitrobenzene.

To ascertain the apparent K_m value of glutathione, a range of subsaturating glutathione concentrations (20–500 μM) were investigated at a constant 1-chloro-2,4-dinitrobenzene concentration (1 mM). Similarly, to determine the apparent K_m of 1-chloro-2,4-dinitrobenzene, a range of subsaturating CDNB concentrations (0.04–2.4 mM) were examined at a constant glutathione concentration (2.5 mM). Data were analyzed as the double-reciprocal plot of substrate concentration versus enzymatic rate of conjugation, with the y intercept = $1/V_{max}$ and the slope = K_m/V_{max}.

Other substrates were utilized to assay for enzymatic activity according to the methods described by Habig *et al.* (1974). All measurements were made in 0.1 M potassium phosphate, pH 6.5, at 25 °C, and in each case, corrections were made for the spontaneous nonenzymatic rate of reaction between glutathione and the hydrophobic substrate employed. The conjugation of *trans*-stilbene oxide (0.1 mM) and glutathione (2.5 mM) was monitored at 232 nm ($\Delta\epsilon = -8.0\ mM^{-1}\ cm^{-1}$). Measurements using ethacrynic acid (0.2 mM) and *trans*-4-phenyl-3-buten-2-one (0.05 mM) were made with a lower glutathione concentration (0.625 mM) because of the relatively large spontaneous nonenzymatic rate of reaction, at 270 nm ($\Delta\epsilon = 5.0\ mM^{-1}\ cm^{-1}$) and 290 nm ($\Delta\epsilon = -24.8\ mM^{-1}\ cm^{-1}$), respectively.

Determination of the apparent K_m for ethacrynic acid was accomplished by measuring the enzymatic rate of conjugation of ethacrynic acid and glutathione (0.625 mM) over a range of ethacrynic acid concentrations (0.025–0.6 mM). As before, data were analyzed as the double-reciprocal plot of substrate concentration versus enzymatic rate of conjugation, with the y intercept = $1/V_{max}$ and the slope = K_m/V_{max}.

Reaction of 4-(Fluorosulfonyl)benzoic Acid with Glutathione S-Transferase. Glutathione *S*-transferase, isozyme 4-4 (0.4 mg/mL), was incubated at 25 °C in 0.1 M potassium phosphate buffer, pH 7.5, with various concentrations of 4-FSB by the addition of appropriate volumes of a 46 mM stock solution of 4-FSB dissolved in DMF. The volume of DMF was adjusted such that this solvent always constituted 10% of the volume of the reaction mixture. When the effect of ligands on the rate of inactivation was studied, the enzyme was preincubated with the ligands for 5 min prior to the addition of 4-FSB. Control enzyme samples were incubated under the same conditions including 10% DMF, but without 4-FSB. At various times, aliquots were withdrawn and diluted 10-fold with 0.1 M potassium phosphate buffer, pH 6.8, at 0 °C. The enzyme was then assayed for residual enzymatic activity. In order to determine the rate of reaction of glutathione *S*-transferase and 4-FSB, the limiting residual activity present at the end of the reaction, E_∞, had to be taken into account, as described under Results. Several concentrations of 4-FSB were investigated, and the average E_∞ was calculated. This E_∞ value was dependent on the hydrophobic substrate employed to assay for enzymatic activity. The k_{obs} value for the reaction was determined from the slope of $\ln[(E_t - E_\infty)/(E_0 - E_\infty)]$ versus time, where E_t and E_0 are the enzymatic velocities at a given time and 0 time, respectively.

In the preparation of modified (and control) enzyme, excess unreacted reagent was removed from the reaction mixture by the gel centrifugation procedure of Penefsky (1979). Aliquots (0.5 mL) of a reaction mixture were withdrawn and applied to two successive 5-mL columns of Sephadex G-50-80 equilibrated with 0.1 M potassium phosphate buffer, pH 7.5. The protein concentration in the filtrate was determined by the Bio-Rad protein assay, which is based on the dye-binding method of Bradford (1976), using a Bio-Rad 2550 RIA reader (600-nm filter). Pure glutathione *S*-transferase, isozyme 4-4, was used to establish the standard protein concentration curve for these determinations.

Measurement of the Incorporation of 4-FSB into Glutathione S-Transferase. Glutathione *S*-transferase (0.4 mg/mL) was incubated with 1.5 mM [3,5-^{3}H]-4-FSB, in the absence and presence of 1 mM bromosulfophthalein, under standard reaction conditions. Aliquots were withdrawn at various times. As described previously, excess reagent was removed by column centrifugation and the protein concentration determined by the dye-binding method. The incorporation of 4-FSB into the 4-4 isozyme was determined as the number of moles of radioactive reagent per mole of enzyme subunit.

Preparation and Separation of Proteolytic Digest of Modified Glutathione S-Transferase. Glutathione *S*-transferase (1 mg) was incubated with 1.5 mM [3,5-^{3}H]-4-FSB, in the absence or presence of 100 μM bromosulfophthalein, for the indicated time. The reaction mixture was then divided into 0.5-mL aliquots and excess reagent removed as described above. In order to block the free cysteine residues of the enzyme, the effluents from all columns were pooled and

incubated with 10 mM N-ethylmaleimide for 5 min at 25 °C; then urea was added to a final concentration of 9 M. After an additional 30-min incubation at 25 °C, the solution was dialyzed against 6 L of 50 mM ammonium bicarbonate, pH 8.0, at 4 °C, with one change for a total of 18 h.

Following dialysis, the solution of modified enzyme was lyophilized. To solubilize the enzyme, 250 μL of 8 M urea in 50 mM ammonium bicarbonate was added. The enzyme was incubated at 37 °C for 2 h before the addition of 750 μL of 50 mM ammonium bicarbonate to give a final urea concentration of 2 M. The modified glutathione S-transferase was digested at 37 °C with two additions of N-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (2.5% w/w) at 1-h intervals. The solution was then centrifuged at 15 000 rpm for 15 min. Aliquots of the supernatant and pellet were withdrawn and mixed with 5-mL portions of Liquiscint, and the samples were monitored for radioactivity; the supernatant was found to contain all of the radioactivity.

The tryptic peptides present in the supernatant were isolated by HPLC using the same system as that employed for determination of the purity of the synthesized 4-FSB. The effluent was monitored continuously at 220 nm, and fractions of 1 mL were collected. Aliquots of fractions were mixed with 5-mL portions of Liquiscint, and the samples were monitored for radioactivity.

