Contribution of Amino Acid Residue 208 in the Hydrophobic Binding Site to the Catalytic Mechanism of Human Glutathione Transferase A1-1†

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ABSTRACT: Glutathione transferases (GSTs) catalyze the nucleophilic attack of the thiolate of glutathione on a variety of noxious, often hydrophobic, electrophiles. The interactions responsible for the binding of glutathione have been deduced in great detail from the 3-dimensional structures that have been solved for three different GSTs, each a member of a distinct structural class. However, the interactions of the electrophilic substrates with these enzymes are still largely unexplored. The contribution of the active-site Met208 to aromatic and benzylic chloride substitution reactions catalyzed by human class Alpha GST A1-1 has been evaluated by comparison of wild-type enzyme with variants mutated in position 208. The results show that the amino acid residue at position 208 primarily affects the aromatic substitution reaction, tested with 1-chloro-2,4-dinitrobenzene as substrate, possibly by interacting with the delocalized negative charge of the substituted ring structure in the transition state.

The glutathione transferases (GSTs)[1] are a group of enzymes involved in the glutathione (GSH)-linked detoxication of electrophilic compounds (Mannervik, 1985). Some of the reactions catalyzed are S_N2-type reactions involving an attack by the nucleophilic thiolate anion of GSH onto an electrophilic center on the second substrate molecule (Armstrong, 1991). When the second substrate is an alkyl- or arylhalogen, the reaction products are the glutathione *S*-conjugate of the hydrocarbon moiety and the free halogenide. Characteristic of the GSTs are broad and partly overlapping specificities for the electrophilic substrates, such that related compounds often can serve as substrates for the same GST isoenzyme.

To date, five structural classes of mammalian GSTs have been identified (Mannervik *et al.*, 1985; Meyer *et al.*, 1991; DeJong *et al.*, 1988). The members of four classes are soluble proteins, structurally distinct but interrelated in a gene superfamily, and the fifth class contains a membrane-bound enzyme (Morgenstern *et al.*, 1989), unrelated in primary structure to the soluble forms. The mammalian soluble GSTs are dimeric proteins and have been subdivided into four classes named Alpha, Mu, Pi, and Theta. Heterodimeric proteins can be formed from subunits within the same structural class (Mannervik & Jensson, 1982).

In recent years, mutagenesis experiments directed at putatively important residues (Stenberg *et al.*, 1991a; Widersten *et al.*, 1991a, Widersten & Mannervik, 1992; Zhang *et al.*, 1991) in combination with increased knowledge about the three-dimensional structure of members of the Pi (Reinemer *et al.*, 1991, 1992), Mu (Ji *et al.*, 1992), and Alpha (Sinning *et al.*, 1993) classes have gradually increased the general understanding of the catalytic action of the soluble GSTs. A conserved Tyr residue has been identified, which in its undissociated form appears to stabilize the nucleophilic thiolate anion of GSH, bound in the active site of the enzyme (Stenberg *et al.*, 1991b; Liu *et al.*, 1992; Kolm *et al.*, 1992; Kong *et al.*, 1992; Karshikoff *et al.*, 1993). Other residues of significance for the binding of the substrate GSH also have been subjected to mutational analyses (Widersten *et al.*, 1992). In summary, the interactions involved in the binding and activation of the obligatory thiol substrate (GSH) have largely become known. In contrast, the residues and interactions involved in binding of the electrophilic, often hydrophobic, second substrate have not been extensively investigated. From the known X-ray structures of GSTs, side chains of amino acid residues can be identified as possible contributors to the hydrophobic binding site based solely on their distance from the sulfur of the cocrystallized GSH or glutathione *S*-adduct. Increased knowledge of the role of the residues in the hydrophobic binding site is of importance, since the amino acid side chains forming this second substrate binding site dictate the range of compounds that can be acted upon by the enzyme.

In the available crystal structure of GST A1-1 [for nomenclature, see Mannervik *et al.* (1992)], *S*-benzylglutathione (the product of the reaction between GSH and a benzylhalide) is bound in the active site of the enzyme, thereby defining the GSH binding residues (Sinning *et al.*, 1993). Furthermore, the surrounding of the benzyl moiety clearly indicates the residues forming the hydrophobic binding site. One particularly close interaction was observed between the phenyl ring of *S*-benzylglutathione and the side chain of Met208[2] in the A1-1 enzyme (Figure 1).

When the known class Alpha primary structures are aligned, this position (no. 208) stands out as being highly variable. Furthermore, the location of this residue at the 'bottom' of the electrophile binding pocket where its side chain can interact with the second substrate indicates that sequence variation in this position of the folded GST Alpha structure may in part be responsible for the differences in substrate specificities among members of the Alpha class (Mannervik & Danielson, 1988).

The present investigation explores the potential of the amino acid residue in position 208 to contribute to the catalysis performed by human GST A1-1. Two different halogen

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[1] Abbreviations: GST, glutathione transferase; GSH, glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; NBC, 4-nitrobenzyl chloride; pCB-SG, *S*-(4-carboxybenzyl)glutathione.

[2] The amino acid residue numbering used includes the initiator Met as residue 1 for both class Alpha and class Mu GSTs.