Analysis of Isolated Peptides. The amino acid sequences of peptides were determined on an Applied Biosystems gas-phase protein (peptide) sequencer, Model 470, equipped with a phenylthiohydantoin analyzer, Model 120, and a Model 900A computer. Typically, 50–800-pmol samples of peptide were analyzed.

Preparation of Modified Enzyme for Affinity Chromatography. Glutathione S-transferase (0.4 mg/mL) was reacted with 1.5 mM 4-FSB for 150 min under standard incubation conditions. The reaction was stopped by the addition of dithiothreitol to yield a final concentration of 10 mM. The solution was incubated for 10 min at 25 °C before dialysis at 0 °C against 6 L of 0.05 M potassium phosphate buffer, pH 7.5, with one change for 18 h. The enzyme was then concentrated to 1 mg/mL using an Amicon concentrator, Model 52, fitted with an Amicon PM 10 ultrafiltration membrane.

Enzyme modified by S-(4-bromo-2,3-dioxobutyl)glutathione was prepared as described by Katusz and Colman (1991). Glutathione S-transferase, isozyme 4-4 (0.4 mg/mL), was incubated for 60 min with 200 μM S-BDB-G in 0.44 M potassium phosphate buffer, pH 6.5. The enzyme exhibited a residual activity of 17% when 1-chloro-2,4-dinitrobenzene was used as the substrate and there was an incorporation of 1.1 mol of reagent/mol of subunit. The solution was dialyzed at 0 °C against 6 L of 0.05 M potassium phosphate buffer, pH 7.5, with one change for 18 h, and then concentrated to 1 mg/mL as described above. The substrate-binding properties of the S-BDB-G-modified enzyme, along with 4-FSB-modified enzyme, were tested in the affinity chromatography experiments described below.

Affinity Chromatography. A 5-mL column of S-hexylglutathione–agarose was equilibrated with 0.05 M potassium phosphate buffer containing 0.2 mM dithiothreitol. A 1-mL sample (1 mg) of modified or unmodified control enzyme was applied to the column. The column was washed with four bed volumes of starting buffer, followed by four bed volumes of starting buffer containing 0.2 M NaCl, and finally by eight bed volumes of 5 mM S-hexylglutathione in the high-salt buffer. The effluent was monitored continuously for absorbance at 280 nm using an ISCO Model UA-5 absorbance monitor equipped with a type 6 optical unit. Fractions of 2 mL were collected using an ISCO Model 328 fraction collector. Fractions were assayed for enzymatic activity using CDNB and the protein concentrations determined by the dye-binding method.

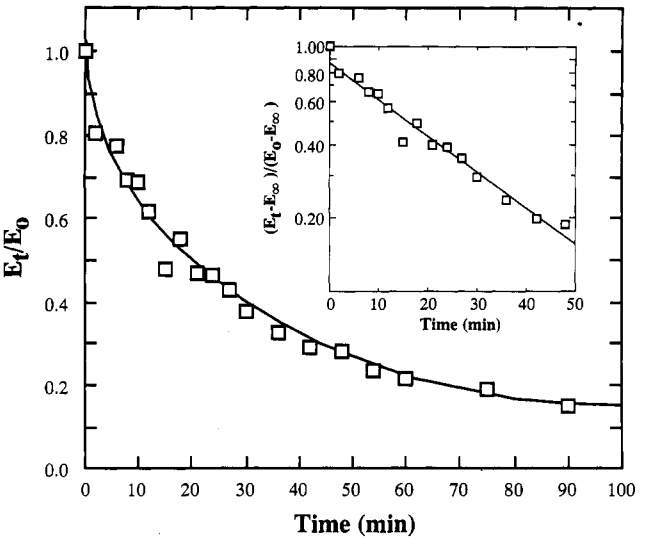

FIGURE 2: Inactivation of glutathione S-transferase, isozyme 4-4, by 4-FSB. Rat liver glutathione S-transferase, isozyme 4-4 (0.4 mg/mL), was incubated with 1.5 mM 4-FSB at pH 7.5 and 25 °C. Residual activity, E/E_0, was measured using CDNB as a substrate, as described under Experimental Procedures. Inset: The 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 enzymatic velocities at time 0 and time t, respectively, and E_∞ is the enzymatic rate at the end of the reaction. In this case, $E_\infty = 0.12E_0$ and $k_{obs} = 0.0342$ min^{-1}.

A 5-mL column of Matrex Gel Blue-A was equilibrated with 0.05 M potassium phosphate buffer, and a 1-mL sample (1 mg) of modified or unmodified control enzyme was applied to the column. The column was washed with four bed volumes of starting buffer, followed by eight bed volumes of 50 μM BSP in the starting buffer. The effluent was monitored continuously for absorbance at 280 nm as described above. As with the S-hexylglutathione affinity column, 2-mL fractions were collected and assayed for activity, and the protein concentrations were determined.

Determination of the Binding Constant of Glutathione for Modified Glutathione S-Transferase. Glutathione S-transferase was incubated with 1.5 mM 4-FSB for 150 min under standard reaction conditions, and excess reagent was removed as described above. The K_d value for glutathione was determined by fluorescence titration using the method of Zhang and Armstrong (1990). The decrease in protein fluorescence of glutathione S-transferase (0.89 μM) was monitored as a function of glutathione concentration (1.0–50 μM). Measurements were made using a Perkin-Elmer fluorescence spectrophotometer, Model MPF-3, with excitation at 280 nm (5-nm slit width) and emission monitored at 355 nm (10-nm slit width).

RESULTS

Inactivation of Glutathione S-Transferase, Isoenzyme 4-4, by 4-(Fluorosulfonyl)benzoic Acid. Incubation of glutathione S-transferase with 1.5 mM 4-FSB at pH 7.5 and 25 °C resulted in a time-dependent inactivation of the enzyme (Figure 2). In contrast, control enzyme, incubated under the same conditions but in the absence of reagent, showed no loss of activity. As seen in Figure 2, the rate of inactivation decreased at longer

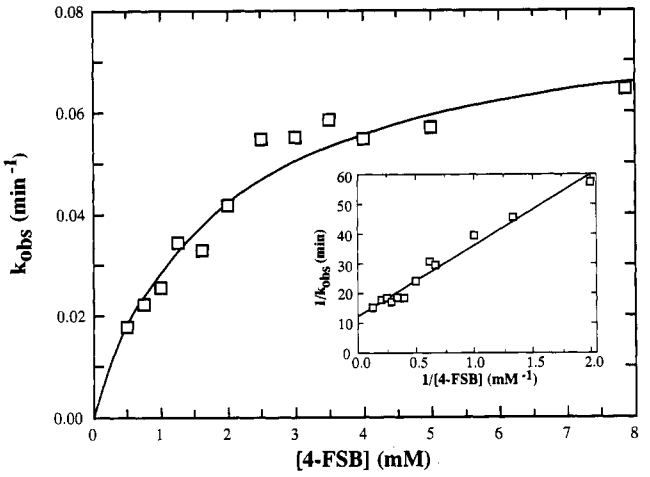

FIGURE 3: Dependence of the rate of inactivation of glutathione *S*-transferase, isozyme 4-4, on the concentration of 4-FSB. Glutathione *S*-transferase was incubated with various concentrations of 4-FSB under the conditions given in Figure 2. For each 4-FSB concentration, k_{obs} was calculated as illustrated in Figure 2, inset, with $E_\infty = 0.12E_0$.

times and the enzyme appeared to be reaching a limiting activity. Several explanations for this observation were considered.

Sulfonyl fluorides can hydrolyze in aqueous solution, and the slowing of the inactivation rate may reflect depletion of the reagent. To test this hypothesis, the rate of decomposition of 4-(fluorosulfonyl)benzoic acid was determined by monitoring its loss of fluoride as described under Experimental Procedures. The rate constant, under the identical incubation conditions, was 0.030 h^{-1} ($t_{1/2}$ = 23 h). Since the decomposition rate is so slow, reagent depletion is excluded as an explanation for the curvature observed in Figure 2.

Although the rate of loss of fluoride from 4-FSB is slow, the possibility exists that the hydrolyzed reagent binds tightly to the enzyme and protects against inactivation. Accordingly, 4-sulfobenzoic acid was added to the incubation mixture with 1.5 mM 4-FSB, and its effects on the rate of inactivation were observed. Addition of 100 μM 4-sulfobenzoic acid had no effect on the rate of inactivation, while inclusion of 1.5 mM 4-sulfobenzoic acid reduced the inactivation rate to half of its original value. These results indicate that 4-FSB and 4-sulfobenzoic acid have comparable affinities for the enzyme; thus protection against inactivation by the very low concentrations of 4-sulfobenzoic acid which are generated by 4-FSB hydrolysis cannot account for the observed kinetics.

A range of 4-FSB concentrations were investigated, and in each case, a limiting residual activity was reached. The average limiting residual activity was 12% when CDNB was used as a substrate. The k_{obs} value for the reaction was determined from the slope of $\ln[(E_t - E_\infty)/(E_0 - E_\infty)]$ versus time, as seen in the inset of Figure 2. E_t and E_0 are the enzymatic velocities at a given time and 0 time, respectively, and the velocity at the end of the reaction E_∞ is $0.12E_0$. As will be discussed later, however, the limiting value is dependent on the substrate selected for assay of enzymatic activity.

Concentration Dependence of the Rate Constant for Inactivation of Glutathione S-Transferase by 4-FSB. Glutathione *S*-transferase, isoenzyme 4-4, was incubated with various concentrations of 4-FSB (0.5–7.85 mM) to determine the dependence of the rate of inactivation on reagent concentration (Figure 3). k_{obs} exhibits a nonlinear dependence on the 4-FSB concentration. This result suggests the initial formation of an enzyme–reagent complex prior to irreversible modification, indicative of an affinity label. The observed rate constant (k_{obs}) at a particular concentration of reagent (R) can be described by

$$1/k_{obs} = 1/k_{max} + (K_I/k_{max})(1/[R]) \quad (1)$$

where k_{max} = the maximum rate of inactivation at saturating concentrations of the reagent and $K_I = (k_{-1} + k_{max})/k_1$ and represents the reagent concentration that results in half of the maximal inactivation rate (Huang & Colman, 1984). The double-reciprocal plot (Figure 3, inset) was used to calculate k_{max} = 0.082 min^{-1} and K_I = 1.95 mM.

Table I: Effect of Substrate Analogues on the Rate Constant for Inactivation by 1.5 mM 4-FSB

	ligand added to reaction mixture	k_{+L}/k_{-L}[a]
1	none	1.00
2	*S*-(4-nitrobenzyl)glutathione (1 mM)	0.18
3	*S*-hexylglutathione (5 mM)	0.30
4	*S*-methylglutathione (5 mM)	0.40
5	*S*-methylglutathione (5 mM) + 2,4-dinitrophenol (10 mM)	0.15
6	*S*-hexylglutathione (5 mM) + 2,4-dinitrophenol (10 mM)	0.06
7	2,4-dinitrophenol (5 mM)	0.39
8	2,4-dinitrophenol (10 mM)	0.22
9	bromosulfophthalein (100 μM)	0.12

[a] k_{-L} = 0.0342 min^{-1}.

Effect of Substrate Analogues on the Inactivation Rate of Glutathione S-Transferase by 4-FSB. The effect of substrate analogues on the reaction rate of 1.5 mM 4-FSB with glutathione *S*-transferase was investigated. The results, shown in Table I, are expressed as the ratio of the inactivation rate constant measured in the presence of a particular ligand (k_{+L}) to the rate constant measured in the absence of any added ligand (k_{-L}). Addition of the product analogue, *S*-(nitrobenzyl)glutathione (Table I, line 2), led to a large decrease in the rate constant for inactivation (low k_{+L}/k_{-L}), indicating that reaction occurs in the active site. *S*-Alkylglutathione derivatives (Table I, lines 3 and 4) also provided substantial protection. However, the protective effect decreases as the alkyl side chain becomes shorter, implying that the reaction target is not the glutathione-binding site. Combination of an alkylglutathione derivative and a hydrophobic substrate analogue (Table I, lines 5 and 6) gives marked protection against inactivation. The k_{+L}/k_{-L} values are similar to that observed in the presence of the product analogue, *S*-(nitrobenzyl)glutathione, indicating that reaction occurs in the hydrophobic substrate-binding site. Inclusion of a hydrophobic substrate analogue, particularly bromosulfophthalein (Table I, lines 7–9), in the reaction mixture causes a large decrease in the rate constant for inactivation by 4-FSB, confirming the hydrophobic substrate-binding site as the reaction site.