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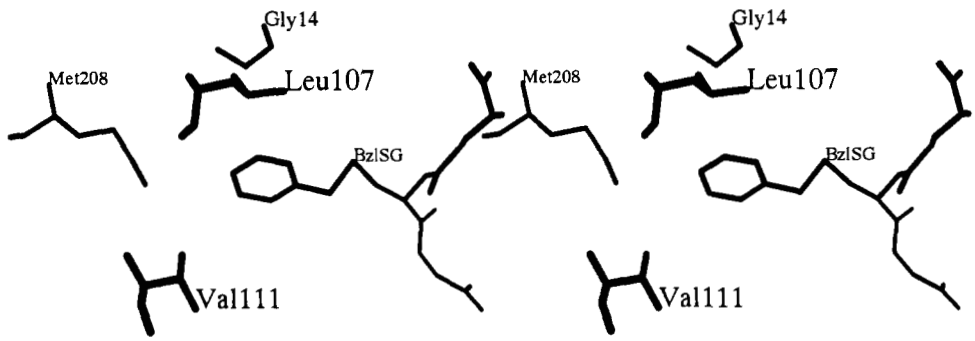

FIGURE 1: Stereo view of the H site in a GST A1 subunit. Residues 3–5 Å from carbon-4 of the benzyl moiety of the cocrystallized *S*-benzylglutathione (Sinning *et al.*, 1993) are shown. The object was constructed with the program *O* (Jones *et al.*, 1991) using the atomic coordinates 1GUH in the Brookhaven Protein Data Bank.

substitution reactions as well as the binding of different 4-substituted *S*-benzyl and *S*-phenylglutathione derivatives were used to monitor functional properties of the enzyme. Substitutions in position 208 have been introduced by means of random mutagenesis, thereby mimicking the naturally occurring sequence variation.

EXPERIMENTAL PROCEDURES

Construction of GST A1-1 M208X Mutants. The *Tac* promoter region together with the cDNA-encoding human glutathione transferase (GST) subunit A1 in plasmid pKHA1 (Stenberg *et al.*, 1992a) was isolated after a *Bam*HI digest and subsequently introduced into *Bam*HI-digested pGEM3Z (Promega Corp. Madison, WI). The pGEM derivative including the expressing unit, where the promoter was flanked upstream by the *Sac*I site and the region encoding the GST A1 subunit was flanked downstream by the *Xba*I site present in the pGEM3Z polylinker, was identified after restriction analysis. This construct was denoted as pGTacA1. pGTacA1 was used as a template in the polymerase chain reaction (PCR) site specifically introducing random mutations in codon 208. The oligonucleotide primers used were obtained from Operon Technologies (Alameda, CA) and their sequences were as follows: 'A1M208X', 5′-CCG *TCT AGA* TTA AAA TCG GAA GAT TTT ACG TGC TTC TTC TAA AGA TTT CTC ATC MNN GGG AGG CTT CCT-3′, where italicized letters indicate an introduced *Xba*I site, M is A or C, and N is A, C, G, or T. As an upstream primer 'M13/pUC universal sequencing primer', 5′-GTA AAA CGA CGG GCA GT-3′ was used. The PCR was performed with 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 μM of the respective primer, and 25 U/mL of *Taq* DNA polymerase (Boehringer Mannheim, Mannheim, Germany). The temperature cycle, repeated 30 times, was as follows: 1 min at 95 °C followed by 2 min at 55 °C and 1 min at 72 °C. The PCR product was digested with *Sac*I and *Xba*I and ligated into *Sac*I/*Xba*I-digested pGEM3Z. The constructed mutant library was transformed by electroporation (Gene-Pulser; Bio–Rad, Richmond, CA) into electrocompetent *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA). Mutant clones were identified by dideoxy sequencing (Sanger *et al.*, 1977). The mutant cDNAs chosen for protein expression were sequenced through their total coding region as well as their *Tac* promoter region to rule out unwanted mutations introduced by the PCR.

Expression and Purification of GST A1-1 M208X Mutants. *E. coli* XL1-Blue carrying the respective mutant GST A1 M208X cDNA was grown in 2TY [1.6% (w/v) tryptone, 1% (w/v) yeast extract, and 0.5% (w/v) NaCl] in the presence of 75 μg/mL ampicillin. Protein expression and protein lysate preparation were performed as previously described (Widersten *et al.*, 1991b). The different mutants were isolated as follows: M208A, M208E, M208H, M208L, M208P, M208W, and M208Y were purified using glutathione Sepharose (Simons & Vander Jagt, 1977); washed with 10 mM Tris-HCl pH 7.8, 1 mM Na_2EDTA (buffer A), and buffer A containing 0.2 M NaCl; and eluted with buffer A supplemented with 5 mM *S*-hexylglutathione (Mannervik & Guthenberg, 1981). M208N was purified as above but washed only with buffer A and eluted with buffer A containing 0.2 M NaCl. M208K was purified on carboxymethyl-Sepharose (Pharmacia-LKB Biotechnology, Uppsala, Sweden), equilibrated, washed with buffer A, and eluted with a linear gradient of 0–0.5 M NaCl in buffer A. The purified proteins were dialyzed against excessive amounts of buffer A containing 0.02% NaN_3. Purity of the isolated mutants was confirmed by SDS–PAGE analysis (Laemmli, 1970) stained with Coomassie Brilliant Blue R-250. Protein concentration was determined from the intrinsic absorbance at 280 nm. The ϵ_{280} values for wild-type GST A1-1 and the M208W mutant were determined from absorbance measurements where the protein concentration had been determined by amino acid analyses. The value obtained for the wild-type enzyme was used to determine the concentration of M208A, M208E, M208H, M208K, M208L, M208N, and M208P. The molar extinction of the M208Y mutant was calculated from the amino acid composition adding the structural contribution to absorbance properties determined from the $\epsilon_{280}^{\text{wild-type}}$ and $\epsilon_{280}^{\text{M208W}}$.

Kinetic Properties. Kinetic parameters, k_{cat} calculated per GST A1 subunit, K_M, and k_{cat}/K_M for two different substrates 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma Chemical Co., St. Louis, MO) and 4-nitrobenzyl chloride (NBC) (Aldrich Chemical Co., Milwaukee, WI) were determined using steady-state kinetic analysis under the following conditions: (i) 150–1500 μM CDNB in the presence of 5 mM glutathione (GSH) (Sigma Chemical Co.) in 0.1 M sodium phosphate, pH 6.5, and 0.1 mM Na_2EDTA at 30 °C; reaction monitored at 340 nm, $\Delta\epsilon = 9600\ M^{-1}\ cm^{-1}$ (Habig *et al.*, 1974); (ii) 50–300 μM NBC in the presence of 5 mM GSH in 0.1 M sodium phosphate, pH 7.2, and 0.1 mM Na_2EDTA at 30 °C; reaction monitored at 310 nm, $\Delta\epsilon = 1900\ M^{-1}\ cm^{-1}$ (Habig *et al.*, 1974). The analysis was performed on a Varian 2290 spectrophotometer. k_{cat} and K_M values were determined by fitting the collected data to the Michaelis–Menten equation by nonlinear regression analysis using the MMFIT program in the SIMFIT program package (Bardsley *et al.*, 1989). The second-order rate constant for the uncatalyzed reaction, k_2, was determined from the slope of v versus [CDNB] or [NBC] graphs divided by the GSH concentration.

The kinetic effect of *S*-(4-carboxybenzyl)glutathione on the CDNB reaction catalyzed by A1-1 M208K was examined

Table 1: GST A1-1 M208X Mutant cDNAs Isolated

amino acid replacement	codon(s)	amino acid replacement	codon(s)
Ala	GCG	Lys	AAG
Asn	AAT	Pro	CCG
Glu	GAG	Ser	TCT
Gly	GGT, GGG	Thr	ACT, ACG
His	CAT	Trp	TGG
Ile	ATT	Tyr	TAT
Leu	CTG, TTG		

by determining k_{cat}/K_M for GSH and CDNB in the absence or in presence of 100 μM *S*-(4-carboxybenzyl)glutathione. k_{cat}/K_M^{GSH} was determined in the presence of 156–5000 μM GSH and 1.5 mM CDNB, and k_{cat}/K_M^{CDNB} was determined in the presence of 150–2000 μM CDNB in the presence of 5 mM GSH in 0.1 M sodium phosphate, pH 6.5, and 1 mM Na_2EDTA at 30 °C. The analysis was performed in a ThermoMax microplate reader. The data were evaluated by nonlinear regression analysis as described under Kinetic Properties to yield the corresponding k_{cat} and K_M values.

Double ligand studies were performed in the presence of 1 mM GSH and 1 mM CDNB in 0.1 M sodium phosphate, pH 6.5, at 30 °C in the presence of varying concentrations of *S*-benzylglutathione and *S*-(4-carboxybenzyl)glutathione.

The rate enhancement from enzymatic catalysis was determined from the ratio of the respective second-order rate constants k_{cat}/K_M and k_2.

Binding Properties of Glutathione S-Adducts. The inhibition of the enzyme-catalyzed reaction between 1 mM GSH and 1 mM CDNB in 0.1 M sodium phosphate, pH 6.5, and 1 mM Na_2EDTA in 30 °C by para-substituted *S*-benzylglutathione derivatives and *S*-(2,4-dinitrophenyl)glutathione was assayed in a ThermoMax microplate reader (Molecular Devices, Menlo Park, CA) by addition or serial dilutions of GSH conjugate. The inhibition was expressed as I_{50}, the concentration of GSH conjugate causing a 50% decrease in enzymatic activity.

The apparent dissociation constants, K_D for *S*-(2,4-dinitrophenyl)glutathione and *S*-(4-nitrobenzyl)glutathione were determined through measuring the quenching of the intrinsic Trp fluorescence. The measurements were performed on an Aminco SPF-500 corrected beam spectrofluorometer in the presence of 0.75 μM GST A1-1 (wild-type or mutant) and 3.9–80 μM *S*-(2,4-dinitrophenyl)glutathione or 7.9–316 μM *S*-(4-nitrobenzyl)glutathione in 0.1 M sodium phosphate, pH 7.2, and 1 mM Na_2EDTA. The excitation wavelength was 280 nm for the dinitrophenyl adduct and 286 nm for the nitrobenzyl adduct. The emission was detected at 340 nm.

The GSH conjugates were synthesized essentially as described by Vince *et al.* (1971), using the α-bromide for benzyl, 4-bromobenzyl, and 4-nitrobenzyl derivatives and the α-chloride for the 4-carboxybenzyl derivative.

RESULTS

Construction of GST A1-1 M208X Mutants. After sequence analysis of 34 clones, a total of 17 different codons corresponding to 13 different amino acid residues and one amber stop codon were identified (Table 1). The replacements of Met208 introduced a range of different side-chain functionalities such as charges (Lys, Glu), amide and other side chains with hydrogen-bonding capacity (Asn, Tyr, Trp, His, Ser, and Thr), or hydrocarbon side chains (Ala, Leu, Ile, and Pro) and Gly.