Incorporation of 4-FSB by Glutathione S-Transferase. Glutathione *S*-transferase was incubated with 1.5 mM [3,5-^{3}H]-4-FSB. A time-dependent incorporation of [3,5-^{3}H]-4-FSB was observed concomitant with an increase in percent maximal inactivation. Figure 4 shows the secondary plot of percent maximal inactivation versus incorporation. Extrapolation to maximal inactivation yields a value of 0.82 mol of reagent/mol of enzyme subunit.

In order to ascertain whether a hydrophobic substrate could prevent reagent incorporation as well as inactivation, glutathione *S*-transferase was incubated with 1.5 mM [3,5-^{3}H]-4-FSB for 90 min in the absence or presence of 1 mM bromosulfophthalein (BSP). In the absence of BSP, 0.77 mol of reagent/mol of enzyme subunit was incorporated while the enzyme reached 96% of its maximal inactivation. In contrast, in the presence of the hydrophobic substrate analogue

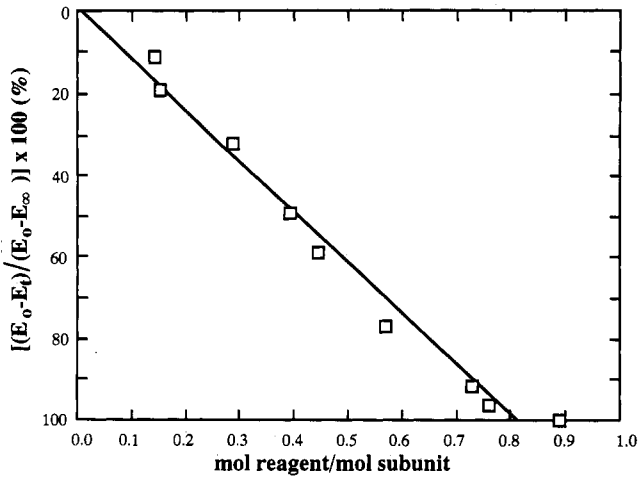

FIGURE 4: Incorporation of 4-FSB into the 4-4 isozyme as a function of percent maximal inactivation. Upon complete modification, glutathione S-transferase reaches a limiting residual activity as assayed with CDNB as the substrate. Loss of activity is therefore expressed as percent maximal inactivation $[(E_0 - E_t)/(E_0 - E_\infty)] \times 100$, with constants defined in the caption to Figure 2.

bromosulfophthalein, only 0.16 mol of reagent/mol of enzyme subunit was incorporated at 90 min, and the enzyme reached only 22% of its maximal inactivation. This result supports the postulate that 4-FSB reacts within the hydrophobic binding site.

Isolation of Tryptic Peptides from Modified Glutathione S-Transferase. Glutathione S-transferase (0.4 mg/mL) was incubated with 1.5 mM [3,5-^{3}H]-4-FSB at pH 7.5 and 25 °C. The modified enzyme was treated with N-ethylmaleimide (NEM) to block its –SH groups and then digested with trypsin as described under Experimental Procedures. The digest was fractionated by HPLC with a C_{18} reverse-phase column by 0.1% trifluoroacetic acid with an acetonitrile gradient, as shown in Figure 5.

Figure 5C (open squares) shows the distribution of radioactivity after a 70-min incubation with 4-FSB. Four major radioactive regions, designated I–IV, are seen. As a control, a sample containing [3,5-^{3}H]-4-FSB and hydrolyzed nonradioactive 4-FSB, but no enzyme, was subjected to the same chromatographic system as the tryptic digest. Hydrolyzed 4-FSB was found in the void volume whereas [3,5-^{3}H]-4-FSB eluted at 37% acetonitrile. Peaks III and IV were therefore identified as hydrolyzed 4-FSB and free 4-FSB, respectively. Thus, peaks I and II were expected to contain the peptides of interest.

Upon a decrease in the incubation time with 4-FSB to 15 min, with the concomitant decrease in maximal inactivation, peaks I and II clearly decreased (Figure 5B). These results further suggested the role of peaks I and II in loss of enzymatic activity. Furthermore, a 70-min incubation with 4-FSB was carried out in the presence of 100 μM bromosulfophthalein (Figure 5C, filled circles). Peaks I and II were almost completely absent, and the enzyme retained nearly all of its activity.

Characterization of Modified Peptides. Peaks I and II were each subjected to gas-phase amino acid sequencing, and representative data are given in Table II. The sequence Leu-Gln-Leu-Ala-Met-Val-(NEM-Cys)-X-Ser-Pro-Asp-Phe-Glu-Arg corresponds to residues 108–121 in the known amino acid sequence of glutathione S-transferase, isozyme 4-4 (Alin *et al.*, 1986; Ding *et al.*, 1986). The X at cycle 8 indicates that the phenylthiohydantoin derivative did not migrate as a standard PTH-amino acid derivative. Since the known amino acid sequence contains a tyrosine at this position, the peptide is most likely modified at the tyrosine (Tyr[115]). In support of this conclusion, the PTH derivative isolated migrates similarly to the PTH derivative of O-[(4-carboxyphenyl)-sulfonyl]tyrosine (CPS-Tyr) (Saradambal *et al.*, 1981) and is the only PTH-amino acid which contains radioactivity. Thus, peaks I and II correspond to the same peptide, as indicated by the amino acid sequences given in Table II.

Table II: Representative Sequences of Modified Peptides Present after Incubation with 1.5 mM [3,5-^{3}H]-4-FSB[a]

	amino acid (pmol)			amino acid (pmol)	
cycle no.	peak I	peak II	cycle no.	peak I	peak II
1	Leu (346)	Leu (155)	8	X (85)[c]	X (44)[c]
2	Gln (153)	Gln (121)	9	Ser (59)	Ser (24)
3	Leu (349)	Leu (147)	10	Pro (162)	Pro (58)
4	Ala (290)	Ala (127)	11	Asp (60)	Asp (28)
5	Met (217)	Met (104)	12	Phe (150)	Phe (54)
6	Val (158)	Val (80)	13	Glu (57)	Glu (22)
7	NEM-Cys[b]	NEM-Cys[b]	14	Arg (16)	Arg (10)

[a] These are representative sequences and do not reflect the relative magnitude of peaks I and II. [b] In gas-phase sequence analysis, NEM-Cys can be detected as a doublet migrating on the HPLC column between the PTH derivatives of Pro and Met (Smyth & Colman, 1991). [c] An X at a given cycle indicates that the phenylthiohydantoin derivative did not migrate as a known PTH-amino acid derivative. The amount of derivative present was determined by measuring radioactivity recovered from sequencing and calculating the pmol of derivative using the specific radioactivity of [3,5-^{3}H]-4-FSB.