Table 2: Kinetic Properties of GST A1-1 M208X Mutants with 1-Chloro-2,4-dinitrobenzene as Electrophilic Substrate

enzyme	k_{cat} (s^{-1})	K_M^{CDNB} (mM)	k_{cat}/K_M^{CDNB} (s^{-1} mM^{-1})	$(k_{cat}/K_M^{CDNB})/k_2^{CDNB}$ [a] ($\times 10^{-6}$)
WT	88 ± 3 (1.0)[b]	0.56 ± 0.04 (1.0)	160 ± 8.0 (1.0)	11 ± 1.6
M208A	110 ± 11 (1.2)	3.4 ± 0.5 (6.1)	34 ± 1.1 (0.21)	2.3 ± 0.33
M208E	–[c]	–	4.4 ± 0.1 (0.03)	0.3 ± 0.042
M208H	17 ± 0.8 (0.19)	0.25 ± 0.05 (0.45)	70 ± 11 (0.44)	4.8 ± 1.0
M208K	50 ± 6[d] (0.57)	6.1 ● 0.9[d] (11)	8.2 ± 1.6[d] (0.05)	0.57 ± 0.13
M208L	64 ± 3 (0.73)	0.75 ± 0.08 (1.3)	85 ± 5.1 (0.53)	5.9 ± 0.88
M208N	66 ± 11 (0.75)	2.3 ± 0.6 (4.1)	29 ± 2.2 (0.18)	2.0 ± 0.31
M208P	–	–	26 ± 0.2 (0.16)	1.8 ± 0.25
M208W	99 ± 2 (1.1)	0.18 ± 0.02 (0.32)	542 ± 36 (3.4)	37 ● 5.1
M208Y	16 ± 2 (0.18)	0.36 ± 0.09 (0.64)	45 ± 7.1 (0.28)	3.1 ± 0.65

[a] The rate constant for the uncatalyzed reaction, k_2, was determined to be 0.0145 ± 0.002 s^{-1} M^{-1} under the conditions described in the Experimental Procedures section. [b] Value in parentheses is a fraction of wild-type value. [c] – indicates that no estimate for k_{cat} or K_M could be obtained since no saturation was observed within the concentration range used. [d] Values determined in the presence of 150–2000 μM CDNB.

Expression and Purification of GST A1-1 M208X Mutants. The mutant proteins chosen for purification and further characterization were M208A, which can be considered as the "parental" structure from which the other mutants can be built by extension of the side chain; M208E, which is carrying a carboxyl group potentially negatively charged [Glu is present in the corresponding position in rat class Alpha GST subunit 2 (Telakowski-Hopkins *et al.*, 1985)]; M208K, which is carrying an amino group potentially positively charged; M208H, M208N, M208W, and M208Y, which have side chains with hydrogen-bonding potential; M208P, which involves folding constraints on the polypeptide backbone and may leave a cavity in the structure [Pro is present in rat class Alpha GST subunit 8 in the corresponding position (Stenberg *et al.*, 1992b)]; M208L, maintaining the volume of Met but introducing a more hydrophobic side chain [Leu is present in a mouse class Alpha GST (Pearson *et al.*, 1988)].

The majority of the mutants could be purified according to the standard protocol. However, M208N was eluted from the affinity matrix already when 0.2 M NaCl was added to the equilibration buffer, and M208K failed to bind to any of the affinity matrices (GSH Sepharose or *S*-hexylglutathione Sepharose) and was purified by carboxymethyl ion-exchange chromatography.

All mutant proteins appeared homogeneous after purification as judged from Coomassie Brilliant Blue R-250-stained SDS–PAGE gels (results not shown). The molar extinction coefficients at 280 nm (ϵ_{280}) determined for wild-type GST A1-1 was 24 700 M^{-1} cm^{-1} and 30 000 M^{-1} cm^{-1} for M208W. The structural contribution to the absorbance at 280 nm was determined to be 4781 ± 149 $M1^{-1}$ cm^{-1}. Using this value, the ϵ_{280} for the M208Y mutant was calculated to be 26 000 M^{-1} cm^{-1}.

Kinetic Properties. The kinetic constants, k_{cat}, K_M, and k_{cat}/K_M determined for the enzyme-catalyzed GSH conjugation of CDNB and NBC, are presented in Tables 2 and 3, respectively. The incremental free energy change for forma-

Table 3: Kinetic Properties of GST A1-1 M208X Mutants with 4-Nitrobenzyl Chloride as Electrophilic Substrate

enzyme	k_{cat} (s^{-1})	K_M^{NBC} (mM)	k_{cat}/K_M^{NBC} (s^{-1} mM^{-1})	$(k_{cat}/K_M^{NBC})/k_2^{NBC}$ [a] ($\times 10^{-4}$)
WT	0.20 ± 0.01 (1.0)[b]	0.15 ± 0.02 (1.0)	1.6 ± 0.2 (1.0)	4.2 ± 0.76
M208A	0.24 ± 0.03 (1.2)	0.31 ± 0.05 (2.1)	0.82 ± 0.05 (0.51)	2.2 ± 0.31
M208E	–[c]	–	0.16 ± 0.02 (0.10)	0.42 ± 0.076
M208H	0.19 ± 0.05 (1.0)	0.17 ± 0.09 (1.1)	1.2 ± 0.3 (0.75)	3.2 ± 0.89
M208K	–	–	0.23 ± 0.07 (0.14)	0.61 ± 0.20
M208L	0.12 ± 0.01 (0.6)	0.058 ± 0.022 (0.39)	2.0 ± 0.6 (1.2)	5.3 ± 1.7
M208N	0.59 ± 0.14 (3.0)	0.97 ± 0.27 (6.5)	0.58 ± 0.05 (0.36)	1.5 ± 0.24
M208P	–	–	0.43 ± 0.03 (0.27)	1.1 ± 0.17
M208W	0.96 ± 0.19 (4.8)	0.55 ± 0.15 (3.7)	1.4 ± 0.14 (0.88)	3.7 ± 0.61
M208Y	0.33 ± 0.06 (1.6)	0.52 ± 0.14 (3.5)	0.63 ± 0.05 (0.39)	1.7 ± 0.25

[a] The rate constant for the uncatalyzed reaction, k_2, was determined to be 0.038 ± 0.005 s^{-1} M^{-1} under the conditions described in the Experimental Procedures section. [b] The value in parentheses is a fraction of wild-type value. [c] – indicates that no estimate for k_{cat} or K_M could be obtained since no saturation was observed within the concentration range used.

Table 4: Substrate Selectivity of GST A1-1 M208X Mutants

enzyme	$(k_{cat}/K_M^{CDNB})/(k_{cat}/K_M^{NBC})$	$\Delta\Delta G$ for transition-state stabilization[a] (kJ/mol)	
		CDNB	NBC
WT	100	–3.9	–1.7
M208A	41	(0)	(0)
M208E	28	+5.2	+4.1
M208H	58	–1.8	–1.0
M208K	35 (267[b])	+3.6 (–1.5[b])	+3.2
M208L	42	–2.3	–2.2
M208N	50	+0.4	+0.9
M208P	60	+0.7	+1.6
M208W	390	–7.0	–1.4
M208Y	71	–0.7	+0.7

[a] Calculated from $\Delta\Delta G = -RT \ln [(k_{cat}/K_M^{Variant})/(k_{cat}/K_M^{M208A})]$. [b] In the presence of 100 μM *S*-(4-carboxybenzyl)glutathione.

tion of the corresponding transition states ($\Delta\Delta G$), as calculated from the formula (Fersht, 1985)

$$\Delta\Delta G = -RT \ln \frac{k_{cat}/K_M^{mutant}}{k_{cat}/K_M^{wild\text{-}type}}$$

are given in Table 4.

Binding Properties and Activation of M208K. When binding of different para-substituted *S*-benzylglutathione derivatives was estimated by measuring the I_{50} values, small differences (1–2-fold) between different enzyme forms were observed. Exceptions were mutants M208K, which showed markedly increased I_{50} values for *S*-(2,4-dinitrophenyl)-glutathione (>52 μM), *S*-benzylglutathione (24 μM), and *S*-(*p*-bromobenzyl)glutathione (30 μM) as compared to the wild-type enzyme (31, 6.4, and 3.9 μM, respectively). M208Y was significantly less sensitive to *S*-(2,4-dinitrophenyl)-glutathione and *S*-(*p*-bromobenzyl)glutathione (I_{50} = >52 and 12 μM) than was the wild-type enzyme. When the ligand *S*-(4-carboxybenzyl)glutathione (pCBSG) was tested for inhibition, larger variations were observed between the different mutants. The I_{50} value for mutants M208H, M208P, and M208Y were increased 5–10-fold as compared to the value of the wild type (720 μM). In the cases of M208A, M208E, and M208K, no inhibition was observed. In contrast, addition of this glutathione adduct to M208A and M208K caused an increased enzyme activity. The activation was approximately 50% for M208A, whereas the effect was more pronounced for M208K (approximately 600% activation). The activation of M208K was concentration dependent and reached a plateau at approximately 100 μM (Figure 2). At high concentrations of pCBSG (1.67–3.33 mM), a slight inhibitory effect was observed. When k_{cat}, K_M^{GSH}, K_M^{CDNB}, k_{cat}/K_M^{GSH}, and k_{cat}/K_M^{CDNB} were determined, the observed effects were different for CDNB and GSH (Figure 3). In the absence of pCBSG, K_M^{CDNB} was about 7-fold higher for the enzyme in the presence of 100 μM pCBSG (6.1 and 0.88 mM, respectively). The k_{cat} value determined in the presence of a constant high concentration (5 mM) of GSH was not significantly affected by the addition of pCBSG (50 and 54 s^{-1}, respectively). K_M^{GSH} was only slightly affected by the addition of an activator (1.1 mM in the absence and 0.86 mM in the presence of pCBSG), whereas the apparent k_{cat} value determined in the presence of 1.5 mM CDNB (not saturating concentrations in the absence of pCBSG) was 6.4-fold increased in the presence of 100 μM pCBSG (8.9 s^{-1} without; 57 s^{-1} with pCBSG). These effects resulted in increased k_{cat}/K_M^{GSH} and k_{cat}/K_M^{CDNB} of 8.3- and 7.6-fold, respectively, in the presence of pCBSG. This corresponds to a decrease in free energy for the formation of the corresponding transition state by approximately 5 kJ/mol. Activation of the NBC reaction by pCBSG was not observed. Furthermore, when *S*-benzylglutathione was tested as an inhibitor in the presence of 100 μM pCBSG, the I_{50} was lowered from 24 to 8 μM. When the inhibition by *S*-benzylglutathione was tested at different fixed concentrations of pCBSG, nonparallel lines in a $1/v$ versus [*S*-benzylglutathione] Dixon plot were obtained (Figure 5).