The same peptide may yield two distinct radioactive peaks because it exhibits mixed modes of interaction with the column (DeCamp and Colman, 1989; Jacobson and Colman, 1984; Batra and Colman, 1986). Peak I, when rechromatographed under the same conditions as employed for its initial isolation, clearly redistributed into two separate peaks corresponding to peaks I and II. Peak II did not contain sufficient material to perform the reciprocal experiment with precision; rechromatography of peak II resulted in redistribution into a single peak eluting at the position of peak I.

Binding Properties of Modified Glutathione S-Transferase. In order to elucidate the site at which 4-FSB attacks glutathione S-transferase, the ability of inactive 4-FSB-modified enzyme to bind glutathione and hydrophobic substrates was evaluated in comparison with active control enzyme. The dissociation constants for GSH from both the 4-FSB-modified and control enzymes were determined by fluorescence titration. It has been demonstrated that upon binding of GSH, the protein fluorescence of glutathione S-transferase decreases, and this change in fluorescence can be employed to determine GSH-binding constants (Zhang & Armstrong, 1990). Quenching of protein fluorescence was monitored as a function of glutathione concentration, as described under Experimental Procedures. Determination of the glutathione concentration that caused half-maximal change of protein fluorescence yielded similar dissociation constants for GSH from modified and control enzymes of 3.2 and 5.3 μM, respectively. These results are consistent with the indistinguishable ability of control enzyme and inactive 4-FSB-modified enzyme to bind to an S-hexylglutathione agarose column (data not shown). In comparison, glutathione S-transferase modified with S-(4-bromo-2,3-dioxobutyl)glutathione (Katusz & Colman, 1991) was unable to bind to the column and eluted in the void volume.

Matrex Gel Blue-A was selected to determine the ability of the 4-FSB-modified enzyme to bind a hydrophobic substrate. This resin was chosen because of its similarity to Cibacron Blue (Figure 1, structure VII), a known inhibitor of glutathione S-transferases, which binds tightly to the hydrophobic sub-

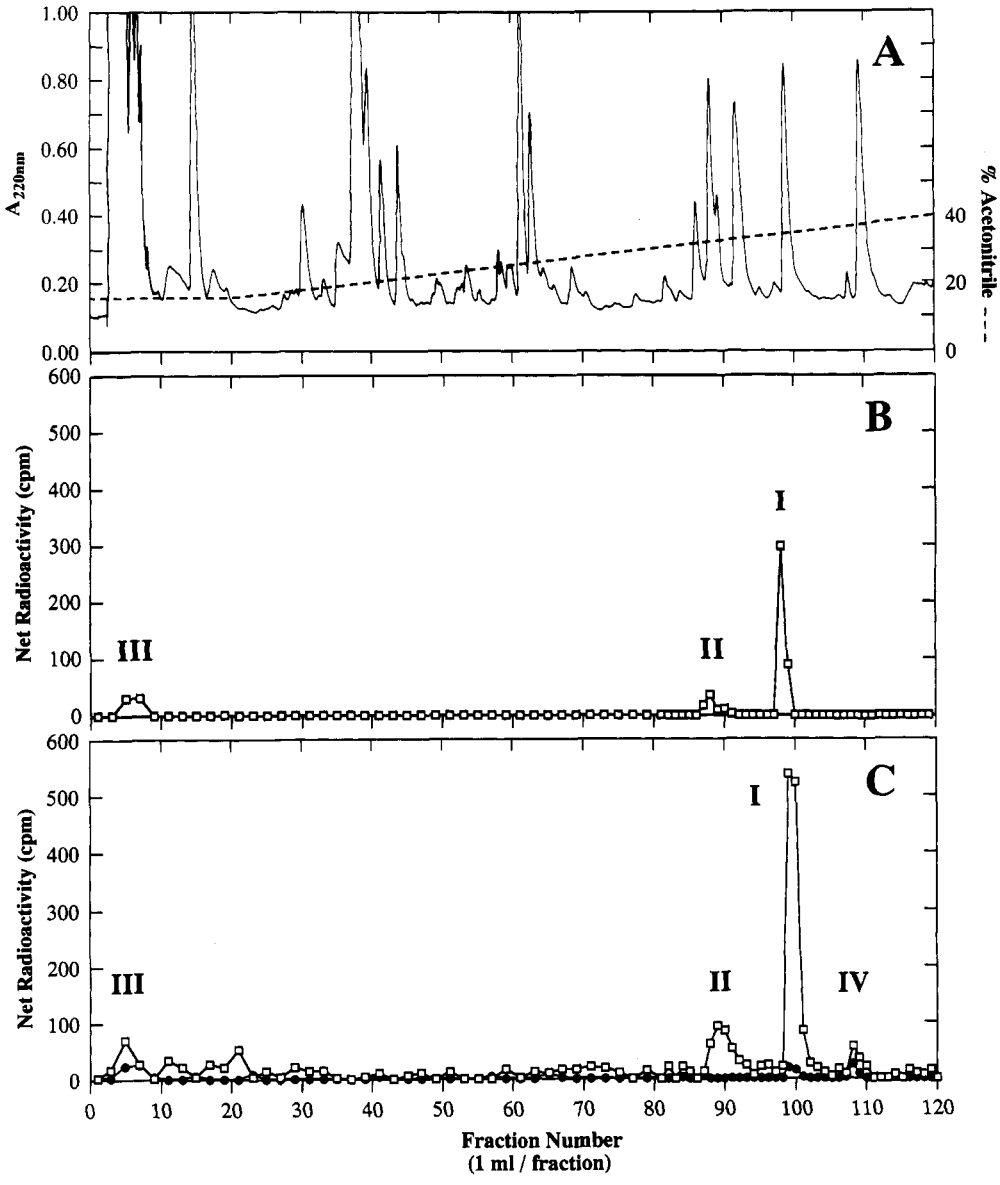

FIGURE 5: Fractionation of tryptic peptides from modified glutathione *S*-transferase by HPLC. Peptides resulting from the modification of glutathione *S*-transferase by 1.5 mM [3,5-^{3}H]-4-FSB were isolated by HPLC using a C_{18} column equilibrated with 0.1% trifluoroacetic acid and a gradient of acetonitrile, as described under Experimental Procedures: (A) A_{220nm} profile of the digest obtained after a 70-min incubation with [3,5-^{3}H]-4-FSB; (B) distribution of the radioactivity in the digest after a 15-min incubation with [3,5-^{3}H]-4-FSB; (C) distribution of the radioactivity in the digest obtained after a 70-min incubation with [3,5-^{3}H]-4-FSB in the absence (open squares) or presence (filled circles) of 100 μM bromosulfophthalein. No significant radioactivity was detected in the regions of the chromatograph not shown.