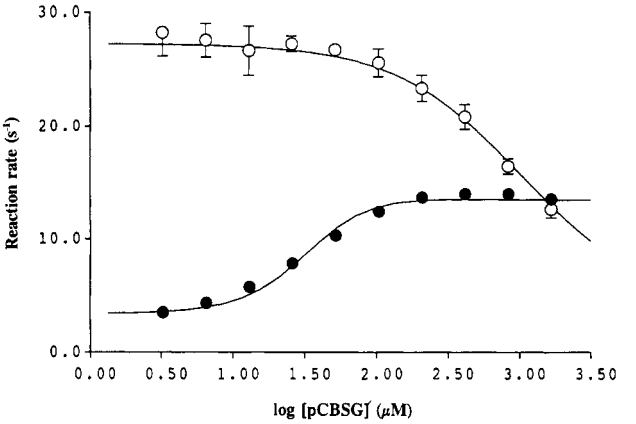

FIGURE 2: Reaction rate versus increasing concentration of *S*-(4-carboxybenzyl)glutathione. The reaction was followed in the presence of 1 mM GSH and 1 mM CDNB in 0.1 M sodium phosphate, pH 6.5, at 30 °C. (○) Wild-type GST A1-1; (●) A1-1 M208K. Error bars indicate standard deviation (n = 5) with 95% confidence limits.

K_D values were determined from fluorescence quenching studies on the wild-type protein and mutants M208W and M208Y. The affinity for both mutants towards *S*-(4-nitrobenzyl)glutathione were decreased about 2-fold from 78 μM (wild type) to 160 μM (M208W) and 120 μM (M208Y). The measured affinities for *S*-(2,4-dinitrophenyl)glutathione were comparable for the three enzyme forms, 29 μM (wild type) and 28 μM (M208W) with a small decrease in affinity for M208Y (45 μM).

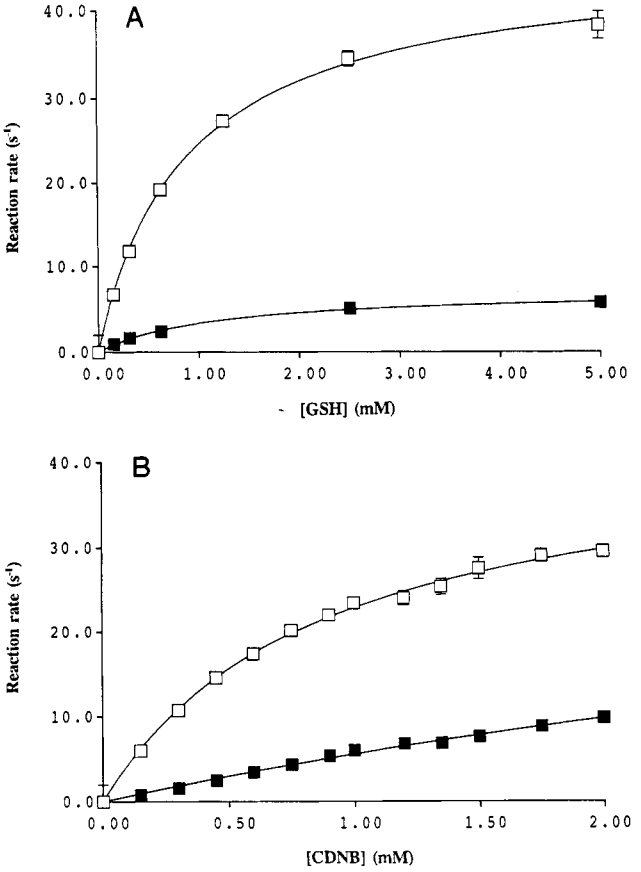

FIGURE 3: Reaction rate versus increasing concentrations of limiting substrate in the absence (■) or presence (□) of 100 μM *S*-(4-carboxybenzyl)glutathione for A1-1 M208K. (A) Reaction rate versus [GSH]. (B) Reaction rate versus [CDNB]. The k_{cat} and K_M values determined in the presence of 100 μM *S*-(4-carboxybenzyl)-glutathione were 43 ± 0.6 s^{-1} and 0.88 mM, respectively. Error bars indicate standard deviation (n = 5) with 95% confidence limits.

DISCUSSION

Residues in the active site of GSTs that contribute to binding of the second hydrophobic substrate and, more importantly, residues contributing to the stabilization of high-energy transition-state structures are still largely unknown. However, in recent investigations, one such residue has been identified; Tyr116[2] in rat class Mu GST 3-3 is involved in the activation of phenanthrene 9,10-oxide for conjugation with GSH (Johnson *et al.*, 1993; Ji *et al.*, 1994) as well as in the stabilization of a Meisenheimer complex, implied as a transition state mimic for the reaction of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) (Ji *et al.*, 1993). Among other interactions in the latter reaction, a hydrogen bond from Tyr116 to one of the nitro groups of the substrate appears to stabilize the Meisenheimer complex.

In the present study of class Alpha GST A1-1, the amino acid residue 208 in the H site of the enzyme has been randomly mutated. This side chain is close to the position of the 4-nitro substituent of the assumed transition state, as judged by model building based on the known structure in which *S*-benzylglutathione is bound at the active site (Figure 1). Although *S*-benzylglutathione as such is not a good analog for a Meisenheimer complex relevant to the GST reaction, this ligand can be used to deduce the approximate distance from a residue at position 208 in the active site of GST A1-1 to a para-positioned nitro group in such a Meisenheimer complex. This is due to the sp^3-type hybridization of carbon-1 in the phenyl ring of the latter complex, which is mimicked by the

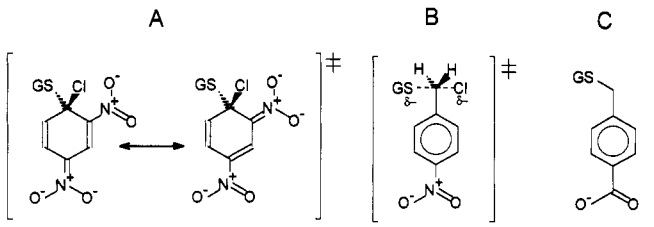

FIGURE 4: Postulated transition-state structures of the CDNB reaction (A) and the NBC reaction (B) and (C) the structure of *S*-(4-carboxybenzyl)glutathione.

methylene group of the benzyl moiety in *S*-benzylglutathione, resulting in similar overall geometry for the two glutathione derivatives. Thus, the estimated position of the planar nitrophenyl system in the Meisenheimer complex is very similar to the position of the phenyl ring in *S*-benzylglutathione, and the position of the nitro group can be deduced by overlaying the available three-dimensional structures of a Meisenheimer complex between GSH and 1,3,5-trinitrobenzene (Ji *et al.*, 1993; constructed from the atomic coordinates in *4GST* in the Brookhaven Protein Data Bank) and *S*-benzylglutathione (constructed from the coordinates in *1GUH* in the Brookhaven Protein Data Bank). In the modeling, carbon-4 of the phenyl ring of *S*-benzylglutathione superimposes on the nitro group nitrogen. Thus, the structure of GST A1-1 with *S*-benzylglutathione in the active site can be used for the interpretation of the various substitutions in the position of amino acid residue 208 in relation to possible contributions to transition-state stabilization in such reactions where the spatial arrangement of atoms is expected to be similar, such as in the reaction between GSH and CDNB (Figure 4).

The functional properties of GST A1-1 and the mutant forms were probed by means of two different nucleophilic substitution reactions involving either CDNB or 4-nitrobenzyl chloride (NBC). The two reactions differ in their transition states, which are distinct both in geometry and electronic configuration (Figure 4). The CDNB reaction involves a Meisenheimer complex (Figure 4A), in which the negative charge originating from the GS^- is delocalized over the phenyl ring and the two nitro groups. The NBC reaction, on the other hand, includes a pentavalent carbon in the transition state (Figure 4B). This creates different geometry in the corresponding transition-state structure where the position of the phenyl ring would be very different from that of the Meisenheimer complex in the CDNB reaction (Figure 4). Therefore, the phenyl group may not be expected to interact directly with a residue in position 208 of the active site, based on comparison with the three-dimensional structure of *S*-benzylglutathione bound to GST A1-1. Further, in the transition state for the NBC reaction, the negative charge is essentially restricted to the atoms surrounding the benzylic carbon and not extending to the ring structure. Therefore, mutations in Met208 are likely to affect primarily the CDNB reaction in which the delocalized negative charge in the transition state would extend into close proximity of the side chain of residue 208.

When assessing the contribution of residue 208 to catalysis, mutant M208A was taken as the reference structure; the methyl group of the Ala side chain is essentially inert, and the structural and chemical contributions of added groups can be evaluated by kinetic studies (Table 4). Focusing on the NBC reaction, with the exception of the mutations introducing potentially charged residues (M208E and M208K), the nature of amino acid 208 alters the stabilization of the transition state only marginally (±2 kJ/mol). However, replacing Met

by Leu decreased K_M^{NBC} significantly (2.6-fold). GSH conjugation of CDNB is more strongly affected by changes in residue 208, in agreement with the discussion above concerning the different transition-state structures. As in the case of NBC, insertion of potentially charged residues (M208E, M208K) is unfavorable for catalysis of the CDNB conjugation. This effect of charge appears to have a counterpart in rat class α GST 2-2, which naturally contains a Glu residue in position 208. GST 2-2 shows a 12-fold lower catalytic efficiency with CDNB as compared to the homologous rat GST 1-1 (Alin *et al.*, 1985), which like human GST A1-1 contains a Met in position 208 (Pickett *et al.*, 1984). In the case of M208K, introduction of positive charge may cause nonoptimal folding of the protein, which decreases the catalytic capability of this mutant. Notably, addition of *S*-(4-carboxybenzyl)glutathione (pCBSG) activates M208K and, to a smaller degree, M208A.

Generally, introduction of a side-chain functionality with the capacity to contribute a proton to a hydrogen bond (His, Trp, Tyr) decreases K_M^{CDNB} in the CDNB reaction, but not K_M^{NBC} in the NBC reaction. This observation is in agreement with the finding that nitro groups of the Meisenheimer complex of 1,3,5-trinitrobenzene and GSH interact with hydrogen bond donors in the active site of rat GST 3-3 (Ji *et al.*, 1993). The lack of effect on K_M^{CDNB} in the M208N mutant may be due to the inability of the amide $N^{\delta}H_2$ group to reach into the binding site due to the short side chain of Asn.