strate-binding site (Alin *et al.*, 1985; Guthenberg *et al.*, 1985). Glutathione *S*-transferase binds very tightly to the column, even at high salt concentrations, and requires the addition of the hydrophobic inhibitor, bromosulfophthalein (see Figure 1, structure **VI**), to effect elution. As shown in Figure 6 (filled circles), control enzyme remains bound to the resin until the addition of BSP. The 4-FSB-modified enzyme (Figure 6, open squares), however, elutes in the void volume of the column. A similar outcome was observed when enzyme modified by *S*-BDB-G was used. These results suggest that the reaction site of 4-FSB is within the hydrophobic substrate-binding site. However, since the *S*-BDB-G-modified enzyme does not bind to either the *S*-hexylglutathione–agarose or the Matrex Gel Blue-A column, these affinity chromatography experiments do not distinguish between the glutathione and hydrophobic substrate sites as the target for *S*-BDB-G modification.

Comparison of the Kinetic Properties of Native and 4-FSB-Modified Enzymes. The catalytic properties of 4-FSB-modified and control enzymes were investigated using as substrates 1-chloro-2,4-dinitrobenzene, ethacrynic acid, *trans*-4-phenyl-3-buten-2-one, and *trans*-stilbene oxide (Figure 1, structures **II–V**). As mentioned previously, when the reaction of glutathione *S*-transferase with 4-FSB is monitored, the percent residual activity depends on the substrate employed to assay for activity. Table III shows that ethacrynic acid yields the highest percent residual activity (20%), and *trans*-4-phenyl-3-buten-2-one, the lowest (2%). Modification of glutathione *S*-transferase clearly affects differentially the ability of the enzyme to utilize various substrates.

The apparent K_m and V_{max} values of modified and control enzymes for glutathione, CDNB, and ethacrynic acid were determined. No significant change in the affinity of the enzyme for glutathione was observed upon modification by 4-FSB; the $K_{m\text{-app}}$ values for glutathione were 67 and 64 μM for control and modified enzymes, respectively. In contrast, the $K_{m\text{-app}}$ for CDNB of 4-FSB-modified enzyme increased nearly 9-fold (Table IV), while the $V_{max\text{-app}}$ value decreased over 4-fold. Overall, the catalytic efficiency of the modified

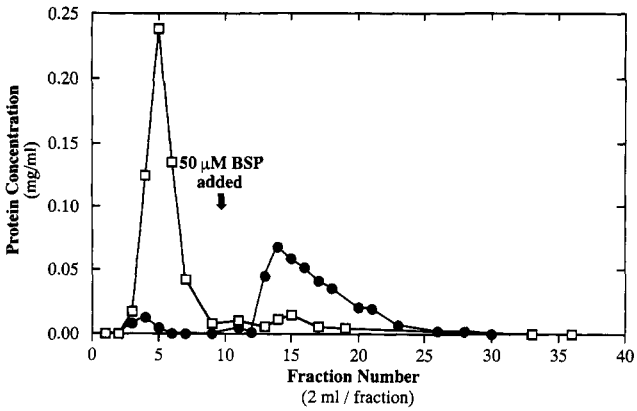

FIGURE 6: Matrex Gel Blue-A affinity column elution profile. Modified (open squares) and control (filled circles) enzymes (1 mg) were each applied to the Cibacron Blue–agarose affinity column. The column was washed with 0.05 M potassium phosphate buffer, pH 7.5, and then with buffer containing 50 μM bromosulfophthalein. The protein concentration in each 2-mL fraction was determined by the dye-binding method described under Experimental Procedures.

Table III: Use of Alternative Substrates by Modified Glutathione S-Transferase, Isozyme 4-4

substrate	enzymatic velocity ((mol of substrate/min)/mg of enzyme) modified enzyme	enzymatic velocity ((mol of substrate/min)/mg of enzyme) unmodified enzyme	% residual activity
ethacrynic acid[a]	0.076	0.382	20
1-chloro-2,4-dinitrobenzene[b]	1.71	15.9	11
trans-stilbene oxide[c]	0.020	0.815	2.5
trans-4-phenyl-3-buten-2-one[d]	0.047	2.40	2

[a] The reaction of ethacrynic acid (200 μM) and glutathione (625 μM) was monitored at 270 nm, pH 6.5, as described under Experimental Procedures. [b] The reaction of 1-chloro-2,4-dinitrobenzene (1 mM) and glutathione (2.5 mM) was monitored at 340 nm, pH 6.5, as described under Experimental Procedures. [c] The reaction of *trans*-stilbene oxide (100 μM) and glutathione (2.5 mM) was monitored at 232 nm, pH 6.5, as described under Experimental Procedures. [d] The reaction of *trans*-4-phenyl-3-buten-2-one (50 μM) and glutathione (625 μM) was monitored at 290 nm, pH 6.5, as described under Experimental Procedures.

Table IV: Determination of Apparent Kinetic Constants for Selected Hydrophobic Substrates

substrate	$K_{m\text{-app}}$ (μM)	$V_{max\text{-app}}$ ((μmol/min)/mg of enzyme)	$k_{cat\text{-app}}$[a] (s^{-1})	$(k_{cat}/K_m)_{app}$ (M^{-1} s^{-1})
1-chloro-2,4-dinitrobenzene				
control enzyme	193	22.90	10.10	52400
modified enzyme	1690	4.95	2.19	1300
ethacrynic acid				
control enzyme	63	0.571	0.252	4000
modified enzyme	132	0.144	0.0636	480

[a] $k_{cat\text{-app}}$ is defined as micromoles of substrate converted per micromole of enzyme per second.

enzyme, as judged by apparent k_{cat}/K_m, decreased nearly 40-fold.

Interestingly, the $K_{m\text{-app}}$ value for ethacrynic acid did not change dramatically: only a 2-fold increase was noted for the modified enzyme as compared to control enzyme (Table IV). Instead, the catalytic step of the modified enzyme seems to have been affected as reflected in its decreased $V_{max\text{-app}}$. The nearly 4-fold decrease in $V_{max\text{-app}}$ and modest change in apparent K_m, equating to an 8-fold decrease in catalytic efficiency, suggest that Tyr[115] may be involved directly in catalysis rather than only facilitating binding of the xenobiotic substrate.

DISCUSSION

4-(Fluorosulfonyl)benzoic acid serves as an effective affinity label of rat liver glutathione S-transferase, isozyme 4-4. A time-dependent decrease in activity is observed upon incubation of this enzyme with 4-FSB. The rate of enzyme inactivation exhibits a nonlinear dependence on 4-FSB concentration, becoming independent of 4-FSB concentration at high reagent concentrations. This observation suggests the formation of an enzyme–reagent complex prior to irreversible modification, indicative of an affinity label. Upon complete modification of glutathione S-transferase, isozyme 4-4, by 4-FSB, nearly 1 mol of reagent/mol of enzyme subunit is incorporated. Protection against incorporation and inactivation is afforded by hydrophobic substrates or their analogues, especially the bulky inhibitor bromosulfophthalein.

A single peptide containing covalently bound 4-FSB is isolated after tryptic digestion of modified enzyme. The peptide, comprising residues 108–121 of the known amino acid sequence of glutathione S-transferase, isozyme 4-4 (Alin *et al.*, 1986; Ding *et al.*, 1986), can be prevented from reacting with 4-FSB by the presence of bromosulfophthalein, indicating that this peptide is located in or near the enzyme active site. Amino acid sequence analysis of the isolated peptide reveals Tyr[115] as the site of reaction of radioactive 4-FSB as demonstrated by identification of the phenylthiohydantoin derivative in cycle 8 as *O*-[(4-carboxyphenyl)sulfonyl]tyrosine, as well as the detection of radioactivity during this cycle. We therefore conclude that reaction of 4-FSB with Tyr[115] is responsible for inactivation of the enzyme. Alignment of representative sequences of the subunits of the isozymes of rat liver glutathione S-transferases shows that Tyr[115] is conserved in subunits 3, 4, and 7, supporting its importance in enzyme function, at least for the μ and π classes.

Tyr[115] was first implicated in the function of rat liver glutathione S-transferase, isozyme 4-4, as a result of affinity labeling studies from this laboratory using S-(4-bromo-2,3-dioxobutyl)glutathione (Katusz & Colman, 1991). On the basis of protection studies, it was postulated that Tyr[115] was important in providing hydrophobic interactions for the non-glutathione substrate. However, because of its glutathione moiety, S-BDB-G presumably also occupied the glutathione site, and reduced glutathione was not expected to bind to the modified enzyme. Thus, it was not surprising that the modified enzyme was rendered completely inactive. As reported in the present study, enzyme modified with S-BDB-G does not bind to either an S-hexylglutathione affinity column or a Matrex Gel Blue-A column, making it difficult to differentiate between a role for Tyr[115] in glutathione binding and a role in the binding of the hydrophobic substrate.

Glutathione S-transferase, isozyme 4-4, modified with 4-FSB is much more amenable to analysis of the role of Tyr[115]. Modified enzyme retains its affinity for glutathione, as demonstrated by protein fluorescence titration. In comparing modified and native enzymes, we observed no significant difference in the K_d values or the apparent K_m values for glutathione. Furthermore, native and 4-FSB-modified enzymes exhibit similar chromatographic behavior on an S-hexylglutathione–agarose affinity column. These results indicate that a free Tyr[115] is not required for efficient glutathione binding. In contrast, glutathione S-transferase modified with 4-FSB does lose its ability to bind to the Matrex Gel Blue-A affinity column, suggesting that Tyr[115] is indeed important in the binding of hydrophobic substrates or, at the very least, is located within the hydrophobic substrate-binding site.

Pennington and Rule (1992), using human class μ muscle glutathione *S*-transferase, mutated Tyr^{116} (equivalent to Tyr^{115} in the 4-4 isozyme isolated from rat liver) to phenylalanine. No decrease in the specific activity of the enzyme was observed when 1-chloro-2,4-dinitrobenzene was used as a substrate. This observation could be interpreted to indicate that the phenolic moiety of Tyr^{116} was not critical for general functioning and that Tyr^{116} instead contributed hydrophobic interactions specifically to the xenobiotic substrate, a contribution that could also be provided by phenylalanine. Johnson *et al.* (1993), using the 3-3 isozyme of rat liver glutathione *S*-transferase, have also shown that the mutation of Tyr^{115} to phenylalanine causes no decrease in specific activity when CDNB is used as the substrate and instead causes a slight increase in enzymatic activity. They postulate that Tyr^{115} forms a hydrogen bond to the hydroxyl group of Ser^{209}, which reduces the rate of product release (the rate-limiting step for the reaction of CDNB with glutathione). Substitution of phenylalanine for Tyr^{115} removes this interaction and causes an increased specific activity.

In contrast, Johnson *et al.* (1993) found that substitution of phenylalanine for tyrosine at position 115 of rat liver glutathione *S*-transferase, isozyme 3-3, results in a mutant enzyme with greatly reduced catalytic properties when phenanthrene 9,10-oxide is used as the substrate. On the basis of X-ray crystallographic studies of the 3-3 isozyme, they proposed that the hydroxyl group of Tyr^{115} stabilizes the developing charge on the oxirane oxygen of phenanthrene 9,10-oxide and thus participates directly in the chemical step, which is rate determining for this substrate. This analysis may shed light on the results of the present investigation.

Examination of the 4-FSB-modified enzyme's ability to utilize a given substrate (Table III) indicates that this modification reaction causes differential effects on various substrates, whose structures are shown in Figure 1. Three general types of reactions catalyzed by glutathione *S*-transferase are examined in Table III: nucleophilic aromatic substitutions, epoxide ring openings, and Michael additions. The enzymatic rates of the Michael addition of glutathione to *trans*-4-phenyl-3-buten-2-one and the ring opening of *trans*-stilbene oxide by glutathione are reduced nearly to zero when enzyme modified with 4-FSB is used. In contrast, the enzymatic rate of catalysis of the nucleophilic aromatic substitution of 1-chloro-2,4-dinitrobenzene by glutathione remains substantial. (The catalytic rates measured for "modified" enzyme may include a contribution from unmodified residual enzyme; however, in view of the differential changes observed using various substrates, this contribution must be small.)