The decrease in k_{cat}^{CDNB} observed for the M208H and M208Y mutants does not appear to be due to the decreased rate of product release (Armstrong *et al.*, 1993), since the binding data rather suggest the opposite; the observed K_D values for M208Y for the same compound is higher than the wild-type value.

The attempt to engineer a mutant GST which offers a more favorable active-site environment for CDNB catalysis than does wild-type GST A1-1 was successful when an indole side chain (M208W) was introduced. This mutation caused a 3.3-fold decreased K_M^{CDNB} with maintained k_{cat}. Evidence for increased stabilization of a Meisenheimer complex by mutant M208W in comparison with wild-type enzyme has been obtained by use of the complex of 1,3,5-trinitrobenzene and GSH (Graminski *et al.*, 1989) as a spectroscopic probe (M. Widersten, R. Björnestedt, and B. Mannervik, unpublished results). The semistable Meisenheimer complex between GSH and trinitrobenzene is regarded as a good mimic of the transition state in an aromatic halogen substitution reaction involving GSH. The detailed molecular interactions underlying the increased affinity have not been established. However, the $N^{\epsilon}H$ of a Trp side chain could be located within less than approximately 3.5–4 Å from the para-positioned nitro group of the trinitrobenzene–GSH Meisenheimer complex (Ji *et al.*, 1993) as judged from model building as described above, suggesting the possibility of hydrogen bonding. Furthermore, as can be concluded from the kinetic analyses (Tables 2–4), the results do not point to a pure effect of the hydrophobicity of residue 208 (Met < Tyr < Leu < Trp) in relation to the catalytic activity of the enzyme with CDNB (Tyr < Leu < Met < Trp).

The mechanism behind the activation of M208K affected by pCBSG is unknown, but double-modifier studies including *S*-benzylglutathione and pCBSG (Figure 5) show that pCBSG acts as a nonessential activator, where the activator, the inhibitor, and the substrate (CDNB) bind to the same enzyme form in the kinetic mechanism (Segel, 1975). Steady-state kinetic studies failed to give evidence for cooperativity in mutant M208K (data not shown). Further studies of this

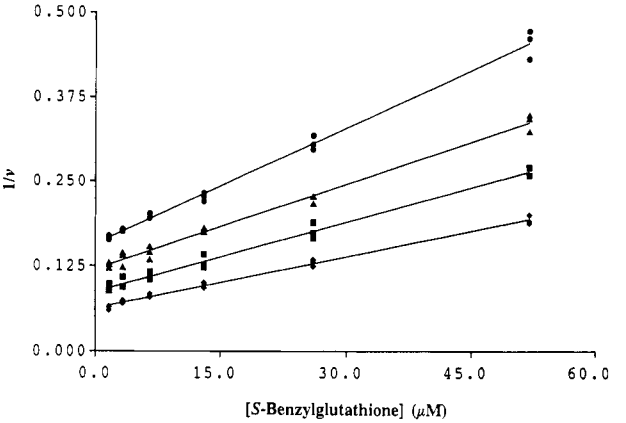

FIGURE 5: Double-modifier study of A1-1 M208K using different concentrations of *S*-benzylglutathione (inhibitor) and *S*-(4-carboxybenzyl)glutathione (activator). The kinetic effect of inhibitor and activator was measured in 1 mM GSH, 1 mM CDNB, and 0.1 M sodium phosphate, pH 6.5. $1/v$ versus [*S*-benzylglutathione] in the presence of (●) 0, (▲) 5, (■) 40, of (◆) 80 μM *S*-(4-carboxybenzyl)-glutathione.

phenomenon are needed to clarify the structural basis for the activation of M208K. From the three-dimensional structure of GST A1-1, it is known that residue 208 is located in the last loop preceding the C-terminal α-helix. One face of this helix contributes five amino acid residues to the H site. Structural changes that could alter the conformation of this helix may also alter the activity of such a mutant. The activation of the M208K mutant by pCBSG occurs by lowering the K_M^{CDNB}, whereas k_{cat} is essentially unaffected. The K_M^{CDNB} value reflects the affinity between free CDNB and enzyme forms not containing this substrate. Since K_M^{CDNB} is decreased by a factor of 7 when pCBSG is present, it is plausible that pCBSG induces a conformational change that favors the binding of CDNB to the enzyme, triggered by an interaction between the para-positioned negative charge on pCBSG and the lysine side chain of Lys208. Similarly, the lowering of the I_{50} value for *S*-benzylglutathione from 24 to 8 μM (close to the wild-type value of 6.4 μM) suggests that the addition of pCBSG to the M208K mutant causes a structural transition favoring binding of the active-site ligand. The crucial role of the negative charge on the carboxylate of pCBSG for the activation is clear, since the isoelectronic, but uncharged, *S*-(4-nitrobenzyl)glutathione did not activate M208K but rather acted exclusively as an inhibitor.

In summary, the presented work demonstrates that changing the amino acid side-chain functionality in position 208 of GST A1-1 can alter catalytic efficiency as well as specificity for the second electrophilic substrate. Although distant from the activated thiol of GSH (the C^{α} of Met208 being approximately 13 Å from the sulfur of GSH), a residue with hydrogen-bonding capacity at position 208 can promote GSH conjugation with CDNB possibly through direct interaction with the transition state. The work also shows that GST A1-1 can be engineered toward altered selectivity for the second substrate by small structural manipulations.

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