It has long been postulated that a functional group of the enzyme may participate in catalysis by stabilizing a transition state, such as the oxyanion formed during epoxide ring opening or the enolate formed during Michael addition to an α,β-unsaturated ketone (Cobb *et al.*, 1983; Mannervik and Danielson, 1988). The differential effects observed upon modification of glutathione *S*-transferase suggest that Tyr^{115} may be the residue that functions as a general acid in epoxide ring opening and Michael addition reactions, such as those involving *trans*-stilbene oxide, *trans*-4-phenyl-3-buten-2-one, and ethacrynic acid as substrates of glutathione *S*-transferase (see Figure 1 and Table III). Attachment of the (carboxyphenyl)sulfonyl to the hydroxyl group of Tyr^{115} would eliminate its ability to hydrogen-bond to the oxyanion or enolate transition state and greatly reduce the enzyme's catalytic rate. Accordingly, as a result of the enzyme's reduced k_{cat}, a large decrease in the specific activity would be observed. As seen in Table III, this decrease is clearly observed. Furthermore, determination of the kinetic constants for the substrate, ethacrynic acid (Table IV), reveals that the decrease in catalytic efficiency is primarily a result of a decreased $k_{cat\text{-}app}$.

These results suggest that Tyr^{115} must be protonated in order for the enzyme to be catalytically active. Lodi and Knowles (1993) have measured the pK values of two histidine residues at opposite ends of an α-helix and have found that the pK of a residue at the C-terminus has a higher than average value. Since Tyr^{115} is located at the C-terminus of α-helix 4 of glutathione *S*-transferase, isozyme 3-3 (Ji *et al.*, 1992), it is likely that Tyr^{115} of the 4-4 isozyme is also located at the C-terminal of an α-helix and thus would be present predominantly in the protonated state at neutral pH.

It has been inferred that product release is the rate-limiting step of nucleophilic aromatic substitutions (Jakobson *et al.*, 1977; Liu *et al.*, 1992; Zhang *et al.*, 1992) and therefore stabilization of the catalytic intermediate by a general acid may not be an important factor in determining the enzymatic rate of the reaction. Inspection of the kinetic constants for the substrate, CDNB, suggests how enzymatic catalysis may be impaired by modification of glutathione *S*-transferase by 4-FSB. Conjugation of glutathione and 1-chloro-2,4-dinitrobenzene has been proposed to involve nucleophilic attack of the glutathione thiolate (GS^-) on the electron-poor center of C(1) of CDNB followed by displacement of Cl^-. For the 3-3 isozyme, the distance between the phenolic oxygen of Tyr^{115} and the thiolate of glutathione has been determined to be 7.5 Å (Ji *et al.*, 1992). Because of the homology between the 3-3 and 4-4 isozymes, it is not unreasonable to regard this distance as similar in the 4-4 isozyme. Tyr^{115} may position C(1) of CDNB in proximity to the thiolate of glutathione by providing hydrophobic interactions for the benzene ring of the substrate. The coupling of a (carboxyphenyl)sulfonyl group to Tyr^{115} introduces not only more bulk but also a negative charge into the hydrophobic substrate-binding pocket. CDNB may no longer bind in its usual position and, instead, may be forced to bind in an unfavorable orientation, which is less efficient for catalysis. The result is a greatly increased apparent K_m and a decrease in $k_{cat\text{-}app}$.

The introduction of a charged, bulky group into the hydrophobic binding pocket is not as detrimental to substrate binding in the case of ethacrynic acid as compared to CDNB; the apparent K_m for ethacrynic acid only increases 2-fold. The site of glutathione addition to ethacrynic acid is the β-carbon of the α,β-unsaturated ketone, which is relatively distant from the benzene ring of ethacrynic acid. We suggest that the benzene rings of CDNB and EA may be bound somewhat differently in order to correctly position the reactive site of the substrate relative to the thiolate of glutathione. Since the reaction site of ethacrynic acid is not directly on the benzene ring, greater flexibility in binding of the ring is tolerated, hence the reduced effect observed in $K_{m\text{-}app}$.

The less dramatic effects in the limiting residual activity attained with ethacrynic acid as compared to *trans*-4-phenyl-3-buten-2-one and *trans*-stilbene oxide (Table III) may be explained by the electron-withdrawing nature of the substituents on the benzene ring of ethacrynic acid. Kubo and Armstrong (1989) demonstrated that the nature of the substituent of para-substituted 4-phenyl-3-buten-2-ones affected the stereoselectivity of the enzyme. Two transition states were proposed: one wherein the enolate was stabilized by additional electrostatic interaction and that was independent

of substituent effects and one that was not stabilized and was more susceptible to the nature of the substituent. The benzene ring of ethacrynic acid (Figure 1, structure III) features three electron-withdrawing groups that would stabilize the enolate intermediate formed during catalysis, even in the absence of enzyme. Comparison of the nonenzymatic rates, determined in order to correct the measured enzyme-catalyzed rates (data not shown), reveals that the spontaneous nonenzymatic rate of the conjugation of ethacrynic acid and glutathione is approximately 15-fold higher than the conjugation rate of either *trans*-4-phenyl-3-buten-2-one or *trans*-stilbene oxide and glutathione. It is therefore not surprising that the 4-FSB-modified enzyme retains as much as 20% residual activity toward ethacrynic acid simply by positioning the thiolate of glutathione and ethacrynic acid in proximity; the additional stabilization of the transition state by a general acid such as the hydroxyl group of Tyr[115] may be less important in this case.

Tyrosine[115] of rat liver glutathione *S*-transferase, isozyme 4-4, has been modified by the affinity label 4-(fluorosulfonyl)-benzoic acid and has been shown to be located within the hydrophobic substrate-binding site of the enzyme. Modification of this residue has dramatic effects on the activity of the enzyme, greatly reducing its specific activity for a number of hydrophobic substrates. This decreased activity is accomplished primarily either by interfering with the substrate binding or by eliminating the stabilization of the transition state afforded by the hydroxyl group of Tyr[115], depending on the substrate.

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